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### RESEARCH ARTICLE

#### INHIBITION OF NADPH OXIDASE ACTIVITY AUGMENTS 5-FLUOROURACIL MEDIATED CELL DEATH IN HUMAN COLON CARCINOMA HCT-116 CELLS

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#### Abstract

5-Fluorouracil (5-FU) is a well known chemotherapeutic agent, which has been most commonly used for the treatment of colon cancer. 5-FU significantly reduces tumor burden, but the progression of metastasis with the phenotype of drug resistance is a major obstacle in successful anti-cancer therapy. The cellular redox balancing genes and proteins participates in cancer metastasis and drug resistance. The cellular redox balance is linked with the level of intracellular ROS and its source, NADPH Oxidase (NOX). Compelling evidences suggest that the activation of various NOX isoforms modulates the development and progression of malignancies. However, the role of NOX enzyme activation during 5-FU treatment has not been investigated. In the present study, we aimed to analyze the role of NOX activity upon 5-FU treatment on HCT-116 cells. We found that the 5-FU inhibits cell proliferation and induces cell death in HCT-116 cells. Moreover elevated NOX activity and subsequent ROS generation was found in 5-FU treated HCT-116 cells. Interestingly, the inhibition of NOX activity using known inhibitor Diphenylene iodonium (DPI) augmented 5-FU mediated cell death. These findings suggest that NOX activation resists 5-FU mediated cell death in HCT-116 cells. Therefore, targeting NOX enzymes may be a potent therapeutic strategy to increase the efficacy of 5-FU treatment in cancer therapy.

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#### Introduction:-

5-Fluorouracil (5-FU) is an anti-metabolite and a potent fluoropyrimidine anticancer agent (Longley et al., 2003). Chemically, 5-FU is a structural analogue of uracil, a major pyrimidine involves in the synthesis of RNA (Zhang et al., 2008). 5-FU rapidly enters the cells and incorporated into RNA and DNA, which disrupts their normal function. Moreover, intracellular 5-FU converts into several metabolites that disrupts RNA synthesis and inhibits the thymidylate synthase (TS) (Longley et al., 2003). 5-FU is a widely used anticancer agent in the treatment of many types of cancers including colorectal, breast and head and neck cancers (Grem, 2000). 5-FU is one of the most frequently used chemotherapeutic drugs in regimens of anti-cancer therapy. However, most chemotherapeutic agents have systemic toxicity and adverse side effects (Fang et al., 2016). Moreover, the development of drug resistance

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and tumor recurrence in colon cancer cells also challenges the conventional chemotherapeutic strategies (Hammond et al., 2016).

Previous reports suggest that the 5-FU influences cellular redox balance in colon cancer cells (Liu et al., 2017). It is known to induce ROS mediated apoptosis (Fan et al., 2013). 5-FU mediated ROS generation regulates p53 activation and promotes apoptosis (Hwang et al., 2001). The ROS is also known to modulate multiple cell signaling events, which promotes the development and progression of malignancies (Liou and Storz, 2010). There are several sources of intracellular ROS including mitochondria, Peroxisome, and various cellular enzyme systems (Holmström and Finkel, 2014). Cancer cells tend to redirect redox imbalance from cell death to cell survival.

NADPH Oxidases (NOXs) are the major sources of cellular ROS. NOXs are the multisubunit enzyme system that catalyzes the transfer of a single electron from NADPH to molecular oxygen and generates free radicals. There are seven NOX isoforms known so far: NOX-1, NOX-2, NOX-3, NOX-4, NOX-5, DUOX-1, and DUOX-2. NOXs are known to play a vital role in various physiological functions and pathological consequences. Recent reports suggest that the NOX mediated ROS generation involves in the progression of various malignancies (Weyemi et al., 2013). Several NOX isoforms: NOX-1 and NOX-2 involve in the development and progression of colon cancer cells (Banskota et al., 2015; Juhasz et al., 2017). Moreover, oncogene such as RAS regulates the expression and activation of NOX-1 via the Raf/MEK/ERK pathway and maintain the oncogenic phenotypes (Adachi et al., 2008).

In the present study, we explored the involvement of NOX during 5-FU mediated ROS generation in consequence with cell proliferation and cell death in human colon carcinoma cells. This study may be useful to improve the therapeutic efficacy of 5-FU by targeting the NOX inhibition in regimens of cancer therapeutics.

## **Materials & Methods:-**

### **Materials:**

All molecular biology grade chemicals were purchased commercially. RPMI 1640 medium, Dulbecco's Phosphate Buffer Saline (DPBS), Fetal Bovine Serum (FBS), PSN (Penicillin, Streptomycin, and Neomycin) antibiotic solution and CFDA dye were purchased from GIBCO, life technologies (USA). 2',7'-Dichlorofluorescein diacetate (H<sub>2</sub>DCFDA), Nitro Blue Tetrazolium (NBT), Thiazolyl Blue Tetrazolium Bromide (MTT), Trypan Blue dye and was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### **Cell lines and culture:**

Human colorectal carcinoma cell line (HCT-116) was obtained from the National center for cell science (NCCS), Pune, India. The cells were grown in RPMI 1640 culture medium (containing L-glutamine (2mmol/l)) supplemented with 10% fetal bovine serum and antibiotics mixture containing penicillin (5 mg/ml), streptomycin (5 mg/ml), and neomycin (10 mg/ml). The cells were kept in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> in a CO<sub>2</sub> incubator at 37°C. Exponentially growing cultured cells were used for all the experiments.

### **Treatment:**

The stock solutions of 5-Fluorouracil (5-FU) (5mM and 50 mM) and DPI (10mM) were freshly prepared in cell culture grade DMSO. HCT 116 cells were treated with 5 µM, 10 µM, 25 µM, and 50 µM of 5-Fluorouracil for 3h, 6h and 48h with or without pretreatment of DPI (10µM) for 2h. Similarly, 0.2% DMSO was used as vehicle control. The respective changes in the treatment have been mentioned in figure legends.

### **MTT Assay:**

The cell proliferation of HCT-116 upon the treatment of 5-FU was determined by MTT assay as described earlier (Waghela et al., 2015). Briefly, HCT 116 cells were treated with a series of concentrations (5 µM, 10 µM, 25 µM, 50 µM, 100 µM, 500 µM) of 5-FU for 48h with or without pretreatment of DPI. The cells were washed with DPBS and incubated with 0.5 mg/ml MTT solution for 4 h at 37°C. Thereafter, 0.1 ml of SDS-HCl (10% SDS in 0.01M HCl) was added to each well, mixed thoroughly, and allowed for incubation in the dark for 20 min at 37°C. Finally, the absorbance was recorded at 570 nm with a reference wavelength of 650 nm using a multimode microplate reader (SpectraMax M2e, Molecular Devices, USA). The results are represented in terms of the percentage of cell proliferation.

**Cell Viability Assay:**

Cell viability assay was performed by trypan blue assay as described earlier (Waghela et al., 2015). Briefly, HCT 116 cells were treated with a series of concentrations (5  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 500  $\mu$ M) of 5-FU for 48h with or without pretreatment of DPI. After completion of incubation, the cells were harvested and the cell suspension was prepared in DPBS. The 10 $\mu$ L of cell suspension was mixed with 10 $\mu$ L of trypan blue and incubated for 3 minutes. The live and dead cells were counted and cell death was determined by the following formula (Percentage of cell death = Number of dead cells/Total number of cells x 100).

**NOX activity Assay:**

NADPH oxidase (NOX) activity assay was performed using NBT as described earlier (Serrander et al., 2007). The NBT assay depends upon the reduction of NBT to formazan by NOX dependent superoxide. Briefly, HCT-116 cells were treated with a series of concentrations (5  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, and 50  $\mu$ M) of 5-FU for 3h and 6h with or without pretreatment of DPI. Thereafter, the cells were incubated with 100  $\mu$ l NBT solution (1 mg/ml in RPMI culture medium) at 37°C for 1h. Then, the NBT solution was removed and the cells were disrupted using 100  $\mu$ l DMSO. Absorbance was detected at 570 nm. The results were represented as the percentage of NOX activation as compared to control.

**Intracellular ROS Assay:**

The intracellular ROS was determined by H<sub>2</sub>DCFDA dye as described earlier. Briefly, HCT-116 cells were treated with a series of concentrations (5  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, and 50  $\mu$ M) of 5-FU for 3h and 6h with or without pretreatment of DPI. Thereafter, the cells were incubated with H<sub>2</sub>DCFDA (25 $\mu$ M) for 20 min at 37°C in the dark. The fluorescence value of DCF molecules was recorded at the excitation 485 nm and emission 525 nm using a Multimode microplate reader (Molecular Devices, USA).

**Cellular Morphological analysis:**

The changes in cellular morphology of HCT 116 cells upon treatment were observed under the DIC filter of bright field microscope. Briefly, HCT 116 cells were treated with the 5-FU for 48h with or without pretreatment of DPI. The cells were observed from three different fields and images were acquired under the DIC filter (DP-71, IX81, Olympus, Japan). The images were acquired by Image-Pro MC 6.1 software (Bethesda, MD, USA) and analyzed by Image J software (NIH, USA).

**Cell Proliferation assay:**

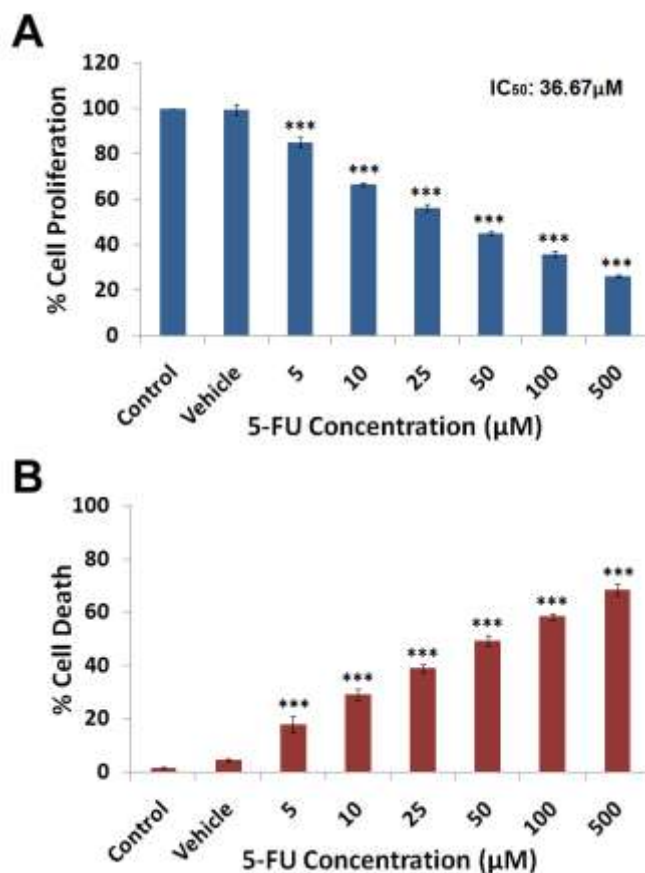
The healthy population of cells was analyzed by CFDA staining. Briefly, HCT 116 cells were treated with the 5-FU for 48h with or without pretreatment of DPI. Thereafter, the cells were stained with CFDA dye (25 $\mu$ M) for 20 minutes and washed twice with DPBS. Subsequently, the stained cells were observed under a fluorescence microscope. The images were acquired by Image-Pro MC 6.1 software (Bethesda, MD, USA) and analyzed by Image J software (NIH, USA).

**Statistical Analysis:**

The data were analyzed by one-way analysis of variance (ANOVA) followed by Student Newman Keulas (SNK) test using Sigma plot 12.0 statistical software. \*p  $\leq$  0.05, \*\*p  $\leq$  0.01, \*\*\*p  $\leq$  0.001 was considered statistically significant.

**Results:-****5-Fluorouracil induces cell death in human colon carcinoma HCT-116 cells:**

The half-maximal inhibitory concentration (IC<sub>50</sub>) value of 5-FU was determined from the dose-response curve after 48h of treatment on HCT-116 cells by the MTT assay. The result showed that IC<sub>50</sub> of 5-FU on HCT 116 cells was ~36.67  $\mu$ M (Figure 1A). Next, cell viability assay using a trypan blue dye was performed to examine the effect of 5-FU on HCT116 cells. The results showed that HCT-116 cells treated with 5  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M and 500  $\mu$ M of 5-FU show significantly higher percentage of cell death as compared to untreated HCT-116 cells after 48h of treatment. Therefore, the 5-Fluorouracil significantly induces cell death in a dose-dependent manner in human colon carcinoma HCT-116 cells (Figure 1B). According to these finding, we selected the doses: 5  $\mu$ M, 10  $\mu$ M, 25  $\mu$ M, and 50  $\mu$ M of 5-FU in HCT116 cells for further experiments.

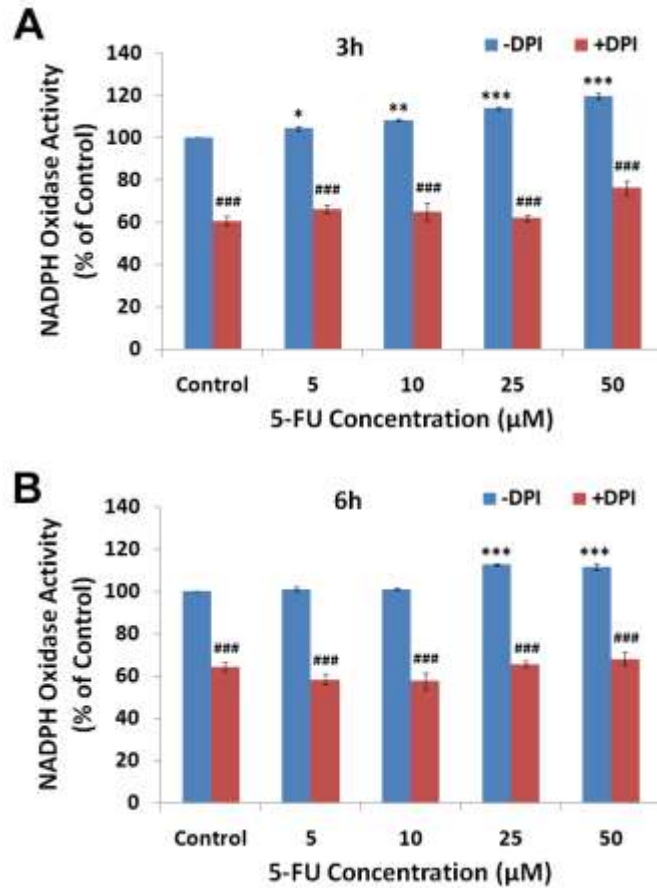


**Figure 1:** 5-FU inhibits cell proliferation and promotes cell death in HCT-116 cells.

HCT-116 cells were treated with 5-FU (5µM, 10µM, 25µM, 50µM, 100µM, and 500µM) for 48h. (A) The effect of 5-FU on cell proliferation of HCT-116 cells was determined by MTT assay. (B) Effect of 5-FU on cell death analysis by trypan blue assay. Error bars represent  $\pm$ SEM of three independent experiments. The significant difference indicated as \*\*\* $p \leq 0.001$  between 5-FU treated and untreated HCT-116 cells (One way ANOVA followed by SNK test).

#### **5-Fluorouracil modulates NOX activation in human colon carcinoma HCT-116 cells:**

Emerging investigations have provided a new understanding of the cellular redox system in cell death and survival. NOX enzymes are one of the major contributors to the cellular redox system. The activation of NOX enzymes plays an essential role in cell survival and death in a tissue-specific manner. Therefore, we were interested to examine the involvement of NOX enzymes after the treatment of 5-FU in HCT 116 cells. The result showed that the 5-FU treatment accelerates NOX activity at 3h and 6h as compared to untreated HCT-116 cells (Figure 2A & 2B). Interestingly, the Diphenylene iodonium (DPI) (a known NOX inhibitor) significantly reduced the NOX activity as compared to 5-FU treated cells (Figure 2A & 2B). Therefore, these results showed that the 5-Fluorouracil influences NOX activity in HCT-116 cells.

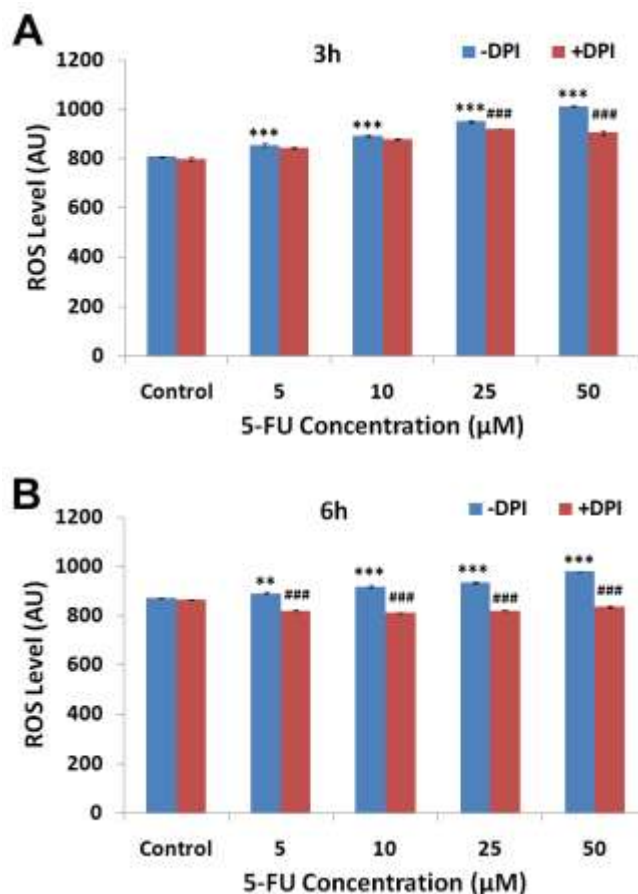


**Figure 2:** 5-FU promotes NOX activity in HCT-116 cells.

HCT-116 cells were treated with 5-FU (5μM, 10μM, 25μM, and 50μM) for 3h and 6h with or without pre-treatment of DPI. The NOX activity was determined by the NBT assay. (A) 3h, (B) 6h. Error bars represent  $\pm$ SEM of three independent experiments. The significant difference indicated as \* $p \leq 0.05$ , \*\* $p \leq 0.01$ , \*\*\* $p \leq 0.001$  between 5-FU treated and untreated HCT-116 cells. ### $p \leq 0.001$  between 5-FU treated with or without pretreatment of DPI (One way ANOVA followed by SNK test).

#### **5-Fluorouracil promotes NOX mediated generation of ROS in HCT-116 cells:**

As the NOX is a potent source of ROS, we were interested to analyze the changes in intracellular ROS level upon the treatment of 5-FU. The results showed that 5-FU significantly induces ROS generation at 3h and 6h in HCT-116 cells (Figure 3A & 3B). In addition, the pre-treatment of DPI showed reduced ROS level as compared to 5-FU treated cells (Figure 3A & 3B). Therefore, these results indicate that 5-Fluorouracil influences NOX mediated ROS generation in HCT-116 cells.



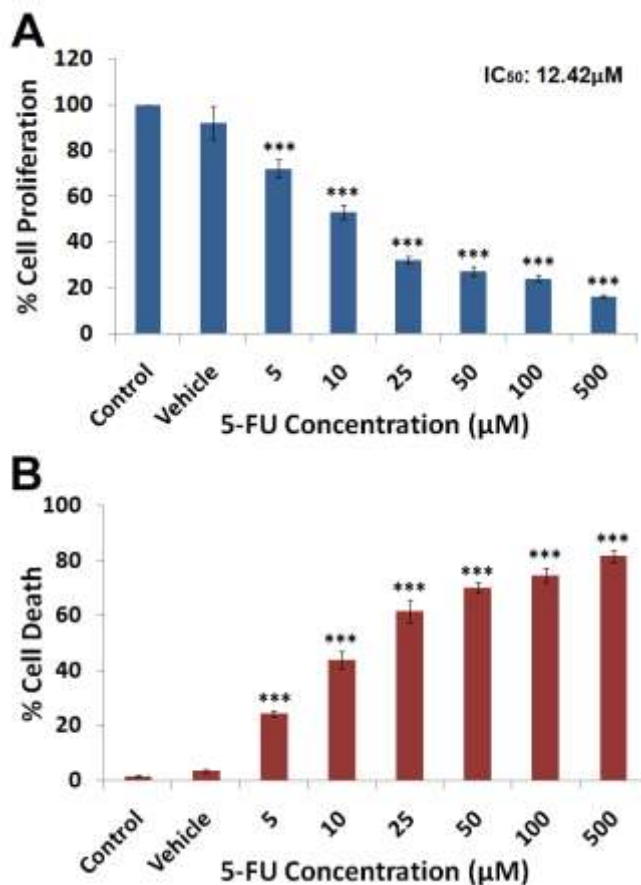
**Figure 3:** 5-FU promotes NOX mediated ROS generation in HCT-116 cells.

HCT-116 cells were treated with 5-FU (5μM, 10μM, 25μM, and 50μM) for 3h and 6h with or without pre-treatment of DPI. The effect of 5-FU on ROS generation was determined using H<sub>2</sub>DCFDA Dye. (A) 3h, (B) 6h. Error bars represent ±SEM of three independent experiments. The significant difference indicated as \*p≤0.05, \*\*p≤0.01, \*\*\*p≤0.001 between 5-FU treated and untreated HCT-116 cells. ###p≤0.001 between 5-FU treated with or without pretreatment of DPI (One way ANOVA followed by SNK test).

#### **Inhibition of NOX activity augments 5-Fluorouracil induced cell death in human colon carcinoma HCT-116 cells:**

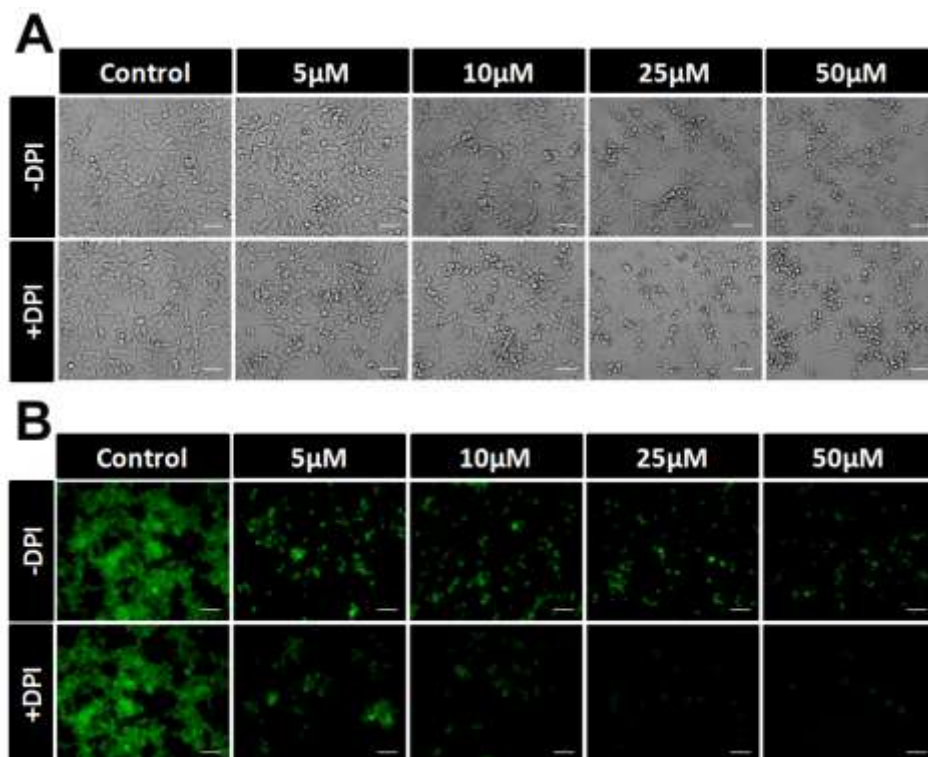
Previous reports suggest that the expression and activation of NOX enzymes play a vital role in the progression of colon cancer (Banskota et al., 2015; Juhasz et al., 2017; Weyemi et al., 2013). Here, we analyzed the role of NOX enzyme in 5-Fluorouracil induced cell death in HCT-116 cells. To identify the role of NOX enzyme in 5-Fluorouracil induced cell death, we have used DPI (a NOX inhibitor). We determined the half-maximal inhibitory concentration (IC<sub>50</sub>) value of 5-FU in DPI pretreated HCT-116 cells. Our results showed that the IC<sub>50</sub> of 5-FU in DPI pretreated cells was ~12.42 μM, which is significantly lower than the IC<sub>50</sub> of 5-FU standalone (Figure 4A & 1A). The IC<sub>50</sub> concentration of 5-FU in DPI pre-treated cells was 3 times lower than the 5-FU standalone treatment (Figure 4A & 1A). Next, we determined the effects on cell death and found that the DPI pretreated cells showed significantly higher percentage of cell death as compared to untreated cells and 5-FU standalone treated cells (Figure 4B & 1B). Further, we also analyzed the change in cellular morphology in cells treated with 5-FU with or without pretreatment of DPI. We found that the 5-FU promotes morphological changes in HCT-116 cells. In addition, the cells pre-treated with DPI showed prominent changes in cellular morphology as compared to the untreated cells and 5-FU standalone treated cells (Figure 5A). Moreover, the healthy population of cells were analyzed by CFDA dye upon 5-FU treatment. We found that the treatment of 5-FU reduces the cell viability of HCT-116 cells. Interestingly, DPI pretreated cells showed lower cell viability as compared to the untreated cells and 5-FU standalone treated cells

(Figure 5B). Thus, these results indicate that the inhibition of NOX activity inhibits cell proliferation and promotes 5-FU mediated cell death in colon carcinoma (HCT-116) cells.



**Figure 4:** Inhibition of NOX promotes 5-FU mediated cell death in HCT-116 cells.

HCT-116 cells were treated with DPI for 2h followed by 5-FU (5µM, 10µM, 25µM, 50µM, 100µM, and 500µM) for 48h. (A) The effect of 5-FU on cell proliferation of HCT-116 cells was determined by MTT assay. (B) Effect of 5-FU on cell death analysis by trypan blue assay. Error bars represent ±SEM of three independent experiments. The significant difference indicated as \*\*\* $p \leq 0.001$  between 5-FU treated and untreated HCT-116 cells (One way ANOVA followed by SNK test).



**Figure 5:** Inhibition of NOX activation promotes 5-FU mediated cell death in HCT-116.

HCT-116 cells were treated with 5-FU (5μM, 10μM, 25μM, and 50μM) for 48h with or without pretreatment of DPI (10μM) in HCT-116. (A) Analysis of cell morphology under the DIC filter. (B) Cell viability analysis by CFDA staining under the fluorescence microscope. Scale Bar: 50μm.

### Discussion:-

5-FU has long been utilized as a potent chemotherapeutic agent in the treatment of colon cancer. It is a structural analogue of uracil, therefore interrupts RNA synthesis. It also inhibits the function of thymidylate synthase (TS). Apart from colon cancer, it also uses in the treatment of various solid tumors including breast, head and neck, etc. However, the therapeutic efficacy of 5-FU, its systemic toxicity and development of drug resistance, as well as tumor recurrence are the major hurdles for the effective therapeutic regimens in colorectal cancer (Fang et al., 2016).

Earlier reports indicate that the dynamic magnitude of intracellular ROS regulates various cellular functions. The cellular level of ROS determines fate of cells. The lower level of ROS regulates cellular physiological processes and a higher level of ROS promotes cell death. The intracellular ROS is generated by various cellular organelles and ubiquitous enzymes found inside the cells. NADPH Oxidase is one of the major group of enzyme which generate ROS as a major product. Recent reports suggest that the NOX mediated ROS generation plays a vital role in cancer development and progression. It also plays a major role in metastasis and drug resistance of various malignancies (Liu et al., 2015; Zhan et al., 2015). 5-FU influences the cellular redox balance and promotes ROS mediated cell death. Therefore, we were keen to analyze whether the 5-FU treatments is associated with NOX activation. Further, we analyzed the involvement of NOX activity in the 5-FU mediated cell death.

In this study, first we examined the effect of 5FU on HCT-116 cells. We found that 5-FU inhibits cell proliferation and promotes cell death in HCT-116 cells. 5-FU is also known to induce ROS mediated apoptosis (Fan et al., 2013; Hwang et al., 2001). The NOX enzymes are a key source of ROS. However, the deregulated NOX activation essentially plays a vital role in the progression of various types of malignancies including colon cancer, prostate cancer, melanoma, and glioblastoma (Weyemi et al., 2013). The previous report suggests that the 5-FU induces ROS generation and apoptosis in human colon carcinoma cells (Yao et al., 2017). The effect of 5-FU on NOX mediated generation of ROS and cell death was not well explored. Therefore, we intended to unravel the influences of 5-FU on NOX mediated generation of ROS and regulation of cell proliferation in colon cancer cells. We monitored the

NOX activity upon 5-FU treatment and found accelerated NOX activity upon 5-FU treatment in HCT-116 cells at 3h and 6h. The treatment of DPI (a NOX inhibitor) showed a significant reduction in NOX activity. We found a similar response in the intracellular ROS level. The level of ROS defines the fate of cell death and survival. Therefore, we monitored the NOX mediated ROS generation and cell death influenced by 5FU. Previous reports suggested that the NOX isoforms play a vital role in the proliferation of colon cancer cells (Juhasz et al., 2017). The switch of NOX isoforms also participates in the invasive phenotypes of colon cancer (Banskota et al., 2015). The NOX isoforms regulate a variety of signaling mechanisms and exert cancer progression (Block et al., 2010; Desouki et al., 2005). The NOX enzymes are also known to participate in chemo-resistance in gallbladder cancer (Zhan et al., 2015). Despite 5-FU remains the first-line chemotherapeutic agent for colon cancer, where gaining the against anti-cancer therapy limits its successful application in cancer therapy (Francipane et al., 2019). However, the role of NOX activation in 5-FU mediated cell death in colon cancer cells was remaining to elucidate. Our results suggested that the inhibition of NOX activity reduces cell proliferation and augments the 5-FU mediated cell death in HCT-116 cells. Moreover, the half-maximal concentration of 5-FU in DPI pre-treated cells was  $\sim 12.42 \mu\text{M}$ , which was almost 3 times lower than the 5-FU. Moreover, our results from morphological analysis and viability staining showed reduced cell viability upon treatment of 5-FU with DPI pretreated HCT-116 cells. Therefore, these results suggested that the inhibition of NOX activity augments 5-FU mediated cell death by inhibiting cell proliferation of colon carcinoma cells. It could be suggest that this approach may be useful to improve therapeutic efficacy of 5-FU by inhibiting the NOX activity using a potent inhibitor of NOX family proteins.

### Conclusion:-

Collectively, we found that the NOX activity is modulated during the treatment of 5-FU. We found elevated NOX activity and subsequent moderate ROS generation during 5-FU treatment. The inhibition of NOX activity augments 5-FU mediated cell death in HCT-116 cells. Thus, the NOX enzymes may be a potent therapeutic target to increase the efficacy of 5-FU.

### Conflict of interest:

The authors declare no conflicts of interest

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