

## Differential oxidative response to fluoropyrimidines in colorectal cancer cell lines

Ufuk ÖZER<sup>1,2,\*</sup>, Karen WOOD BARBOUR<sup>1</sup>

<sup>1</sup>Department of Biological Sciences, Faculty of Science, University of South Carolina, Columbia, SC, USA

<sup>2</sup>Department of Molecular Biology and Genetics, Faculty of Science, Dicle University, Diyarbakır, Turkey

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**Abstract:** Colorectal cancer (CRC) is the second most common human malignancy in women and the third in men, accounting for approximately 8% of cancer-related deaths and 1.3 million newly diagnosed cases annually worldwide. CRC cell lines differ in morphological features, growth rate, and their genetic alterations, implicating the extent of variability among the lines. The goal of the current study was to investigate the phenotypes of CRC lines related to NADPH oxidase (NOX) activity and expression of NOX2 subunits in response to fluoropyrimidines. NOX activity in response to 5'-fluoro-2'-deoxyuridine (FdUrd) and VAS2780 was measured in CRC cell lines. Cells were treated with FdUrd in order to determine apoptotic cell death and mRNA expressions of NOX2 cytoplasmic subunits. We find that both basal and drug-induced NOX activity differ in the lines. Similarly, drug-mediated apoptotic indices vary in the cell lines at basal levels. Among NOX1 and NOX2 subunits, only NOX2 accessory subunits, p67phox, p40phox, and p47phox, differ in the lines. Our results show that CRC cells have diverse oxidative background and their oxidative response to anticancer drugs vary, while they all exhibit increases in NOX activity.

**Key words:** NADPH oxidase, fluoropyrimidines, oxidative stress, CRC cells

### 1. Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide in males and the disease rate is the highest in developed countries, including the US, Australia, Canada, and Europe. It accounts for 8% to 9% of the estimated cancer deaths in the US in 2015 (Siegel et al., 2015). Treatment of CRC depends upon the degree of progression of the cancer and it can be curative if caught at an early stage (Cunningham et al., 2010; Stein et al., 2011). Survival rate (5 years) has not changed with the recent advances and chemotherapy is still the mainstay of treatment (Lucas et al., 2011; Platell et al., 2011). Fluoropyrimidines, in particular, FUra and its nucleoside analogue 5'-fluoro-2'-deoxyuridine (FdUrd), are widely used agents and remain a major component of many standard regimens for various cancer types (Sotos et al., 1994; Milano et al., 2004; Soong and Diasio, 2005; Soong et al., 2008). FUra and FdUrd inhibit thymidylate synthase (TS), which blocks thymidylate (dTTP) production (Berger and Berger, 2006; Wyatt and Wilson, 2009) and leads to genome damage through misincorporation of uracil into DNA, and shuts off DNA synthesis and repair, triggering apoptosis (Dananberg, 1977; Spears et al., 1988; Krokan et al., 2002; Longley et al., 2003). It has been suggested that generation of reactive

oxygen species (ROS) is one of the consequences of TS inhibition. ROS production is increased by FUra, and the inhibition of ROS reverses its cytotoxicity (Ueta et al., 1999; Hwang et al., 2001; Laurent et al., 2005; Alexandre et al., 2006; Hwang et al., 2007; Shibata et al., 2008). As a result of drug treatment, ROS are released and partially trigger apoptosis in cancer cells (Gallego 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; Bognoux et al., 2009). It is known that the activity of ROS generating enzymes like NADPH oxidases is increased to kill cancer cells (Lambeth, 2004; Kumar et al., 2008). The family of NADPH oxidases (NOX); NOX1-5 and dual oxidases (Duox), Duox1 and Duox2 produce ROS as their primary and sole function (Lambeth, 2004; Bedard and Krause, 2007; Altenhöfer et al., 2012). 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). We have previously reported that augmentation of NOX2 activity via induction of p67phox mRNA expression is the proximate cause of oxidative cell death in HCT116 cells (Ozer et al., 2015).

\* Correspondence: ufuk.ozer@dicle.edu.tr

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). Established and characterized cell lines were classified into different groups based on their morphological features, growth rate, and other functional parameters, implicating a broad range of systemic heterogeneity and drug response (Leibovitz et al., 1976; Brattain et al., 1981; McBain et al., 1984; Ribas et al., 2003; Flatmark et al., 2004; 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). In spite of these interesting findings, understanding of how these cells indicate diverse response to drugs is still lacking. In the present study, we examined levels of NOX induced-oxidative stress and -apoptotic cell death in response to TS inhibitors in different colon cancer cells. We found variations regarding NOX activity, expression of NOX2 subunits, and the role of NOX in apoptotic response to TS inhibitors.

## 2. Materials and methods

### 2.1. Materials

FUra and FdUrd were from Sigma-Aldrich Co. (St. Louis, MO, USA). NADPH was purchased from Calbiochem/EMD Biosciences (San Diego, CA, USA). Lucigenin and VAS-2870 were provided by Enzo Life Sciences Inc. (Plymouth Meeting, PA, USA). Paraformaldehyde was from Alfa Aesar (Ward Hill, MA, USA). Oligonucleotides were from Integrated DNA Technologies Inc. (Coralville, IA, USA).

### 2.2. Cell culture

Human colon tumor cell lines HCT116 (originally obtained from Dr. Michael G. Brattain), SW480, HCT15, DLD-1, LoVo, MOSER, and LS180 (obtained from American Type Culture Collection, Manassas, VA, USA) were grown in RPMI-1640 medium (Cellgro, Manassas, VA, USA) supplemented with 10% heat-activated fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) at 37 °C in a humidified 5% CO<sub>2</sub> atmosphere.

### 2.3. Measurement of apoptotic cell death

TS inhibitors and inhibitory agents were applied to cells at the indicated concentrations and times. Apoptotic indices were determined by TUNEL assays, performed using the In Situ Cell Death Detection Kit, POD (Roche Applied Science, Indianapolis, IN, USA). Cells were stained according to the manufacturer's instructions, counterstained with hematoxylin, and viewed under a light microscope at 400× magnification. Pictures from each treatment were taken with camera installed on top of

the microscope. Apoptotic nuclei were counted manually, based on staining and morphology, and the apoptotic index was calculated as the ratio of apoptotic/total cells. In each determination, at least 1000 cells from several microscopic fields were counted.

### 2.4. NADPH oxidase assay

For each reaction, 10<sup>5</sup> cells were suspended in 500 µL of reaction buffer [50 mM phosphate buffer (pH 7.0), 1 mM EGTA, 150 mM sucrose]. NADPH oxidase activity was detected by lucigenin-derived chemiluminescence with 100 µM NADPH as substrate and 5 µM lucigenin. Cells were incubated at 37 °C for 10 min. Chemiluminescence was measured using a luminometer (Promega, Madison, WI, USA) and expressed as arbitrary light units per 10<sup>5</sup> cells.

### 2.5. RNA extraction and RT-PCR

Total RNA was isolated using RNeasy Mini Kit (Qiagen, MD, USA) with the addition of RNase Free DNase (Qiagen, Hilden, Germany) to eliminate contaminating genomic DNA. The RNA concentration was determined by measuring the absorbance at 260 nm and 280 nm (NanoDrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, USA). For each reaction, 1 µg of RNA was reverse transcribed using an iScript cDNA synthesis kit (Biorad, Hercules, CA, USA) according to the manufacturer's instructions. For quantitative PCR (qPCR), cDNA (1 µL) prepared as described above was amplified using Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The PCR thermal profile was one cycle at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s, 50 °C for 15 s, and 72 °C for 40 s using an Applied Biosystems 7300 Real Time PCR System. Relative mRNA levels were normalized to GAPDH, and calculated by the 2<sup>-ΔΔCt</sup> method. Relative changes in expression of each gene in response to TS inhibitors were expressed as fold-induction compared with the basal level of expression in nontreated cells. Gene-specific primer sets (Integrated DNA Technologies) used for qPCR are listed in the Table. Controls with no reverse transcriptase and no template RNA were used to monitor contamination.

### 2.6. 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 standard. Lysates (100 µg) were run on 12.5% Tris/HCl (Bio-Rad), electrophoresed in running SDS-PAGE buffer, transferred to PVDF membranes, and probed with anti-p67phox goat polyclonal antibody (sc7662, C-19, Santa Cruz, Dallas, TX, USA) at a dilution of 1:200 in 3% nonfat milk in PBS/0.05% Tween for 1 h at room temperature. The membrane was washed three times for 10 min with PBS/0.05% Tween before and after

**Table.** Sequence of the primers for quantitative RT-PCR.

Genes	Primers (5' to 3')
p67phox	sense: 5'- ACCAGAAGCATTAACCGAGAC -3' antisense: 5'- TTCCCTCGAAGCTGAATCAAG -3'
p47phox	sense: 5'- GCTGGTGGGTCATCAGGAA -3' antisense: 5'- GCCCCGACTTTTGCAGGTA -3'
p40phox	sense: 5'- GCTTCACCAGCCACTTTGTT -3' antisense: 5'- TCCTGTTTCACACCCACGTA -3'
GAPDH	sense: 5'- TCCCTGAGCTGAACGGGAAG -3' antisense: 5'- GGAGGAGTGGGTGTCGCTGT -3'

incubation with appropriate secondary antibody for 1 h at room temperature. An 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.

### 2.7. Statistical analysis

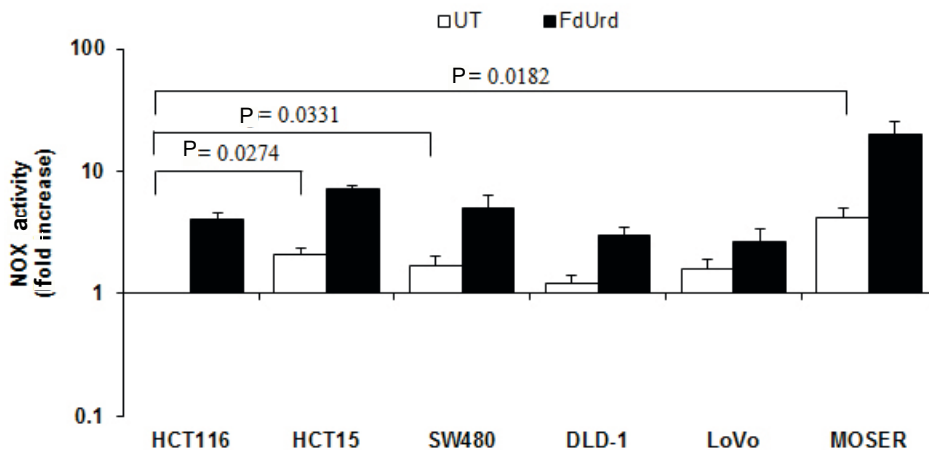
All data were reported as the mean  $\pm$  SEM. Statistical significance of the mean for each group was determined using Student's t-test. Differences with  $P \leq 0.05$  were considered statistically significant.

## 3. Results

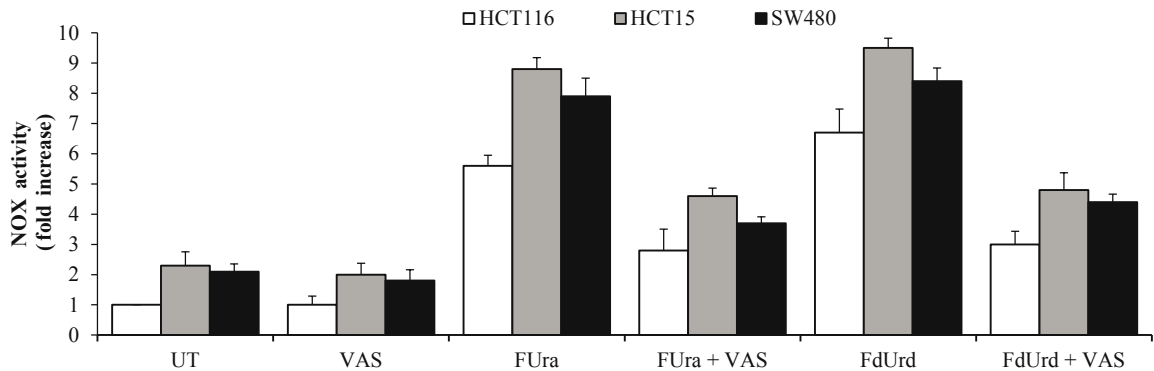
### 3.1. Human colon cancer cell lines exhibit different levels of NOX activity

Human colon cancer cell lines show a wide range of genetic, morphological, and functional heterogeneity (Leibovitz et al., 1976; Brattain et al., 1981; McBain et al., 1984). 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 10  $\mu$ M FdUrd for 24 h, and NOX activity was measured. HCT116 basal activity levels were set as 1 and the fold-increase in 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) and drug-inducible (4.8-fold,  $P = 0.0174$ ) 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,  $P = 0.025$  and 1.7-fold, respectively) activity levels (Figure 1).

In order to verify whether NOX activity was decreased by a specific NOX inhibitor, VAS-2870, in cell lines, we examined NOX activity in response to FUra and FdUrd in human colon cancer cell lines: HCT116, HCT15, and SW480. NOX activity was induced ( $P < 0.05$ ) by drugs for all 3 colon cancer lines; furthermore, the activation of NOX in all 3 cell types was diminished ( $P < 0.05$ ) by VAS-2870 (Figure 2).



**Figure 1.** NOX activity levels vary in human colon cancer cell lines. HCT116, HCT15, SW480, DLD-1, LoVo, and MOSER cell lines were grown  $\pm$  10  $\mu$ M FdUrd for 24 h and then were subjected to NOX activity assay. Lucigenin derived chemiluminescence was measured by luminometer. Relative fold increase was calculated in reference to HCT116 control, which was set as 1. Bars represent fold increase in chemiluminescence  $\pm$  SEM from 3 experiments.



**Figure 2.** VAS reduces NOX activity increased by drugs in colon cancer cell lines. HCT116, SW480, and HCT15 cells were treated with both TS inhibitors (10  $\mu$ M Fura, 10  $\mu$ M FdUrd) and 10  $\mu$ M VAS for 24 h. NOX activity was measured by luminometer. Relative fold increase was calculated in reference to HCT116 control, which was set as 1. Bars represent fold increase of chemiluminescence  $\pm$  SEM from 3 experiments.

### 3.2. 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) and 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 h, and apoptotic indices were evaluated by TUNEL assay. We observed that drug-mediated cells were swelled and apoptotic cells were dark brown (Figure 3A). It was observed that basal levels of apoptosis change from cell line to cell line. LoVo exhibited 3-fold higher ( $P = 0.009$ ) basal levels relative to HCT116 cells and HCT15. Drug-mediated increases in apoptotic indices were greater in HCT116 and HCT 15 cells (16-fold,  $P = 0.0076$  and 14-fold,  $P = 0.0084$ ) respectively than SW480 and LoVo cells (8-fold,  $P = 0.0088$  and 6.5-fold,  $P = 0.0092$ ), respectively (Figure 3B).

### 3.3. mRNA expressions of NOX2 accessory subunits, p67phox, p40phox, and p47phox, 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 in NOX activity levels and apoptotic responses in human colon cancer cells, we determined whether mRNA levels of NOX2 accessory subunits, p67phox, p40phox, and p47phox, vary in different colon cancer cells. Cells were treated with FdUrd for 24 h and expression of the subunits was assessed by qPCR. Human colon cancer cell lines (HCT116, HCT15, SW480, DLD-1, LoVo, MOSER,

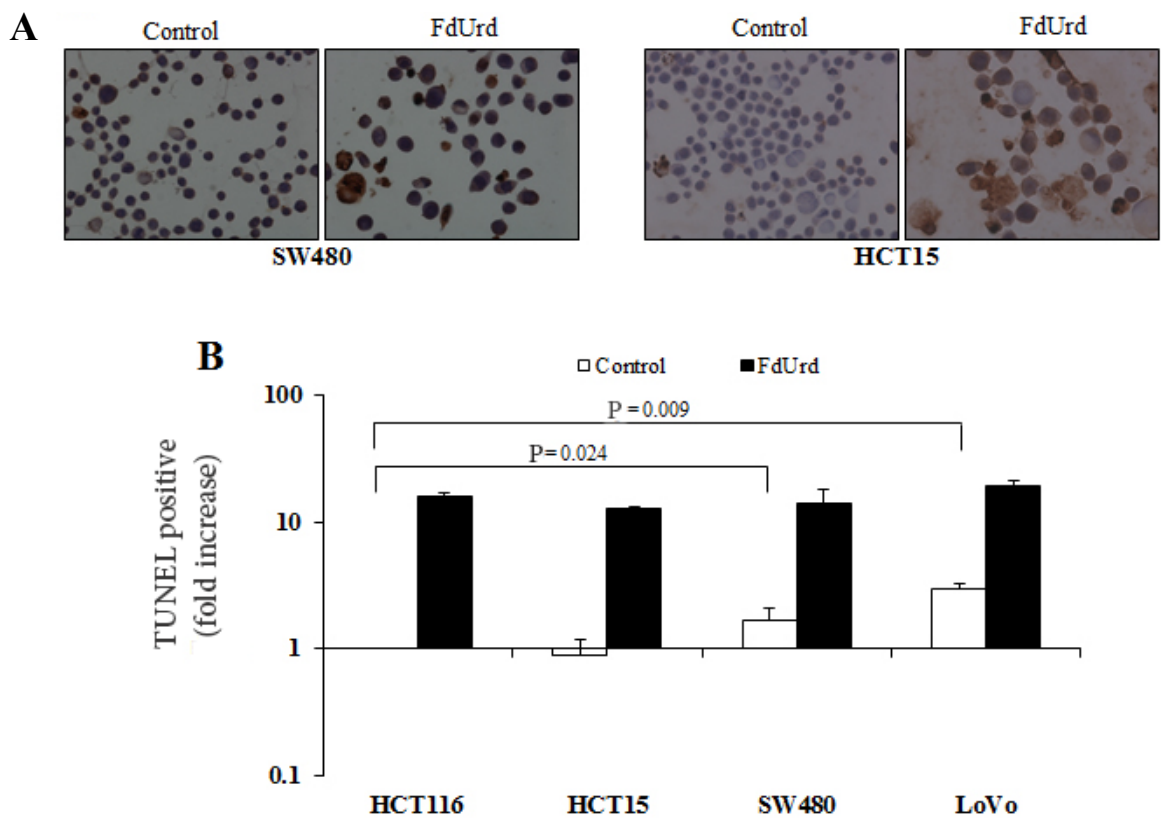
and LS180) showed different basal and drug-inducible mRNA levels for p67phox (Figures 4A). LoVo, MOSER and LS180 exhibited high basal levels of p67phox ( $P < 0.05$ ) while SW480 exhibited intermediate levels ( $P < 0.05$ ). HCT116, HCT15 and DLD-1 expressed low basal levels of p67phox. FdUrd-induced p67phox levels were seen in HCT116 and DLD-1, about 23-fold ( $P = 0.0045$ ) and 9-fold ( $P = 0.0076$ ), respectively (Figure 4A). Protein levels of p67phox subunits in colon cancer cells exhibited the same trend as seen in its mRNA expression (Figure 4B). Low basal levels of p67phox in several lines could not be detected by western-blot analysis.

Expression of p40phox was low in HCT15, LoVo, and MOSER cells compared to HCT116, SW480, DLD-1, and LS180. FdUrd-induced p40phox levels in HCT15 and LoVo cells were 8-fold ( $P = 0.025$ ) and 10-fold ( $P = 0.021$ ), respectively. HCT116 and MOSER cells exhibited low levels of drug-induced p40phox levels while SW480, DLD-1, and LS180 expressed intermediate levels (Figure 5).

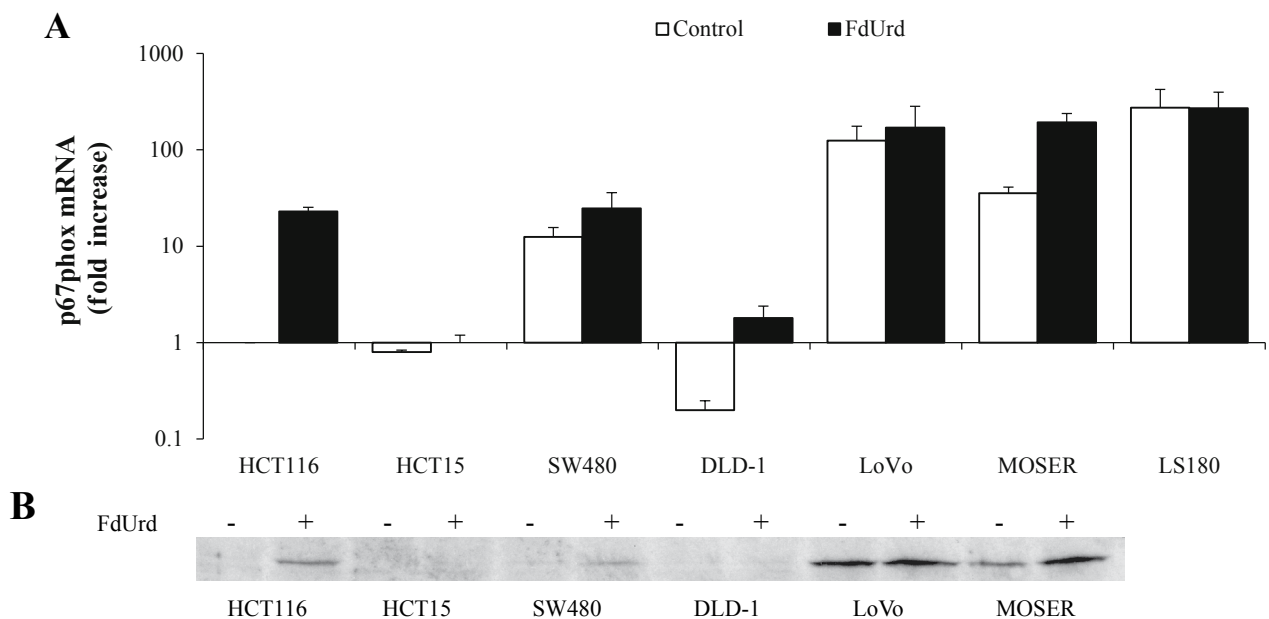
Basal and drug-inducible mRNA levels of p47phox also vary among the lines. HCT116 and SW480 showed similar basal and drug-inducible mRNA levels of p47phox. Although MOSER and LS180 exhibited similar basal levels of p47phox, drug-induced levels were unchanged in MOSER, but were induced 8.5-fold ( $P = 0.019$ ) in LS180 cells (Figure 6).

## 4. Discussion

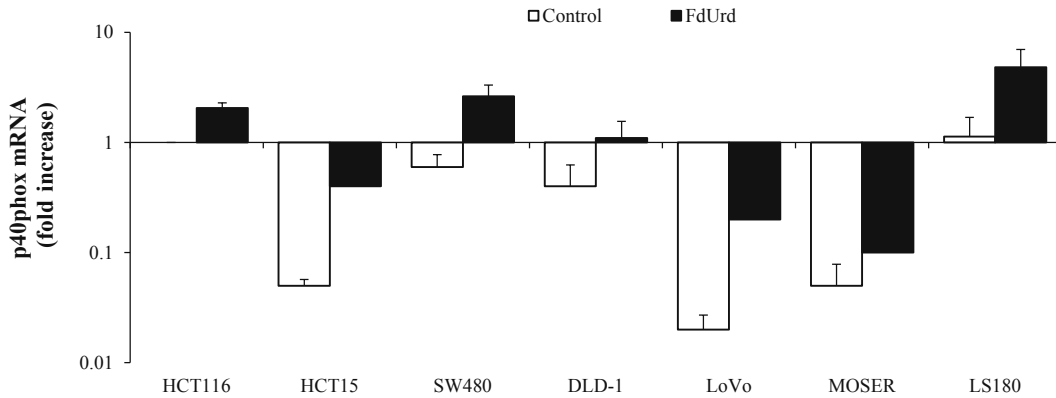
Cancer laboratories establish a large number of human colorectal cancer cell lines from surgical specimens in the pathology laboratories. Based on their morphological features, growth rate, karyotypes, ability to synthesize carcinoembryonic antigen (CEA), etc., these lines were classified into different groups (Leibovitz et al., 1976; McBain et al., 1984). Well-described colon cancer cells



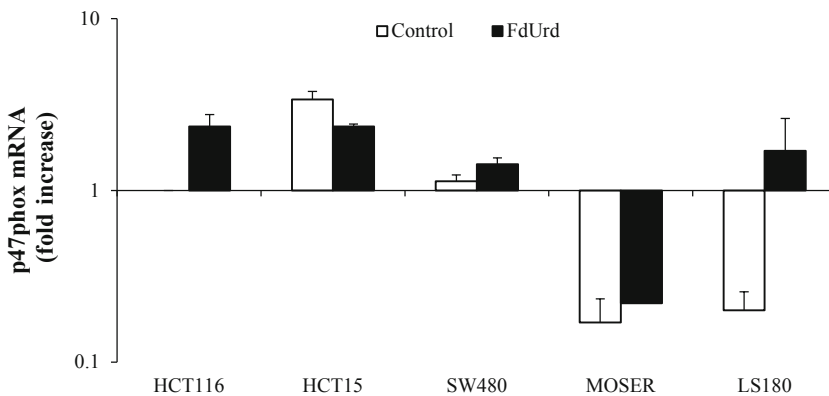
**Figure 3.** FdUrd diversely induces apoptosis in human colon cancer cells. HCT116, HCT15, SW480, and LoVo cells were grown with and without 100  $\mu$ M FdUrd for 24 h. Extents of apoptosis were determined by TUNEL assay. A. Cells were photographed at 400 $\times$ . B. Relative fold increase was calculated in reference to HCT116 control, which was set as 1. Bars represent fold increase of apoptotic indices  $\pm$  SEM from 3 experiments.



**Figure 4.** mRNA levels of p67phox vary in human colon cancer cell lines. A. qPCR was used to measure mRNA levels of p67phox in total RNA isolated from HCT116, HCT15, SW480, DLD-1, LoVo, MOSER, and LS180 cultured for 24 h  $\pm$  10  $\mu$ M FdUrd. Relative fold increase was calculated in reference to HCT116 control, which was set as 1. GAPDH was tested as a loading control. Bars represent an average of fold increase  $\pm$  SEM from 3 experiments. B. Protein levels of p67phox were measured for the lines cultured for 24 h  $\pm$  10  $\mu$ M FdUrd.



**Figure 5.** mRNA levels of p40phox vary in human colon cancer cell lines. qPCR was used to measure mRNA levels of p40phox in total RNA isolated from HCT116, HCT15, SW480, DLD-1, LoVo, MOSER, and LS180 cultured for 24 h  $\pm$  10  $\mu$ M FdUrd. GAPDH was tested as a loading control. Relative fold increase was calculated in reference to HCT116 control, which was set as 1. Bars represent an average of fold increase  $\pm$  SEM from 3 experiments.



**Figure 6.** mRNA levels of p47phox vary in human colon cancer cells. qPCR was used to measure mRNA levels of p47phox in total RNA isolated from HCT116, HCT15, SW480, DLD-1, LoVo, MOSER, and LS180 cultured for 24 h  $\pm$  10  $\mu$ M FdUrd. GAPDH was tested as a loading control. Relative fold increase was calculated in reference to HCT116 control, which was set as 1. Bars represent an average of fold increase  $\pm$  SEM from 3 experiments.

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). Heterogeneity resulting from any system in cancer cells may bring about resistance to chemotherapeutic drugs (de Anta et al., 2006). However, understanding the mechanism of the heterogeneity creates an opportunity to increase the efficacy of drugs in the treatment of tumor cells. It is possible that a number of biomarkers differing in cell lines might be relevant to variations. Knowledge augmentation of heterogeneity in cell lines will lead to improved therapy.

In the present study, we demonstrated that FdUrd induced apoptosis 6.5- to 16-fold change in human colon cancer cell lines. These different inductions result from

variation in basal apoptosis levels of cell lines, reflecting the heterogeneity in cell lines. Our findings make it clear that FdUrd induces cell death in all cell lines, but its effects on their survival vary because of different backgrounds.

We have indicated that increases in NOX activity by TS inhibitors were in HCT116, SW480, and HCT15 cell lines and VAS2870 attenuated drug-induced activity (Figure 2). In order to expand the results, 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. Thus, cell lines differ in the oxidative environment and exhibit, in the presence of fluoropyrimidines, different mechanisms to modulate it.



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). As shown in Figure 4A, p67phox expression in SW480, LoVo, MOSER, and LS180 is much higher than in HCT116 while it is lower in DLD-1 and HCT15. Therefore, drug inducible expression levels of p67phox are hardly seen in SW480 and LoVo. However, p67phox expression levels were further induced by FdUrd in MOSER cells despite very high basal expression levels. These results are not consistent with basal NOX activity levels in cell lines (Figure 1), indicating the importance of other subunits in the activation. Then we looked at expression of other subunits like p40phox and p47phox. Basal p40phox expression was much lower in HCT15, LoVo, and MOSER cells compared to HCT116 cells. FdUrd-induced p40phox levels are easily seen in all cell lines, especially in HCT15 and LoVo cells, thereby showing a potential contribution of p40phox in activation (Figure 5). In MOSER and LS180 cells, expression of p47phox is lower than in HCT116 cells, however; it is higher in HCT15 cells. FdUrd significantly induced p47phox expression in only LS180 and HCT116 cells, implicating the role of p40phox in the activation in these cells (Figure 6). The logic behind these experiments was profiling various colon cancer cell lines in terms of their NOX metabolism. Experiments were done for all NOX1 and NOX2 subunits, and variable expression levels for each subunit were plotted as shown in Figures 4A, 5, and 6. Among all subunits, only NOX2 cytosolic subunits, p67phox, p40phox, and p47phox, show alteration in basal and FdUrd-inducible levels. Although expression levels of subunits are changed differently in colon cancer cell lines,

they all work for the activation of NOX2 enzyme. Therefore, targeting this enzyme may enhance therapeutic approaches in FdUrd-mediated cancer therapy.

CRC cell lines were genetically and epigenetically characterized for mutations in oncogenes including *KRAS*, *BRAF*, *PIK3CA*, *PTEN*, and *TP53* (Ahmed et al., 2013). HCT15, DLD-1, HCT116, LoVo, and SW480 cell lines showed no mutations on *BRAF* and *PTEN* genes; however, *KRAS* mutations were found in all lines. There were no genetic differences in HCT15 and DLD-1 cells derived from the same patient. In our study, these cells also show similar patterns for NOX activity, protein, and mRNA expression levels of p67phox (Figures 1, 4A, and 4B). *PIK3CA* mutations were not found in LoVo and SW480 cell lines, while DLD-1, HCT15, and HCT116 were characterized for the mutations. Accordingly, basal p67phox expression was higher in LoVo and SW480 compared to DLD-1, HCT15, and HCT116 cells (Figure 4A).

Based on our results, the antitumor effects of FdUrd appear to vary among colon cancer cell lines, reflecting phenotypic 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.

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