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Evaluation of the Potential HMGA1-EF24 Nexus in Human Colon Cancer

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ABSTRACT

The architectural chromatin binding proteins High Mobility Group A1 (HMGA1) are proteins expressed at high levels in malignant cancers and induce neoplastic transformation. The protein is increased as the last step of the Wnt/ β -catenin/TCF-4 pathway and mediates drug resistance, therefore correlating with a poor patient prognosis. HMGA1-mediated chemoresistance results from a self-protective process called cellular senescence. Analogs of the antioxidant, curcumin, when used in combination with traditional chemotherapeutic agents, are useful treatment options for drug resistant tumors. This study had two specific aims. The first being to investigate how colon cancer cells HCT116 respond to treatment with EF24. The second aim was to evaluate how *hmg1* expression changed as a result of treatment with EF24. Our preliminary findings showed that cell viability decreased after 24-hour treatment with low-dose EF24, as indicated by an MTS assay, with notable discrepancies between cells that underwent a pulsed treatment regimen versus those that underwent continuous treatment. Furthermore, we demonstrated that cells exhibited fragmented DNA when treated with low-dose EF24, which is characteristic of apoptotic cells. At higher, pulsed doses, senescence activity increased, indicating the induction of a senescence pathway. Lastly, gene expression studies indicated that *hmg1* was significantly down regulated in cells treated with continuous, low-dose EF24. Further investigation of this pathway could lead to decreased toxicity and increased viability of combination cancer therapies.

INTRODUCTION

The High Mobility Group A1 (HMGA1) family of proteins are non-histone binding architectural proteins. They are aptly named based on their high mobility in polyacrylamide gel (Reeves, 2001). The sub-family consists of two isoforms, HMGA1a and HMGA1b, which are the result of alternately spliced mRNA transcripts derived from the common *hmg1* gene. The HMGA1 proteins are characterized by a prominent binding domain that preferentially binds to AT-rich regions on the minor groove of DNA (Reeves, 2001). This allows the protein to alter shape of B-form DNA and control the transcription of a variety of genes and the activity of a variety of proteins (Thanos *et al.*, 1992). Depending on the context, HMGA1 can promote or repress a variety of molecular pathways. As a result, the HMGA family participates in a number of cellular processes including cellular proliferation, neoplastic transformation, aging and metastasis (Reeves, 2001).

Hmg1 was identified as a gene that is induced by growth factors, and is required by cells to pass through the G1/S cell cycle checkpoint (Fedele *et al.*, 2001). More importantly, HMGA1 functions as an oncogene when overexpressed in those same cells (Resar, 2010). Therefore, it follows that *hmg1* is up-regulated in a number of malignant cancers including, but not limited to: ovarian, breast, lung, thyroid, and colon cancers (Scala *et al.*, 2000). When tissue samples from normal, unaffected mice were compared to their metastatic counterparts, the HMGA1 levels were almost undetectable; suggesting that HMGA1 plays an important role in the transformation of cells from normal to malignant (Xu *et al.*, 2004). Furthermore, when compared to localized tumors, *hmg1* was vastly up-regulated in the metastatic counterparts. Similarly, malignant tissues in which *hmg1* is overexpressed exhibit increased resistance to common chemotherapeutic drugs. When *hmg1* expression is inhibited, sensitivity to the same

drugs increased significantly (D'Angelo *et al.*, 2014). Taken together, these factors suggest that there is a correlation between high levels of *hmga1* expression and poor patient prognosis.

When a malignant cell undergoes therapeutic treatment, it triggers a number of responses; the two most prominent responses are apoptosis and a protective cellular process known as senescence (Childs *et al.*, 2014). Previous research shows that HMGA1 disrupts normal cell cycle regulation by preventing malignant cells from undergoing apoptosis while simultaneously propelling it through mitosis (Fedele *et al.*, 2001). Contrastingly, HMGA1 also promotes cellular senescence (Narita *et al.*, 2006). Senescence is characterized by arrest of the cell cycle, expression of SA- β -galactosidase and induction of tumor suppressors p16 and p21. Senescent cells can then induce a change in phenotype in surrounding cells through emission of an intercellular signal (Narita *et al.*, 2006).

Curcumin, the active ingredient of the turmeric plant, possesses novel antioxidant, anti-inflammatory and anti-proliferative properties. Studies show that malignant tissues treated with curcumin exhibited elevated levels of HMGA1 and subsequently induced apoptosis and senescence (Kuo *et al.*, 1996; Jin *et al.*, 2016 and Mosieniak *et al.*, 2016). However, a clinical study showed that curcumin presents with low bio-availability (Ramasamy *et al.*, 2015 and Cheng *et al.*, 2001). EF24 is a chemically synthesized analog of curcumin, however its exact anti-proliferative mechanism remains relatively unknown.

Taken together, we sought to investigate the relationship between *hmga1* expression and treatment with EF24, with two specific questions in mind. How does *hmga1* expression change upon treatment with EF24? How do HCT116 cells respond to treatment with EF24? It was shown that upon treatment with low-dose, continuous EF24, *hmga1* expression was down-regulated. Furthermore, cell viability decreased significantly upon treatment with both continuous and pulsed EF24. Lastly, at low concentrations of EF24, there was evidence of apoptosis, whereas at

higher concentrations there was increased evidence of senescence. Further investigation into this nexus could lead to the discovery of a viable, targeted combination cancer therapy.

METHODS

Cell Culture

The HCT116 human colon cancer cell line was obtained from the American Type Culture Collection (ATCC). Cells were maintained in McCoy's 5A media supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin-Amphotericin B. Cells were maintained in a humidified incubator at 37°C and 5% CO₂.

RNA Extraction and RT-PCR

HCT116 cells were treated with varying concentrations of EF24 (0.0, 0.25, 0.5 μ M) for 24h. After 24h, total RNA was isolated using the QuickRNA Mini Prep kit following the manufacturer's instructions. GAPDH was used as a positive control. The forward and reverse primers for both HMGA1 and GAPDH are shown below:

<u>Primer</u>	<u>Sequence</u>
HMGA- Forward	5'- GAT GGG ACT GAG AAG CGA-3'
HMGA1-Reverse	5'-CTT CTC CAG TTT CTT GGG TC-3'
GAPDH- Forward	5'-TGC ACC ACC AAC TGC TTA GC-3'
GAPDH-Reverse	5'-GGC ATG GAC TGT GGT CAT GAG-3'

The assembled reactions were run using the following thermal cycler program:

<u>Temperature (°C)</u>	<u>Time</u>	<u># of cycles</u>
50	15 min	1
95	15 min	1
95	20 s	25
55	30 s	
72	1 min	
72	5 min	1

Cell Viability MTS Assay

Approximately 6000 cells were plated per well in a 96-well plate, and were incubated

for 24h. Cells were then treated with increasing concentrations of EF24 for 24h. For continuous treatment, the treated cells were assayed for cell viability following 24h EF24 treatment using CellTiter Aqueous One Solution Cell Proliferation Assay. For pulsed treatment, the treated media was removed, replaced with drug-free media and cells were incubated for an additional 48h. Following the 48h recovery, cells were assayed for cell viability. Cell viability was quantified by measuring absorbance at 490nm.

Apoptosis Assay

HCT116 cells were plated in a 6-well plate and treated with increasing EF24 concentrations (0.0, 0.05, 0.125 μM) for 24h. Treated media was then removed, and cells were cultured in drug-free media for an additional 48h. Genomic DNA was then isolated and the fragments were resolved on a stained agarose gel.

SA- β -galactosidase Activity Senescence Assay

Approximately 10,000 cells were plated per well in a 6-well plate and incubated for 24h. Cells were then treated with increasing concentrations of EF24 (0.0, 0.05, 0.125 μM) and incubated for 24h. Treated media was removed and replaced with drug free media and incubated for an additional 48h. Cells were lysed and β -galactosidase activity was assayed using the 96-well Cellular Senescence Assay Kit (Cell Biolabs Inc.) Enzyme activity was quantified by measuring absorbance at 360nm.

RESULTS

*EF24 Treatment Down-Regulates *hmga1* Expression*

HMGA1 has a well-established link to the phenotype of colon cancer cells. Therefore, we initially analyzed the effect EF24 treatment had on *hmga1* expression. Treatment with EF24 had a significant effect on the expression of *hmga1* in HCT116 cells. As the concentration of EF24 increased, the expression of the gene was three to four-fold down regulated (**Figure 1**).

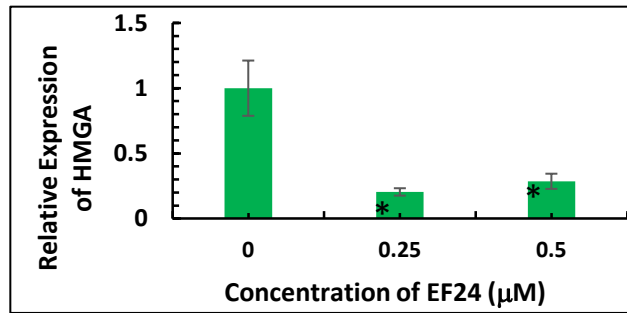


Figure 1. HMGA1 mRNA Levels in HCT116 Cells Treated with Low-Dose EF24 are Down-Regulated Quantitative real time RT-PCR shows HMGA1 mRNA levels in treated HCT116 cells are significantly down-regulated when compared to untreated cells ($p < 0.05$)

Continuous EF24 Treatment Decreases Cell Survival

The HMGA1 is a key player in oncogenic transformation because it allows cells to ignore normal growth pathways and cell cycle regulations, resulting in abnormal proliferation. Upon observing that treatment with EF24 resulted in a down-regulation of *hmga1*, we decided to test for cell viability after a continuous 24h treatment. **Figure 2** indicates that there was a significant decrease in cell viability from 100% to approximately 65% at low doses of EF24. With a gradual decrease in cell viability as the concentration of EF24 increases.

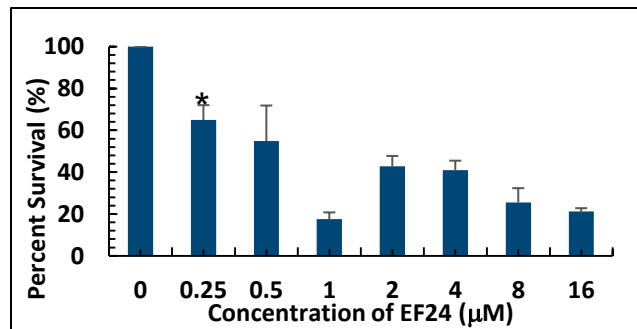


Figure 2. HCT116 Cells Treated with Continuous Low-Dose EF24 Experienced Decreased Cell Survival Cell proliferation assays showed treated HCT116 cells were significantly less viable when compared to untreated cells ($p < 0.05$)

Pulsed EF24 Treatment Decreases Cell Survival and Induces Apoptosis

Chemotherapeutic treatment follows a treatment-recovery model. Patients undergo treatment for a period, which is then directly followed by a period of recovery. We sought to mimic that model by allowing the cells to recover post-treatment. **Figure 3A** indicates that there was a significant decrease in cell viability from 100% to 15% at low doses of EF24. Cell viability was noticeably low across the board, indicating that cells were unable to recover after the treated media was removed regardless of EF24 concentration.

The prominent decrease in cell viability prompted the need to observe the mechanism by which the cells were dying, particularly at low doses. DNA isolated from untreated cells and from cells treated with 0.05 μM EF24 presented with fragmented DNA. Contrastingly, cells treated with 0.125 μM presented with solely whole DNA (**Figure 3B**).

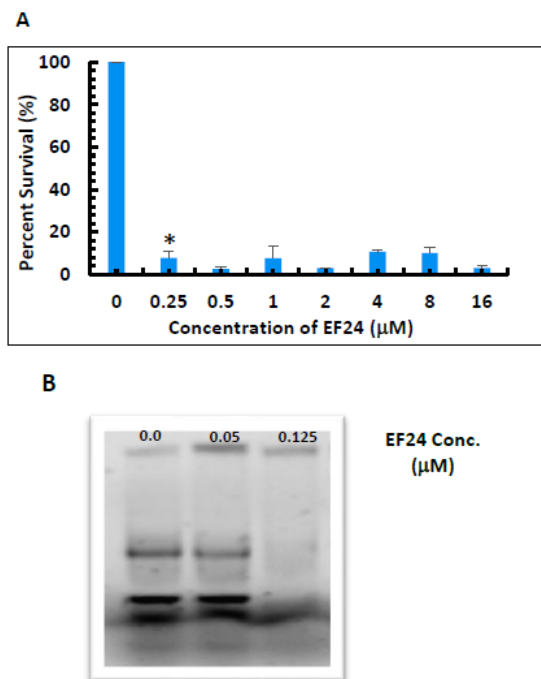


Figure 3. HCT116 Cells Treated with Pulsed Low-Dose EF24 Experienced Apoptosis (A) Cell viability assays show that there was a significant decrease in cell viability between treated cells and untreated cells ($p < 0.05$) (B) Isolated DNA from treated cells was resolved to show the presence of fragments.

Increased Concentrations of EF24 Induce Senescence

It has been shown that curcumin, the naturally occurring counterpart of EF24, has the capacity to force cells into a protective process known as senescence. The absence of fragmented DNA upon treatment with higher concentrations of EF24 leads us to believe that the cells may have been forced out of the cell cycle into cellular senescence. **Figure 4** is indicative of an increase in senescence associated β -galactosidase activity from untreated to treated. However, the difference in activity was not found to be statistically significant.

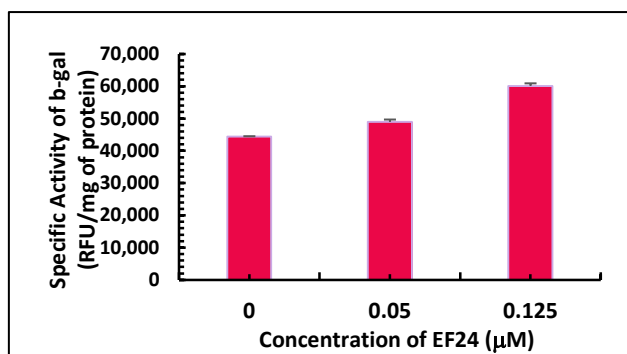


Figure 4. HCT116 Cells Treated with Pulsed Low-Dose EF24 Exhibited Increased SA- β -galactosidase Activity SA- β -galactosidase assays showed that enzymatic activity increased as concentration of EF24 treatment increased. However, results were not statistically significant.

DISCUSSION

HMGA1 is overexpressed in a variety of different malignant tumors (Scala *et al.*, 2000). Not only does it facilitate the neoplastic transformation of normal cells, it also facilitates drug resistance and abandonment of the cell cycle while simultaneously inhibiting programmed cell death (Xu *et al.*, 2004; D'Angelo *et al.*, 2014; and Fedele *et al.*, 2001). Recent studies have shown that HMGA1 also plays a role in the promotion of cellular senescence (Narita *et al.*, 2006). The present study investigated the relationship between HMGA1 expression and EF24 in colon cancer cells. The data indicates that treatment with EF24 significantly decreases cell viability at low-doses and down-regulates *hmga1* expression. Furthermore, upon pulsed treatment with low

doses, both apoptotic and senescent activity are exhibited.

As previously mentioned, expression of *hmga1* results in the inhibition of p53 mediated apoptosis (Fedele *et al.*, 2001). Therefore, it follows that upon treatment with therapeutic agent, EF24, *hmga1* expression should decrease. This hypothesis was confirmed by PCR analysis. Upon down-regulation of *hmga1*, the normal cell cycle should resume resulting in a decrease in overall cell viability. This trend is observed in the cells treated with continuous EF24. Therefore, EF24 is effective in reducing the viability of colon cancer cells as a first wave attack. However, this model of treatment does not accurately represent most modern chemotherapy treatments.

Chemotherapy follows a treatment-recovery model. Patients are treated for a short period of time and allowed to recover. This method is employed as a means of protecting non-malignant cells from repetitive attack by harmful chemotherapeutic drugs. Therefore, an optimal chemotherapeutic compound should not continue to kill cells during the recovery period. Doing so would put vital stem cells at risk of being compromised. Keeping this in mind, the colon cancer cells were treated with EF24 using a pulsed treatment model. The results indicated that cell viability decreased significantly despite the 48-hour recovery period. This prompted us to investigate exactly what was happening to the cells to compromise their viability. The apoptosis assay showed evidence of fragmented DNA in both the untreated cells and cells that were treated with the lowest concentration of EF24. However, at higher concentrations, the band that was indicative of fragmented DNA disappears. Taken together with the data from the senescence assay leads us to postulate that at higher, pulsed concentrations, cells forego the apoptotic pathway in favor of the protective cellular senescence pathway. When cells are continuously treated with drugs, some will die and some will enter senescence (Childs *et al.*, 2014). However, the 24-hour treatment time might not be long enough to allow the senescent cells to signal neighboring cells to enter senescence. Contrastingly, when cells are

treated using a pulsed regimen, the cells that have entered senescence can continue to signal neighboring cells even after the treated media is removed resulting in the drastic decrease in cell viability observed in **Figure 3A**.

Continuing to study the role of HMGA1 in the cellular response of colon cancer following treatment with EF24 may contribute to the discovery and development of less toxic combination cancer therapies.

FUTURE WORK

To confirm the hypothesis that treatment using a pulsed regimen promotes cellular senescence, both apoptosis and senescence assays need to be conducted using cells that have been treated with a continuous treatment model. Furthermore, *hmga1* expression studies need to be done using cells treated with a pulsed treatment model.

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