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## Combined effects of chemotherapy and Nrf2 activation in colorectal cancer cells *in vitro*

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### ABSTRACT

Colon cancer is the third leading type of cancer diagnosis in the United States (Siegel et al, 2017). Common treatments include chemotherapy, which can be toxic to the patient and produce multiple adverse side effects (Sarkar, 2008). Combination therapies with chemotherapy drugs and other compounds have been reported to decrease tumor growth in breast and colon cancer by increasing efficacy of chemotherapeutic agents at lower doses, thus reducing off-target adverse effects (Borcharding et al, 2015; Chen et al 2017). Both activation of Nrf2, a transcription factor that induces expression of anti-oxidant genes, and dopamine receptor agonists, have been shown to reduce tumor growth in multiple cancer types (Borcharding et al, 2015; Melba et al, 2013). Thus, we examined whether combining a common chemotherapy drug, Doxorubicin, with a Nrf2 activator, CDDO-ME, or a dopamine-type-1 receptor agonist, Fenoldopam, improved efficacy of chemotherapy. Treatment of HT29 and HCT116 colorectal cancer cells *in vitro* with or CDDO-ME in conjunction with Doxorubicin augmented the effects of Doxorubicin alone, as determined by MTT assay. The results support that Doxorubicin had an effect on both cell lines above concentrations of 100 nM. However, Fenoldopam, a dopamine-type-1 receptor agonist, did not significantly affect cell viability. Therefore, the effects of Doxorubicin may be achieved at a lower dose when administered with CDDO-ME.

### INTRODUCTION

Colorectal cancer (CRC) is the third highest diagnosed cancer and the second leading cause of cancer-related deaths across the United States. In fact, one in twenty people are diagnosed with this disease and over 50,000 people died from CRC in 2017. Although the causes of a recent increase in CRC cases diagnosed in patients under the age of 50 are not well defined, risk factors include excess body weight, family history of CRC and past medical history of

Inflammatory Bowel Disease (IBS) (Siegel et al, 2017; Janakiram & Rao, 2014).

CRC is commonly treated with surgical removal of the tumor. However, most cases also require chemotherapy treatment to ensure any remaining cells are eliminated to prevent recurrent growth of a tumor (Chen et al, 2017; Sarkar, 2008). Although current chemotherapy drugs used to treat CRC are effective, the side effects associated with these drugs are numerous and chemotherapeutic resistance is

becoming more of an issue in oncology (Sarkar, 2008). Doxorubicin, a widely used anti-cancer chemotherapy compound, often causes cardiotoxicity (Gustafson & Thamm, 2010). Therefore, the importance of discovering new treatment regimens for CRC to improve both the patient's well-being during treatment and their overall outcome is significant.

Dopamine is primarily known as a neurotransmitter and hormone in the brain and central nervous system (CNS). However, Dopamine receptors (DR) are commonly located on cell membranes in blood vessels, the gastrointestinal tract and in the kidney (Borcherding et al, 2015). When the activity of these receptors is inhibited, the homeostatic mechanisms of these tissues are disrupted, leading to conditions like hypertension and gut hypermotility (Borcherding et al, 2015). However, activation of D1Rs with dopamine and dopamine-type-1 receptor (D1R) agonists has been shown to stabilize tumor blood vessel formation and decrease the size of tumors resulting from human breast and colon cancers in tumor bearing mice (Borcherding et al, 2015; Chen et al, 2017). The D1R agonist Fenoldopam, which is a drug commonly used to treat renal hypertension, was successful in suppressing not only breast cancer cell viability *in vitro*, but also breast cancer tumor growth in mice *in vivo* (Borcherding et al, 2015). Activation of the D1R/cGMP/protein kinase G (PKG) pathway caused apoptosis and necrosis of the cancer cells, which suggests that Fenoldopam could be utilized as a chemotherapeutic drug for tumor cells that express D1R receptors (Borcherding et al, 2015).

In addition to these findings, Dopamine also significantly inhibited tumor growth in mice when used in conjunction with chemotherapeutic drugs (Sarkar, 2008). 5-FU is the most widely used CRC chemotherapy drug on the market (Chen et al, 2017). In a study using HT29 cells and human breast cancer cells, dopamine was shown to significantly augment the antitumorigenic effects of 5-FU and doxorubicin (Sarkar, 2008). These effects were attributed to dopamine's ability to decrease vascular permeability factor/vascular endothelial growth factor (VPF/VEGF), which stabilized blood supply to the tumor cells (Sarkar, 2008). The findings of this study suggest that dopamine, and potentially other D1R agonists, could be useful adjuvants to chemotherapy agents *in vitro* (Sarkar, 2008).

Another target for CRC chemotherapeutic agents is Nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that alerts the cell to electrophilic and oxidative stress (Sadeghi, 2018). Expression of Nrf2 stimulates the transcription of genes that encode proteins responsible for producing anti-inflammatory and anti-oxidant effects (Sadeghi, 2018). This signaling pathway, facilitated by the interactions between Nrf2 and its primary inhibitor, Keap1, protects the cell from harmful substances and genetic changes, which can lead to cancer (Melba et al, 2013). However, there have been conflicting reports concerning the effectiveness of Nrf2 agonists as chemotherapy treatments (Melba et al, 2013; Sporn & Liby, 2012).

The conflicting studies have been attributed to the "good side and the dark side of Nrf2"

(Melba et al, 2013). As mentioned before, the Nrf2 pathway is one of the cell's major defense mechanisms. Studies have shown that this defense mechanism protects healthy cells from damage caused by carcinogens, thereby classifying Nrf2 as a tumor suppressor protein (Melba et al, 2013). However, this system can also be utilized by cancer cells to protect them from chemotherapeutic agents (Sporn & Liby, 2012). The role of Nrf2 in cancer continues to be a common area of study in the research community.

In order to build on the current understanding of CRC treatment options, the goals of this study were to test whether Doxorubicin, a chemotherapeutic agent that is known to be successful for treating breast cancer could also be effective in treating colorectal cancer. Additionally, Fenoldopam, a peripheral D1R agonist, and CDDO-ME, an Nrf2 activator, were used to treat CRC cells in conjunction with Doxorubicin to determine if the combination therapy could produce an additive or synergistic effect.

## **MATERIALS AND METHODS**

### *Cell Culture*

HT29 and HCT116 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in McCoy's 5a growth media containing 10% fetal bovine serum (Atlanta Biologicals, Flowery Branch, GA, USA) and 5% Penicillin-Streptomycin that were stored in a 37°C incubator and were passaged every three to four days using sterile technique. They were also routinely evaluated for bacterial infection.

### *Cell Plating*

HT29 and HCT116 cells were plated in sixty wells (6x10 grid) on a 96 well plate (two plates per cell line). Growth media was removed from the respective cell culture flasks and the cells were trypsinized with 3 mL TrypLE Express for ten to fifteen minutes in a 37°C incubator. Once the cells were mobile in the media, 3 mL of supplemented McCoy's 5a growth media (described above) was added to each flask. The cells and the media were transferred to a 15mL conical and centrifuged for 3 minutes (100xg, 4°C). The supernatant was removed and the cells were resuspended in 10 mL of supplemented McCoy's 5a growth media. The number of cells per milliliter was calculated using a hemocytometer (10 uL cell solution, 10 uL Trypan Blue). Using the average number of cells per milliliter, the cell/media mixture was diluted to the proper amount in order plate 1000 cells per 100 microliters in each well. 100 microliters of growth media was added to the outside rows. The plated cells were incubated in a 37°C incubator for twenty-four hours prior to treatment.

### *Cell Treatments*

HT29 and HCT116 cells were treated with a range of doses of Doxorubicin (100 uL) combined with Fenoldopam (20 uM, 100 uL) or CDDO-ME (HT29: 100 nM, 100 uL; HCT116: 200nM, 100 uL). After the plated cells had incubated for twenty-four hours, the growth media was removed. One column of six replicates was treated with either the control (McCoy's 5a Growth Media, 2.5% FBS, 1% Pen-Strep with DMSO, 100 uL), Fenoldopam or CDDO-ME as well as Doxorubicin. Doxorubicin was added to the plates in the following concentrations

for both the controls and the Fenoldopam/CDDO-ME treatment groups: 2 nM, 20 nM, 200 nM and 2  $\mu$ M. The cells were placed back in the 37°C incubator for three days.

#### *Cell viability Assay*

After three days, MTT (20  $\mu$ L) was added to the plates of HT29 and HCT116 cells treated with Doxorubicin and either Fenoldopam or CDDO-ME. The plates were incubated at 37°C for one hour. The media was aspirated off the cells and the crystals were solubilized with DMSO (100  $\mu$ L). The plates were put on a plate shaker for three minutes and the absorbance was measured using a plate reader with an optical density of 570 nm.

## **RESULTS**

### *Doxorubicin*

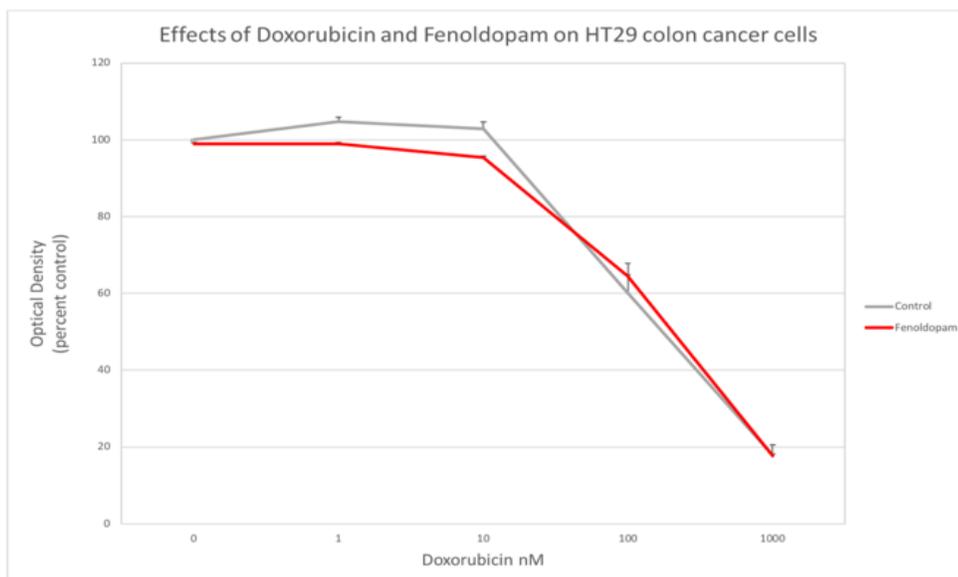
Doxorubicin had a significant effect on HT29 cell viability at concentrations of 1nM, 100nM and 1000nM (Figure 1, Figure 3). Doxorubicin had a significant effect on HCT116 cell viability at concentrations of 100nM and 1000nM (Figure 2, Figure 4).

### *Doxorubicin and Fenoldopam*

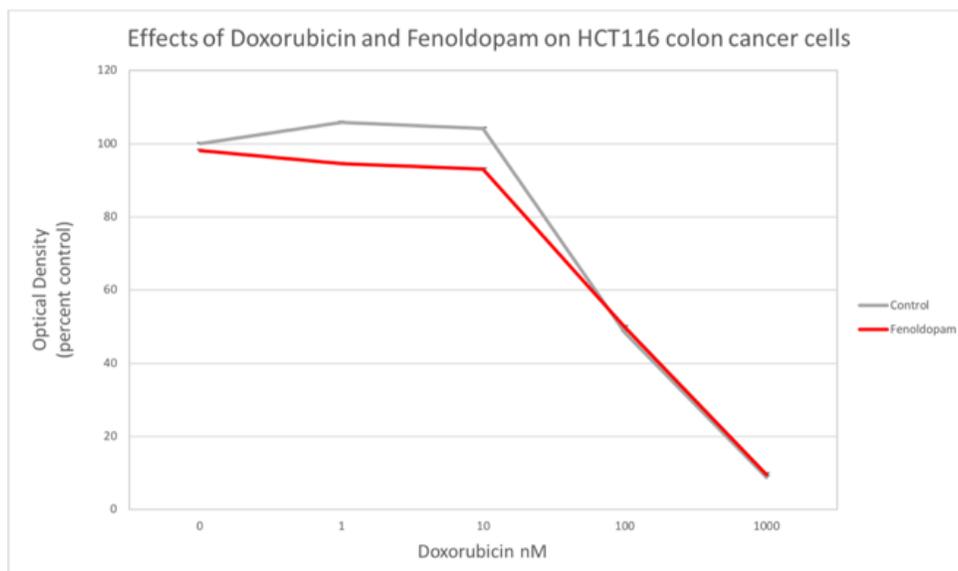
For both the HT29 and HCT116 cell lines, there was not a significant difference between the control group treated with only Doxorubicin and the test group treated with Doxorubicin and Fenoldopam at any concentrations of Doxorubicin (Figure 1, Figure 2).

### *Doxorubicin and CDDO-ME*

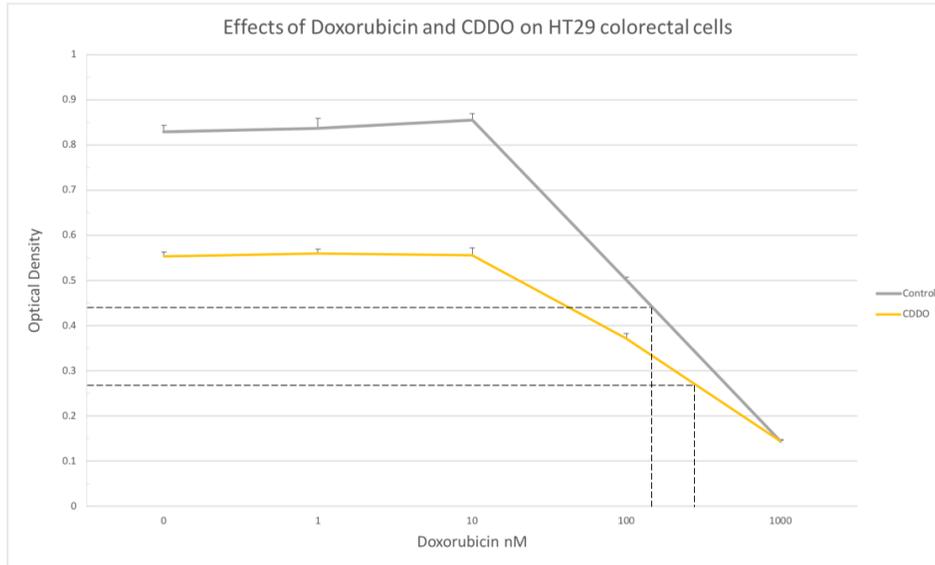
For both cell lines, there was a significant difference between the control group treated with only Doxorubicin and the test group treated with Doxorubicin and CDDO-ME at Doxorubicin concentrations of 0nM, 1nM, 10nM and 100nM (Figure 3, Figure 4).



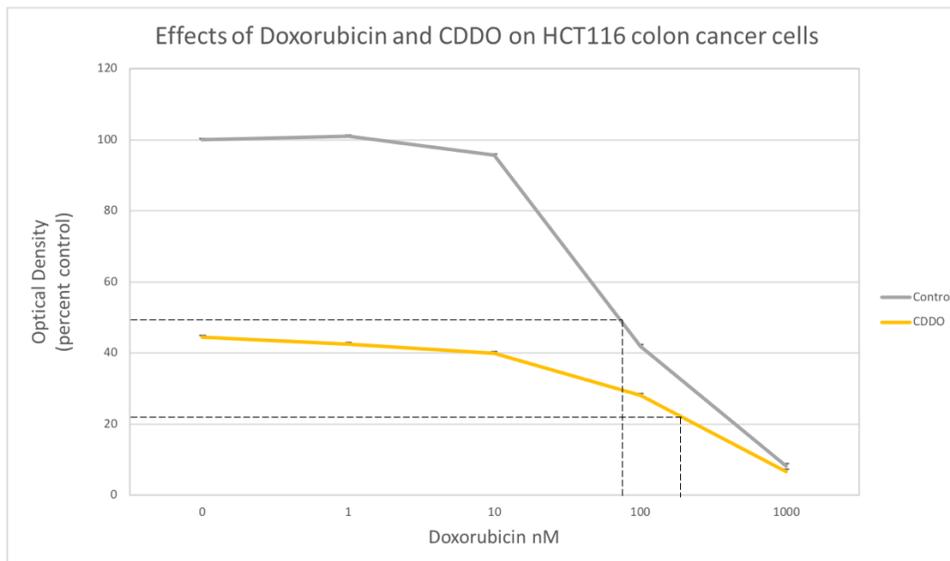
**Figure 1. Effects of Doxorubicin and Fenoldopam on HT29 colon cancer cells.** HT29 colon cancer cells were treated with four different concentrations of Doxorubicin. Each sample was treated with the same concentration of Fenoldopam. Cell viability was evaluated using the MTT Assay and absorbance was measured. The percent control values for each experiment were compiled and statistical analyses were conducted. Doxorubicin had a significant effect on cell viability in the control group at the concentrations of 1nM, 100nM and 1000nM (p-values: 0.029, 0.014,  $1.43E^{-5}$ ). The cell viability of samples treated with Fenoldopam was not statistically different from that of the control group at any concentration of Doxorubicin (all p-values > 0.05).



**Figure 2. Effects of Doxorubicin and Fenoldopam on HCT116 colon cancer cells.** HCT116 colon cancer cells were treated with four different concentrations of Doxorubicin. Each sample was treated with the same concentration of Fenoldopam. Cell viability was evaluated using the MTT Assay and absorbance was measured. The percent control values for each experiment were compiled and statistical analyses were conducted. Doxorubicin had a significant effect on cell viability in the control group at the concentrations of 10nM, 100nM and 1000nM (p-values: 0.026,  $3.03E^{-5}$ ,  $2.43E^{-9}$ ). The cell viability of samples treated with Fenoldopam was not statistically different from that of the control group at any concentration of Doxorubicin (all p-values > 0.05).



**Figure 3. Effects of Doxorubicin and CDDO on HT29 colon cancer cells.** HT29 colon cancer cells were treated with four different concentrations of Doxorubicin. Each sample was treated with the same concentration of CDDO. Cell viability was evaluated using the MTT Assay and absorbance was measured. Statistical analysis were conducted using the optical density of each sample. Doxorubicin had a significant effect on cell viability in the control group at the concentrations of 100nM and 1000nM (p-values:  $3.18 \times 10^{-8}$ ,  $1.15 \times 10^{-12}$ ). The cell viability of samples treated with CDDO was statistically different from that of the control group at the Doxorubicin concentration of 0nM, 1nM, 10nM and 100nM (p-values:  $4.2 \times 10^{-8}$ ,  $8.83 \times 10^{-7}$ ,  $1.71 \times 10^{-7}$ ,  $3.37 \times 10^{-7}$ ).



**Figure 4. Effects of Doxorubicin and CDDO on HCT116 colon cancer cells.** HCT116 colon cancer cells were treated with four different concentrations of Doxorubicin. Each sample was treated with the same concentration of CDDO. Cell viability was evaluated using the MTT Assay and absorbance was measured. The percent control values for each experiment were compiled and statistical analyses were conducted. Doxorubicin had a significant effect on cell viability in the control group at the concentrations of 100nM and 1000nM (p-values:  $3.15 \times 10^{-6}$ ,  $1.41 \times 10^{-8}$ ). The cell viability of samples treated with CDDO was statistically different from that of the control group at the Doxorubicin concentrations of 0nM, 1nM, 10nM and 100nM (p-values:  $1.91 \times 10^{-6}$ ,  $4.82 \times 10^{-5}$ ,  $2.32 \times 10^{-4}$ ,  $5.63 \times 10^{-3}$ ).

## DISCUSSION

### *Doxorubicin*

As mentioned before, Doxorubicin is a chemotherapeutic drug that is commonly used to treat breast cancer, however, its effectiveness in treating colon cancer has not been well researched (Sarkar, 2008). Therefore, one of the goals of this study was to determine whether the use of Doxorubicin is a viable treatment for colorectal cancer. The findings from the cell viability studies demonstrate that Doxorubicin does decrease the viability of colorectal cancer cells *in vitro*. These effects were not noted in concentrations below 100nM, but were significant at both 100nM and 1000nM concentrations.

### *Doxorubicin and CDDO-ME*

Studies of Nrf2 activators including CDDO-ME have supported the ability of Nrf2 to function as a tumor suppressor by activating anti-inflammatory and anti-oxidant systems of defense to protect the cells from carcinogens and other harmful cellular changes (Melba et al, 2013). In addition, colorectal cancer progression is linked to inflammatory processes and patients with a history of IBS have a higher risk of developing colorectal cancer (Janakiram & Rao, 2014).

The MTT cell viability assay indicates that CDDO-ME, an Nrf2 activator, had a significant inhibitory effect on HCT116 and HT29 cells as well as an additive effect with Doxorubicin. At concentrations below 10nM, Doxorubicin did not appear to be effective in decreasing the viability of either HT29 or HCT116 cells. However, there was a significant difference between the cells treated with CDDO and those treated with the control at all concentrations of

Doxorubicin except the highest dose. This is supported by the estimated OD50 of each treatment group. Because Doxorubicin appears to be ineffective below a concentration of 100nM, the significant difference between the treatment groups in this range can be attributed to CDDO-ME. At the concentration of 100nM Doxorubicin, there is a significant difference noted in both the control group and the CDDO-ME test groups when compared to their respective controls as well as when the two groups are compared to one another. Therefore, the decrease in cell viability at this concentration of Doxorubicin can be attributed to the additive effect of CDDO-ME with Doxorubicin.

In the HT29 cell line, there was less of an effect of CDDO in conjunction with Doxorubicin, which is demonstrated by the estimated OD50. Preliminary studies with HT29 cells had demonstrated that the concentration of CDDO used on HCT116 cells had been too concentrated to solidify evidence supporting the additive effect of CDDO. Therefore, the concentration of CDDO used on HT29 cells in this study was half of that of the HCT116 cells. The results suggest that the concentration of CDDO used to treat HT29 cells may have been too low to demonstrate an equivalent additive effect.

### *Doxorubicin and Fenoldopam*

In breast cancer cells, D1R agonist Fenoldopam alone was able to surmount an anti-tumor effect *in vitro* and *in vivo* (Borcherding et al, 2015). This was attributed to Fenoldopam's ability to stabilize tumor angiogenesis and promote apoptosis (Borcherding et al, 2015). Therefore, this study sought to evaluate

whether the same conclusions might be true with colorectal cancer cells *in vitro*. Results from the cell viability assay of the control groups treated with Fenoldopam and Doxorubicin suggest that Fenoldopam did not have an effect on cell viability *in vitro*. While the data from the Doxorubicin and Fenoldopam experiments demonstrate significant difference with an increase in Doxorubicin concentration, there is not a statistical difference between the treatments with or without Fenoldopam. This suggests that the changes in cell viability were attributed to Doxorubicin only and not an additive or synergistic effect of Fenoldopam and Doxorubicin. However, because the mechanism of Fenoldopam in reference to inhibition of tumor growth also pertains to angiogenesis, further studies of Fenoldopam *in vivo* may provide more similar results to those of previous studies that demonstrated the therapeutic effects of this drug on breast cancer. Preliminary studies (data not shown) that demonstrated negative findings with combination of Dopamine and Doxorubicin might have also resulted for similar reasons to Fenoldopam. In this case, future *in vivo* studies with Dopamine might also be beneficial.

### CONCLUSIONS

Based on the results of this study, it appears that Doxorubicin has the potential to be an effective treatment of colorectal cancer and should be further investigated *in vivo* to solidify this conclusion. Likewise, CDDO-ME appears to have both an individual and additive effect on colorectal cancer cells. In addition, Fenoldopam was not demonstrated to be an effective individual or additive treatment of either cell line.

Although the MTT assay is effective in evaluating cell viability, it is not specific enough to evaluate whether the decrease in viability is a result of apoptosis or a decrease in cell proliferation. Because of this limitation, future experiments to determine whether apoptosis or decreased cell proliferation was the mechanism responsible for the noted decrease in cell viability would be beneficial. In addition, studies to determine the effects of these drugs *in vitro* using mouse models would be useful to evaluate whether the conclusions made in this study transcend to organ systems and to determine whether the negative results concerning Fenoldopam and Dopamine present differently *in vivo*.

### CONFLICT OF INTEREST

The authors of this study do not declare any conflicts of interest.

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