THE ROLE OF REACTIVE OXYGEN SPECIES IN CELL DEATH INDUCED BY THYMIDYLATE SYNTHASE INHIBITORS

by

Ufuk Ozer

Bachelor of Science
İnönü University, 2004

Master of Science
İnönü University, 2006

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Accepted by:

Franklin G. Berger, Major Professor

David Reisman, Committee Chairperson

Deanna S. Smith, Committee Member

Richard G. Vogt, Committee Member

Caryn E. Outten, Committee Member

Lacy Ford, Vice Provost and Dean of Graduate Studies

DEDICATION

This work is dedicated to:

My advisor, *Dr. Berger*, who led me with the great excitement.

My parents, *Ahmet & Yeter Özer*, who have provided me the strength to accomplish this work. I would not do it without your sacrifices. I want to thank my brothers and sisters for their endless motivation. Thank you all for enduring this heavy yearning without revealing.

My wife, *Hatice*, who was always proud of me with my little achievements and who stood by me in any little troubles.

My daughter, *Erva*, who rose to my life like a sun and who made my last PhD year more enjoyable.

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ABSTRACT

Thymidylate synthase (TS) catalyzes the reductive transfer of a methyl group from N^5,N^{10}-methylenetetrahydrofolate (CH_2-THF) to dUMP, forming dTMP and dihydrofolate (DHF). It is generally accepted that inhibition of the enzyme leads to a deficiency of dTMP, followed by genome damage and programmed cell death. As such, TS has long been viewed as an important target of anti-neoplastic agents, such as 5-fluorouracil (5-FU) and raltitrexed (RTX), which lead to inhibition of the enzyme. Oxidative stress is well-recognized as having a central role in cellular response to a number of DNA damaging agents, and may be a proximate cause of therapy-induced cell death. Despite such recognition, little detail exists on the origin and regulation of reactive oxygen species (ROS) during TS-directed chemotherapy. We have undertaken a detailed examination of the origin, nature, and role of ROS in cell death mediated by TS inhibitors. In colon tumor cell line HCT116, fluorescence detection of H_2O_2 and $O_2^{\bullet -}$ (using H_2DCFDA and DHE, respectively) showed that profound increases in ROS levels occur during drug exposure. These increases are attenuated by treatment with the antioxidant N-acetylcysteine (NAC), by addition of thymidine to the medium, and by TS overproduction, indicating that it is indeed TS inhibition and the resulting dTMP deficiency that is responsible for the increased ROS levels. Apoptotic indices, as measured by TUNEL assays, parallel changes in ROS levels, i.e., they are induced in response to drug, and are inhibited by NAC. We have identified NADPH oxidase (NOX) as the primary source of increased ROS following exposure to TS-directed agents, as

indicated by the observation of decreased ROS production and apoptotic indices in presence of NOX inhibitors. We have found that in response to TS inhibitors, NOX enzyme activity increases in association with induction of the transcript for $p67^{phox}$, which specifically regulates the NOX2 isoform. These effects were reduced by thymidine and by TS overproduction. To substantiate and expand results obtained from HCT116, we used other colorectal cancer cells, including HCT15, SW480, DLD-1, LoVo, MOSER and LS180. We have determined that colon cancer cells exhibit diverse responses in both basal and drug-inducible levels of apoptosis, NOX activity and mRNA of NOX2 regulatory subunits, implicating the heterogeneity in colorectal cancer cells. To understand whether other factors are involved in TS inhibitors-mediated ROS generation, we investigated the effects of NFκB on drug-mediated ROS accumulation in HCT116 cells. Loss of NFκB has no effects on increase in NOX activity mediated by TS inhibitors. It has no effects on drug-induced ROS and $p67^{phox}$ mRNA expression, either. However, we have determined that NFκB is an important suppressor for basal ROS formation in correlation with the induction of $p67^{phox}$ mRNA. Based on these results, we conclude that augmentation of NOX2 activity via induction of $p67^{phox}$ mRNA expression is the proximate cause of programmed cell death elicited by dTMP deficiency in HCT116 cells. NFκB has no role in such effects. Each colorectal cancer cells might have different mechanism to undergo ROS-mediated cell death.

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LIST OF ABBREVIATIONS

5-FU 5-fluorouracil

ATZ ..... Aminotriazole

APO.....Apocynin

BSA..... Bovine Serum Albumin

CH_2-THF.....N^5,N^{10}-Methylenetetrahydrofolate

CRC.....Colorectal Cancer

DHE .....Dihydroethidium

DHF.....Dihydrofolate

dnIkBα .....dominant negative Inhibitor of kappa-B-α

DPI.....Diphenylene Iodonium

dTMP .....Deoxythymidine Monophosphate

dUMP..... Deoxyuridine Monophosphate

Duox.....Dual Oxidase

FACS.....Fluorescence-Activated Cell-Sorting

FdUMP.....5-Fluoro-2'-Deoxyuridylic Acid

FdUrd ..... 5'-Fluoro-2'-Deoxyuridine

FR.....Ferredoxin Reductase

GAPDH.....Glyceraldehyde 3-Phosphate Dehydrogenase

GEF.....Guanine-nucleotide Exchange Factor

H_2DCFDA.....2',7'-Dichlorodihydrofluorescein Diacetate

HCT116, HCT15, SW480, DLD-1, LoVo, MOSER, LS180 .....Colon Cancer Cell Lines

IKKβ .....Inhibitor of Kappa B Kinase-β

IκBα .....Inhibitor of kappa-B-α

LV ..... leucovorin

mRNA.....Messenger Ribonucleic Acid

NAC .....N-acetylcysteine

NADPH.....Nicotinamide Adenine Dinucleotide Phosphate

NFκB.....Nuclear Factor Kappa B

NOX.....NADPH Oxidase

NOXA1.....NOX Activator 1

NOXO1.....NOX Organizer 1

PBS .....Phosphate-Buffered Saline

PVDF .....Polyvinylidene Fluoride

qPCR..... Quantitative Polymerase Chain Reaction

Rac1 .....Ras-related C3 botulinum toxin substrate 1

ROS.....Reactive Oxygen Species

RTX.....Raltitrexed

SDS-PAGE ..... Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SEM ..... Standart Error of the Mean

SSAT..... Spermidine/Spermine N^1-Acetyltransferase

Thy ..... Thymidine

TS .....Thymidylate Synthase

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

VAS..... VAS-2870

GENERAL INTRODUCTION

Colorectal cancer (CRC) is a disease that starts in glands in the lining of the colon (the longest part of the large intestine) or the rectum (the last several inches of the large intestine closest to the anus). It is the third most common cancer worldwide and the disease rate is the highest in developed countries, including the USA, Australia, Canada and Europe. Worldwide, every year, more than 1.2 million individuals develop CRC, accounting for a mortality over 600,000 and 9.7% of total cancer deaths (Ferlay et al., 2010). According to the National Cancer Institute, estimated new cases in the USA in 2012 numbered 103,170 in the colon and 40,290 in the rectum, leading to approximately 51,690 deaths from CRC (www.cancer.gov/cancertopics/types/colon-and-rectal). The development of inflammatory bowel disease (ulcerative colitis), having a family history of CRC and age over 50 increase the lifetime risk of colorectal carcinogenesis (Keane and Johnson, 2012).

Treatment of CRC depends upon the degree of progression of the cancer and can be curative if caught in an early stage (Stein et al., 2011; Cunningham et al., 2010). Treatments may include surgery (removal of cancer cells), chemotherapy (to kill cancer cells) and radiation (to destroy cancerous tissue). The prognosis still remains modest in spite of recent advances at the molecular level of CRC. Survival rate (5 years) has not changed with the recent advances and chemotherapy still depends upon the mainstay of

treatment (Platell et al., 2011; Lucas et al. 2011). There are several drugs, including 5-fluorouracil (5-FU), irinotecan, and oxaliplatin that can be used to treat CRC. These drugs are often combined to get more efficient chemotherapy. Fluoropyrimidines, in particular, 5-FU and its nucleoside analog 5'-fluoro-2'-deoxyuridine (FdUrd), are widely used agents and remain a major component of many standard regimens for various cancer types (Figure I1) (Soong and Diasio, 2005; Milano et al. 2004). Metabolism and cytotoxic mechanisms of 5-FU involve multiple pathways. These pathways show anti-proliferative effects of 5-FU and its metabolites through incorporation into DNA or RNA and/or inhibition of thymidylate synthase (TS) (Berger and Berger, 2006; Wyatt and Wilson, 2009). Investigating and understanding potential determinants of fluoropyrimidine metabolism could help to establish a strong rationale for improving therapeutic response. TS inhibition is known as a major mechanism of 5-FU cytotoxicity; the degree of its inhibition could provide potential indicators of efficacy (Kessel et al., 1966, Myers et al., 1975).

TS Inhibition and Cancer Chemotherapy

TS (EC 2.1.1.45) catalyzes the reductive transfer of a methyl group from N^5, N^{10}-methylenetetrahydrofolate (CH_2-THF) to the C5 of deoxyuridylate monophosphate (dUMP) to form thymidylate (dTMP) whose subsequent phosphorylation to dTTP provides a precursor for DNA synthesis (Figure I2) (Carreras and Santi, 1995). In the absence of exogenous thymidine, the enzyme is the sole source of thymidylate. To form dTMP, a covalent ternary complex is formed by the enzyme, dUMP and CH_2-THF. 5-FU and FdUrd are converted to the efficient TS inhibitor 5-fluoro-2'-deoxyuridylic acid

(FdUMP), which is an analogue of the natural TS substrate dUMP, and forms an inhibitory ternary complex with the enzyme and CH_2-THF (Santi et al., 1987; Longley et al., 2003). This inhibitory complex is comprised of two covalent linkages as in the catalytic complex, resulting in considerably stable inhibition of the enzyme. Inhibition of TS blocks dTMP production and leads to genome damage through misincorporation of uracil into DNA, and shuts off DNA synthesis and repair, triggering apoptosis (Danenberg, 1977; Spears et al., 1988; Krokan et al., 2002; Longley et al., 2003). As a consequence, TS is an important target for anticancer chemotherapy by anti-neoplastic agents, including 5-FU and antifolate inhibitors, in treatment of colorectal and other tumors (Takezawa et al., 2010).

The important role of the folate co-substrate in TS activation prompted structural investigations into the synthesis and design of antifolates (folate analogs) that inhibit TS (Jackman et al., 1996). Antifolates might potentially be more useful than dUMP analogs because TS-inhibitiory antifolates are not sensitive to the increased levels of dUMP arising from the inhibition. dUMP analogs compete with elevated dUMP to bind the enzyme's active site (Gmeiner, 2005; Winder and Lenz, 2010). Larger structure gives a chance to antifolates for various chemical modifications. Addition to these advantages, they have different toxicity profiles from fluoropyrimidines, particularly 5-FU, that has RNA-mediated effects making contribution to cytotoxicity as well. Several antifolates, including ZD1694 (raltitrexed (RTX), tomudex) (Figure I1), CB3717, LY231514, GW1843 and AG337, are currently being evaluated for the treatment of cancer (McGuire, 2003; Gmeiner, 2005; Takemura and Jackman, 1997; Bertino, 1997).

Fluoropyrimidines have been integrated into numerous clinical trials and have exhibited anti-tumor activity in the chemotherapy of various neoplasms. In particular, the antimetabolite 5-FU is widely used in chemotherapy of CRC and remains one of the most common anticancer drugs in use today (Soong et al., 2008). At current doses, adjuvant treatment with 5-FU shows clinical response in stage III CRC patients with ~10-15% survival rate (Moertel et al., 1995). Incremental evidence shows that 5-FU-based therapies are beneficial for stage II colon carcinoma patients as well (Midgley and Kerr, 2005). Biomodulation of the anti-cancer effects of 5-FU has been attempted and studies based upon known metabolic interactions have improved the drug-based therapy. One of the current standard treatment options for advanced CRC is usage of 5-FU in combination with leucovorin (LV) (N6-formyl tetrahyrofolate), a reduced folate, that is converted to CH2-THF and enhances the interaction between FdUMP and TS, prolonging the inhibition (Sotos et al., 1994; Berg et al., 2002). Other drug combinations, such as 5-FU/ LV with platin analogue oxaliplatin (FOLFOX), or DNA topoisomerase I inhibitor irinotecan (FOLFIRI) have enhanced the therapeutic capability of 5-FU-LV treatment (Berg et al., 2002; Folprecht and Kohne, 2004; Vincenzi et al., 2004; Rose et al., 2002; Wilson et al., 2006; Davis et al., 1995).

The X-ray structure studies of TS ternary complexes from E. coli and human have been useful in designing novel TS-inhibitory drugs (Matthews et al., 1990; Montford et al., 1990; Schiffer et al., 1995). These approaches have given rise to delineation of the chemistry of the enzyme's active site, the interaction of enzyme and ligands, and the understanding of the structural basis for TS mutations that generate resistance to TS inhibitors (Almog et al., 2001; Phan et al., 2001; Tong et al., 1998; Fantz et al., 2000).

These efforts are accelerated by the availability of more sophisticated computational tools.

Cellular Response to TS Inhibitors

CRC is a malignant disease that results from accumulation of genetic and epigenetic alterations that lead to derangements in cell proliferation, and differentiation, giving rise to transformation of normal colonic epithelium to colon adenocarcinoma (Vogelstein et al., 1988). Many studies have shown that alterations in TS gene expression and structure are strong markers for the prediction of response to 5-FU-based chemotherapy in CRC (Popat et al., 2004; Popat et al., 2005; Pullarkat et al., 2001). TS overexpression can mediate drug resistance due to elevated mRNA and protein levels (Jenh et al., 1985; Berger et al., 1985). Sensitivity to TS-targeting drugs can be modified by several events including altered metabolism of 5-FU, diminished cellular uptake and polyglutamylation of anti-folates and elevated drug efflux (Gorlick and Bertino, 1999; Berg et al., 2002; Mader et al., 1998). It is essential to comprehend the multiple mechanisms of action of TS inhibitors in order to understand drug resistance.

There are other factors and mechanisms that can play roles in drug-based cytotoxicity following TS inhibition. These act downstream of the inhibition to regulate either apoptosis or DNA repair, and include apoptotic enzyme caspases (Backus et al., 2003), death receptors FAS and KILLER/DR5 (Petak et al., 2000; Wang and El-Deiry, 2004), tumor suppressor protein p53, cell-cycle kinase inhibitor p21 (Berg et al., 2002; Van Triest et al., 2000; Geller et al., 2004), the thymine glycosylase MBD-4 (Millar et

al., 2002; Sansom et al., 2003), SMUG1 glycosylase, (Wyatt and Wilson, 2009), and the mismatch repair protein MLH-1 (Meyers et al., 2005; Wyatt and Wilson, 2009).

It has been suggested that generation of reactive oxygen species (ROS) is one of the consequences of TS inhibition. ROS production is increased by 5-FU, and the inhibition of ROS reverses its cytotoxicity (Alexandre et al., 2006b, Hwang et al., 2007; Laurent et al., 2005; Hwang et al., 2001; Shibata et al., 2008; Ueta et al., 1999). As a result of drug treatment, ROS are released and partially trigger apoptosis in cancer cells (Gallego et al., 2008). Chemoresistance in these cells is induced by high levels of anti-oxidants (Ramanathan et al., 2005; Derdak et al., 2008). Accordingly, a significant strategy involves modulation of oxidative stress in cancer cells in order to sensitize them to anticancer drugs and generate novel protocols for improved clinical responses (O'Dwyer et al., 1996; Bougnoux et al., 2009).

A Biomarker of Cell Death Induced by TS Inhibitors: ROS

ROS is a common term that delineates a group of reactive chemical species that originate from the incomplete reduction of oxygen, including radical oxidants such as superoxide anion ($O_2^{\cdot -}$), the hydroxyl radical ($OH^{\cdot}$) and stable non-radical oxidants such as hydrogen peroxide (H_2O_2). They are formed by several different mechanisms. They can be an inevitable byproduct of cellular respiration in mitochondria due to electron leakage. They may be synthesized by devoted enzymes like NADPH oxidase, xanthine oxidase, myeloperoxidase, or through the transition metals such as iron (Fe) and copper (Cu). Finally, they can result from the interplay of biological molecules in response to ionizing radiation (Balaban et al., 2005; D'Autreaux and Toledano, 2007). ROS regulate various

signal transduction pathways through reaction with proteins, lipids, genes and transcription factors, resulting in modification of their structures and modulation of their functions (Birben et al., 2012; Trachootham et al., 2009). They also regulate intracellular signaling pathways mediated by cytokines, leading to removal of pathogens (Klebanoff, 2005). If ROS levels elevate severely, it might inexorably cause oxidative damage, following cell death. In contrast, slight elevation of ROS might lead to temporary cellular alteration (Perry et al., 2000). So, it is crucial to maintain ROS homeostasis in normal cell proliferation and survival.

ROS may function as a double-edged sword. A mild increase in ROS levels may promote cell growth and survival, whereas excessive ROS may overwhelm the antioxidant resistance of the cell (Schafer and Buettner, 2001). The shift between oxidants and antioxidant in response to excessive ROS is named “oxidative stress”, which contributes to several pathological diseases such as cancer, hypertension, diabetes, asthma (Valko et al., 2006; Kerr et al., 1999; Asami et al., 1997; Andreadis et al., 2003). It has been observed that diverse types of cancer cells have higher ROS levels than their normal counterparts (Szatrowski and Nathan, 1991; Kawanishi et al., 2006). Under physiological conditions, the balance between pro-oxidants (ROS production) and antioxidants (ROS detoxification) is controlled by maintaining basal level of ROS, sustaining redox homeostasis in normal cells (Tiligada, 2006). Tolerance to ROS stress can be provided by antioxidant system until it reaches to the cell-death threshold. Upregulation of ROS in cancer cells because of genetic instability and metabolic abnormalities could promote an adaptation to oxidative stress by inducing antioxidant mechanisms, keeping cells below the toxic threshold (Pelicano et al., 2004).

Consequently, antioxidant-targeting agents that produce further ROS makes cancer cells more dependent upon antioxidant capacity and more vulnerable to oxidative stress than normal cells. This causes ROS levels to increase above a cellular tolerability threshold, resulting in cell death (Figure I3) (Schumacker, 2006; Trachootham et al., 2009). Such vulnerability to oxidative stress represents an Achilles heel that offers potential strategies to direct anti-cancer agents.

The fate of cancer cells is thus bound up by the level of ROS induced by anticancer drugs. Upon genome damage, including that caused by TS inhibitors, cell cycle is arrested by the machinery called the checkpoint response (Zhou and Elledge, 2000). The response could be either repair of the damage or annihilation of the cell by apoptosis. Several studies have shown that genome damage induced by TS inhibitors, 5-FU and FdUrd, causes G1/S and G2/M arrest, and activates the checkpoint signaling enzymes like Chk1, ameliorating the DNA damage (Robinson et al., 2006; Xiao et al., 2006; Parsels et al., 2004). However, the checkpoint response can escalate the damage if it is excessive. Oxidative stress via TS inhibitors may lead to this checkpoint response. As such, the checkpoint response to TS inhibitors can occur in two opposite ways: cell survival through DNA repair and cell death via augmentation of genome damage induced by ROS. In both scenarios, a decline in the number of damaged cells occurs. Efficiency in treatment of TS inhibitors depends on the balance between these two responses.

As stated above, it has recently been shown that oxidative stress is a significant cellular response to anticancer drugs, including TS inhibitors (Alexandre et al., 2006b; Hwang et al., 2007; Laurent et al., 2005; Hwang et al., 2001). However, mechanisms by

which these drugs induce ROS generation in cancer cells are not well defined. It is known that the activity of ROS generating enzymes like NADPH oxidases is increased to kill cancer cells (Kumar et al., 2008; Lambeth, 2004).

NADPH Oxidases (NOX): A Potential Target of TS Inhibitors

The generation and accumulation of ROS can physiologically occur as a by-product of functioning or damaged mitochondria, by enzyme systems such as peroxisomal oxidases and lipoxygenases, and in response to ROS-producing environmental exposures or inflammatory conditions (Balaban et al., 2005; Schrader and Fahimi, 2004). In contrast, the family of NADPH oxidases (NOX) produce ROS as their primary and sole function (Bedard and Krause, 2007; Lambeth, 2004). This enzyme family occurs as multi-protein complexes consisting of a membrane-spanning catalytic NOX subunit and regulatory subunits that are localized in the cytosol and the membrane. The NOX family is comprised of seven isoforms: NOX1-5 and dual oxidases (Duox), Duox1 and Duox2 (Lambeth, 2004; Bedard and Krause, 2007; Altenhöfer et al., 2012).

All NOX family members are trans-membrane proteins that generate $O_2^{\cdot -}$ by transporting electrons (e^-) across the membrane to reduce oxygen (O_2). NOX4 appears to generate essentially H_2O_2 (Takac et al., 2011). Expression and regulation of NOX isoforms vary in tissues and are differentially localized in subcellular compartments (Brown and Griendling, 2009; Altenhöfer et al., 2012). It has been reported that all NOX isoforms bind to membrane and/or cytosolic proteins to mediate their activation. Among them, NOX1 and NOX2 subunits are the best characterized. Catalytic NOX1 and NOX2 subunits bind to a common protein, $p22^{phox}$ in the membrane, forming a membrane

complex termed flavocytochrome b_{558}. The flavocytochrome b_{558} complex of NOX1 binds to NOX activator 1 (NOXA1) and NOX organizer 1 (NOXO1), while the complex of NOX2 binds the respective subunits $p67^{phox}$ and $p47^{phox}$, as well as subunit $p40^{phox}$. Rac, a small GTPase, binds to NOXA1 and $p67^{phox}$ upon activation of enzymes (Hayes and Knaus, 2013; Wingler et al., 2011; Altenhöfer et al., 2012).

NOX2, also known the phagocyte NOX (neutrophils and macrophages) or $gp91^{phox}$, was the prototypical NADPH oxidase (Babior et al., 2002). In recent past, its biochemical properties have been comprehensively studied (Robinson et al., 2004; Vignais, 2002). Under normal conditions, NOX2 and $p22^{phox}$ are localized in the plasma membrane or the membrane of intracellular vesicles in neutrophils, existing in close association. Cytosolic regulatory subunits ($p40^{phox}$, $p47^{phox}$ and $p67^{phox}$) are triggered by signaling enzymes such as protein kinases and lipid-metabolizing enzymes. Phosphorylation by protein kinases results in conformational changes that cause the movement of cytosolic subunits $p40^{phox}$, $p47^{phox}$ and $p67^{phox}$ to the membrane, and binding to flavocytochrome b_{558}. Assembly of subunits involves binding of $p22^{phox}$ to the bis-SRC-homology 3 (SH3) domain of $p47^{phox}$ and of NOX2 to the activation domain (AD) of $p67^{phox}$. RAC also binds to the tricodecapeptide (TPR) domain of $p67^{phox}$ due to a conformational change. Assembly of all subunits brings about the activation of NOX2 catalytic function, involving the transportation of electrons from cytoplasmic NADPH to FAD, first and second heme groups, respectively and finally to extracellular or phagosomal oxygen to produce $O_2^{\cdot -}$ (Figure I4) (Hayes and Knaus, 2013; Bedard and Krause, 2007; Lambeth, 2004).

It has been suggested that human tumor cells generate their characteristically elevated ROS levels by NOX, indicating the contribution of this enzyme to carcinogenesis for various types of cancer (Kamata, 2009; Laurent et al., 2008; Lassegue and Griendling, 2010; Banfi et al., 2000). This contribution might be because of ROS-dependent signaling and promotion of cell growth. In colorectal cancer, ROS generation occurs within both colon epithelial cells themselves and derivation from NOX1 and NOX2 enzymes (Kikuchi et al., 2000; Perner et al., 2003). The excessive amount of ROS derived by NOX1 and NOX2 raises the question to which extent overactivation is balanced by the antioxidant capability of cells. It has been suggested that NOX2-derived ROS may have a significant role in the defense system against inflammatory colon cancer (McKenzie et al., 1996; Huang et al., 2004).

NOX1 and NOX2 are potentially important targets of TS inhibitors that induce ROS formation, since they are highly expressed in colon tissue (Hwang et al., 2001; Juhasz et al., 2009). For decades, TS inhibitors have been widely used in chemotherapy of colorectal cancer because of their strong cytotoxic impacts. Greater efficacy in causing apoptotic programmed cell death may be achieved by activating ROS-producing systems. Therefore, the NOX enzyme family by virtue of its ability to produce the excessive ROS in colon cancer cells may be an attractive target of TS inhibitors.

The primary aim of the studies presented in this work was to increase an understanding of the role of ROS in cell death induced by TS inhibitors. Particularly, the goal was to examine the origin and nature of ROS produced in response to TS inhibitors. The specific goals were to: (1) determine ROS generation in response to TS inhibitors,

(2) identify the source of ROS formed by TS inhibitors and (3) determine the role of ROS in cell death induced by TS inhibitors in human colon cancer cell lines.

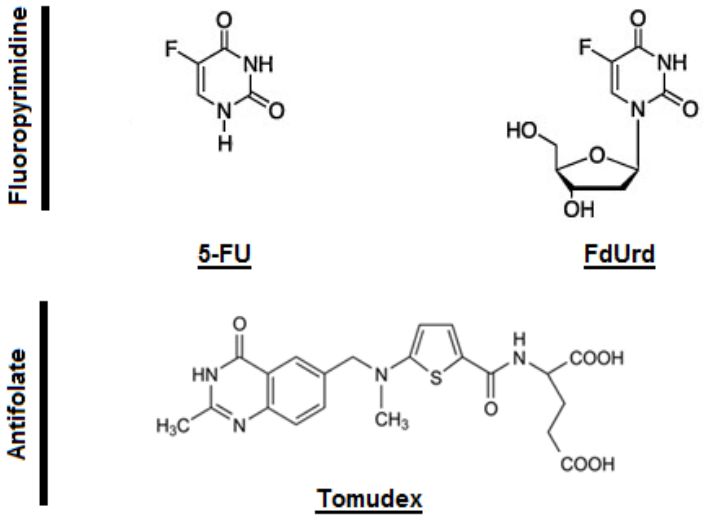

Figure I 1. **Chemical structure of TS inhibitors; 5-FU, FdUrd and RTX.**

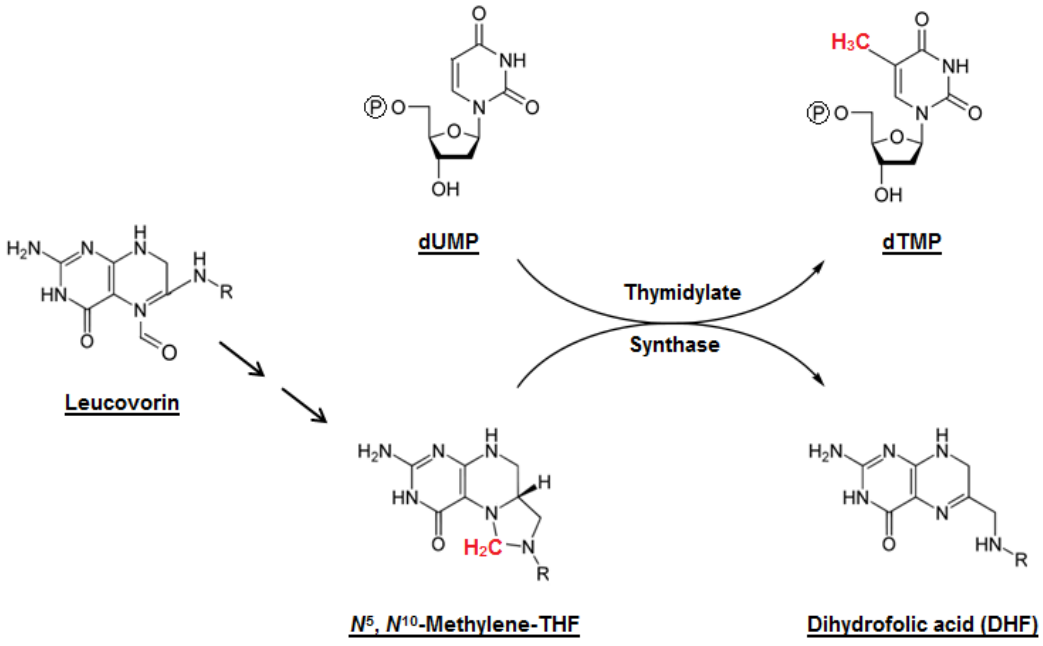

Figure I 2. **Thymidylate synthase (TS) reaction.** TS catalyzes transfer of a methyl group from N^5, N^{10}-methylenetetrahydrofolate (CH_2-THF) to deoxyuridylate monophosphate (dUMP) to form deoxythymidylate monophosphate (dTMP). Leucovorin is converted to CH_2-THF and enhances the interaction between FdUMP and TS.

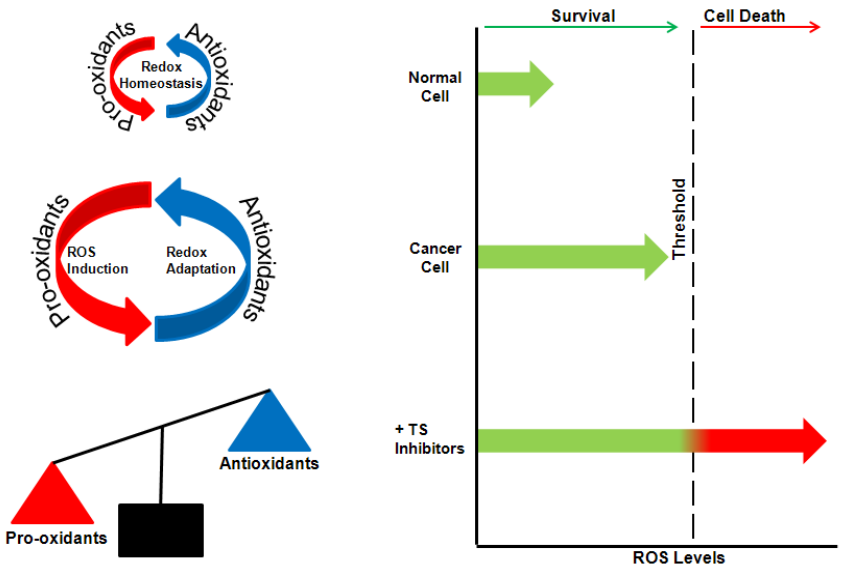

Figure I 3. **Redox homeostasis in normal and cancer cells.** Normal cells have a low level of basal ROS to sustain redox homeostasis by virtue of balance between pro-oxidants (ROS induction) and antioxidants (ROS elimination). In cancer cells, redox homeostasis is sustained with high pro-oxidants resulting from metabolic abnormalities and high antioxidants resulting from high levels of basal ROS, holding ROS levels below the toxic threshold. Further ROS increase by TS inhibitors results in an imbalance favoring pro-oxidants, thereby making cancer cells vulnerable to cell death.

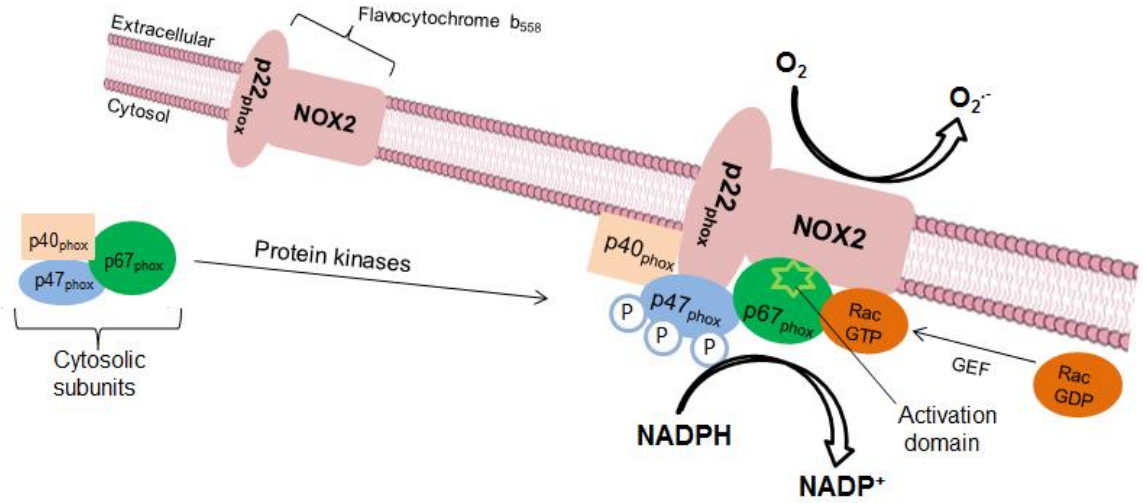

Figure I 4. **ROS production by assembly of NOX2 regulatory proteins.** Activation of NOX2 system mediated by protein kinases and guanine-nucleotide exchange factor (GEF) results in the assembly of flavocytochrome b_{558}, $p22^{phox}$ and NOX2, and cytosolic regulatory proteins, $p40^{phox}$, $p47^{phox}$, $p67^{phox}$ and RAC-GTP. Phosphorylation of $p47^{phox}$ allows it to bind $p22^{phox}$, leading to binding of $p67^{phox}$ to NOX2 and binding of $p40^{phox}$ to lipids. RAC binding to $p67^{phox}$ helps to assemble the active complex. The activation domain of $p67^{phox}$ induces e- transfer from NADPH to O_2 (the rate limiting step) to generate $O_2^{\cdot-}$.

CHAPTER 1

THE EFFECTS OF TS INHIBITORS ON ROS GENERATION

1.1. INTRODUCTION

Anticancer drugs including TS inhibitors kill cancer cells by disrupting genome stability and metabolic normality (Pelicano et al., 2004). In general, TS inhibitors lead to genome damage via dTMP depletion and loss of DNA synthesis (Longley et al., 2003; Santi et al., 1987). There are other cellular responses to drug-based cytotoxicity following TS inhibition, regulating either apoptosis or DNA repair (Backus et al., 2003, Van Triest et al., 2000; Wyatt and Wilson, 2009).

It has been previously demonstrated that oxidative stress is an important cellular response to TS inhibitors (Hwang et al., 2007; Laurent et al., 2005; Hwang et al., 2001). ROS have an essential role in regulation of diverse signal transduction pathways. They react with many molecules such as protein, lipids to modify their structures, resulting in alteration of their functions. Therefore, maintaining ROS homeostasis in normal cell survival is so important. It is known that cancer cells have more ROS than normal cells, making them vulnerable to oxidative stress (Trachootham et al., 2009).

It has been previously shown that 5-FU, in a p53-dependent manner, induces the mitochondrial enzyme ferredoxin reductase (FR) that transfers electrons from NADPH to cytochrome P450, resulting in the generation of ROS in mitochondria (Hwang et al., 2001; Liu and Chen, 2002). It has also been demonstrated that colorectal tumors have about 2-fold more FR expression than normal cells, indicating contribution of FR and oxidative stress to tumor development (Yu et al., 2003).

Another determinant of 5-FU-mediated oxidative stress is spermidine/spermine N^1-acetyltransferase (SSAT), which is a rate-limiting enzyme in polyamine catabolism that forms H_2O_2 as a by-product. 5-FU induces SSAT in a p53-dependent manner and acts synergistically with SSAT inducers like N^1, N^{11}-diethylnorspermine (DENSPM) to kill colon carcinoma cells. These synergistic effects are abolished by antioxidants (Allen et al., 2007; Choi et al., 2005).

Recently, a novel mitochondrial protein called reactive oxygen species modulator 1 (Romo1) was identified and shown to be highly expressed in various cancer cells (Chung et al., 2006). Following upregulation of Romo1 in cancer cells by 5-FU, ROS formation is elevated. This 5-FU-based induction of Romo1 is abolished by Romo1 siRNA (Hwang et al., 2007).

Overexpression of superoxide dismutase (SOD) increases H_2O_2 levels in human cancer cells. It has been demonstrated that two nonpeptidyl mimics of SOD, copper [II] diisopropylsalicylate (CuDIPS) and manganese [III] tetrakis-(5,10,15,20)-benzoic acid porphyrin (MnTBAP), enhance the cytotoxicity of anticancer drugs through augmentation of H_2O_2 levels (Laurent et al., 2005). In addition to these redox modulators, mangafodipir, a chelate of manganese [II] and the ligand fodipir, a vitamin B6 derivative, increases H_2O_2 generation via its SOD-, glutathione reductase- and catalase-like properties. It was shown that 5-FU-induced ROS production and glutathione depletion was elevated by CuDIPS, MnTBAP and mangafodipir in CT26 mouse colon cancer cells, and inhibited by N-acetylcysteine (NAC), an antioxidant that has catalase- and glutathione reductase-like effects on redox metabolism (Alexandre et al., 2006a).

Activation of manganese superoxide dismutase (Mn-SOD) by 5-FU was seen in squamous cell carcinoma (SCC) cells (OSC-1 to OSC-4). Furthermore, 5-FU induced the intracellular H_2O_2 and $O_2^{\cdot -}$ levels and decreased the mitochondrial membrane potential ($\Delta\psi_m$) in these cells, indicating action of 5-FU at multiple steps of redox metabolism (Ueta et al., 1999).

It was shown that 5-FU treatment induced mitochondrial oxidative stress in human colon cancer cell lines HT-29 and SW-620, by virtue of increased mitochondrial lipid peroxidation and protein carbonylation and decreased levels of uncoupling protein-2 (UCP2) and -5 (UCP5), mitochondrial ROS suppressors (Santandreu et al., 2011). Overall, accordingly, these observations have implied that oxidative stress has an important role in the cytotoxic response to TS inhibitors.

The mechanism that drives the accumulation of ROS by TS inhibitors is not well defined. It is of value to know the type of ROS induced by TS inhibitors and whether or not this induction is mediated by various TS inhibitors. Here, we demonstrate that TS inhibitors, 5-FU, FdUrd and RTX, induce generation of H_2O_2 and $O_2^{\cdot -}$, which is inhibited by the antioxidant NAC. We also demonstrated that the pro-oxidant aminotriazole (ATZ), a catalase inhibitor, induces H_2O_2 generation, but not apoptosis.

The best known action of TS inhibitors is the inhibition of TS enzyme. The dependency of ROS accumulation on TS inhibition is not known. Here, we demonstrate that exogenous supplement of thymidine inhibits TS inhibitor-mediated ROS formation, indicating the dependency of ROS induction on TS inhibition. In order to verify this

dependency, we also use TS-overexpressing cells (Berger et al., 1988) that are resistant to TS inhibitors.

1.2. RESULTS

ROS formation is induced by TS inhibitors

ROS are formed by a variety of mechanisms, including mitochondrial electron leakage, oxidative enzymes like NADPH oxidase, xanthine oxidase, metal (Fe, Cu) transition, etc. (Balaban et al., 2005; D'Autreaux and Toledano, 2007). Previous work has shown that 5-FU increases levels of ROS in various cancer cells by virtue of its capability to modulate expression of ROS-inducer proteins like Romo1, UCP2 and enzymes like FR, SSAT, MnSOD (Hwang et al., 2001; Yu et al., 2003; Allen et al., 2007; Hwang et al., 2007; Ueta et al., 1999; Santandreu et al., 2011). In order to substantiate results found in other laboratories, 5-FU and other two TS inhibitors, the fluoropyrimidine FdUrd and the antifolate RTX, were added to subconfluently plated HCT116 cells, for 24 hours, after which the cells were harvested and stained with ROS indicator dyes, H_2DCFDA (H_2O_2 marker) and DHE ($O_2^{\cdot -}$ marker). The 24h time point and 10μM drug concentration were selected due to the observed high levels of H_2O_2 in response to FdUrd (Figure 1.1 C).

Cells treated with TS inhibitors exhibited increased H_2O_2 formation, as indicated by green fluorescence resulting from the interaction of H_2DCFDA and H_2O_2. Also, the drug induced $O_2^{\cdot -}$ formation, as indicated by red fluorescence resulting from the interaction of DHE and $O_2^{\cdot -}$ (Figure 1.1 A). In order to verify these findings, cells stained by H_2DCFDA and DHE were assayed by flow cytometry. As observed in fluorescence

microscopy, cells treated with drugs exhibited a shift to the right, indicating increased H_2O_2 and $O_2^{\cdot -}$ generation, thereby implicating TS inhibitors as a ROS inducer in colon cancer cells (Figure 1.1 A).

In order to modify intracellular redox status, cells were treated with NAC for 24 hours, and analyzed by fluorescence microscopy and flow cytometry. In presence of NAC, TS inhibitors have no effect on H_2O_2 and $O_2^{\cdot -}$ generation, indicating the inhibition of ROS production by NAC (Figure 1.1 B). We conclude that all three drugs upregulated both H_2O_2 and $O_2^{\cdot -}$ formation in HCT116 cells, and that this upregulation was ameliorated by NAC (Figure 1.1 A and B). Cells were also treated with ATZ, a catalase inhibitor that increases H_2O_2 levels, for 24 hours. Fluorescence microscopy and flow cytometry assays were used to analyze H_2O_2 generation. Combination of ATZ to FdUrd has no effects on H_2O_2 generation, meaning that both ATZ and FdUrd may induce H_2O_2 generation from the same source in HCT116 cells (Figure 1.5 A).

ROS generation is due to TS inhibition

TS is the sole source of dTMP whose subsequent phosphorylation provides dTTP, a precursor for DNA synthesis (Carreras and Santi, 1995). Inhibition of TS enzyme abolishes dTMP production and results in genome damage by virtue of misincorporation of uracil into DNA, promoting apoptosis and loss of DNA synthesis (Danenberg, 1977; Spears et al., 1988; Krokan et al., 2002; Longley et al., 2003). In presence of exogenous thymidine, dependency on TS diminishes, decreasing the cytotoxic effects of TS-directed drugs. In order to delineate the role of TS inhibition on ROS generation, thymidine was supplemented to HCT116 cells for 24 hours. Cells treated with thymidine exhibited no

induction of either H_2O_2 and $O_2^{\cdot -}$ following treatment with in combination of 5-FU, FdUrd and RTX compared to cells treated with drugs alone (Figure 1.2 A and B).

To extend this conclusion, we utilized FdUrd-resistant cell line HCT116/200 that overproduces a variant structural form of TS, causing decreased affinity for FdUMP and CH_4-THF. HCT116/200 cells are treated with 5-FU, FdUrd and RTX for 24 hours. They showed only about two fold induction of ROS formation in treatment of drugs, thereby implicating TS inhibition as important in ROS production mediated by TS inhibitors (Figure 1.3 A and B).

The role of ROS in drug-mediated apoptosis

Previous work from our lab demonstrated that the apoptotic response to increasing concentrations of TS inhibitors never exceeds 10-25% in HCT116 cells (Barbour and Berger, 2008). Mechanisms of cell death following dTMP deprivation in cells treated with TS inhibitors have been studied for decades. A number of such studies have focused on signaling pathways such as the FAS pathway with caspase activation (Longley et al., 2003; Petak et al., 2000); however, others have examined pathways that are caspase-independent (Fiers et al., 1999; Broker et al., 2005). In order to determine direct role of ROS augmentation on cell death, HCT116 cells were treated with 5-FU, FdUrd or RTX, with and without NAC for 48 hours, and apoptotic indices were determined by TUNEL assay. From each treatment, pictures were taken of representative areas of the cultures and at least 500 cells both apoptotic (dark brown) and non-apoptotic (purple) were counted (Figure 1.4 A). NAC inhibited drug-mediated apoptosis by more than 50% compared to cells treated with drugs alone, thereby implicating a contribution

of ROS accumulation to apoptosis (Figure 1.4 B). Cells were also treated with TS inhibitors, with and without ATZ for 48 hours to assay apoptotic response by TUNEL assay. ATZ slightly increased apoptosis itself; however, it has no effect on drug-mediated apoptosis, thereby implicating that ROS generation by itself does not induce apoptosis (Figure 1.5 B). Some other consequence of exposure to TS inhibitors, in addition to ROS, is necessary.

1.3. DISCUSSION

The current data shows that TS inhibitors, fluoropyrimidine 5-FU, FdUrd and antifolate RTX increase the levels of ROS, as indicated by DCF (H_2O_2) and HE fluorescence ($O_2^{\cdot -}$), in colon cancer cells (Figure 1.1 A). This drug-mediated ROS augmentation was abrogated with a general antioxidant, NAC, confirming oxidative stress caused by TS inhibitors (Figure 1.1 B). Supplementation of thymidine abolished ROS formation induced by TS inhibitors, showing the relation between TS inhibition and ROS induction. This suggests that dTMP depletion by TS inhibition brings about oxidative stress that is counteracted by thymidine addition. Furthermore, fluoropyrimidine-resistant HCT116/200 cells, overexpressing TS protein, produce only moderate increases in ROS following with TS inhibitors. This resistance decreases drug-mediated oxidative stress, indicating a strong connection between oxidative stress and TS inhibition.

Previous work from our lab demonstrated that apoptosis induced by TS inhibitors reaches only 10-25% in HCT116 cells even they are treated with drug concentrations far in excess levels (Barbour and Berger, 2008). Our results indicate that apoptosis mediated by TS inhibitors is diminished above 50% by NAC, thereby implicating importance of

oxidative stress in drug-mediated cell death. Additionally, apoptosis is not induced by ATZ either alone or in combination with TS inhibitors. These results show that ROS is necessary for apoptosis following drug exposure, but it is not sufficient to kill cells.

In summary, my data suggests that TS inhibitors induce ROS generation in a TS dependent manner and that this induction sensitizes cells to apoptotic death. This gives a general indication about how drug-mediated ROS induction takes place. In order to understand the mechanism behind the induction, it is necessary to determine the source of ROS source, as affected by TS inhibitors.

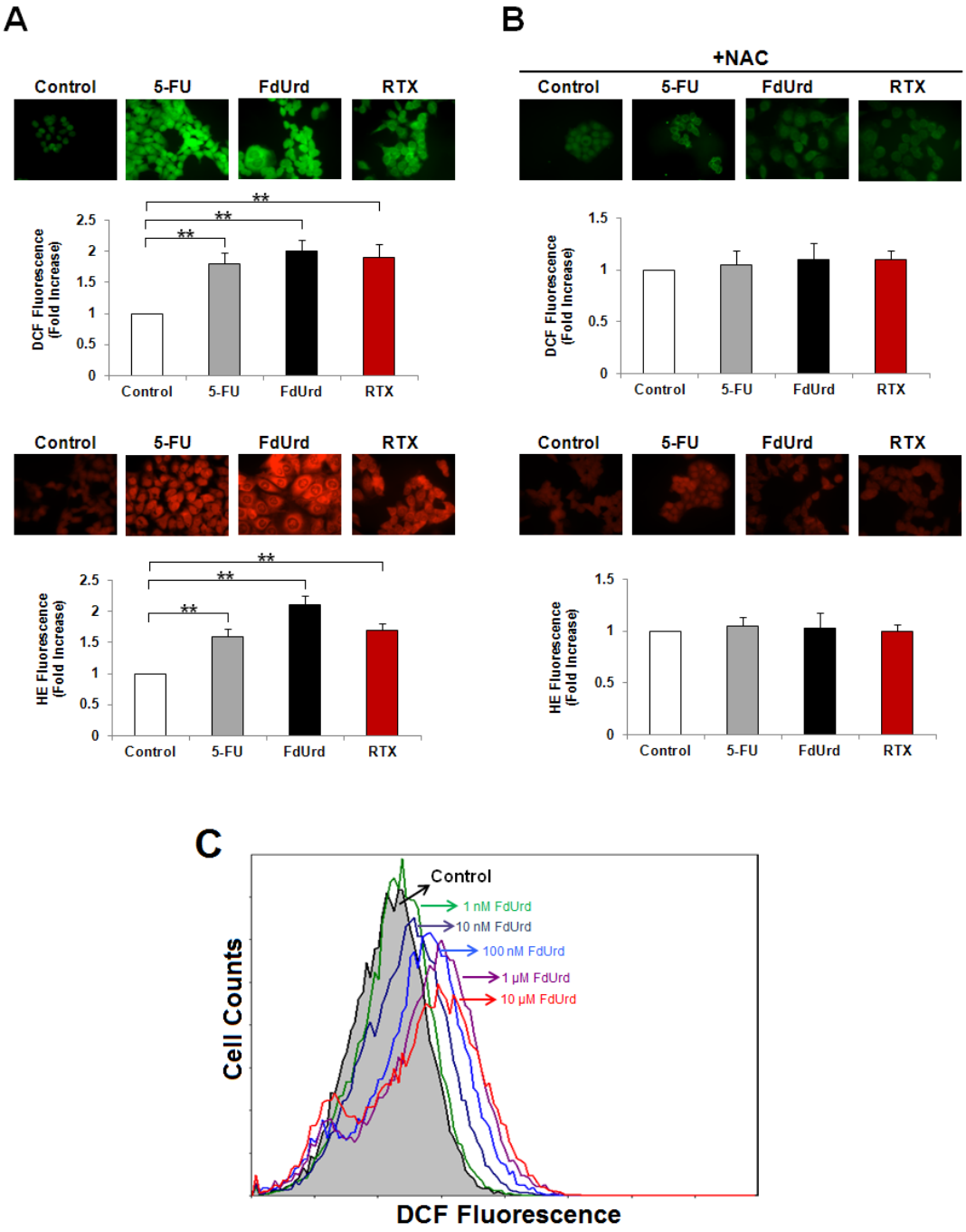

Figure 1.1. **NAC inhibits ROS generation mediated by TS inhibitors.** (A) HCT116 cells were treated with 10µM FdUrd, 10µM 5-FU and 1µM RTX for 24 hours and then stained by 5µM H2DCF-DA and DHE. ROS detection was done in both fluorescence microscopy and flow cytometry. Pictures represent H_2O_2 formation (green) and $O_2^{\cdot -}$ (red). (B) 1mM NAC is combined with TS inhibitors and treated to cells for 24h. (C) HCT116 cells were treated with increasing numbers of FdUrd concentrations, 1nM, 10nM, 100nM, 1µM, 10µM and then stained by 5µM H2DCF-DA to measure dose response by flow cytometry. Bars represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (HE fluorescence) formation ± SEM from 3 experiments (** $p<0.01$).

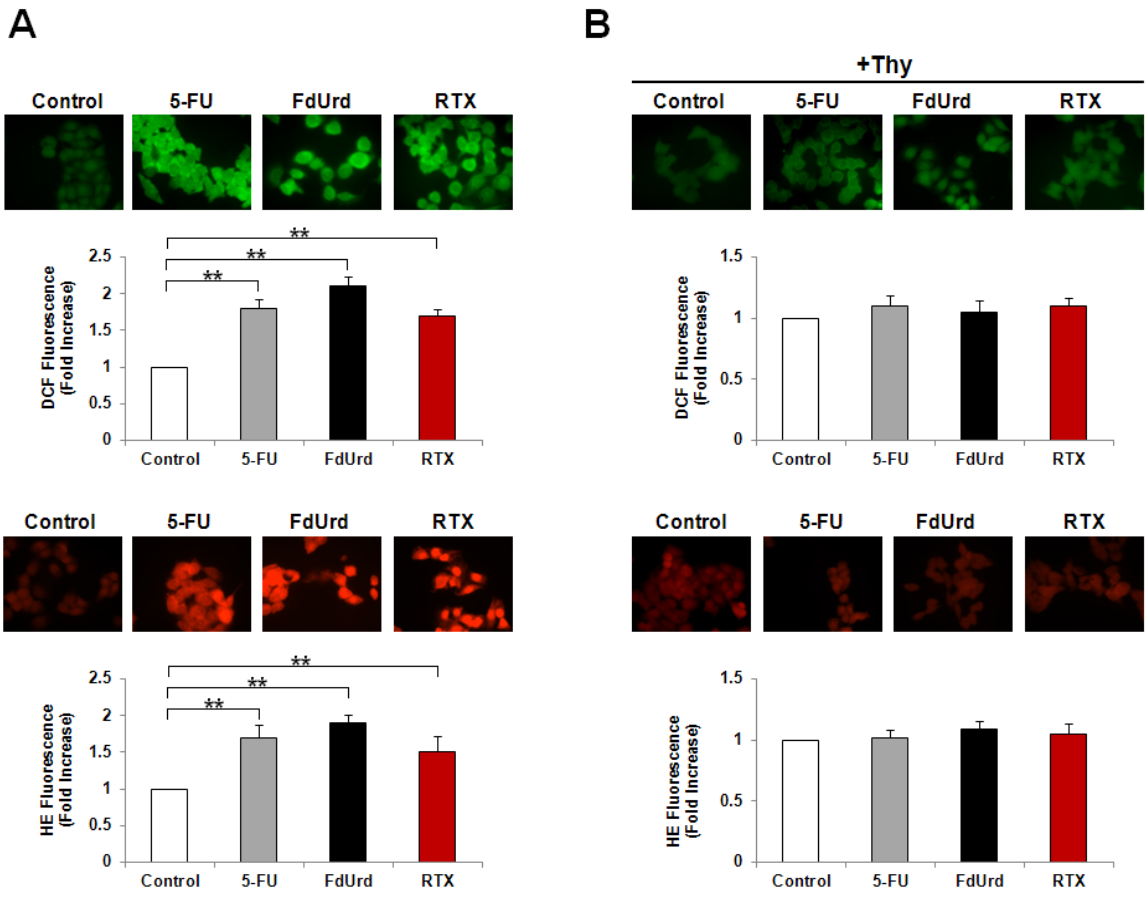

Figure 1.2. **Rescue of drug-induced ROS by thymidine.** (A) HCT116 cells were treated with 10μM FdUrd, 10μM 5-FU and 1μM RTX for 24 hours and then stained by 5μM H2DCF-DA and DHE. (B) 10μM Thy is combined with TS inhibitors and treated to cells for 24h. Bars represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (HE fluorescence) formation ± SEM from 3 experiments (** $p<0.01$)

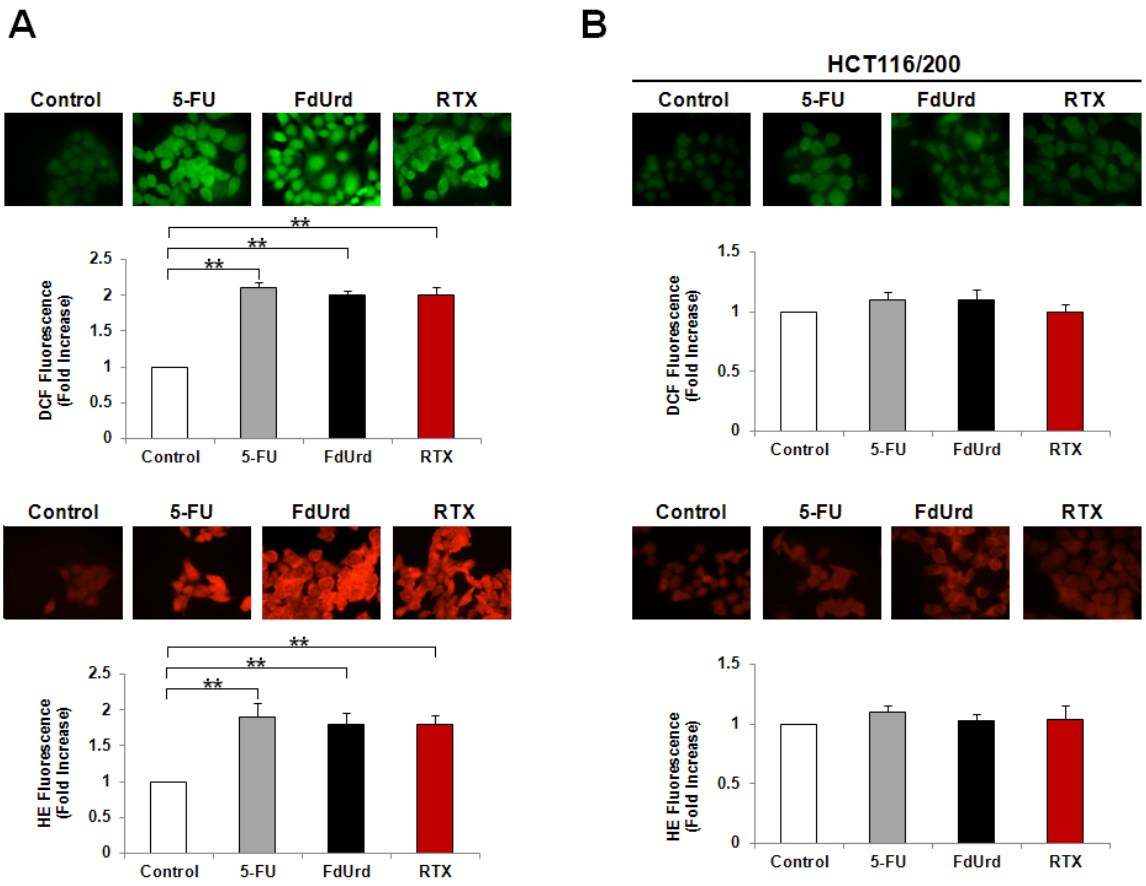

Figure 1.3. **Rescue of drug-induced ROS by TS overproduction.** (A) HCT116 cells were treated with 10μM FdUrd, 10μM 5-FU and 1μM RTX for 24 hours and then stained by 5μM H2DCF-DA and DHE. (B) HCT116/200 cells were treated with TS inhibitors for 24h. Bars represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (HE fluorescence) formation ± SEM from 3 experiments (** $p<0.01$)

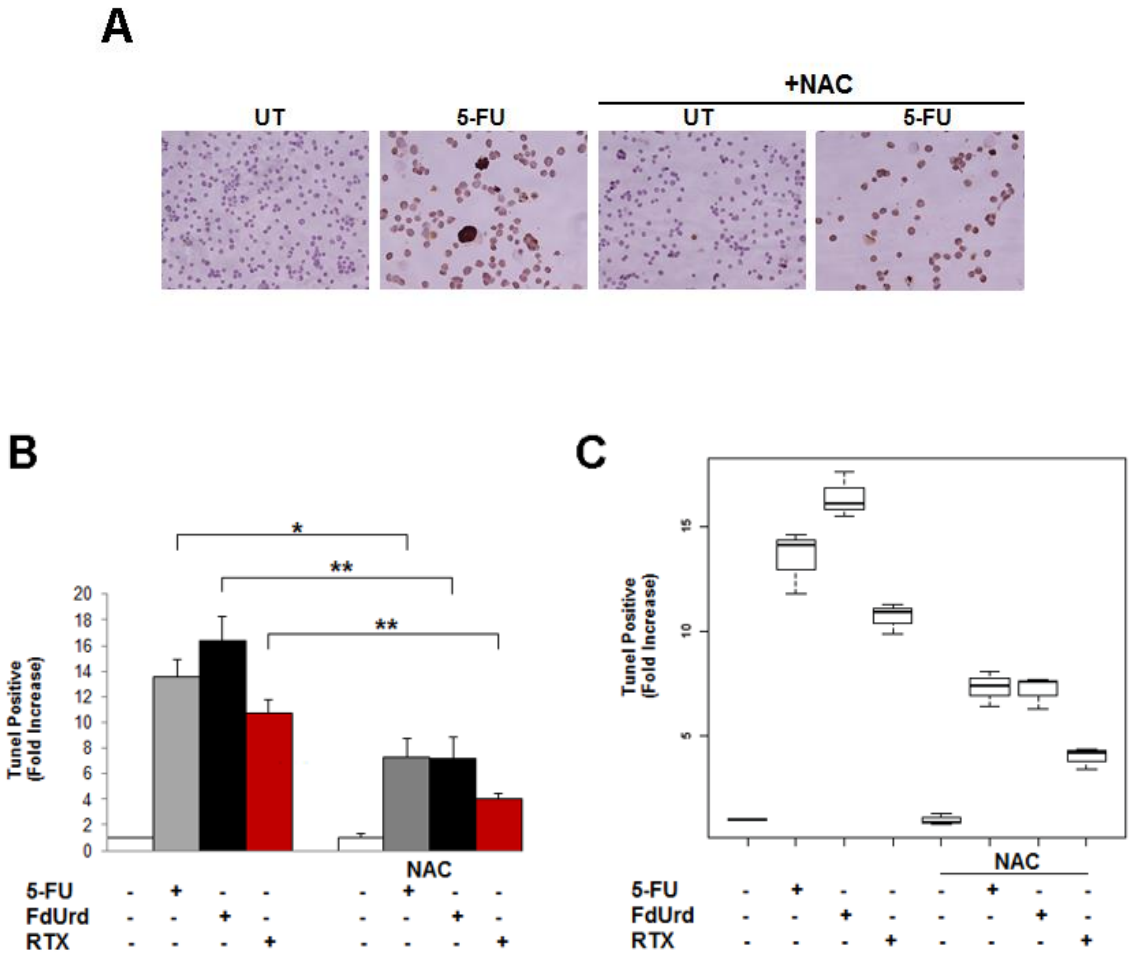

Figure 1.4. **Effects of NAC on drug-induced cell death.** (A) HCT116 cells were treated with 1mM NAC and 10μM 5-FU for 48 hours and then extents of apoptosis were determined by TUNEL assay. Pictures for with and without (UT) 5-FU treatment and its combination with 1mM NAC were taken under light microscopy at 400X. (B, C) 1mM NAC combined with 10μM 5-FU, 100μM FdUrd and 1μM RTX treated to cells for 48 hours. Bars represent fold increase of apoptotic indices ± SEM from 3 experiments (* $p<0.05$, ** $p<0.01$).

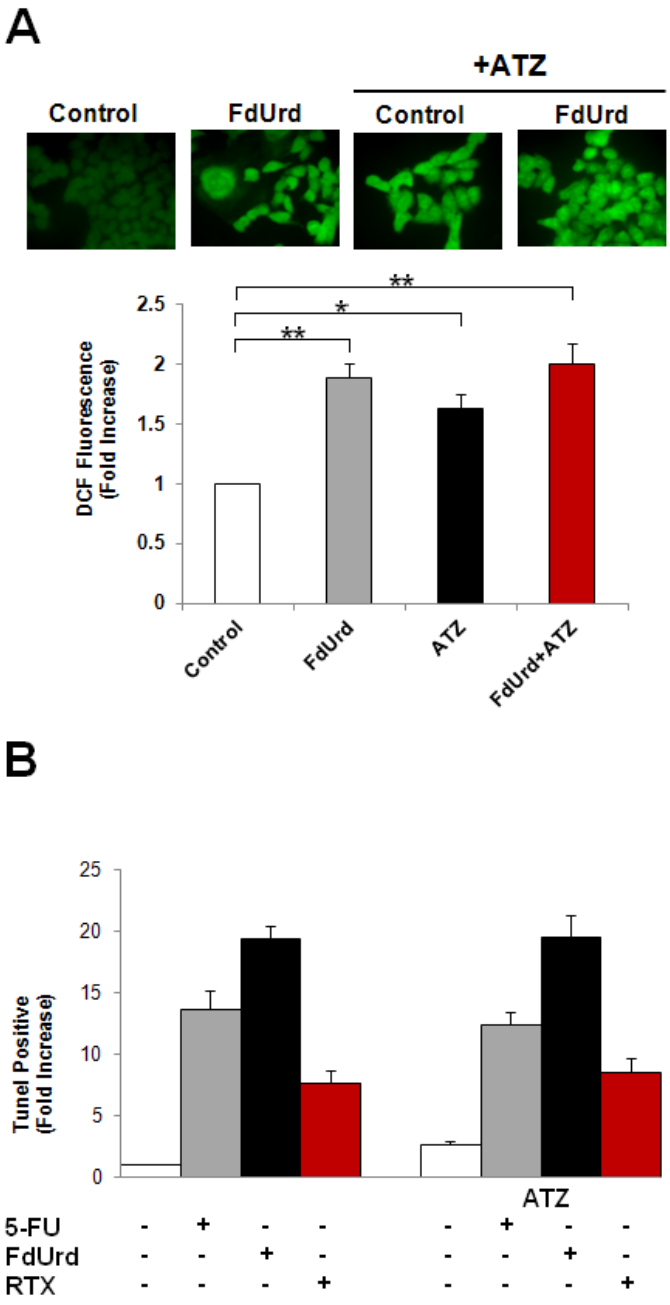

Figure 1.5. **ATZ induces H_2O_2 generation, but not apoptosis.** (A) HCT116 cells were treated with 10μM FdUrd and 1mM ATZ for 24 hours and then stained by 5μM H2DCF-DA. ROS detection was done in both fluorescence microscopy and flow cytometry. Pictures represent H_2O_2 formation (green). Bars represent fold increase of H_2O_2 (DCF fluorescence) formation ± SEM from 3 experiments (* $p<0.05$, ** $p<0.01$). (B) HCT116 cells were treated in combination of 1mM ATZ with 10μM 5-FU, 100μM FdUrd and 1μM RTX for 48 hours and then extents of apoptosis were determined by TUNEL assay. Bars represent fold increase of apoptotic indices ± SEM from 3 experiments.

CHAPTER 2

ROS INDUCED BY TS INHIBITORS IS ASSOCIATED WITH UPREGULATION OF P67^{PHOX} SUBUNIT OF NADPH OXIDASE 2

2.1. INTRODUCTION

In TS-directed therapy, the origin and regulation of ROS is not known (Hwang et al., 2001; Liu and Chen, 2002; Yu et al., 2003; Allen et al., 2007; Choi et al., 2005; Chung et al., 2006; Hwang et al., 2007; and Ueta et al., 1999). Work described in Chapter 1 revealed that NAC inhbits ROS generation mediated by TS inhibitors, but the origin of ROS in response to TS inhibitors was not determined. Here, we show that the enzyme NADPH oxidase (NOX) generate profound amount of ROS in response to TS inhibitors in human colon cancer cells through an increase in NOX enzyme activity.

Expression of NOX isoforms is diverse in tissues (Brown and Griendling, 2009; Altenhöfer et al., 2012). NOX1 and NOX2 could be important sources of ROS in response to TS inhibitors since they are highly expressed in colon tissue (Hwang et al., 2001; Juhasz et al., 2009). Here, we demonstrate expression of NOX1 and NOX2 subunits in human colon cancer cell, HCT116. Among all NOX regulatory subunits, $p67^{phox}$ is the only one whose expression is increased by TS inhibitors, thereby indicating that it may be a key subunit in activation of NOX2 in HCT116 cells following exposure to TS inhibitors.

$p67^{phox}$ has an activation domain whose functional role is to interact with the membrane-bound NOX2 subunit. Because of its significant role in activation, it is of interest to examine whether it is responsible for ROS induction induced by TS inhibitors. Here, we determined that NAC and NOX inhibitors abrogated the induction of $p67^{phox}$ mRNA and protein levels, indicating the role of $p67^{phox}$ in drug-directed ROS generation.

Furthermore, we show that the induction of mRNA and protein levels of $p67^{phox}$ was abolished by TS overproduction or thymidine supplementation.

2.2. RESULTS

NOX inhibitors abolish ROS induction mediated by TS inhibitors

The sole function of NOX is formation of superoxide (Bedard and Krause, 2007; Lambeth, 2004). It is suggested as a major source of ROS in human cancer cells (Kikuchi et al., 2000; Perner et al., 2003); however, it is not known if it is a source of ROS in TS-targeted therapy. To test whether NOX is involved in ROS generation in response to TS inhibitors, we utilized pharmacologic NOX inhibitors, DPI, APO and VAS-2870. By using fluorescence microscopy, we found that elevated levels of H_2O_2 in response to 5-FU, FdUrd and RTX are abrogated by NOX inhibitors in HCT116 cells (Figure 2.1, 2.2 and 2.3 upper panel).

NOX inhibitors also reduce induction of $O_2^{\cdot -}$ formation by 5-FU, FdUrd and RTX in HCT116 cells. Findings obtained from fluorescence microscopy were also verified by flow cytometry (Figure 2.1, 2.2 and 2.3 lower panel).

NOX activity induced by TS inhibitors is reduced by NOX inhibitors, addition of thymidine and TS-overproduction

After seeing the modulation of TS inhibitor-mediated ROS by NOX inhibitors, the question of how do TS inhibitors mediate NOX-derived ROS was raised. To answer this question, we tested whether TS inhibitors induce NOX enzyme activity. We utilized a

chemiluminescence assay to measure NOX activity in cells exposed to TS inhibitors. This activity assay does not distinguish NOX isoforms, meaning that all NOX isoforms are responsible for measured activity levels. First, we used 5-FU and FdUrd to test whether they activate the NOX enzyme. Following treatment with 5-FU and FdUrd, NOX activity increased 5-6 folds in HCT116 cells; this increase was attenuated by DPI treatment, showing a significant effect of TS inhibitors on NOX activity (Figure 2.4).

We next tested whether thymidine addition and TS overproduction modulate the activation of NOX activity mediated by TS inhibitors. NOX activation by 5-FU and FdUrd was decreased by thymidine addition and TS overproduction, thereby indicating that the increase in NOX activity is due to TS inhibition (Figure 2.5).

In order to understand whether this activation of NOX activity occurs in cell lines other than HCT116, we examine of NOX activity in response to 5-FU and FdUrd in human colon cancer cell lines; SW480 and HCT15. Drug-induced NOX activation was about the same for all 3 colon cancer lines; furthermore, the activation of NOX in all 3 cell types was diminished by specific NOX inhibitor, VAS-2870, thereby implicating NOX as a main driver of ROS formation in response to TS inhibitors in human colon cancer cells (Figure 2.6).

NOX inhibitors decrease cell death in response to TS inhibitors

As seen in Chapter 1, apoptotic indices by TS inhibitors are abrogated by NAC, showing involvement of ROS in drug-mediated apoptosis. In order to determine whether NOX inhibitors cause an effect on apoptosis induced by TS inhibitors, we treated HCT116 cells

with DPI, APO and VAS-2870. As seen in Figure 2.7, DPI and APO abrogated cell death in response to 5-FU, FdUrd and RTX by >80% ($p<0.01$). The specific NOX inhibitor VAS-2870 decreased drug derived-apoptotic indices by about 50% ($p<0.05$), thereby showing the contribution of NOX enzyme to apoptosis induced by TS inhibitors (Figure 2.7). Decrease in apoptotic indices mediated by TS inhibitors is more in treatment of DPI and APO than that of VAS, implicating that there are other enzymes mediating drug-induced apoptosis in addition to NOX enzyme.

Association of $p67^{phox}$ with NOX-derived ROS formation in response to TS inhibitors

The NOX enzyme family comprises multi-protein complexes consisting of a membrane-spanning catalytic NOX subunit and regulatory subunits localized in both the cytosol and the membrane. There are 7 members of the family: NOX1-5 and dual oxidases (Duox), Duox1 and Duox2 (Lambeth, 2004; Bedard and Krause, 2007; Altenhofer et al., 2012).

It has shown that expression of NOX isoforms varies in tissues (Brown and Griendling, 2009; Altenhöfer et al., 2012). In colon tissue, NOX1 and NOX2 are highly expressed and may be important sources of oxidative stress following exposure to TS inhibitors (Hwang et al., 2001; Juhasz et al., 2009). We tested whether NOX1 and NOX2 are targeted by TS inhibitors. To test this, HCT116 cells were treated with 5-FU and microarray analysis was done to measure expression of various NOX1 and NOX2 subunits. We have found that about 3000 genes are differentially expressed in response to 5-FU. Among them, there are genes that are targeted by nuclear factor (erythroid-derived 2)-like 2 (Nrf2) whose antioxidant response is the major defense system against oxidative

stress in human cells (Table 2.1). With the exception of $p67^{phox}$, expression of all NOX1 and NOX2 subunits were unchanged by 5-FU (Figure 2.8). Similarly, mRNA levels detected semi-quantitatively of all NOX1 and NOX2 subunits, except $p67^{phox}$, were unaltered by FdUrd treatment (Figure 2.9). Among all subunits, only mRNA levels of $p67^{phox}$ was induced by about 28-fold ($p<0.01$) (Figure 2.10). Thus, $p67^{phox}$ subunit may be a key regulator of NOX enzyme in response to FdUrd.

In order to determine if $p67^{phox}$ plays a role in NOX-derived ROS formation in response to TS inhibitors, we treated HCT116 cells with FdUrd and NOX inhibitors and determined mRNA levels of $p67^{phox}$ by quantitative PCR. As expected, mRNA levels of $p67^{phox}$ induced by FdUrd were decreased by DPI, VAS-2870 and NAC, suggesting a correlation between $p67^{phox}$ and NOX derived-ROS formation in response to FdUrd (Figure 2.11 A).

In order to determine whether the induction of $p67^{phox}$ by FdUrd depends upon TS, we added thymidine to growth medium and also used TS-overproducing cells. Consistent with our previous results, thymidine addition and TS overproduction attenuated $p67^{phox}$ induction (Figure 2.12 A). Consistent with mRNA levels, we also measured protein levels of $p67^{phox}$ following FdUrd. NOX protein levels were increased; this increase was markedly reduced by NAC, DPI, VAS-2870 and thymidine addition (Figure 2.11 C and 2.12 C). Taken together, these results implicate NOX2 as a major source and $p67^{phox}$ as a key regulator of ROS generation in response to TS inhibitors. Activation of NOX enzyme and induction of $p67^{phox}$ in treatment of TS inhibitors depend

on TS inhibition. This shows a correlation of NOX enzyme and $p67^{phox}$ in drug-induced ROS generation.

2.3. DISCUSSION

Oxidative stress is an important cellular response to TS inhibitors (Hwang et al., 2001; Laurent et al., 2005; Alexandre et al., 2006b; Hwang et al., 2007). As indicated in Chapter 1, TS inhibitors increase ROS levels in HCT116 colon cancer cells and this increase is associated with thymidine stress and cell death. However, the origin and nature of ROS generation induced by TS inhibitors is unknown. NAC is a general antioxidant, but provides no information on the source of ROS.

Here, we demonstrate that NOX inhibitors attenuate the induction of ROS mediated by TS inhibitors, and decrease the activation of NOX enzyme induced by TS inhibitors, implicating NOX as a major source of ROS production in response to TS inhibitors. The activation of NOX enzyme in response to TS inhibitors showed the same trend in 3 different human colon cancer cell lines, HCT116, HCT15 and SW480, thereby implicating the common activation spectrum in distinct colon cancer cells. Additionally, the induction of apoptosis by TS inhibitors is inhibited by NOX inhibitors, showing the role of NOX in ROS-derived cell death.

We examined gene expression of NOX1 and NOX2 subunits in response to 5-FU and found that the expression of $p67^{phox}$ is induced by 5-FU. Similarly, the mRNA level of $p67^{phox}$ was induced in response to FdUrd.

$p67^{phox}$ is the cytosolic subunit of the NOX2 enzyme and, during activation of the enzyme, it interacts with the NOX2 subunit in the membrane via its functional activation domain (Lambeth, 2004). We have shown that the activation of NOX by TS inhibitors correlates with the induction of $p67^{phox}$ expression. The correlation between the inhibition of drug-induced ROS and $p67^{phox}$ mRNA and protein by NAC and NOX inhibitors suggests the role of $p67^{phox}$ in ROS formation in response to TS inhibitors. Examination of mRNA levels of $p67^{phox}$ in addition of thymidine and TS overproduction also indicate a possible role for $p67^{phox}$ in TS-directed therapy.

This study is the first to reveal the role of NOX2 with its cytosolic subunit $p67^{phox}$ in ROS generation and in cell death mediated by TS inhibitors in HCT116 cells. Although further studies are necessary, NOX enzyme may be a novel source in the field of cancer chemotherapy.

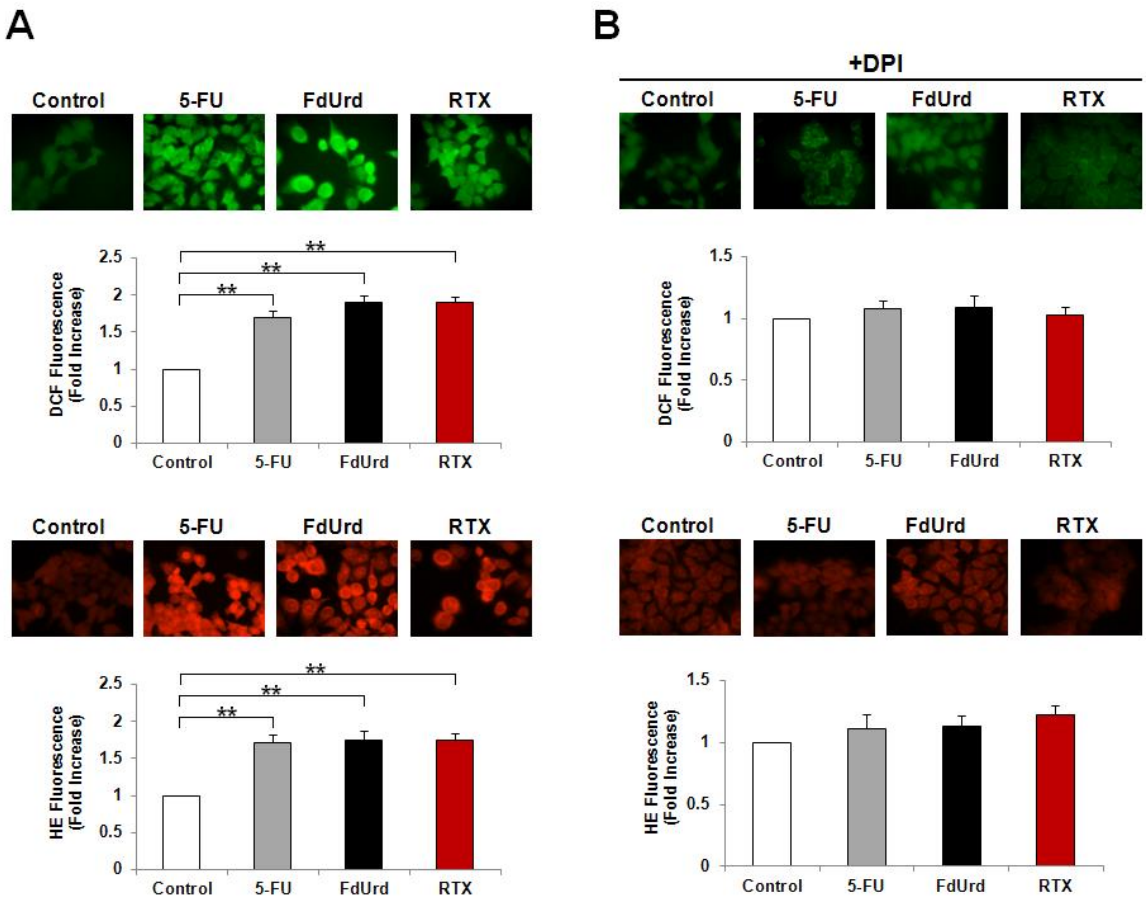

Figure 2.1. **DPI abrogates ROS generation induced by TS inhibitors.** (A) HCT116 cells were treated with 10µM FdUrd, 10µM 5-FU and 1µM RTX for 24 hours and then stained by 5µM H2DCF-DA and DHE. (B) 10µM DPI is combined with TS inhibitors and treated to cells for 24h. Bars represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (HE fluorescence) formation ± SEM from 3 experiments (** $p<0.01$)

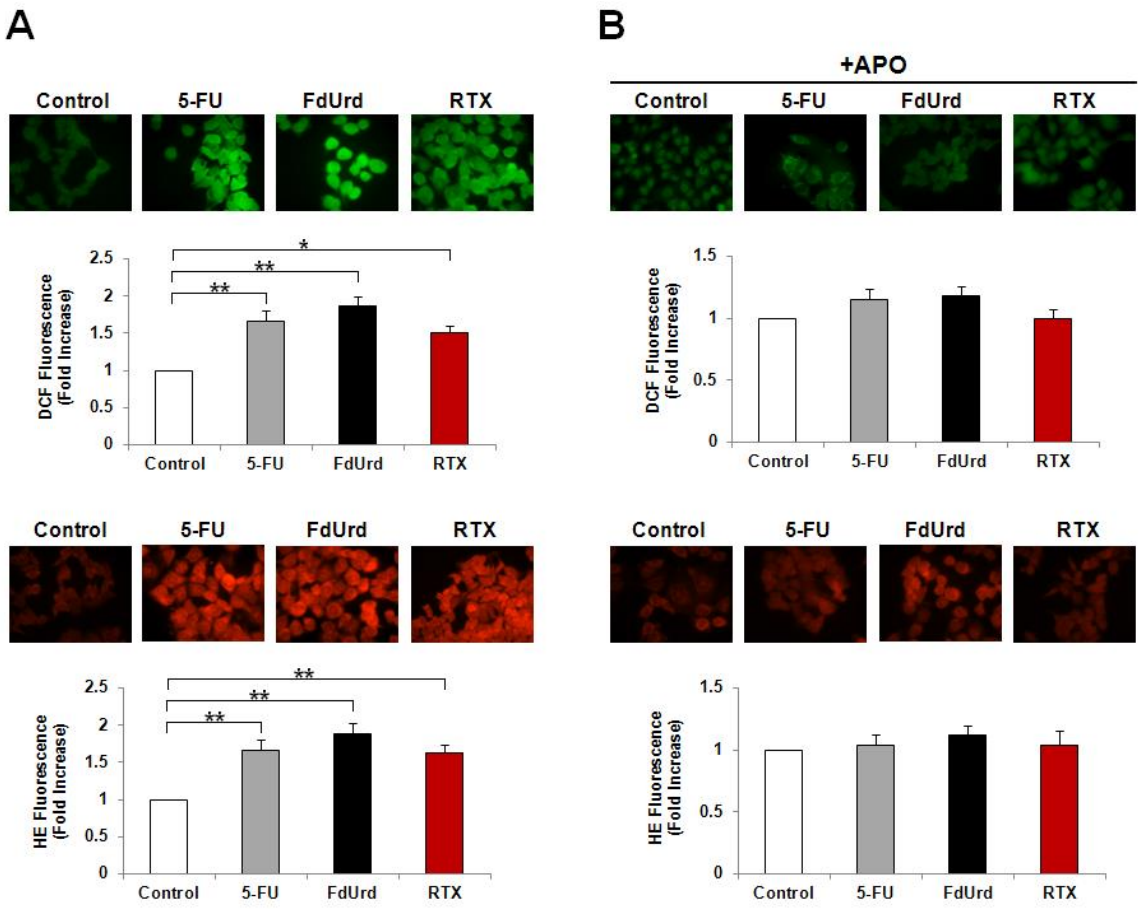

Figure 2.2. **APO reduces ROS generation mediated by TS inhibitors.** (A) HCT116 cells were treated with 10μM FdUrd, 10μM 5-FU and 1μM RTX for 24 hours and then stained by 5μM H2DCF-DA and DHE. (B) 1mM APO is combined with TS inhibitors and treated to cells for 24h. Bars represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (HE fluorescence) formation ± SEM from 3 experiments (* $p<0.05$, ** $p<0.01$)

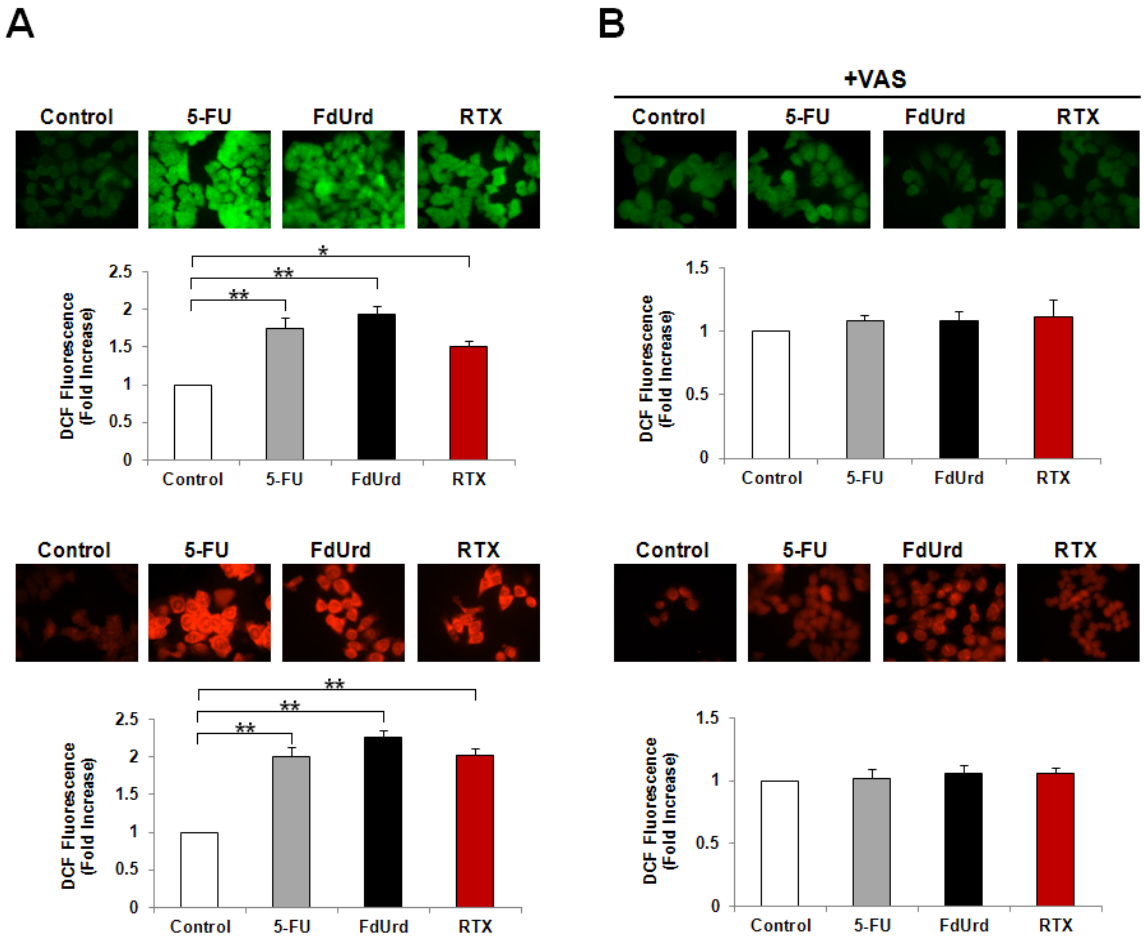

Figure 2.3. **VAS decreases ROS generation induced by TS inhibitors.** (A) HCT116 cells were treated with 10μM FdUrd, 10μM 5-FU and 1μM RTX for 24 hours and then stained by 5μM H2DCF-DA and DHE. (B) 10μM VAS is combined with TS inhibitors and treated to cells for 24h. Bars represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (HE fluorescence) formation ± SEM from 3 experiments (* $p<0.05$, ** $p<0.01$).

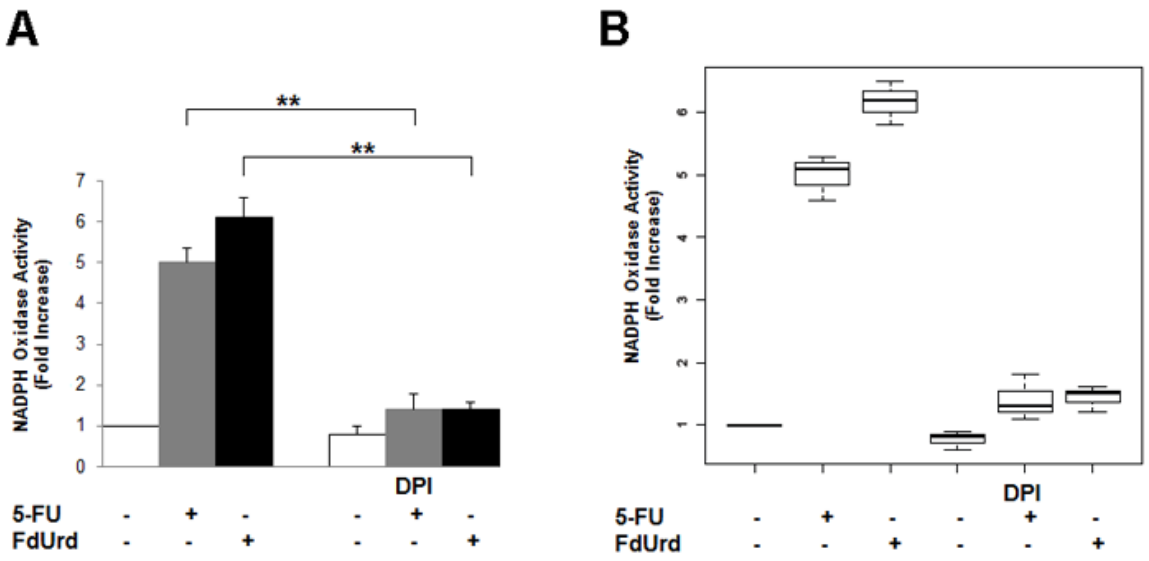

Figure 2.4. **DPI decreases NOX activity increased by TS inhibitors.** (A, B) HCT116 cells were treated with 10μM 5-FU and 10μM FdUrd for 24 hours and then were subjected to NOX activity assay as described in "materials and methods". 10μM DPI was added to reaction buffer. Lucigenin derived chemiluminescence was measured by luminometer. Bars represent fold increase of chemiluminescence ± SEM from 3 experiments (** $p<0.01$).

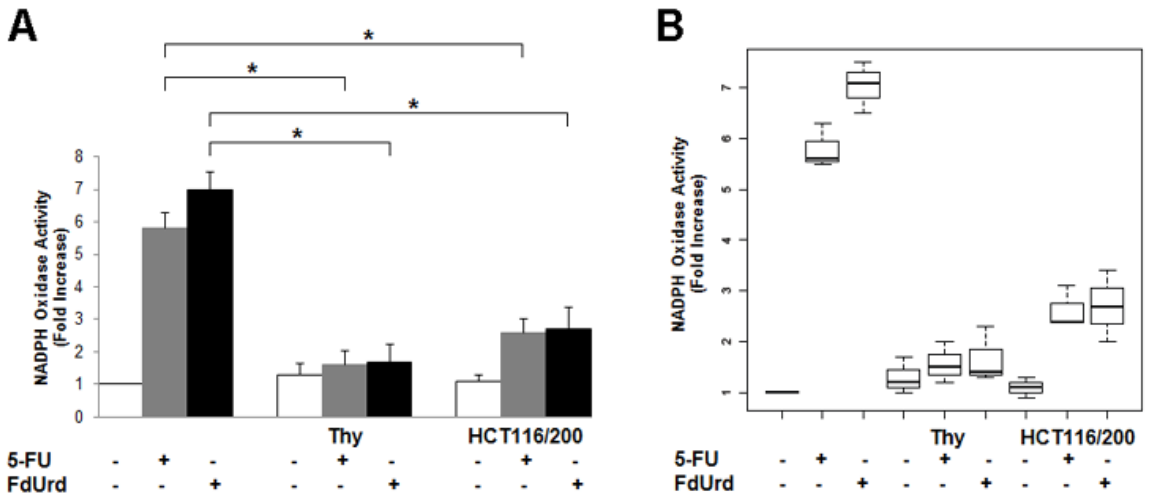

Figure 2.5. **NOX activity increased by drugs depends upon TS inhibition.** (A, B) HCT116 cells were treated with both TS inhibitors (10μM 5-FU, 10μM FdUrd) and 10μM thymidine for 24 hours. HCT116/200 cells were treated with 10μM 5-FU, 10μM FdUrd for 24 hours. NOX activity were measured by luminometer. Bars represent fold increase of chemiluminescence ± SEM from 3 experiments (* $p<0.05$).

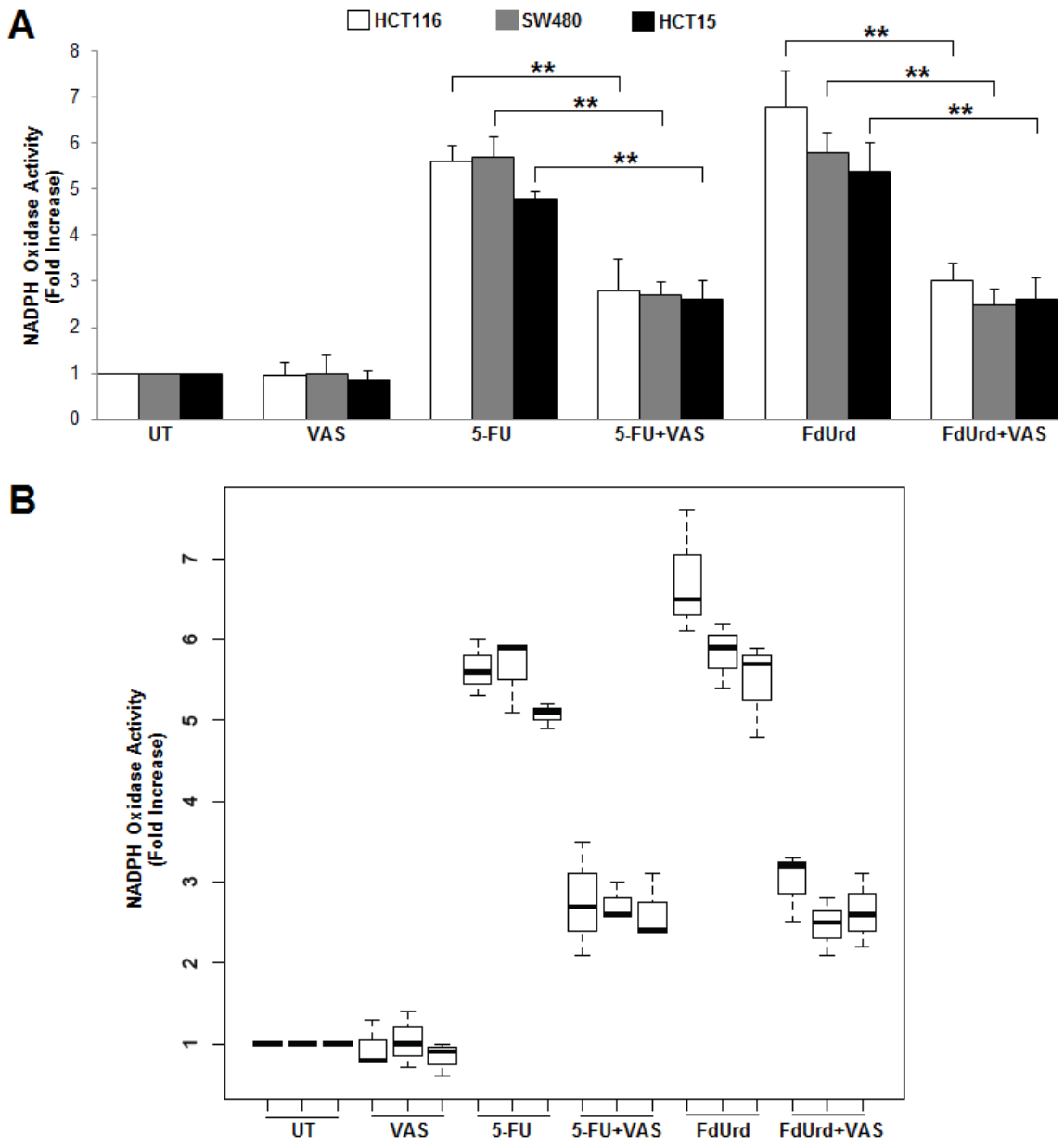

Figure 2.6. **VAS reduces NOX activity increased by drugs in colon cancer cell lines.** (A, B) HCT116, SW480 and HCT15 cells were treated with both TS inhibitors (10μM 5-FU, 10μM FdUrd) and 10μM VAS for 24 hours. NOX activity were measured by luminometer. Bars represent fold increase of chemiluminescence ± SEM from 3 experiments.

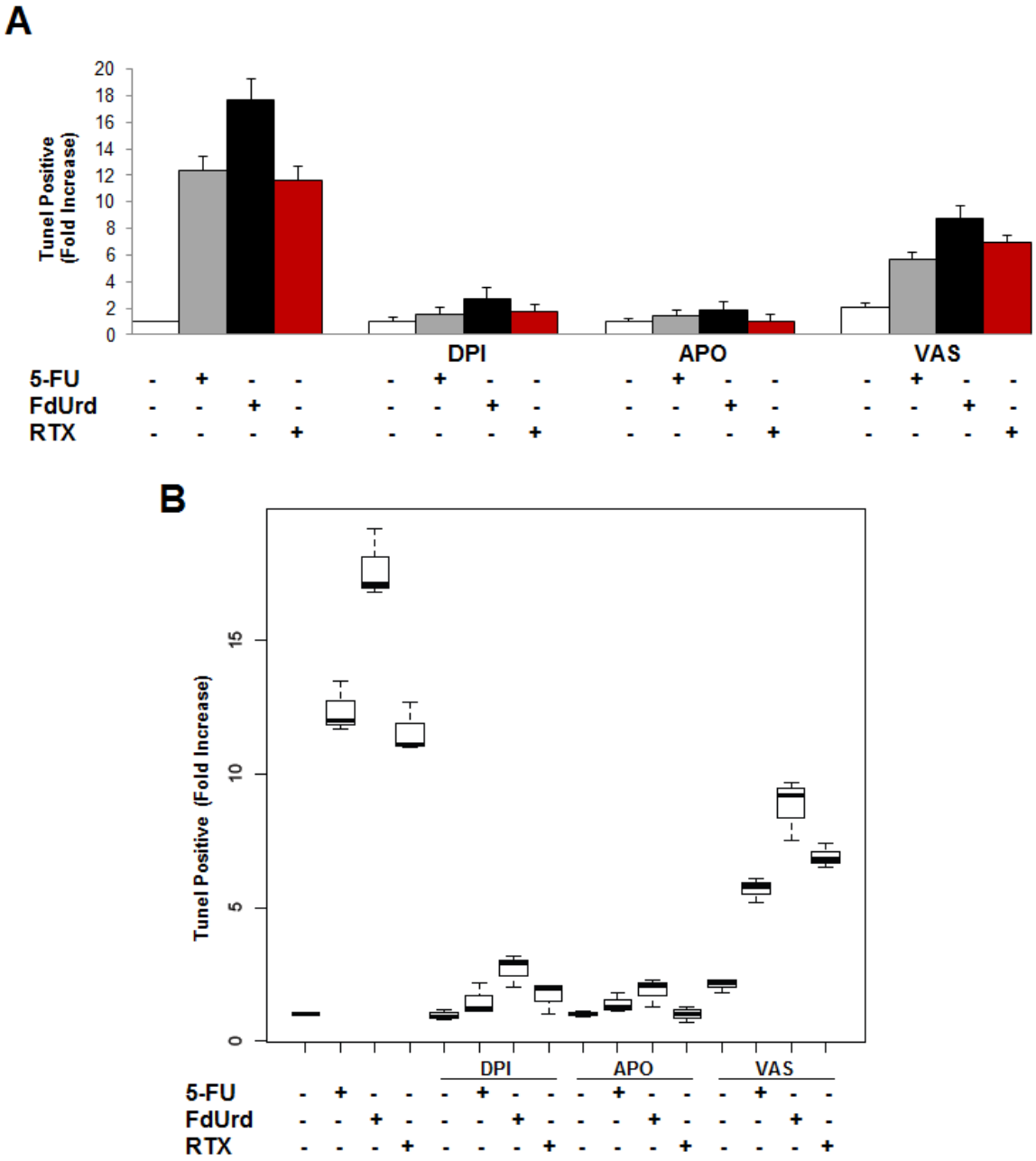

Figure 2.7. **NOX inhibitors decrease drug-induced cell death.** (A, B) HCT116 cells were grown in 10µM DPI (3h), 1mM APO (48h) and 10µM VAS (48h) with and without 10µM 5-FU, 100µM FdUrd and 1µM RTX for 48 hours. Extents of apoptosis were determined by TUNEL assay. Cells were photographed at 400X. Bars represent fold increase of apoptotic indices ± SEM from 3 experiments.

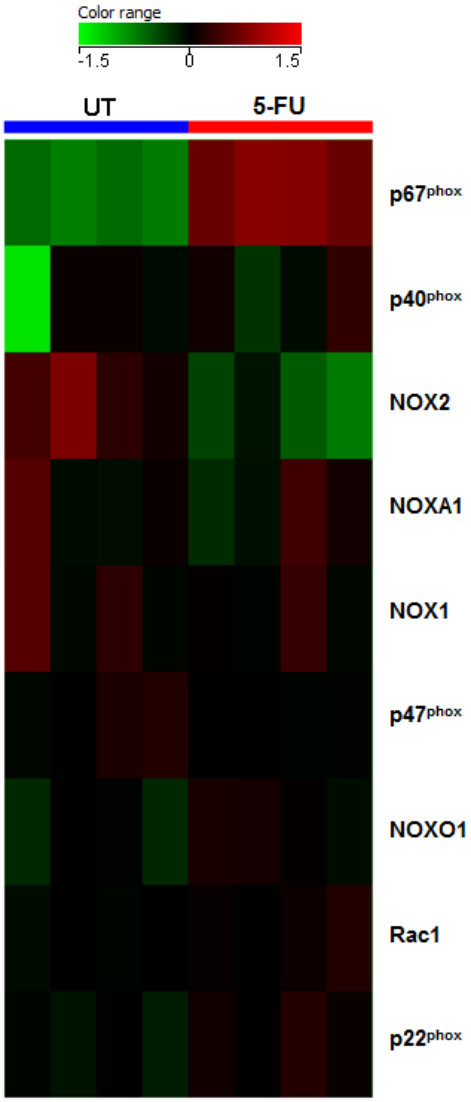

Figure 2.8. **Gene expression of NOX1 and NOX2 subunits in response to 5-FU.** HCT116 cells were treated in quadruplicate with ± 10μM 5-FU for 24h. cDNAs are synthesized from isolated mRNA samples. T7 RNA polymerase was added to cDNA samples to amplify original mRNA molecules and was added to cDNA samples and to simultaneously incorporate cyanine 3- or cyanine 5-labeled CTP (cRNA) into the amplification product. Labeled cRNA samples were hybridized to Agilent Human GE 4 x 44K v2 Microarrays at 65°C for 17 hours. Arrays were scanned using an Agilent DNA Microarray Scanner System.

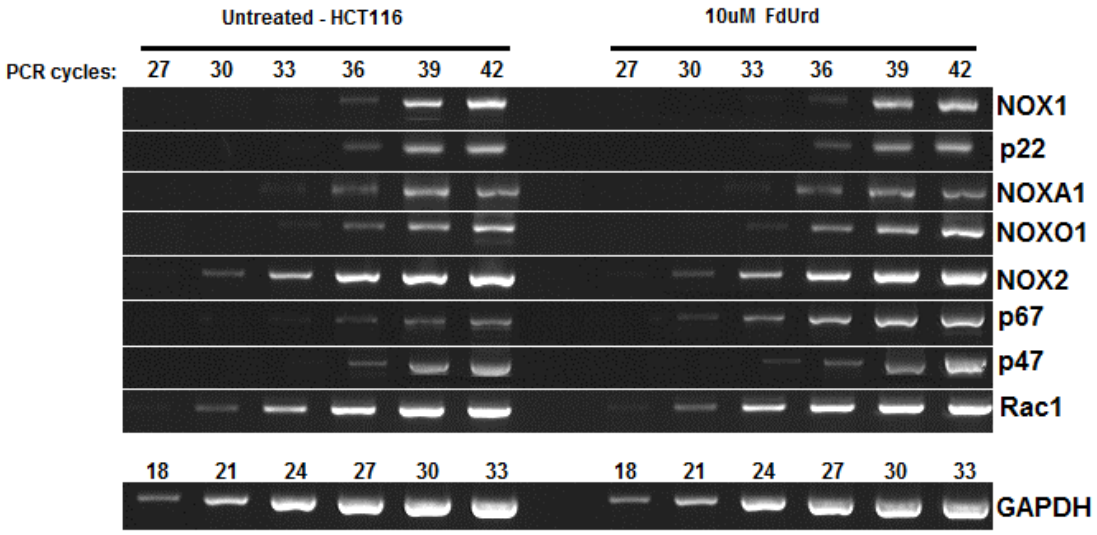

Figure 2.9. **mRNA levels of NOX1 and NOX2 subunits in response to FdUrd.** HCT116 cells were treated with ± 10μM FdUrd for 24h. Semi-quantitative RT-PCR was used to determine relative levels of NOX1 and NOX2 subunit's mRNAs in total RNA isolated from cells. Indicated subunits were examined to show relative levels of mRNAs in untreated and FdUrd treated cells. GAPDH was tested as a loading control.

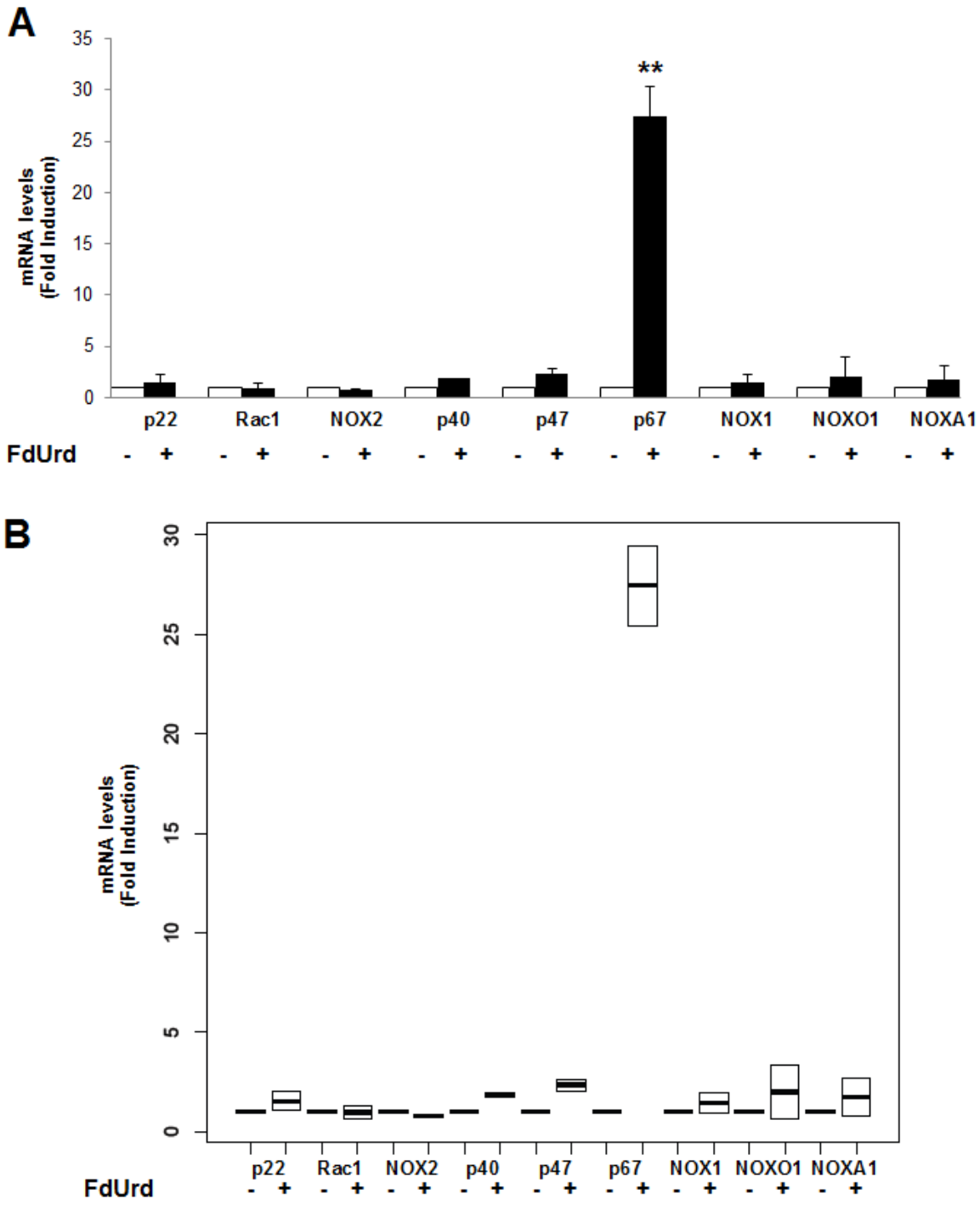

Figure 2.10. **Only p67^{phox} mRNA is induced in treatment of FdUrd.** HCT116 cells were treated with ± 10μM FdUrd for 24h. mRNA levels of NOX1 and NOX2 subunits were assayed by qPCR using GAPDH as a loading control. Bars represent an average of fold increase ± SEM from 2 separate experiments (** $p<0.01$).

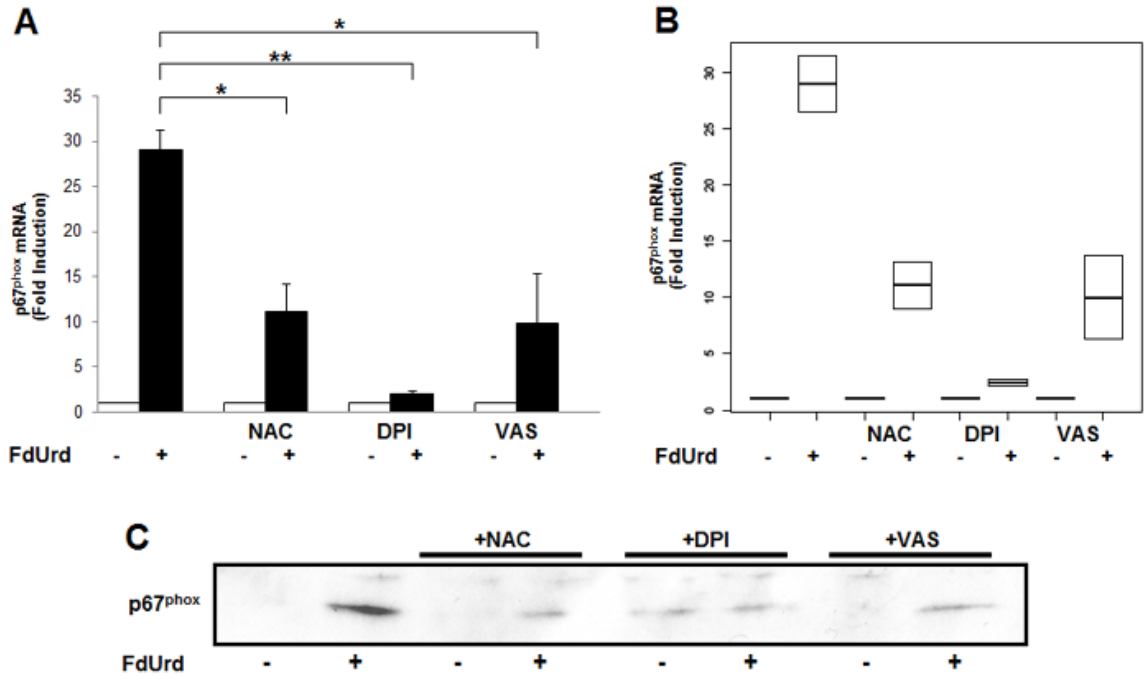

Figure 2.11. **Expressions of p67^{phox} mRNA and protein levels in treatment of FdUrd and antioxidants.** (A, B) HCT116 cells were cultured for 24h ± 10μM FdUrd and addition of 1mM NAC (24h), 10μM DPI (3h), and 10μM VAS (24h). p67^{phox} mRNA levels were assayed by qPCR using GAPDH as a loading control. Bars represent an average of fold increase ± SEM from 2 separate experiments (** $p<0.01$, * $p<0.05$). (C) Protein levels of p67^{phox} were determined in treatment of ± 10μM FdUrd for 24h and its combinations with 1mM NAC (24h), 10μM DPI (3h), and 10μM VAS (24h).

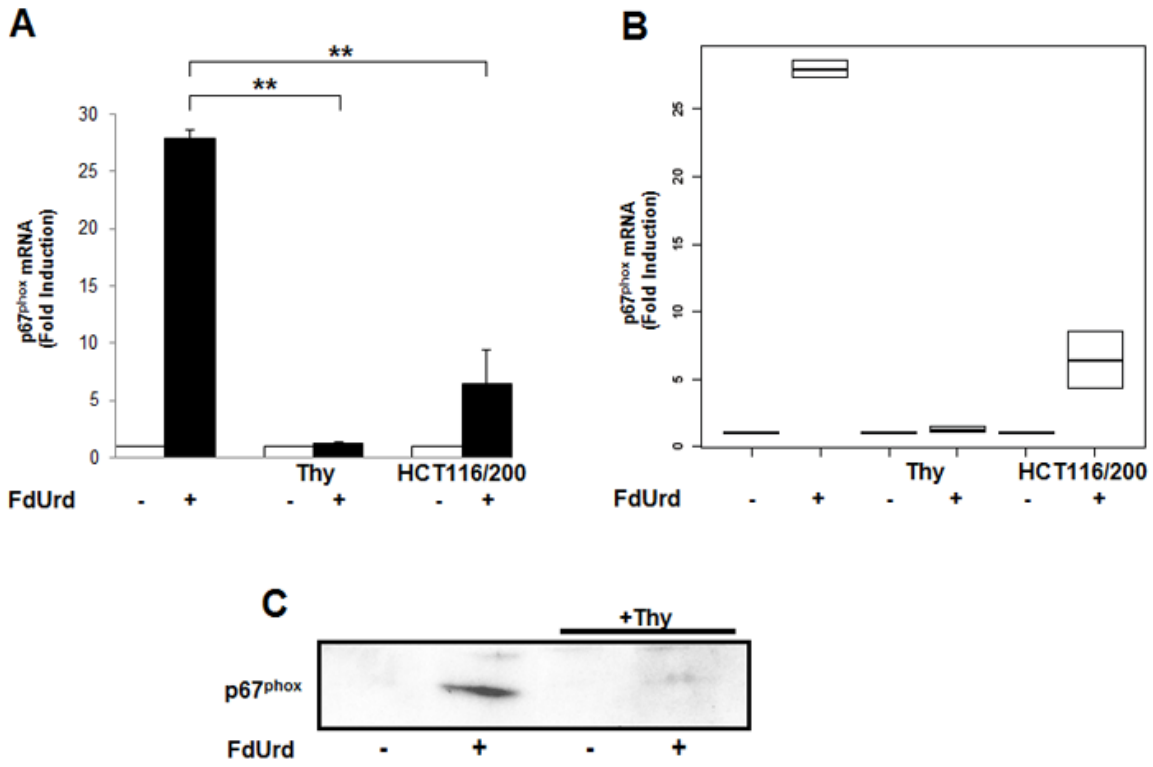

Figure 2.12. **Addition of thymidine and TS overproduction decrease induction of p67^{phox} mRNA by FdUrd.** (A, B) HCT116 cells were treated with ± 10μM FdUrd and its combination with 10μM thymidine for 24h. HCT116/200 cells were treated with ± 10μM FdUrd for 24h. p67^{phox} mRNA levels were assayed by qPCR. Bars represent an average of fold increase ± SEM from 2 separate experiments (** $p<0.01$). (C) Protein levels of p67^{phox} were determined in treatment of ± 10μM FdUrd for 24h and its combinations with 10μM Thy (24h).

Table 2.1. **5-FU-regulated Nrf2 target genes in HCT116 cells**

Nrf2 Target Genes	Gene Symbol	Fold Change	
Aldehyde dehydrogenase 3 family, member A1	ALDH4A1	2.0	up
Aldo-keto reductase family 1, member B10	AKR1B10	6.4	up
Aldo-keto reductase family 1, member C1	AKR1C1	2.3	up
ATP-binding cassette, sub-family C, member 2	ABCC2	2.6	up
Growth arrest and DNA-damage-inducible, alpha	GADD45A	2.1	up
Heat shock 22kDa protein 8	HSPB8	2.8	up
Heme oxygenase 1	HMOX1	2.0	up
Serpin peptidase inhibitor, clade E, member 1	SERPINE1	6.0	up
Spermidine/spermine N1-acetyltransferase 1	SAT1	2.5	up
v-maf musculoaponeurotic fibrosarcoma oncogene homolog F	MAFF	2.1	up
Cyclin-dependent kinase inhibitor 3	CDKN3	2.2	down
Glutamate-cysteine ligase, catalytic subunit	GCLC	1.8	down
NFKB repressing factor	NKRF	1.8	down
RAB11 family interacting protein 4	RAB11FIP4	2.6	down

CHAPTER 3

TS INHIBITOR-MEDIATED INDUCTION OF APOPTOSIS AND EXPRESSION OF NOX SUBUNITS VARY IN COLORECTAL CANCER CELL LINES

3.1. INTRODUCTION

Cancer laboratories establish a large number of human colorectal cancer cell lines from surgical specimens in the pathology laboratories. Established and characterized cell lines were classified into different groups based on their morphological features, growth rate, karyotypes, ability to synthesize carcinoembryonic antigen (CEA), etc. (McBain et al., 1984; Leibovitz et al., 1976). It has been shown that there is no correlation among these classification parameters and that genetic alteration in each tumor is not similar, implicating high levels of variability among the lines (Leibovitz et al., 1976). It has been also shown that the individual colon cancer exhibit a broad range of morphological and functional heterogeneity (McBain et al., 1984; Brattain et al., 1981). Cell lines may undergo these changes during their long-term culture, suggesting that properties of the cells may not reflect those of their original tumors (Brattain et al., 1981)

It has been shown that well-described colon cancer cells characterized *in vivo* and *in vitro* with regard to drug response, genetic abnormalities, expression of cancer-associated genes, etc. display important differences in tumor growth and metastatic capacity, leading to heterogeneity, possibly in a clinical relevant manner (Flatmark et al., 2004). Studies done to characterize structural instability in chromosomes of colon cancer cells revealed that it plays a significant role in the formation of genetic heterogeneity in different colon cancer cell lines (Ribas et al., 2003). Heterogeneity resulting from any system in cancer cells may bring about resistance to chemotherapeutic drugs (de Anta et al., 2006).

Heterogeneity is also apparent in apoptosis of different colon cancer cells and is a major drawback in identifying treatments (Fidler and Goste, 1985). Heterogeneity in apoptosis originates from variations in protein expression and protein interactions in different colon cancer cells (Schmid et al., 2012).

Expression of NOX isoforms and regulatory proteins varies among human colorectal cell lines. For instance, LS180 and LS174T cell lines expressed $p67^{phox}$ mRNA at intermediate levels, while Caco2 and HT-29 cell lines expressed it in very low levels (Juhasz et al., 2009). In addition, human colon cancer cells exhibit heterogeneity in response to iodonium-class flavin dehydrogenase inhibitors (Doroshow et al., 2013).

In this chapter, we demonstrate that human colon cancer cell lines HCT116, HCT15, SW480, DLD-1, LoVo, and MOSER vary in basal levels of NOX enzyme activity. Furthermore, treatment of FdUrd results in differential induction of levels of the enzyme, implicating a diverse response to TS inhibitors. Additionally, we demonstrate that both basal and drug-induced apoptotic indices in response to FdUrd vary. Furthermore, mRNA levels of NOX2 regulatory subunits—$p67^{phox}$, $p40^{phox}$ and $p47^{phox}$ vary significantly in both basal and FdUrd-inducible levels, thereby implicating considerable variation among colon cancer cell lines.

3.2. RESULTS

Human colon cancer cell lines exhibit different levels of NOX activity

Human colon cancer cell lines show the wide range of genetic, morphological and functional heterogeneity (Leibovitz et al., 1976; McBain et al., 1984; Brattain et al.,

1981). In order to determine whether these cell lines show variations in NOX activity in response to FdUrd, several, including HCT116, HCT15, SW480, DLD-1, LoVo and MOSER were treated with 10μM FdUrd for 24 hours, and NOX activity measured. HCT116 basal activity levels were set as 1 and the fold-increase of the activity in other cell lines in the absence and presence of FdUrd was calculated. Basal levels of NOX activity in these cell lines range from 1.2- to 4.2-fold. Following FdUrd, the activity was increased 1.7- to 4.8-fold in lines. MOSER had the highest basal (4.2 fold, $p<0.05$) and drug-inducible (4.8 fold, $p<0.05$) activity level among these cells. However, DLD-1 and LoVo cell lines showed low basal (1.2- and 1.6-fold, respectively) and drug-inducible (2.5-fold, $p<0.05$ and 1.7-fold, respectively) activity levels (Figure 3.1).

Apoptotic response to FdUrd varies in human colon cancer cells

Heterogeneity arising among tumor cells can lead to resistance to chemotherapeutic drugs (de Anta et al., 2006), may be a significant impediment in effective chemotherapeutic treatments (Fidler and Goste, 1985). It has been previously shown that apoptotic protein expression and interactions differ in colon cancer cells (Schmid et al., 2012). Thus, we determined if apoptotic response to FdUrd varies among different colon cancer cells. In order to test this, HCT116, HCT15, SW480 and LoVo cells were treated with FdUrd for 24 hours, and apoptotic indices were evaluated by TUNEL assay. It was observed that basal levels of apoptosis change from cell line to cell line. LoVo exhibited 3-fold higher ($p<0.05$) basal levels relative to HCT116 cells and HCT15. Drug-mediated increases in apoptotic indices were greater in HCT116 and HCT 15 cells (16-fold, $p<0.01$ and 14-

fold, $p<0.01$) respectively than SW480 and LoVo cells (8-fold, $p<0.01$ and 6.5-fold, $p<0.01$) respectively (Figure 3.2).

mRNA expressions of NOX2 accessory subunits—$p67^{phox}$, $p40^{phox}$ and $p47^{phox}$ differ in human colon cancer cell lines

It has previously been demonstrated that expression of NOX isoforms and their accessory subunits varies in human tumor cells (Juhasz et al., 2009). Given the variation of NOX activity levels and apoptotic responses in human colon cancer cells, we determined whether mRNA levels of NOX2 accessory subunits—$p67^{phox}$, $p40^{phox}$ and $p47^{phox}$ vary in different colon cancer cells. Cells were treated with FdUrd for 24 hours, and expression of the subunits was assessed by qPCR. Human colon cancer cell lines—HCT116, HCT15, SW480, DLD-1, LoVo, MOSER and LS180 showed different basal and drug-inducible mRNA levels for $p67^{phox}$ and $p40^{phox}$ (Figure 3.3 and 3.4). LoVo, MOSER and LS180 exhibited high basal levels of $p67^{phox}$ ($p<0.05$) while SW480 exhibited intermediate levels ($p<0.05$). HCT116, HCT15 and DLD-1 expressed low basal levels of $p67^{phox}$. FdUrd-induced $p67^{phox}$ levels were seen in HCT116 and DLD-1, about 23-fold ($p<0.01$) and 9-fold ($p<0.05$), respectively (Figure 3.3 A).

Expression of $p40^{phox}$ was low in HCT15, LoVo and MOSER cells compared to HCT116, SW480, DLD-1 and LS180. FdUrd-induced $p40^{phox}$ levels in HCT15 and LoVo cells, were 8- and 10-fold ($p<0.05$), respectively. HCT116 and MOSER cells exhibited low levels of drug-induced $p40^{phox}$ levels while SW480, DLD-1 and LS180 expressed intermediate levels (Figure 3.4).

Basal and drug-inducible mRNA levels of $p47^{phox}$ also vary among the lines (Figure 3.5). HCT116 and SW480 showed similar basal and drug-inducible mRNA levels of $p47^{phox}$. Although MOSER and LS180 exhibited similar basal levels of $p47^{phox}$, drug-induced levels were unchanged in MOSER, but were induced 8.5-fold ($p<0.05$) in LS180 cells.

Protein levels of $p67^{phox}$ subunits in colon cancer cells exhibited the same trend as seen in its mRNA expression (Figure 3.3 A). Low basal levels of $p67^{phox}$ in several lines could not be detected by western-blot analysis.

3.3. DISCUSSION

In chapter 1 and 2, we showed that TS inhibitors mediate increases in apoptosis about 10 to 18-fold in HCT116 colon tumor cells (Figure 1.4 B and Figure 2.7). Here, we demonstrate that FdUrd induce apoptosis 6.5- to 16-fold change in human colon cancer cell lines. This different inductions result from variation in basal apoptosis levels of cell lines, reflecting the heterogeneity in cell lines.

In chapter 2, we indicated that increases in NOX activity by TS inhibitors were similar in HCT116, SW480 and HCT15 cell lines (Figure 2.6). However, we don't know how basal levels of their NOX activity change. Therefore, we determined basal and drug-increased levels of NOX activity in six different cell lines. Here, we demonstrate that they vary in basal NOX activity levels, reflecting different backgrounds among the lines. In response to FdUrd, increases in NOX activity were profoundly exhibited in most cell lines, with DLD-1 and LoVo showing only slight increases.

Our results are consistent with earlier studies, which revealed that human colon cancer cell lines exhibit different expression levels for NOX genes (Juhasz et al., 2009). Based on our results, the anti-tumor effects of FdUrd appear to vary among colon cancer cell lines, reflecting phenotypic and heterogeneities. Each cell line shows different expression levels of regulatory subunits, and different levels of NOX activity and apoptosis levels. Therefore, deeper understanding of phenotypic features of each colon cancer cell line could be essential to therapy of this deadly disease.

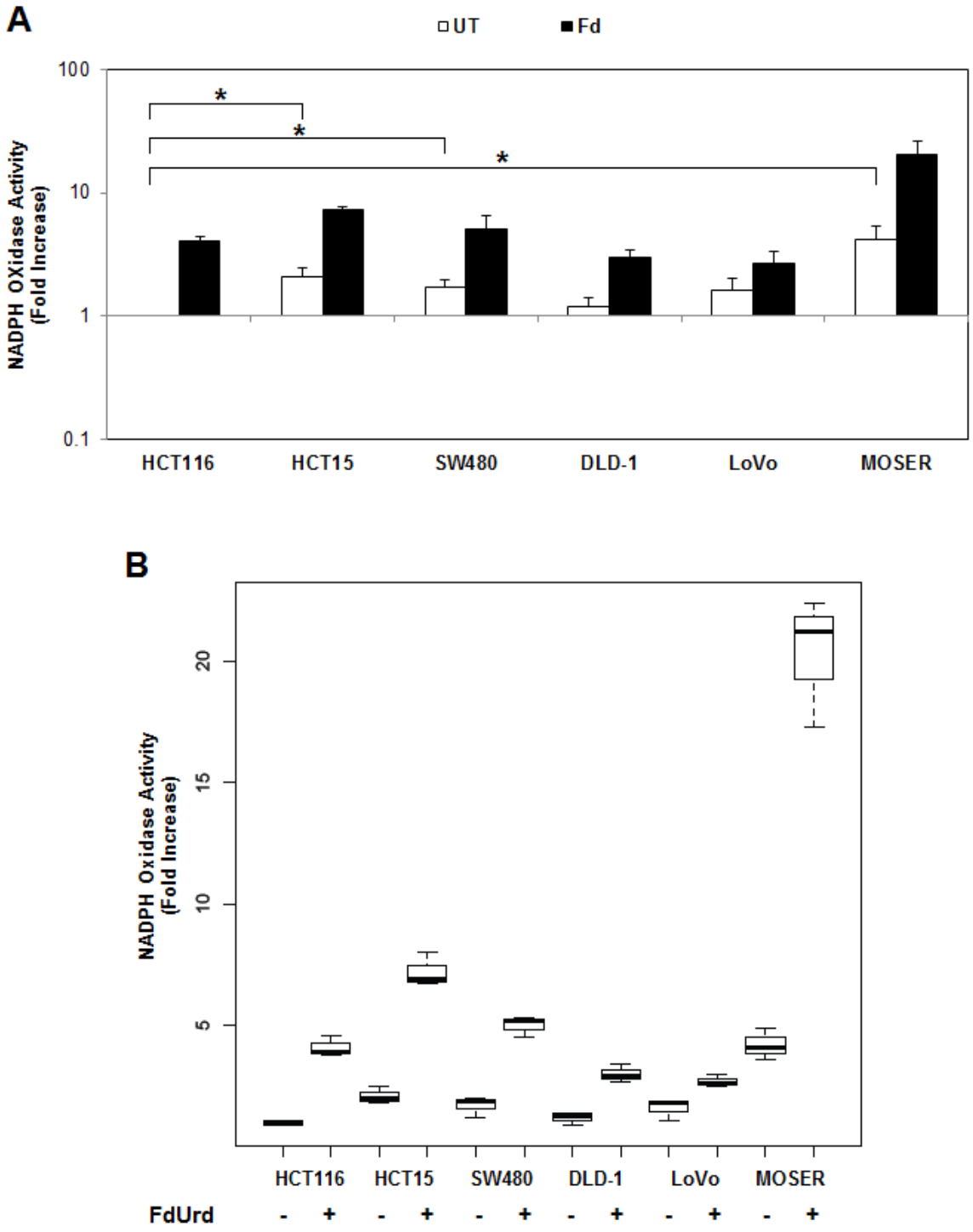

Figure 3.1. **NOX activity levels vary in human colon cancer cell lines.** (A, B) HCT116, HCT15, SW480, DLD-1, LoVo and MOSER cell lines were grown ± 10μM FdUrd for 24 hours and then were subjected to NOX activity assay. Lucigenin derived chemiluminescence was measured by luminometer. Bars represent fold increase of chemiluminescence ± SEM from 3 experiments (* $p<0.05$).

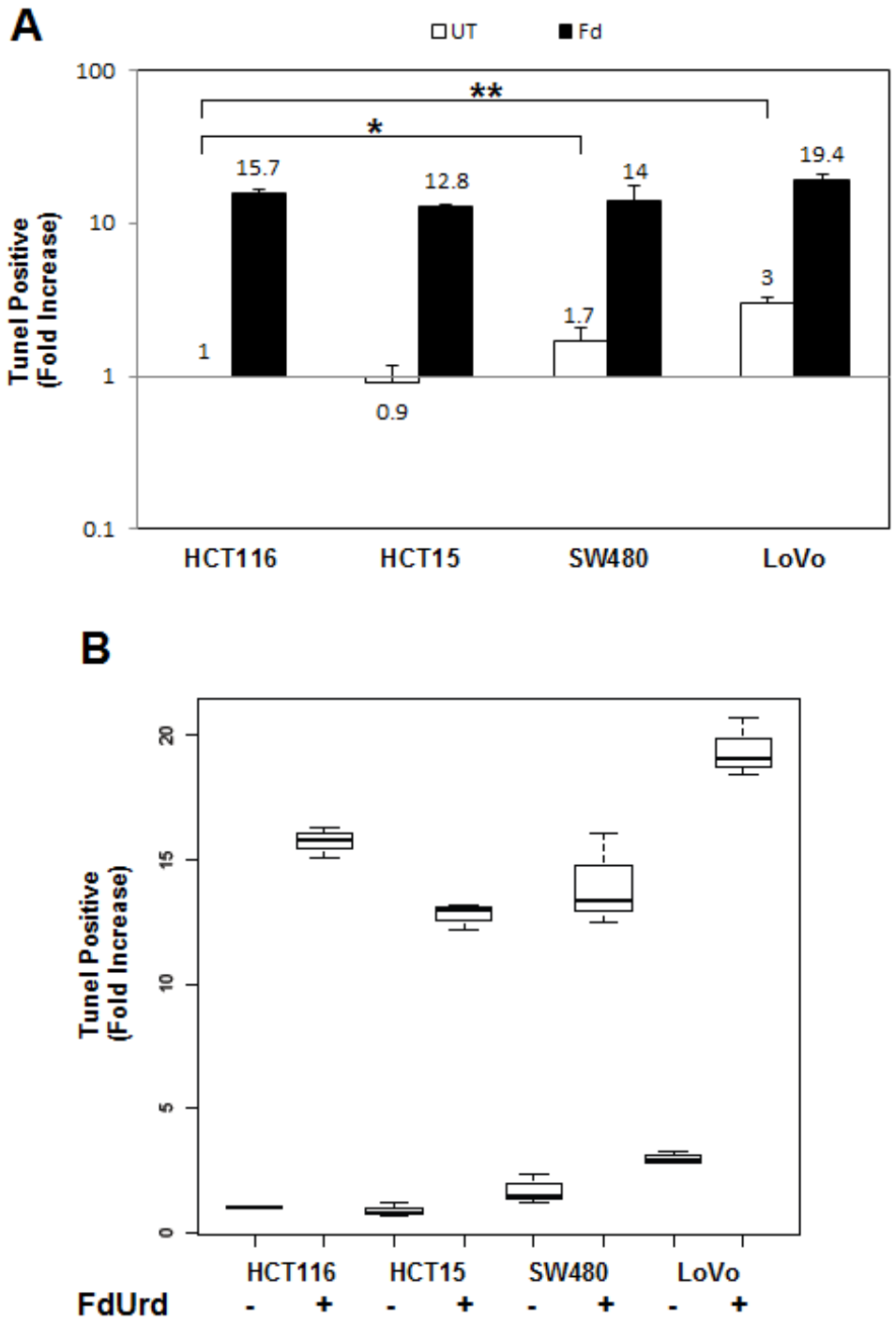

Figure 3.2. **FdUrd diversely induces apoptosis in human colon cancer cells.** (A, B) HCT116, HCT15, SW480 and LoVo cells were grown with and without 100µM FdUrd for 24 hours. Extents of apoptosis were determined by TUNEL assay. Cells were photographed at 400X. Bars represent fold increase of apoptotic indices ± SEM from 3 experiments (** $p<0.01$, * $p<0.05$).

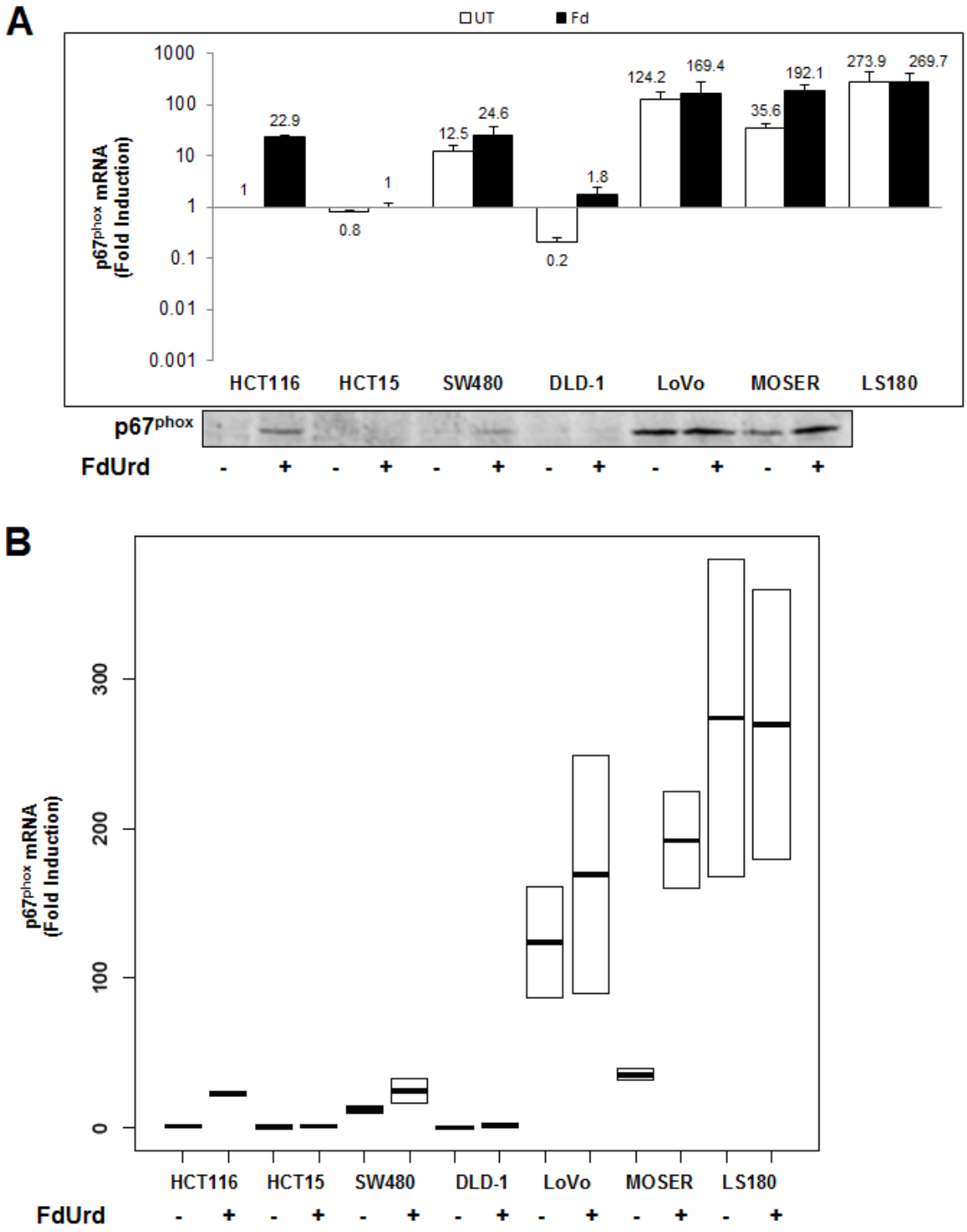

Figure 3.3. **mRNA levels of p67^{phox} vary in human colon cancer cell lines.** (A, B) qPCR was used to measure mRNA levels of p67^{phox} in total RNA isolated from HCT116, HCT15, SW480, DLD-1, LoVo, MOSER and LS180 cultured for 24h ± 10μM FdUrd. GAPDH was tested as a loading control. Bars represent an average of fold increase ± SEM from 2 experiments.

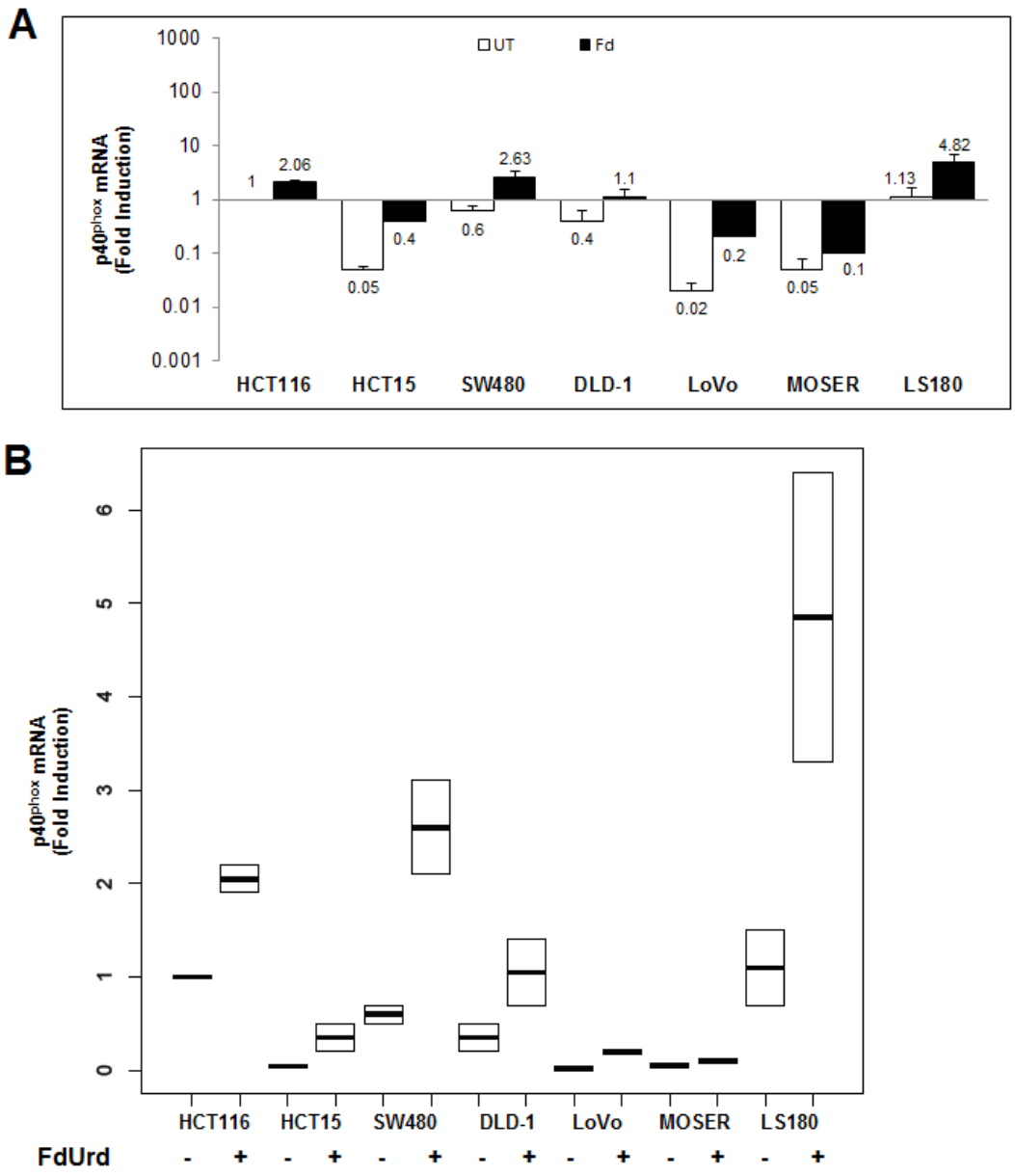

Figure 3.4. **mRNA levels of p40^{phox} vary in human colon cancer cell lines.** (A, B) qPCR was used to measure mRNA levels of p40^{phox} in total RNA isolated from HCT116, HCT15, SW480, DLD-1, LoVo, MOSER and LS180 cultured for 24h ± 10µM FdUrd. GAPDH was tested as a loading control. Bars represent an average of fold increase ± SEM from 2 experiments.

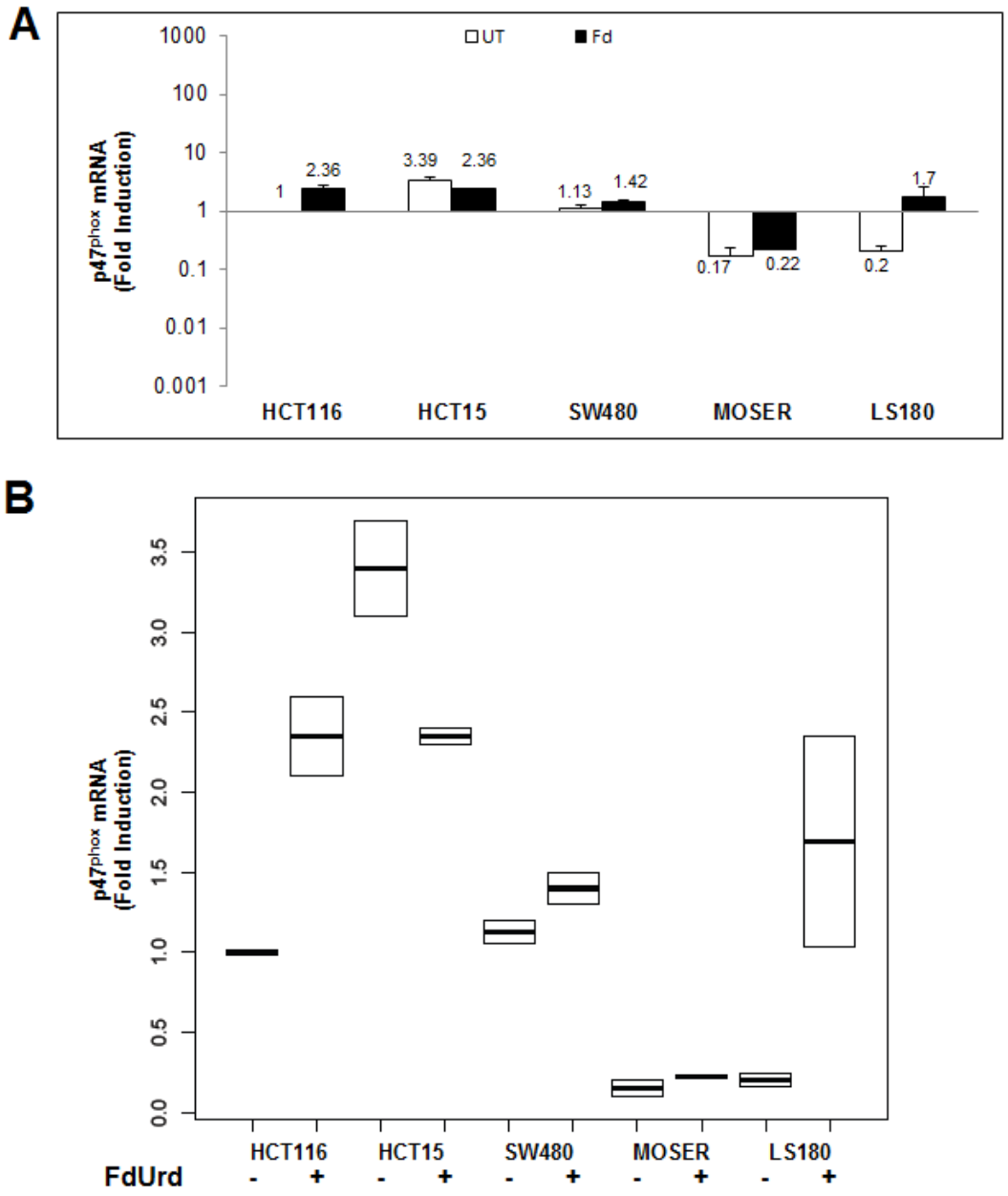

Figure 3.5. **mRNA levels of p47^{phox} vary in human colon cancer cells.** (A, B) qPCR was used to measure mRNA levels of p47^{phox} in total RNA isolated from HCT116, HCT15, SW480, DLD-1, LoVo, MOSER and LS180 cultured for 24h ± 10μM FdUrd. GAPDH was tested as a loading control. Bars represent an average of fold increase ± SEM from 2 experiments.

CHAPTER 4

NFκB PLAYS A ROLE IN BASAL LEVELS OF ROS IN HCT116 CELLS

4.1. INTRODUCTION

Nuclear factor kappa B (NFκB) is a transcription factor that binds to specific sites on DNA and influences downstream expression of many genes involved in immune response, apoptosis, cell proliferation, adhesion and differentiation (Perkins, 2000; Beinke and Ley, 2004). Its function is inhibited by binding to the so-called Inhibitor of kappa-B-α (IκBα). Activation of NFκB follows the 26S proteasome-mediated degradation of IκBα that results from phosphorylation of IκBα by IκB kinase-β (IKKβ) (Beinke and Ley, 2004). NFκB signaling is a central coordinator of the innate and adaptive immune system. It is widely accepted that NFκB functions as an anti-apoptotic factor. Consistent with this, many investigations have shown that low levels of NFκB make cells vulnerable to the cytotoxic effects of TS inhibitors (Konishi et al., 2006; Tafani et al., 2013; Camp et al., 2004; Li et al., 2006; Voboril et al., 2004).

In direct opposition to the generally accepted view, our laboratory has shown that NFκB promotes apoptosis during thymidylate deprivation, implicating a pro-apoptotic role in cells responding TS inhibitors (Barbour and Berger, 2008; Harwood et al., 2000). Other studies have also shown a pro-apoptotic function for NFκB, implicating its dual role in the regulation of apoptosis (Ravi et al., 2001; Kasperczyk et al., 2005). The activation of NFκB is therefore a double-edged sword, and its function depends on context of signaling pathways.

To show that the apoptotic response to TS inhibitors is regulated by NFκB in human colon carcinoma cells, our lab utilized a NFκB deficient cell line, HCT116-dnIkBα, and its revertent, HCT116-dnIkBα-R (Barbour and Berger, 2008). HCT116-

dnIkBα cell line is produced by stably transfecting HCT116 cells with a plasmid that expresses a dominant-negative mutant form of IkBα. To produce HCT116-dnIkBα-R cell line, HCT116-dnIkBα cells were passaged in non-selective media for 4 months and a revertent clone was obtained. Experiments described in Chapters 1 and 2 indicated that cell death mediated by TS inhibitors is ROS-dependent, and that NOX enzyme is involved. Whether there is a link between ROS and transcription factor NFκB in apoptotic response to TS inhibitors is unknown. Here, we demonstrate that basal ROS levels were increased in HCT116-dnIkBα cells compared to HCT116 and HCT116-dnIkBα-R cells, however; the induced levels of ROS by TS inhibitors was not significantly changed in these cells. In addition to this finding, mRNA levels of $p67^{phox}$ were increased in HCT116-dnIkBα cells compared to HCT116 and HCT116-dnIkBα-R cells while the upregulation of $p67^{phox}$ mRNA by FdUrd was not significantly changed, indicating parallel changes with levels of ROS. We also demonstrate that drug-induced increases in NOX activity were similar in all three cells. Overall, these observations have led to the conclusion that NFκB acts on basal, but not drug-mediated ROS levels.

4.2. RESULTS

ROS formation mediated by TS inhibitors is not NFκB-dependent

NFκB regulates the expression of many genes for immune response, cell proliferation and differentiation, etc. Imbalance between NFκB and its inhibitor IκB has been associated with progress of many diseases, including cancer (Beinke and Ley, 2004). It is suggested that NFκB plays a pro-apoptotic role in apoptotic response to TS inhibitors (Barbour and Berger, 2008; Harwood et al., 2000). To determine whether it acts on oxidative or ROS-

derived apoptotic response to TS inhibitors, we exposed parental line HCT116, NF-κB deficient cell line, HCT116-dnIkBα, and its revertent, HCT116-dnIkBα-R to TS inhibitors. Basal H_2O_2 and $O_2^{\cdot -}$ levels were higher in HCT116-dnIkBα cells as compared to HCT116, and were rescued in HCT116-dnIkBα-R cells. However, both H_2O_2 and $O_2^{\cdot -}$ levels induced by TS inhibitors were unchanged in all three lines (Figure 4.1).

Loss of NFκB does not alter mRNA levels of $p67^{phox}$ induced by FdUrd

As seen in Chapter 2, $p67^{phox}$ induction is associated with in drug-mediated ROS generation in HCT116 cells (Figure 2.11 and 2.12). In order to determine if NFκB plays a role in the induction of $p67^{phox}$ mRNA induced by TS inhibitors, we treated HCT116, HCT116-dnIkBα, and HCT116-dnIkBα-R cells with inhibitors and found that loss of NFκB increased basal levels of $p67^{phox}$ mRNA in HCT116-dnIkBα relative to HCT116 and HCT116-dnIkBα-R cells. However, NFκB deficiency did not significantly change mRNA levels of $p67^{phox}$ in drug-treated cells (Figure 4.2).

NFκB deficiency does not affect increases in NOX activity mediated by TS inhibitors

In order to examine whether drug-mediated increases in NOX activity are altered by NFκB, we exposed HCT116, HCT116-dnIkBα, and HCT116-dnIkBα-R cells to TS inhibitors and measured NOX activity. We determined that loss of NFκB did not alter increases in NOX activity mediated by TS inhibitors in any of the three cells (Figure 4.3).

4.3. DISCUSSION

NFκB is a multipotent transcription factor that regulates cell proliferation and differentiation, immune response, apoptosis and other integral cellular functions (Perkins, 2000; Beinke and Ley, 2004). Although several studies have reported it as having a pro-apoptotic role (Barbour and Berger, 2008; Harwood et al., 2000), most investigations have indicated it to be an anti-apoptotic factor (Karin, 2006; Konishi 2006; Tafani et al., 2013), showing its dual functions in the control of cell functions.

It is known that NFκB promotes cell death due to thymidylate deprivation resulting from TS inhibition. However, the mechanism underlying NFκB-mediated cell death is not well understood. Our data suggests that this does not occur via alterations in ROS induction mediated by TS inhibitors. We have shown that HCT116-dnIkBα, NFκB deficient, cells have high basal levels of ROS compared to HCT116 and HCT116-dnIkBα-R cells. This might suggest that absence of NFκB results in abnormalities in cells because it affects expressions of many genes in cellular metabolism, giving rise to oxidative stress. Due to loss of NFκB, TS inhibitors do not further induce ROS generation in HCT116-dnIkBα cells compared to HCT116 and HCT116-dnIkBα-R cells, indicating the effect of NFκB on drug-mediated ROS generation.

We have also shown that basal levels of $p67^{phox}$ mRNA are increased in HCT116-dnIkBα cells relative to HCT116 and HCT116-dnIkBα-R cells, but induced mRNA levels by TS inhibitors is similar between these 3 cells. Additionally, increases in NOX activity are also similar in these cells, indicating that under the conditions of our experiments, NFκB has no role in NOX activity in response to TS-directed agents.

Under certain conditions, it is suggested that pro-apoptotic role of NFκB in thymidylate deficiency might be ROS-dependent, but it is not due to $p67^{phox}$ or NOX enzyme. It will be important to reveal the mechanism by which it promotes apoptosis in future work and to determine how the anti-apoptotic responses were silenced.

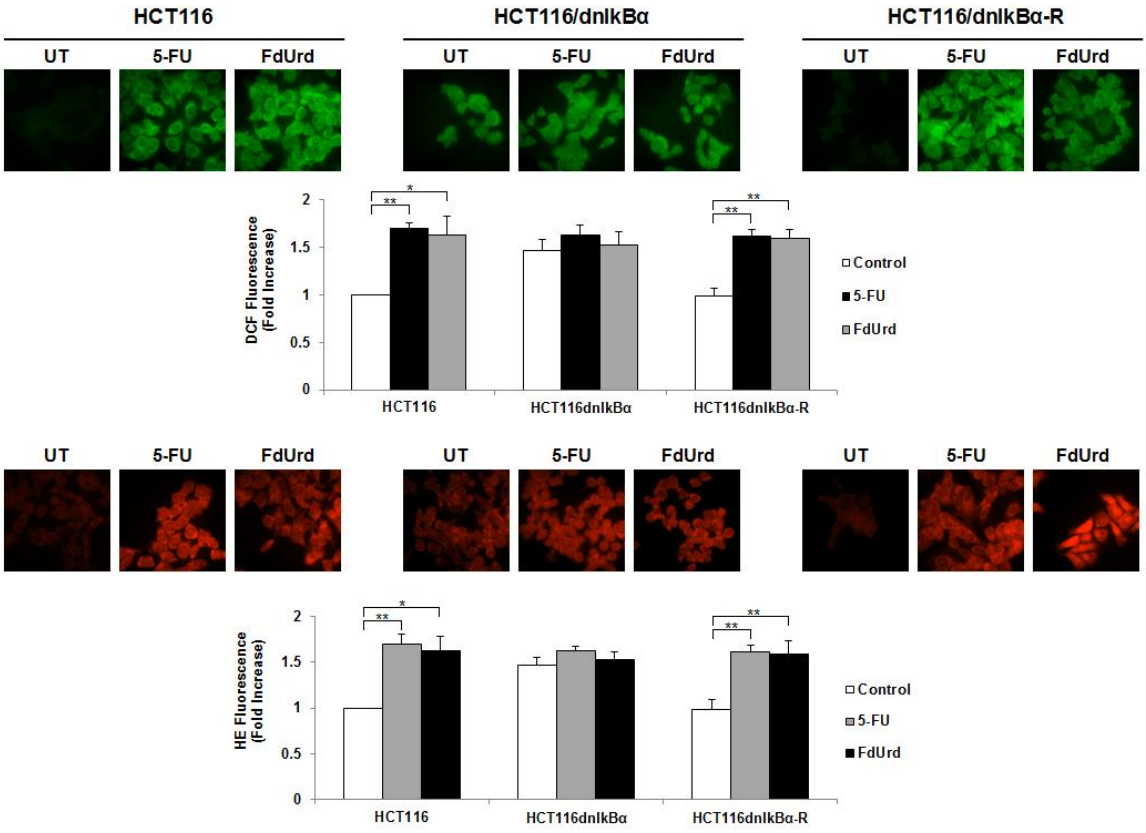

Figure 4.1. **Loss of NFκB increases basal ROS levels.** HCT116, HCT116/dnIkBα and HCT116/dnIkBα-R cells were treated with 10μM 5-FU, and 10μM FdUrd for 24 hours and then stained by 5μM H2DCF-DA and 5μM DHE. ROS detection was done in both fluorescence microscopy and flow cytometry. Pictures from fluorescence microscopy represent H_2O_2 formation (green) and $O_2^{\cdot -}$ (red). Bars from flow cytometry represent fold increase of H_2O_2 (DCF fluorescence) and $O_2^{\cdot -}$ (HE fluorescence) formation ± SEM from 3 experiments (* $p<0.05$, ** $p<0.01$).

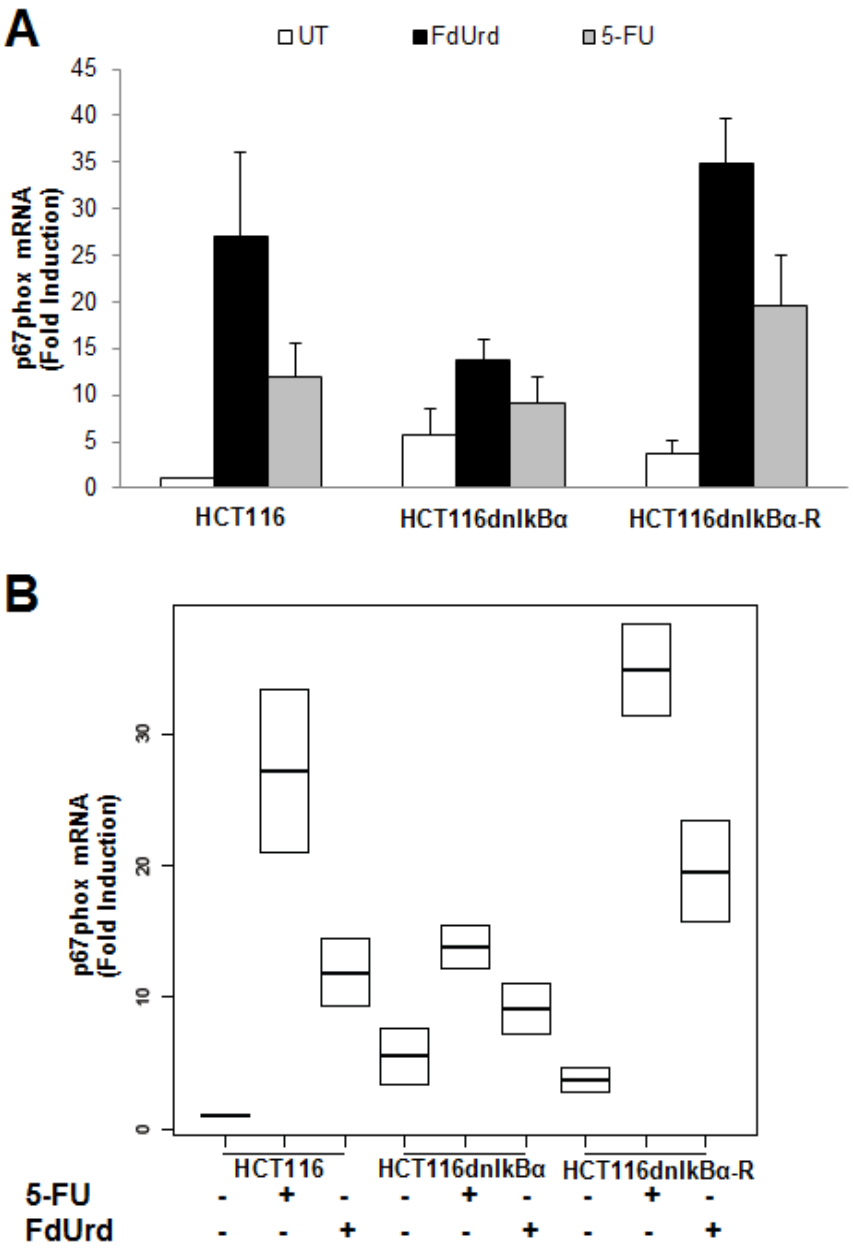

Figure 4.2. **Basal levels of p67^{phox} mRNA is increased by loss of NFκB.** (A, B) qPCR was used to measure mRNA levels of p67^{phox} in total RNA isolated from HCT116, HCT116-dnIkBα and HCT116-dnIkBα-R cell lines cultured for 24h ± 10μM 5-FU and 10μM FdUrd. GAPDH was tested as a loading control. Bars represent an average of fold increase ± SEM from 2 experiments.

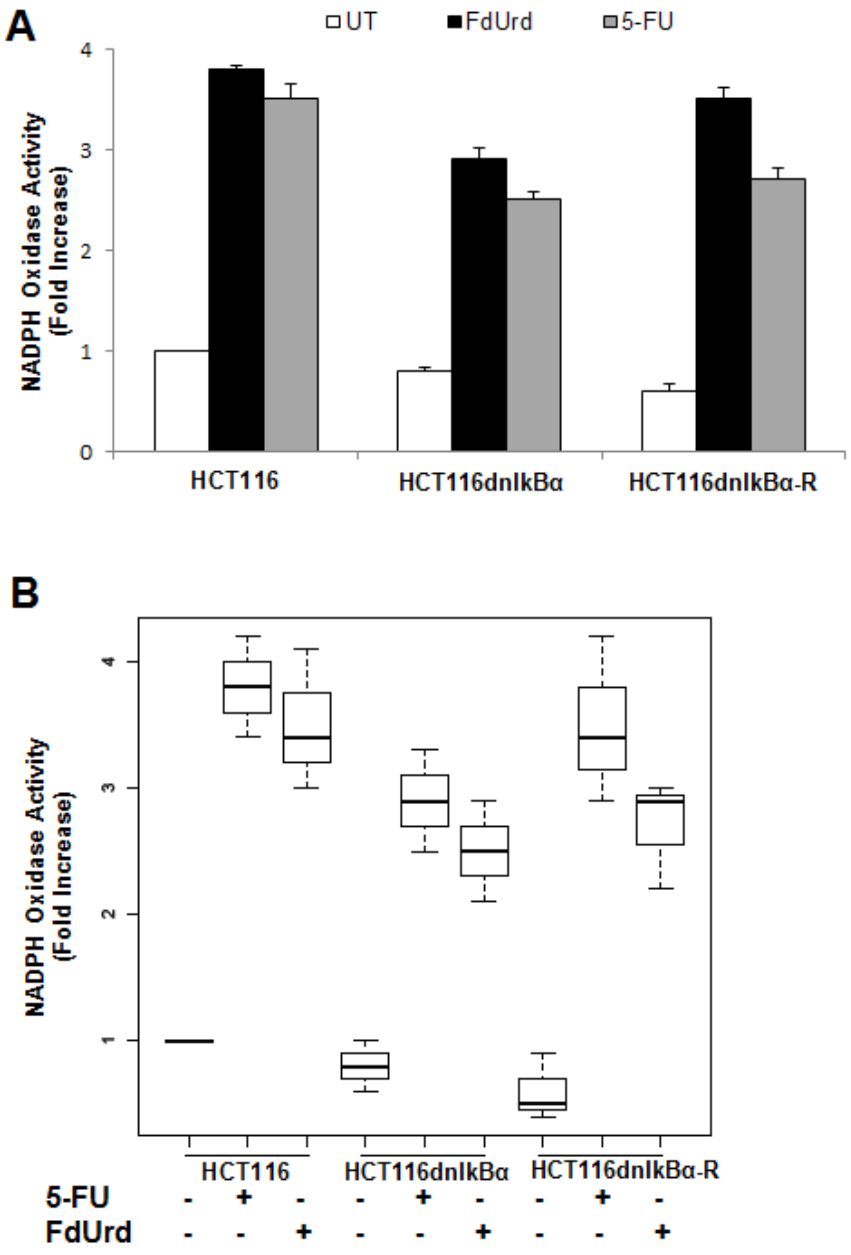

Figure 4.3. **Drug-increased NOX activity is unchanged in NFκB deficiency.** (A, B) HCT116, HCT116-dnIkBα and HCT116-dnIkBα-R cell lines were grown ± 10μM 5-FU and 10μM FdUrd for 24 hours and then were subjected to NOX activity assay. Lucigenin derived chemiluminescence was measured by luminometer. Bars represent fold increase of chemiluminescence ± SEM from 3 experiments.

CONCLUDING STATEMENTS

TS is the sole source and indispensable for *de novo* synthesis of thymidylate associated with DNA synthesis and cell growth in absence of exogenous thymidine. Therefore, it has been viewed as a significant target in the treatment of cancer for quite some time. TS-targeted therapy by anti-metabolites, including TS inhibitors—5-FU, RTX, has been effective for decades and is still mainstay in the cancer chemotherapy. Drug-mediated TS inhibition leads to thymine-less cell death, misincorporation of uracil and thymine into DNA and RNA, genome damage, oxidative stress and apoptosis. Effects of oxidative stress on TS inhibitors-directed cell death are not well understood. Here, we demonstrate that 5-FU, FdUrd and RTX induce ROS formation in human colon tumor cell line HCT116 and the induction is attenuated by NAC, addition of thymidine and TS overproduction, showing dependence of oxidative stress to TS inhibition. NAC also inhibits drug-mediated cell death. NOX enzyme is identified as the primary source of ROS following drug exposure because NOX inhibitors decreased ROS accumulation in combination of drugs. Additionally, TS inhibitors increase the enzyme activity in correlation with induction of $p67^{phox}$ mRNA expression that specifically regulates the NOX2 isoform. Addition of thymidine and TS overproduction diminish these effects.

Other colon tumor cell lines showed variation in basal and drug-induced levels of NOX activity and of gene expressions of NOX2 accessory subunits—$p67^{phox}$, $p40^{phox}$ and $p47^{phox}$. They also indicate diverse basal and drug-inducible levels of apoptosis, implicating cell specific responses to drugs. Drug-induced ROS generation in correlation with induction of $p67^{phox}$ mRNA expression is not NFκB dependent. In absence of NFκB,

basal levels of ROS and $p67^{phox}$ mRNA expression are increased in HCT116 cells, showing independent oxidative stress resulted from different mechanisms.

Based on the findings herein reported, a model was developed to illustrate the origin and role of ROS in cell death mediated by TS inhibitors (Figure C1). These findings shed light on understanding of drug effects in cell death since how TS inhibitors kill cells is ill-defined. While our results clearly demonstrate a correlation between drug-induced ROS generation and apoptosis, NOX activity, and $p67^{phox}$ expression, they also raise several questions. First, it is not clear how these results are linked to each other. It would be necessary to knockdown or overexpress the $p67^{phox}$ subunit in order to determine if it regulates drug-induced ROS production, apoptosis, and NOX activity. Second, we don't know how upregulation of $p67^{phox}$ subunit by drugs impacts protein function. Is there a linear relationship between protein expression and function? If not, what are the factors mediating the function?

A third question rising from our results is whether or not expression of other NOX isoforms, i.e., NOX3-5, is altered in response to drugs. We also don't know which isoform(s) results in drug-increased NOX activity. It would be interesting to investigate which isoform(s) is targeted by TS inhibitors.

Our results also showed that, in addition to ROS induction, drug exposure increased expressions of some Nrf2 target genes which are the major defense response against oxidative stress, implicating dual impacts of drugs in colon cancer cells. If TS inhibitors are combined with drugs that inhibit the defense system, cells may become more sensitive to ROS-derived apoptosis. Future studies should reveal detailed

mechanisms of drug-mediated ROS generation and the antioxidant responses in colon cancer cells. At the mechanistic level, future studies should focus on other ROS sources, in addition to NOX, involved in oxidative stress-derived apoptotic response to drugs.

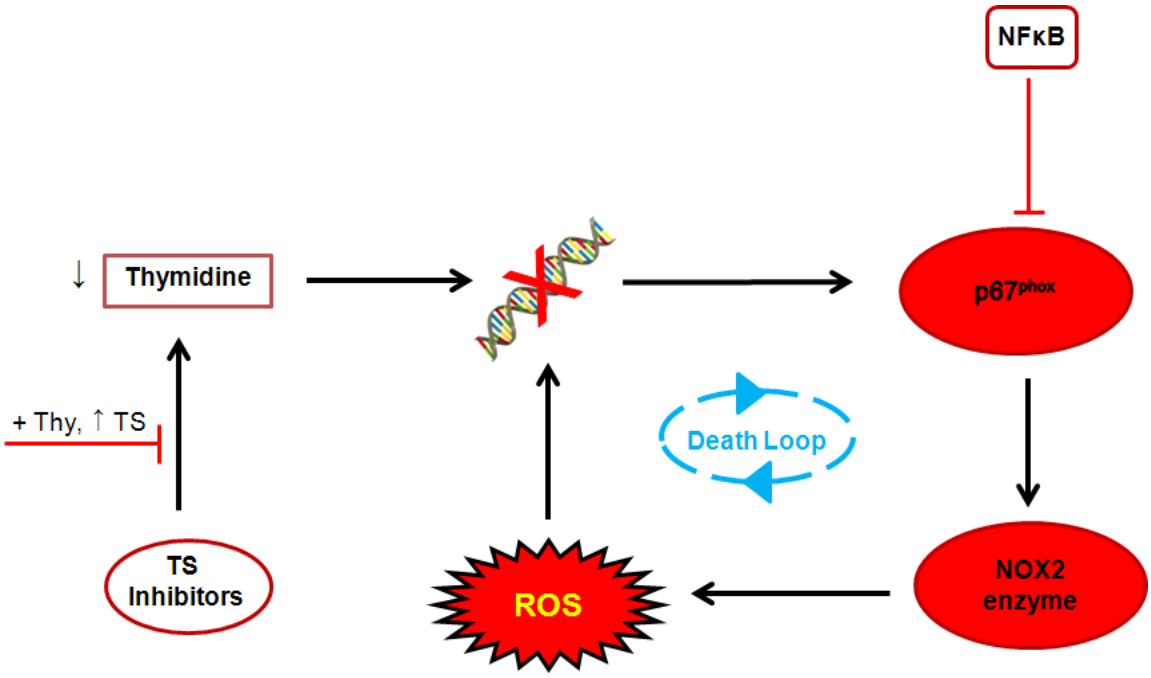

Figure C1. **Model for the role of drug-mediated ROS in cell death.** TS inhibitors deplete thymidylate levels following DNA damage. Increase in NOX2 activity correlated with induction of $p67^{phox}$ results in further ROS generation and genome damage. If such damage mediated by "feed-forward" death loop is over tolerable threshold, cells undergo programmed cell death. These effects are inhibited by exogenous addition of thymidine or TS overproduction. NFκB inhibits ROS formation induced by NOX2 in correlation with the induction of $p67^{phox}$.

CHAPTER 5

MATERIALS AND METHODS

Cell culture

Human colon tumor cell line HCT116 was kindly obtained from Dr. Michael G. Brattain. HCT116/200 is TS-overproducing derivative of HCT116 and has been previously described (Berger et al., 1988). Other human colon cancer cell lines SW480, HCT15, LoVo, DLD-1, MOSER and LS180 are obtained from American Type Culture Collection (ATCC); (Manassas, VA). NFκB-deficient cell line, HCT116/dnIκB, and its revertent cell line, HCT116/dnIκB-R were previously described (Barbour and Berger, 2008). Cells were grown in RPMI-1640 medium (Cellgro) supplemented with 10% heat-activated fetal bovine serum (FBS, Atlanta Biologicals) at 37°C in a humidified 5% CO2 atmosphere. Additionally, HCT116/dnIκB cells were grown with 1mg/ml G418 (FisherBiotech, Fair Lawn, N.J.) to stabilize clone.

Drug Treatment

HCT116 and HCT116/200 cells were treated with TS inhibitors; 5-FU, FdUrd (Sigma-Aldrich Co., St. Louis, MO, USA) and RTX (AstraZeneca, Macclesfield, Cheshire, UK) for the indicated concentrations and times. 10μM folinic acid (leucovorin, Sigma-Aldrich Co., St. Louis, MO, USA) was combined with 5-FU and FdUrd treatments in every time. NAC, Thy (Sigma-Aldrich Co., St. Louis, MO, USA), Apocynin (APO, Acros Organics Inc., Geel, Belgium), ATZ (Chem Service, West Chester, PA, USA) and VAS-2870 (Enzo Life Sciences Inc., Plymouth Meeting, PA, USA) are combined with TS inhibitors and co-incubated. Diphenyleneiodonium (DPI, Calbiochem/EMD Biosciences, San Diego, CA, USA) is pretreated to HCT116 cells for 3h. After that, cells were washed

twice with Hanks solution (Cellgro Mediatech Inc., Manassas, VA) and treated with TS inhibitors for 24h.

Fluorescence Microscopy

Intracellular ROS production was measured by oxidation of cell permeable dyes H_2DCFDA and DHE (Invitrogen/Molecular Probes Inc., Eugene, OR, USA) to fluorescent DCF by H_2O_2 and to fluorescent 2-hydroxyethidium (HE) by $O_2^{\cdot -}$ respectively. Cells were grown to 60% confluence in chamber slides (VWR International LLC., Suwanee, GA, USA). After incubation, they were washed twice with PBS and were fixed in 4% paraformaldehyde (Alfa Aesar, Ward Hill, MA, USA) for 30 min at room temperature. Cells were washed twice with PBS and separately stained with 5µM H_2DCFDA and DHE in dark followed incubation at 37°C for 30 min. Slides were kept in dark after this point. After incubation, cells were washed twice with PBS and chambers were separated from slides by slide separator. Coverslips were mounted on glass slides using permount medium (Fisher Scientific, Fair Lawn, New Jersey, USA). Images were acquired at room temperature using an inverted microscope (Axiovert 200, Carl Zeiss) equipped with a Plan-Apo 63×/1.40 objective and a charge-coupled camera (AxioCam HRm, Carl Zeiss) linked to AxioVision software (version 4.7, Carl Zeiss). To get the best visual reproduction, the image input levels were adjusted by stretching the histogram. In this point, Image brightness and linearity were sustained. To analyze changes in DCF and HE fluorescence, ImageJ software (National Institutes of Health) was used to get fluorescence in numbers. Analysis of DCF and HE fluorescence was done by selecting cells with a pixel range for brightness and by subtracting background stained.

Flow Cytometry

Cells were grown to 80% confluence in 60mm plates. After incubation, they were washed twice and suspended with fluorescence-activated cell-sorting (FACS) staining buffer (PBS with 1% BSA). 0.5μM H_2DCFDA and DHE dyes were separately applied to cells for at 37°C for 15min in dark. After this point, cells were kept in dark. They were washed twice, suspended with FACS buffer and analyzed by flow cytometer (FC 500 by Beckman Coulter Fort Collins Co). Histograms were analyzed in CXP software by obtaining X-mean value for each treatment. From 3 separate experiments, average X-mean was calculated for each treatment and data was normalized in fold increase of drug treatments to control.

Measurement of Apoptotic and Necrotic Cell Death

To determine apoptotic cells, 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 stained with regard to the manufacturer's instructions following hematoxylin counterstaining and observed under a light microscope at 400X magnification. Cells exhibited brown nuclear staining that might be very dark after labeling since they contain fragmented nuclear chromatin characteristic of apoptosis. Based on staining, 1000 cells, both attached and non-attached, were counted manually for each treatment so as to calculate apoptotic content as the proportion of apoptotic/total cells.

NADPH Oxidase Assay

Cells were washed twice with PBS after incubation. 10^5 cells for each cell line and treatment were suspended in 500µl reaction buffer [50mM phosphate buffer (pH 7.0), 1mM EGTA (Research Organics, Cleveland, OH, USA), 150mM sucrose (Mallinckrodt Chemical, Paris, KY, USA)]. 100µM NADPH (Calbiochem/EMD Biosciences, San Diego, CA, USA) was added as substrate. Light sensitive 5µM lucigenin (Enzo Life Sciences Inc., Plymouth Meeting, PA, USA) was added to reaction mixture in dark. Cells were kept in dark after this point and incubated at 37°C for 10min. Negative controls like adding only NADPH to the reaction mixture without cells and adding only cells to the mixture without NADPH were prepared. After incubation, NADPH oxidase activity was immediately detected by lucigenin-derived chemiluminescence using a luminometer (GloMax-20/20 luminometer, Promega, Madison, WI, USA). The levels of emitted chemiluminescence in every 15s for 1min of measurement were saved for each treatment. The signal reading was normalized by subtracting chemiluminescence obtained from negative controls and expressed as arbitrary light units per 10^5 cells.

Microarray Analysis

RNA Isolation from HCT116 Cells: Cells were treated in quadruplicate with 10 µM of 5-FU for 24 hours. According to the recommendations of the manufacturer, total RNA was isolated from 6 x 10^6 cells using RNeasy Mini Kit (Qiagen, Maryland, USA) by additionally applying RNase Free DNase Set (Qiagen, Hilden, Germany) to eliminate contaminating genomic DNA. The integrity and concentration of the total RNA was

assessed on a 2100 Bioanalyzer (Agilent Biotechnologies), using the RNA 6000 Nano LabChip. The RNA integrity number range from 9.8 to 10.0.

mRNA Labeling and Hybridization: Microarrays experiments were performed using Agilent's platform. Total RNA samples isolated from HCT116 cells were amplified and labeled using Agilent's Low Input Quick Amp Labeling Kit (Cat. # 5190-2306) according to the manufacturer recommendations. Briefly, mRNA contained in 200ng of total RNA was converted into cDNA using a poly-dT primer that also contained the T7 RNA polymerase promoter sequence. Subsequently, T7 RNA polymerase was added to cDNA samples to amplify original mRNA molecules and to simultaneously incorporate cyanine 3- or cyanine 5-labeled CTP (cRNA) into the amplification product. In addition, Agilent RNA spike-in controls (Cat. # 5188-5279) were added to samples prior to cDNA synthesis and were used as experimental quality control. In the next step, labeled RNA molecules were purified using Qiagen's RNeasy Mini Kit (Cat. # 74104). After spectrophotometric assessment of dye incorporation and cRNA yield, samples were store at -80°C until hybridization. Labeled cRNA samples were hybridized to Agilent Human GE 4 x 44K v2 Microarrays (Cat. # G4845A) at 65°C for 17 hours using Agilent's Gene Expression Hybridization Kit (Cat. # 5188-5242) according to the manufacturer's recommendations. Four (4) control sample replicates we hybridized against four (4) 5-FU treated sample replicates in a dye swap design. After washes, arrays were scanned using an Agilent DNA Microarray Scanner System (Cat. # G2565CA).

Data analysis: Data was extracted from images with Feature Extractor Software version 10.7.3.1 (Agilent). In this process, background correction using additive and

multiplicative detrending algorithms was performed. In addition, linear and LOWESS methods were used for dye normalization. Subsequently, background-corrected, dye-normalized data was uploaded into GeneSpring GX version 11.5.1 for analysis. In this process, data was log2 transformed, quantile normalized and base line transformed using the median of all samples. Then, data was filtered by flags in a way that 100% of the samples in at least one of the two treatment groups have a "detected" flag. Differentially expressed genes were determined by analysis of the data using an unpaired t-test statistics that was corrected for multiple testing using the Benjamini-Hochberg algorithm. A cutoff p-value of 0.005 was used. Additionally, a fold change cutoff value of 1.5 was used to filter the data

Semi-quantitative RT-PCR

After each treatment, total RNA was isolated using RNeasy Mini Kit (Qiagen, Maryland, USA) by additionally applying RNase Free DNase Set (Qiagen, Hilden, Germany) to eliminate contaminating genomic DNA. RNA concentration was spectrophotometrically measured at 260 and 280nm absorbance (NanoDrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, USA). For each reaction, 1µg RNA was reversely transcribed in a Mycycler Thermal Cycler (Biorad, Hercules, CA) with iscript cDNA synthesis kit (Biorad, Hercules, CA, USA) according to manufacturer procedures. The reactions were incubated for 5 min in 25°C, 30 min in 42°C, heated for 5 min in 85°C and hold at 4°C. The relative expression of each gene was assessed in indicated PCR cycles. Controls with no reverse transcriptase enzyme and no template RNA were used to check contamination in each gene. The different PCR tubes within each series were set up such that they went

through PCR for increasing numbers of amplification cycles. The PCR reactions for each gene were run with 1 µl of cDNA and 250ng primers (Table 1, Integrated DNA Technologies Inc., Coralville, IA, USA) in a total volume of 25 µl, using GoTaq Hot Start Green Master Mix (Promega, Madison, WI, USA).

All PCR reactions comprised an initial denaturation step for 2 min at 95°C and followed by denaturation (30s at 95°C), extension (30s at 72°C) and final extension step (5 min at 72°C). Annealing step was 30s at 50°C for GAPDH, Rac1, NOXA1, gp91 and $p67^{phox}$; at 49.5°C for $p47^{phox}$; at 60°C for NOXO1 and at 52°C for NOX1. Amplification cycles were from 18 to 33 for GAPDH and Rac1 and 32 to 44 for other genes. The amplified cDNA products were resolved by 1.5% (w/v) agarose gel electrophoresis and visualized by staining with ethidium bromide (Sigma-Aldrich Co., St. Louis, MO, USA).

Quantitative PCR

According to the manufacturer's suggestions, cDNA (1µl) was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Reactions were run for one cycle at 95°C for 10 min, and 40 cycles of 95°C for 15s, 50°C for 15s and 72°C for 40s using an Applied Biosystems 7300 Real Time PCR System. All mRNA levels were quantified relative to GAPDH by measuring SYBR green incorporation during quantitative PCR using the relative standard curve method. Statistical analyses and calculations were done as described in the manufacturer's protocol. Relative changes in each gene levels between drug-treated and nontreated cells are expressed as fold induction compared with the basal level of expression in nontreated cells. Primers

(Integrated DNA Technologies Inc., Coralville, IA, USA) used for qPCR are listed in Table 2.

Western Blotting

Cells were lysed in M-PER® mammalian protein extraction reagent (Thermo scientific, Rockford, IL, USA) and protein content was determined colorimetrically using the Bradford assay (Bio-Rad, Hercules, CA, USA) with BSA as protein standart. Lysates (100 µg) were run on by 12.5% Tris/HCl (Bio-Rad), electrophoresed in running SDS-PAGE buffer, transferred to PVDF membranes and probed with anti-$p67^{phox}$ goat polyclonal antibody (sc7662, C-19, Santa Cruz, Dallas, TX, USA) at a dilution of 1:200 in 3% non-fat milk in PBS/0.05% Tween for 1 hour at room temperature. The membrane was washed three times for 10 min with PBS/0.05% Tween before and after incubated with appropriate secondary antibody 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.

Statistical Analysis

All data were reported as the mean ± SEM. Statistical significance of the mean for each groups were determined using student's t-test. Differences with $P \leq 0.05$ were considered statistically significant.

Table 5.1. **Primers used for semi-quantitative RT-PCR**

mRNA	Primer (F:5'-3', R:5'-3')	Product Size (bp)
p22	F: ATGGAGCGCTGGGGACAGAAGTACATG R: GATGGTGCCTCCGATCTGCGGCCG	252
gp91	F: TGGTACACACATCATCTCTTTGTG R: AAAGGGCCCATCAAGCGCTATCTTAGGTAG	558
NOX1	F: TGAAGGACCTCTCCAGAATC R: CAGGTGTGCAATGATGTG	434
$p47^{phox}$	F: ACCCAGCCAGCACTATGTGT R: AGTAGCCTGTGACGTCGTCT	767
$p67^{phox}$	F: CGAGGGAACCAGCTGATAGA R: CATGGGAACACTGAGCTTCA	747
NOXO1	F: GGCAGCCCTGGTGCAGATCAAGAGGC R: CAGTCGCCAGCAGCCTCCGAGAATAGG	281
NOXA1	F: CCATCGACTACACGCAGCT R: GTAGGCAGTCGACGTGCAGC	467
Rac1	F: GGTGAATCTGGGCTTATGGG R: CTAGACCCTGCGGATAGGTG	280
GAPDH	F: ACCACCATGGAGAAGGCTGG R: CTCAGTGTAGCCCAGGATGC	528

Table 5.2. **Primers used for qPCR**

mRNA	Primer (F:5'-3', R:5'-3')	Product Size (bp)
p22	F: ATGGAGCGCTGGGGACAGAAGTACATG R: GATGGTGCCTCCGATCTGCGGCCG	77
gp91	F: TGGTACACACATCATCTCTTTGTG R: AAAGGGCCCATCAAGCGCTATCTTAGGTAG	94
NOX1	F: CTCCCTTGCCTCCATTCTC R: AGGCTATTGTCATGATCACTCC	149
$p40^{phox}$	F: AAAGTCAAGAGCGTGTCCC R: GAGGAAGATCACATCTCCAGC	132
$p47^{phox}$	F: ACACCTTCATCCGTCACATC R: GAACTCGTAGATCTCGGTGAAG	143
$p67^{phox}$	F: CGAGGGAACCAGCTGATAGA R: CATGGGAACACTGAGCTTCA	131
NOXO1	F: AGATCAAGAGGCTCCAAACG R: AGGTCTCCTTGAGGGTCTTC	117
NOXA1	F: CAGGCTGTGGATCGTGG R: CACGGCTTGGTCAAATGC	150
Rac1	F: GGTGAATCTGGGCTTATGGG R: CTAGACCCTGCGGATAGGTG	82
GAPDH	F: ACCACCATGGAGAAGGCTGG R: CTCAGTGTAGCCCAGGATGC	218

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