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Anti-Cancer Effects Of Tocotrienols In Nsclc

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ANTI-CANCER EFFECTS OF TOCOTRIENOLS IN NSCLC

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DEDICATION

"I dedicate this to my parents, wife, my family members and everyone who has supported my education so far."

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ΔCT	Delta (normalized) cycle threshold value
1H NMR	Proton nuclear magnetic resonance spectroscopy
4EBP1	Eukaryotic translation initiation factor 4E-binding protein
ALK	Anaplastic lymphoma kinase
ANOVA	Analysis of one way variance
Bcl-2	B-Cell CLL/Lymphoma 2
CCND1	Cyclin-D1
cDNA	Complimentary deoxyribonucleic acid
CT	Computerized tomography
DNA	Deoxyribonucleic acid
dNTP	nucleotide triphosphate
EGFR	Epidermal growth factor receptor
ECGC	Epigallocatechin gallate
EGF	Epidermal growth factor
FID	Free induction decay
K-Ras	V-Ki-ras2 kirsten rat sarcoma
LAT-1	L-type amino acid transporter 1
MMP9	Matrix metallopeptidase-9
mRNA	Messenger ribonucleic acid
miRNA	micro ribonucleic acid
mTOR	Mechanistic target of rapamycin
NF- κB	Nuclear factor kappa-light-chain-enhancer of activated ß cell
Notch1	Notch homolog 1

NTC	Non-template control
NSCLC	Non-small cell lung cancers
OPLS	Orthogonal projections to latent structures regression
OPLS-DA	Orthogonal partial least squares discriminant analysis
PCA	Principle component analysis
PLS-DA	Partial least square discriminant analysis
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
SCLC	Small cell lung cancers
S6K1	p70 S6 kinase
αT	Alpha Tocotrienols
γT	Gamma Tocotrienols
δT	Delta Tocotrienol
TRF	Tocotrienol Rich Fraction
TRMC	Tocotrienol Rich Mixture in Capsule
uPA	Urokinase-type plasminogen activator
VIP	Variable of projection

CHAPTER 1 INTRODUCTION

Cancer

Cancer is a disease characterized by the growth of abnormal cells that divide uncontrollably and have the capability to penetrate and destroy normal body tissue [1]. The growth of any cancer is a multistep process. Normal cells gradually turn into malignant cells by acquiring cancer capabilities such as uncontrolled cell proliferation, resisting cell death (apoptosis), and activating invasion and metastasis after somatic mutation [2].

Cancer remains the second leading cause of death in the United States, surpassed only by heart disease, and accounts for nearly one of every four deaths [3]. Approximately 14.5 million Americans were diagnosed with cancer as of January 1, 2014,. About 1,685,210 new cancer cases are estimated to be diagnosed in 2016, and nearly 595,690 Americans are projected to die from cancer in 2016, a mortality rate of about 1,630 fatalities per day [3].

Many external factors raise the risk of cancer, such as exposure to radiation, environmental pollutants, certain infections, tobacco use, certain dietary factors, obesity, and lack of physical activity. These factors can either directly influence genes or combine with existing genetic errors within the cells to form mutations [4]. Mutations in cancer cells lead in developing main traits ("hallmarks") that Weinberg et al. [5] highlighted. The main traits or “hallmarks" of cancer cells are [5]: (1) self-sufficiency in growth signals; (2) insensitivity to anti-growth signals; (3) evading apoptosis; (4) limitless replicative potential (5) sustained angiogenesis; and (6) tissue invasion and metastasis.

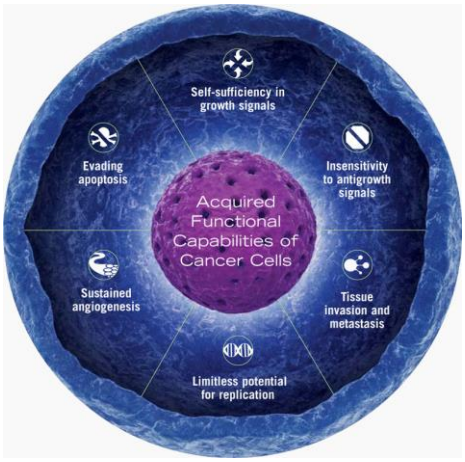


Figure 1.1: The Six Hallmarks of Cancer, As Published In 2013 By Weinberg and Hanahan.

Cancer etiology

Carcinogenesis process can be divided theoretically into four steps [6]: initiation, promotion, progression, and metastasis. Initiation is the first step in the process of carcinogenesis; it involves the alteration, change, or mutation of chromosomes arising spontaneously or induced by exposure to a carcinogenic agent. Each cell can repair the mutations before passing into new cells [7]. However, under certain situations, cells lose their ability to fix the mutations. As the mutations build up over time, the damaged cell is more likely to become cancerous [8]. Those mutations can result in dysregulation of biochemical signaling pathways associated with cellular proliferation, survival, and differentiation [6]**.**

Promotion is the phase between a premalignant lesion and the development of invasive cancer [7]. This step is a lengthy, reversible process. Additional and recurrent damage needs to happen before cancer progresses. Agents, such as hormones or some drugs, cause this further damage and are known as promoters [8]. This Promoter speeds up the pace of cell division process and creates more genetic mutations.

Progression is the final development of invasive cancer where genetic and phenotypic changes and cell proliferation arise in the cells [7]. This implicates a rapid increase in the tumor size, where cells may undergo further mutations with invasive and metastatic potential. The production of tumor cells, known as tumorigenesis, is a process by which mutated cells receive constant signals that command them to divide at initial proliferation cycles; abnormal cells form a cluster in a single mass commonly known as a tumor (or neoplasm).Body conditions like genetic factors, hormones, etc. have an effect on the progression of cancer[6].

As cancer cells proliferate, at late proliferation cycles, tumor cells acquire the ability to invade surrounding tissue for energy and nutrients; these invaders, termed malignant cells, enter the bloodstream or lymphatic vessels [7] by destroying basal membranes and forming secondary tumors at distant locations in the body [8] by a process known as metastasis.

Signal transduction process in cancer

Signal transduction pathways convert environmental stimuli to changes in cell behavior and are thus central to the control of all biological processes [9]. Most human cancers have aberrant signal transduction elements in their development process. It is the communication processes of cells that usually involve a cascade of protein phosphorylation events, ultimately resulting in the activation (or inactivation) of transcriptional regulators and changes in target genes controlling cell processes of growth, differentiation, and survival. Abnormalities in signal transduction elements can lead to increased proliferative potential, raised invasion and metastasis, and apoptosis inhibition [10].

Cells are regularly opened to a variety of exterior signals, ranging from soluble endocrine and paracrine factors to signaling molecules in neighboring cells [11]. The cell-surface receptor is the environmental signal receiver most often located on the cell surface and interacts with internal

cellular components [10]. After receiving information at the receptor, the information is converted into other chemical forms. Changes in the concentration of chemical forms, called second messengers, create the next step in the molecular information network. Particularly important second messengers include cyclic AMP, cyclic GMP, calcium ion, inositol 1,4,5-trisphosphate, (IP_3), and diacylglycerol (DAG). Many second messengers elicit responses by activating protein kinases [10]. The protein kinases transfer phosphoryl groups from ATP to specific serine, threonine, and tyrosine residues in cytoplasmic proteins. These cytoplasmic proteins either activate transcription factors or proteins that activate transcription factors [12]. Transcription factors are small proteins which regulate the expression of genes within the cell, and they ultimately change the regulation of its target gene expression [13]. In normal cell processes, transcription factors function as a single protein or as part of a complex in the regulatory region of the gene [13]. Some of the transcription factors have been identified as oncogenic, meaning they regulate the function of cancer genes AP-1, NF-κB, and STAT3. Most of the transcription factors maintain genes in an "on" position in cancer cells whereas shutting off these genes by inactivation of transcription factors will result in cancer cell death[13]. Sometimes cell-surface receptors also directly activate transcription factors or target genes without activating protein kinases. They can send a signal into the nucleus and alter the transcription pattern of the cells. Thus, it is extremely important that the cell accurately interprets these extracellular signals via Signal transduction pathways to create the appropriate developmental or proliferative-related gene responses [14].

Genes in cancer

Mutations in the cellular genome are considered to be one of the leading causes of the carcinogenesis process by expressing or controlling cell growth [15]. Accumulation of mutations

in somatic genome results in increasing the risk of cancer development in humans. Based on their functions, genes that contribute to the expansion of cancer fall into two broad categories:

(1) An oncogene is a modified gene or a group of nucleotides that encodes for proteins, increasing the malignancy of tumor cells. A proto-oncogene is an original form of a gene that can convert into an oncogene either after mutation or improved expression [16]. Proto-oncogenes provide information for proteins often involved in carcinogenic signal transduction pathways, generally in cell differentiation and cell growth regulation. Upon activation, it (or its product) develops a tumor inducing agent, known as an oncogene. RAS, WNT, MYC, ERK, and TRK are some of the proto-oncogenes that may become oncogenes after activation [16].

(2)Tumor suppressor genes are the second category of cancer genes that prevent and inhibit the development of tumors by regulating the growth of cells [17]. Following a modification or inactivation due to a mutation, they lose the ability to make the protein that regulates cell growth, resulting in uncontrolled cell division which develops into cancer. Some suppressor genes slow down or stop cell proliferation, inducing apoptosis, whereas other suppressor genes are responsible for repairing damages. Retinoblastoma susceptibility gene (RB), Wilms' tumors (WT1) and P53 are some examples for common tumor suppressor genes [17].

Lung cancer

Lung cancer is a malignant lung tumor characterized by uncontrolled cell growth in tissues of the lung. It is currently the second leading cause of cancer death in both men and women in the U.S in 2016. An estimated 224,390 new cases of lung cancer are expected in 2016, accounting for about 14% of all cancer diagnoses in the United States. Of the estimated 158,080 deaths estimated for 2016, cancer will account for approximately 25% of those fatalities [3]. From 2008 to 2012, death rates from cancer were reduced by 2.9% per year in men and by

1.9% per year in women. The 5-year average survival rates for lung cancer is 17% and Only 16% of lung cancers are diagnosed at a localized stage, for which the 5-year survival is 55% [3].

Lung cancers are classified into three types: non-small cell lung cancers (NSCLC), small cell lung cancers (SCLC) and lung carcinoid tumors. The classification is based on the microscopic appearance of the tumor cells, treatment options, and growth.

Non-small cell lung cancer

Non-small cell lung cancer (NSCLC) is manifested as aggressive tumors arising from the airway epithelial cells (majority) and interior parts of the lungs [18]. It is the most common type of lung cancer, comprising 85% of all lung cancers. The current therapeutic options for NSCLC, which include surgery, radiotherapy, and chemotherapy [18], have slightly improved NSCLC survival rate at some developmental stages in both men and women. In the last 30 years, these therapies have resulted in the plateauing of the overall five-year survival rate, which has remained between 12-18% from 1975 -2011 [19]. Further, the five-year survival rate of patients diagnosed with early stage NSCLC is 50%, but the rate is significantly reduced to less than 5% in patients diagnosed with distant metastasis [20]. Despite such a wide 5-year survival rate ranging from 5% -50%, NSCLC remains the leading cause of global cancer death [20]. Also, several studies report that there is a high probability of reoccurrence and development of resistance to drug therapies in NSCLC after treatment with chemotherapeutic agents [21]. Plateauing of the overall five-year survival rate along with a high probability of reoccurrence and development of resistance to drug therapies warrants efforts to identify novel therapeutic agents and targets for preventing and treating NSCLC.

Non-small cell lung cancer is further divided into three groups: adenocarcinoma, squamous cell carcinoma and large cell carcinoma. Adenocarcinoma arises from epithelial tissue which has

a glandular origin, glandular characteristics, or both. It is the most common form of lung cancer in people. Nearly 40% of all lung cancers in the U.S. are adenocarcinomas that originated from peripheral lung tissues. Inhalation of cigarette smoke causes peripheral lesions, which are usually adenocarcinomas of the lung [22]. These types of cancer grow more slowly and form smaller masses than the other subtypes. However, they tend to form metastases at an early stage. Squamous cell carcinoma arises in cells that line the central airways of the lungs. It accounts for about 15% of all lung cancer cases. This type of cancer is the most aggressive and grows rapidly in the lungs. Large cell carcinoma grows and spreads more quickly making it difficult to treat. It accounts only for about 10% to 15% of all lung cancers.

Other types of lung cancer

Small cell lung cancer makes up about 10%-15% of all lung cancers. This type of lung cancer tends to spread quickly. Less than 5% of lung cancers are lung carcinoid tumors, also called lung neuroendocrine tumors. Most of these tumors develop slowly and rarely spread.

Mechanistic aberrations in lung cancer

It is becoming more accepted now that the identification of genomic alterations in lung cancer can impact therapeutics, especially when the alterations represent “oncogenic drivers” in the processes of tumorigenesis and progression. Mutations in oncogenic drivers, namely epidermal growth factor receptor (*EGFR*), *KRAS*, and anaplastic lymphoma kinase (*ALK*) are mutually exclusive in patients with NSCLC. However, more than 35% of mutations in oncogenic drivers of NSCLC are still unknown. Aberrant Notch-1 and mTOR expressions are considered as emerging oncogenic drivers in the pathobiology of NSCLC. The mutations in oncogenic drivers of NSCLC activates the genes, transcription factors and/or miRNA that is controlling cell proliferation,

metastasis or inducing apoptosis. Therefore, targeting for these mutations and tailoring therapy accordingly is widely accepted.

Notch signaling pathway

Most of the signaling pathways controlling cell growth and differentiation, including Notch, are commonly altered in cancer. Notch signaling plays a major role in the communication process of controlling cell proliferation, differentiation, and apoptosis in cells. Notch is one of the signaling pathways whose dysregulation has been found to contribute to many types of human cancers [23] including of NSCLC patients [24, 25]. It shows both tumor promotion and inhibition function in lung cancer by inducing different anti-cancer activities, namely cell proliferation, apoptosis, and invasion. For instance, Notch-1 shows a growth-promoting function on NSCLC, whereas in SCLC it plays a tumor-suppressive role [26]. Baumgart et al. 2010 [27] reported that Notch-1 expression from excessive ADAM17 activities leads to subsequent regulation of the EGFR expression and tumorigenicity of NSCLC cells. Overexpression of Notch-1 also has been reported to inhibit apoptosis in lung adenocarcinoma [28]. Inhibiting Notch signaling by Gamma secretase inhibitors (GSI) also prompted with apoptosis in lung squamous cell carcinoma cells [29].

Four Notch receptors (Notch 1, 2, 3 and 4) and five ligands, including delta-like ligand (DLL) 1, 3, and 4, and Jagged (JAG) 1 and 2 in mammals, have been identified [30]. The Notch transmembrane receptors and their ligands play a vital role in cancer development [30]. Once a ligand binds to the receptor, two proteolytic enzymes, namely ADAM metalloprotease and a γ-secretase complex, make two proteolytic cleavages. Then it releases to the Notch intracellular domain (NICD) [31]. The activated form of Notch, NICD, translocate to the nucleus and binds to the transcriptional repressor to induce transcription of Notch target downstream genes such as

the Hes family, Hey family, NF-kB, VEGF, BcL family, c-myc, and cyclin D1[32, 33]. These Notch target downstream genes are involved in various cellular and developmental contexts [33]. Also, Notch-1 supports invasion and metastasis via regulating MMP9 expression. The elevated expression of MMP9 induced by Notch ligand namely delta-like 1 homolog (DLK1) could be significantly decreased by inhibiting Notch signaling using notch inhibitor namely gamma secretase inhibitors (GSI) [34].

Notch-1 has been reported to cross-talk with NF-κB , which plays a major role in many physiological processes [35-37]. Constitutive levels of Notch activity are vital in maintaining NF-κB activity in various cell types [38]. Reduced level of Notch expressions in mice have shown to significantly lower NF-κB activity [38]. Additionally, the downregulation of the Notch pathway was associated with miR expression. For example, up-regulation of miR-34a inhibits NSCLC cell proliferation, induces apoptosis and inhibits the invasion in NSCLC [39]. These results suggest that the modification of Notch signaling may be a potential therapeutic utility in treating NSCLC.

MMP9 and uPA

Matrix metallopeptidase 9 (MMP-9), is a matrix, a class of enzymes that belong to the zinc-metalloproteinases family. MMP-9 has a metastasis-promoting role [40, 41] and it has been shown to be involved in degradation of the extracellular matrix and promotion of tumor growth and metastasis by its angiogenic properties namely cell migration, aggregation, adhesion and invasion in cancer [41-43]. MMP-9 expression increases with tumor size and its expression is significantly higher in NSCLC cases with metastasis as compared to those without metastasis.

uPA is a serine protease which converts inactive plasminogen to active plasmin. The active plasmin is responsible for the activation of MMP-9 which degrades extracellular matrix (ECM), allowing cell invasion. uPA was also found to be involved in cell adhesion and migration.

Together, MMP-9 and uPA play a major role in tumor invasion and the metastasis process. Therefore, modulating uPA and MMP-9 could be an excellent therapy for preventing cell invasion and metastasis in NSCLC.

NF-κB (Nuclear factor kappa-light-chain-enhancer of activated B cells)

NF-κB is one of nuclear transcription factors responsible for regulating expression of genes involved in apoptosis, replication, tumorigenesis, inflammation, and many other physiological processes [44]. Five *NF-κB* family proteins bound to IκB family proteins have been identified in mammalian species. The IκB family proteins prevent translocation of NF-κB: IκB complex into the nucleus. After external stimuli, NF-κB: IκB complex undergoes a phosphorylation process and divides the NF-κB: IκB complex into individual IkB and NF-κB complexes. Individual IκBs are recognized by the ubiquitin ligase enzyme and degraded at the IkB kinase enzyme complex. Activated NF-κB then translocates to the nucleus and binds with different DNA-binding domains of specific genes that are responsible for physiological responses such as an inflammatory or immune response, a cell survival response, or cellular proliferation [44].

Constitutive activation of NF-κB is attributed to the carcinogenic function in mammalian species as NF-κB play a key role in controlling genes in cell proliferation, cell survival [45] and apoptosis. Some of the NF-κB transcription factors target anti-apoptotic genes, include TRAF1, TRAF2, and BCL-2[46]. A variety of solid tumors, including prostate, breast, cervical, pancreatic and lung cancer has shown to activate NF-kappaB expression constitutively [44, 47]. Lung cancer patients also showed high levels of NF-kappaB activation in both small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC) [44, 47]. Elevated levels of NF-κB are significantly associated with poor prognosis in lung cancer patients, whereas inhibition of NF-kappaB using siRNA, IKK inhibitors, and IkappaB tumor suppressor showed high survival rate

and low proliferation rate [48, 49]**.** Thus, lowering NF-κB activity in cancer cells could be potential molecular targets for lung cancer treatments.

MicroRNA-mediated regulation of lung cancer

MicroRNAs (miRNAs) are small, non-coding regions in RNAs (usually 20–22 nucleotides) that play a significant role in controlling basic biological functions, including mammalian. The mechanism for miRNAs-mediated gene regulations functions by either straight cleavage of a targeted mRNA or interfering with translation by interacting with the 3' untranslated region (UTR) of a target mRNA.

Usually, miRNAs have multiple target genes. Therefore, a single miRNA can often affect multiple cellular processes including tumorigenesis [50]. Some of the microarray-based analyses of miRNA expression studies have identified many lung cancer-associated miRNAs [51]. Aberrant expression of miRNAs has been found to show a significant role in cancer as either oncogenes or tumor suppressor genes [52].

miRNAs that exhibit tumor suppressing function are frequently underexpressed in lung tumors, particularly NSCLC, compared to normal lungs, and their reduced expression has also been associated with poor prognosis [53]. MiR-29a/b/c, miR-34a/b/c, miR-16, *and* miR-126 [51] are examples for tumor suppression miRNAs. Oncogenic miRNAs are usually over-expressed in lung cancer and the miR-17-92, a cluster of seven miRNAs, miR-21, miR-93, miR-98, miR-197, miR-221/222, *and* miR-155 are reported to be involved in oncogenic miRNA in lung cancer [53].

Recent evidence shows a strong link between miRNAs and invasion and metastasis. Several miRNAs have been found to regulate key regulators of EMT, a process central to cancer metastasis [53]. These include miR-10b (through inhibition of *HOXD10),* miR-126, and the

miRNA-200 family (which inhibit EMT inducers ZEB1 and ZEB2[53]. Therefore, manipulation of miRNAs may be a possible therapeutic target in cancer treatment for NSCLC.

mTOR Pathway

The mechanistic target of rapamycin (mTOR) is a key regulator of cell growth and activator of protein translation in response to amino acid abundance and growth factor signaling [54]. It senses and integrates various nutritional and environmental cues into the cells via external stimuli. Aberrant mTOR signaling is involved in many diseases including cancer, cardiovascular disease, and diabetes.

The two functionally distinct protein complexes, namely mTOR complex 1, and the mTOR complex, are key compasses in mTOR [55]. The mTORC1 is made with an mTOR-raptor, mLST8, and two negative regulators, PRAS40 and DEPTOR [56]. The basic function of Raptor is to regulate mTOR activity and functions by recruiting mTORC1 substrates [56]. Activated mTORC1 from external stimuli phosphorylates two main downstream effectors, S6K1 and 4EBP1[57-59]. The targets of S6K1 include ribosomal proteins, elongation factors, and insulin growth factor 2 [60] whereas 4EBP1 inhibits the protein translation by binding and inactivating eIF4E (eukaryotic translation initiation factor 4E)[61]. S6K1 and 4EBP1 are used to evaluate mTORC1 activity *in vivo* as they have been shown to be directly regulated by mTOR in several studies.

Cumulative evidence shows that the lysosome at the core of mTOR complex 1 (mTORC1) activated by EAA and glutamine via two parallel pathways, Rag GTPase-Regulator and Vps34-phospholipase D1 (PLD1). For instance, knockdown of glutamine transporter SLC1A5 in cells altered glutamine uptake and export, EAA uptake and mTORC1 activation. *In vitro* treatment of cancer cells with L-γ-glutamyl-p-nitroanilide (GPNA; an inhibitor of SLC1A5) or with 2-

aminobicyclo-(2,2,1)heptane carboxylic acid (BCH; an inhibitor of SLC7A5–SLC3A2) inhibits glutamine-dependent activation of mTORC1 and induce autophagy [62] suggesting that glutamine uptake and export is required for EAA activation of mTORC1. Therefore, controlling EAA uptake and mTORC1 activation could be an excellent therapy for preventing NSCLC.

Cancer, nutrients, and phytochemicals

Deeper understanding of the pathobiology of NSCLC has led to the discovery of novel small molecules that target genetic mutations known to play critical roles in the progression to metastatic disease. Nutraceuticals, plant-based small molecules, contributed to drug discovery and development processes in cancer over the last four decades[63, 64]. Plant-based small molecules show minimum cytotoxicity and simultaneously target multiple signaling pathways involved in cell growth, apoptosis, invasion, angiogenesis and metastasis in cancer cells [65, 66].Recent studies on humans and animals also confirmed that phytochemicals found in the diet suppress the inflammatory processes which would otherwise lead to transformation and initiation of carcinogenesis. The anti-cancer function of curcumin (turmeric), resveratrol (red grapes), lycopene (tomato), catechins (green tea), vitamin C (citrus fruits), Beta-carotene (carrot), indole 3-carbinol, tea polyphenols, isothiocyanates and coenzyme Q10 have been confirmed in several studies. Since cancer is the outcome of a dysregulation of multiple signaling pathways, and natural products provoke multi-targeted activities, natural products hold great potential for treatment of human tumors given they are specific to multiple therapeutic targets [67].

Vitamin E

Vitamin E is a fat-soluble vitamin with two major isomers: tocopherols (α-, β-, γ-, δ-) and tocotrienols (α-, β-, γ-, δ-). Vitamin E acts as a potent antioxidant, capable of neutralizing free radicals directly by donating hydrogen from its chromanol ring. It has also been identified to have

a role in the prevention and inhibition of cancer [68, 69]. The anti-cancer properties of vitamin E, attributed from either a combination or individual isomers, have been demonstrated in colon, lung, prostate, and breast cancer [70, 71]. Apart from antioxidant and anti-cancer properties, Vitamin E plays a role in protecting bone, cardiovascular, eye, nephrological and neurological diseases. The sources of Vitamin E includes vegetable oils, including rice bran oil and palm oil, wheat germ, barley, anatto, and certain other types of seeds, nuts, grains [72].

Heath benefits in tocotrienols

Tocotrienols were first identified in 1964 [77]. Despite its discovery in 1964, it was not until the 1980s that tocotrienols emerged in the spotlight prior to research interest in tocopherol; tocopherols were regarded as the major isoform with the most potent antioxidant and biological activity [79, 80]. The unsaturated side chain of tocotrienol permits for more efficient diffusion into tissues that have saturated fatty layers such as the brain and liver [15]. Experimental research examining the antioxidant, free radical scavenging, effects of tocopherol and tocotrienols have found that tocotrienols appear superior due to their better distribution in the lipid layers of the cell membrane [80] [16]. Cumulative evidence from recent investigations have shown that tocotrienols may be superior to tocopherols in their anti-cholesterolemic (unique to tocotrienols only), antioxidant, anti-cancer, anti-inflammatory, cardioprotective and neuroprotective properties [77]. Additional pharmacological potential of tocotrienols has also been summarized in Table 1. Table 01 has been adapted from [81], modified, and used in this thesis.

Table 01: Biological properties of tocotrienols

Protective activity	Tocotrienol type	Proposed mechanism of action
Anti-diabetic	TRF from palm oil and rice bran oil	Prevents the formation of advanced glycation endproducts in diabetic rats

Protective activity	Tocotrienol type	Proposed mechanism of action
	α-T, γ-T, δ-T	Reduces hyperglycemia and hyperlipidemia in diabetic rats
	α-T, γ-T, δ-T	Inhibition of NF-κB signaling pathway
	α-T, γ-T, δ-T	Inhibition of oxidative-nitrosative stress
	α-T, γ-T, δ-T	Inhibition of TNF-α, IL-1β, TGF-β1 and caspase-3 activity
	TRF from palm and rice oil	Reduction of glucose-insulin index
	α-T, γ-T, δ-T	Increase in insulin sensitivity
Anti-inflammatory	α-T, γ-T, δ-T	Suppression of NF-κB, TNF-α, IL-1, IL-6, IL-8 and iNOS
	α-T, γ-T, δ-T	Suppression of cyclooxygenase-2 activity
	α-T, γ-T, δ-T	Suppression of STAT-3 signaling pathway
Antioxidant	α-T, γ-T, δ-T	Increase in the activity of antioxidant enzymes
	TRF from palm oil and rice bran oil, α-T, γ-T, δ-T	Quenching and scavenging of free radicals
	α-T, γ-T, δ-T	Inhibition of lipid peroxidation
Immuno-stimulatory	α-T, δ-T	Induction of antibody production
	α-T, γ-T, δ-T	Induction of IFN-γ, IL-4, IL-1β production
	δ-T	Suppression of TNF-α
Cardio-protective	α-T, γ-T	Inhibition of HMG-CoA reductase activity

Protective activity	Tocotrienol type	Proposed mechanism of action
	α-T, γ-T	Inhibition of expression of cell adhesion molecules
	α-T, γ-T	Reduction in the levels of blood cholesterol
	TRF from palm oil and rice bran oil, δ-T	Inhibition of lipid peroxidation
	γ-T, δ-T	Downregulation of c-Src expression
	γ-T, δ-T	Upregulation of phosphorylation of Akt
	TRF from palm oil	Reduction in the production of apolipoprotein B, platelet derived factor-4, thromboxane B2
	TRF from palm oil and rice bran oil	Downregulation of TGF-β
Neuro-protective	α-T	Inhibition of PP 60 (c-Src) kinase activity and phosphorylation of Erk
	α-T, γ-T	Inhibition of 12-lipoxygenase activity
	α-T, γ-T, δ-T	Reduction of oxidative stress
Hepato-protective	α-T, γ-T	Inhibition of lipid peroxidation and oxidative damage
	γT, δT	Induction of the expression of CYP450, UGT1A1 and MDR-protein 1
	TRF from palm oil and rice bran oil, αT, γT, δT	Induction of hepatic antioxidant status
Nephroprotective	TRF from rice bran oil, α-T, γT	Inhibition of oxidative-nitrosative stress

Protective activity	Tocotrienol type	Proposed mechanism of action
	TRF from palm oil and rice bran oil, αT, γT	Downregulating the expression of NF-κB, TGF-β, TNF-α and caspase-3

Tocotrienols and cancer

In addition to above properties, tocotrienols display anti-cancer properties via anti-proliferative, anti-survival, pro-apoptotic, antiangiogenic, and anti-inflammatory activities *in in-vitro* and *in-vivo* studies. Several cell culture studies have demonstrated the ability of tocotrienols to inhibit cancerous cell growth/proliferation of breast, prostate, lung, bladder, liver, colorectal, and pancreas origin [70, 73]. In an animal study, mice treated with tocotrienols showed significant tumor suppression as monotherapy [73] or in combination with chemotherapeutic agents [74]. Tocotrienols inhibited metastatic B16 melanoma in female mice, and δT was more active than γT in this study [70]. In another study, γT and δT also delayed tumor growth in mice implanted with hepatoma [70]. Further cumulative evidence has reported that tocotrienol isomers individually or in combinations exhibit anticancer properties by inducing apoptosis [75-77]. For instance, apoptosis induced by tocotrienols involves mitochondrial depolarization [78] and is facilitated via the increased expression of Bax [79-81], cleavage of Bid [80], the release of cytochrome C [82], and activation of caspase-9, which in turn leads to activation of caspase-3 [82, 83].

Anti-invasive effects of δT in cancer cells have been reported in several studies. Yap et al. [17] used a Matrigel invasion assay to show that δT have the ability to halt the invasion of prostate cancer cells [84]. In another study, a 2-fold suppression on cell invasion using annatto extracted from δ-tocotrienols was observed at a concentration ranging from 20 to 30 μM in non-small cell lung carcinoma (NSCLC) [85]. Combinational treatment of cisplatin and δ-tocotrienol has also been found to effectively reduce the invasive capability of NSCLC cells in comparison to either

agent alone [85]. The inhibition of angiogenesis by tocotrienols is observed by inhibition of VEGF expression [86] and VEGF receptor signaling [76] in some studies. All these studies suggest that tocotrienols have the potential to both prevent and treat cancer.

CHAPTER 2 SPECIFIC OBJECTIVES

NSCLC is one of the leading causes of death among cancers in the USA. The current main therapies for lung cancer are surgery, chemotherapy, radiation and molecular-targeted or combined modalities. Despite these treatments, the five-year survival rate has not changed over the last four decades. This plateauing five-year survival could be attributed to late diagnosis, tumor metastasis, chemo/radioresistance, and tumor recurrence. Poor outcomes suggest that it is important to identify newer and novel therapeutic agents for improving treatments and survival rates for NSCLC patients.

All cancers, including NSCLC, show aberrant cellular signaling pathways that are required for the complex communication networks between the interior and exterior of cells. Cellular signaling pathways transmit basic information to regulate diverse processes, such as protein synthesis, cell growth, cell architecture, migration, differentiation, and programmed cell death. Understanding these cellular signaling pathways and how they differ in cancer will help to identify the novel therapeutic target. Notch signaling pathways have been designated as one of functional signaling pathways in the normal development of many tissues and cell types, through diverse effects on differentiation, proliferation, and survival. The up-regulated Notch expression has been observed in many types of cancers including NSCLC patients. Notch-1 has shown cross-talk with nuclear factor NF-κB in different studies and indicates that it plays a major role in many physiological processes, such as cell proliferation, cell death, inflammation, apoptosis and immune response in cancers. MMP-9 is a metalloprotein downstream to Notch-1 and NF-κB that regulates cell migration, angiogenesis, adhesion, aggregation and immune response in cancer. The urokinase-type plasminogen activator (uPA) pathway is one of the major pathways that is involved in modulating MMP-9 activities. Down-regulation of Notch-1 has shown anti-invasive effects on

some cancer cells by inhibiting NF-κb and matrix metalloproteinase-9. Therefore, modulating uPA and/or MMP-9, along with NF-κb, Notch -1 and miR451, from a single compound could be an excellent therapy for preventing cell invasion and metastasis in NSCLC. The first specific aim of this study is proposed to investigate the effect of δT on the NSCLC cell line with the working hypothesis that the MMP-9 dependent invasion and metastasis of non-small cell lung cancer (NSCLC) cell would be inhibited by suppressing Notch-1 mediated NF-κb and uPA pathways.

Studying the metabolome of NSCLC is a potentially informative mirror of the signaling and biochemical dynamics which is strongly geared toward the discovery of new biomarkers of disease onset, progression and effects of treatment regimes. Although anti-cancer effects of δT were investigated using molecular biology techniques, in our previous studies and in specific aim one, the change in the metabolomics patterns produced due to the introduction of δT in non-small cell lung carcinoma cell lines has not been investigated yet. Further, the primary energy source of NSCLC is glutamine, and this cancer exhibits a high rate of glutamine dependency during its growth and development. Glutamine and essential amino acids (EAA) in NSCLC are reported to upregulate mTOR, a bioenergetics sensor which regulates cell growth, cell survival, and protein synthesis. SLC1A5/SLC7A5 transporters that allow glutamine and EAA to enter the proliferating tumors and send a regulatory signal to mTOR are novel concepts in cancer cell growth and development. Therefore, inhibiting glutamine uptake via blocking or downregulating glutamine transporters would be an excellent therapeutic target for NSCLC treatment. Therefore, the second specific aim is proposed to study the effect of δT on the metabolite dysregulation of glutamine and its derivatives in NSCLS using 1H-NMR based metabolomics, with expression of NSCLC cell growth, development, glutamine transporters and mTOR pathway.

Our previous studies showed the potential of δT against NSCLC prevention via downregulating notch-1 and other genes in cell culture studies. As a next step, we want to start a clinical trial and investigate the effect of tocotrienol on human lung cancer patients. However, pure δT isomer to treat cancer is not plausible since it is difficult to isolate, is expensive, and pure δT isomers are presently not available in quantities required for animal or clinical studies. The specific aim three is performed to investigate the anti-cancer effects of commercially available tocotrienols isomer rich capsules on NSCLC cell lines and explore molecular mechanisms behind anti-cancer regulations by testing the expression of Notch-1 and its downstream stream genes.

Hypothesis

The overall goal of the study is to evaluate tocotrienols as an anticancer nutraceutical. To achieve this goal, we hypothesize that δT, acting either as a single agent or in combination with other tocotrienol isomers, will be an effective therapeutic strategy for treating NSCLC.

SPECIFIC AIM 01

To investigate the effect of on the NSCLC cell line with the working hypothesis that the MMP-9 dependent invasion and metastasis of non-small cell lung cancer (NSCLC) cell is inhibited by suppressing Notch-1 mediated NF-κb and uPA pathways.

The effect of δT on cell migration, invasion, and cell adhesion and aggregation capability was investigated using a suitable assay to investigate functional activities of MMP-9 upon the δT intervention. MMP-9 enzyme activity with δT was also determined using gel zymography. The various proteins, genes, and miRNA involved in the MMP-9 activation pathways namely Notch-1, NF-κb and uPA, was investigated by using RT-PCR and Western blot analysis.

SPECIFIC AIM 02:

To investigate the effect of δT on the metabolite dysregulation of glutamine and its derivatives in NSCLS using 1H-NMR based metabolomics, with an expression of NSCLC growth, development, glutamine transporters and mTOR pathway.

The endo-metabolome of the cells was determined using 1H-NMR Spectroscopy and Multivariate statistical analyses methods namely Principal component analysis (PCA), PLS-DA and OPLS-DA, was performed with SIMCA+ and MetaboAnalyst 3.0 to identify differences among treated and non-treated NSCLC cells. By using partial least squares (PLS) regression analysis, the correlation between the metabolomic profile and anticancer effects namely anti-proliferation, apoptosis, and invasion, was performed to identify how the metabolites mechanistically affect cell behavior. Changes in specific metabolite concentrations due to intervention with δT was quantified by Chenomx-NMR Suite and Chenomx data further analyzed with metboanyst 3.0 software by using different analysis methods such as pathway analysis, heat maps, etc. The altered metabolites in cells were used to gain insight into biochemical pathways and differences in response to tocotrienol interventions. In addition, altered metabolites identified from Chenomx-NMR suite were further analyzed using metaboanalyst software and fitted into appropriate pathways. Finally, impacted pathways were validated using western blot analysis.

SPECIFIC AIM 03

To investigate the effect of a commercially available tocotrienol-rich capsule directly extracted from palm oil on growth inhibition of NSCLC cells and on induction of apoptosis by inhibition of Notch-1 signaling via the NF-kB pathway.

MTS assay was performed to determine the δT IC50 (half inhibition concentration) on H520 and A549 and data from MTS assay was confirmed from clonogenic (or colony forming) assay. Induction of apoptosis in these cell lines by treating with δT was explored by using flow

cytometry and histone ELISA. The cell invasion and migration capabilities after induction of tocotrienol was also investigated by using wound healing and cell invasion assays. Molecular mechanisms behind these regulations were explored by testing the expression of Notch-1 and its downstream genes at the protein and mRNA levels from RT-PCR and western blot analysis. Finally, NF-κB activity was measured by ELISA assay.

CHAPTER 3 DELTA TOCOTRIENOL INHIBITS MMP-9 DEPENDENT INVASION AND METASTASIS OF NON-SMALL CELL LUNG CANCER (NSCLC) CELLS.

Introduction

With current advances in the understanding of mechanisms of cancer invasion and metastasis, it is very clear that matrix metalloproteinases (MMPs), an enzyme including 21 subtypes in humans [87], show a strong association with metastasis [87]. Several study designs ranging from cell culture investigation[88] to clinical investigations [89] have shown inhibition of MMPs involved in decreasing metastasis in NSCLC. Matrix metalloproteinase 9 (MMP9), a subtype of MMPs, regulates cell migration, angiogenesis, adhesion, aggregation and immune response in cancer [90, 91]. In this process, MMP9 is primarily responsible for degrading collagen type IV and elastin in basal membranes, facilitating the way for lung cancer metastasis. Elevated levels of MMP-9 also have been found in the serum of lung carcinomas patients [92].Therefore, modulating MMP 9 protein expressions and their activities would be excellent therapeutic targets for the inhibition of local invasion or distant metastases in NSCLC.

The urokinase-type plasminogen activator (uPA) pathway is one of the major pathways that can directly modulate MMP-9 activities in cancer [93]. The urokinase-type plasminogen activator (uPA) system includes serine protease, urokinase-type plasminogen activator receptor (uPAR), and the endogenous inhibitors, plasminogen activator inhibitors 1 and 2[94]. The uPA, a plasminogen activator, facilitates extracellular matrix degradation by converting zymogen plasminogen into plasmin[95]. Then, plasmin facilitates the conversation of inactive pro MMP-9 into active MMP-9. Higher expression of the uPA system was reported in NSCLC tissue as compared to normal lung tissue[96]. Rao, et al. [97] using antisense technology showed that the inhibition of uPA and MMP-9 might be a promising anti-invasion and anti-metastatic strategy for cancer gene therapy in lung cancer. Although inhibition of uPA and/or MMP-9 is a possible

therapeutic target for preventing local invasion or distant metastases in lung cancer, uPA, and MMP-9 pathways have shown cross talks with external factors, namely transcription factors, signaling pathways, and miRNA; these cross-talks have made it more complex them to modulate directly. Tong**,** et al. [98]showed that nuclear factor-κb (NF-κb), a well-known transcription factor for cancer initiation and progression, directly bonded to the uPA promoter *in vitro*, and that the inhibition of NF-κB activity impaired the cell invasion and reduced the uPA production in NSCLC cells. Recently, accumulating evidence has suggested that the NF-κb signaling pathway contributes to the progression of metastasis by regulating MMP9 in colorectal cancer[99], prostate cancer[100], renal cancer[101], ovarian cancer[102]**,** and head and neck cancer [103]. However, the role of NF-κb mediated uPA and/or MMP-9 function in cell invasion and metastasis in lung cancer remains generally unclear.

Elevated miR-451 expression levels, an abundant class of small non-coding RNAs that modulates gene expression in a posttranscriptional manner through sequence-specific binding to target mRNA, were also reported to decrease cell invasion and metastasis, accompanied with the decrease of matrix metalloproteinase-9 (MMP-9) expression levels in primary liver cancer[104]. Elevated miR-451 expressions were also found to suppress cell proliferation and metastasis in A549 lung cancer cells lines[105]. However, the impact of miR-451 on NSCLC invasion and metastasis has not been investigated via target MMP-9, uPA, NF-κb and Notch-1 signaling pathway. Therefore, modulating uPA and/or MMP-9 along with NF-κb, Notch -1 and miR451, from a single compound could be a possible therapy for preventing cell invasion and metastasis in NSCLC.

Previous studies from our laboratory showed that δT, an isomer of vitamin E, found naturally in palm oils, inhibit NF- κb signaling pathways via the down-regulation of Notch-1

thereby decreasing the proliferation and at the same time inducing the apoptosis of NSCLC cells in a dose and time-dependent manner [8, 24, 106, 107]. The present study aims to investigate the effect of δT on the NSCLC cell line with the working hypothesis that the MMP-9 dependent invasion and metastasis of non-small cell lung cancer (NSCLC) cell would be inhibited by suppressing Notch-1 mediated NF-κb and uPA pathways.

Materials and methods

Reagents and antibodies.

δT was a kind gift from American River Nutrition (DeltaGold® MA, USA). DMSO, TNF-α, bovine serum albumin (BSA), was purchased from Sigma-Aldrich (St. Louis, Mo., U.S.A.); the protein assay kit was obtained from Bio-Rad Labs. (Hercules, Calif., U.S.A.). NSCLC cell lines of human A549, H1299 were obtained from ATCC. RPMI medium (Mediatech, Manassas, VA) used as a growth medium for cells. Antibodies namely β-actin, MMP-9, HES-1, Notch-1 (Cell Signaling Technology, Danvers, MA) and uPA (Santa Cruz Biotechnology, USA) used for western blot analysis.

Cell Culture and treatments.

NSCLC cell lines, namely A549 and H1299, were cultured in RPMI medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in 5% CO_2 and 37 C^0. The culture medium was renewed every 2 to 3 days. Adherent cells were detached by incubation with trypsin-EDTA and centrifuged at 1500 RPM. The treatment media was prepared by mixing δT (<.01% DMSO as a vector) in the RPMI medium whereas the control was treated only with RPMI media containing <.01% DMSO. The δT, 10μM, 20μM and 30μM concentration containing <.01% DMSO, was chosen as the treatment concentration.

Cell viability assay.

MTS assay was conducted to analyze the anti-proliferative effects of δT on the above NSCLC cell lines. $5x10^5$ of A549 and H1299 cells were seeded in a 96-well plate and incubated overnight. After incubation, the medium was replaced, and cells were treated with fresh medium containing < 0.10% DMSO (control) and treatment medium containing 10μM, 20μM, 30μM and 40μM of δT. After 72 hours of treatment, 20μl of CellTiter 96 Aqueous One Solution Reagent from Promega, (Madison, WI) was added to each well and incubated for 2 hours at 37°C in a humidified 5% CO_2 atmosphere. Then the absorbance at 490nm was measured using the Bio-Tek ELx800 plate reader (Winooski, VT). Each variant of the experiment was performed with six replicates.

Cell invasion assay.

The ability of A549 and H1299 lung cancer cells to pass through filters coated with Matrigel was assessed by BD Biocoat invasion kit (BD, San Jose, CA). 2.5×10^5 both A549 and H1299 cells were extracted in an aqueous environment with PBS and resuspended into each upper chamber of six well plates with a serum-free medium in the presence or absence of treatment. 3ml of culture medium with 15 % FBS was added to each lower chamber of the six-well plate and incubated for 20 h. After the incubation, the cells in the upper chamber were removed using a cotton swab, and the cells invading across the Matrigel to the lower surface of the membrane were fixed with 4% Paraformaldehyde and stained with 2% crystal violet. The invading cells on the lower surface of the membrane filter were counted and pictured with the camera attached to the light microscope. The data were presented as cells attached to the bottom surface from randomly chosen fields. Each experiment was carried out in triplicate. Again, the same procedure was continued up to 20 h incubation in a BD Biocoat invasion kit; then the cells on the upper chamber were removed using a cotton swab, and 50uL of MTS reagent was added into the lower chamber.

The absorbance of the media contained in the lower chamber was measured at 450nm using the Bio-Tek ELx800 plate reader (Winooski, VT). Each experimental condition was performed in triplicates.

Cell migration assay.

To detect the migration speed of a cells using wound healing assay, A549, and H1299 cells were seeded in a six-well plate at the concentration of 1 X 10^6 cells per well. After the cells, had been incubated for 36 hrs., the culture media was removed and treated with or without treatment media. Scratches were made using a pipette tip (200µl) and photographed immediately (time 0). After 20 h incubation, all the wound areas were washed with PBS three times to make sure that no loosely held cells were attached. The plate was imaged, and the width of the scratch was measured by a Nikon H 600L microscope connected to the microscope.

Cell adhesion assay.

The cell lines A549, and H1299 (one million) were pre-treated with or without different concentrations of δT (control, 10µM, 20µM, and 30µM) for 72 hrs. The cell lines were suspended in an RPMI medium to form a single-cell suspension and 2 x 10^6 cells/ml (2 x 10^5 cells/well) were seeded into a 96-well microtiter cell culture plate pre-coated with Matrigel®. After 1h of incubation at 37^0C, the wells were washed three times with PBS to remove non-adherent cells and 100mL of RPMI medium with 20µM MTS reagent was added into each well for an additional 2h. As a measure of cell viability, the absorbance at 570 nm was taken on anELx800 plate reader (Bio-Tek, Winooski, VT). Significant differences between control and treatments were evaluated using ezANOVA.

Cell aggregation assay.

To investigate the anti-aggregation capabilities of δT, a single cell suspension was obtained using standard trypsinization procedure. A total of 2 x 10^5 cells in 1 ml of RPMI medium with or without different concentrations of δT (10μM, 20μM, and 30μM) were placed in polystyrene micro tubes and were gently shaken every 5 min for 1 hr. at 37^0C. At the end glutaraldehyde (at a final concentration 2% (v/v)) was added to stop the aggregation process. Homotypic aggregation was evaluated and photographed by a Luna automated cell counter (Logos Biosystems, USA). Aggregates were counted per square cm, and significant differences between control and treatments were evaluated using ezANOVA

Zymogram gel assay.

To investigate the activity of MMP-9, A549, and H1299 cells were seeded in a six-well plate with or without treatment by δT in FBS-free RPMI media. The media of all groups was removed and concentrated into 10X media by using 3KDa amicon centrifugal filters (EMD Millipore, USA). Then the total protein concentrations of the concentrated media were estimated by Pierce BSA Protein Assay kit and normalized into 2000mg into the sample. Zymogram precast gel (Bio-Rad, Hercules, CA) with gelatin was used as a separation gel. One-part sample was mixed with two parts of zymogram sample buffer. Samples were loaded in the gel, and the gel was run with 1x Tris-Glycine-SDS running buffer according to the standard running conditions (100V, constant voltage). Run time was for 90 min or when the bromophenol blue tracking dye reached the bottom of the gel. After running, the Zymogram renaturing buffer (10x) was diluted (1:9) with double distilled water and the gel was incubated in the buffer (100 ml for one or two mini-gels) with gentle agitation for 30 minutes at room temperature. Later zymogram renaturing buffer was decanted and replaced with 1x zymogram developing buffer (100 ml for one or two mini-gels). The gel was equilibrated for 30 minutes at room temperature with gentle agitation, and then the

buffer was replaced with fresh 1x zymogram developing buffer and again incubated at 37ºC overnight for maximum sensitivity. The gel was stained with Coomassie blue R-250 for 30 minutes to 1 hour the next day. For maximum contrast, stain concentration of 0.5% (w/v in 40% methanol and 10 % acetic acid) was used instead of the usual concentration of 0.1%. Gels were destained with an appropriate coomassie R-250 destaining solution (161-0438) (Methanol: Acetic acid: Water (40: 10: 50). Areas of protease activity appeared as clear bands against a dark blue background where the protease had digested the substrate.

Real-time quantitative PCR

One million A549 and H520 cells were seeded in 100mm dish per plate and incubated for 24 hours. Subsequently, culturing media was replaced with treatment or control medium, incubated for another 72 h. Then, total RNA was isolated using RNeasy Mini Kit from QIAGEN (Valencia, CA) according to the manufacturer's protocols. 1000ng of total RNA from each sample was subjected to first strand cDNA synthesis using high capacity RNA to cDNA master mix (Applied Biosystems, Foster City, CA) in a total volume of 50 ml. qRT-PCR was performed as part of gene expression analysis. The appropriate primers sense of MMP-9, 5′-CAACATCACCTATTGGATCC-3′, antisense of MMP-9, 5′- CGGGTGTAGAGTCTCTCGCT-3′ (antisense). sense of Bactin, 5′-ACCAACTGGGACGACATGGAGAAG-3′; antisense of 5′-TACGACCAGAGGCATACAGGGACT-3′ere used for PCR amplifications. Diluted cDNA (2 uL) and 2 uL of each reverse and forward primer and 12.5 uL master mix (SYBR GREEN PCR Master Mix; Applied Biosystems, Warrington, UK) were used in each 25 uL PCR reaction performed in Eppendorf master cycler realplex 4 (Eppendorf, Hauppauge, NY) at 25C for 10 min, followed by 48 C^0 for 30 min and 95 C^0 for 5 min. Expression values were normalized with B-actin). Each gene expression was tested in triplicate.

Western blot analysis.

To investigate the protein expression of A549 and H1299 with δT treatment, the one million cells were seeded in 100mm dish per plate and incubated for 24hours; then the cells incubated for 72 hours with treatment and control medium. Subsequently, the cells were lysed in the cold 1X cell lysis buffer (Cell Signaling Technology Danvers, MA) for 30 min on ice with 1X protease inhibitor (Cell Signaling Technology Danvers, MA). Then protein concentrations were calculated by using Pierce BSA Protein Assay kit (Bio-Rad Laboratories, CA). Subsequently, 50 mg of total cell lysates was mixed with equal amounts of 4X lemma buffer (Bio-Rad Laboratories, CA) and samples were loaded on 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel electrophoretically was transferred to a nitrocellulose membrane (Whatman, Clifton, NJ) using a transfer buffer (25mM Tris, 190mM glycine, 20% methanol) in a Hoefer TE70XP transfer apparatus (Holliston, MA). The membranes were incubated for 2 h at room temperature with 5% BSA in 1x TBS buffer containing 0.1% Tween. After that, the membranes were incubated overnight at 4C with primary antibodies (1: 1,000). The membranes were washed three times with TBS-T and subsequently incubated with the secondary antibodies (1:5,000) containing 2% BSA for 2 h at room temperature. The signal intensity was then measured by chemiluminescent image with chemiDoc XRS (Bio-Rad Laboratories, CA).

NF-κB binding activity.

To determine the NF-κB transcription factor binding activity, A549 and H1299 were seeded in Petri dishes and incubated for 24 hours. Cells were then treated with or without δT. After 72 hours of treatment, cells were collected and washed with the PBS. The nuclear protein extraction was performed with NE- PER ® Nuclear and cytoplasmic extraction reagent kit (Thermo Scientific, USA) based on the manufacturer's protocols. Nuclear protein concentrations were

determined using the Pierce BCA protein assay kit (Rockford, IL). Then NF-κB Filter Plate Assay Kit obtained from Signosis (Sunnyvale, CA) and used to determine the NF-κB DNA binding ability. The assay was conducted according to the manufacturer's protocol. Briefly, the biotin-labeled DNA sequence of NF-κB was mixed with 3µg of nuclear extract to make an NF-κB-DNA binding complex. Then, 10µl TF binding buffer mix, 2µl NF-κB probe, 3µg and distilled water was added to each sample and brought the total volume up to 20µl. The filter plate was used to retain the bound NF-κB probe while the unbound NF-κB probe was filtered out. The bound, pre-labeled NF-κB probe was then eluted from the filter, collected and transferred to a hybridization plate for quantitative analysis. NF-κB probe was further detected using streptavidin-HRP, and the luminescence of the probe was measured using an Ultra Multifunctional Microplate Reader from Tecan (Vienna, Virginia).

miRNA 451 expression analysis.

To investigate the miRNA 451 expression of A549 and H1299, the total RNA was extracted from the cultured cells using RNeasy Mini Kit from QIAGEN according to the manufacturer's protocols. The concentration and purity of the RNA were determined using ultraviolet spectrophotometry using the Nanodrop 2000 system (Thermo, Japan). The quantitative real-time PCR was performed using SYBR GREEN PCR Master Mix (Applied Biosystems, Warrington, UK) according to the manufacturer's instructions. U-6 primers from miScript miRNA PCR (Valencia, CA) was used as an endogenous reference, and its levels were used for normalization.

Results

Anti-proliferative effect of δT.

The MTS assay showed a dose-dependent decrease in cell growth and proliferation in both A549 and H1299 cells with addition of δT. After 72 hours of incubations with 10, 20, 30 and 40 μM of δT doses, ≃ 10, 40, 80 and 85% of cell growth inhibition was observed, respectively, compared to control in A549 cells. Similarly, the H1299 cell line showed ≃ 25, 20, 55 and 87% cell growth inhibition, respectively, with the same doses and conditions. (Figure 3.1.)

Inhibition of cell invasion and migration by δT.

We observed dose-dependent anti-invasive effects of δT in A549 and H1299 cells at the concentration levels ranging from 0 – 30μM in Matri gel invasion assay (Figure 3.2AB). At 10, 20 and 30 μM concentrations, invasion was reduced by ~ 10%, 35% and 65% in A549 cell lines, respectively, for 72 h incubation; similarly, H1299 cells also showed ~ 35% 45% and 70 % inhibition of cell invasion under the same conditions. For further confirmation, microscopic images of the migrated cell through the membrane were taken, stained and analyzed. In A549, we observed that a low number of cells migrated through the membrane as compared with untreated cells. Also, we hardly observed any invaded cells at 30 μM treatments in H1299 ($P < 0.05$ at 30 μM treatment) (Figure 3.2CD). The wound healing assay was performed to investigate the migration of A549 and H1299 after δT treatment. As showed in (Figure 3.3AB), δT significantly inhibited the migration of cells after 20 h treatment with 30 μM δT.

Inhibition of cell aggregation and adhesion capabilities by δT.

As shown in Figure 3.4, δT inhibits cell aggregation at 30μM of δT in both A549 and H1299 cells, respectively. To test the effects of δT on cell adhesion to the matrigel surface, A549 and H1299 cells were treated with δT for 72 h and adhesion capacities to Matrigel surface were measured. We observed a dose-dependent inhibition of cell adhesion in both A549 and H1299 cells. (Figure 3.5.)

δT suppressed secretion and expression of MMP-9.

Since metalloproteinase plays a crucial role in cell invasion, we then carried out the zymography assay to compare the activity of MMPs in treated and control cells. A dose-dependent reduction in the enzyme activity of MMP-9 was observed in zymography assay with δT (Figure 3.6) at concentrations of 0μM, 10μM, 20μM, and 30μM. TNF- α was used as a positive control. The results of the Western blot analysis indicates that δT inhibits MMP-9 protein expressions in a dose and time-dependent manner compared to the control group in both A549 and H1299 cells (Figure 3.7) In order to verify whether the inhibition of MMP-9 protein expression in Western blot analysis was the result of decreasing the m-RNA expression, real time-PCR analysis was performed.We observed that the mRNA level of MMp-9 decreased significantly in a dose-dependent manner, compared to the control group (Figure 3.8AB), in both A549 and H1299 cells, after the treatment with δT for 72 h. The δT mediated change in the mRNA levels of MMP-9 correlated with the protein levels expression of MMP-9, as indicated by the results from the Western blot analysis, suggesting that δT may regulate MMP-9 expression at transcription levels, up steam gene or other factors such as miRNA.

δT inhibits expression uPA/Notch-1 pathway proteins.

To investigate the possible metastatic link between MMP-9 and uPA/Notch pathways, the protein expression of uPA/Notch-1 pathways protein in A549 and H1299 cells treated with various concentrations of δT were examined from western blot analysis. Based on our western blot data, we observed the dose-dependent inhibition of uPA protein expression, in both A549 and H1299 lines tested, with δT (Figure 3.7). In addition, δT significantly inhibited the Notch-1 and its downstream signaling molecule Hes-1 in A549 and H1299 cells, compared to Control (Figure

3.7). The simultaneous inhibition of uPA/Notch-1 and MMp-9 with δT in protein levels suggests a possible metastatic link between MMP-9 and uPA/Notch-1.

δT increase expression of miR-451.

To investigate the differences in miR-451 expression in A549 and H1299 cells upon treatment by δT, we conducted a miRNA array analysis using the RT^2 miRNA PCR array system (SABiosciences, MD). We found that miR-451 expression was 2-fold and 3.5-fold higher in the in A549 and H1299 cells, respectively, with δT treated cells compared to control (Figure 3.8CD). These results suggest that δT could inhibit the progression of NSCLC through induction of miR-451 A549 and H1299 cells.

Inhibition of NF-κB DNA-binding activity with δT.

we investigated whether δT modulates the nuclear translocation of NF-κB and influences the transcription of uPA/MMp9. Based on NF-kB Filter Plate Assay results, δT significantly inhibited the DNA-binding activity of NF-κB in a dose-dependent manner for both A549 and H1299 lines tested (Figure 3.9).

Discussion

In previous studies, we have reported that δT inhibits uncontrolled cell proliferation and invasion and induces cell apoptosis [24, 106, 108]. Lim *et al.* [109] suggested that δT is the most effective and efficient apoptotic executor compare to other tocotrienols isomers. Liu *et al.* [110] showed that γ-tocotrienol inhibits cell invasion and metastasis of the human gastric system by down-regulating MMP-9 and MMP-2. However, anti-metastasis functions of δT on lung cancer, such as invasion, migration, adhesion, aggregation and enzyme functions, have not been investigated to date. In this study. We demonstrated that δT decreased migration and invasion in lung cancer cells by suppressing the Notch 1 signaling pathway, inhibiting the nuclear

translocation and DNA binding activity of NF-κB, and reducing the level of uPA expression and activities of MMP-9.

Based on the data, 0-30 uM of δT suppresses the cell migratory and invasive abilities of A549 and H1299 cells at non-necrotic concentrations in this study. In our previous study, by performing histone ELISA and ANNEXIN V stain based flow cytometry analysis, we clearly demonstrated that the 0-30 uM range of δT was not necrotic to A549 and H1299 cells, and it induced apoptosis in A549 and H1299 cell lines dose-dependently [24]. δT was also reported to inhibit the proliferation of human liver cancer cell line (HepG2)[111], breast cancer[112], prostate cancer[112, 113], human leukemia cells, murine melanoma cells and malignant epithelial cells[112], in a dose- and time-dependent manner. We also observed a dose-dependent decrease in cell proliferation within 0-30 uM of δT for 72 h in MTS assay in this study. Therefore, 0-30 uM of δT for 72hrs appears to be sufficiently efficacious enough to exert apoptotic effects in lung cancer cell lines and decrease proliferation, which could lead to a decrease in invasion and migration of lung cancer cells.

The metastasis process starts from local invasion to distant metastasis and involves multiple distinct steps: migration/invasion through the ECM/basement membrane and entry into the circulatory system; survival without contact with the ECM; adhesion to the wall of lymphatic or blood vessels, and exit from the vessels (extravasation); and survival and proliferation in target tissues. [114] We tested all these steps from *in vitro* assays, namely wound healing assay, cell invasion assay, cell adhesion assay, aggregation assay and MTS assay. Based on our data, δT inhibited the aforementioned metastasis steps *in vitro* in a dose- and time-dependent manner suggesting δT as a potential anti-metastasis facilitator in A549 and H1299 cells.

The extracellular matrix is proteolytically degraded to allow the migration and invasion of cancer cells. Single cells, detached from a tumor and released to their intercellular junctions during metastasis [115], are capable of making cell aggregates. Naturally, non-tumor single cells are prone to anoikis, a form of apoptosis which is induced by inadequate or inappropriate cell–ECM interactions or attachments[116]. However, cancer cells show resistance to anoikis; as a result, they can survive after detachment from their primary site [114]. Cancer cell aggregates exhibit lower levels of anoikis compare to regular apoptosis. Therefore, the formation of cell aggregates may induce regular cell apoptosis in cancer cells. However, we observed a dose-dependent decrease in cell aggregate formation as well as a decrease in adhesion cell adhesion capabilities with δT treatment. This may have occurred due to a stimulation of anoikis in non-adhesive cancer cells/ single migrating cells and an induction of regular apoptosis in cell aggregates. Therefore, future studies are needed to explain δT based apoptosis approaches in A549 and H1299 cells.

Effective cell migration process requires disturbing the interaction between cells and the ECM. In this process, several proteolytic enzymes (MMP-9) or their activators (uPA) play a key role in degrading the interaction between cells and the ECM[117]. Thus, identifying new agents with the capability of inhibiting proteolytic enzymes (MMP9) and/or their activators (uPA) is a key factor for preventing tumor cell metastasis. Infection with Ad-uPAR-MMP-9 showed a decrease in the expression of uPa and MMP9 by >90% and also observed reduced levels of cell invasion in A549 and H1299 cells[97]. In the same study, they showed that inhibition of uPAR and MMP-9 expression had a more pronounced effect in inhibiting capillary formation in endothelial cells compared with inhibiting uPAR alone[97]. Also, high expression levels of the uPA-system and MMP-9 were reported in NSCLC tissue as compared to normal lung tissue[118]. Elevated uPA and MMP-9 expression are also considered as a prognostic biomarker in lung cancer

[119, 120].This implies the cooperation between uPA/uPAR and MMP-9 is required for lung cancer metastasis. In our study, we discovered δT inhibits the expression of MMP-9 and uPA does dependently in A549 and H1299 cells. Some studies have also reported that the anti-metastatic effect of other nutraceuticals is linked to uPA and MMP9 activity. For example, carnosic acid, silibinin, curcumin, EGCG, quercetin and baicalein, are some nutraceuticals, which inhibited cell migration and invasion by downregulating uPA and/or MMP-9 expression in cervical cancer cells, melanoma cells, human breast epithelial cells, oral cancer cells, and prostate and liver cancer cells, respectively [121-125]. Consistent with these findings, our results also demonstrated that anti-metastastic properties of δT on the inhibition of invasion and migration in lung cancer cells are due to suppression of uPA /MMp9 expression.

The regulation of uPA/MMP-9 expression involves multiple signaling cascades and miRNAs. Notch signaling is a signaling system that plays important roles in cell fate decision and stem cell maintenance in embryonic and postnatal tissues[126, 127]. Lung cancer has shown an aberrant expression of Notch -1 signaling cascades**.** Notch-1 inhibition reduces the formation of brain metastases from breast cancer [128]. Abnormal Notch-1 expression also strongly correlated with metastatic tumor disease in HCC patients.[129] However, some studies showed Notch-1 signaling acts as a tumor suppressor role in B-cell malignancies [127], neural crest tumors[130], and skin cancer. Therefore, we can consider that the Notch-1 signaling seems to function as an oncogene or a tumor suppressor in different types of cancer. In our study, we found that δT dose-dependently inhibited the Notch -1 expressions compared to the control. It is well documented that the activated Notch-1 pathway can increase the expression of its downstream Hes-1 activities. In this study, δT down-regulated HES-1 protein expression dose dependently alone with Notch -1

expression in A549 and H1299 cell, which suggests δT as a potential inhibitor for the Notch -1 signaling pathway downstreamly.

Many studies have established that miR-451 is widely dysregulated in human malignancies, including in lung cancer [131, 132], gastric cancer [133], breast cancer[134], glioma [135], and leukemia[136], indicating that miR-451 might play a critical role in oncogenesis. The downregulation of miR-451 was reported in gastric and colon cancers and childhood B-cell precursor acute lymphoblastic leukemia[137]. miR-451 functions as a tumor suppressor and regulates survival in NSCLC cells [56]. It is also one of the most down-regulated miR in lung cancer patients [56]. Our results showed that δT treatment is induced the expression of miR-451 in a concentration-dependent manner in A549 and H1299 cell lines. Moreover, repression of tumor suppressor miR-451 showed Notch1-induced oncogenesis in T-ALL[136]. Similarly, we observed that δT inhibited the Notch-1 signaling pathway at the same time with inducing miR 451 expression in A549 and H1299 cells. These results suggest possible cross talk between Notch -1 signaling and miR-451 for regulation of cell migration and invasion in A549 and H1299 cell lines.

In considering the activation of NF-κb, a variety of stimuli from different signaling pathways are needed. Notch -1, one type of cellular signaling pathway**,** activates the NF-κb pathway via its ligands [138]. The down-regulation of Notch-1 has inhibited invasion by inactivation of NF-κb and MMP-9 in pancreatic cancer cells[139], lung cancer [106] and prostate cancer[140]. Similarly, we observed that δT inhibited the Notch-1 signaling pathway at the same time with inhibiting the expression of MMP9, NF-κb, and uPA in A549 and H1299 cells. Therefore, the results suggest possible cross-talks among Notch -1, NF-κb, MMP9 and uPA for regulation of cell migration and invasion in A549 and H1299 cell lines.

These results provide insight into the approach in which δT modulates *in vitro* invasion and metastasis in lung cancer. Figure 3.10 shows the proposed mechanism that δT suppresses as the cell migration and invasion in A549 and H1299 cells. This study suggests that δT as a potential natural therapeutic approach to prevent lung cancer invasion and metastasis at *in vitro* level. Therefore, further studies to establish efficacy of δT in preventing lung cancer migration and invasion at the animal and clinical level are highly recommended.

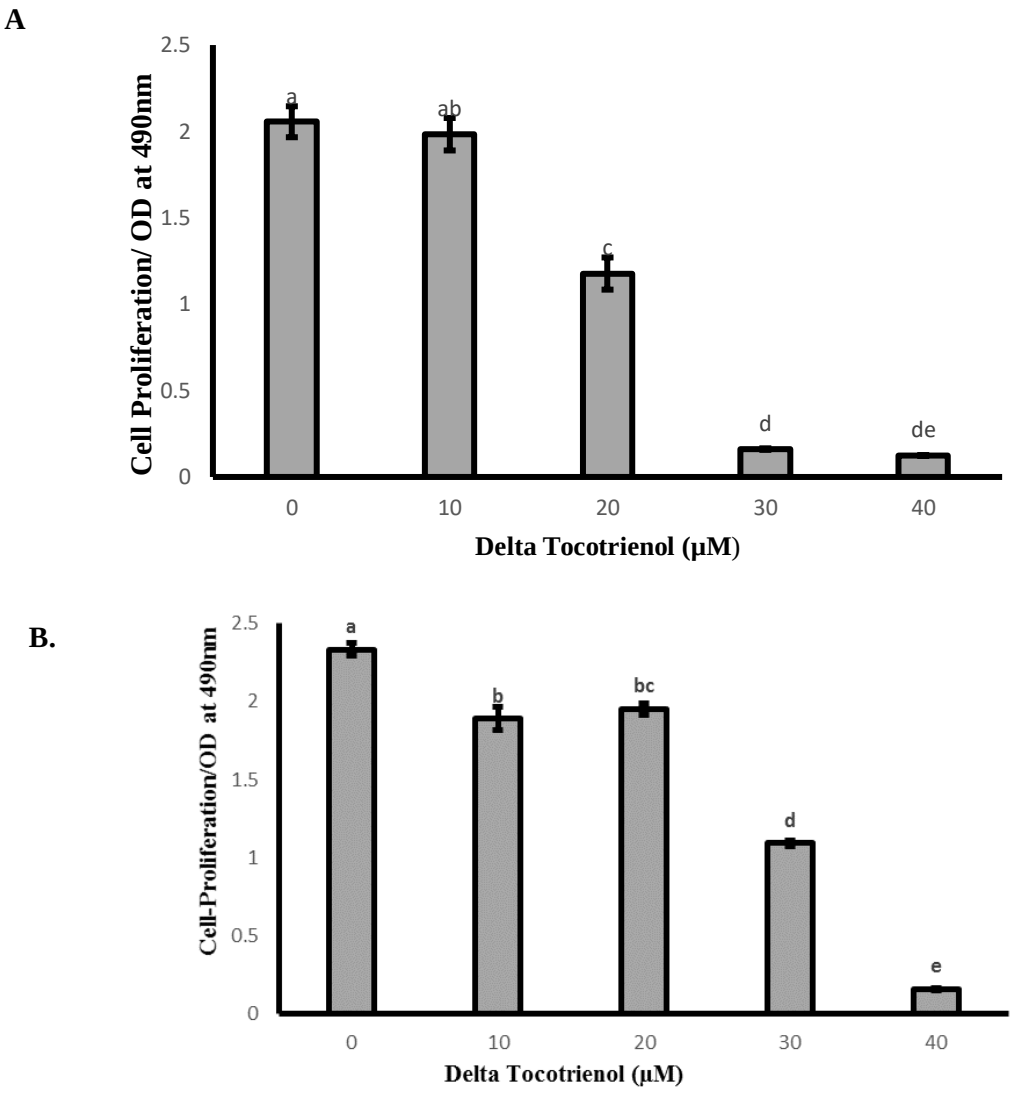


Figure 3.1. Antiproliferative effects of delta-tocotrienol on A549 (A) and H1299 (B) Cells.

Viable A549 (A) and H1299 (B) cell number was determined using the MTS colorimetric assay after delta-tocotrienol treatment. Both A549 (A) and H1299 (B) were initially plated at a density of 5×10^3 cells/well (3 wells/group) in 96-well plates and grown in experimental medium containing 0, 10, 20, 30 µM of delta-tocotrienol for 72 hrs. The vertical bars indicate the mean absorbance ± SEM (n = 6) where the mean absorbance, represented by different letters, is significantly different (one-way ANOVA followed by TukeyHSD multiple comparison test, $p < 0.05$).

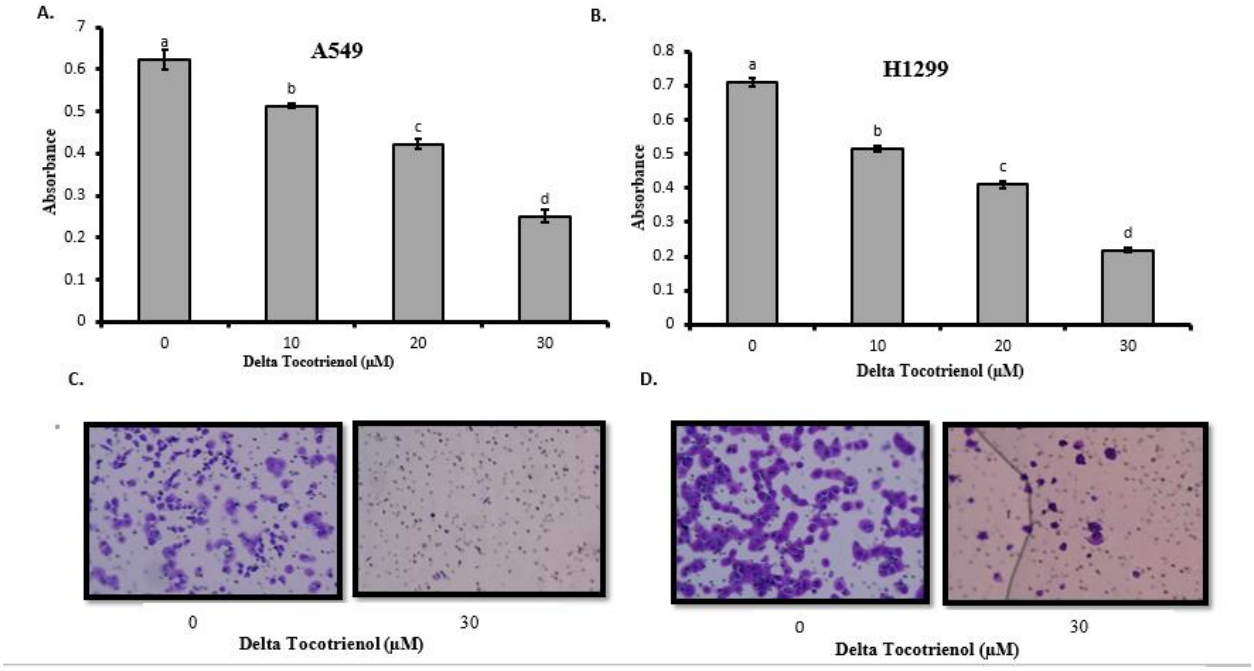
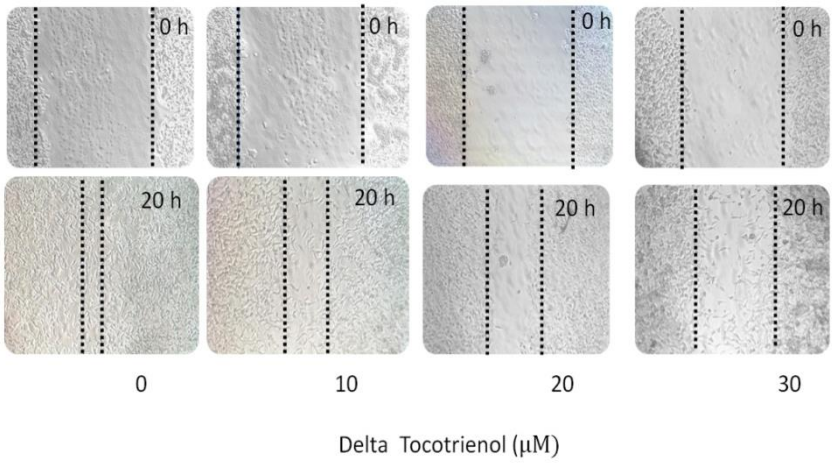


Figure 3.2. **Delta Tocotrienol inhibits cell migration and invasion in NSCLC cells.**

A549 and H1299 cells were seeded and treated in Matrigel-coated inserts with or without delta-tocotrienol. A549 (A) and H1299 (B) cells that invaded the lower surface of the insert over a period of 20 h were measured from color development after adding MTS . Vertical bars indicate the mean absorbance ± SEM (n = 3) where mean absorbance, represented by different letters, is significantly different (one-way ANOVA followed by TukeyHSD multiple comparison test, $p < 0.05$). A549 (C) and H1299 (D) cells (purple color patches) that invaded to the lower surface of the insert over a period of 20 h were stained with crystal violet dye and captured from microscope. Purple color patches indicate invaded cells whereas small dots indicate the pores in Matrigel surface.

A



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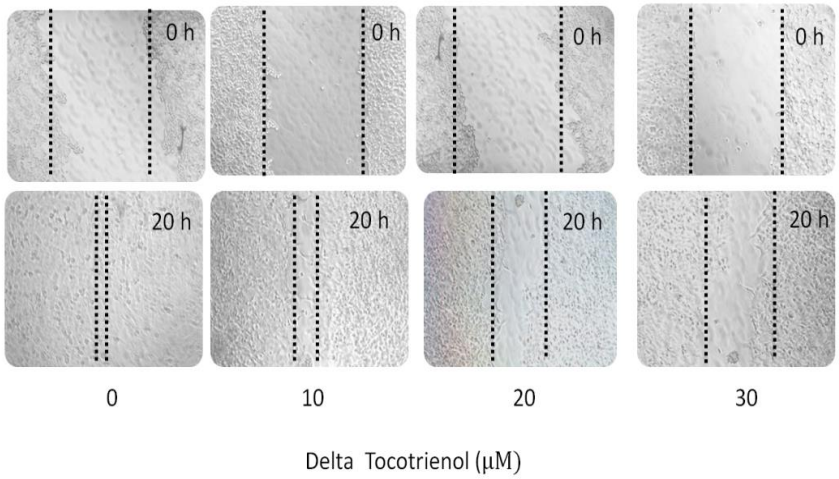


Figure 3.3. Delta Tocotrienol inhibits A549 (A) and H1299 (B) cell migration dose-dependently in wound healing assay.

Dose-dependent inhibition of (A) A549 and H1299 (B), migration by delta-tocotrienol at 0, 10, 20, 30 μM using the wound healing assay. Uniform wounds were created by scratching in confluent cultures which were treated with delta-tocotrienol over 20 h. Wound healing images were captured using a microscope at 10× objective.

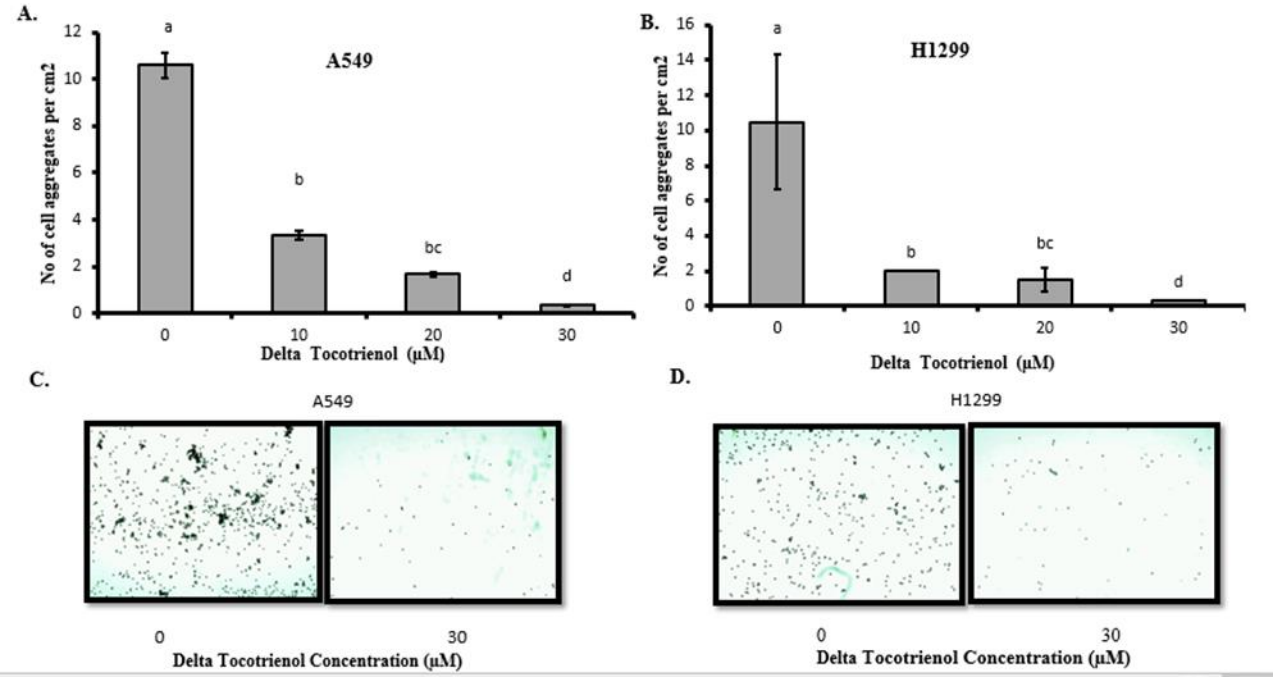


Figure 3.4. Delta tocotrienol inhibits cell aggregation in A549 and H1299 cells.

A dose-dependent reduction in no of cell aggregates per cm ^{2} in A549 (A) and H1299 (B) cells in cell aggregation assay. Vertical bars indicate the cell aggregates per cm ^{2} ± SEM (n = 2) where mean absorbance, represented by different letters, is significantly different (one-way ANOVA followed by TukeyHSD multiple comparison test, $p < 0.05$). All values are significantly different with each other ($p < 0.05$). Photographic view of A549 (C) and H1299 (D) cell aggregates after treating with/without delta tocotrienol were shown in lower panel.

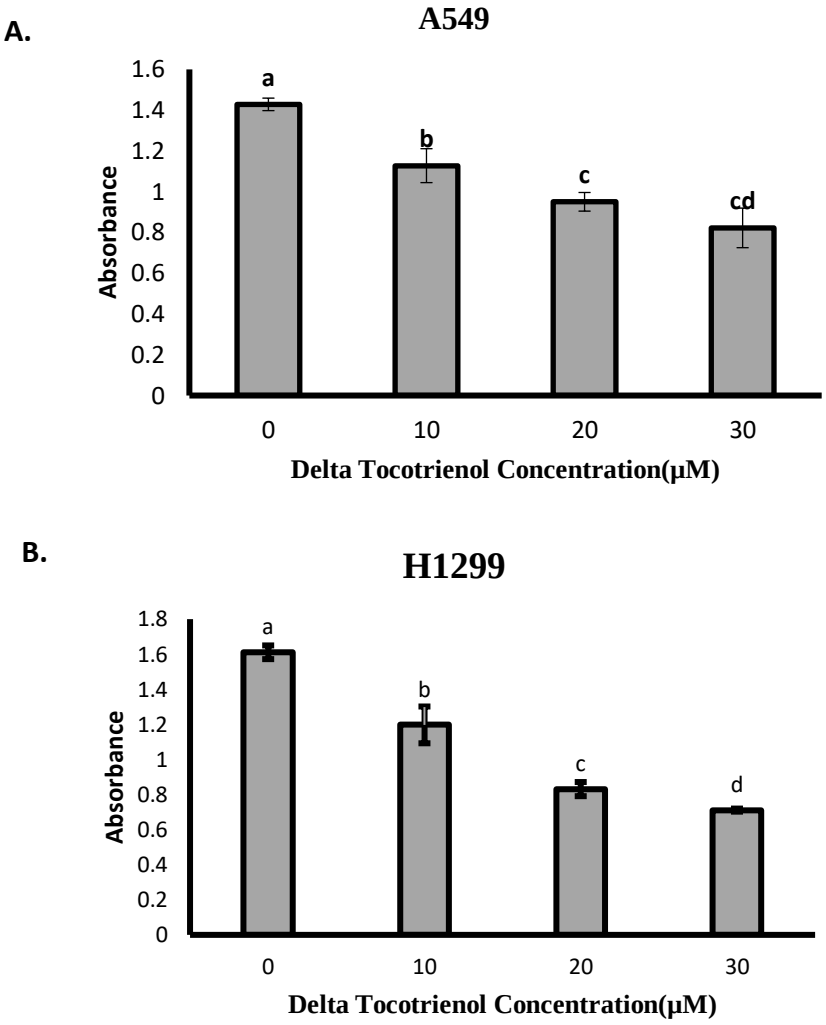


Figure 3.5. Delta-tocotrienol inhibits cancer cell adhesion in A549(A) and H1299 cells

A549 (A) and H1299(B) cells were treated with 0, 10, 20, 30 µM concentrations of delta-tocotrienol for 18 h and placed into wells precoated with Matrigel®. After 45 min incubation at $37C^0$, the percentage of adhering cells was determined by the MTS method. The experiments were repeated three times, each with similar results. Vertical bars indicate the mean absorbance (number of cells) ± SEM (n = 3) where mean absorbance, represented by different letters, is significantly different (one-way ANOVA followed by TukeyHSD multiple comparison test, $p < 0.05$).

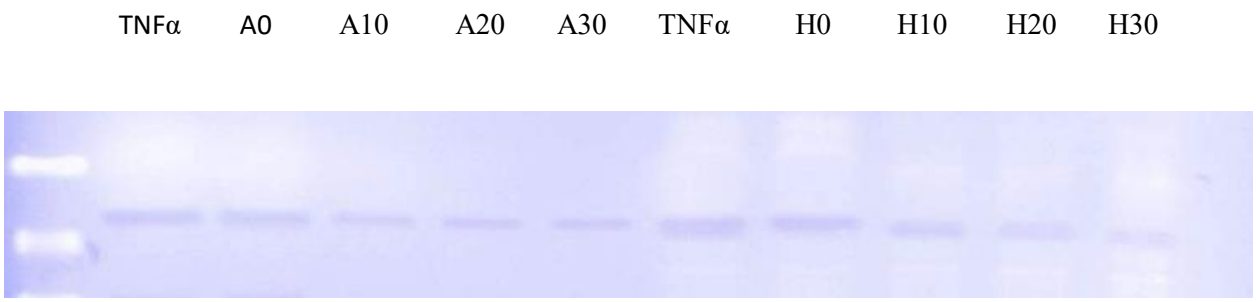


Figure 3.6. Effect of Delta -tocotrienols on the expression of MMP-9 enzyme activity in A549 (A) and H1299(B).

MMP-9 activities of A549 (A) and H1299(B) cells were determined by gelatin zymography method after treating with 0, 10, 20, 30 μM concentrations of delta-tocotrienol for 72 h. TNF-treated cell medium used as a positive control for this study. A0, A10, A20 and A30 indicates different treatment concentrations namely 0, 10, 20, 30 μM in A549 cells similarly H0, H10, H20 and H30 indicates same treatment concentrations in H1299 cells.

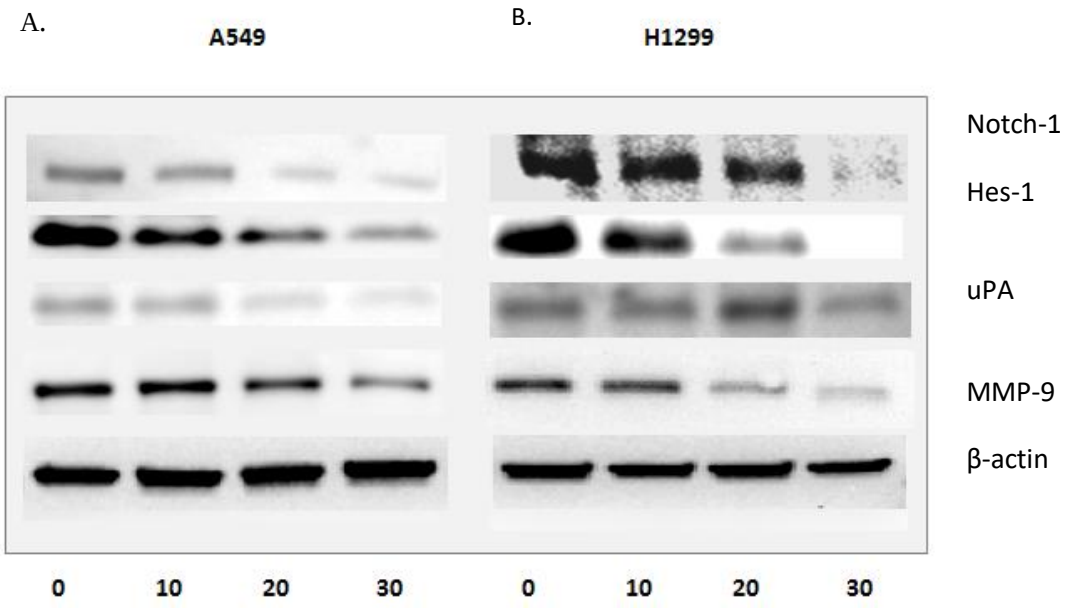


Figure 3.7: Down-regulation of Notch-1/uPA pathway proteins by delta-tocotrienol in A549 (A) and H1299 (B) cells.

A549 (A) and H1299 (B) cells were treated with 0, 10, 20, 30 µM concentrations of delta-tocotrienol for 72 h. The expressions of Notch-1, Hes-1, uPA, MMp-9 and Beta actins protein were detected by western blot analysis.

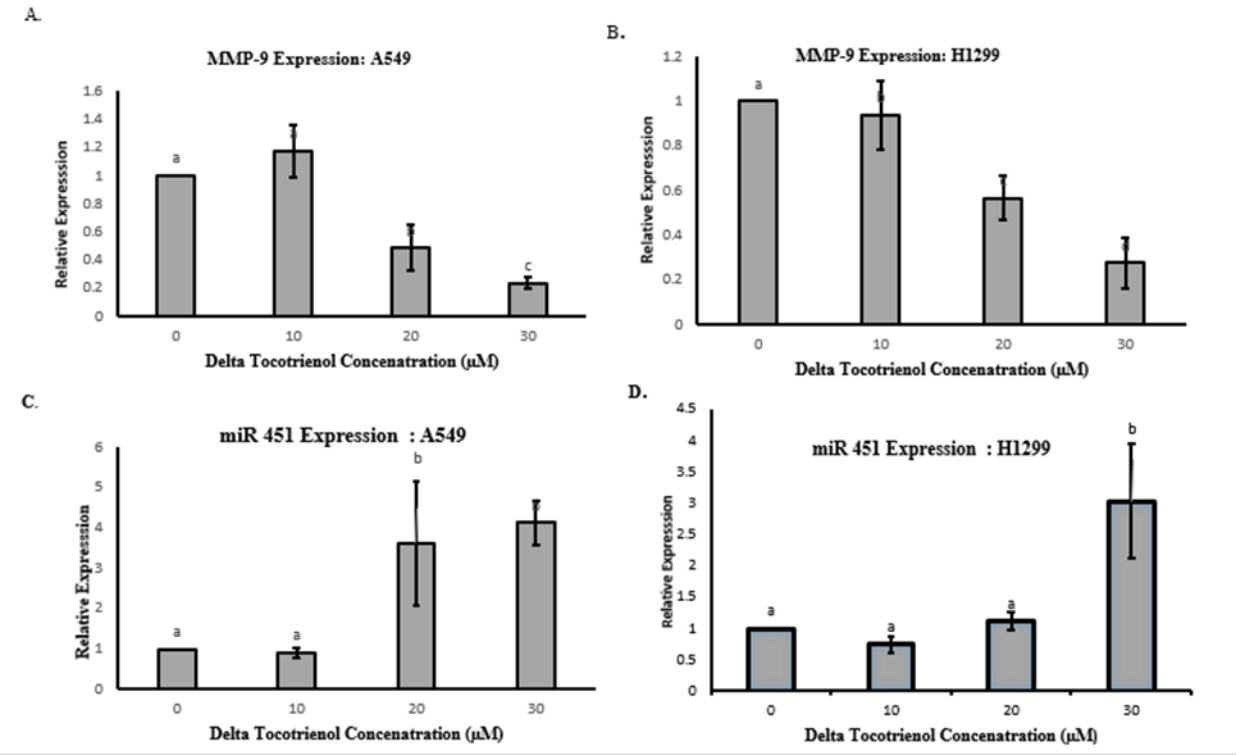


Figure 3.8. Delta Tocotrienol inhibits MMP-9 gene expression in A549 (A) and H1299(B) and increases the miR451 in A549 (C) and H1299 (D).

qRT-PCR analysis was carried out after the cell were treated with or without different doses of delta tocotrienol. The procedure is described in the "Materials and Methods". These experiments were repeated independently three times (for each group, n = 3). Both control and treatment expressions were relative to those of the internal control gene beta-actin or U-6. The vertical bars indicate the mean relative expression ± SEM (n = 3) of MMP-9 (relative to B actin) in A549 (A) and H1299(B) and miR 451 (relative to U-6) in A549 (C) and H1299(D). The mean relative expression, represented by different letters, is significantly different (one-way ANOVA followed by TukeyHSD multiple comparison test, $p < 0.05$).

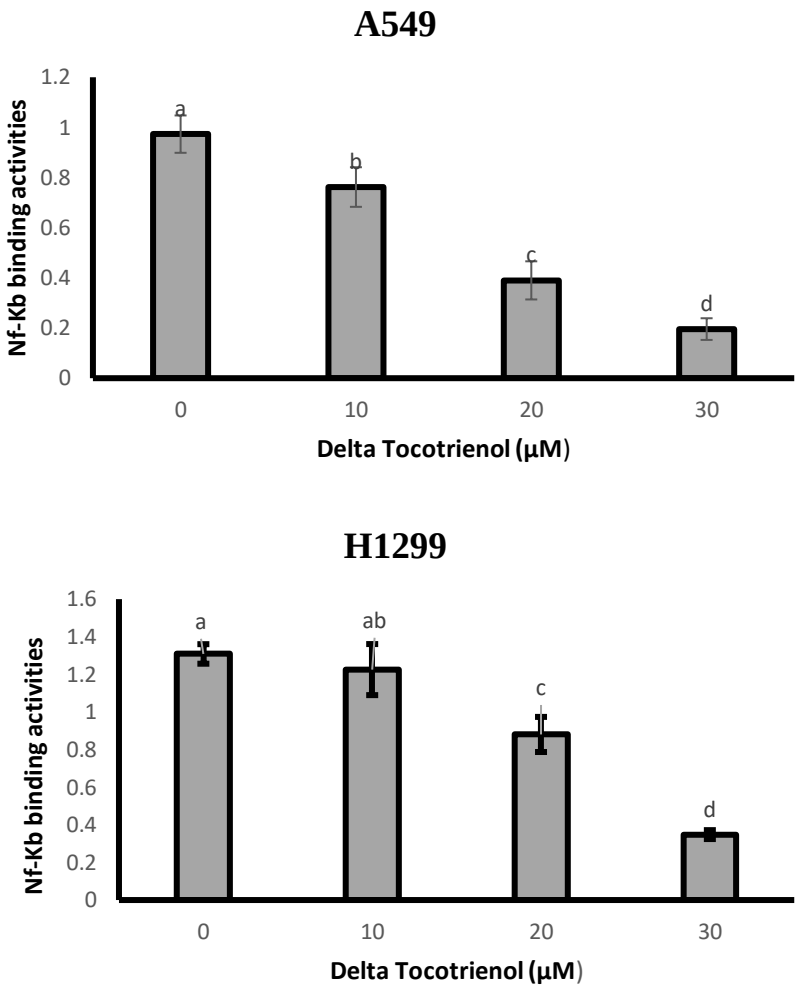


Figure 3.9. Dose-dependent down-regulation of NF-kB binding activity by delta-tocotrienol in A549 (A) and H1299(B) cell lines.

A549 (A) and H1299(B) **c**ells were incubated with increasing concentrations of delta-tocotrienol or DMSO-control for 72 h, and nuclear proteins were extracted and evaluated NF-kB binding activity in ELISA plate. The vertical bars indicate the mean absorbance ± SEM (n = 6) where the mean absorbance, represented by different letters, is significantly different (one-way ANOVA followed by TukeyHSD multiple comparison test, $p < 0.05$).

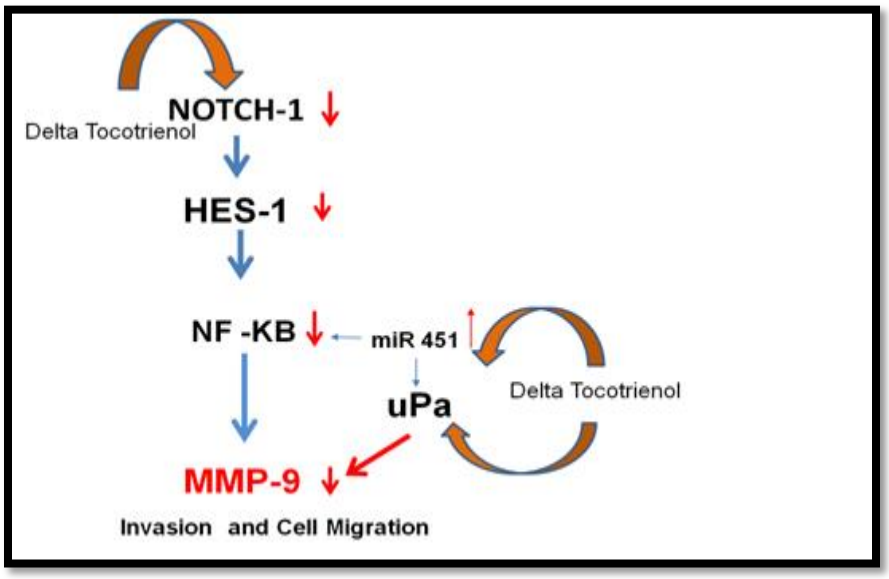


Figure 3.10. Proposed mechanism by which delta-tocotrienol suppresses cell migration and invasion in A549 and H1299 cells.

CHAPTER 4 1H-NMR METABOLOMICS REVEALS DELTA-TOCOTRIENOL AS A NOVEL LAT-1 INHIBITOR FOR CONTROLLING GLUTAMINE DEPENDENCE IN NON-SMALL CELL LUNG CANCER (NSCLC) CELL LINES.

Introduction

Metabolomics, a novel, versatile, and comprehensive approach can provide unbiased information about the dysregulated metabolites, altered signaling pathways, and their interactions [26]. The NSCLC metabolome is a potentially informative mirror of the impact of disease and its dynamics which could lead to promising developments in cancer research, strongly geared toward the discovery of new biomarkers of disease onset, progression and effects of treatment regimens. Most current cancer metabolomics studies focus on finding diagnostic biomarkers and understanding fundamental mechanisms in cancer [26]; nevertheless, this approach could also be used for identifying the efficacy of treatments. Cell culture metabolomics can be used as a bottom-up approach to identify biomarkers of all effects of treatment regimens, [27] and can also be used to investigate the change in the metabolomics patterns produced due to the introduction of tocotrienols in NSCLC cell lines.

It is well known that cancer cells, including NSCLC, show aberrant energy metabolism [141, 142] leading to dysregulation of the metabolome. Healthy cells utilize glucose to produce ATP (adenosine-triphosphate) through aerobic glycolysis, regardless of oxygen availability, in a controlled manner, whereas most of the cancer cells access indirect energy sources to support uncontrolled cell proliferation. Glutamine plays a role as a indirect energy source in NSCLC, which produces ATP through glutamine-driven oxidative phosphorylation [143]. Extra consumption of glutamine in tumors is used for generating metabolic precursors for uncontrolled cell proliferation. These precursors include elevated levels of nucleic acids, lipids, and proteins for proliferation [144], and increased GSH production for cell death resistance.

Current research provides further evidence that glutamine in cancer facilitates exchanging of EAAs (essential amino acids) into proliferating cells via glutamine transporters, which induces mTOR (mammalian target of rapamycin) activation in NSCLC and other types of cancer [145, 146]. Activated mTOR then promotes protein translation and cell growth via activation of its downstream genes such as S6k1 and 4EBP1. ASCT2 and LAT1 are the two primary transporters for glutamine uptake [147, 148]. LAT1 transporter enables transport of the essential amino acids to enhance cancer cell growth via mTOR-stimulated translation, and ASCT2 maintains the cytoplasmic amino acid pool to drive LAT1 function [149]. This cooperation of ASCT2 and LAT1 suppresses apoptosis and fuels the energy economy via net delivery of glutamine [149]. Therefore, inhibiting glutamine uptake via blocking or downregulating glutamine transporters would be an excellent therapeutic target for NSCLC treatment. The aim of this study was to investigate the effect of δT on the metabolite dysregulation of glutamine and its derivatives in NSCLS using 1H-NMR based metabolomics, with an expression of NSCLC growth, development, glutamine transporters and mTOR pathway.

Material and Method

Cell culture and treatment with δ-tocotrienol

NSCLC cell lines, namely A549 and H1299, were cultured in RPMI medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in 5% CO_2 at 37 C ^{0}. The culture medium was renewed every 2 to 3 days. Adherent cells were detached by incubation with trypsin-EDTA and centrifuged at 1500 RPM. The treatment media was prepared by mixing δT (<.01% DMSO as a vector) in the RPMI medium whereas the control was treated only with RPMI media containing <.01% DMSO. The δT, 10μM, 20μM and 30μM

concentration containing <.01% DMSO, was chosen as the treatment concentration. δT was gifted from the American River Nutrition for this experiment.

Intracellular metabolite extraction and determination

A549 and H1299 lines were seeded at a density of 2×10^6 per 100-mm dishes with for 24 hours followed by replacement of media absent or fortified with different δT concentrations (10 µM, 20 µM, and 30µM) at 37°C. Then, cells were incubated for another 72 hours before extracting metabolites. For extracting intercellular metabolites, 10 mL of ice-cold ethanol was added and shaken for 30 min at 4 C^0. After 30 min shaking, ice-cold ethanol was transferred into tubes, and all the extraction solvents were readily removed before NMR analysis by Speed Vac at room temperature. Then, Intracellular metabolites powder was made by evaporating with ethanol, redissolved in 450 µl D_2O with 0.5 µM 2,2-Dimethyl-2-silapentane-5-sulfonic acid (DSS) and 10 µM imidazole used as a reference for chemical shift and concentration determination. An additional set of petri dishes was prepared, and samples were normalized to total protein contained within the additional Petri dishes before using for NMR.

1H- NMR spectroscopy

High-resolution 1H-NMR spectra of intracellular metabolites were obtained on a variant 600 spectrometers operating at 700.13 MHz after normalizing the samples into total protein concentrations. H-NMR spectra of intracellular extracts were acquired using a 6-kHz spectral width and 64 K data point. The acquisition time was 5.44 s, relaxation delay 14.56 s, and 64 scans.

1H- NMR spectroscopy processing

After NMR analysis, Free Induction Decay (FID) files were obtained and processed using NMR processing software ACD (Advanced Chemistry Development, Inc. Toronto, Ontario, Canada). NMR spectra for all the samples were stacked on top of each other like

a single batch and processed simultaneously. First, FID files were Fourier Transformed (mathematical algorithm) to visualize spectra followed by phasing, baseline correction and binning with the auto option of the software. After completing these steps, the whole spectra was divided into 1000 bins, giving a numerical value for corresponding peaks and converted into a data table. The processed data table was used for further statistical analysis using SIMCA-P + 13.0 multivariate data analysis software (Umetrics, Umea, Sweden).

Multivariate data analysis: PCA, PLS, PLS-DA and OPLS-DA

The Processed NMR spectrum data table from ACD software was imported into the SIMCA P+ software (Umetrics Academy, Sweden) for Multivariate data analysis (MVDA). After importing, the data table was transposed and labeled accordingly. The integrals corresponding to the spectral region from 4.5 to 6 ppm were excluded as this region contains water peaks and exchangeable protons. Spectral regions displaying no peaks, and some of the very common spectral regions to all the samples, were also excluded from the data set. After editing, mathematical models were created based on predictability (Q2) and variability (R2) in the data set using autofit option in the software. Samples were labeled and colored for identification purposes, and all data was Pareto-scaled before analysis. Based on the Hotelling T^2 test (95 % confidence interval) and DMOX test results, some samples were removed as they were recognized as extreme outliers for the model.

Principal component analysis (PCA), a classical unsupervised multivariate pattern recognition method, was used to determine differences in clustering behavior among treated and non-treated NSCLC cells. Subsequently, PLS-DA, a supervised pattern recognition method, was further applied to maximize the variation and clustering behavior amongst the groups. Additionally, OPLS-DA, a supervised pattern recognition method, was performed to maximize the

variation between groups and to determine the variables that contributed to this variation. The qualities of models were validated by determining R2 (goodness of fit parameter) and Q2 (goodness of prediction parameter) values and the variables that contributed to this variation. Finally, by using a partial least square (PLS) regression analysis, we observed a correlation between the metabolomic profile and anticancer effects, namely anti-proliferation and apoptosis. The results from PCA PLS, PLS-DA, and OPLS-DA analysis were presented in the score plots, where each point represents an individual sample (to show the group clusters), and loading plots or S-plots, where each coordinate represents one 1H-NMR spectral region (to identify the variables contributing to the classification).

Metabolite identification and quantification from Chenomx NMR suite.

The metabolites spectrum from NMR was identified using Chenomx NMR suite (CHENOMX INC, Edmonton, Alberta). The fid files from the 1D 1H –NMR spectra were imported into the Chenomx software. This software has its own processing interface where spectra were fourier transformed and baseline corrected. Phasing was done using DSS reference peak at 0.0 ppm, and water peak was also deleted. The processed spectra analyzed in the profiler module of the software. The 600 MHz library with the corresponding pH was selected. Identification and concentrations of different metabolites were calculated by fitting the set of peaks for those compounds in the sample spectrum. If the area was crowded with many peaks, then multiple metabolites were adjusted at one time to match the reference spectrum closest to the sample spectrum. The identified and quantified compounds were then exported into the Excel sheet.

Statistical analysis and metabolic pathway identification on metabolites

MetaboAnalyst 3.0 software, a web-based metabolomics data processing tool [150]was use to statistically analyze the metabolites identified from Chenomx NMR suite. Quantified data

from Chenomx NMR suite was normalized to DSS reference peak at 0.0 ppm followed by range scaling. T-test was performed, and Manhattan plot and heat maps were generated using Metabo Analyst 3.0 software. The p-values less than 0.05 were considered statistically significant.

Metabolic pathway identification was performed with the pathway analysis option of MetaboAnalyst 3.0 software. Briefly, quantified data from Chenomx NMR suite was normalized to DSS reference peak at 0.0 ppm followed by range scaling. Then, Homo Sapiens pathway library was selected as a reference, and to generate Pathway Analysis output on all matched pathways, the pathway analysis was performed according to p values from pathway enrichment analysis and pathway impact values from pathway topology analysis. Further, metabolites that were changing most between the control and 30μM treatment were traced back to their origin, and the pathways were interpreted for metabolism changes using current biochemistry.

Western blot for protein expression analysis

Based on data from Chenomx analysis, altered metabolites were fitted into appropriate biochemical pathways and dysregulated protein expression associated with altered metabolites were explored using Western blot analysis. One million cells of each A549 and H1299 were seeded in 100mm dish per plate and incubated for 24hours; then, the media with/without δT was replaced with original media and incubated for another 72 hours. Then, the cell was extracted and lysed in the cold 1X cell lysis buffer (Cell Signaling Technology Danvers, MA) for 30 min on ice with 1X protease inhibitor (Cell Signaling Technology Danvers, MA) and kept at -80C overnight.

Protein concentrations were estimated by using Pierce BSA Protein Assay kit (Bio-Rad Laboratories, CA) and 40 μg of total cell lysates mixed with equal amounts of 6x lemma buffer (Bio-Rad Laboratories, CA), followed by boiling at 100 C for 5 min. Samples were loaded on 10% SDS-polyacrylamide gel electrophoresis, and the gel was electrophoretically transferred to a

nitrocellulose membrane (Whatman, Clifton, NJ) in transfer buffer (25mM Tris, 190mM glycine, 20% methanol) using a Bio-Rad Trans-Blot® Turbo™ Transfer System. The membranes were incubated for 1 h at room temperature with 5% BSA in 1x TBS buffer containing 0.1% Tween. After that, the membranes were incubated overnight at 4C with primary antibodies (1: 1,000). The following proteins antibodies SLC7A5, SLC1A5, mTOR, S6K and c-MYC (Cell Signaling Technology, Danvers, MA) were used in the analysis. The membranes were washed three times with TBS-T and subsequently incubated with the secondary antibodies (1:5,000) containing 2% BSA for 2 h at room temperature. The signal intensity was then measured by chemiluminescent image with chemiDoc XRS (Bio-Rad Laboratories, CA).

Results

δT inhibit the cell proliferation in A549 and H1299 cell lines

To further evaluate and validate the antiproliferative effects of δT, an MTS assay was performed after 72 hours of incubation with 10, 20 and 30μM of δT doses, respectively. We observed a dose-dependent decrease in cell proliferation of A549 and H1299 cells, and data was displayed as a whisker plot in figure 4.1.

δT changes metabolite profiles in A549 and H1299 cells

To investigate the changes in metabolism and metabolites with δT intervention, unsupervised PCA analysis was initially performed using NMR spectral data acquired from intracellular metabolome of cell lysate. The score plot showed clear separation with different treatment doses along PC 1 and PC2 in A549 cells, whereas separation was not very clear in H1299 cells (Figure 4.2 AB). When data for all groups were combined in PLS-DA scores, the separation of the control from different treatment groups further improved at a 2D view of the score plot and

it validated the impact of δT on cellular metabolite profile of above cell lines in a dose-dependent manner. (Figure 4.3 AB).

All subsequent analyses were then carried out by separate comparisons of control and 30μM δT treatment after 72 hours incubation using OPLS-DA. The OPLS-DA score plot of cellular NMR metabolic profile of 30μM δT treated and control cells lines are shown in Figure 04AB. The OPLS-DA score plot exhibited clear segregation between control and treatment groups in A549 cells and H1299 cells with treatment of δT; the significantly high Q^2 indicated a considerable difference in the cellular metabolic profile of treated cells compared to control cells. To identify the NMR spectral regions (bins) that varied significantly between control and treated groups, the loading S-plot from the model was generated. Figure 04 CD shows a representative S-plot (correlation of spectral bins with group separation) corresponding to the score plot of Figure 4.4AB. These bins numbers were further analyzed to identify the significant metabolites that contributed to the separation of the control and treatment groups seen in the OPLS-DA model. Based on the analysis of S-plot bin numbers, the bin numbers responsible for the differences could be attributed to glutamine, glutamate and glutathione and lactate in both cell lines.

Cellular metabolites show a significant correlation between anti-proliferative and apoptotic properties.

The PLS plot (Figure 4.5 ABCD) gives an indication of correlation between metabolite changes in the cellular metabolome with δT and anti-proliferative and apoptosis effects. The treatment with δT for 72 hours at various concentrations (10,20,30) induced a significant coefficient of determination R2 = 0.77 in A549 and R2= 0.49 in H1299 when the data were correlated with the anti-proliferative effects of δT (Figure 4.5 AB). Furthermore, as determined by the PLS analysis, apoptotic effects were correlated with the plasma metabolomics profile (R2 =

0.78 in A549 and R2 = 0.56 in H1299), indicating that δT induces apoptosis in both cell lines (Figure 4.5 CD).

Quantification of metabolites reveals that δT alter the glutamine metabolism

Chenomx 7.6 Suite NMR software was used to probe the metabolome profiles in treatment and control groups. H NMR spectra provided information on over 45 metabolites, including amino acids, intermediates of the tricarboxylic acid cycle (TCA), energy molecules, and nucleic acid associated molecules (Figure 4.7 AB). Among all the metabolites analyzed, we identified several metabolites in glutamine metabolism that were significantly decreased ($p < 0.05$) in the treatment group as compared to controls (Figure 4.6AB). In addition, we recognize the metabolites such as leucine and other essential amino acids significantly decreased in both cell lines after δT treatment (Figure 6AB). Moreover, the metabolites related to cell proliferation such as 2- oxoglutarate, citrate, and uracil also significantly decreased ($p < 0.05$) in the treatment group as compared to controls (Figure 4.6AB).

To recognize the metabolic pathways and biological relevance of the identified metabolites from chenomix analysis, we performed pathway analysis using MetaboAnalyst 3.0 software [25]. Some of the key altered pathways include lysine biosynthesis, purine metabolism, alanine, aspartate and glutamate metabolism, D-glutamine and D-glutamate metabolism, Citrate cycle (TCA cycle) and pyruvate metabolism for both cell lines. The ranking of pathway analysis is based on statistical p values from enrichment analysis alone with multiple adjustments. The total number of compounds in the pathway; the hits are the -matched numbers from the uploaded data; the raw p is the original p-value calculated from the enrichment analysis; the holm p is the p-value adjusted by holm-bonferroni method; the FDR p is the p-value adjusted using false discovery rate; the impact is the pathway impact value calculated from pathway topology analysis.

δT inhibits glutamine transporter (LAT-1) profiles in A549 and H1299 cells

The key differences in metabolite spectrums of glutamine metabolism, leucine, and essential amino acids were observed in S- plot, Chenomx based quantification of metabolites and pathway analysis. Thus, we performed a Western blot analysis for protein analysis of mTOR pathway and glutamine transporters as current literature provides the solid evidence that glutamine uptake and EAA including leucine are associated with the mTOR pathway activation. Surprisingly, we observed a dose-dependent inhibition of glutamine transporter LAT-1 and mTOR pathway proteins (P-mTOR, mTOR, S6K, c-MYC) in the Western blot analysis.

Discussion

In this study, we observed a dose-dependent inhibition of cell proliferation with δT in NSCLC, namely A549 and H1299. The cumulative evidence shows that the uncontrolled cell proliferation in cancer cells require macromolecular synthesis to create new biomass, including DNA, proteins, and lipids [28]. In the proliferating process, glucose based ATP is not the only production source; glucose and glutamine both provide substrates by supplying both ATP and carbon skeletons for macromolecular synthesis [29]. Basically, glucose and glutamine contribute to glycolysis and the TCA cycle where carbon skeletons from glycolysis and the TCA cycle contribute to macromolecular synthesis for the growing process of the cancers. Glutamine mainly replenishes the TCA cycle intermediates removed by the process called Glutaminolysis, and for GSH synthesis in cancer [30, 31]. In the process of Glutaminolysis, glutaminase enzyme (GLS1) catalyzes the conversion of glutamine to glutamic acid and the subsequent conversion of glutamate to α-ketoglutarate (2-oxoglutarate), catalyzed by glutamate dehydrogenase [32]. In cancer cells, the enhanced production of α-ketoglutarate (2-oxoglutarate) from glutamine metabolism can be observed, as it helps to maintain the citric acid cycle and energy production [32]. Remarkably, in

the metabolomics analysis of this intervention, we observed a significant reduction of glutamine, glutamate, and 2-oxoglutarate after treating with 30 μM of δT on NSCLC cells tested (Figure4. 6). The S-plot from OPLS-DA analysis also showed that glutamine and glutamate concentrations were the major differences in metabolomes of NSCLC after treating with 30 μM of δT. Further, we found that some precursors, namely aspartate, glutamine, and uracil (Figure 4.9), needed for DNA replication were significantly decreased by the δT treatment on NSCLC (Figure 4.6 and Table 4.1). All this evidence suggests δT may attenuate the glutamine uptake or conversion of glutamate into α-ketoglutarate where these attenuations of above processes may inhibit the uncontrolled cell proliferation.

In PLS correlation analysis for both cell lines, the strong correlation between apoptosis effects and cellular metabolome with increasing δT concentrations were observed in the PLS score plot. Further, in the previous study, we demonstrated that δT induce apoptosis in a dose-dependent manner in NSCLC from Annexin based flow cytometry analysis and histone ELISA[24]. The current literature provides strong evidence that GSH depletion in cancer cells induces apoptosis *in vitro* and *in vivo[151]*. Dalton TP showed GSH-depleted knock-out mouse of γ-GCS died from massive apoptotic cell death[152]. Further elevated levels of GSH are also connected with apoptotic resistant phenotypes in several models of apoptosis in previous studies [153, 154], and GSH depletion by itself has been observed to either induce or stimulate apoptosis [153, 155]. Surprisingly, GSH quantification, after treating with δT in A549 and H1299 cells, shows a clear decline in intercellular GSH levels in both cell lines (Figure 4.6). The results reveal there may be an association between GSA levels and induction of apoptosis in NSCLC cells after treating with tocotrienol.

The Western blot and PCR data from our previous study revealed that δT dose dependently inhibits the BCL-2 and BcL-xl expressions in A549 and H1299 cells. Flow cytometry analysis with Annexing stain also indicated that δT treatment induced apoptotic cell proportion in A549 and H1299 cells [38]. The anti-apoptotic role of Bcl-2 has been linked to GSH content in some studies. In one study, provoked by GSH-depleting reagents, Bcl-2 overexpression increases GSH levels and inhibit mitochondrial-induced cell death. [39]. Bcl-2 has also been shown to regulate GSH content in different cellular compartments [40]. Bcl-2 expressions with decreasing in cellular GSH concentration is reported in maintaining a high nuclear GSH concentration [41], activating death receptor induced by apoptotic cascade [42] and generating mitochondrial apoptotic signaling [43]. Moreover, Bcl-2-mediated resistance to apoptosis was prevented with depletion of intracellular GSH [44], and anti-apoptotic effect of Bcl-xl has also been attributed to the regulation of GSH homeostasis by preventing GSH loss. [45] However, these effects seem to be cell type-specific and context-dependent [46]. Thus, decreasing GSH in A549 and H1299 cells after treating with δT may be attributed to inhibition of the BCL-2 and BcL-xl expression and induction of apoptosis.

MetaboAnalyst 3.0 online metabolomics data analysis software aided to identify impacted pathways and networks analysis (Figure 4.8). The key aberrant pathways identified from the above tool include D-glutamate and Glutamine, alanine, aspartate, glutathione metabolism, and the TCA cycle, etc. (Figure 4.8). The current literature provides solid evidence that increasing levels of glutamine were detected in lung cancer tissue especially in non-small lung cancer (NSCLC) when compared to other cancer types such as colon or stomach cancer [47]. In this study, we compared glutamine concentrations in cell lysates with 20 reference libraries by using MetaboAnalyst 3.0. The results from analysis indicated that glutamine and some of the metabolites were lower than

reference levels in other studies (data is not presented). The reason is that all the databases were based on urine and plasma glutamine levels, and we extracted metabolites from cell lysates.

All major aberrant pathways included shared metabolites within the network. For instance, the statistical analysis identified several significant pathways as indicated by low p-value and low FDR, such as leucine, asparagine, glutathione, glutamate and glutamine. Among the highly-impacted metabolites, metabolites related to glutamine metabolism were significant. Further, aberrant glutamine and its related metabolites were also identified in the S-plot of OPLS-DA analysis. Based on the information in this study, we recognized glutamine and glutamine based pathways are highly impacted after treatment with δT.

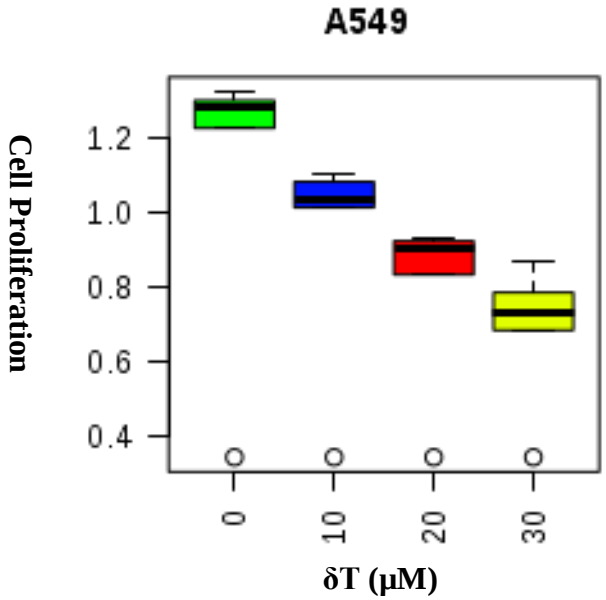
As glutamine plays a key role in normal cell physiology, alterations in glutamine transporters and metabolizing enzymes that are involved in cancer cells are expected to be highly impacted [156]. In this study, we found that cellular glutamine levels significantly decrease after treatment with δTin NMR analysis. This may be due to inhibition of glutamine transporters, and we thus tested the protein expression levels of glutamine transporters, namely the bidirectional L-type amino acid transporter 1 (LAT1) and solute carrier family A1 member 5 (SLC1A5) in Western blot analysis. Above transporters play a fundamental role in glutamine uptake process in normal cell physiology. LAT-1 also known as SLC7A5/SLC3A2 facilitate glutamine efflux in exchange for the influx of L-leucine and other essential amino acids (EAA) across the cell membrane; similarly, SLC1A5 mediates uptake of neutral amino acids including glutamine [157]. LAT1 expression is a significant element indicating a poor prognosis in various human cancers including NSCLC [158-162]. We observed dose-dependent inhibition of LAT-1 expressions with δT, whereas SLC1A5 expression was not changed with tocotrienol concentrations. In addition to facilitating the transport of essential amino acids (EAA) for protein

synthesis, LAT1 stimulated the growth of cancer cells via mammalian target of rapamycin (mTOR)[163-165]. mTOR functions include stimulation of cell growth and proliferation by promoting a variety of anabolic processes, including biosynthesis of proteins, lipids, and organelles, and limiting catabolic processes such as autophagy [166]. In head and neck squamous cell carcinoma cell lines, inhibition of LAT-1 transporter using an inhibitor lowered the levels of phosphorylation of mTOR and its downstream signaling molecules [167]. Similarly, we observed lower expression of mTOR along with LAT-1 in Western blot analysis after treating with δT. We quantified EAA including leucine in cell lysates after treating with δTas LAT-1 facilitating glutamine efflux in exchange for the influx of L-leucine and other essential amino acids (EAA). Indeed, we observed lower concentrations of EAA including leucine after treating with 30 µm of δT. Moreover, mTOR functions are mediated by two downstream proteins, the eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and the p70 ribosomal S6 kinase 1 (p70S6K1, S6K1) [166]. S6K1 is a serine-threonine kinase activated by mTORC1-mediated phosphorylation, which in turn phosphorylates the 40S ribosomal protein S6, enhancing cap-dependent mRNAs translation [166]. These two proteins regulate F-actin reorganization, focal adhesion formation, and tissue remodeling through the proteolytic digestion of extracellular matrix via up-regulation of matrix metalloproteinase 9 (MMP-9)[168]. Interestingly in our previous study, we observed δT reduced cell migration, invasion and adhesion in a dose and time-dependent manner and MMP-9 expressions in NSCLC cell [8]. In summary, we are suggesting that ΔT inhibit LAT-1 glutamine transporter, which then interferes in glutamine efflux in exchange for the influx of L-leucine and other essential amino acids (EAA) in A549 and H1299 cells. The interference of glutamine and EAA exchange result in inhibition of mTOR-1 and its downstream proteins such as

S6. This inhibition of the mTOR pathway may be one of cause for the inhibited cell proliferation and induced apoptosis in A549 and H1299 cells.

In the present work, anti-cancer effects of tocotrienols were confirmed by H-NMR metabolomics analysis. We employed NMR-based metabolomics approaches to reveal metabolism regulations of tocotrienol on NSCLC, namely A549 and H1229 cell lines. The metabolomics profiling of NSCLC lysates showed that changes in the glutamine related metabolisms are highly affected after treating with δT. Further, δT works by inhibiting glutamine uptake into proliferating cells through glutamine transporter inhibition, thus resulting in inhibition of cell proliferation and induction of apoptosis via downregulation of the mTOR pathway. Through this work, NMR-based cellular metabolomics proves possible capacities for evaluating the therapeutic effect of phytochemicals/ bioactive and reveal holistic regulations on mechanisms.

A.



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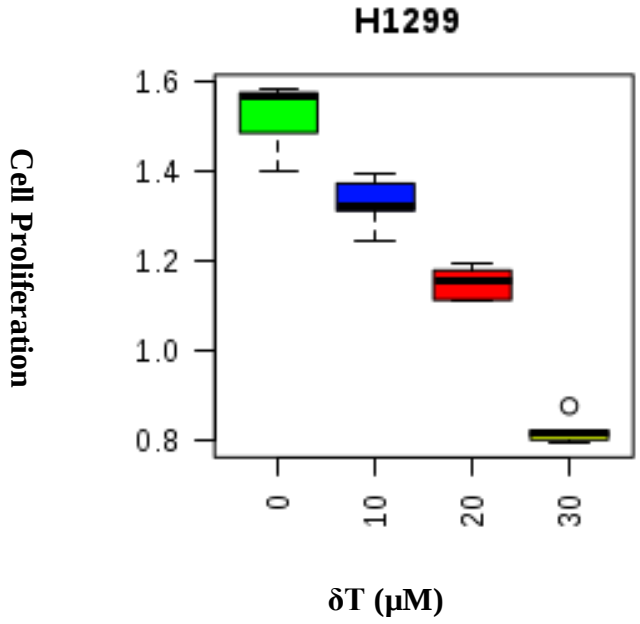


Figure 4.1 : Antiproliferative effects of tocotrienol on NSCLC cells. Cell viability of human NSCLC cell lines A549 (A) and H1229 (B) cells.

Both A549 and H520 cells were initially plated at a density of 5×10^3 cells/well (5 wells/group) in 96-well plates and grown in experimental medium containing 0, 10, 20, 30 μM of δT treatment media for 72 h. Viable cell number was determined using the MTS colorimetric assay. Absorbance data from MTS data further analyzed using metalanalystat software, and above whisker plots were generated.

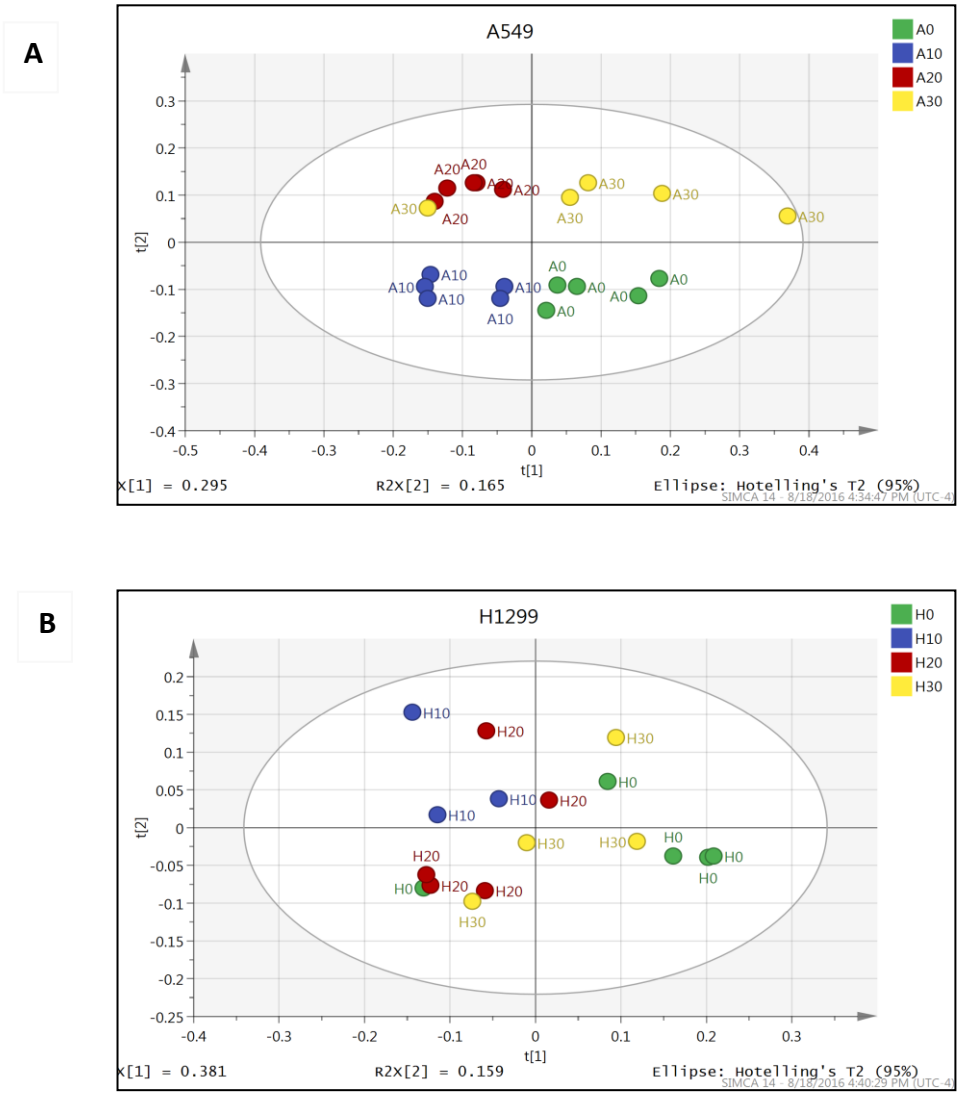
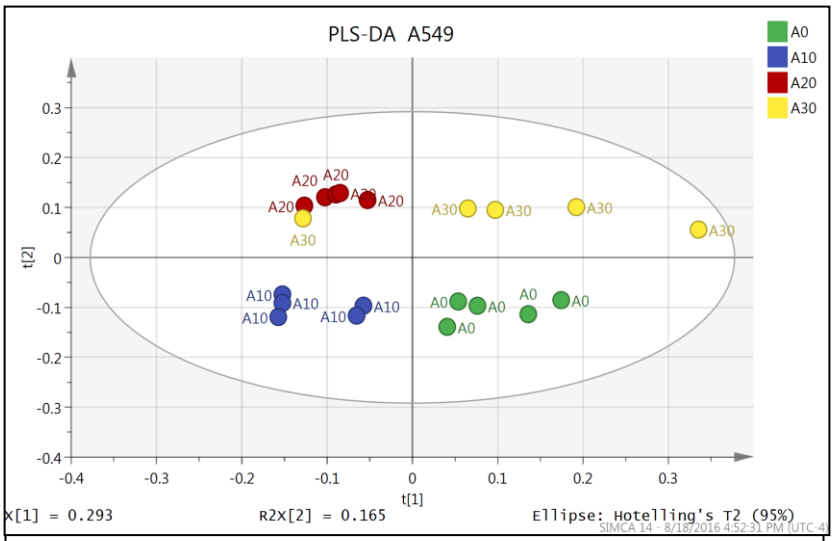


Figure 4.2:Effects of δT on A549 (A, C) and H1299 (B, D) on the metabolome of lung cancer cell lines.

Principal component analysis (PCA) scores plot(A and B), PC1 (t[1]) versus PC2 (t[2]) showing the unsupervised separation of metabolites profiles among the δT treatments 0(Green), 10(blue), 20(Red),30 (yellow) after 72 hours incubation. Each symbol represents metabolites from the one Petri dish described at the method section; The ellipses shown in A and B represents the Hotelling's T2 95% confidence interval for the multivariate data. Data is parito scaled and mean centered.

A



B

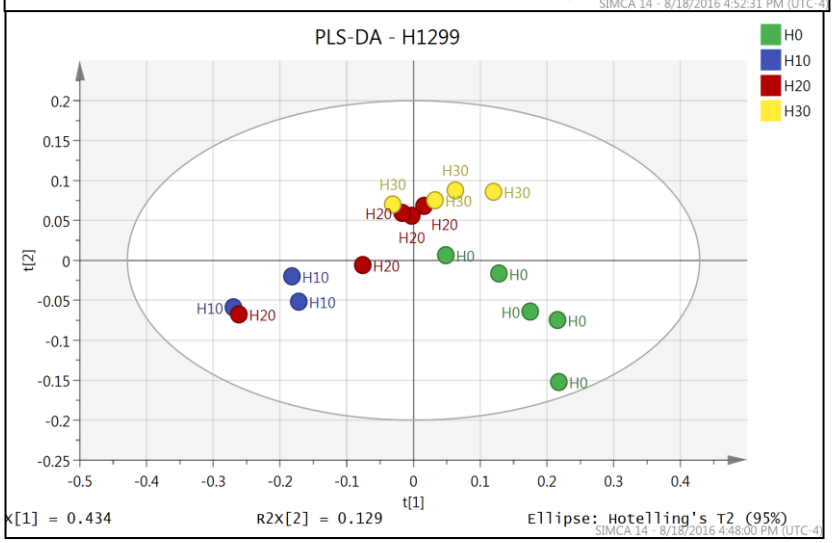


Figure 4.3: PLS-DA scores plots of A549(A) and H1299 (B) lung cancer cell line metabolomes after treating with/without δT for 72 hours.

Partial least squares Discriminant Analysis (*PLS-DA*) scores plot(A and B), PC1 (t[1]) versus PC2 (t[2]) showing supervised separation between the four sample classes based on the δT treatments namely 0(Green), 10(blue) , 20(Red),30 (yellow) after 72 hours incubation period . The ellipses shown in A and B represents the Hotelling's T2 95% confidence interval for the multivariate data. Details of the experimental procedures are given in Materials and Methods.

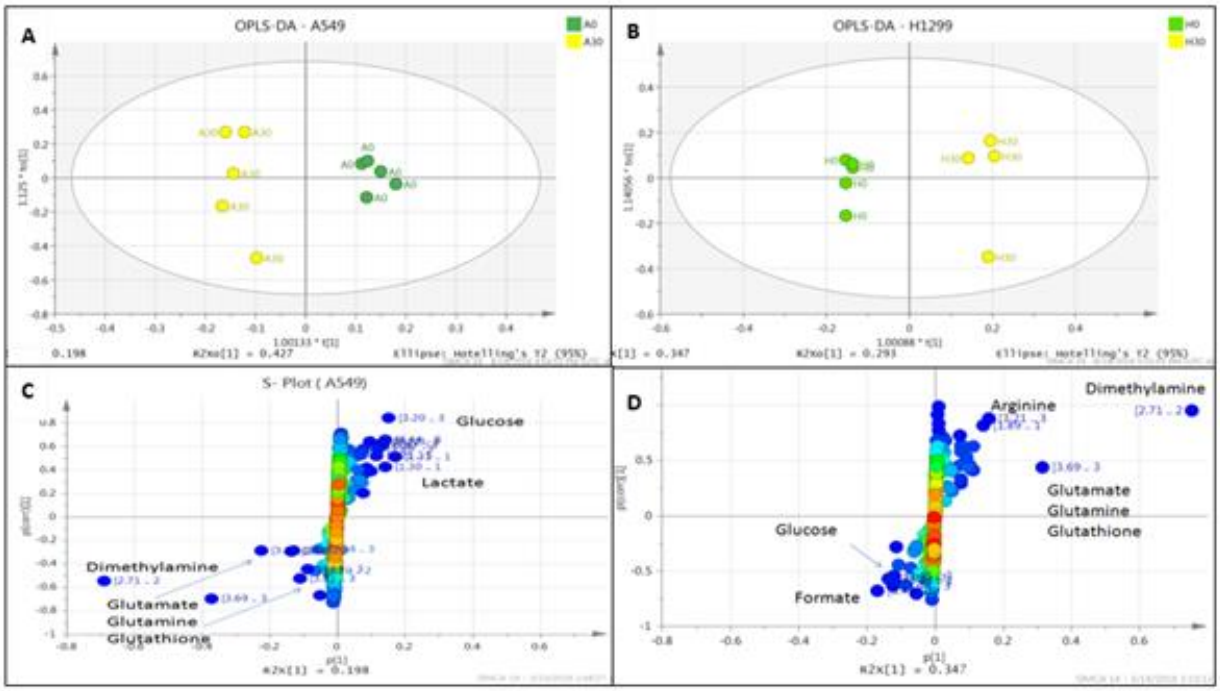


Figure 4.4: OPLS-DA analysis of A549 and H1299 the metabolome of lung cancer cell lines after treating with/without δT for 72 hrs.

OPLS-DA score plot based on the cellular metabolic profiling of lung cancer cells lines namely A549 (A) and H1299 (B), 30μM treatment (Green) and control (Yellow); the results indicated that cellular metabolic profiling of lung cancer cells lines was significantly changed after δT treatment for 72 hours. The S-plots of OPLS-DA analysis of A549(C) and H1299 (D) from Treatment (30μM) and control (0μM) cells. The axes plotted in the S-plot from the predictive component are p [1] vs. p(corr) [1], representing the magnitude and reliability, respectively. Variables in labeled compound name could be exploited as potential biomarkers.

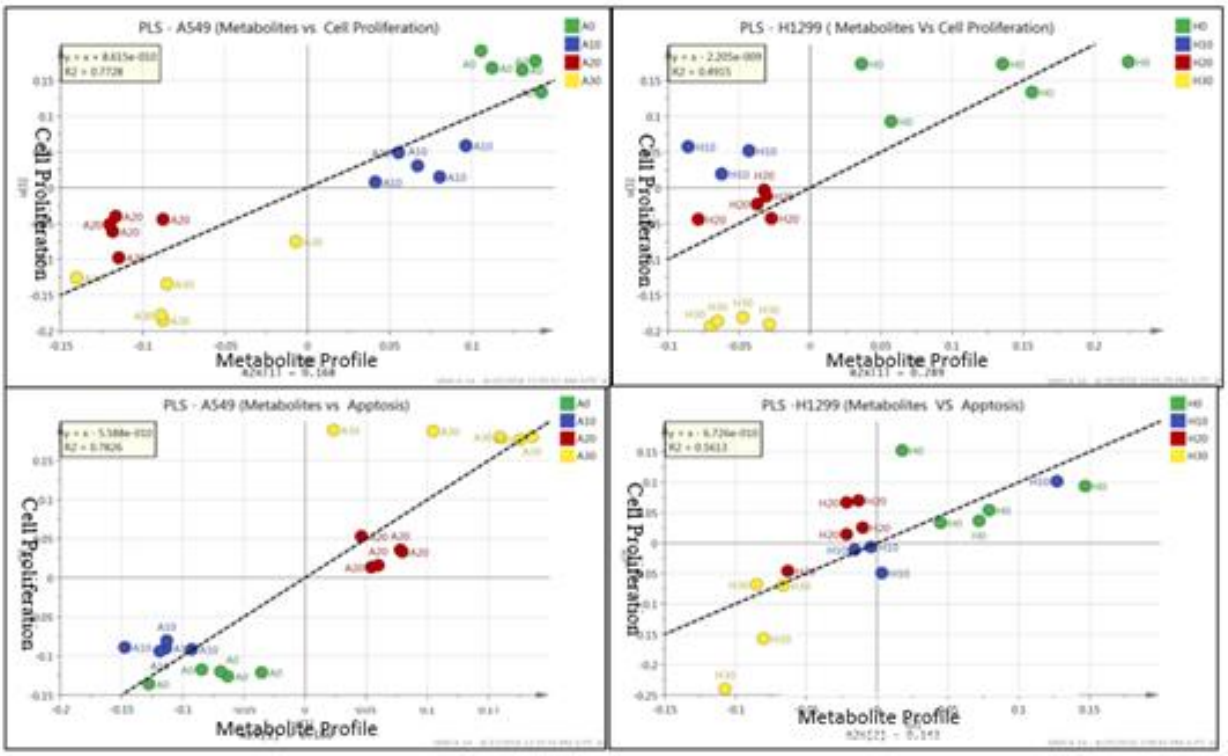


Figure 4.5: Correlation of lung cancer cell line's metabolomes (with/without tocotrienol) between antiproliferative and apoptosis effects.

Changes in the metabolomic score values ([t1] or[t2]) of individual cells treated with/without δT are plotted along with the corresponding anti-proliferative and apoptosis effects ([u1] or [u2]), as determined by the PLS analysis. The coefficient of determination (R^2) among ([t1] or [t2]) and ([u1] or [u2]) that was calculated after linearization of the relationship is indicated. The relationship between the cellular metabolomic profile with/without δT and antiproliferative in A549 (A) ($R^2 = 0.7$) and H1299 (B) ($R^2 = 0.7$) 3b**.** The relationship between cellular metabolomic profile with/without δT and apoptosis effects in A549 (A) ($R^2 = 0.7$) and H1299 (B) ($R^2 = 0.7$). Each symbol in different colors namely 0(Green), 10(blue), 20(Red), 30 (yellow) represents metabolites from the one Petri dish described at the method section;

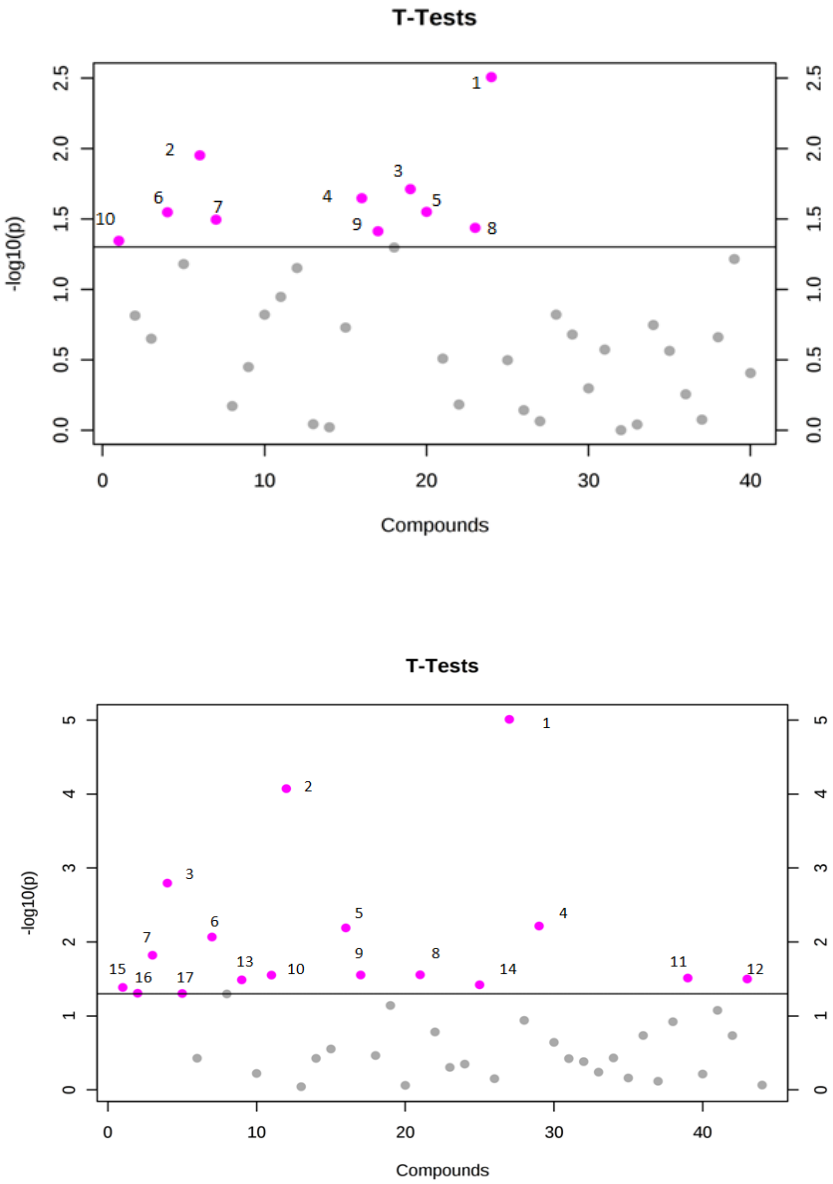


Figure 4.6: The effects of δT on a cancer cell metabolites were measured with H-NMR and analyzed in Chenormix and MetaboAnalyst 3.0 software.

The Important metabolites selected by t-tests with threshold 0.05 in Manhattan Plot (A) A549 and (B) H1299. The red circles represent features above the threshold. The p values are transformed by -log10 so that the more significant features (with smaller p values) is plotted higher on the graph. The table below shows the detailed results from the T-test. The number in table is correspondence to the metabolites label in in Manhattan Plot.

Table 4.1: List of Significantly impacted metabolites in NSCLS after δT treatment

The table below shows the detailed results from the T-test in (A) A549 and (B) H1299. The number (#) in table is correspondence to the metabolites labeled in Manhattan Plot figure 4.6.

A.

Compound	#	T. stat	value	-log 10(p)	FDR
Leucine	1	6.3729	0.0031094	2.5073	0.12437
Asparagine	2	4.4595	0.011165	1.9522	0.17165
Glutathione	3	3.7816	0.01941	1.712	0.17165
Glutamate	4	3.6127	0.022502	1.6478	0.17165
Glycine	5	3.3659	0.028151	1.5505	0.17165
Alanine	6	3.3592	0.028325	1.5478	0.17165
Aspartate	7	3.2306	0.031958	1.4954	0.17165
Lactate	8	3.0906	0.036552	1.4371	0.17165
Glutamine	9	3.0342	0.038622	1.4132	0.17165
2-Oxoglutarate	10	2.8766	0.045169	1.3452	0.18068

B.

Compound	#	T. stat	p.value	Log 10(p)	FDR
Leucine	1	27.941	9.76E-06	5.0105	0.000429
Dimethylamine	2	16.224	8.44E-05	4.0734	0.001858
AMP	3	7.612	0.0015987	2.7962	0.023448
Malate	4	5.3022	0.0060782	2.2162	0.056716
Glutamate	5	5.216	0.006445	2.1908	0.056716
Aspartate	6	4.8139	0.0085607	2.0675	0.062779
Alanine	7	4.0807	0.01509	1.8213	0.094849
Histamine	8	3.3817	0.027741	1.5569	0.11002
Glutamine	9	3.375	0.027915	1.5542	0.11002
Cystine	10	3.3708	0.028024	1.5525	0.11002
Succinate	11	3.2728	0.030707	1.5128	0.11002
Uracil	12	3.2413	0.031634	1.4998	0.11002
Citrate	13	3.2127	0.032505	1.488	0.11002
Isoleucine	14	3.0541	0.037876	1.4216	0.11904
2-Oxoglutarate	15	2.9704	0.041127	1.3859	0.12064
ADP	16	2.7906	0.04928	1.3073	0.12313
Arginine	17	2.7832	0.049656	1.304	0.12313

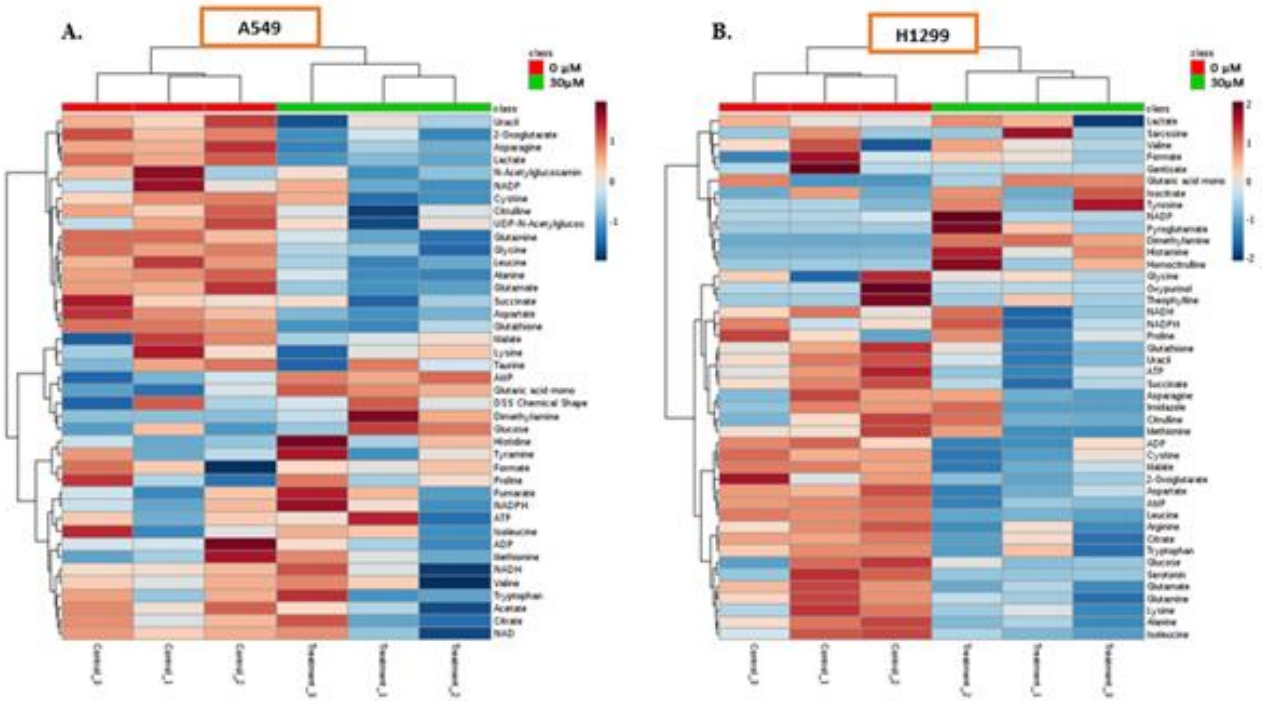
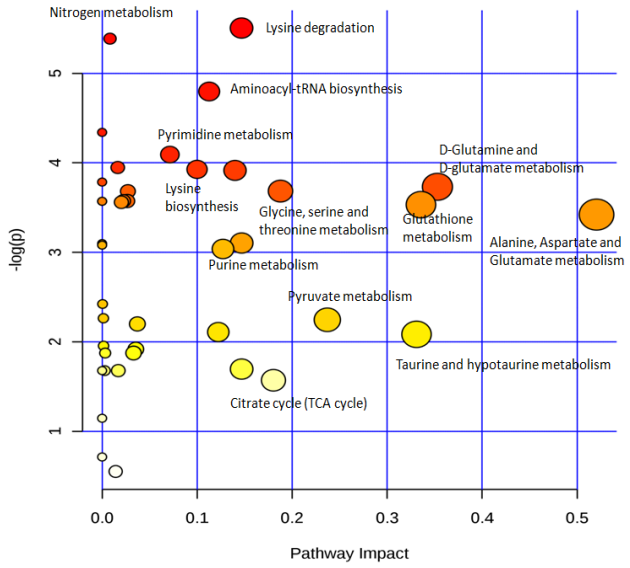


Figure 4.7: Heat map of metabolites significantly differentially expressed between control and treatment in lung cancer cell lines.

The metabolites are detected at chenomix software analysis of NMR spectrums of A549 cells (A) and H1299 (B) after incubating with or without δT for 72 hours. Each column represents a sample, and each row represents the expression profile of metabolites. The fold changes from the overall mean concentration are shown in a color-coded way. Blue color represents a decrease, and red color an increase. The very Top row with Green color indicates the control samples and red color row indicates the samples with the 30µM treatment of δT.

A.



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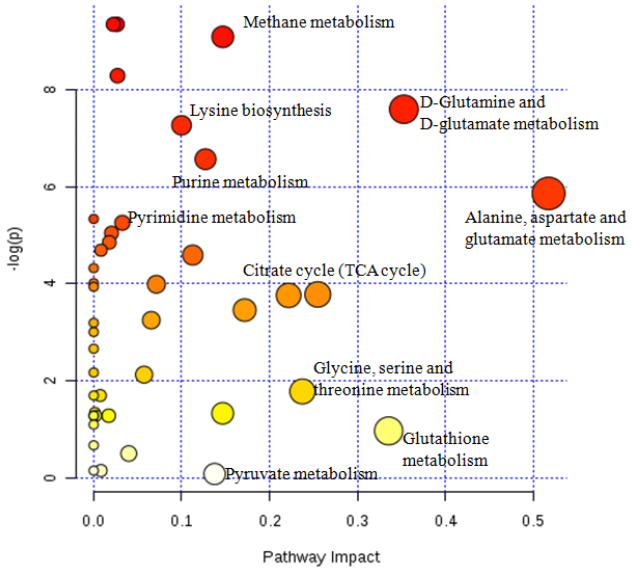


Figure 4.8: The most predominant altered metabolic pathways and the biochemical linkage among the metabolites were analyzed by pathway analysis of MetaboAnalyst 3.0 in A549 (A) and H1299(B) cell lines after treating with/without δT for 72.

The Ranking of pathway analysis is based on statistical p values from enrichment analysis alone with multiples multiple adjustments. The Total is the total number of compounds in the pathway; the Hits is the matched number from the uploaded data; the Raw p is the original p-value calculated from the enrichment analysis; the Holm p is the p-value adjusted by Holm-Bonferroni method; the FDR p is the p-value adjusted using False Discovery Rate; the Impact is the pathway impact value calculated from pathway topology analysis.

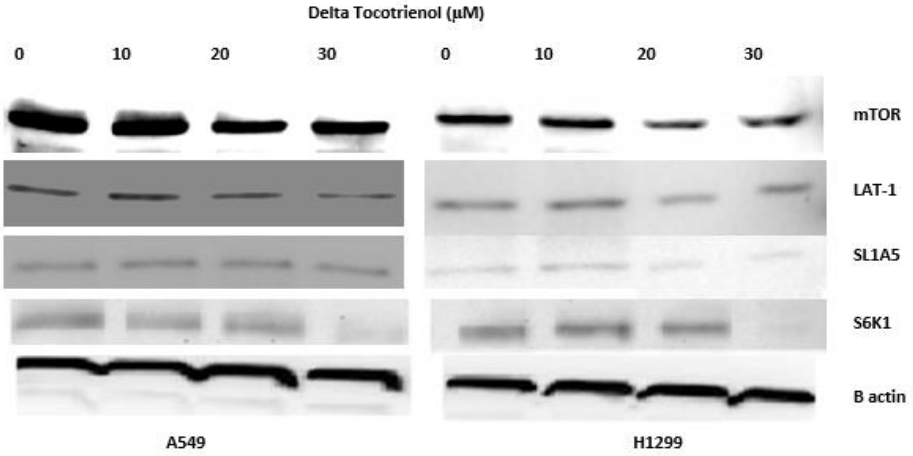
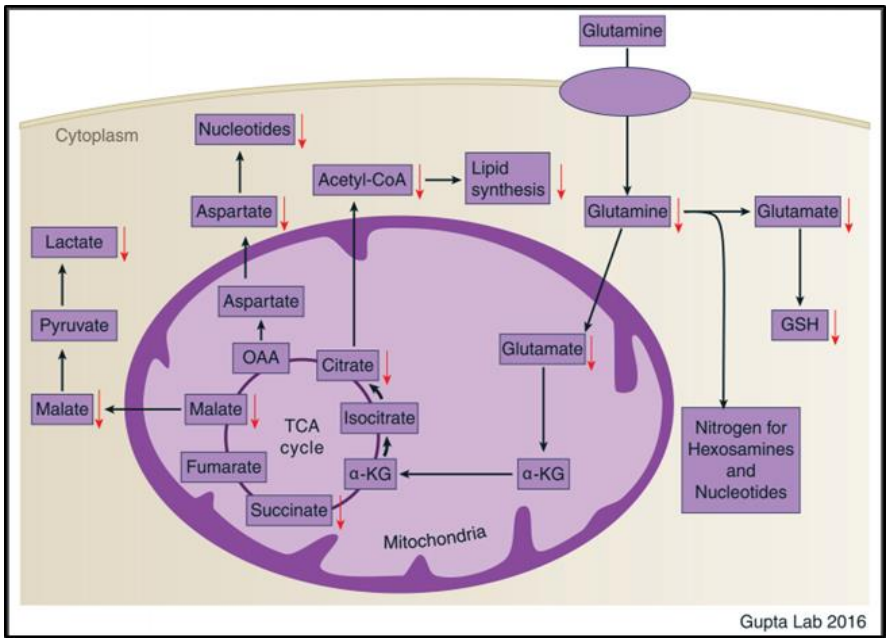


Figure 4.9: Effect of δ- tocotrienol on expressions of the mTOR pathway and glutamine transporters.

A549 (A) and H1299 (B) cells were treated with varying concentrations of δT for 72 h. The expressions of protein were detected by western blot analysis including mTOR, LAT-1, SLC1A5, S6K1, and B-Actin.

A.



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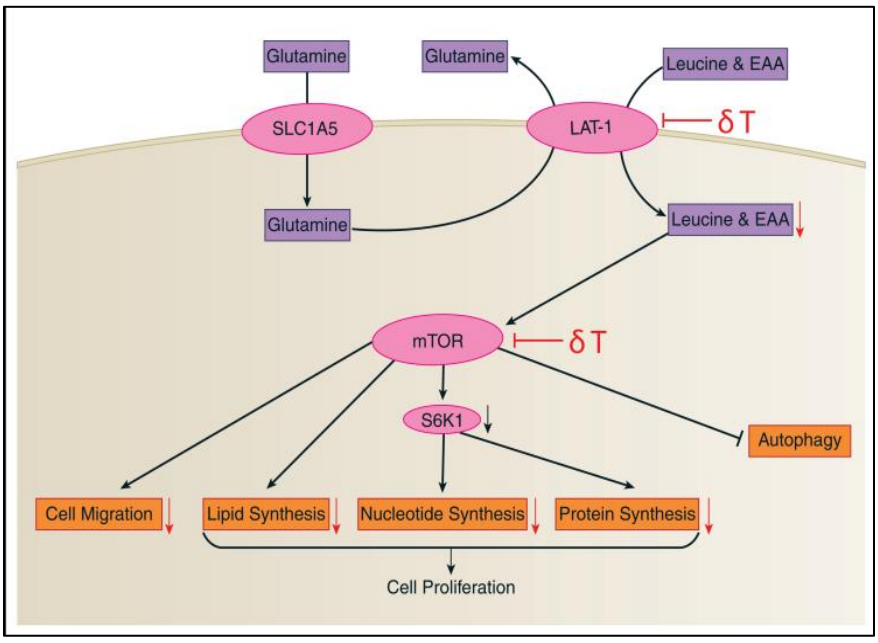

Fig 4.10. Effect of δT on glutamine transporters, mTOR pathway and glutamine metabolism.

CHAPTER 5 TOCOTRIENOLS RICH MIXTURE INHIBITS CELL PROLIFERATION AND INDUCES APOPTOSIS VIA DOWN-REGULATION OF THE NOTCH-1/NF-KB PATHWAYS IN NON-SMALL CELL LUNG CANCER CELLS (NSCLC).

Introduction

Tocotrienols and tocopherols together comprise vitamin E. Most commercially available vitamin E supplements contain tocopherol isomers as their key active ingredient. However, studies have shown that tocotrienols are more potent in their antioxidant and antitumor properties [169]. Tocotrienol isomers namely α, β, γ, and δ are found naturally in cereal grains, vegetable oils, and palm oil, and have demonstrated a strong association with the prevention of cancer and inhibition of tumors, both *in vitro* and *in vivo* [170]. Tocotrienols have displayed anti-tumor effects on different human cancer cells including prostate, breast, colon, melanoma, and lung, via induction of apoptosis by inhibiting multiple signaling pathways including the Notch and NF- κB pathway. Our previous work clearly showed that δT inhibits NF- κB signaling pathways via down-regulation of Notch-1, thereby inhibiting the proliferation, metastatic potential while inducing apoptosis of NSCLC adenocarcinoma cells in a dose-dependent manner [8, 24, 107, 171]. However, overall effects of tocotrienols on NSCLC are still not well understood.

Using δT to treat cancer is not efficient since it is both difficult to isolate and expensive. In addition, individual isomers are not currently available in quantities required for animal or clinical studies. Thus, it becomes necessary to investigate the therapeutic targets of naturally available tocotrienols rich mixtures. The present study aims to investigate the effect of commercially available tocotrienol rich mixture in capsules (TRMC) extracted directly from palm oil with the working hypothesis that this treatment would inhibit NSCLC cell proliferation and induce apoptosis by inhibition of Notch-1 signaling via the NF-kB pathway.

Materials and Methods

Cell culture and treatment with tocotrienols

Two different NSCLC cell lines, representing squamous cell carcinoma (H520) and adenocarcinoma (A549), were cultured in RPMI medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin in 5% CO_2 and 37°C ^{0}. Tocotrienols rich capsule, a gift from Carotino (Kuala Lumpur, Malaysia), containing 21.3 % tocopherol and 78.7% tocotrienol, were used in this study. Tocotrienols in the capsule consist of 26.7% α-tocotrienol, 3.3% ß-tocotrienol, 38.1% y-tocotrienol and 10.6% δ-tocotrienol whereas the remainder 21.3% is α- tocopherol isomer. The medium containing DMSO (vehicle control) or different concentrations of TRMC diluted from a 100mg/mL stock solution were used as experimental treatment media for cell culture. The final concentration of treatment media is expressed as amount of TRMC (ng) in l mL of RPMI media (ng/mL).

Anti-proliferative effects of TRMC

The anti-proliferative effects of TRMC on NSCLC cell lines were analyzed using MTS assay. $5x10^5$ of A549 and H520 cells were seeded in a 96-well plate and incubated overnight. After incubation, the medium was replaced, and cells were treated with fresh medium containing < 0.10% DMSO (control) and different concentrations of TRMC (treatment). After 72 hours of treatment, 20µl of Cell Titer 96 Aqueous One. Solution Reagent from Promega, (Madison, WI) was added to each well and incubated for 2-hour at 37°C in a humidified, 5% CO2 atmosphere. Then absorbance at 490nm was measured using the Bio-Tek ELx800 plate reader (Winooski, VT). Each variant of the experiment was performed in triplicate.

In colongenic assay**,** 1 x 10^5 of A549 and 1 x 10^6 of H520 cells were seeded in 100mm dish and incubated overnight. Subsequently, culturing media was replaced with treatment (different concentrations of TRMC) and control medium and then incubated for another 72 h. The

viable cells were counted by an automated cell counter (Innovating Standards, USA) and 2000 cells were transferred per 100mm dishes with 10mL growing media. Then, cells could grow for 25 days at $37C^0$ in a 5% CO2 incubator. After subsequent incubation, all the colonies were fixed in 4% Paraformaldehyde and stained with 2% crystal violet.

Cell death detection

Cell death detection histone /DNA ELISA Kit from Roche (Palo Alto, CA) was used to detect apoptosis in NSCLC cells. 1 x 10^5 of A549 and 1 x 10^6 of H520 cells were seeded into six-well plates. After an overnight incubation, cells were treated with control medium or treatment medium (different concentrations of TRMC) for 72 hours. Cytoplasmic histone/DNA fragments were extracted from lysed cell extract and incubated in microtiter plate modules coated with anti-histone antibody. Next, peroxidase conjugated anti-DNA antibody was used to detect the immobilized histone/DNA fragment. Bound antibodies were detected by the intensity of color development in microtiter plate modules, after washing with ABTS substrate. The absorbance of the samples was measured at 405nm using the Bio-Tek ELx800 plate reader (Winooski, VT).

Annexin V-FITC apoptosis detection kit (BD, San Jose, USA) was used for apoptosis analysis. A549 and H520 cells were incubated in the control or treatment (0.6ng/mL concentration of TRMC) media for 72 h. After, cells were extracted by scraping and collected to ice-cold PBS. Then, cells were spun down and resuspended in 1x binding buffer at a concentration of 10^5 /ml cells in a total volume of 100 µl. Subsequently, five µl of Annexin V-FITC and five µl of PI (Propidium Iodide) were added. All cells were kept in the dark for 20 min at room temperature. Finally, 400 µl of 1X binding buffer was then added to each tube, and the number of apoptotic cells was analyzed by flow cytometry (BD, San Jose, CA).

Cell migration assay

A549 and H520 cells were seeded in a six-well plate at the concentration of 2 X 10^5 and 1 X 10^6 cells per well respectively. After the cells had been incubated for 36 hrs., the media was removed, and a scratch wound across each well was made using a 100ul pipette tip. All the wound areas were washed with PBS three times to ensure that no loosely held cells were attached. The width of the scratch was imaged and measured by a Nikon H 600L microscope connected to the camera at five places along the scratch. Subsequently, the cells were cultured in control or treatment media (different concentrations of TRMC) for 30 hrs. Then the width of the scratch was reimaged and measured to find the progress of cells that had migrated into the wound.

Cell invasive assay

The tumor invasive ability in the above cell lines was assessed by BD Biocoat invasion kit (BD, San Jose, CA). 2.5 x 10^5 A549 cells and 5 x 10^5 H520 were seeded with basal media in each six well upper chamber in the presence or absence of treatment media (different concentrations of TRMC). In the meantime, 3ml of culture medium with 10% FBS was added to each lower chamber of the six-well plate. After a 20 h incubation, the cells in the upper chamber were removed using a cotton swab. Then cells were fixed in 4% Paraformaldehyde and stained with 2% crystal violet. Then cell unbound crystal violet was washed with PBS before they dried. After that, the stained crystal violet (cell bound) was washed with 20 % acetic acid, and then the absorbance of the dissolved crystal violet was measured at 405nm using the Bio-Tek ELx800 plate reader (Winooski, VT). Each experimental condition was performed in triplicate.

Real-time quantitative PCR for gene expression analysis

One million A549 and H520 cells were seeded in 100mm dish per plate and incubated for 24 hours. Subsequently, culturing media was replaced with treatment (different concentrations of TRMC) or control medium and then incubated for another 48 h. Total RNA was isolated using

RNeasy Mini Kit from QIAGEN (Valencia, CA) according to the manufacturer's protocols. 1000ng of total RNA from each sample was subjected to the first strand cDNA synthesis using High Capacity RNA to cDNA master mix (Applied Biosystems, Foster City, CA) in a total volume of 50 µl. qRT-PCR was performed to explore the Notch-1expression. Diluted cDNA (2 uL) and 2 uL of each reverse primer (5′-GTT GTA TTG GTT CGG CAC CAT-3′) and forward primer (5′-CAC TGT GGG CGG GTC C-3′) and 12.5 uL master mix (SYBR GREEN PCR Master Mix; Applied Biosystems, Warrington, UK) were used in each 25 uL PCR reactions performed in Eppendorf Master cycler realplex 4 (Eppendorf, Hauppauge, NY) at 25C for 10 min, followed by 48 C^0 for 30 min and 95 C^0 for 5 min. Expression values were normalized with a β-actin.(sense (5′-ACCAACTGGGACGACATGGAGAAG-3′; antisense (5′-TACGACCAGAGGCATACAGGGACT-3′)). Each gene expression was tested in triplicate.

Western blot for protein expression analysis

Western blot analysis was performed as part of a protein expression analysis using the following antibodies: Poly (ADP-ribose) polymerase (PARP), b-actin, Survivin, Bcl-XL and Notch 1(Cell Signaling Technology, Danvers, MA) in cell signaling pathways. One million A549 and H520 cells were seeded in a 100mm dish per plate and incubated for 24hours. Then cells were treated for 72 hours with treatment (different concentrations of TRMC) and control medium and incubated for 72 h. Cells were lysed in the cold 1X cell lysis buffer (Cell Signaling Technology Danvers, MA) for 30 min on ice with 1X Protease inhibitor (Cell Signaling Technology Danvers, MA). Then protein concentrations were calculated by using Pierce BSA Protein Assay kit (Bio-Rad Laboratories, CA). Subsequently, 50 mg of total cell lysates were mixed with equal amounts of 4X lemma buffer (Bio-Rad Laboratories, CA) and samples were loaded on 10% SDS-polyacrylamide gel electrophoresis. After electrophoresis, the gel electrophoretically was

transferred to a PVDF (Trans-Blot Turbo Mini PVDF system, Bio-Rad) using Trans-Blot® Turbo™ Transfer System (Holliston, MA). The membranes were incubated for two hours at room temperature with 5% Casein. After that, membranes were incubated overnight at 4C˚ with primary antibodies (1: 1,000-4000). The membranes were washed three times with TBS-T and subsequently incubated with the secondary antibodies (1:5,000) containing 2% BSA for two h at room temperature. The signal intensity was then measured by a chemiluminescent imager with chemiDoc XRS (Bio-Rad Laboratories, CA).

NF-κB filter plate assay for measuring NF-κB binding activity

NF-κB filter plate assay kit was obtained from Signosis (Sunnyvale, CA) and used to determine the NF-κB DNA binding ability of each sample. A549 and H 520 were seeded in petri dishes and incubated for 24 hours. Cells were then treated with or without different concentrations of TRMC. After 72 hours of treatment, cells were collected and washed, and nuclear protein extraction was performed with a NE- PER ® Nuclear and Cytoplasmic Extraction reagent kit (Thermo Scientific, USA) according to the manufacturer's protocols.

Protein concentrations were determined using the Pierce BCA protein assay kit (Rockford, IL). Standard samples were prepared according to the manufacture's protocol. The absorbance of both standards and samples was measured at 562nm using a UV-1800 spectrophotometer from Shimadzu Scientific Instruments (Kyoto, China). The assay was conducted according to the protocol using a biotin-labeled DNA sequence of NF-κB mixed with 3µg of nuclear extract to form an NF-κB-DNA binding complex. For each sample, 10µl TF binding buffer mix, 2µl NF-κB probe, 3µg and distilled water was added to bring the total volume up to 20µl. A filter plate was used to retain bound NF-κB probe, while the unbound NF-κB probe was filtered out. The bound, pre-labeled NF-κB probe was then eluted from the filter, collected and transferred to a hybridization

plate for quantitative analysis. NF-κB probe was further detected using streptavidin-HRP, and luminescence of the probe was measured using an Ultra Multifunctional Microplate Reader from Tecan (Vienna, Virginia).

Data analysis

Significant difference between treatment and control groups was analyzed using a one-way ANOVA (eZanova , USA). Values of <0.05 were considered statistically significant.

Results

Anti-Proliferative effect of tocotrienols on A549 and H520 Cells

To evaluate and compare the cell viability and proliferative effects after *in vitro* exposure of tocotrienols, MTS and the traditional clonogenic assays were used. Results from the MTS assay showed a dose-dependent decrease in cell growth and proliferation for both A549 and H520 cells. A549 cells on treatment with 0.04, 0.08, 0.12 and 0.16 ng/mL concentrations of TRMC demonstrated 13, 15, 38 and 88% cell growth inhibition respectively, relative to control, after 72 hours' incubation (Figure 5.1-A). Similarly, the H520 cell line on treatment with 0.04, 0.08, 0.12 and 0.16 ng/mL concentrations of TRMC also exhibited a respective 0, 12, 33 and 84 % cell growth inhibition relative to control at same conditions (Figure 5.1-B). Inhibition of cell growth was significant at every concentration for A549 cells and for H520 cell inhibition was significant at a concentration of TRMC above 0.08 ng/mL.

The clonogenic assay was performed to investigate the enduring proliferative effect of tocotrienols. Exposure of TRMC on A549 and H520 cells for 72 h irreversibly inhibited 80% clonogenic growth compared with untreated cells (Figure 5.1 CD). For both cell lines, colony formation was greatly reduced at 0.12 ng/mL of TRMC. In this study, there were similar trends in

both MTS and clonogenic assays, suggesting that the commercially available mixture of tocotrienols from capsule significantly inhibited the growth of NSCLC cells.

Tocotrienols induce apoptosis in lung cancer cell lines

Histone/DNA ELISA assay and Annexin V/PI staining were used to evaluate the apoptotic effects of TRMC on A549 and H520 cells. Results from ELISA showed significantly increased apoptosis with concentrations of TRMC on A549 cells and H520 cell lines (Figure 02-AB). To further confirm the results from our histone ELISA data, flow cytometry based quantification was performed after Annexin V/PI staining. Quantitation of apoptotic cells from flowcytometry analysis after treatment with 0.06ng/mL of TRMC for 72 hours showed increased apoptosis in both cell lines (Figure 5.2 CD). Thus, these outcomes evidently suggested that tocotrienols caused a statistically significant increase in the percentage of apoptotic cells in lung cancer cell lines.

Inhibition of cell invasion and migration by tocotrienols

The effect of TRMC on tumor cells invasion and migration were evaluated using Matrigel invasion and wound healing assays. TRMC concentrations (0.4-0.12 ng/mL) resulted in significant decreased penetration of lung cancer cells through the Matrigel-coated membrane as compared with the control cells (Figure 5.3 AB), confirming TRMC reduced the invasion capacity of lung cancer cells. For further confirmation of anti-migratory effects of TRMC, the wound healing assay was performed. The results of the wound healing assay revealed that there was reduced cell migration from custom made wounds with 0.8ng/mL of TRMC after 30 hrs. incubation (Figure 5.3 CD). However, wound healing was significant without TRMC at same incubation conditions.

Down-regulation of the Notch-1 and its target genes expressions by tocotrienols

Investigations of molecular mechanisms behind tocotrienols inhibited cell growth, induced apoptotic cell death, cell invasion and migration in NSCLC cells were evaluated using RT-PCR

and western blot analysis. Dose-dependent response of TRMC showed a significant decrease in Notch-1 m-RNA expressions in A549 and H520 cells after incubating 48 hours (Figure 5.4 AB). Moreover, results from protein expressions in Notch -1 downstream genes, namely HES 1, BcL-XL and Survivin, PARP, were given a dose-dependent decrease with TRMC in western blot analysis (Figure 5.5).

Inhibition of NF-κB DNA-binding activity with tocotrienols

NF-κB and Notch pathways have shown cross talks in many types of cancer including lung cancer [1,2]. Thus, we explored whether the downstream effect of Notch-1 down-regulation was mechanistically linked to the NF-κB pathway. Nuclear proteins from treated and control cells from A549 and H520 were analyzed for NF-κB DNA-binding activity as measured by NF-κB Filter Plate Assay. As demonstrated in Figure 6 A and B, compared to the control, TRMC significantly inhibited the DNA-binding activity of NF-κB for both cell lines.

Discussion

Despite new findings in molecular pathways involved in lung cancer biology and the application of new therapeutic results, NSCLC remains one of the foremost reasons for cancer deaths worldwide [172]. The treatment methods currently available for lung cancer include surgery, target therapy, and different modalities of chemotherapy and radiation therapy. However, these treatment methods have not significantly impacted the five-year survival rate of NSCLC over the last four decades[173]. Poor survival rates are mainly attributed to late diagnosis and tumor metastasis and current chemotherapeutic drugs also have resulted in several side effects, drug resistance, and recurrence among treated NSCLC patients[173]. Therefore, new therapeutic modalities with minimal side effects are urgently needed in improving the treatment outcome to include better long-term survival of patients diagnosed with lung cancer.

PCR and western blot data from in this study clearly demonstrated that TRMC targeted and dose-dependently inhibited expression of Notch-1 in A549 and H520 cell lines. Aberrant expression of Notch has been reported in many types of cancer, including pancreatic, colon, lung, cervical, breast, and skin cancers[174-179]. Notch signaling is also reported to play important roles in regulating cancer cell proliferation, differentiation, invasion, and apoptosis[180-183]. Aberrant Notch 1 expression also exhibited tumor promotion under hypoxia conditions in lung cancer[184]. In another clinical study, Notch 1 expression levels were varying from low to high in some NSCLC patients, and Notch 1 expression was found to be associated with TNM stage in histological grading,[185] suggesting Notch 1 may play key roles in the advancement of NSCLC. Remarkably, we observed TRMC inhibited the cell proliferation dose-dependent manner in MTS and Clonogenic assay alone with Notch-1 inhibition, suggesting that expression of Notch-1 with TRMC may prevent the advancement of A549 and H520 cells. In our previous studies, we clearly demonstrated that δT inhibit cell proliferation by inhibiting different therapeutic targets including Notch-1 [8, 24, 107, 171]. Therefore, TRMC could be a potential Notch-based therapeutic target therapy method in preventing NSCLC.

Furthermore, blockage of the Notch pathway using γ-secretase inhibitor suppressed osteosarcoma growth *in vitro* and *in vivo* [186, 187]. Preclinical studies also showed the therapeutic efficacy of Notch inhibitors against NSCLC in some studies[188]. Stabilized peptides was another approach which interferes with receptor/ligand interactions in Notch signaling pathway [189]. Although these approaches have shown potential in inhibiting Notch-1 expressions, their inhibitory potential has not been evaluated at a clinical level, warranting the importance of exploring novel natural Notch-1 inhibitors with minimal side effects. In this study, we used TRMC which is directly isolated from palm oil with minimum processing. Tocotrienols

have been widely consumed by humans for a long time, and GRAS status for tocotrienols is also granted by FDA [190] . Thus, our approach on inhibition of Notch-1 using TRMC could be a promising strategy to achieve better treatment outcome with minimum side effects for NSCLC patients.

We further observed that TRMC dose-dependently inhibited the HES-1 expressions in A549 and H520 cell lines. It is well documented that Hes-1 is a transcriptional target of the Notch signaling pathway [191], suggesting TRMC dose dependently inhibits the Notch-1 pathway in a downstream manner. Further insight into the molecular mechanism of Notch 1 pathway and its target genes, Notch-1/Hes-1 pathways have been reported to be upstream to NF- κB activation in lung cancer and leukemia cells [24, 192]. In this study, the results from NF- κB filter plate assay clearly showed a dose-dependent decrease in NF- κB DNA-binding activity in A549 and H520 cell with different concentrations of TRMC. According to current evidence, Notch ligands induced NF-κB activation in leukemia cells and decreased Notch-1 lowered NF-κB DNA binding activity [193]. Another study also reported that mice with reduced Notch activities had significantly decreased NF-κB activity [194]. Schwarzer1 *et al.* reported Notch had exerted its effects through regulation of NF-κB in human lymphomas [195]. Our previous study also demonstrated the cross talk between the Notch pathway and NF-κB pathway in adenocarcinoma lung cancer cell lines, which was induced by δT [8, 24, 107, 171]. Therefore, our data alone, in conjunction with current evidence, strongly supports that TRMC inhibits notch-1 mediated NF-Kb pathways.

TRMC induced apoptosis in A549 and H520 cells dose dependently in this study. The TRMC consisted of *α*-tocotrienol, ß-tocotrienol, y-tocotrienol and δ-tocotrienol isomers. Some studies have shown that y-tocotrienol induced apoptosis in neuroblastoma SH-SY5Y cells[196] and human gastric cancer cells [79]. In our previous study, we clearly showed δT induced the

apoptosis in NSCLC in a dose and time-dependent manner at 10 -30 µM concentration range. In another study, gamma and δT exerted a more potent anticancer effect on breast cancer cell lines as compared to α-tocotrienol[197]. Numerous results from recent studies on tocotrienols also indicated that γ- and δ-tocotrienols exhibited greater anticancer activity than α- or β-tocotrienols; where δT shows a higher efficacy and effectiveness in the induction of apoptosis in both A549 and U87MG cancer cells as compared to alpha- and gamma-tocotrienols [109]. Therefore, induction of apoptosis in A549 and H520 cells with TRMC could be the result of individual γ- and δ-tocotrienol isomers, or from their cumulative effects.

Bcl-2 and Bcl-X_L inhibitor proteins play a significant role in apoptosis [70, 198]. We observed a dose decrease in Bcl-2 gene expression by PCR and Bcl-X_L protein expression by western blot analysis. Also, we found inhibition of survivin protein with TRMC in Western blot analysis where survivin, a member of the inhibitor of apoptosis, inhibited caspase activation, thereby leading to negative regulation of apoptosis [199]. Moreover, a down-regulation of Notch-1 was observed to decrease Bcl-XL apoptosis protein expression in pancreatic cancer cells. In breast cancer, down-regulation of Notch-1 is associated with the lower expression of Bcl-2 and Bcl-XL [200]. Activated Notch-1 pathway can increase the expression of survivin expression [201]. Furthermore, Survivin, Bcl-2, and Bcl-XL are downstream targets of the NF-Kb in several cancers cells. Consistent with above, we suggest that TRMC inhibits survivin, Bcl-2, and Bcl-X_L via down-regulation of Notch-1 and NF-Kb that it lead in inducing apoptosis in NSCLC.

Further studies need to be done to assess the anti-cancer activity and bioavailability of vitamin E capsules that are readily available. Bioavailability is always a potential concern for all nutraceuticals. Although *in vitro* experimental evidence has been very promising, oral supplementation of tocotrienols in animal and human studies has produced varying results [202].

Oral absorption of tocotrienols into the circulation is mediated by a carrier transporter system that displays saturation and down-regulation when exposed to high concentrations of tocotrienols [24, 202]. To compensate for these limitations in oral absorption of tocotrienols, investigators have developed new derivatives and nanoparticle delivery systems that significantly enhance tocotrienol bioavailability and, therefore, the therapeutic effects of tocotrienols on cancer [202]. In addition to bioavailability, timing and dosage are also concerns, and these factors will be different for cell cultures versus animals versus humans. Further experiments need to be conducted to investigate whether this capsule can show the same results in animals before it can be taken to a human trial, which is the ultimate goal.

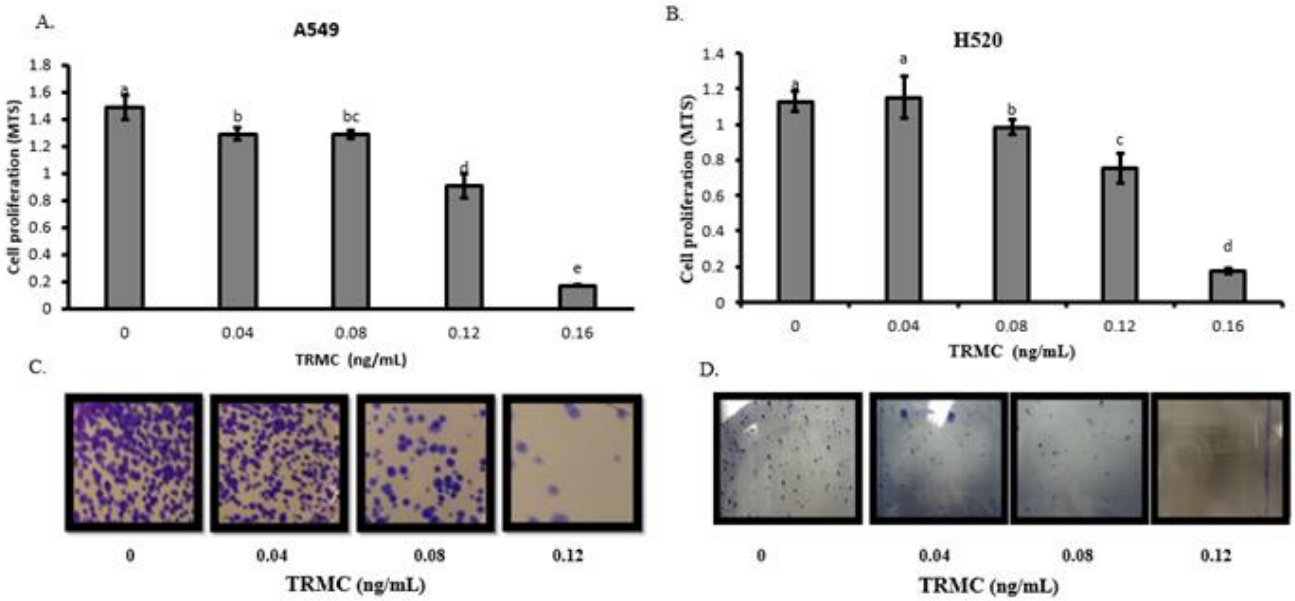

Figure 5.1: Antiproliferative effects of TRMC on A549 (A) and H520 (B) cells were determined using MTS assay.

Both A549 and H520 cells were initially plated at a density of 5×10^3 cells/well (3 wells/group) in 96-well plates and grown in experimental medium containing 0, 0.04,0.08, 0.12 and 0.16 ng/mL of TRMC for 72 h. Viable cell number was determined using the MTS colorimetric assay. Vertical bars indicate the mean absorbance ± SEM (n = 3) where mean absorbance, represented by different letters, is significantly different ($P < 0.05$). Cell survival of human NSCLC cell lines, A549 (C) and H520 (D) cells, in clonogenic assay. A549 and H520 cells treated with different concentration of TRMC (0, 0.04, 0.08, and 0.12 ng/mL) were evaluated by the clonogenic assay. The

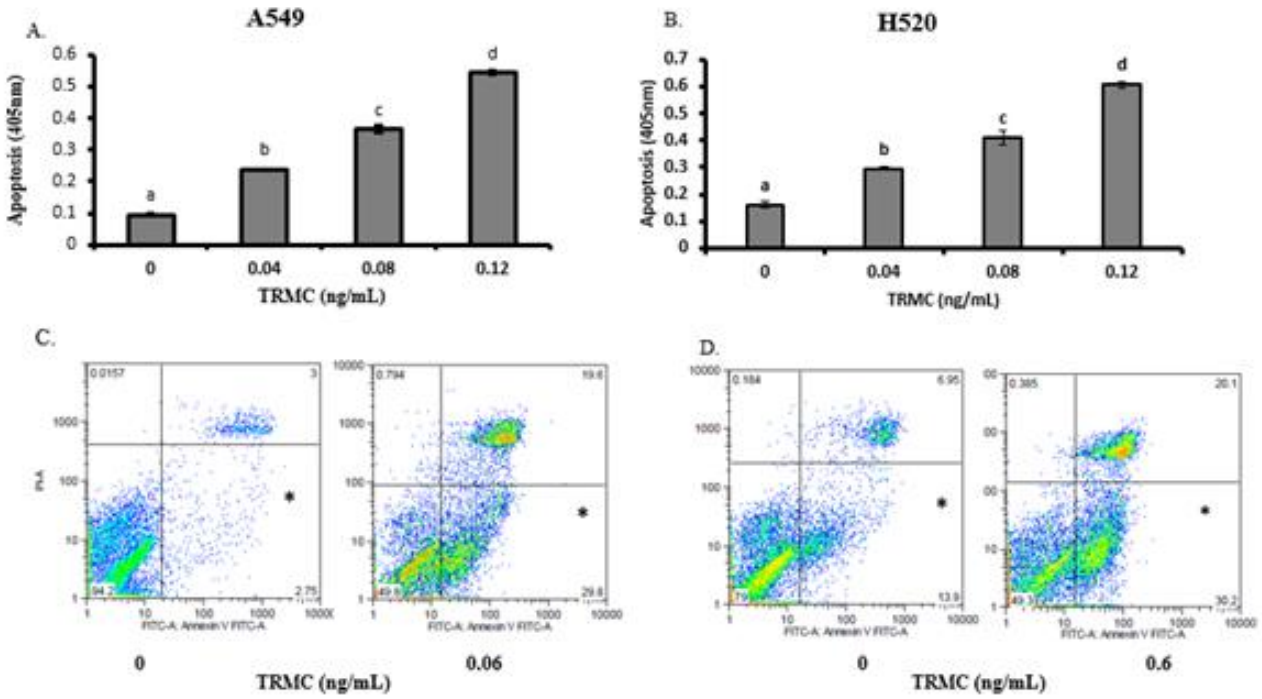

Figure 5.2: Apoptotic effects of TRMC on A549 (A) and H520 (B) were determined using histone/DNA ELISA.

Cells were treated with increasing concentration of TRMC for 72h. Vertical bars indicate the mean absorbance ± SEM (n = 3) where mean absorbance, represented by different letters, is significantly different ($P < 0.05$). Apoptosis of A549 (C) and H520 (D) cells were determined by Annexin V-FITC based flow cytometry analysis. Cells were treated with 0.6ng/mL of TRMC for 48 h and Apoptotic cells were detected from flow cytometry. Quadrant with (*) indicates early apoptotic cells after with or without treatment.

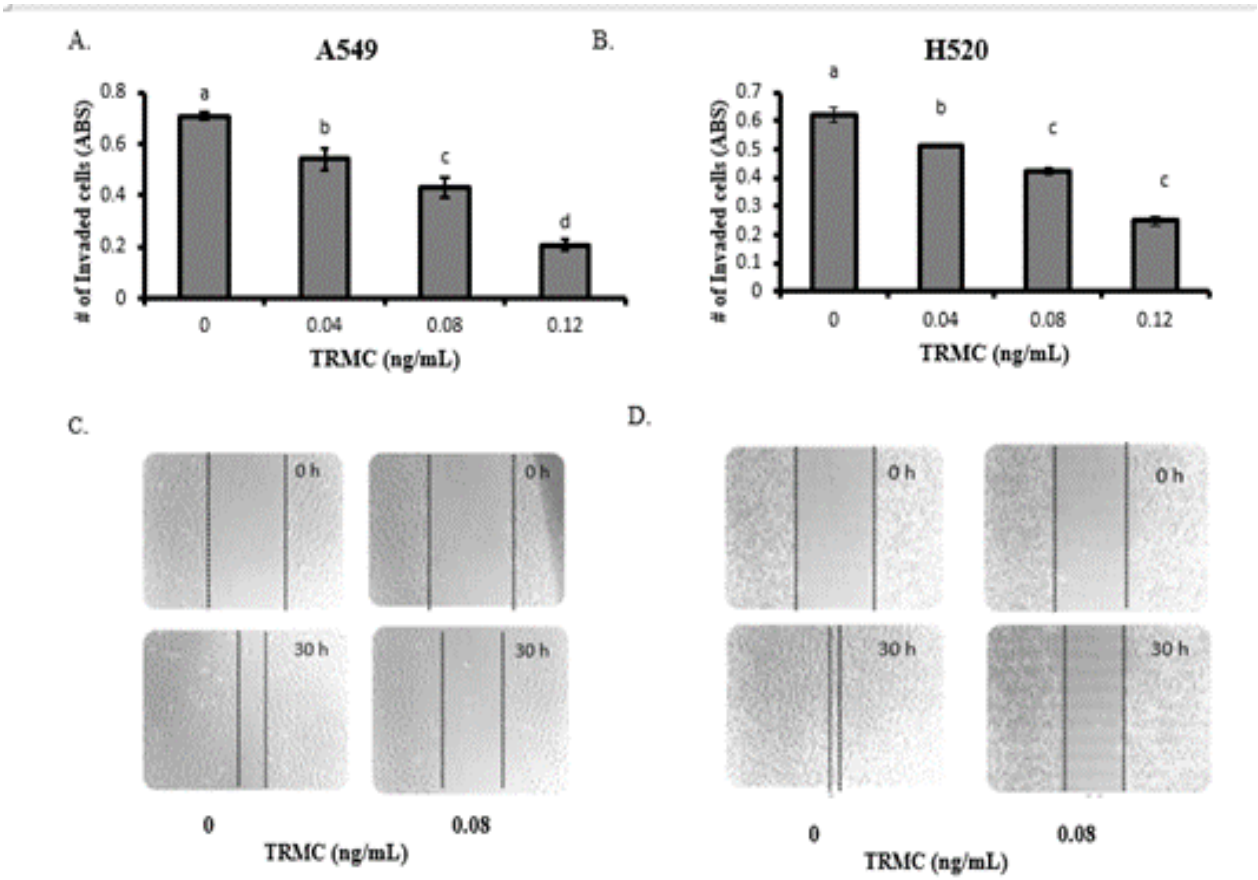

Figure 5.3: TRMC inhibits cell migration and invasion in NSCLC cells.

A549 (A) and H520 (B) cells were seeded treated seeded into Matrigel-coated inserts with TRMC or DMSO. Cells that invaded the lower surface of the insert over a period of 20 hours were stained with crystal violet dye, followed by the absorbance reading. Vertical bars indicate the mean absorbance ± SEM (n = 3) where mean absorbance represented by different letters is significantly different (one-way ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$). (C and D), dose-dependent inhibition of NSCLC cells migration by TRMC using the wound healing assay. Uniform wounds were done by scratching in confluent cultures which were treated with TRMC over 30 h. After that, the wound healing images were captured using a microscope at 10× objective.

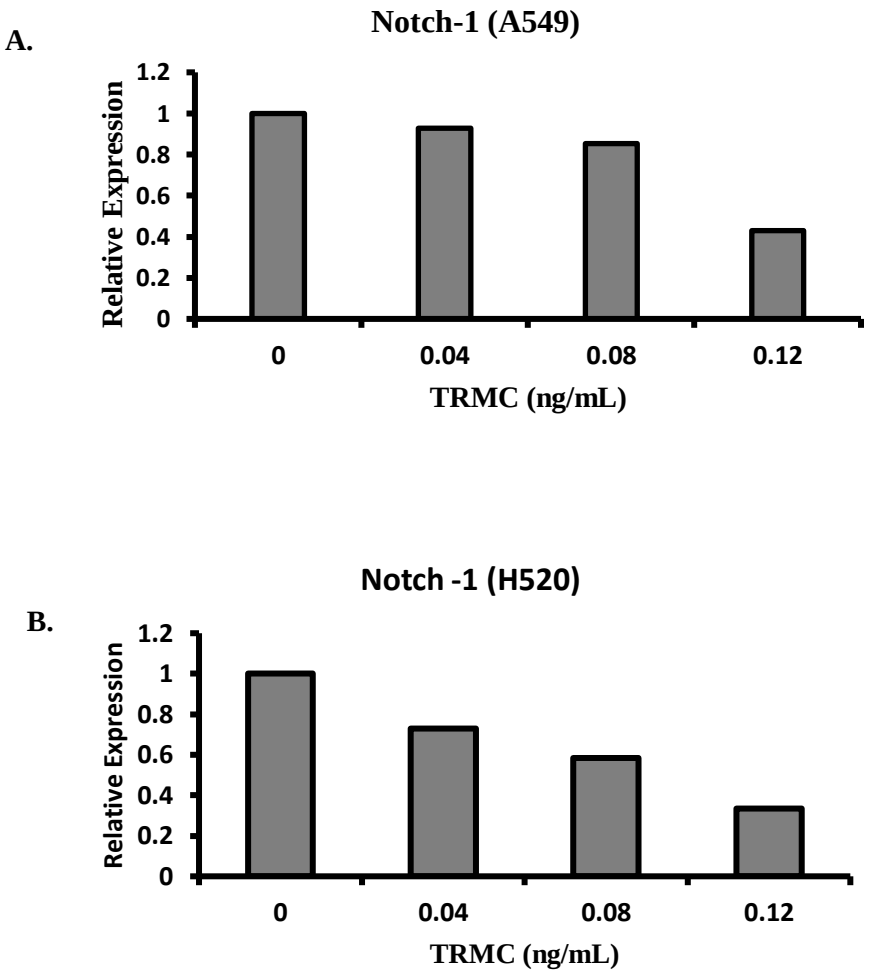

Figure 5.4: Dose-dependent down-regulation of Notch-1 gene expression by TRMC.

A549 (A) and H520 (B) cells were treated with or without TRMC for 72 h. Data are expressed as delta C_T values normalized against β-actin (mean±SE).

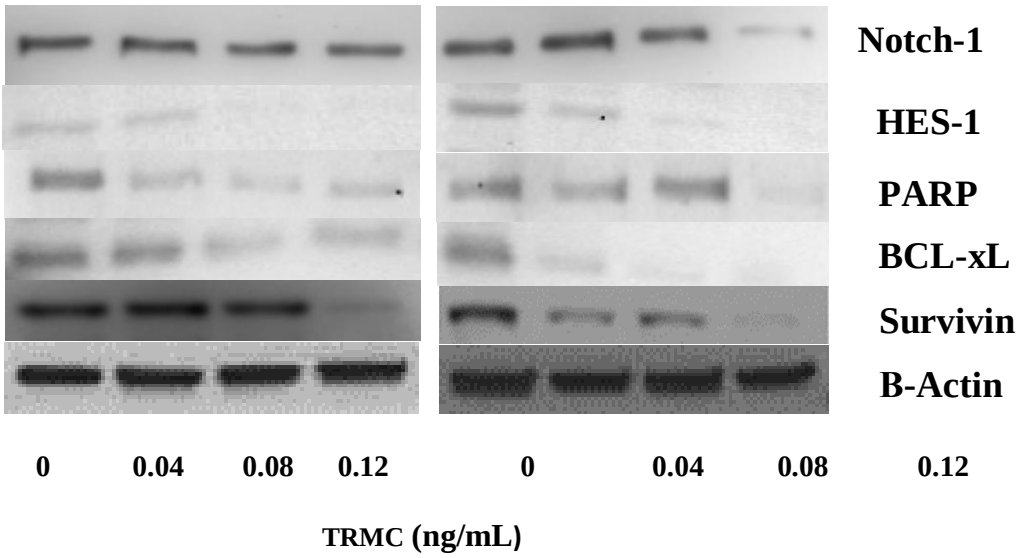

Figure 5.5: Down-regulation of Notch-1, Hes-1, PARP, Survivin and BCL-2 by TRMC.

The expressions of protein were detected by western blot analysis in A549 (A) and H520 (B) cells after treating with or without TRMC for 72 hours.

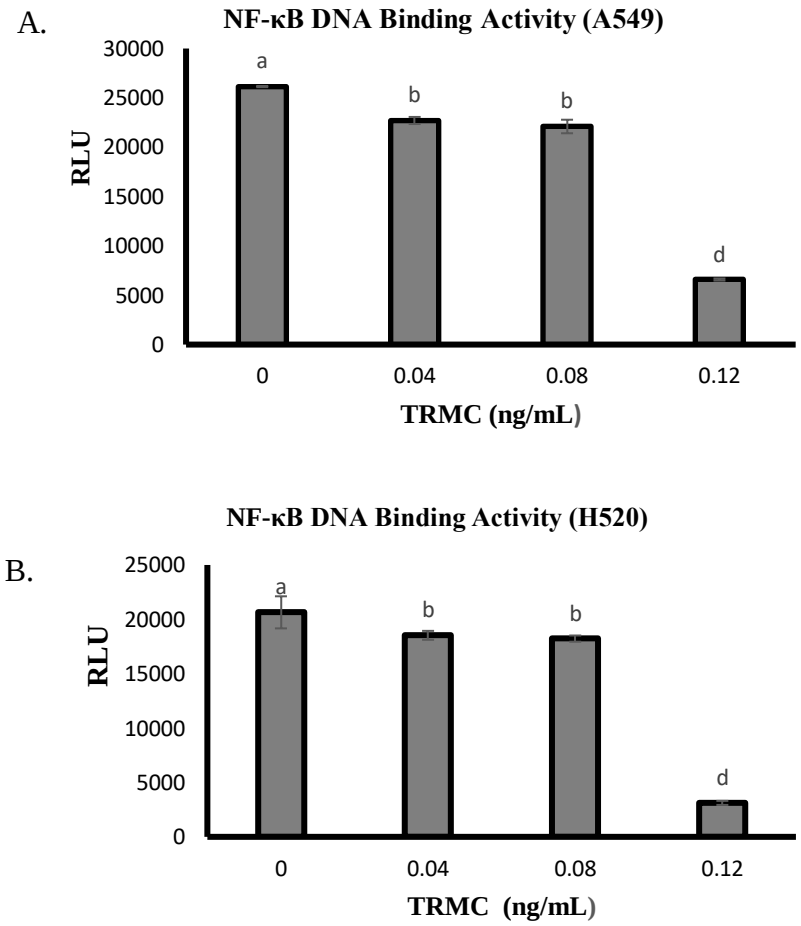

Figure 5.6: Dose-dependent down-regulation of NF-κB DNA Binding Activity by TRMC.

A549 (A) and H520(B) cells were incubated with increasing concentrations of TRMC or DMSO-control for 72 h, and nuclear proteins binding activities were evaluvated by ELISA Vertical bars indicate the mean absorbance ± SEM (n = 3) where mean absorbance represented by different letters is significantly different (one-way ANOVA followed by Dunnett's multiple comparison test, $p < 0.05$).

CHAPTER 6 CONCLUSIONS AND FUTURE DIRECTIONS

NSCLC has shown a 5-year survival rate of 50% at early stages, but the rate is significantly reduced to less than 5% for cases diagnosed with distant metastasis in late stages. The current evidence clearly suggests that MMP-9 mediated metastasis is one of the key invasion and metastasis factors in lung cancer patients. In this study, we clearly showed that δT inhibits MMP-9 expression along with its key functions namely cell migration, cell invasion, cell adhesion, cell aggregation and cell proliferation. We also explored the underlying mechanism for those regulations as suppression of Notch-1 activation through reducing translocation to the nucleus and DNA-binding activities of NF- κB, leading to the down-regulation of uPA and MMP-9 expression. Further, δT induced the miR 451 expression in A549 and H1299 cells which show possible crosstalk between miR 451, Notch -1 and NF- κB. Thus, we can suggest that δT inhibits the MMP-9 mediates cell metastasis *in vitro*. However, further studies on the effect of δT on animal models and a human clinical trial are need be established to understand the efficacy of δT in inhibiting metastasis at in *in-vivo* levels. Moreover, MMP-9 is not the only pathway that it is involved in regulating metastasis in NSCLC. Therefore, further investigation of tocotrienol involvement in other metastasis pathways is needed to investigated.

The current research provides substantial evidence that the primary energy source of NSCLC is glutamine, and that NSCLC exhibits a high rate of glutamine dependency during growth and development. Furthermore, excess glutamine and essential amino acids in NSCLC are also reported to upregulate mTOR, a bioenergetics sensor which regulates cell growth, cell survival, and protein synthesis in NSCLC. In the present work, we employed NMR-based metabolomics approaches to reveal metabolism regulations of tocotrienol on NSCLC, namely A549 and H1229 cell lines. The metabolomics profiling of NSCLC lysates showed that changes in the glutamine

related metabolisms are highly affected after treating with δT. Further, δT works by inhibiting glutamine uptake into proliferating cells through glutamine transporters (LAT-1 and inhibition, thus resulting in inhibition of cell proliferation and induction of apoptosis via downregulation of the mTOR pathway. As future directions, investigation of glutamine metabolism using stable isotopes based flux analysis after treating with/without tocotrienols is needed as it provides a detailed overview of biochemical pathways of glutamine fate and help in identification of complete mechanistic pathway. Also, clinical or animal trail may also recommend in performing with δT supplementation among NSCLC patients to investigate the effect of tocotrienol on glutamine metabolism at *in-vivo* levels.

Previously, we reported that δT downregulates the Notch-1 via NF -KB. However, the pure isomers are presently not available in quantities required for animal or clinical studies. Therefore, the objective of this study was to investigate the interactions and effects of commercially available tocotrienols (a mixture of isomers) on the Notch-1 pathway in adenocarcinoma (A549) and squamous cell carcinoma lung cancer (H520) cell lines. Interestingly, we observed the same effect with a mixture of tocotrienol isomers directly isolated from the palm oil compared to pure δT. Briefly, tocotrienol isomers in capsule inhibit cell growth, migration, and tumor cell invasiveness via down-regulation of Notch 1 and NF -KB while inducing apoptosis. Hence, these commercially available tocotrienol rich capsule could be an effective therapeutic for lung cancer prevention at *in vivo.*

Before going for the *in vitro* studies, the bioavailability of vitamin E capsules need to be studied as bioavailability is always a potential concern for all nutraceuticals. Although experimental *in-vitro* evidence has been very promising, oral supplementation of tocotrienols in animal and human studies has produced inconsistent results [202]. Oral absorption

of tocotrienols into the circulation is mediated by a carrier transporter system that displays saturation and down-regulation when exposed to high concentrations of tocotrienols [24, 202]. To compensate for these limitations in the absorption of tocotrienols, a new delivery system such as new derivatives and nanoparticle delivery systems could be developed as they significantly enhance tocotrienol bioavailability. In addition to bioavailability, timing and dosage are also concerns, and these factors will be different for cell cultures versus animals versus humans. Further experiments need to be done to investigate whether or not this capsule can show the same results in animals before it can be taken to a human trial, which is the ultimate goal.

Research on nutraceuticals and their influence on the processes involved in preventing tumor development, to lower the incidence risk of common cancers, is lacking at the clinical level. Despite the dearth of clinical data, many cancer patients turn to nutraceuticals from a belief that they will not cause pain, and may benefit their clinical outcome. Caution is paramount when considering certain bioactive including tocotrienol, as they may antagonize the activity of therapeutic agents, or, in some cases, alter their metabolism from efficacious forms. There is a promise on the horizon, in that there are some molecular targets in common for nutraceuticals including tocotrienols that have inhibited, at least experimentally *in vivo*, some aspects of cancer. Tocotrienols may be generally safe to use during therapy, but this area of research is still immature. The long-term plan should be to identify novel therapeutic targets of tocotrienols that would allow innovative utilization of tocotrienols as adjunct to both conventional chemotherapy and radiation therapy, in order to improve the overall quality of life and survival of patients diagnosed with cancers.

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ABSTRACT

ANTI-CANCER EFFECTS OF TOCOTRIENOLS IN NSCLC

by

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Lung cancer is one of the leading causes of death among cancers, with non-small cell lung cancer (NSCLC) accounting for 80-85% of all lung cancers and a five-year survival rate of 5 % at stage IIIB. Delta-tocotrienol (δT) including other tocotrienol isomers has been shown to exhibit anti-tumor activity via inhibition of different signaling pathways in tumors including NSCLC. Previously we reported that δT reduced cell invasion via inhibition of the Notch-1 and NF-κb pathway.

Matrix metallopeptidase 9 (MMP9) dependent cell migration and invasion are key processes in cancer metastasis. Hence, its suppression is a promising strategy for cancer therapeutics. The objective of specific aim 01 was to investigate the possibility of MMP9 inhibition as the underlying mechanism behind the anti-metastatic effects of δT on NSCLC cell lines A549 and H1299. Effects of δT on cell migration, invasion, cell adhesion and aggregation capability were investigated. MMP-9 activity was determined using gel zymography. The various 129 proteins, genes, and miR involved in the Notch-1 and uPA signal transduction pathways have been

studied for anti-metastatic activity by RT-PCR and western blot. Our findings showed that δT reduced cell migration, invasion, and adhesion in a dose and time-dependent manner. δT significantly inhibited MMP-9 activity in gel zymography. Further, δT inhibited Notch-1 mediated NF-κb and urokinase plasminogen activator (uPA) pathways which lead to the down-regulated expression of MMP-9 and increased miR 451 expressions. Our data suggests that δT attenuates tumor aggressiveness, invasion, and metastasis by down-regulation of the MMP-9 gene via Notch1 and uPA pathways

The primary energy source of NSCLC is glutamine, and this cancer exhibits a high rate of glutamine dependency during its growth and development. Glutamine and essential amino acids (EAA) in NSCLC are reported to upregulate mTOR, a bioenergetics sensor which regulates cell growth, cell survival, and protein synthesis. SLC1A5/SLC7A5 transporters that allow glutamine and EAA to enter the proliferating tumors and send a regulatory signal to mTOR are novel concepts in cancer cell growth and development. Therefore, inhibiting glutamine uptake via blocking or downregulating glutamine transporters would be an excellent therapeutic target for NSCLC treatment. The Specific Aim:2 of this study, was to verify the metabolic dysregulation of glutamine and its derivatives in NSCLC using cellular 1H-NMR metabolomics approach while investigating the effect of δT on NSCLC growth and development, glutamine transporters, and mTOR pathway. Endometabolome of NSCLC was analyzed followed by cellular metabolomics analysis using SIMCA+ multivariate analyzing software. The results in metabolomics analysis showed significant inhibition in the uptake of glutamine, its derivatives; glutamate and glutathione, and some EAA in both cell lines with δT treatment providing their potential use as robust surrogate biomarkers for δT intervention in NSCLC. To further validation, NMR spectrums were quantified 130 using Chenomx NMR Suite and metabolites were further analyzed using metaboanalyst 3.0

software. The results in metaboanalyst 3.0 indicated that δT directly impacted on the metabolism of glutamine and its derivatives where further validating results at previous analysis. Therefore, expression of glutamine transporters and mTOR pathway proteins were explored, and dosedependent inhibition of glutamine transporters (SLC7A5 and SLC1A5) and mTOR pathway proteins (P-mTOR, mTOR, S6K, c-MYC and Bcl-XL) was evident in western blot analysis. Our findings suggest that δT works by inhibiting glutamine uptake into proliferating cells through glutamine transporter inhibition thus resulting in inhibition of cell proliferation and induction of apoptosis via downregulation of the mTOR pathway.

As specific aim, one and two reported that δT shows anti-cancer properties by targeting different anti-cancer mechanisms. However, the δT are presently not available in quantities required for animal or clinical studies. Therefore, the objective of specific aim 03 is to investigate the interactions and effects of commercially available tocotrienols mixture directly isolated from palm oil (a mixture of isomers) in adenocarcinoma (A549) and squamous cell carcinoma lung cancer (H520) cell lines. A dose-dependent decrease in all growth was observed in both cancer cell lines with the addition of tocotrienols by MTS and colonogenic assay. Furthermore, a significant reduction in cell migration and tumor invasiveness was seen in both cell lines. Additionally, a significant induction of apoptosis was observed in Annexin V stain in flow cytometry analysis. Since tocotrienols showed effects against proliferation, apoptosis, migration, and invasiveness, RT-PCR and western blot analysis were used to explore molecular mechanisms behind above regulations by testing the expression of Notch-1 and its downstream stream genes. A dose-dependent decrease in expression was observed in Notch-1, Hes-1, Survivin, MMP-9, VEGF, and Bcl-XL proteins. Also, we found a mechanism linking the NF-kB pathway and Notch-131 1 down-regulation from NF- Kb colorimetric assay. Thus, our data suggests that commercially

available tocotrienols inhibits cell growth, migration, and tumor cell invasiveness via downregulation of Notch 1 and NF -KB while inducing apoptosis. Hence, these commercially available tocotrienol rich capsule could be an effective therapeutic for lung cancer prevention as same as pure δT. These anticancer effects and mechanisms of δT warrant further investigation of δT as a potential natural therapeutic approach to prevent NSCLC.

AUTOBIOGRAPHICAL STATEMENT

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EDUCATION

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- Selected among the 100-future young bio-leaders to present at the world's first global leadership summit in Biotechnology at Cambridge University, United Kingdom. 2014
- PhD Achievement Award, IFT- Great lake Section, IFT, USA. 2014
- Best poster award in Diet and Cancer Section, American Nutrition Society. 2014
- Summer Destination Fellowship, Wayne State University. 2016
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