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Effects of Select Vitamin E Isoforms on the Production of Polyunsaturated Fatty Acid
Metabolites in Colorectal Cancer

A thesis
presented to
the faculty of the Department of Chemistry
East Tennessee State University

In partial fulfillment
of the requirements for the degree
Master of Science in Chemistry

by
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May 2015

Dr. Sharon Campbell, Chair
Dr. William Stone
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Keywords: Polyunsaturated fatty acids, hydroxyeicosatetraenoic acids,
hydroxyoctadecaenoic acids, alpha tocopherol, gamma tocopherol, gamma tocotrienol,
alpha-gamma tocopherol mixture

ABSTRACT

Effects of Select Vitamin E Isoforms on the Production of Polyunsaturated Fatty Acid Metabolites in Colorectal Cancer

by

Martha Akuorkor Borketey

Vitamin E exhibits anti-tumor activity by regulating pathways in cancer cells, potentially the lipoxygenase (LOX) pathway. We studied the effects of alpha tocopherol (AT), gamma tocopherol (GT), gamma tocotrienol (GT3), and an alpha-gamma tocopherol mixture (ATGT) on the production of the LOX metabolites 13-hydroxyoctadecaenoic acid (HODE), 15-hydroxyeicosatetraenoic acid (HETE), 12-HETE, and 5-HETE in colorectal cancer. These metabolites were examined in the HCT-116 cell line after 24 h treatment with select vitamin E isoforms and quantified by LC/MS/MS. Under physiological conditions, we find that treatment with varying vitamin E isoforms have different effects on the production of 13-HODE, 15-HETE, 12-HETE, and 5-HETE. GT increases 13-HODE and decreases 12-HETE. AT reverses the effects of GT regulation on the LOX pathway, while GT3 has no significant effect on the metabolites tested. GT shows superiority in regulating the LOX pathway as it increases 13-HODE and decreases 12-HETE for possible prevention of colorectal cancer.

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DEDICATION

I dedicate this thesis to my husband Matthew Essandoh and my family.

ACKNOWLEDGEMENTS

I thank the good Lord for bringing me to a successful completion of this program. I also thank my supervisor, Dr. Sharon Campbell for directing me through this research, her collaborator Dr. Stacy Brown for helping with the analysis of our samples, and Drs. Cassandra Eagle and William Stone for serving on my committee.

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CHAPTER 1

INTRODUCTION

Mechanisms of Cancer Development

Cancer is a disease consisting of a mass of cells that grow and divide faster than normal cells of the corresponding tissue type. Some features of cancer cells include the fact that they are poorly differentiated compared with normal cells. Cancer cells also have the ability to metastasize as compared to normal cells which are confined to their position within an organ by cell adhesion molecules¹.

The development of cancer involves multiple processes which are marked by four successive stages: initiation, promotion, progression and malignancy². The first stage, initiation, often occurs as a result of continual deoxyribonucleic acid (DNA) damage by reactive oxygen species (ROS) such as hydrogen peroxide, superoxide radicals, and hydroxyl radicals. These ROS are produced by exogenous and endogenous sources such as environmental pollutants, radiation (e.g. X-rays, ultraviolet light (UV) light and gamma rays), metals (i.e. by Fenton reactions), activation of inflammatory cells (e.g. macrophages and neutrophils), cytochrome P450 enzyme etc^{2,3,4}. Mutations to genes can result in the formation of oncogenes and the loss of function of tumor suppressor genes⁵ which may be prevented, in part, by antioxidant intake. Tumor suppressor genes are genes that hinder the proliferation of cells. Malfunction or inactivation of these genes can promote carcinogenesis⁶. Inactivation of

tumor suppressor genes occurs through mechanisms such as point mutations (change of a single nucleotide in DNA) or deletions⁷.

Oncogenes are genes that are formed as a result of mutations in proto-oncogenes (i.e. proteins that regulate cell proliferation)⁸. Oncogenes can be activated by mechanisms such as gene amplification (i.e. production of several copies of a gene due to continuous replication of a section of the DNA), point mutation and chromosome rearrangements or translocations (i.e. attachment of a section of chromosome to a different chromosome)^{9,7}. These mutations cause proto-oncogenes to be more highly expressed in cells than normal which enhance carcinogenesis^{10,1,11}.

Promotion, the second stage of cancer development, involves proliferation of the altered cells to produce benign tumors resulting from group of compounds called promoters². Progression and malignant stages in carcinogenesis occur when some of these benign tumors further develop to form invasive tumors^{7,2}.

Carcinogenesis occurs due to a malfunctioning of genes that usually regulate the growth and proliferation of cells which leads to continual progression of the cell cycle⁷. Alterations of genes that control the checkpoints of the cell cycle from Gap1 (G₁) to S (DNA synthesis) or from Gap 2 (G₂) to Mitotic (M) phase may cause cells to rapidly divide (enhance cell proliferation) or may cause cells to refuse to undergo apoptosis. Mutations in these checkpoint genes arise in many human cancers¹². These genes include cyclins, retinoblastoma gene (RB), p53 gene, cyclin-dependent kinases (cdks)⁷, etc.

Cyclin-dependent kinase 4 (Cdk4) and cyclin D1 complex (proto-oncogenes) are also genes that control the G₁ phase. These genes phosphorylate RB causing the continuation of the cell cycle in normal cells¹. The over expression of cyclin D1 or Cdk4 enhance cell proliferation and are observed in various human cancer cells including breast cancer¹³.

RB is a tumor suppressor gene that is hypophosphorylated in the initial stages of G₁ and bonds to E2F proteins, nullifying E2F activity by joining to promoter genes in which the E2F/DP complex bind. The E2F/DP complex regulates proteins involved in the development of the cell cycle such as cyclins and cdks. When RB is hypophosphorylated, it inhibits the untimely production of genes that control the cell cycle^{1, 7} and allows for DNA replication. Functional RB protects the fidelity of the DNA by preventing DNA replication of mutated DNA. Dysfunction of the RB gene results in replication of mutated DNA which creates genetic instability and allows for uncontrolled cell proliferation in several cancers¹³. Defects in the RB gene in germ line cells can also cause familial RB cancers⁷.

The p53 gene is another tumor suppressor gene which functions as a pro-apoptotic gene by preventing multiple mutations from being integrated into DNA, by up-regulating apoptotic proteins that cause the death of the cells when severe DNA damage occurs¹. Malfunctioning of the tumor suppressor gene p53 prevents apoptosis from occurring and leads to an increase in mutations, which enhances the development of cancer. Apoptosis is a process that allows organisms to control the number of cells in a tissue by removing cells that are aged or damaged¹². The significance of apoptosis

in the development of cancer is observed when mutated cells continue to proliferate instead of dying¹². The refusal of cancer cells to undergo apoptosis plays a significant role in the progression of tumors¹⁴.

The p16 gene is a tumor suppressor gene whose function is to hinder the continuation of the cell cycle when cells are damaged by inhibiting the activity of cyclin D1 and Cdk4 complex. A defect in the p16 gene is observed in many human cancers. Mutations in other genes (e.g. p15 and p18) that inhibit the production of cyclin D1 and Cdk4 complex are also observed in various human cancers^{13,12}. However, over expression of B-cell lymphoma 2 (bcl-2) protein, an anti-apoptotic protein, impedes apoptosis and enhances the survival of cancer cells¹.

Carcinogenesis may also occur as a result of poor differentiation of cells. Cell differentiation is the process by which specialized cells are formed from non-specialized stem cells. These specialized, differentiated cells generally perform the work of tissues in an organism. Tumors may develop as a result of incomplete differentiation of these stem cells, which may occur as a result of mutations in their DNA, or dedifferentiation of fully differentiated cells. Accumulation of mutations in cells may prevent cells from undergoing complete differentiation which could lead to cancer development^{12,1,15}.

The carcinogenesis process is influenced by environmental and hereditary factors¹⁶. Hereditary factors typically range from 5% to 10% of all cancers while environmental factors account for 90% to 95% of cancers¹⁷. Environmental factors include obesity, poor nutrition, physical inactivity, radiation, tobacco usage, and exposure to pollutants from the environment¹⁷.

Colorectal Cancer and its Risk Factors

Colorectal cancer is ranked as the third most common cancer in both males and females¹⁶. In 2014, approximately 136,830 colorectal cancer cases with 50,310 deaths are anticipated to occur in the United States. Colorectal cancer risk increases with age and 90% of colorectal cancer cases are identified in people who are 50 years and older. Alcohol intake, chronic smoking, lower intake of fruits and vegetables as well as a diet rich in meat (red or processed) are environmental factors that enhance the risk of developing colorectal cancer¹⁶.

Tobacco smoking is a major cause of cancer worldwide¹⁸. More than 60 carcinogens have been confirmed to be present in cigarette smoke¹⁹. These include aldehydes, volatile organic hydrocarbons, *N*-nitrosamines, aromatic amines, metals, and polycyclic aromatic hydrocarbons (PAHs). Among these, the major carcinogens in human cancers are *N*-nitrosamines, aromatic amines, and PAHs¹⁹. The relationship between tobacco products and cancer development in humans is due to the presence of these carcinogens^{20,21}. Cancer associated with tobacco consumption includes bladder, lung, stomach, kidney, colorectal, and pancreatic cancers¹⁸. It has been shown that smokers have a greater risk of developing colorectal cancer compared with non-smokers²². In addition, the risk of developing colorectal cancer is linked to the number of years of cigarette smoking²².

Cigarette smoke extract enhances the growth of colorectal tumors by up-regulating the expression of cyclooxygenase-2 (COX-2) and 5-LOX proteins as well as their metabolites prostaglandins E2 (PGE₂) and leukotriene B4, in colorectal tumors²³.

Nicotine, a primary constituent of cigarettes increases cell proliferation in human colorectal cancer cell lines²⁴. These findings indicate that tobacco constituents play a critical role in colorectal tumorigenesis.

Dietary factors have also been associated with colorectal cancer development. For example, high intake of fruits (e.g. banana, fruit juice, pear, citrus fruits, and apple) and vegetables (e.g. spinach, tomato, carrot, cabbage) are inversely linked to colorectal cancer development²⁵. The intake of fruit fiber is also shown to be inversely linked to the risk of developing colorectal cancer²⁶.

However, a high intake of red (e.g. lamb, beef and pork) and processed meat (e.g. corned beef, chicken, ham, sausages, bacon etc.) is reported to be significantly linked to enhanced risk of colorectal cancer development^{27,26}. Animal fat including monounsaturated fat and saturated fat is positively linked to the risk of developing colorectal cancer²⁶. In addition, a significant inverse link is observed between polyunsaturated fatty acids and colorectal cancer development²⁸.

The Role of Polyunsaturated Fatty Acid Metabolites and Their Enzymes in Cancer

Bioactive products such as PGs, HETEs, and HODEs produced through the metabolism of arachidonic acid (AA) and linoleic acid (LA) by LOX and COX enzymes are associated with carcinogenesis^{29,30,31}.

COX enzymes metabolize AA to produce thromboxane A₂ (TXA₂) and PGs (D₂ and E₂)³². LOX enzymes also metabolize AA to produce hydroperoxyeicosatetraenoic acids, which are subsequently reduced to form HETEs. The main LOX enzymes

present in humans are 15-LOX, 5-LOX, and 12-LOX which metabolize AA to produce 15-HETE, 5-HETE, and 12-HETE, respectively^{33,34}. 15-LOX also metabolizes LA to produce 13-HODE and 9-HODE³⁴. Production of LOX and COX metabolites are illustrated in Figures 1, 2, and 3^{35,36}.

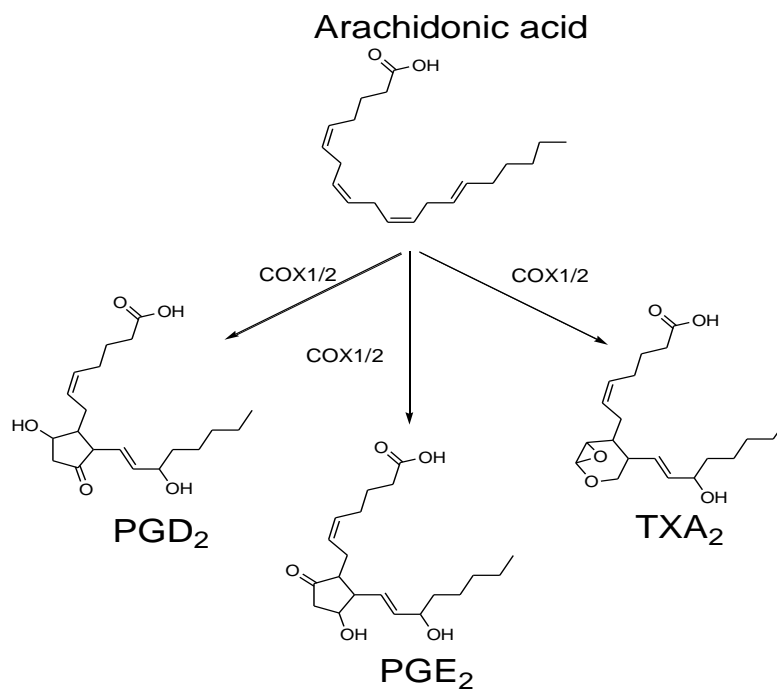


Figure 1. The production of TXA₂, PGD₂, and PGE₂ through the metabolism of AA by COX enzymes

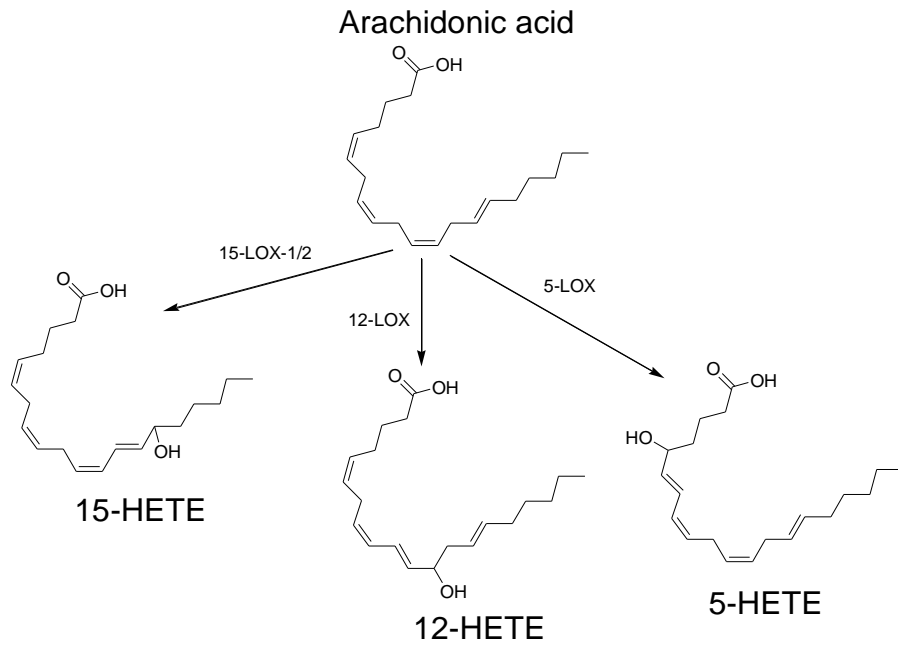


Figure 2. The production of 15-HETE, 12-HETE, and 5-HETE through the metabolism of AA by LOX enzymes 15-LOX-1, 12-LOX, and 5-LOX respectively

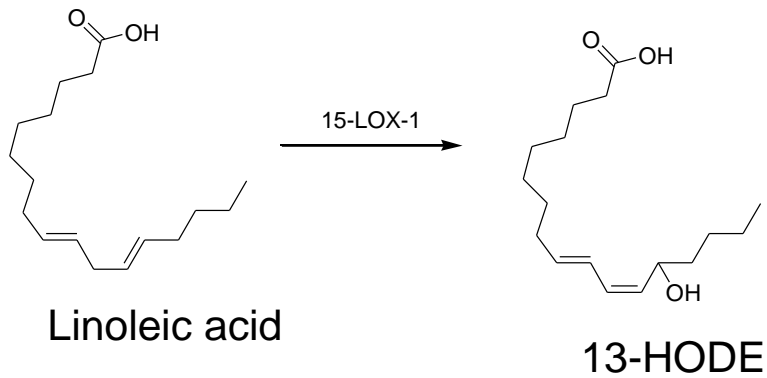


Figure 3. The production of 13-HODE through the metabolism of LA by 15-LOX-1 enzymes

A number of studies indicate that COX and LOX pathways play important roles in cancer development^{37,38}. For instance, COX-2 is highly expressed in various tumors including prostate and colorectal tumors^{39,37,40}. Also, 12-LOX and its metabolite 12-HETE are shown to be highly expressed in colorectal tumors compared with the normal colorectal tissue, indicating that over expression of the 12-LOX pathway enhances colorectal carcinogenesis^{41,42,43}. High levels of 12-HETE in colorectal cancer cells enhances cell proliferation which is important to colorectal cancer development^{41,44}. Hence, inhibiting the production of 12-HETE may slow proliferation in colorectal cancer cells. This was demonstrated when the reduction of 12-HETE production by sulindac (a non-steroidal anti-inflammatory drug (NSAID)) decreased the colon tumor growth in Fischer 344 rats⁴². Also, a decrease in 12-HETE production by baicalein (a 12-LOX inhibitor) hindered cell proliferation in Caco-2 human colon cancer cells⁴⁴. These studies indicate that, blocking the 12-LOX pathway is important in preventing colorectal cancer development.

In addition to colorectal cancer cells, 12-LOX is also shown to be highly expressed in prostate cancer cells^{45,46}. This was demonstrated in a study that analyzed the expression of 12-LOX in the cancer and normal tissue collected from the prostate of 122 prostate cancer patients. Approximately half of prostate cancer patients had high levels of 12-LOX in their prostate cancer tissues compared to their corresponding normal prostate tissues, suggesting that 12-LOX may contribute to the progression of prostate tumors⁴⁵. Blocking 12-LOX pathway by its inhibitors (i.e. N-benzyl-N-hydroxy-5-phenylpentamide (BHPP) and baicalein) was shown to enhance apoptosis, decrease cell proliferation and cause cell cycle arrest in prostate cancer cells (DU-145 and

PC3)⁴⁶. It was observed that BHPP and baicalein inhibited about 50% of DU-145 and PC3 cells from proliferating in a concentration dependent manner (5–50 μ M). Subsequently, inhibition of 12-LOX results in cell cycle arrest as observed by an increase in the number of cells in G₀/G₁ phase, a decreased expression of G₁ phase proteins (Cdk2/4 and cyclins D1 and D2), a decrease in cell number in S phase and G₂-M phase, and decreased expression of the anti-apoptotic protein, bcl-2⁴⁶. These results indicate that 12-LOX plays an important role in enhancing cell proliferation and preventing apoptosis in prostate cancer and hence, blocking this pathway may be important in suppressing both prostate and colon cancer development. 12-LOX and its metabolite 12-HETE is also expressed in other cancer cell lines such as W256 cells (a rat Walker carcinoma cells)^{31,43}. In the W256 cells, it was observed that treatment with the 12-LOX inhibitor, BHPP hindered cell growth³¹. These studies suggest that blocking the 12-LOX pathway may result in suppressing tumorigenesis in various tissue types.

5-LOX is also linked to cancer development^{38,47}. For example, 5-LOX is highly expressed in colorectal tumors as compared with normal colorectal tissues^{48,47}. Higher levels of 5-HETE, a 5-LOX metabolite are reported in colorectal cancer cells^{44,42}. These observations suggest that the 5-LOX pathway may also enhance colorectal tumorigenesis. It is observed that the treatment of colorectal cancer cells (Caco-2 and LoVo cells) with 5-LOX inhibitors (1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]- α,α -dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid (MK 886) and α -pentyl-3-(2-quinolinylmethoxy)-benzenemethanol (Rev5901)) decrease the proliferation of the cells,

indicating that colorectal carcinogenesis may be suppressed as the 5-LOX pathway is blocked^{44,47}.

In addition to colorectal cancer cells, high levels of 5-HETE are present in the prostate cancer cells PC3 and LNCaP³⁸. The presence of 5-HETE enhances cell proliferation in these prostate cancer cells suggesting the key role for 5-HETE in prostate cancer development⁴⁹. Treatment of PC3 and LNCaP cells with 5-LOX inhibitors (MK886 and 2-(12-Hydroxydodecane-5, 10-diynyl)-3, 5, 6-trimethyl-p-benzoquinone (AA861)) induces apoptosis and inhibits cell growth^{49,38} indicating that, the 5-LOX pathway is essential in prostate cancer progression.

13-HODE, another metabolite of LOX enzymes is produced when LA is metabolized by the 15-LOX-1 enzyme⁵⁰. Higher levels of 13-HODE are found to be present in normal colorectal tissues compared with colorectal cancer tissue^{51,52}, suggesting a protective effect of 13-HODE against colorectal cancer. 15-LOX-1, the enzyme which metabolizes LA to produce 13-HODE, is reduced in colorectal tumors compared with normal colorectal tissues, indicating that loss of 15-LOX-1 expression enhances colorectal tumorigenesis⁵².

13-HODE plays a significant role in preventing colorectal cancer progression by inducing apoptosis, reducing cell proliferation, and inhibiting the continuation of the cell cycle in colorectal cancer cells (RKO and HT-29 cells)⁵². These results indicate that 13-HODE up regulation suppresses colorectal tumorigenesis. As a result NSAIDs such as sulindac, sulindac sulfone, and N-[2-(cyclohexyloxy)-4-nitrophenyl]-methanesulfonamide (NS-398) have been found to increase 13-HODE production as well as 15-LOX-1

expression in colorectal cancer cell lines (DLD-1, RKO, and HT-29 cells)^{53,54}. Apart from colorectal cancer cells, the level of 13-HODE production in esophageal, breast, and lung cancer cells is also reduced compared with their normal cell counterparts^{55,56,57}, suggesting that 13-HODE formation is crucial in suppressing the development of these cancers.

LOX metabolites have been identified as endogenous ligands of peroxisome proliferator-activated receptor γ (PPAR γ)^{58,59}. PPAR γ is a nuclear receptor identified to be highly present in adipose tissues and controls adipocyte differentiation⁶⁰. PPAR γ is also seen to be highly expressed in cancer cells such as colon, breast, bladder and prostate cancers^{61,62,63,64,65}. Ligand activation of PPAR γ causes differentiation, hinders cell proliferation, and enhances apoptosis of cancer cells^{65,61,62}. PPAR γ is activated by synthetic ligands such as thiazolidinediones (e.g. rosiglitazone (BRL 49653) and troglitazone)⁶¹ and endogenous ligands such as 15-HETE, 13-HODE and 9-HODE^{66,59}.

In colorectal cancer cells (Moser and HT-29 cells), it is reported that ligand activation of PPAR γ by 15-deoxy-delta-12, 14-prostaglandin J2, BRL49653, and troglitazone reduces cell proliferation, enhances cell differentiation, and impedes G₁ cycle progression^{61,65}. In breast cancer cells (MCF7), ligand activation of PPAR γ by an all trans-retinoic acid and troglitazone mixture decreases the tumor volume of MCF7 cells in mice and enhances apoptosis in MCF7 cells observed by a reduction in bcl-2 protein expression⁶². PPAR γ activation by 15-HETE reduces cell proliferation in prostate cancer cells⁶⁶. These findings suggest that up regulating endogenous PPAR γ

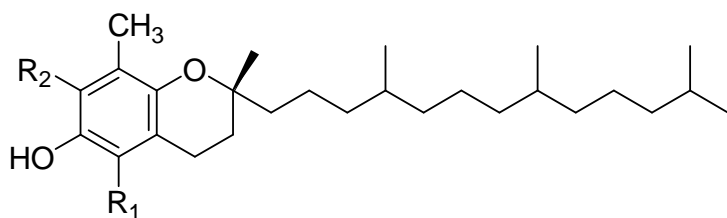
ligands such as 13-HODE could repress the progression of cancers such as colon, esophageal, breast, and lung cancer, which have decreased levels of 13-HODE.

15-HETE, a 15-LOX-1 metabolite is also identified in colorectal cancer tissues^{44,67}. Higher levels of 15-HETE are shown to be present in colorectal tumors compared with the normal colorectal tissues^{42,44}. The presence of 15-HETE in colorectal cancer cells (Caco-2 cells) has also been demonstrated to enhance the growth of the cells, indicating that high levels of 15-HETE may enhance colorectal carcinogenesis⁴⁴.

Vitamin E and its Role in Cancer Development

Vitamin E is a family of compounds that include isoforms of tocopherols and tocotrienols. Tocotrienols and tocopherols have four different isoforms that vary from each other by the number and arrangement of methyl groups on the chromanol head. These isoforms include alpha, beta, gamma, and delta⁶⁸. Both families of compounds possess a chromanol head with tocopherol having a phytyl tail and tocotrienols having an isoprenoid tail⁶⁹. Vitamin E is obtained from nuts, vegetable oil, and grains⁷⁰. The structures of the different vitamin E isoforms are shown in Figure 4⁷¹.

A. Tocopherol



B. Tocotrienol

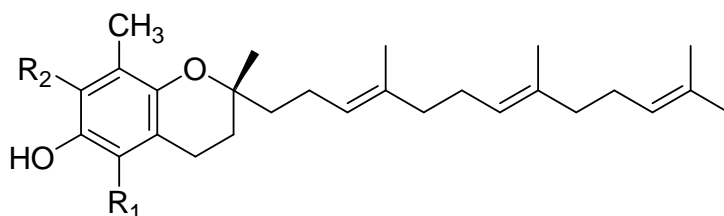


Figure 4. The structure of tocopherols and tocotrienols. Alpha isoform when R_1 and R_2 represent CH_3 and CH_3 , beta isoform when R_1 and R_2 represent CH_3 and H, gamma isoform when R_1 and R_2 represent H and CH_3 , delta isoform when R_1 and R_2 represent H and H

Vitamin E is recognized by its ability to function as an antioxidant by donating its phenolic hydrogen to primarily quench peroxy radicals, as well as other ROS such as hydroxyl radicals etc., and hence prevent these ROS from attacking lipids, proteins, cell membranes, and nucleic acids (e.g. DNA)^{72,73,4,2}. Scavenging of these ROS by vitamin E may prevent continual DNA damage which could lead to cancer initiation^{3,4}.

A number of studies have shown an inverse link between vitamin E and the risk of developing cancer. For example, it has been reported in a study that mice fed 600 mg of vitamin E (form not specified, but likely all-rac-alpha-tocopheryl acetate) per kilogram of diet, the vitamin E group developed low number of colon tumors compared to a non-vitamin E-supplemented group⁷⁴. It has also been reported that high amounts

of AT in human serum is associated with a reduced risk of developing gastric cancer⁷⁵. A mixture of synthetic vitamin E (all-rac-alpha-tocopheryl acetate) and lycopene (5 mg/kg body weight each) has also been shown to reduce the growth of prostate tumors as well as enhance the survival of the mice in a PC-346C mouse xenograft model⁷⁶.

Alpha-tocopheryl succinate (2-20 μ M), a synthetic vitamin E ester has also been reported to decrease proliferation and cause cell cycle arrest in prostate cancer cells (LNCaP cells). Cell cycle arrest in LNCaP cells was observed by an increase in the number of cells in G₁ phase and a decrease in cell number in S and G₂ phase. In this same study, a decrease in the expression of cycle proteins such as cdk4, cdk2, cyclin D3, cyclin D4, and cyclin E (G₁/S proteins) was observed after treating the LNCaP cells with alpha-tocopheryl succinate⁷⁷. Over expression of the proteins cdk4, cdk2, cyclin, D3 cyclin D4, and cyclin E enhances cell proliferation, hence, a decrease in these proteins by alpha tocopheryl succinate would impede progression in LNCaP cells. These findings suggest that vitamin E esters exhibit anti-cancer properties and may play a significant role in suppressing cancer development.

Although a majority of studies conducted with vitamin E in relation to cancer prevention have been performed with AT or AT derivatives^{75,76,16,77}, GT exhibits better anti-cancer activity than AT. For example, GT is shown to decrease human breast and mammary tumors (MDA-MB-231-GFP and 66cl-4-GFP cells) and induce apoptosis in mice, while neither AT nor a mixture of AT and GT had any effect on the tumor volume nor induced apoptosis in these mice^{78,79}.

In colorectal cancer cell lines (HCT-116, HT-29, and CaCo-2 cells), GT decreases cell proliferation better than AT^{80,81}. It was observed that GT significantly causes cell death at a concentration as low as 25 μ M while AT only results in cell death at a concentration of 200 μ M compared to the control. GT also induced apoptosis in HCT-116 and SW480 cells better than AT⁸¹. GT has also been shown to increase PPAR γ expression better than AT in SW480 cells colorectal cancer cells⁸².

In the LNCaP and DU-145 cells prostate cancer cells, 25 μ M GT was shown to effectively reduce cell proliferation compared to 25 μ M AT. In this study, GT causes more than a 70% decrease in cell proliferation while AT causes about a 50% reduction in proliferation⁸⁰. GT decreases the cell proliferation in PC-3 cells as well as the expression of cyclin D1, D3, and E (G₁ check point proteins) in prostate cancer cell lines PC-3, LNCaP, and DU-145^{80,83}. Over expression of the proteins cyclin D1, D3, and E mediates cell proliferation. Hence a decrease in these proteins by GT would impede the progression of the cell cycle at G₁ phase and hence slow proliferation of the prostate cancer cells^{80,83}.

These findings suggest that GT regulates pathways in cancer cells such as cell proliferation, cell cycle, and apoptosis in order to suppress cancer progression. These findings, also demonstrate that GT is more potent than AT in regulating these pathways (i.e. cell proliferation, cell cycle and apoptosis) to prevent cancer development.

GT3, an isoform of vitamin E containing an isoprenoid tail, has demonstrated potent anti-cancer potential. For example, in MCF-7 and MDA-MB 231 breast cancer cells both α -tocotrienol (AT3) and GT3 hindered proliferation of the cells in a

concentration dependent manner (10-40 μM)⁸⁴. However, the proliferation of the cells in GT3-treated sample was less than the proliferation in the AT3-treated cells suggesting that GT3 is a more potent anti-cancer agent than AT3. In addition, GT3 decreases G₁ phase check proteins such as Cdk4 and cyclins D1/D3 in MCF-7 cells which indicates that GT3 can cause cell cycle arrest at G₁ phase⁸⁴.

In other cancer tissue types such as the human cervical cancer HeLa cell line, both AT3 and GT3 decrease cell proliferation with GT3 showing a greater inhibitory effect than AT3⁸⁵. Both AT3 and GT3 also impede the progression of the cell cycle in HeLa cells as observed by an increase in the number of cells in G₀/G₁ phase and decrease in cell number in the S phase⁸⁵.

Finally, in the RKO colorectal cancer cell line, the tocotrienol-rich fraction consisting of a mixture of AT, beta tocopherol (BT), and GT isoforms hinders cell growth, induces apoptosis, causes cell cycle arrest at G₁, and increases the expression of p53 proteins in the RKO cells⁸⁶. These studies indicate that tocotrienols also regulate various pathways such as cell cycle, cell proliferation, and apoptosis in cancer cells to prevent cancer progression. In addition, these studies suggest that GT3 exhibits greater anti-cancer activity than AT3.

Based on these observations, vitamin E (tocopherols and tocotrienols) can be said to exhibit anti-tumor activity by regulating various pathways in cancer cells such as cell proliferation and apoptosis. These studies also suggest that the development of colorectal cancer is enhanced by low production of 13-HODE and high production of 15-HETE, 12-HETE, and 5-HETE. Therefore, we hypothesize that vitamin E (tocopherols

and tocotrienols) can increase the production of 13-HODE and decrease the production of 15-HETE, 12-HETE, and 5-HETE in colorectal cancer cells.

CHAPTER 2

METHODS

Cell Culture

Human colon cancer cells (HCT-116 cell line) were cultured in McCoy's 5A media which is enriched with 10% fetal bovine serum (FBS) and 50 IU penicillin/streptomycin in an environment with 5% carbon dioxide at 37 °C⁸³.

Determination of Concentrations of AT, GT, GT3, and a Mixture of Alpha (5%) and Gamma Tocopherol (95%)(ATGT)

The concentrations of AT, GT, GT3, and ATGT were determined using an HP-8542A diode array spectrophotometer. The wavelength of maximum (max) absorbance (λ_{\max}) for GT, GT3, and ATGT is 298 nm and that of AT is 292 nm. The molar absorptivity (ϵ) for GT and ATGT is 3810, AT is 3270, and GT3 is 4230. The Beer-Lambert Law was used to calculate the concentration of the vitamin E isoforms using the absorbance and the ϵ at the appropriate λ_{\max} ⁸³.

Sample Preparation for Liquid Chromatography Mass spectrometry Analysis (LC/MS/MS)

Treatment of Cells. HCT-116 cells were seeded at a concentration of 2.5×10^6 in a T-75 mL flask or 10 mm round dishes for 24 hours before treatment. The cells were then treated with 5 μ M of AT, GT, GT3, and ATGT for 24 hours. The untreated cells

(vehicle) were also prepared with a volume of ethanol corresponding to the volume of vitamin E added for 24 hours⁸³.

Extraction of Fatty Acid Metabolites from Cells. After 24 hours of treatment, the media was removed from the cells and stored in a -80 °C freezer for a period of no longer than 1 week before the analysis was performed. The cells were harvested with 5 mL of trypsin (0.25%) with balanced salts (Hyclone SH30042.02) and collected by centrifugation. The cell pellet was washed with 5 mL of phosphate buffered saline (PBS), and collected by centrifugation. The cells were re-suspended in 500 µL of PBS with 1 mM calcium chloride solution and incubated at 37°C for 2 minutes⁸⁷. As previously described, an aliquot 2.5 µL of calcium ionophore A23187 (1 mM) was added to the mixture, followed by addition of 100 µM of AA⁸⁷. The ionophore in this reaction will demonstrate the activity of the LOX pathway, and in the presence of additional substrate (AA) it will show that the LOX enzyme is still active upon production of metabolite. The reaction mixture was incubated at 37°C for 10 minute under minimal light⁸⁷. After incubation, the cells were then collected by centrifugation. Using an enzyme-linked immunosorbent assay (ELISA) assay (Enzo Life Sciences, Farmingdale, NY) according to the manufacturers' protocol, the cell pellets were then washed with 1 mL of ice cold PBS, and recollected by centrifugation. The cells were lysed with ice cold lysis buffer (200 µL) and acidified to pH 3.5-4.0 with 0.2 N HCl. The fatty acid metabolites were extracted from the reaction mixture three times using a 3-fold excess of ethyl acetate. The upper layer (organic phase) were collected into a fresh tube and dried under a stream of nitrogen gas. The dried samples were stored in a -80 °C freezer

no longer than one week before analysis was performed. The dried samples were dissolved in 100 μ L of methanol and filtered for LC/MS/MS analysis immediately before analysis.

Extraction of Fatty Acid Metabolites from Media. A solid phase extraction (Sep-Pak) cartridge was conditioned with methanol and equilibrated with water under gravity, then loaded with 3 mL of sample. The column was then washed with 3 mL of the following solvents, water, 15% ethanol, and hexane. Following the washes, 1 mL of methanol was added to the cartridge and centrifuged for 10 minutes to collect the analytes. The eluent containing the analytes was dried under nitrogen gas and stored in a -80°C freezer no longer for a period of one week before analysis. Just prior to the analysis, the dried sample was dissolved in 100 μ L of methanol and filtered for LC/MS/MS analysis⁸⁸.

Analysis of Fatty Acid Metabolites Using Gradient LC/MS/MS

Separation and detection of metabolites was achieved using the Shimadzu IT-TOF (ion trap-time of flight) LC/MS/MS with an ultrasphere C18 4.6 mm * 250 mm column (5 μ m, Beckman)⁸⁹. Mobile phase A contains 30% acetonitrile/70% 0.1% formic acid in water and mobile phase B contains of 0.1% formic acid in acetonitrile. Mobile phase A and B were delivered at a flow rate of 0.5 mL/min with the following gradient: 75-90% B in 10 min: 90% B in 2 min: 75 % B in 5 min. The volume of sample injected was 10 μ L and the column temperature was kept at 40°C . The metabolites were detected with the Shimadzu IT-TOF mass spectrometer (Columbia, MD), which is run in electrospray negative mode with a source temperature of 200°C . The metabolites were

identified based on their m/z ratios and their retention times. The m/z of HETEs (12-HETE, 15-HETE, and 5-HETE) was 319.22 and 13-HODE was 295.22⁸⁹. All samples and external standards (12-HETE, 15-HETE, and 5-HETE prepared standards) were analyzed in duplicate.

Sample Preparation for ELISA Analysis

Treatment of Cells. HCT-116 cells were cultured to about 50-75% confluence. The cells were then treated with 5 μ M of GT, AT, ATGT, or GT3 for 24 hours. The untreated cell (vehicle) was also prepared for 24 hours.

Extraction of Fatty Acid Metabolites. The treated cells were harvested with 5 mL of trypsin (0.25%) with balanced salts (Hyclone SH30042.02) and collected by centrifugation. The resulting cell pellets were washed in 5 mL of PBS. The cells were counted with a hemocytometer and a total of 10 million cells collected by centrifugation and were resuspended in 500 μ L of PBS with 1 mM calcium chloride solution and incubated at 37 °C for 2 minutes⁸⁷. An aliquot of 2.5 μ L of calcium ionophore A23187 (1 mM) was added to the mixture, followed by addition of 100 μ M of AA⁸⁷. The reaction mixture was incubated at 37 °C for 10 minutes at minimum light⁸⁷. After incubation, cells were collected by centrifugation. Using an ELISA assay (Enzo Life Sciences, Farmingdale, NY) according to the manufacturers' protocol, cell pellets were then washed with 5 mL of ice cold PBS, and recollected by centrifugation. The cells were lysed with 200 μ L of ice cold lysis buffer and the lysate was acidified to pH 3.5-4.0 with 0.2 N HCl. The fatty acid metabolites were extracted from the reaction mixture three times using 3-fold excess of saturated ethyl acetate. The upper layer (organic phase)

were collected into a fresh tube and dried under nitrogen gas. The dried samples were stored in -80 °C freezer for a period of no longer than one week before prepared for analysis by dissolving them in 44 µL of ethanol and 176 µL of assay buffer.

Analysis of Fatty Acid Metabolites Using ELISA

The amount of 13-HODE in HCT-116 cells were analyzed using an ELISA assay (Enzo Life Sciences, Farmingdale, NY) according to the manufacturers' protocol. The optical density was read at 405 nm with ultraviolet-visible (UV-VIS) spectrophotometer plate reader (Molecular Devices, Spectra Max Plus 384).

CHAPTER 3

RESULTS

LC/MS/MS Chromatogram for Fatty Acid Metabolites in HCT-116 Cells

The amount (nmoles) of 12-HETE, 5-HETE, 15-HETE, and 13-HODE in the cells and the media of HCT-116 cell line were determined from the concentrations obtained from peak areas using the Shimadzu IT-TOF (ion trap-time of flight) LC/MS/MS. The concentration of 12-HETE, 5-HETE, 15-HETE, and 13-HODE corresponding to each peak was determined from a calibration graph prepared from a series of standards (0.05 to 4.5 $\mu\text{g/mL}$). Stock solutions (100 $\mu\text{g/mL}$) of 12-HETE, 5-HETE, 15-HETE, and 13-HODE standards were used to prepare series of standards (0.05 to 4.5 $\mu\text{g/mL}$) for the calibration graph. A chromatogram showing the separation of these fatty acid metabolites in HCT-116 cells is shown in Figure 5. The intensities of the peaks are measured by the mass spectrometer based on their ion masses to charge ratio (m/z).

The detection of these metabolites is based on their retention time and mass to charge ratio (m/z). The m/z of HETEs (12-HETE, 15-HETE, and 5-HETE) is 319.22 and 13-HODE is 295.22. The retention times of 13-HODE, 15-HETE, 12-HETE and 5-HETE are 8.135, 8.22, 8.851 and 9.321 min, respectively. Since the retention time of 13-HODE (8.135 min) and 15-HETE (8.22 min) are very close, their identification is made easier by the mass spectrometry using their molecular weight differences. Mass spectrometry was chosen as the method of analysis because the concentrations of these metabolites in the HCT-116 cells are low, ranging from 0.05 - 4.5 $\mu\text{g/mL}$ and

require a more sensitive instrument for detection. Since the mass spectrometry is highly sensitive compared to optical methods such as a UV-VIS spectrophotometry⁹⁰, it allows the injection of very low sample volume (10 μ L) preventing the broadening and overlapping of peaks.

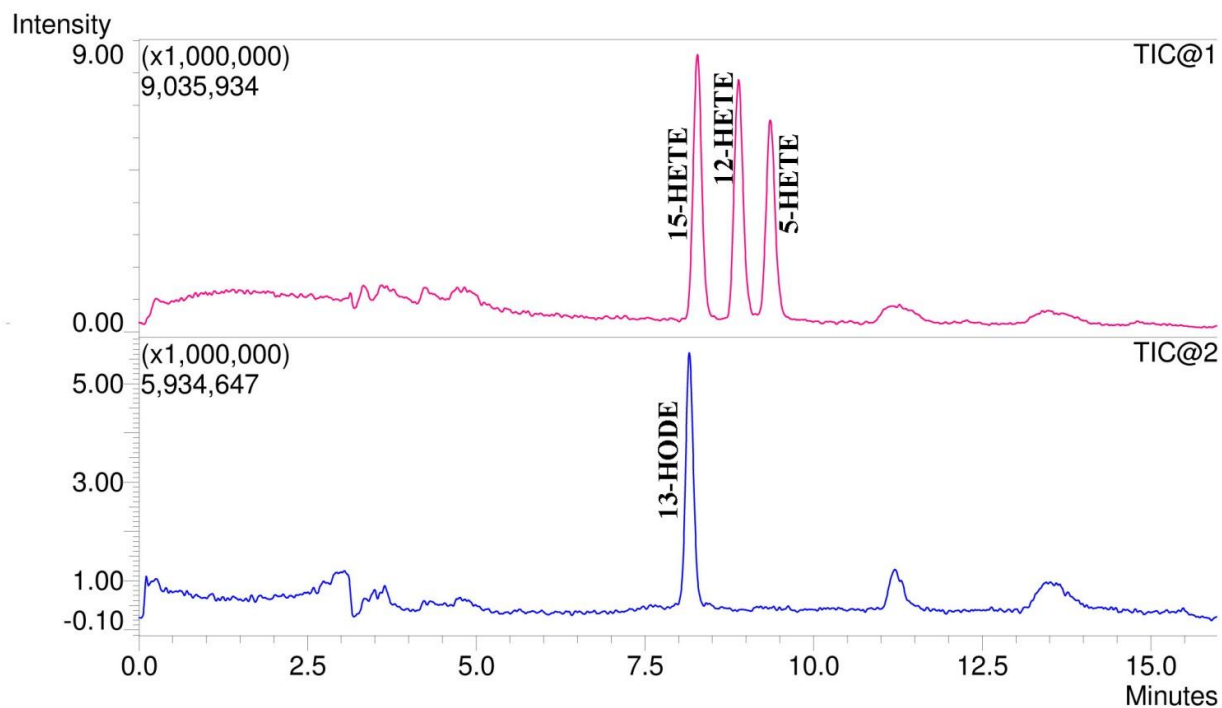


Figure 5. The chromatogram of fatty acid metabolites (13-HODE, 15-HETE, 12-HETE, and 5-HETE) in HCT-116 cells obtained by the Shimadzu LC/MS/MS. This chromatogram represents a 10 μ L injection of 4.5 μ g/mL solution of 15-HETE, 12-HETE, 5-HETE, and 13-HODE. The 4.5 μ g/mL standard solutions were prepared from a 100 μ g/mL stock solution in ethanol. These metabolites were separated using mobile phase A (30% acetonitrile/70% 0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetonitrile). Mobile phase A and B were delivered at a flow rate of 0.5 mL/min with the following gradient: 75-90% B in 10 min: 90% B in 2 min: 75 % B in 5 min

Amounts of 13-HODE, 15-HETE, 12-HETE, and 5-HETE, Secreted into the Media of HCT-116 Cells Following Treatment of HCT-116 Cells with GT, AT, ATGT, and GT3.

The metabolites 13-HODE, 15-HETE, 12-HETE, and 5-HETE accumulate in the cells and then are secreted out of the cells into the extracellular space or the media. Hence, the amounts of the metabolites (13-HODE, 15-HETE, 12-HETE, and 5-HETE) in the media of HCT-116 cells were analyzed using the LC/MS/MS method and used as a measure of metabolite production. The formation of the metabolites and secretion into the media occur under normal physiological conditions.

The Amount of 13-HODE Secreted into the Media of HCT-116 Cells is Increased by GT or ATGT but not GT3 nor AT under Physiological Conditions.

13-HODE has been shown to inhibit cell proliferation and induce apoptosis in colorectal cancer cells⁵², but the level of 13-HODE in colorectal cancer cells is low compared to normal colorectal cells⁵¹. By increasing the level of 13-HODE, a decrease in cell proliferation in colorectal cancer cells is possible⁵². Hence, we decided to investigate whether GT, ATGT, AT, and GT3 can increase the amount of 13-HODE secreted into the media of HCT-116 cells.

The treatment of HCT-116 cells with 5 μ M GT significantly increases the amount of 13-HODE as compared with the vehicle in the media of HCT-116 cells (Figure 6; p = 0.038). Under physiological conditions, the amount of 13-HODE secreted into the media of the GT treated samples is more than a five-fold increase over the vehicle treated samples.

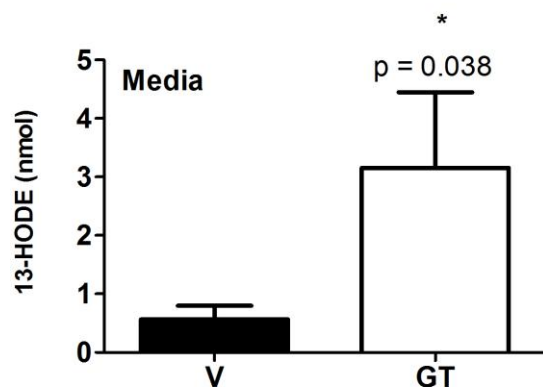


Figure 6. The effect of ATGT on the amount of 13-HODE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 13-HODE in the media was determined by stand curve analysis. The amount of 13-HODE in the media of HCT-116 cells treated with ATGT demonstrate a significant increase over the vehicle-treated cells (n =7; p = 0.045). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

Some studies have shown that the inclusion of AT with GT decreases the ability of GT to inhibit cell proliferation and induce apoptosis in cancer cells⁷⁸. Other studies demonstrate that AT is less effective at reducing cell proliferation and enhancing apoptosis in cancer cells^{78,79,80,81}, therefore we investigated the effect of ATGT mixture on the production of fatty acid metabolites (12-HETE, 5-HETE, 15-HETE, and 13-HODE). The treatment of HCT-116 cells with 5 μ M ATGT mixture (95% GT and 5% AT) under physiological conditions, also increases the amount of 13-HODE significantly as compared with the vehicle in the media of HCT-116 cells (p = 0.045) as shown in Figure 7. While the amount of 13-HODE secreted into the media in the ATGT treated samples was less than a two- fold increase over the vehicle, the 5 μ M GT treatment of HCT-116 cells increases the amount of 13-HODE greater than five-fold over the vehicle in the media (Figure 6). The decrease in amount of 13-HODE in the ATGT treated

samples compared with the GT treated samples may be due to the presence of the 5% AT. This was confirmed when the addition of 5 μ M pure AT to HCT-116 cells did not significantly increase the amount of 13-HODE in the media of HCT-116 cells after treatment with 5 μ M pure AT as compared with the vehicle (Figure 8; $p < 0.068$), suggesting that AT does not increase the amount of 13-HODE in the media of HCT-116 cells and that the addition of AT decreases the ability of GT to increase the amount of 13-HODE in the media of HCT-116 cells.

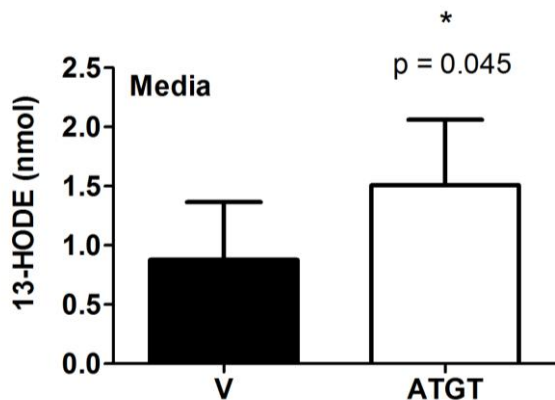


Figure 7. The effect of ATGT on the amount of 13-HODE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 13-HODE in the media was determined by stand curve analysis. The amount of 13-HODE in the media of HCT-116 cells treated with ATGT demonstrate a significant increase over the vehicle-treated cells ($n = 7$; $p = 0.045$). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

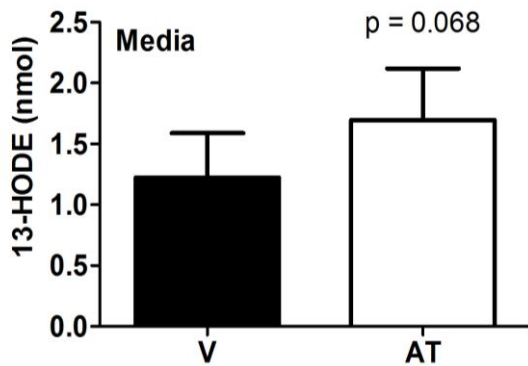


Figure 8. The effect of AT on the amount of 13-HODE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M AT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 13-HODE in the media was determined by stand curve analysis. The amount of 13-HODE in the media of HCT-116 cells treated with AT cells demonstrated no statistical difference between the AT treated sample compared with the vehicle treated sample ($n = 8$; $p = 0.068$). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample

While both GT and ATGT significantly increase the amount of 13-HODE in the media of HCT-116 cells, the amount of 13-HODE secreted into the media of HCT-116 cells did not significantly change following treatment with 5 μ M GT3 compared to the vehicle (Figure 9; $p = 0.37$). These data (Figures 8 and 9) suggest that neither GT3 nor AT may be the best isoform of vitamin E to induce an up-regulation of 13-HODE in the media of HCT-116 cells. Other isoforms of vitamin E such as GT, as well as the combination of AT and GT (ATGT) should be considered when an increase in 13-HODE production in HCT-116 cells is to be achieved.

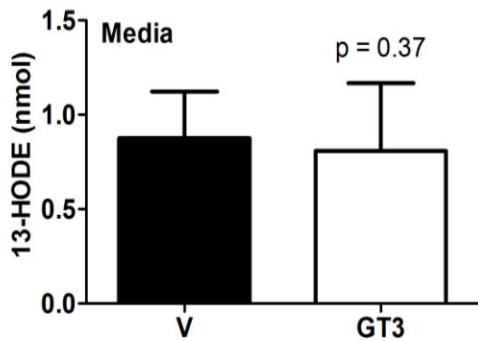


Figure 9. The effect of GT3 on the amount of 13-HODE secreted into the media of HCT-116 cells analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT3 for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 13-HODE in the media was determined by stand curve analysis. The amount of 13-HODE in the media of HCT-116 cells treated with GT3 demonstrated no statistical difference between the GT3 treated sample compared with the vehicle treated sample ($n = 7$; $p = 0.37$). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

GT, ATGT, AT or GT3 did not Regulate the Amount of 15-HETE in the Media of HCT-116 Cells under Physiological Conditions.

15-HETE, a 15-LOX-1 metabolite, has been shown to be high in colorectal cancer cells⁴⁴. Higher levels of 15-HETE have been observed to enhance the growth of colorectal cancer cells^{44,42}. We, therefore, investigated whether vitamin E isoforms, GT, GT3, AT, and ATGT can cause a reduction in the amount of 15-HETE in the media of HCT-116 cells. The amount of 15-HETE in the media of HCT-116 cells did not show a statistical difference after treatment with 5 μ M GT under physiological conditions as compared with the vehicle (Figure10; $p = 0.36$). Further, the amount of 15-HETE in the media of HCT-116 cells following treatment with 5 μ M ATGT under physiological conditions did not show a statistical difference as compared with the vehicle (Figure 11; $p = 0.25$). In addition, the amount of 15-HETE in the media of HCT-116 cells following

treatment with 5 μ M AT did not show a statistical difference after treatment as compared with the vehicle (Figure 12; $p = 0.49$). Alongside GT (Figure 10), ATGT (Figure 11), and AT (Figure 12), the amount of 15-HETE in the media of HCT-116 cells following treatment with 5 μ M GT3 did not show a statistical difference after treatment with 5 μ M GT3 as compared to the vehicle (Figure13; $p = 0.47$). These data demonstrate that the production of 15-HETE under physiological conditions is not regulated by any of the vitamin E isoforms tested (GT, AT, GT3) nor the ATGT mixture.

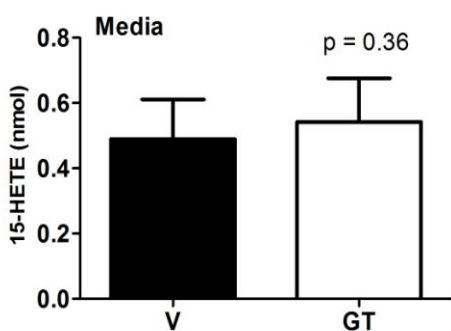


Figure 10. The effect of GT on the amount of 15-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 15-HETE in the media was determined by stand curve analysis. The amount of 15-HETE in the media of HCT-116 cells treated with GT demonstrated no statistical difference between the GT treated sample compared with the vehicle treated sample ($n = 10$; $p = 0.36$). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

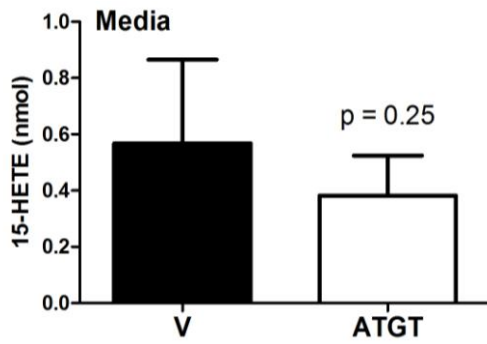


Figure 11. The effect of ATGT on the amount of 15-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 15-HETE in the media was determined by stand curve analysis. The amount of 15-HETE in the media of HCT-116 cells treated with ATGT demonstrated no statistical difference between the ATGT-treated sample compared with the vehicle-treated sample (n = 8; p = 0.25). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

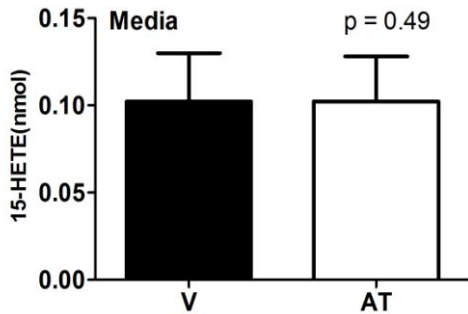


Figure 12. The effect of AT on the amount of 15-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M AT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 15-HETE in the media was determined by stand curve analysis. The amount of 15-HETE in the media of HCT-116 cells treated with AT demonstrated no statistical difference between the AT-treated sample compared with the vehicle-treated sample (n = 8; p = 0.49). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

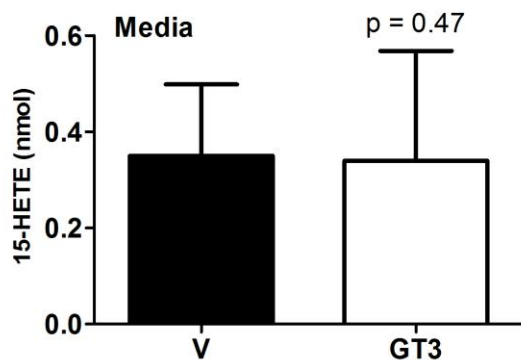


Figure 13. The effect of GT3 on the amount of 15-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT3 for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 15-HETE in the media was determined by stand curve analysis. The amount of 15-HETE in the media of HCT-116 cells treated with GT3 demonstrated no statistical difference between the GT3-treated sample compared with the vehicle-treated sample ($n = 8$; $p = 0.47$). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

GT, but not GT3, AT, or ATGT Treatments Result in the Reduction of the 12-LOX

Metabolite, 12-HETE in HCT-116 Cells under Physiological Conditions.

12-HETE is produced through the metabolism of AA by the 12-LOX enzyme. 12-HETE is found to play a role in the survival of cancer cells^{43,31}. Higher levels of 12-HETE is found to be present in the colorectal cancer cells compared to the normal colorectal cells^{41,44}. This high level of 12-HETE is shown to enhance cell proliferation in cancer cells including colorectal and prostate cancers^{44,41,16,46}. Reduction in the level of 12-HETE by its inhibitors (i.e. flavonoid, baicalein, and cinnamyl-3, 4-dihydroxy-a-cyanocinnamide), decreased cell proliferation in cancer cells^{43,31}. We have, therefore, investigated whether the amount of 12-HETE secreted into the media of HCT-116 cells can be reduced by treatment with vitamin E isoforms GT, GT3, AT, and ATGT.

The amount of 12-HETE secreted into the media under physiological conditions was significantly decreased following treatment of HCT-116 cells with 5 μ M GT as compared to the vehicle (Figure 14; $p = 0.0014$). However, the amount of 12-HETE secreted into the media of HCT-116 cells after treatment of the cells with 5 μ M ATGT under physiological conditions did not show a statistical difference as compared with the vehicle (Figure 15; $p = 0.30$). In addition, the amount of 12-HETE secreted into the media following treatment of HCT-116 cells with 5 μ M AT under physiological conditions did not show statistical difference compared with the vehicle (Figure 16; $p = 0.45$). Treatment of HCT-116 cells with GT3 did not show a statistical significance difference in the amount of 12-HETE secreted into the media compared with the vehicle (Figure 17; $p = 0.29$). These data show that the amount of 12-HETE produced into the media of HCT-116 is reduced only by GT treatment.

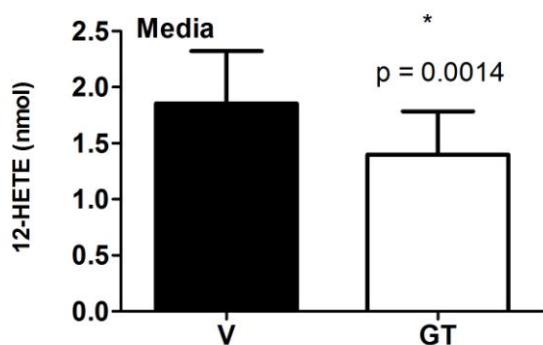


Figure 14. The effect of GT on the amount of 12-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 12-HETE in the media was determined by stand curve analysis. The amount of 12-HETE in the media of HCT-116 cells treated with GT demonstrates a significant decrease over the vehicle-treated cells ($n = 7$; $p = 0.0014$). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

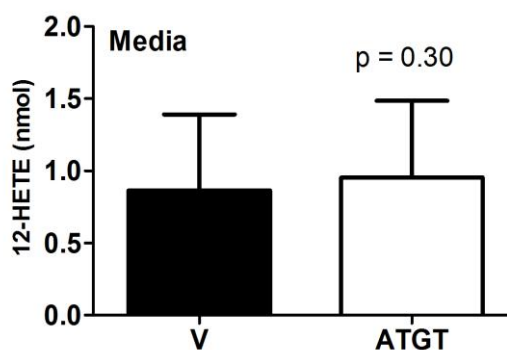


Figure 15. The effect of ATGT on the amount of 12-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 12-HETE in the media was determined by stand curve analysis. The amount of 12-HETE in the media of HCT-116 cells treated with ATGT demonstrated no statistical difference between the ATGT treated sample compared with the vehicle treated sample (n =7; p = 0.30). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

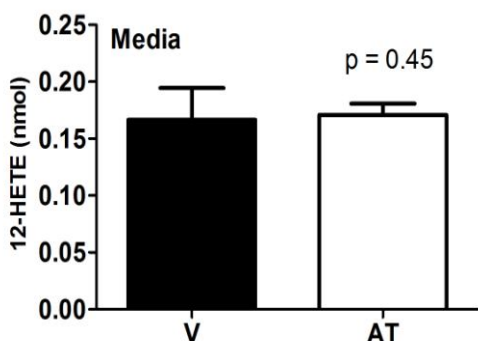


Figure 16. The effect of AT on the amount of 12-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M AT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 12-HETE in the media was determined by stand curve analysis. The amount of 12-HETE in the media of HCT-116 cells treated with AT demonstrated no statistical difference between the AT treated sample compared with the vehicle treated sample (n =8; p = 0.45). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

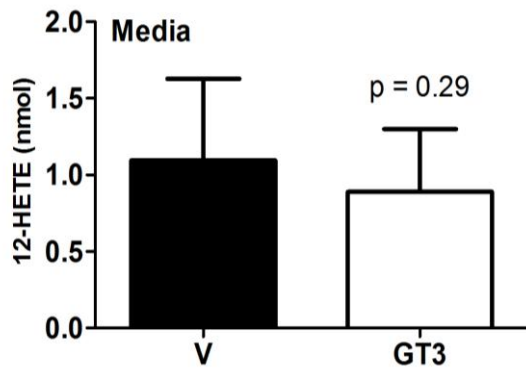


Figure 17. The effect of GT3 on the amount of 12-HETE secreted into the media of HCT-116 cells analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT3 for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 12-HETE in the media was determined by stand curve analysis. The amount of 12-HETE in the media of HCT-116 cells treated with GT3 demonstrated no statistical difference between the GT3 treated sample compared with the vehicle treated sample (n =8; p = 0.29). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

The Amount of 5-HETE Secreted into the Media of HCT-116 Cells was not Affected by GT, AT, ATGT, or GT3 under Physiological Conditions.

5-LOX, the enzyme which metabolizes AA to produce 5-HETE has been shown to be highly expressed in colorectal cancer cells⁴⁷. High levels of 5-LOX enzyme in colorectal cancer cells is linked to increased tumor size and growth⁴⁸. Addition of 5-LOX inhibitors to colorectal cancer cells decreases cell proliferation⁴⁷. We therefore chose to investigate whether the addition of vitamin E isoforms, GT, AT, GT3, and ATGT mixture can decrease the amount of 5-HETE (5-LOX metabolite) secreted into the media of HCT-116 cells.

The amount of 5-HETE secreted into the media did not show any significant change after treatment of HCT-116 cells under physiological conditions with 5 μ M GT as compare with the vehicle (Figure 18; $p = 0.49$). Further, the amount of 5-HETE secreted into the media following treatment of HCT-116 cells with 5 μ M ATGT under physiological conditions did not show a statistically significant difference compared with the vehicle (Figure 19; $p = 0.24$). Neither did the amount of 5-HETE secreted into the media following treatment of HCT-116 cells under physiological conditions with 5 μ M AT show a statistically significant difference as compared with the vehicle Figure 20; $p = 0.072$. The amount of 5-HETE in the media following treatment of HCT-116 cells under physiological conditions with 5 μ M GT3 did not show a significance difference as compared with the vehicle (Figure 21; $p = 0.24$). These data suggest that treatment of HCT-116 cells with vitamin E isoforms GT, AT, ATGT mixture as well as GT3, did not reduce the amount of 5-HETE secreted into the media.

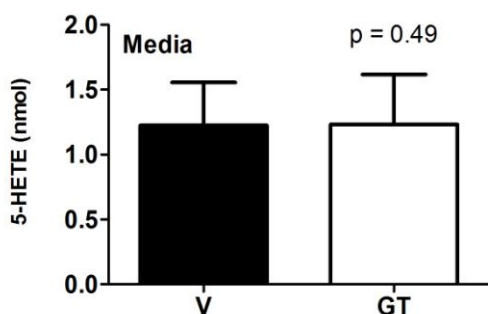


Figure 18. The effect of GT on the amount of 5-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 5-HETE in the media was determined by stand curve analysis. The amount of 5-HETE in the media of HCT-116 cells treated with GT demonstrated no statistical difference between the GT-treated sample compared with the vehicle-treated ($n = 11$; $p = 0.49$). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

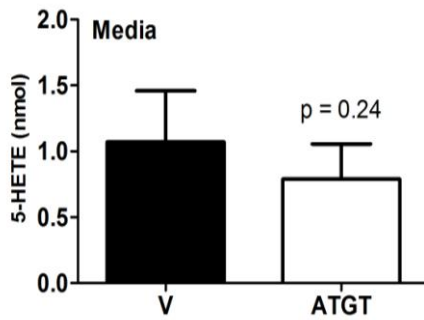


Figure 19. The effect of ATGT on the amount of 5-HETE secreted into the media of HCT-116 cells analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 5-HETE in the media was determined by stand curve analysis. The amount of 5-HETE in the media of HCT-116 cells treated with ATGT demonstrated no statistical difference between the ATGT treated sample compared with the vehicle treated sample ($n = 9$; $p = 0.24$). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

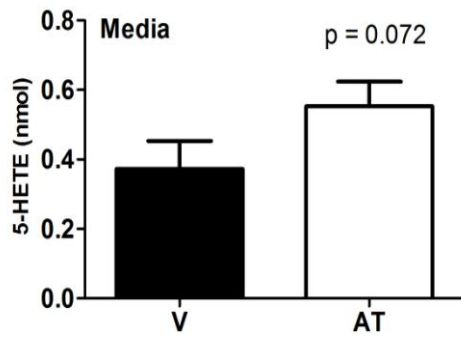


Figure 20. The effect of AT on the amount of 5-HETE secreted into the media of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M AT for 24 hours. The lipid content was isolated from the media and analyzed by LC/MS/MS. The amount of 5-HETE in the media was determined by stand curve analysis. The amount of 5-HETE in the media of HCT-116 cells treated with AT demonstrated no statistical difference between the AT treated sample compared with the vehicle treated sample ($n = 8$; $p = 0.072$). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

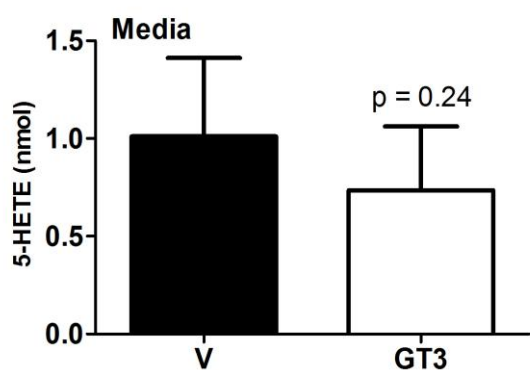


Figure 21. The effect of GT3 on the amount of 5-HETE secreted into the media of HCT-116 cells analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT3 for 24 hours. The lipid extract was isolated from the media and analyzed by LC/MS/MS. The amount of 5-HETE in the media was determined by stand curve analysis. The amount of 5-HETE in the media of HCT-116 cells treated with GT3 demonstrated no statistical difference between the GT3 treated sample compared with the vehicle treated sample (n =9; p = 0.24). Statistical differences were determined by paired t-test. p values < 0.05 were determined to be statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

The Amount of 13-HODE, 15-HETE, 12-HETE, and 5-HETE Produced in HCT-116 Cells Following Treatment with GT, AT, ATGT, or GT3 and then Activation of the Lipoxygenase Pathway with Calcium Ionophore A23187 and Addition of Arachidonic Acid.

The amount of polyunsaturated fatty acid metabolites, 13-HODE, 15-HETE, 12-HETE, and 5-HETE were also measured in HCT-116 cells following treatment of HCT-116 cells with vitamin E isoforms, GT, AT, ATGT, and AT and activation of the LOX pathway with the calcium ionophore A23187 and addition of exogenous AA. If the corresponding LOX enzyme was still active after the treatment under physiological conditions, the exogenous AA that was added to the cells provides enough substrate to be used by the LOX enzymes for the production of 5-HETE, 15-HETE, and 12-HETE in

HCT-116 cells. If the enzyme is inactivated following the secretion of the metabolites into the media, additional HETE metabolites will not be produced by the cells.

Under LOX Activation Conditions, GT3, but not GT, ATGT, or AT Reduce the Production of 13-HODE in HCT-116 Cells.

To obtain a concentration range of 13-HODE in the cells for standard preparation and to ensure that the extraction method was valid, the amount of 13-HODE in HCT-116 cells was initially measured using ELISA method. This was carried out by preparing 13-HODE standards and a calibration graph. Using the ELISA method, amount of 13-HODE in HCT-116 cells following treatment with 5 μ M GT after 24 hours did not show a statistically significant difference as compared to the vehicle (Figure 22; $p = 0.096$). Using LC/MS/MS, the amount of 13-HODE produced in HCT-116 cells following treatment with 5 μ M GT did not show statistical difference as compared with the vehicle (Figure 23; $p = 0.39$).

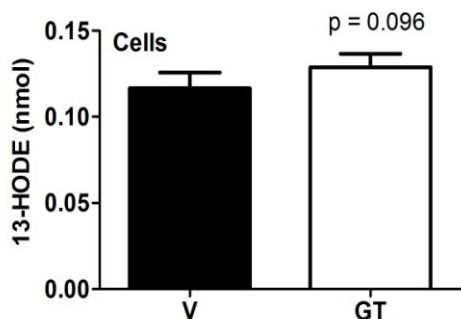


Figure 22. The effect of GT on the amount of 13-HODE in HCT-116 cells using the ELISA method. HCT-116 cells were treated with 5 μ M GT for 24 hours. The lipid content was isolated from HCT-116 cells following treatment with GT. Activation of cells with the calcium ionophore A23187 and addition of exogenous AA was carried out and the lipids were analyzed by the ELISA method. The amount of 13-HODE was determined by stand curve analysis. The amount of 13-HODE in HCT-116 cells shows no statistical difference between the vehicle-treated and the GT-treated sample ($n=7$; $p = 0.096$). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. Error bars are representative of SEM of sample

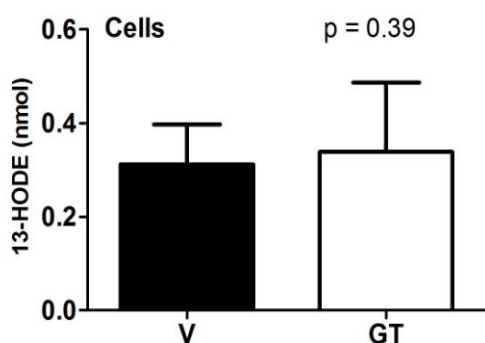


Figure 23. The effect of GT on the amount of 13-HODE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT for 24 hours. The lipid content was isolated from HCT-116 cells following treatment with GT. Activation of cells with the calcium ionophore A23187 and addition of exogenous AA was carried out and the lipids were analyzed by LC/MS/MS. The amount of 13-HODE in HCT-116 cells was determined by stand curve analysis. The amount of 13-HODE in HCT-116 cells treated with GT demonstrated no statistical difference between the GT- treated sample compared with the vehicle-treated sample ($n = 11$; $p = 0.39$). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

Treatment of HCT-116 cells with 5 μ M ATGT and 5 μ M AT did not show statistical differences as compared with the vehicle (Figures 24 and 25) with p values of 0.14 and 0.16, respectively. These data suggest that, after secretion of 13-HODE into the media, there is less substrate (linoleic acid) for the 15-LOX-1 to act upon or the 15-LOX-1 enzyme has been inactivated following the treatment with GT or ATGT (Figures 23, 24, and 25).

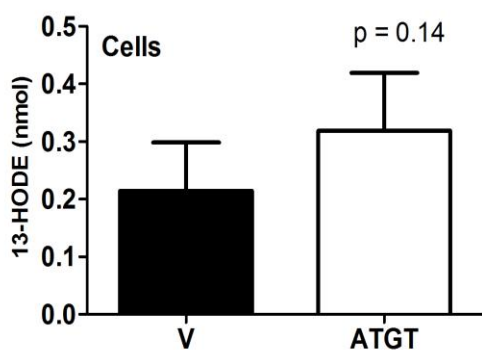


Figure 24. The effect of ATGT on the amount of 13-HODE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with ATGT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA were carried out, and the lipids were analyzed by LC/MS/MS. The amount of 13-HODE in the HCT-116 cells was determined by stand curve analysis. The amount of 13-HODE in HCT-116 cells shows no statistical difference between the ATGT-treated sample and the vehicle-treated sample treated sample (n =9; p = 0.14). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

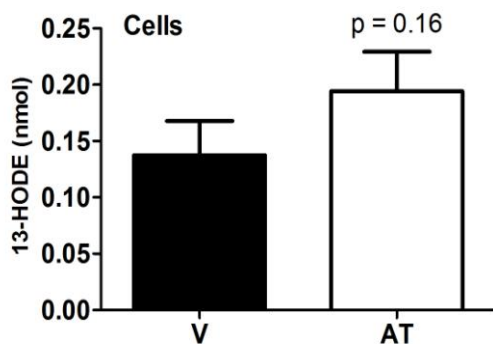


Figure 25. The effect of AT on the amount of 13-HODE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M AT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with AT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA were carried out, and the lipids were analyzed by LC/MS/MS. The amount of 13-HODE in the HCT-116 cells was determined by stand curve analysis. The amount of 13-HODE in HCT-116 cells shows no statistical difference between the AT-treated sample and the vehicle-treated sample (n =8; p = 0.16). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

While the treatment of the tocopherols showed no significant increase in 13-HODE production, treatment of HCT-116 cells with GT3 significantly decreased the production of 13-HODE as compared with the vehicle (Figure 26; $p = 0.0092$). This decrease is likely due to the decrease in the limited substrate availability (linoleic acid), if GT3 were decreasing the activity of the 15-LOX-1 enzyme there would be a significant decrease in the production of 15-HETE in the presence of GT3, but that does not happen (Figure 31). The decrease in the amount of 13-HODE in HCT-116 cells after treatment with 5 μM GT3 was also observed during the preliminary analysis with ELISA method (Figure 27).

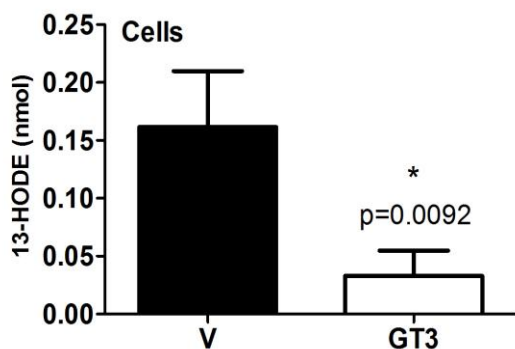


Figure 26. The effect of GT3 on the amount of 13-HODE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μM GT3 for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT3. Activation of the cells with the calcium ionophore A23187 and the addition of exogenous AA was carried out, and the lipids were analyzed by LC/MS/MS. The amount of 13-HODE in the HCT-116 cells was determined by stand curve analysis. The amount of 13-HODE in HCT-116 cells show a statistically significant decrease in the GT3-treated sample compared with the vehicle ($n=7$; $p = 0.0092$). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

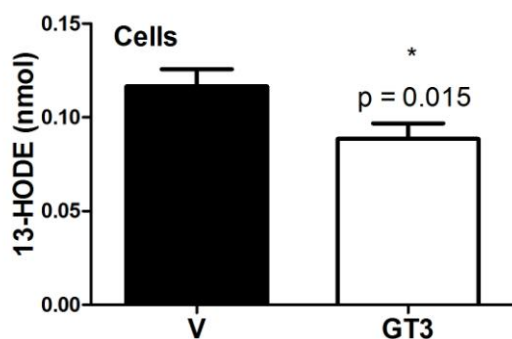


Figure 27. The effect of GT3 on the amount of 13-HODE in HCT-116 cells as analyzed by the ELISA method. HCT-116 cells were treated with and without 5 μ M GT3 for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT3. Activation of the cells with the calcium ionophore A23187 and the addition of exogenous AA was carried out, and the lipids were analyzed by the ELISA method. The amount of 13-HODE in the HCT-116 cells was determined by stand curve analysis. The amount of 13-HODE in HCT-116 cells show a statistically significant decrease in the GT3-treated sample compared with the vehicle (n=7; p = 0.015). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. Error bars are representative of SEM of sample.

GT, ATGT, AT, nor GT3 Regulate the Production of 15-HETE in HCT-116 Cells.

The amount of 15-HETE produced in HCT-116 cells following treatment with 5 μ M GT shows no significant difference as compared with the vehicle (Figure 28; p = 0.081). In addition, the production of 15-HETE following treatment with 5 μ M ATGT shows no significant difference as with the vehicle (Figure 29; p = 0.39). Further, treatment of HCT-116 cells with 5 μ M AT and 5 μ M GT3 shows no statistical difference in the amount of 15-HETE as compared with the vehicle (Figures 30 and 31) with p values of 0.12 and 0.21, respectively. These data indicate that neither GT, ATGT, AT, nor GT3 significantly regulate the production of 15-HETE in HCT-116 cells after activation of the LOX pathway with the calcium ionophore A23187 and addition of exogenous AA to the cells.

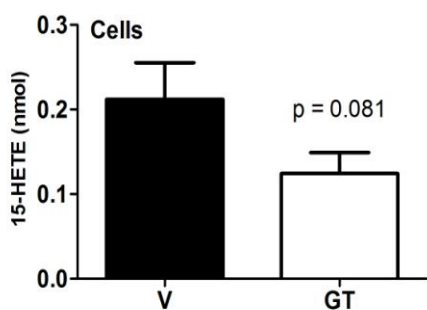


Figure 28. The effect of GT on the amount of 15-HETE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA were carried out, and the lipids were analyzed by LC/MS/MS. The amount of 15-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 15-HETE in HCT-116 cells shows no statistical difference between the GT-treated sample and the vehicle-treated sample (n =10; p = 0.081). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values were obtained at 95% confidence level. Error bars are representative of SEM of sample.

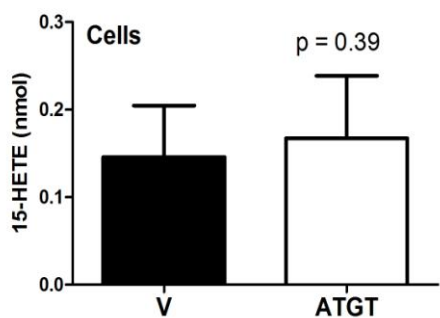


Figure 29. The effect of ATGT on the amount of 15-HETE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from HCT-116 cells following treatment with ATGT Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA was carried out, and the lipids were analyzed by LC/MS/MS. The amount of 15-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 15-HETE in HCT-116 cells shows no statistical difference between the ATGT-treated sample and the vehicle-treated sample treated (n =8; p = 0.39). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

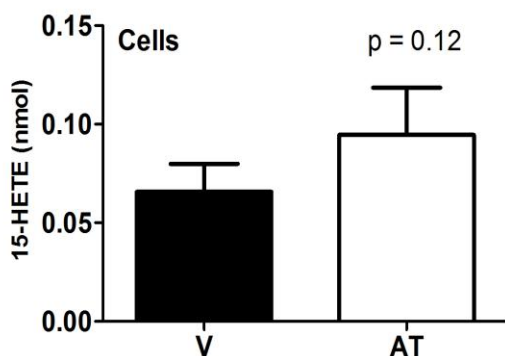


Figure 30. The effect of AT on the amount of 15-HETE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M AT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with AT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA were carried out, and the lipids were analyzed by LC/MS/MS. The amount of 15-HETE in HCT-116 cells was determined by stand curve analysis. The amount of 15-HETE in HCT-116 cells shows no statistical difference between the AT-treated sample and the vehicle-treated sample (n =8; p = 0.12). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

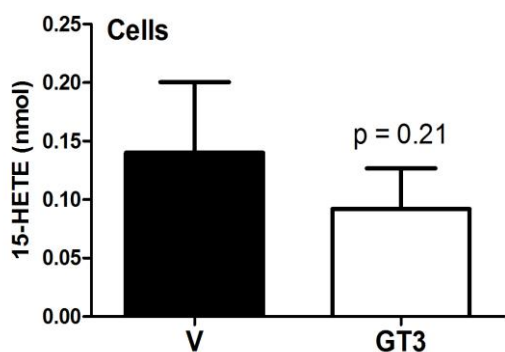


Figure 31. The effect of GT3 on the amount of 15-HETE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT3 for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT3. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA were carried out, and the lipids were analyzed by LC/MS/MS. The amount of 15-HETE in CT-116 cells was determined by stand curve analysis. The amount of 15-HETE in HCT-116 cells shows no statistical difference between the GT3-treated sample and the vehicle-treated (n =8; p = 0.21). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

ATGT or GT3 Decrease the Production of 12-HETE in HCT-116 Cells after LOX Activation while AT Increases the Production but GT Does not Change the Production of 12-HETE.

The amount of 12-HETE produced in HCT-116 cells after treatment with GT, AT, ATGT, and GT3 followed by activation of calcium ionophore and addition of exogenous AA was measured. The production of 12-HETE in HCT-116 cells after treatment with 5 μ M GT did not show a statistical difference as compared with the vehicle (Figure 32; $p = 0.47$). Interestingly, treatment of HCT-116 cells with 5 μ M ATGT significantly decreases the production of 12-HETE in HCT-116 cells as compared with the vehicle (Figure 33; $p = 0.024$). However, treatment of the cells with 5 μ M AT significantly increased the production of 12-HETE in HCT-116 cells as compared with the vehicle (Figure 34; $p = 0.034$). In addition, treatment of the HCT-116 cells with 5 μ M GT3 significantly decreased the production of 12-HETE as compared with the vehicle (Figure 35; $p = 0.027$). These data indicate that the activity of the 12-LOX is decreased by ATGT mixture and GT3 treatments.

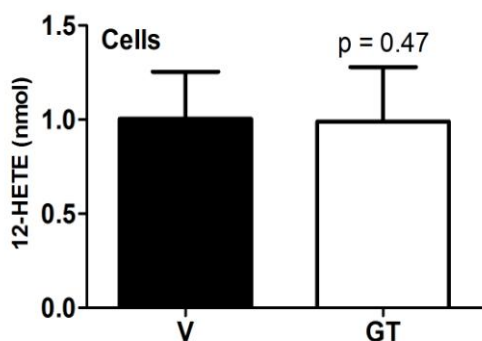


Figure 32. The effect of GT on the amount of 12-HETE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA was carried out. The lipids were analyzed by LC/MS/MS. The amount of 12-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 12-HETE in HCT-116 cells treated shows no statistical difference between the GT-treated sample and the vehicle-treated sample (n =10; p = 0.47). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

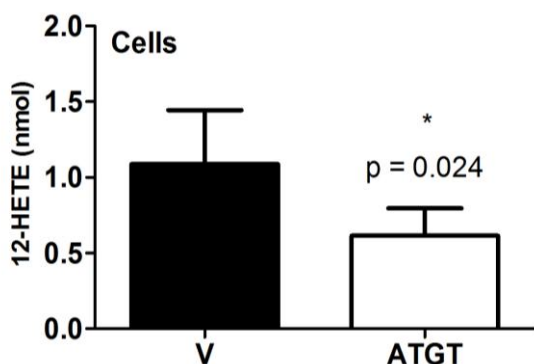


Figure 33. The effect of ATGT on the amount of 12-HETE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with ATGT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA were carried out. The lipids were analyzed by LC/MS/MS. The amount of 12-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 12-HETE in HCT-116 cells show a statistically significant decrease in the ATGT-treated sample compared with the vehicle treated sample (n =8; p = 0.024). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

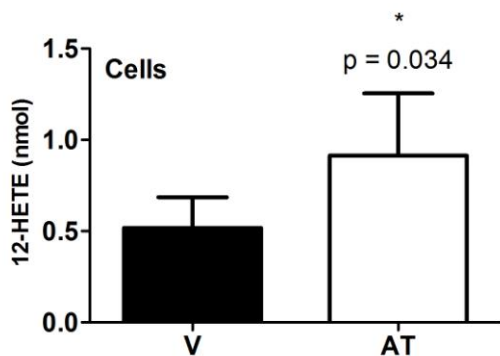


Figure 34. The effect of AT on the amount of 12-HETE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M AT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with AT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA were carried out. The lipids were analyzed by LC/MS/MS. The amount of 12-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 12-HETE in HCT-116 cells show a statistically significant increase in the AT-treated sample compared with the vehicle treated sample (n =7; p = 0.034). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

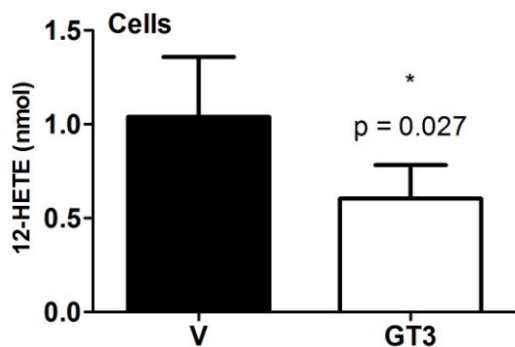


Figure 35. The effect of GT3 on the amount of 12-HETE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT3 for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT3. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA was carried out, and the lipids were analyzed by LC/MS/MS. The amount of 12-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 12-HETE in HCT-116 cells show a statistically significant decrease in the GT3-treated sample compared with the vehicle (n =8; p = 0.027). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

5-HETE is Decreased by GT3, Increased by AT and not Changed by GT or ATGT Treatments after Activation of LOX Pathway by the Calcium Ionophore (A23187) and Arachidonic Acid.

The amount of 5-HETE produced in HCT-116 cell following treatments with vitamin E isoforms, GT, AT, GT3, and ATGT mixture and further activation of the cells with calcium ionophore and addition of AA was also investigated. 5-HETE in HCT-116 cells is not significantly decreased by treatment with 5 μ M GT as compared with the vehicle (Figure 36; $p = 0.11$). Treatment of HCT-116 cells with 5 μ M ATGT did not significantly decrease the production of 5-HETE in HCT-116 cells as compared with the vehicle (Figure 37; $p = 0.11$). However, treatment of HCT-116 cells with 5 μ M AT significantly increased the production of 12-HETE as compared with the vehicle (Figure 38; $p = 0.037$). In addition, 5 μ M GT3 significantly decreased the production of 5-HETE in HCT-116 cells (Figure 39; $p = 0.046$). These data indicate that only GT3 allowed for a significant decrease in the production of 5-HETE production after activation of HCT-116 cells with the calcium ionophore and the addition of exogenous AA. The increase in the production of 5-HETE by AT after activation of HCT-116 cells with calcium ionophore and addition AA suggest that the 5-LOX enzyme is still active allowing for the production of 5-HETE.

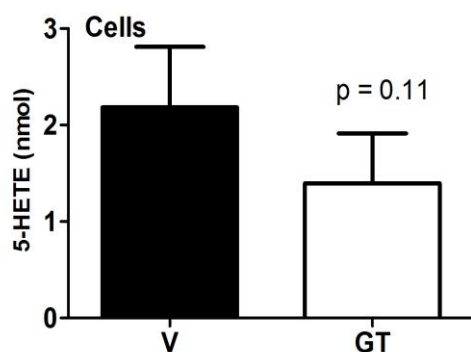


Figure 36. The effect of GT on the amount of 5-HETE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M GT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT. Activation with calcium ionophore and the addition of exogenous AA were carried out. The lipids were analyzed by LC/MS/MS. The amount of 5-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 5-HETE in HCT-116 cells shows no statistical difference between the GT-treated sample and the vehicle-treated sample (n =11; p = 0.11). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

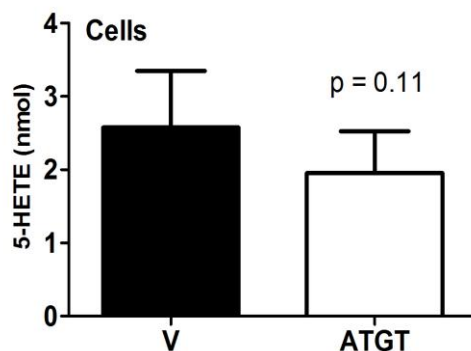


Figure 37. The effect of ATGT on the amount of 5-HETE of HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μ M ATGT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with ATGT. Activation with calcium ionophore and the addition of exogenous AA were carried out. The lipids were analyzed by LC/MS/MS. The amount of 5-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 5-HETE in HCT-116 cells shows no statistical difference between the ATGT-treated sample and the vehicle-treated sample (n= 9; p = 0.11). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

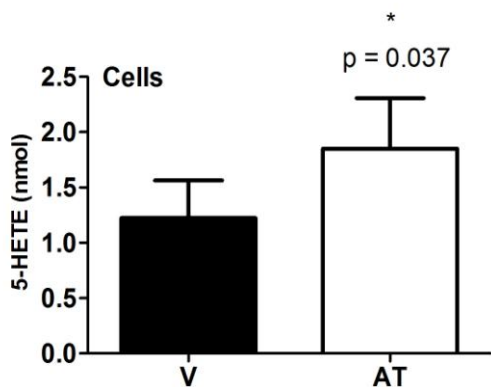


Figure 38. The effect of AT on the amount of 5-HETE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μM AT for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with AT. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA was carried out, and the lipids were analyzed by LC/MS/MS. The amount of 5-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 5-HETE in HCT-116 cells shows a statistically significant increase in the AT-treated sample compared with the vehicle (n =8; p = 0.037). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

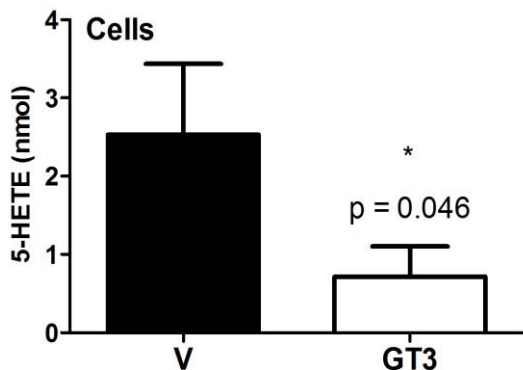


Figure 39. The effect of GT3 on the amount of 5-HETE in HCT-116 cells as analyzed by LC/MS/MS. HCT-116 cells were treated with and without 5 μM GT3 for 24 hours. The lipid content was isolated from the HCT-116 cells following treatment with GT3. Activation of cells with the calcium ionophore A23187 and the addition of exogenous AA was carried out, and the lipids were analyzed by LC/MS/MS. The amount of 5-HETE in the HCT-116 cells was determined by stand curve analysis. The amount of 5-HETE in HCT-116 cells shows a statistically significant decrease in the GT3-treated sample compared with the vehicle (n =7; p = 0.046). Statistical differences were determined by paired t-test. p values < 0.05 were determined statistically significant. p values are obtained at 95% confidence level. Error bars are representative of SEM of sample.

CHAPTER 4

DISCUSSION

In this study we found that, under both, physiological conditions and LOX induction by calcium ionophore A23187, each isoform of vitamin E tested (GT, AT, GT3 and ATGT mixture) exhibited different effects on the production of polyunsaturated fatty acid metabolites (LOX metabolites). Since these metabolites (13-HODE, 15-HETE, 12-HETE, and 5-HETE) have previously been associated with colorectal cancer development^{52,44,67}, we were interested in determining whether vitamin E isoforms have the ability to regulate the LOX pathway through the production of the LOX metabolites.

Under normal physiological conditions, we discovered that GT increases the amount of 13-HODE (Figure 6), and decreases the amount of 12-HETE in the media of HCT-116 cells (Figure 14), but does not regulate the amount of 15-HETE or 5-HETE in the media of HCT-116 cells (Figures 10 and 18).

NSAIDs like sulindac and NS398 enhance 13-HODE production in colorectal cancer cells^{53,54}. GT mirrors this function of NSAIDs in colorectal cancer cells. The flavonoid baicalein and sulindac are 12-LOX inhibitors and thereby inhibiting the production of 12-HETE in colorectal cancer cells^{42,41}. GT also mirrors the function of 12-LOX inhibitors under physiological conditions as it down regulates the production of 12-HETE into the media of HCT-116 cells treated under physiological conditions (Figure 14).

We found that under physiological conditions, the ATGT mixture (95% GT and 5% AT) increases the production of 13-HODE secreted into the media from HCT-116 cells (Figure 7). However, the ATGT mixture does not regulate the amount of 15-HETE (Figure 11), 12-HETE (Figure 15), or 5-HETE (Figure 19) produced and secreted in the media of HCT-116 cells.

This study demonstrates that, under normal physiological conditions, AT decreases the ability of GT to regulate the production of 13-HODE and 12-HETE secreted into the HCT-116 media. This is evidenced by the fact that the ATGT mixture demonstrated less than a 2 fold increase in 13-HODE compared to the vehicle (Figure 7) while pure GT demonstrated a 5 fold increase in 13-HODE compared to the vehicle (Figure 6). Further, the ATGT mixture (Figure 15) did not significantly decrease the amount of 12-HETE secreted in the HCT-116 media as did pure GT (Figure 14). Pure AT did not significantly regulate the production of 13-HODE (Figure 8) or 12-HETE (Figure 16) under physiological conditions. In fact, AT under physiological conditions had no effect on any of the metabolites (13-HODE (Figure 8), 12-HETE (Figure 16), 5-HETE (Figure 12) or 5-HETE (Figure 20).

Our study shows that, under normal physiological conditions, GT3 does not significantly affect the amount of 13-HODE (Figure 9), 15-HETE (Figure 13), 12-HETE (Figure 17), and 5-HETE (Figure 21) secreted in the media of the HCT-116 cells.

Hence, under normal physiological conditions, GT shows a greater effect in regulating the production of polyunsaturated fatty acid metabolites as it increases the

amount of 13-HODE and decreases the amount of 12-HETE secreted in the media of the HCT-116 cells.

When examining the production of the metabolites under the LOX activation conditions (addition of the calcium ionophore A23187 with exogenous AA to the cells post treatment), we observed that the 12-LOX enzyme was no longer active. The production of 12-HETE (Figure 32) in the cells as compared with the vehicle were not significantly increased. A possible reason for the reduction may be a lack of substrate availability or enzyme activity. We would expect that if the 12-LOX enzyme was still active, the amount of 12-HETE produced after activation of cells with calcium ionophore A23187 and addition of AA should increase following treatment with GT. However, the amount of 12-HETE produced in the GT-treated samples did not significantly change as compared with the vehicle under conditions that would activate the LOX pathway, which suggests that the 12-LOX activity is reduced by the GT pretreatment. The decrease in activity of the 12-LOX enzyme by GT was also observed as the amount of 12-HETE produced and secreted in the media under physiological conditions was significantly lower when compared with the vehicle (Figure 14).

As previously observed in the media (Figure 11 and 19), ATGT did not significantly decrease the amount of 15-HETE (Figure 29) and 5-HETE (Figure 37) in HCT-116 cells under LOX Activation conditions, thus confirming that ATGT does not significantly regulate the amount of 15-HETE or 5-HETE in HCT-116 cells neither under physiological conditions nor with LOX activation by the calcium ionophore A23187 and additional AA.

In addition, AT was observed to significantly increase the production of 12-HETE (Figure 34) and 5-HETE (Figure 38) after addition of calcium ionophore A23187 and exogenous AA to the cells, although under normal physiological conditions AT did not significantly increase the amount of 12-HETE or 5-HETE produced into the media (Figures 16 and 20). It is possible that under normal physiological conditions, there is not enough substrate (AA) present in the cells to be metabolized by 12-LOX or 5-LOX enzyme after treatment with AT. It is also possible that the addition of calcium ionophore A23187 activated the LOX pathway enhancing the activity of the 12-LOX or 5-LOX enzyme and allowing AT to produce more 12-HETE or 5-HETE in the cells.

Further, we observed that GT3 has no effect on LOX metabolism under physiological conditions, but significantly decreases the production of 13-HODE (Figures 26 and 27), 12-HETE (Figure 35), and 5-HETE (Figure 39) conditions of LOX activation. Thus, it is possible that the activity of 15-LOX-1, 12-LOX, and 5-LOX in HCT-116 cells are reduced by GT3 treatment. The addition of AA to the cells will provide the substrate necessary to produce the 12-HETE and 5-HETE. It is also possible that GT3 prevents the activation of the LOXs on AA. Additional studies are necessary to determine the role that GT3 plays on the statistically significant inhibition of 12-HETE and 5-HETE production following the activation of the LOX pathway with the calcium ionophore A23187 and AA.

Finally, we found that none of the vitamin E isoforms tested (GT, AT, ATGT and GT3) significantly regulates the production of 15-HETE in HCT-116 cells either under normal physiological conditions or LOX activation conditions.

In summary:

Table 1. Amount of 13-HODE, 15-HETE, 12-HETE, and 5-HETE Secreted into the Media of HCT-116 Cells after Treatment of Cells with 5 μ M GT, AT, ATGT, or GT3 under Physiological Conditions.

Vitamin E isoforms	13-HODE	15-HETE	12-HETE	5-HETE
GT	Increase	Not statistically significant	Decrease	Not statistically significant
ATGT	Increase	Not statistically significant	Not statistically significant	Not statistically significant
AT	Not statistically significant	Not statistically significant	Not statistically significant	Not statistically significant
GT3	Not statistically significant	Not statistically significant	Not statistically significant	Not statistically significant

Table 2. Production of 13-HODE, 15-HETE, 12-HETE, and 5-HETE Following 24 hour Pretreatment of HCT-116 Cells with 5 μ M GT, AT, ATGT, or GT3 and Subsequent LOX Activation with the Addition of Calcium Ionophore A23817 and Exogenous Arachidonic Acid.

Vitamin E isoforms	13-HODE	15-HETE	12-HETE	5-HETE
GT	Not statistically significant	Not statistically significant	Not statistically significant	Not statistically significant
ATGT	Not statistically significant	Not statistically significant	Decrease	Not statistically significant
AT	Not statistically significant	Not statistically significant	Increase	Increase
GT3	Decrease	Not statistically significant	Decrease	Decrease

In conclusion, the vitamin E isoforms, GT, GT3, AT as well as ATGT mixture did not have the same effect on the production of polyunsaturated fatty acid, 13-HODE, 15-HETE, 12-HETE, and 5-HETE under physiological conditions or LOX activation conditions. Under normal physiological conditions, GT is the best isoform of vitamin E as it increases the amount of 13-HODE and decreases 12-HETE in media of HCT-116 cells. It was also determined that under normal physiological conditions, AT has the ability to decrease the activity of GT in regulating the production of 13-HODE and 12-HETE in the media of HCT-116 cells.

Analytical Problems and Future Directions

Problems. Random error was observed in the data collected during this project. Hence, high number of replicates was done to enhance the precision of the data. A paired t-test was used to examine whether the metabolites obtained from the vehicle and treated samples are statistically significant.

Analytes obtained from extract initially precipitated at higher pH values (5.5-7.4) when dissolved in the following solvent system: ethanol, methanol, hexane, and ammonium acetate. The pH of the solution was therefore decreased to about 3.5-4.0 which prevented precipitation of the analyte in the solvent (methanol) used for dissolving these analytes. This is because the analytes obtained after extraction are fatty acids and exhibit high solubility as the pH of the solution is decreased.

Future Work. We would like to investigate the concentrations of HETEs (12-HETE, 15-HETE, and 5-HETE) and 13-HODE when LA is added during the isolation rather than AA. This experiment would be aimed at determining if reduction of 13-HODE production observed with GT and ATGT in the cells is due to lack of substrate or reduced enzyme activity.

We would also like to investigate the concentrations of HETEs (12-HETE, 15-HETE, and 5-HETE) and 13-HODE in the presence of chemical inhibitors for 15-LOX (e.g. 6, 11-dihydro-[1]benzothiopyrano[4,3-b]indole(PD146176)), 12-LOX (e.g. caffeic acid), and 5-LOX (e.g. AA861). These experiments would enable us to know the predominant LOX pathway that causes cell death in colorectal cancer cells.

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APPENDICES

Appendix A

Tables of Confidence Limits and p-values

Table 3. Confidence Intervals (CI) for 13-HODE, 15-HETE, 12-HETE, and 5-HETE in the Media of HCT-116 Cells Obtained at 95% Confidence Level

Vitamin E isoforms	13-HODE	15-HETE	12-HETE	5-HETE
GT	Figure 6 CI = - 5.5 to 0.40	Figure 10 CI = - 0.37 to 0.27	Figure 14 CI = 0.23 to 0.69	Figure 18 CI = -1.0 to 1.0
ATGT	Figure 7 CI = - 1.4 to 0.13	Figure 11 CI = - 0.60 to 1.17	Figure 15 CI = - 0.50 to 0.32	Figure 19 CI = - 0.60 to 1.2
AT	Figure 8 CI = - 1.1 to 0.20	Figure 12 CI = - 0.10 to 0.10	Figure 16 CI = - 0.079 to 0.070	Figure 20 CI = - 0.44 to 0.079
GT3	Figure 9 CI = - 0.39 to 0.53	Figure 13 CI = - 0.37 to 0.27	Figure 17 CI = - 0.64 to 1.0	Figure 21 CI = - 0.57 to 1.1

Table 4. Confidence Intervals (CI) for 13-HODE, 15-HETE, 12-HETE, and 5-HETE in HCT-116 Cells Obtained at 95% Confidence Level

Vitamin E isoforms	13-HODE	15-HETE	12-HETE	5-HETE
GT	Figure 23 CI = - 0.23 to 0.18	Figure 28 CI = -0.043 to 0.22	Figure 32 CI = - 0.44 to 0.47	Figure 36 CI = - 0.57 to 2.14
ATGT	Figure 24 CI = - 0.31 to 0.10	Figure 29 CI = - 0.19 to 0.15	Figure 33 CI = 0.0072 to 0.94	Figure 37 CI = - 0.47 to 1.7
AT	Figure 24 CI = - 0.19 to 0.071	Figure 30 CI = - 0.081 to 0.024	Figure 34 CI = - 0.83 to 0.04	Figure 38 CI = -0.44 to 0.079
GT3	Figure 26 CI = 0.031 to 0.23	Figure 31 CI = - 0.083 to 0.18	Figure 35 CI = - 0.0079 to 0.88	Figure 39 CI = - 0.41 to 4.0

Table 5. Probability Test Values (p-value or p) of 13-HODE, 15-HETE, 12-HETE, and 5-HETE in the Media of HCT-116 Cells Obtained at 95% Confidence Level

Vitamin E isoforms	13-HODE	15-HETE	12-HETE	5-HETE
GT	Figure 6 p = 0.038	Figure 10 p = 0.36	Figure 14 p = 0.0014	Figure 18 p = 0.49
ATGT	Figure 7 p = 0.045	Figure 11 p = 0.25	Figure 15 p = 0.30	Figure 19 p = 0.24
AT	Figure 8 p = 0.067	Figure 12 p = 0.49	Figure 16 p = 0.45	Figure 20 p = 0.072
GT3	Figure 9 p = 0.37	Figure 13 p = 0.47	Figure 17 p = 0.29	Figure 21 p = 0.24

Table 6. Probability Test Values (p-value or p) of 13-HODE, 15-HETE, 12-HETE, and 5-HETE in HCT-116 Cells Obtained at 95% Confidence Level

Vitamin E isoforms	13-HODE	15-HETE	12-HETE	5-HETE
GT	Figure 23 p = 0.39	Figure 28 p = 0.081	Figure 32 p = 0.47	Figure 36 p = 0.11
ATGT	Figure 24 p = 0.14	Figure 29 p = 0.39	Figure 33 p = 0.024	Figure 37 p = 0.11
AT	Figure 25 p = 0.16	Figure 30 p = 0.12	Figure 34 p = 0.034	Figure 38 p = 0.037
GT3	Figure 26 p = 0.0092	Figure 31 p = 0.21	Figure 35 p = 0.027	Figure 39 p = 0.046

Table 7. Confidence Intervals (CI) and Probability Test Values (p-value or p) for the Amount of 13-HODE in HCT-116 Cells Using ELISA Method.

Vitamin E isoforms	13-HODE
GT	Figure 22 p = 0.096 CI = -0.033 to 0.0081
GT3	Figure 27 p = 0.015 CI = 0.0039 to 0.052

Appendix B

Buffers and Solutions

1. PBS: Prepared using 8 g sodium chloride, 0.2 g potassium chloride, 0.2 g potassium dihydrogen phosphate, and 1.15 g disodium hydrogen phosphate in 1 L of distilled water.
2. Lysis buffer: Prepared using 98 mL of PBS, 1 mL of 10% triton X-100, 1 mL of 10% sodium dodecyl sulphate.
3. Calcium chloride (1 mM): 0.0147 g of calcium chloride dihydrate was weighed and dissolved in 100 mL of PBS.
4. Calcium ionophore (1 mM): 0.001 g of calcium ionophore was dissolved in 2 mL of PBS solution.
5. 0.2 N HCl: 1.65 mL of 12.1 N HCl was diluted to 100 mL with deionised water.
6. Wash Buffer: 5 mL of the supplied concentrate was diluted with 95 mL of deionized water.
7. Conjugate (1:10) dilution: 50 μ L of the supplied conjugate was diluted with 450 mL of standard diluents (assay buffer).
8. Arachidonic acid (10000 μ M): 10000 μ M was prepared from 0.8210 M arachidonic acid stock solution in ethanol.

Appendix C

List of Instruments

1. HP- 8452A diode array spectrophotometer
2. UV-VIS spectrophotometer plate reader (Molecular devices, Spectra Max Plus 384).
3. Shimadzu IT-TOF LC/MS/MS

Appendix D

Lists of Abbreviations

AA	- arachidonic acid
AA-861	- 2-(12-Hydroxydodecane-5, 10-diynyl)-3, 5, 6-trimethyl-p benzoquinone
AT	- alpha tocopherol
AT3	- alpha tocotrienol
ATGT	- alpha tocopherol – gamma tocopherol mixture
Bcl-2	- B-cell lymphoma 2
BHPP	- N-benzyl-N-hydroxy-5-phenylpentamide
BRL49653	- rosiglitazone
Cdk	- cyclin-dependent kinases
COX-2	- cyclooxygenase-2
DNA	- deoxyribonucleic acid
ELISA	- enzyme-linked immunosorbent assay
FBS	- fetal bovine serum
G ₁	- gap 1
G ₂	- gap 2
GT	- gamma tocopherol

GT3	- gamma tocotrienol
HCl	- hydrochloric acid
HETE	- hydroxyeicosatetraenoic acid
HODE	- hydroxyoctadecadienoic acid
13-HODE	-13-hydroxyoctadecadienoic acid
15-HETE	-15-hydroxyoctadecadienoic acid
12-HETE	-12-hydroxyeicosatetraenoic acid
IT-TOF	- ion trap time of flight
IU	- international unit
Kg	- kilogram
LOX	- lipoxygenase
LC/MS/MS	- liquid chromatography/tandem mass spectrometry
LA	- Linoleic acid
μM	- micromolar
Max	- maximum
M	- mitotic
MD	- Maryland
mg	- milligram
min	- minutes

mL	- milliliter
mM	- millimolar
MK 886	- 1-[(4-chlorophenyl)methyl]-3-[(1,1-dimethylethyl)thio]- α,α - dimethyl-5-(1-methylethyl)-1H-indole-2-propanoic acid
μ M	- micromolar
nM	- nanometer
NSAID	- non-steroidal anti-inflammatory drug
NY-	-New York
P	- protein
PAH	- polycyclic aromatic hydrocarbons
PBS	- phosphate buffered saline
PG	- prostaglandins
PPAR γ -	- peroxisome proliferator-activated receptor γ
R	- rectus
RB	- retinoblastoma
S	- synthesis
UV-VIS	- ultraviolet-visible

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