

**INSIGHTS INTO THE EFFECT OF TRIGONELLINE IN THE P53  
PATHWAY**

An Undergraduate Research Scholars Thesis

by

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## ABSTRACT

Insights Into the Effect of Trigonelline in the p53 Pathway. (May 2014)

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Colon cancer is the second leading cause of cancer-related deaths in the United States. Early screening has improved detection of precancerous polyps, however, colon cancer still claims over fifty thousand lives per year. Statistics have shown a strong correlation between an increased level of estrogen and a decreased risk of colon cancer. Data collected in our laboratory has shown that estradiol (E<sub>2</sub>) is protective through its ability to increase apoptosis of non-malignant colonocytes that are at risk for becoming cancerous due to stress. This is mediated through a mechanism involving estrogen receptor  $\beta$  (ER  $\beta$ ) in mice, and the protein p53 has been found to be protective in some mechanisms.

Trigonelline (Trig) is a product of niacin metabolism and accounts for ~1% dry matter in roasted coffee beans. Data from our laboratory has shown the ability of Trig to induce an estrogenic response through the activation of nuclear reporter assays. We investigated the effect of Trig by analyzing the apoptotic and growth activity of young adult mouse colonocytes (YAMCs) in response to non-permissive conditions and the role that the p53 protein plays in the apoptotic mechanism. RT-PCR analysis was used to measure protein levels that are associated with pro-apoptotic p53 target genes (PUMA, Bax, and Noxa) in YAMCs after treatment to demonstrate the protective estrogenic effect of Trig.

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# CHAPTER I

## INTRODUCTION

Women from all racial backgrounds have a lower risk of developing colon cancer as compared to their male counterparts [1, 3, 8]. This gender correlation has been seen in animal models as well [2]. When young adult mice were exposed to a carcinogen that targets the colon, male mice had twice the rate of colorectal cancer development as compared to females under identical conditions [2]. An even lower risk has been observed in premenopausal women, in contrast to post-menopausal women who are not receiving Hormone Replacement Therapy (HRT) [25]. In one of the largest cohort studies, the Nurses Health Study, observed a strong protective effect against colon cancer when post-menopausal women were treated with HRT [2]. Data collected in our laboratory has shown that  $E_2$  is protective through its ability to increase apoptosis of non-malignant colonocytes that are at risk for becoming cancerous due to stress [8]. This is mediated through a mechanism involving estrogen receptor  $\beta$  ( $ER\beta$ ) in mice, and has been found to be protective in the p53 mechanism [14].

It is well known that  $E_2$  binds to estrogen receptors (ERs) and that this interaction is capable of altering various physiological responses throughout the body in a wide range of tissues [14].

There are two types of ERs that have been identified,  $ER\alpha$  and  $ER\beta$  [20]. Although these receptors are similar in many ways, they have vastly different roles in cellular modulation [20]. Both subtypes are found within the cytosol, but migrate in response to the presence of hormones [14].  $ER\beta$ 's role in cell cycle arrest and apoptosis has been established [8]. The varied ratio of  $ER\alpha$  and  $ER\beta$  is believed to contribute to differing tissue response to  $E_2$  and

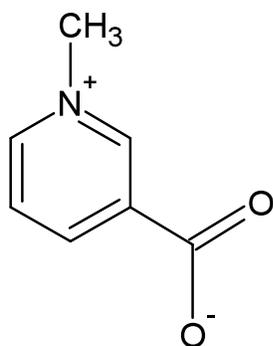
phytoestrogens. Because of the antagonistic roles of ER $\alpha$  and ER $\beta$ , elucidating their mechanisms and varied tissue effects can be difficult to model.

Understanding the mechanistic involvement of p53 in cell apoptosis and the influence of ER $\beta$  is on the most heavily studied mechanisms. p53 is known as the ‘guardian of the genome’ because of its ability to induce cell cycle arrest and apoptosis in response to various stimuli [8, 18]. p53 is a tumor suppressor protein that has the ability to increase or decrease the transcription of various target proteins in response to internal or external signals [18]. Damage to or inhibition of p53 can lead to unchecked cell growth and eventually tumor evolution [8, 18]. The loss of a functioning p53 is a marker for increased cancer risk and tumor progression in various tissues [18].

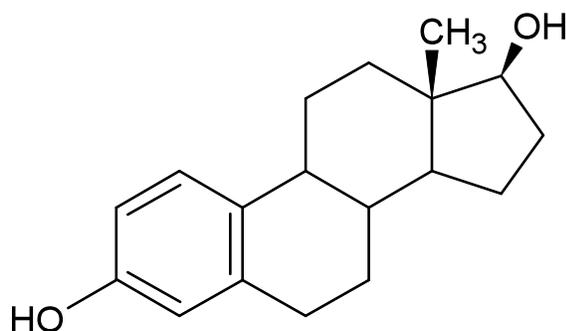
Recently, the study of the involvement of ER $\beta$  has been analyzed via downstream targets of p53 such as BCL-2 associated X protein (Bax), p53 up-regulated modulator of apoptosis (PUMA), and Phorbol-12-myristate-13-acetate-induced protein 1 (NOXA) [8, 12]. A study showed that the up-regulation of p53 via E<sub>2</sub> increases the levels of Bax, PUMA and NOXA in non-malignant colonocytes, which is a sign of increased apoptotic activity [8].

Phytoestrogens, such as genistein found in soy, are compounds that are plant-derived and estrogenic in structure and in function. Phytoestrogens are being studied for their ability to influence cell growth in colonocytes in response to stress through the induction of apoptosis [19, 21, 27]. There have been multiple observational studies on the effects of coffee within the body, and the correlation between increased coffee intake and a reduced colon cancer risk has been demonstrated [11, 22, 23]. To further understand this effect, research of bioactive compounds

found in roasted coffee beans and their relationship to colon cancer has increased. However, little research has been done to analyze the effect of Trigonelline (Trig) within the colon. Trig is a niacin-related compound that is found in the highest concentration in dry roasted coffee beans, as well as hemp seed and garden peas [12]. Despite Trig (Figure 1a) having a dissimilar structure from E<sub>2</sub> (Figure 1b) it has been shown to modulate estrogen receptor activity.



**Figure 1a** Structure of Trigonelline



**Figure 1b** Chemical Structure of Estradiol

The estrogenic properties of Trig have been demonstrated in estrogen-dependent human breast cancer (MCF-7) cells by their ability to induce an increase in expression of ER target genes [12]. However, the effects of Trig on colonocytes, which primarily express ER $\beta$ , have not been investigated. Additionally, the relationship between Trig and p53 is not understood. This is a new area and could lead to a better understanding of how bioactive molecules can protect against colon cancer development.

The goal of this study is to establish the ability of Trig induce apoptosis through p53 mechanisms. Success in our study would also establish a link between ER  $\beta$  molecular pathways and Trig.

## CHAPTER II

### MATERIALS & METHODS

#### **Chemicals**

$\beta$ -Estradiol ( $E_2$ ) and trigonelline (Trig) were purchased from Sigma Alderich. Reagents were dissolved in dimethyl sulfoxide (DMSO, Sigma-Alderich).

#### **Cells**

Young adult mouse colonocytes (YAMCs) bleo/neo and mp53/neo cells were provided by Dr. Hartmut Land (University of Rochester Medical Center). For general maintenance, cells were cultured in RPMI 1640 (Sigma Aldrich) with 10% fetal bovine serum (FBS; HyClone); 0.1% insulin, transferrin, and selenious acid (ITS; BD Biosciences); and 1% gentamicin (GIBCO). Cells were maintained under permissive conditions, 33°C with 5 units  $\gamma$ -interferon ( $IFN\gamma$ )/mL medium (Roche) on plates coated with rat-tail collagen type I (BD Biosciences). Forty-eight hours before plating for all experiments, YAMC bleo/neo and mp53/neo cells were transferred to medium containing 10% charcoal-dextran stripped FBS, 1% gentamicin, and 0.1% ITS.  $\beta$ -Estradiol was diluted in dimethyl sulfoxide (DMSO) as 1000 $\times$  stocks and delivered as 1  $\mu$ L/mL medium to achieve the final dose listed.

#### **Cell growth assay**

YAMC bleo/neo and mp53/neo cells were seeded at 15,000 cells per well on six-well plates (Grenier bio-one) and grown under permissive conditions, 37 °C and presence of  $IFN\gamma$ . Three wells per treatment per experiment were used. Cells were exposed to individual treatments of vehicle 1nM  $E_2$  positive control, 100 pm Trig or 0.1% DMSO and transferred to non-permissive

conditions. The medium was changed 48 hours after the first treatment and a second dose of the given treatments was delivered at this time. At the end of the 96-h treatment period, cells were trypsinized and prepared for counting using a coulter counter. Cell numbers were counted using a Beckman Coulter particle counter. 20  $\mu$ l of cells were diluted in 10 mL Isotone II diluent (Beckman Coulter) and samples were counted in triplicate.

### **RNA extraction and RT-PCR**

Cells were seeded at a concentration of  $1.4 \times 10^5$  cells/dish in 100 mm dishes (Corning) and grown at permissive conditions with IFN- $\gamma$  for 24 hours. YAMC bleo/neo and mp53/neo cells (25,000/well) were seeded in 6-well plates and treated with E<sub>2</sub> or Trig at the appropriate concentrations or 0.1% DMSO for 24 hours and maintained under non-permissive conditions in stripped serum media. Cells will be trypsinized and centrifuged, and RNA isolation conducted using the Direct-201 RNA MiniPrep Kit (Zymo). 1  $\mu$ g total RNA will be used for cDNA synthesis using the Transcriptor First Strand cDNA Synthesis kit. RT-PCR will be run on a Roche Lightcycler for 45 cycles.

RNA was isolated by the manufacture's protocol for Direct-zol RNA MiniPrep Kit (Zymo Research). In brief, 950  $\mu$ l TRIzol reagent was added to trypsinized cells to lyse cell membrane. After washing with 70% Ethanol, mixtures were loaded into column with a collection tube and spun down. Flow-through was discarded and collection tube was replaced. 400  $\mu$ l of Direct-zol RNA PreWash was added to the column and collected after being spun-down, followed by the addition of 700  $\mu$ l of RNA Wash Buffer. Finally, the RNA was eluted via 30  $\mu$ l DNase/RNase-Free Water and collected in RNase-free tubes. Isolated RNAs were stored at -70°C.

cDNA synthesis was performed with Transcriptor First Strand cDNA synthesis kit (Roche) using the manufacturer's protocol. In brief, 0.8 µg of RNA was incubated at 65°C for 10 min with 1 µl oligo dT primer and 2 µl random Hexamer primer. After the addition of 4 µl reaction buffer, 0.5 µl RNase inhibitor, 2 µl dNTP and 0.5 µl reverse transcriptase, thermo cycling was performed; 25°C for 10 min, 50°C for 60 min and 85°C for 5 min. cDNA was stored at -20°C.

Real-Time PCR was performed with FastStart Universal SYBR Green Master mix (Roche). The sequence of the PCR primers were as follows: 18S (Forward: TCA AGA ACG AAA GTC GGA GGT, Reverse: GGA CAT CTA AGG GCA TCA CAG), Bax (Forward: CAC CAG CTC TGA GCA GAT G, Reverse: GCG AGG CGG TGA GCA CTC C), NOXA (Forward: GAA ATG CCT GGT ATT GGA TGG A, Reverse: GAA CTC AT CCT ATC TCC TTC ATC AT), or PUMA Forward: GCG GCG GAG ACA AGA AGA, Reverse: GGA GTC CCA TGA AGA GAT TGT ACA). 18S was utilized as an internal control. RT-PCR was run on a Roche LightCycler: 95°C 10 min, 45 cycle of 15 sec at 95°C and 30 sec at 60°C.

### **Statistical analysis**

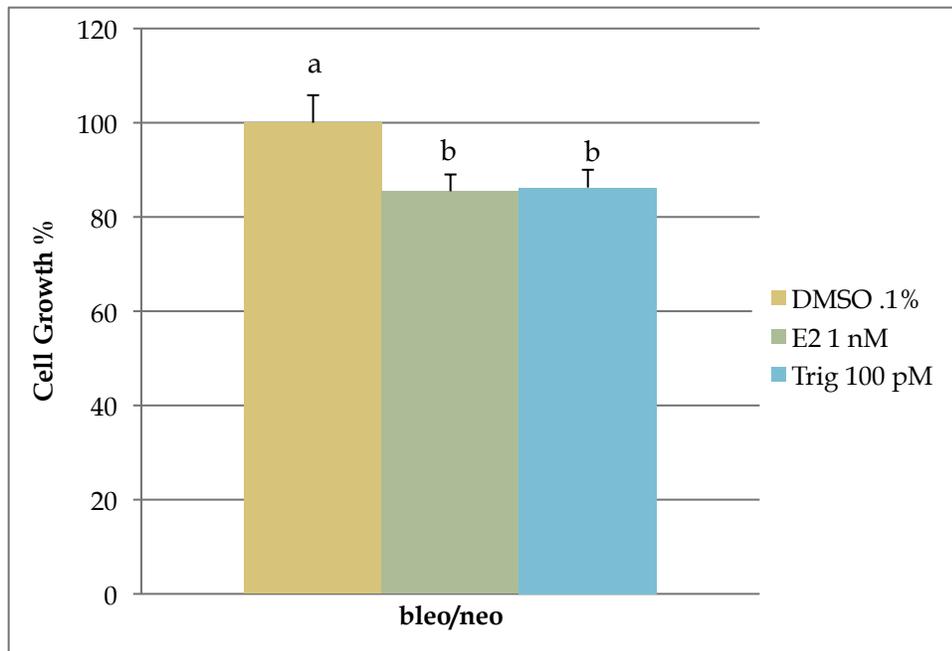
The data are expressed throughout as the mean ± SD, which was calculated from at least three different experiments. The statistical significance among the test groups was determined by One-way ANOVA. A p-value of less than 0.05 was considered significant.

## CHAPTER III

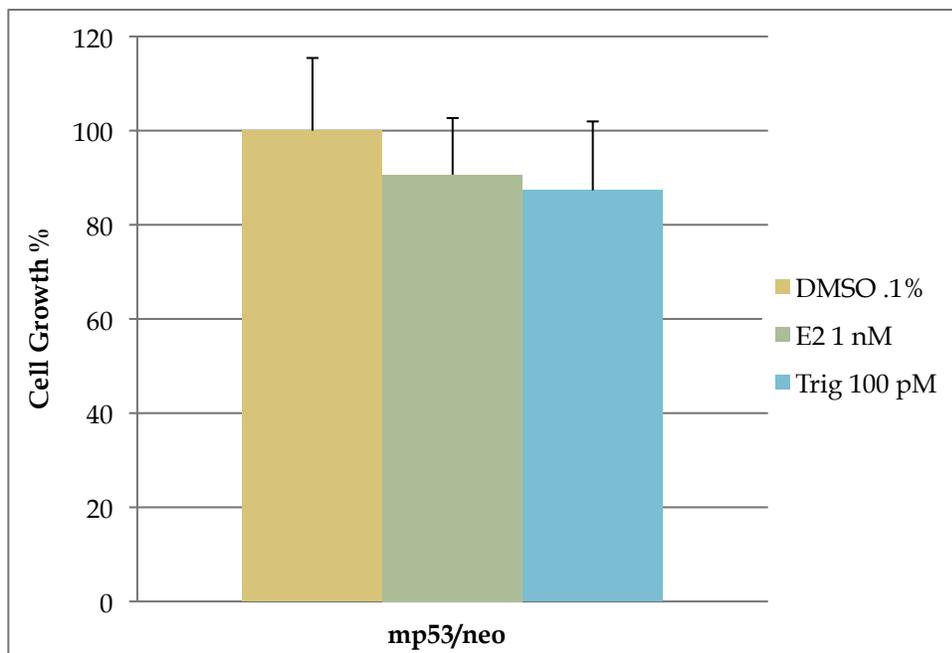
### RESULTS

#### **E<sub>2</sub> and Trig cell growth regulation requires a wild type p53 protein**

To explore the role p53 plays in the physiological response of non-malignant colonocytes to E<sub>2</sub> as compared to Trig 100 pM, we examined the growth of YAMCs in response to both treatments. Both Trig and E<sub>2</sub> suppressed cell growth in YAMCs with wild-type p53 (Figure 1a), but the effect of Trig and E<sub>2</sub> is lost in YAMCs with mutant p53 (Figure 1b). In the wild-type p53 colonocytes (bleo/neo), E<sub>2</sub> 1 nM was included as a positive control, while .1% DMSO was used as a negative control. Both Trig and E<sub>2</sub> suppressed cell growth significantly as compared to DMSO. YAMCs with a mutant p53 (mp53/neo) were run through an identical protocol to determine if modulation via Trig required a functioning p53 for growth suppression. Results were not significant, but as compared to bleo/neo cell growth, it is obvious that the effect of Trig and E<sub>2</sub> was not as profound without a mutant p53 present.



**Figure 2a.** *Effect of E<sub>2</sub> or Trig on bleo/neo cell number.* Values are means +/- SEM. Bars without a common letter differ, p-values < 0.05.

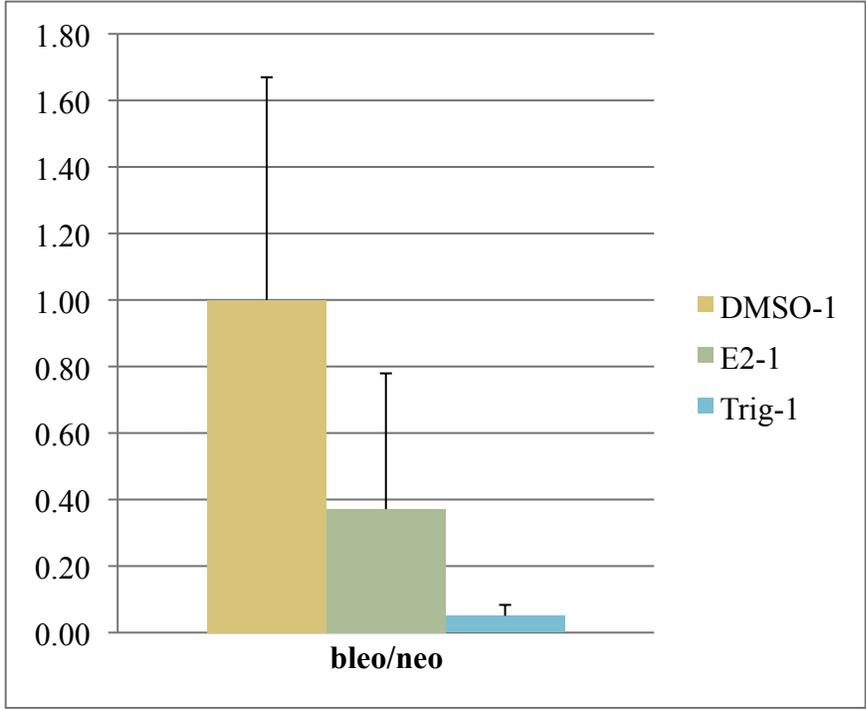


**Figure 2b.** *Effect of E<sub>2</sub> or Trig on mp53/neo cell number.* Values are means +/- SEM. Bars without a common letter differ, p-values < 0.05.

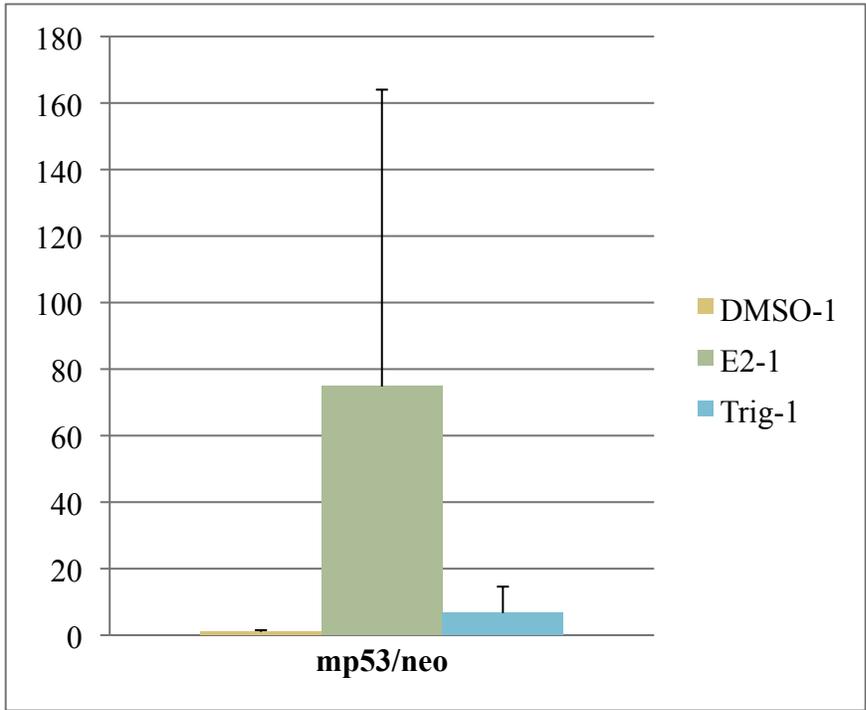
### **Trig and E<sub>2</sub> decrease expression of pro-apoptotic targets in cells with wild type p53**

Investigation of p53 downstream targets in non-malignant colonocytes was analyzed by quantifying response to E<sub>2</sub>, Trig and DMSO treatments. Through the analysis of gene expression of p53 downstream targets PUMA (Figure 3a and 3b), NOXA (Figure 4a and 4b), and Bax (Figure 5a and 5b) via RT-PCR would demonstrate the ability of E<sub>2</sub> and Trig treatments to induce apoptosis. E<sub>2</sub> 1 nm was used as a positive control and .1% DMSO was used as a negative control.

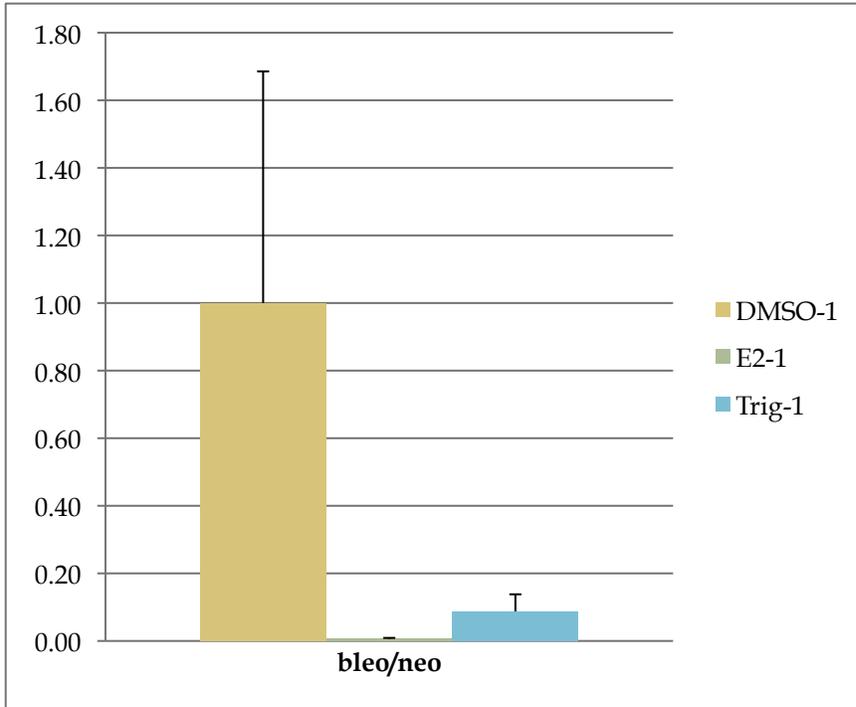
E<sub>2</sub> and Trig decreased the expression of all three measured pro-apoptotic target proteins of p53 in cells with a functioning p53 as compared to DMSO. The opposite effect was seen in mp53/neo cells, E<sub>2</sub> and Trig increased the expression of pro-apoptotic proteins in all three of the target proteins. None of the protein measurements for bleo/neo or mp53/neo were statistically significant when compared to the negative control, DMSO.



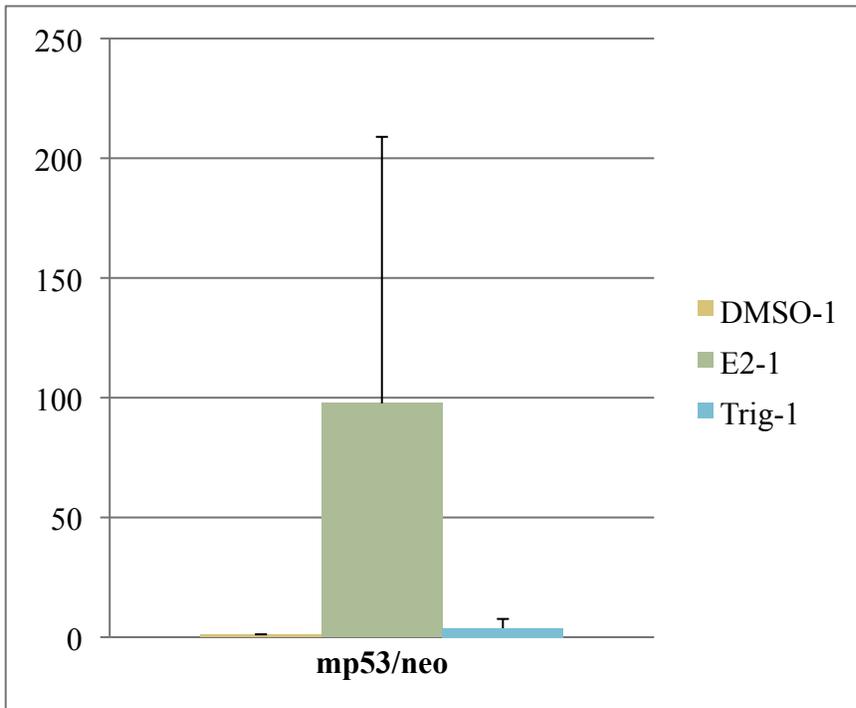
**Figure 3a.** *Effect of E<sub>2</sub> and Trig on levels of PUMA.*



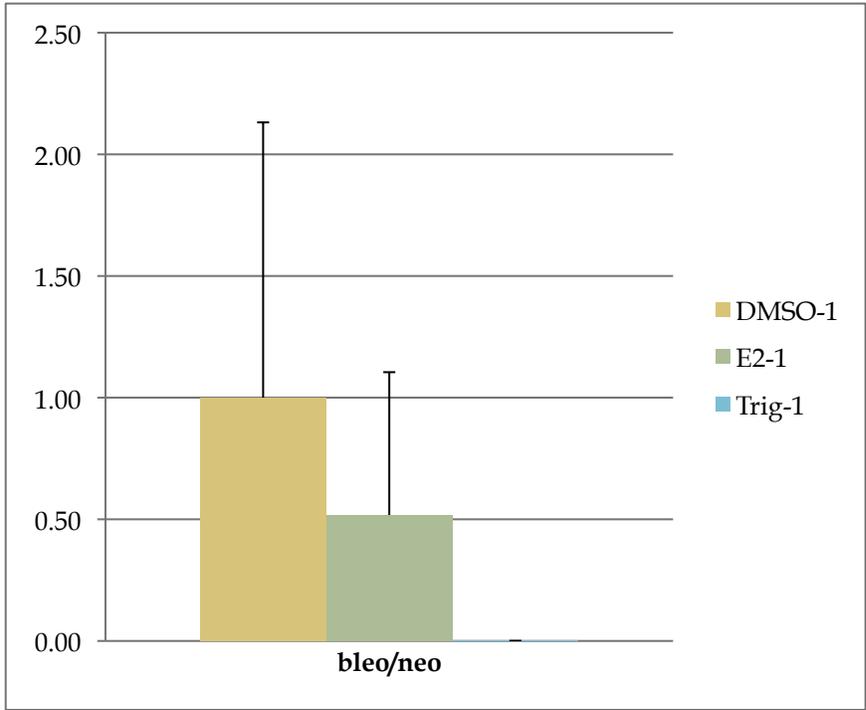
**Figure 3b.** *Effect of E<sub>2</sub> and Trig on levels of PUMA.*



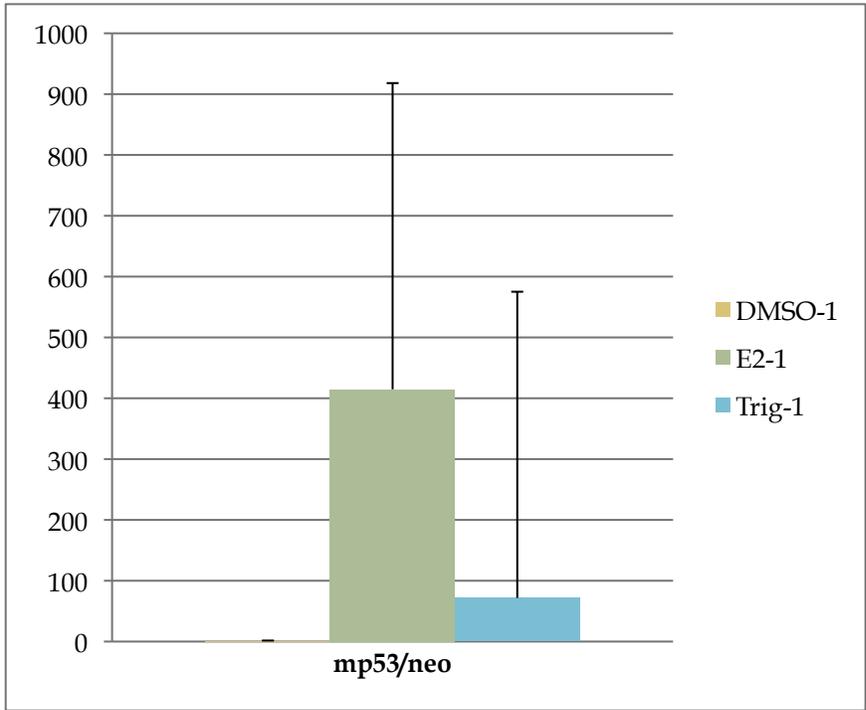
**Figure 4a.** Effect of  $E_2$  and Trig on levels of NOXA.



**Figure 4b.** Effect of  $E_2$  and Trig on levels of NOXA.



**Figure 5a.** *Effect of E<sub>2</sub> and Trig on levels of Bax.*



**Figure 5b.** *Effect of E<sub>2</sub> and Trig on levels of Bax*

## CHAPTER IV

### DISCUSSION

Epidemiological and clinical data suggest that E<sub>2</sub> has a protective effect against the development of colon cancer [8, 9, 11, 13, 15]. However, the mechanism to explain this correlation remains unclear. Due to the lack of consistency of these results with data that is statistically significant, my findings are not conclusive. Earlier data shows that levels of the pro-apoptotic genes Bax, and PUMA were significantly increased following E<sub>2</sub> treatment in YAMC bleo/neo cells [8]. This is the opposite of the effect seen in all three of the proteins measured in this study.

Because of the documented estrogenic activity of Trig, it was not surprising that both E<sub>2</sub> and Trig it suppressed cell growth in colonocytes with a wild type p53 [12]. Our laboratory has continually demonstrated that E<sub>2</sub> increases the expression of pro-apoptotic target proteins in cells with a functioning p53. However, my results showed that E<sub>2</sub> and Trig decreased the expression of all three measured pro-apoptotic target proteins of p53. This was the opposite effect of what was expected in the study design, and what has been seen in previous E<sub>2</sub> studies. The lack of consistency with previous studies, and the large error observed in the RT-PCR data makes it difficult to draw any solid conclusions from this study. It has been demonstrated that Trig is a novel phytoestrogen and requires a functioning ER to effect cellular activity. The expected results would have indicated that Trig has the potential to have an effect similar to E<sub>2</sub> through p53 mediation.

In conclusion, it is unclear how much or if there is any modulation on cell growth and apoptosis by Trig. The concentration or bioavailability of Trig in coffee and other plants is also unclear. Exposure to phytoestrogens also has the potential to be a problem for other tissues, and further risks should be analyzed. Additional research is needed to determine the long-term health risks and benefits of Trigonelline.

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