EFFECTS OF NOVEL COMPOUND DERIVED FROM VITAMIN E ON PROSTATE CANCER CELL LINE

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EFFECTS OF NOVEL COMPOUND DERIVED FROM VITAMIN E ON PROSTATE CANCER CELL LINE

submitted by **NURİ ERGEN** in partial fulfillment of the requirements for the degree of **Doctor of Philosophy of in Biological Sciences Department, Middle East Technical University** by,

Prof. Dr. Gülbin Dural Ünver _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. Orhan Adalı _____
Head of Department, **Biological Sciences**

Prof. Dr. Feride Severcan _____
Supervisor, **Biological Sciences Dept., METU**

Examining Committee Members:

Prof. Dr. Mesut Muyan _____
Biological Sciences Dept., METU

Prof. Dr. Feride Severcan _____
Biological Sciences Dept., METU

Assoc. Prof. Dr. Çağdaş Devrim Son _____
Biological Sciences Dept., METU

Assist. Prof. Dr. Bala Gür Dedeoğlu _____
Biotechnology Institute, Ankara University

Assist. Prof. Dr. Özgür Şahin _____
Molecular Biology and Genetics Dept., Bilkent University

Date: 9.12.2016

I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

Name, Last name: Nuri ERGEN

Signature :

ABSTRACT

EFFECTS OF NOVEL COMPOUND DERIVED FROM VITAMIN E ON PROSTATE CANCER CELL LINE

Ergen, Nuri

Ph.D., Department of Biological Sciences

Supervisor: Prof. Dr. Feride Severcan

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Cancer is a complex disease characterized by uncontrolled cell proliferation, invasion and metastasis. It is known that vitamins having high antioxidant capacity, like vitamin A, C and E, play important roles in adjuvant therapy and as cancer protective agents. Vitamin E is a group of fat soluble antioxidant nutrients consisting of tocopherols and tocotrienols. Various research has been performed on the utility of vitamin E derivatives on cancer treatment. Energy metabolism of cells alters during cancer progression due to the increased energy need of cells. Changes in fatty acid, phospholipid and triglyceride amounts in cancer cells were reported in previous studies. Prostate cancer cells, which are regulated by the lipid derivative molecules like androgen have remarkable changes in lipid metabolism. A novel vitamin E derived compound, which has modification on the side chain, was used in this study in order to reveal its roles on cancer progression and cancer lipid metabolism.

Based on several experiments performed in this study, it has been concluded that alpha tocopherol derived compound had less toxicity to normal prostate epithelium and compound treatment caused reduction in proliferation and increase in apoptosis of the prostate cancer cell line LNCaP by causing mitochondrial membrane depolarization. Also, cells treated with the novel compound had a remarkable reduction in their motility and migration capacity implying anti metastatic activity and compound treatment led to alterations on lipid metabolism which were detected at both spectroscopic and protein, mRNA levels. Additionally, cells treated with compound show decreased lipid peroxidation as well as increased lipid order that leads to changes in cellular membrane structure. These properties of alpha tocopherol

derived compound emphasize the importance of this molecule as a possible therapeutic option in prostate carcinogenesis.

Keywords: Vitamin E, alpha tocopherol derivatives, prostate cancer, lipid metabolism

ÖZ

E VİTAMİNİ TÜREVLİ YENİ BİR MOLEKÜLÜN PROSTAT KANSERİ HÜCRE HATTI ÜZERİNDEKİ ETKİLERİ

Ergen, Nuri
Ph.D., Biyolojik Bilimler Bölümü

Tez Yöneticisi: Prof. Dr. Feride Severcan
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Kanser, kontrol dışı hücre çoğalması, invazyon ve metastaz ile ilişkilendirilen kompleks bir hastalıktır. Antioksidan özelliğe sahip olan A, C ve E vitaminlerinin, destekleyici (adjuvan) terapide ve kansere karşı koruyucu olarak kullanıldıkları bilinmektedir. E vitamini yağda çözünen, antioksidan özelliğe sahip, tokoferol ve tokotrienollerden oluşan bir vitamin grubudur. E vitamini ve E vitamini türevlerinin kanser tedavisinde kullanımı ile ilgili yapılmış birçok çalışma bulunmaktadır. Kanser gelişimi sırasında, artan enerji ihtiyacına bağlı olarak hücrelerin enerji metabolizmaları değişikliğe uğramaktadır. Kanser hücrelerinde, glikoz, yağ asidi, fosfolipit ve trigliserit miktarındaki değişimler rapor edilmiştir. Androjen gibi lipit temelli moleküller tarafından regüle edilen prostat kanseri hücrelerinde lipit metabolizmasında çarpıcı değişimler meydana gelmektedir. Yan zincirinde değişiklik yapılan yeni bir E vitamini türevi bu çalışmada kullanıldı ve bu molekülün kanser gelişimi ve kanser lipit metabolizması üzerindeki etkileri belirlendi.

Bu çalışmada yapılan birçok deney sonucunda şu sonuçlar elde edildi: E vitamini türevi bileşik, normal prostat epitel hücrelerinde daha düşük seviyede toksisiteye sebep olmuştur ve molekül muamelesi sonucunda hücre proliferasyonunda azalma ve apoptozda mitokondriyel membran depolarizasyonuna bağlı olarak artış meydana gelmiştir. Ayrıca bu bileşik, hücrelerin hareket ve göç kabiliyetlerinin azalmasına sebep olmuştur ve kanser hücrelerinin lipit metabolizmasında anlamlı değişikliklere yol açmıştır. Bunlara ek olarak, molekül lipit peroksidasyonunda azalmaya neden olup, lipit düzenlenmesinde artışa yol açarak membran yapısında değişikliğe sebep

olmuştur. E vitamini türevli molekülün belirtilen tüm bu özellikleri, molekülün prostat kanseri tedavisinde, bir destekleyici tedavi seçeneği olarak kullanılma olasılığını artırmaktadır.

Anahtar kelimeler: E Vitamini, alfa tokoferol türevleri, prostat kanseri, lipit metabolizması

Dedicated to my unique and distinguished family and my beloved grandmothers who passed away during this PhD study,

Sevim Ergen and Hikmet Aktaş

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LIST OF ABBREVIATIONS

ATR	Attenuated Total Reflectance
FTIR	Fourier Transform Infrared Spectroscopy
ESR	Electron Spin Resonance
HCA	Hierarchical Cluster Analysis
PCA	Principal Component Analysis
VES	Vitamin E Succinate
VEA	Vitamin E Analogs
PUFA	Poly Unsaturated Fatty Acids

CHAPTER 1

INTRODUCTION

1.1. Cancer

Cancer is a group of disorder characterized by unusual cellular proliferation with the ability to spread into the distant parts of the organism (Hanahan and Weinberg, 2011). Cancer is among the leading reasons of mortality worldwide, with approximately 14 million new cases and 8.2 million cancer related deaths in 2012 (Torre et al., 2015). The five most prevalent sites of cancer diagnosed in 2010 were lung, prostate, colorectal, stomach and liver cancer among men (Jemal et al., 2011). On the other hand, among women, the five most widespread cancers were breast, colorectal, lung, cervix and stomach (Jemal et al., 2011). Potential signs and symptoms of cancer involve a mass, unusual bleeding, continuous cough, weight loss and alterations in intestinal activity. It is known that more than 100 different types of cancer affect human beings (Duffy, 2013). Not all the tumors are considered as cancer, because benign tumors do not migrate to the other parts of the body (Hanahan and Weinberg, 2000).

Carcinogenesis, which is known as cancer development process, is a multi-stage event triggered by various carcinogens (Dorai and Aggarwal, 2004). Smoking and tobacco usage are the cause of approximately 22% of cancer deaths in the world, another 10% is because of consuming alcohol, obesity, poor diet and lack of physical activity (World Health Organization, Last accessed on August 2016). Other potential risk factors include environmental pollutants, exposure to ionizing radiation and infections (Anand et al., 2008). Cancer causing viral infections including HBV/HCV and HPV are responsible for up to 20% of cancer deaths in low and middle income countries (de Martel et al., 2012). Therefore, we can summarize these risk factors in 3 different groups: (1) physical carcinogens, such as ultraviolet and ionizing

radiation; (2) chemical carcinogens, such as asbestos, components of tobacco smoke, aflatoxin and arsenic ; and (3) biological carcinogens, such as infections from different viruses, bacteria or parasites. These factors act by altering the genetic structure of a cell (World Health Organization, Last accessed on August 2016). Basically, many genetic changes are required before the cancer develops. Nearly 5–10% of cancers are because of the inherited genetic mutations from a patient's parents (American Cancer Society, Last accessed on September 2016). The altered genes are divided into two main categories. Firstly, oncogenes are the genes promoting cell growth and reproduction. Secondly, tumor suppressors are the genes inhibiting cell division and survival. Changes in several genes are required to transform a normal cell into a cancer cell and malignant transformation can occur through the emergence of novel oncogenes, the abnormal over expression of normal oncogenes, or by the under expression or loss of function of tumor suppressor genes (Knudson, 2001). These various mutations may occur via deletions, insertions, point mutations, missense mutations, nonsense mutations and translocations. Large-size mutations contain the deletion or insertion of a portion of a chromosome. On the other hand, small-size mutations containing point mutations, insertions and deletions, which may occur in the promoter region of a gene and influence its transcription, or may take place in the gene's coding sequence and affect the function of its protein product. Besides DNA sequence changes, aberrant epigenetic changes lead to malignant transformation due to loss of cellular control (Hanahan and Weinberg, 2011).

1.2. Hallmarks of Cancer

Throughout the multi-stage progress of cancer, several biological capabilities are acquired by tumor cells (Hanahan and Weinberg, 2011). These biological features are given below, also the image with the drugs used to interfere with each capability are demonstrated in Figure 1.

- Sustaining proliferative signaling
- Evading growth suppressors
- Avoiding immune destruction
- Enabling replicative immortality
- Tumor promoting inflammation
- Activating invasion and metastasis
- Inducing angiogenesis
- Genome instability and mutation
- Resisting cell death
- Deregulating cellular energetics

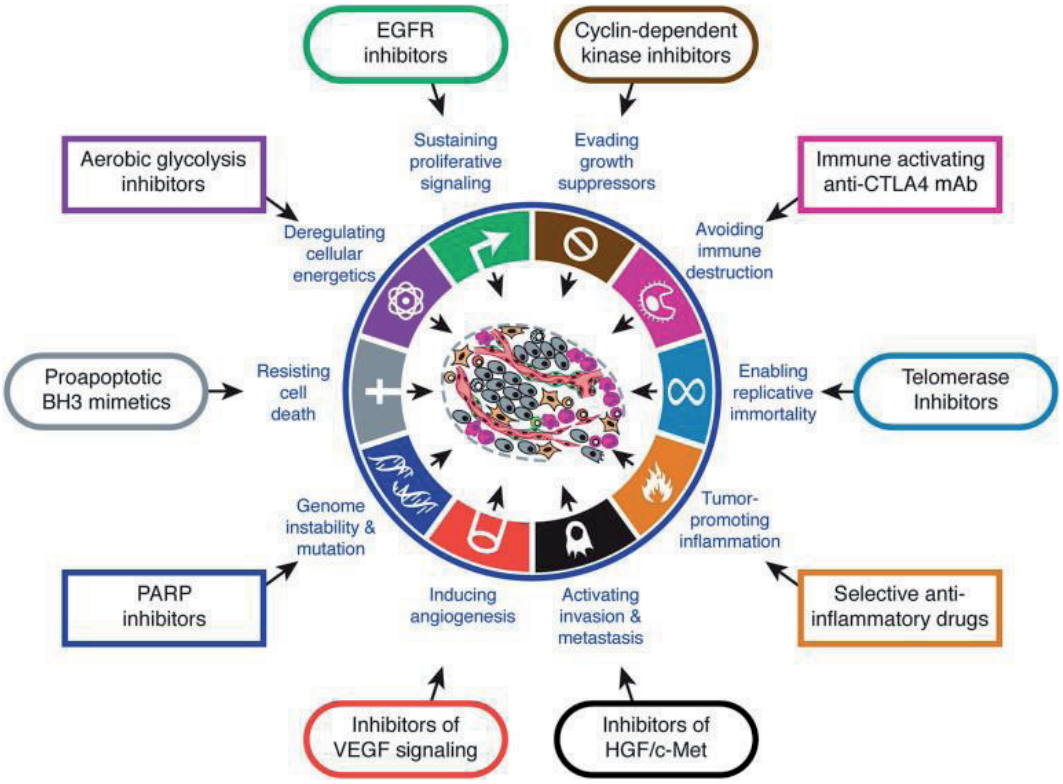


Figure 1: The hallmarks of cancer and the drugs used to interfere with each acquired biological capability, adapted from (Hanahan and Weinberg, 2011)

Normal healthy cells precisely control the production and secretion of mitogenic signals that direct entry into the cell cycle, so that providing a homeostasis of cell number and maintenance of normal tissue structure and function. Cancer cells disrupt this process and decide to their own fate. The growth signals are conveyed into the cell by transmembrane receptors which bind to different classes of signaling molecules: growth factors, extracellular matrix components, and cell to cell adhesion molecules. Tumor cells can gain the ability to keep proliferating by activating different mechanisms. For example, they may produce their own growth factor signals, thus getting rid of requirement for stimulation by microenvironment (Witsch et al., 2010). They may respond to their own produced growth factor signals through the activation of cognate receptors in an autocrine manner (Lemmon and Schlessinger, 2010).

In an alternative way, cancer cells may send messages to trigger normal cells within the stroma. Eventually, normal cells reply this stimulation by producing and providing growth factors to cancer cells (Cheng et al., 2008). Proliferative signaling may also be disrupted by upregulation of receptor proteins located at the tumor cell surface. Similar stimulation can result from structural changes in the receptors facilitating ligand independent activation. Somatic mutations on the genes associated with proliferative signaling such as PI3K, Akt and MAPK lead to dysregulation in cell growth and results in cancer (Davies and Samuels, 2010; Jiang and Liu, 2009).

Besides sustaining proliferative signaling, cancer cells must evade growth suppressors which are under control of tumor suppressor genes. Under normal conditions, negative feedback mechanisms work to attenuate proliferation signaling (Amit et al., 2007). Aberrations in these feedback systems causes enhancement of mitotic signaling. Two different mechanisms exist which can prevent cellular proliferation. First of which involves sending the cells out of cell cycle into an inactive G_0 state (quiescent mode). The other mechanism can be explained by inducing cells to post-mitotic states called senescence. The two key tumor suppressor molecules P53 and Retinoblastoma (RB) acts at a crucial point to decide whether to proliferate or to go senescence or apoptosis (Hanahan and Weinberg, 2000).

Apoptosis is one of the most important cellular processes that provides homeostasis. In case of cellular damage, some specific mechanisms are activated and cells undergo apoptosis to keep homeostasis. However, in cancer cells this mechanism is dysregulated and tumor cells start to evade from apoptosis by activating or inhibiting different pathways (Adams and Cory, 2007). Under normal circumstances, apoptosis can be initiated through one of two pathways. In the intrinsic pathway, the cell goes to apoptosis due to the fact that it senses cellular stress, while in the extrinsic pathway the cell kills itself because of the signals coming from outside (Uguz et al., 2009). Both pathways induce cell death by activating caspases or enzymes that degrade proteins. The intrinsic pathway of caspase activation is induced by the events such as DNA damage, growth factor withdrawal, or loss of contact with the extracellular matrix. These events cause alterations in the integrity of the mitochondrial membrane, which is regulated by Bcl-2 family proteins.

In non-apoptotic cells, phosphorylated Bad is kept in the cytoplasm by the 14-3-3 protein, and Bcl-2, Bcl-xL bind to the pro-apoptotic Bax and BAK proteins to inhibit apoptosis. When cytoplasmic levels of free Bad increase, Bcl-2 and Bcl-xL bind to Bad and release Bax and BAK. Bax and BAK can then insert into the mitochondrial membrane, disrupting its integrity. Loss of mitochondrial integrity culminates in the release of pro-apoptotic proteins including Cytochrome c, Endonuclease G, Apoptosis-Inducing Factor (AIF) and Smac/Diablo. In the cytoplasm, Cytochrome c interacts with APAF-1, which recruits Pro-Caspase-9 to form the apoptosome (Tait and Green, 2010). But in case of cancer, the whole mechanism explained above is disrupted (Lowe et al., 2004). Cancer cells develop a number of strategies to avoid controlled cell death. The most prevalent mechanism is the loss of P53, which is a critical DNA damage sensor. Lack of P53 function causes disruption in apoptotic mechanism. Also, elevation at expression levels of antiapoptotic proteins (Bcl-2, Bcl-xL) leads to apoptosis prevention by downregulating proapoptotic molecules (Bax, Puma, Bim) or dysregulation of extrinsic ligand induced apoptosis pathway (Hanahan and Weinberg, 2011).

Under normal conditions, propagation of the cells is limited because of senescence and crisis mechanisms. These events are considered as proliferation barriers. Senescent cells, which are still viable, go into an irrecoverable non- proliferative condition. On the other hand, crisis state involves cell death. While the cells are grown in culture, replicated times of cell division causes triggering of senescence. Then the cells which are able to overcome this barrier, enter into a crisis state in which most of the cells in culture die. Rarely, cells show unlimited replicative activity. This remarkable alteration has been named as immortalization. Telomeres having roles in preserving the ends of chromosomes are implicated in enabling of replicative immortality (Blasco, 2005). Telomerase enzyme, which is implicated in adding specific repeated sequences to the ends of chromosomes, are lacking in most of the non-immortalized cells. But they are significantly expressed in immortalized cells, including tumor cells. Therefore, induction of this enzyme provides resistance to replicative barrier mechanisms such as senescence and crisis (Hanahan and Weinberg, 2011).

Cancer cells, just like other healthy cells require a vascular system in order to provide nutrients, oxygen and exhaust metabolic wastes and carbon dioxide. New vasculature is formed, which is called angiogenesis by virtue of these special needs. During cancer development, angiogenic switch is kept on which eventually leads to producing new blood vessels that feeds tumor tissue (Hanahan and Folkman, 1996). These newly produced blood vessels help tumor cells to spread distant parts of the body. Defined angiogenic regulators are molecules binding to stimulatory or inhibitory receptors expressed at vascular endothelial cell surfaces. Vascular endothelial growth factor-A (VEGF-A) and thrombospondin-1 (TSP-1) are a crucial molecules controlling angiogenesis event. VEGF-A expression is stimulated in case of oncogenic signaling (Carmeliet, 2005).

Invasion and metastasis is an essential point for morbidity and mortality of cancer. Invasion to local tissues and metastasis to distant sites of the body are the most important features of malignant transformation. The malignant tumor cells usually demonstrate changes in their shape and attachment to other cells. Interaction with extracellular matrix (ECM) is disrupted as well (Hanahan and Weinberg, 2000).

These alterations remarkably change the behavior of tumor cells. One of the most crucial cell to cell adhesion molecule E-cadherin is lost upon malignant transformation. E-cadherin establishes adherent junctions with adjacent epithelial cells, thereby reducing detachment potential (Berx and van Roy, 2009). Epithelial-mesenchymal transition (EMT), which is a developmental regulatory program is involved in metastasis. Invasion and metastasis include several steps, starting with invasion to local tissues, followed by intravasation into blood and lymph vessels. Then the cells travel through circulation and invade to distant tissues (extravasation) and they generate small masses and finally forming colonies (Talmadge and Fidler, 2010).

Another capability involves the facilitation of tumor cell invasion by inflammatory cells that are present at the vicinity of tumors. They generate ECM degrading enzymes and some other molecules carrying out invasive growth (Kessenbrock et al., 2010). Inflammation may support tumor development by supplying mitogenic factors that promote proliferation, survival factors that prevent cell death, angiogenic

proteins, ECM- degrading enzymes facilitating angiogenesis and metastasis (DeNardo et al., 2010).

Well known theory of immune surveillance mechanism asserts that cells are continuously checked by immune system cells and emerging cancer cells are destroyed by this control process. However, malignant cells succeded to avoid detection by immune system, hence evading suppressing effects of immunity. Problems in the function of CD8+ cytotoxic T lymphocytes , CD4+ helper T cells or natural killer (NK) cells give rise to considerable increases in tumor incidences. The studies indicate that immune system contribute notably to immune surveillance and eventually tumor prevention (Teng et al., 2008). Highly metastatic cancer cells escape immune destruction by inhibiting the components of immune system. For instance, they secrete TGF- beta and several other immune suppressive molecules to get rid of immunity (Yang et al., 2010).

1.3. Cancer Metabolism

Proliferating cells reorganize their metabolism to support growth. Quiescent cells break down fuels such as glucose, glutamine, and fatty acids for complete oxidation to carbon dioxide through the Krebs cycle . The chemical energy of these fuels is utilized by reducing electron carriers NAD+ and FAD to NADH and FADH2, respectively. These high-energy electrons are used to produce large amounts of adenosine triphosphate (ATP) by mitochondrial electron transport chain. In this manner, quiescent cells efficiently convert nutrients into energy to carry out basic cellular processes. On the other hand, highly proliferating cells must produce proteins, lipids and nucleic acids necessary to make a daughter cell (Hsu and Sabatini, 2008). Eventually, cells uptake large amounts of nutrients such as glucose and glutamine which are used to perform cell growth. Intermediates of glycolysis and the Krebs cycle provide the building blocks for nucleic acids, amino acids, and fatty acids. The signaling pathways controlling nutrient uptake are frequently activated in cancer and directly fuel anabolic pathways. For example, PI3K/Akt pathway regulates the import and retention of glucose, thus providing substrates for glycolysis and the biosynthetic pathways. mTOR, downstream of Akt signaling,

regulates the rate of protein translation. Also, MYC regulates several steps in glutamine uptake and catabolism, ensuring a steady supply of nitrogen for nucleotide biosynthesis and a-ketoglutarate to fuel the Krebs cycle.

A large number of metabolic enzymes are amplified, alternatively spliced, or mutated during the pathogenesis of cancer. Together, these findings show that oncogenic events affect the pathways of nutrient uptake and critical points of metabolism in order to sustain anabolic growth (Hanahan and Weinberg, 2011). A detailed picture of cancer cell metabolism is demonstrated in Figure 2.

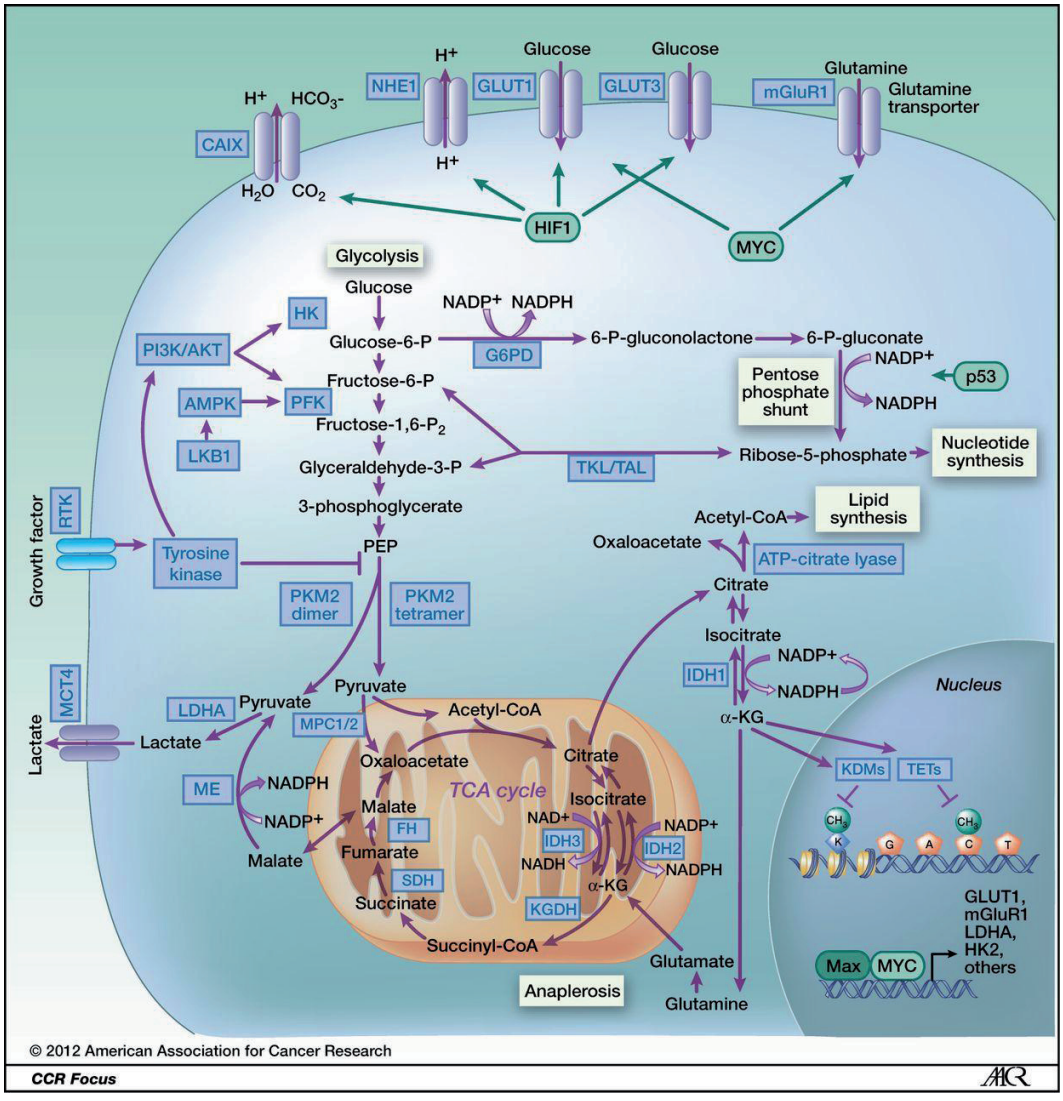


Figure 2: Altered cancer metabolism, adapted from (Teicher et al., 2012)

As it is stated above, lipid metabolism is dysregulated in cancer cells. Alterations in lipid metabolism has been considered as a carcinogenesis signal for many cancer types including breast and prostate cancer (Hilvo et al., 2011; Patra, 2008). Therefore it has been thought that research concerning the changes in lipid metabolism, could yield productive outcomes. Prostate tissues, which are regulated by lipophilic androgen hormone, are influenced by altered lipid metabolism. The role of lipid metabolism in the development of prostate cancer has been examined on several levels. In a study carried out by using Raman spectroscopy it was shown that, the changes occurred in cholesterol metabolism induced prostate cancer development (Yue et al., 2014). It is known that an upregulation exists in fatty acid, phospholipid, cholesterol and triglyceride levels (Clarke and Brown, 2007; Suburu and Chen, 2012). Also, there is an induction of fatty acid beta oxidation levels due to high energy need of cancer cells (Carracedo et al., 2013; Liu, 2006). Thus, increased number of anabolism and catabolism reactions of lipids are associated with cancer. A detailed picture of cancer cell lipid metabolism is shown in Figure 3.

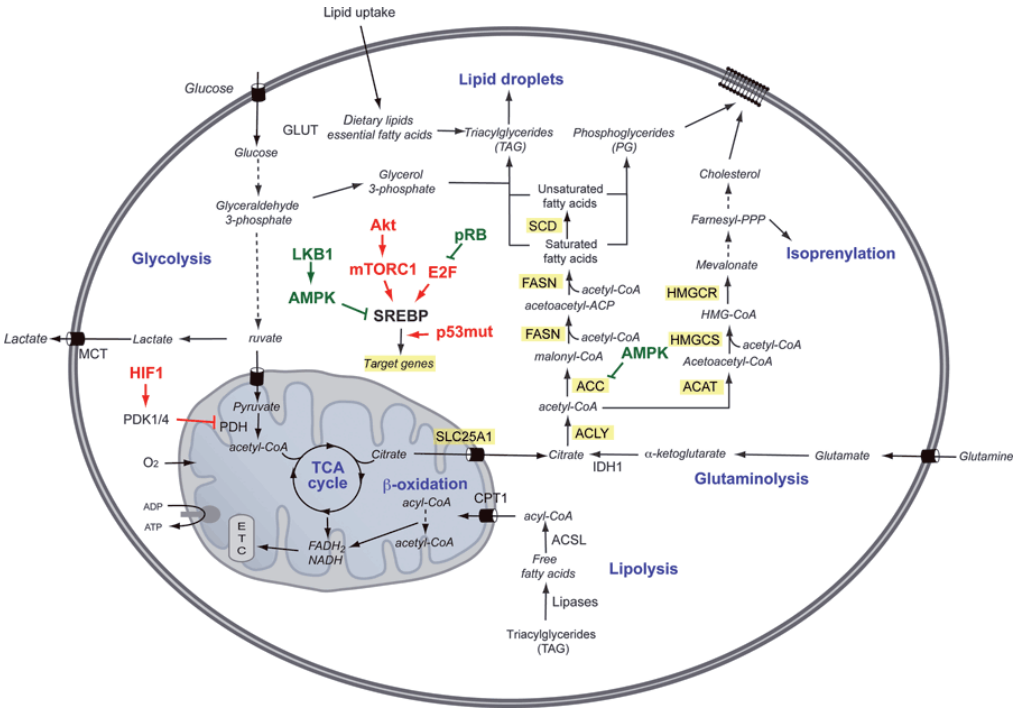


Figure 3: Cancer lipid metabolism, adapted from (Santos and Schulze, 2012)

Although increased number of lipid usage fuels cancer development, it is found that excessive lipid accumulation leads to apoptosis in various cancers. Alterations toward accumulation of lipids or cholesterol trigger apoptosis in cancer cells (Beloribi-Djefaflia et al., 2016; Huang and Freter, 2015). For instance, saturated fatty acid accumulation caused apoptosis in MDA-MB-231 breast cancer cells (Hardy et al., 2003). Also, cholesterol overload was shown to induce cell cycle arrest in glioblastoma cells (Rios-Marco et al., 2013). Moreover, increased levels of ceramide, which is composed of sphingosine and fatty acid, can also promote cell death in pancreatic adenocarcinoma and glioma (Carracedo et al., 2006; Ponnusamy et al., 2010).

1.4. Prostate Cancer and Treatment Options

Prostate cancer or alternatively prostate carcinoma, is the development of tumor in prostate gland which has role in male reproductive system (National Cancer Institute, Last accessed on September 2016). Although most of the prostate tumors progress slowly; some of them are found to grow very aggresively (National Cancer Institute, Last accessed on September 2016). Prostate tumors may migrate from the prostate tissue to the distant parts of the body especially bones and lymph nodes (National Cancer Institute, Last accessed on September 2016). At the onset of disease, it may cause no symptoms. In the further stages, it can cause to difficulty in urinating, blood in the urine, or pain in the pelvis and back. Benign prostatic hyperplasia may cause similar symptoms as well (National Cancer Institute, Last accessed on September 2016). The potential risk factors for developing prostate cancer are stated as old age, a familial history of prostate cancer and race. Approximately 99% of cases occur in people over 50 years old. Prostate cancer is the third most common cause of cancer in Europe for males, and the 6th most common cause of cancer death overall, with around 92,300 deaths from prostate cancer in 2012 (Cancer Research UK, Last accessed on August 2016).

As it is mentioned above, genetic factors contribute to the development of prostate cancer. No single gene is responsible for the emergence of prostate cancer; several different genes have been involved in the development process. Mutations in BRCA1

and BRCA2, important risk factors for ovarian cancer and breast cancer in women, have also been associated with prostate cancer (Struewing et al., 1997). Other associated genes include; androgen receptor, hereditary prostate cancer gene 1 (HPC1) and vitamin D receptor (Gallagher and Fleshner, 1998).

Also, loss of tumor suppressor genes such as PTEN, E-cadherin, p53 as well as activation of proto-oncogenes like PI3K/Akt, androgen receptor and focal adhesion kinase are associated with progression of prostate cancer (Attard et al., 2016).

Prostate contains many small glands which produce about 20% of the fluid constituting semen. In prostate cancer, the cells of these prostate gland are transformed into cancer cells. Male hormone androgens are necessary for the precise working of prostate tissue. Dihydrotestosterone is produced from testosterone within the prostate tissue. Androgen hormones are responsible for the development of secondary sex characteristics such as facial hair and enhanced muscle mass (Attard et al., 2016).

Prostate cancer staging is based on how far the tumor has spread. Biopsies, PET CT Scans, pathological examinations and blood tests are used in determining the stage of prostate cancer. Different treatment strategies are developed for different cancer stages. Treatment options for different stages of prostate cancer are demonstrated in Table 1.

Table 1: Treatment options by stage for prostate cancer, adapted from National Cancer Institute, Last accessed on September 2016

Stage (TNM Staging Criteria)	Standard Treatment Options
Stage I Prostate Cancer	Watchful waiting or active surveillance
	Radical prostatectomy
	External-beam radiation therapy (EBRT)
	Interstitial implantation of radioisotopes
Stage II Prostate Cancer	Watchful waiting or active surveillance
	Radical prostatectomy
	EBRT with or without hormonal therapy
	Interstitial implantation of radioisotopes
Stage III Prostate Cancer	EBRT with or without hormonal therapy
	Hormonal manipulations (orchiectomy or luteinizing hormone-releasing hormone [LH-RH] agonist)
	Radical prostatectomy with or without EBRT
	Watchful waiting or active surveillance
Stage IV Prostate Cancer	Hormonal manipulations
	Bisphosphonates
	EBRT with or without hormonal therapy
	Palliative radiation therapy
	Palliative surgery with transurethral resection of the prostate (TURP)
	Watchful waiting or active surveillance
Recurrent Prostate Cancer	Hormone therapy
	Chemotherapy for hormone-resistant prostate cancer
	Immunotherapy
	Radiopharmaceutical therapy/alpha emitter radiation

As it can be seen from table 1, in early stages of prostate cancer surgery and radiation therapy is preferred as a treatment option. In later stages, hormonal therapies and chemotherapeutic drugs are used to treat prostate cancer.

If it can not be removed by surgery, radiotherapy and chemotherapy, it is called as recurrent prostate cancer. In this case, new treatment strategies such as immunotherapy and biological therapies are used (Crawford and Flaig, 2012). Drugs used in the treatment of prostate cancer are shown in Table 2.

Table 2: Drugs approved for prostate cancer treatment, adapted from National Cancer Institute, Last accessed on September 2016

Drugs approved for prostate cancer treatment
Abiraterone Acetate
Bicalutamide
Cabazitaxel
Casodex (Bicalutamide)
Degarelix
Docetaxel
Enzalutamide
Flutamide
Goserelin Acetate
Jevtana (Cabazitaxel)
Leuprolide Acetate
Lupron (Leuprolide Acetate)
Lupron Depot (Leuprolide Acetate)
Lupron Depot-3 Month (Leuprolide Acetate)
Lupron Depot-4 Month (Leuprolide Acetate)
Lupron Depot-Ped (Leuprolide Acetate)
Mitoxantrone Hydrochloride
Provenge (Sipuleucel-T)
Radium 223 Dichloride
Sipuleucel-T
Taxotere (Docetaxel)
Viadur (Leuprolide Acetate)
Xofigo (Radium 223 Dichloride)
Xtandi (Enzalutamide)
Zoladex (Goserelin Acetate)
Zytiga (Abiraterone Acetate)

1.5. Vitamin E and Cancer

The role of vitamins for cancer therapy has been widely studied so far and it has been known that vitamins that have high antioxidant capacity (like vitamin A, C and E) play important roles in adjuvant therapy and as cancer protective agents (Mamede et al., 2011). There are also many research concerning the applicability of vitamin E and its derivatives, like α, β, γ and δ tocopherols, on cancer therapy (Wada, 2012; Yang et al., 2012). Vitamin E is a group of fat-soluble antioxidant nutrients consisting of tocopherols, tocotrienols and alpha-tocopherol is the most abundant form. Although the classical chemotherapeutic agents have toxic effects on non-cancerous normal tissues, it was demonstrated that some Vitamin E forms have very low side effects on healthy tissues (Zhao et al., 2009).

It has been shown that vitamin E prevents the progression of prostate cancer both in animal models and in vitro studies (Huang et al., 2014; Wada, 2012; Yang et al., 2013). It has also been demonstrated that different vitamin E derivatives such as ɣ-tocopherol and ɣ-tocotrienol suppressed prostate cancer growth by inhibiting cell cycle proteins cyclin D1, E (Agarwal et al., 2004; Gysin et al., 2002). In another animal study, alpha and gamma tocopherol were found to inhibit cancer development by downregulating Akt signaling pathway (Huang et al., 2013). Vitamin E mainly demonstrates its anti-cancer effects by carrying out antioxidant activity that prevents membrane poly unsaturated fatty acids (PUFAs) from oxidation (Akinloye et al., 2009; Yang and Suh, 2013). However, in clinical studies some inconsistent and contradictory results were obtained (Heinonen et al., 1998; Klein et al., 2011; Lippman et al., 2009; Wada, 2012). Animal study and clinical trial results of Vitamin E in various cancers are given in Table 3 and 4 respectively. In literature, there is no comprehensive study about the influences of vitamin E on prostate cancer lipid metabolism.

Table 3: Animal studies of different Vitamin E derivatives in various cancers, adapted from (Wada, 2012)

Effect	Effective Isoforms	No or Slight Effect	Type of Tumor
Protective effect	α-T1, γ-T1		Colon, lung, prostate, mammary gland tumor
Life-prolonging effect	γ-T3>α-T3>α-T1		Sarcoma (Inoculated)/mouse
Reduction of tumor nodules	tocotrienol-rich vitamin E		Liver, lung tumor/mouse
Retarded onset and tumor growth	TRF		Breast cancer (inoculated)/mouse
Life-prolonging effect	γ-T3>α-T3>α-T1		Sarcoma (Inoculated)/mouse
Suppression of vessel formation	TRF	α-T1	Colorectal adenocarcinoma (implanted)
Inhibition of tumor growth	α-TOS, α-TEA		Breast cancer (injection)/ mouse
Enhanced tumorigenesis	α-tocopheryl acetate		Duodenum, cecum, colon, rectum, and anus/ mouse

T1: tocopherol, T3: tocotrienol, TRF: tocotrienol-rich fraction, α-TOS: α-tocopheryl succinate, α-TEA: α-tocopheryloxyacetic acid.

Table 4: Clinical trials of different Vitamin E derivatives in various cancers, adapted from (Wada, 2012)

Trial	Study Population	Effective Isoforms	Type of Tumor	Supplementation Period
ATBC	Age 50-69 Men smokers	α-tocopheryl acetate 50 mg/day	Reduced prostate cancer incidence and mortality	6.1 years
			Increased colorectal adenoma	6.3 years
		No supplement after the trial	the anti-cancer effects was absent	6 years (post intervention)
SELECT	Age 50 or older Men	α-tocopheryl acetate 400 IU/day	Increase prostate cancer but not significant	5.46 years
New Hampshire Study	Age 25-74 Men and women Bladder cancer diag- Nosed	Dietary Vitamin E	High intake of vitamin E tended to reduce Bladder cancer	
Linxian Trials	gge 44-60 Men and women	β-carotene 15 mg, selenium 50 μg and α-tocopherol 60 mg	Reduced gastric cancer incidence and mortality, cancer mortality, and total mortality	5.25 years

1.6. Vitamin E Analogs

Selective anti-cancer activities of Vitamin E analogs were shown by several studies (Zhao et al., 2009). Several anticancer molecules have been discovered to induce cell death by targeting mitochondria. These molecules are called as mitocans which are involved in apoptosis by destabilizing the mitochondrial outer membrane permeabilization (MOMP), eventually leading to the release of soluble apoptosis modulators (Alirol and Martinou, 2006; Armstrong, 2007; Galluzzi et al., 2006). Some analogues of vitamin E belong to mitocans family. Alpha-Tocopheryl succinate (α-TOS) promotes apoptosis in a variety of malignant cells including prostate and breast cancer via mitochondria-dependent apoptotic signaling (Shiau et al., 2006; Yu et al., 2003). Some other analogs of vitamin E such as; alpha-tocopheryl maleyl amide (α-TAM), alpha tocopherol with ether-linked acetic acid (α-TEA), alpha-Tocopheryl oxalate, alpha-tocopheryl malonate and alpha-Tocopheryloxybutyric acid (α- TOB) also demonstrate apoptotic properties (Zhao et al., 2009). Besides inducing mitochondrial destabilization, vitamin E analogs stimulate non-mitochondrial related apoptosis by promoting death receptor pathways, involvement of JNK activity, decreased PKC activity and inactivation of NF-κB signaling (Zhao et al., 2009). Vitamin E analogs mediated apoptosis signaling pathways are shown in Figure 4.

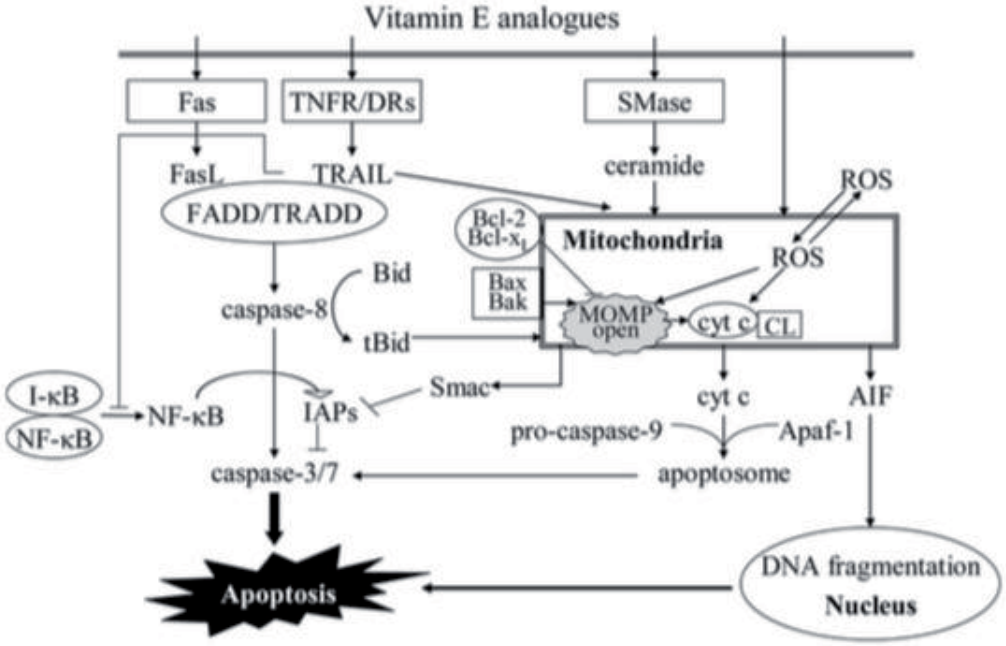


Figure 4: Vitamin E analogs related apoptosis signaling pathways, adapted from (Zhao et al., 2009)

The molecule of interest in this thesis study is the new vitamin E derivative compound, possessing additional biological and medical properties compared to the native vitamin E. It was proposed that new vitamin E derivative can be used for prophylaxis, as well as for treatment of variety of pathological states with oxidative stress etiology. The product provides effective protection against pathologic conditions, such as hypoxia, vitamins deficiency, intoxication, cancer, etc. This derivative of vitamin E can be used as an independent product, as well as the component of an existing medications and therapies for treatment of disease and pathological states in human or in animals. Innovative aspects and main advantages of the product are the following:

a) increased viability at stress conditions; b) normalized lipid structure of the cell membrane; c) regulation of free-radical processes in cells and organism; d) effect on activity of the antioxidant enzymes.

This molecule is synthesized by our collaborator Dr. Oleksandr Kuzmenko, who is a member of Department of Vitamins and Coenzymes Biochemistry of the Palladin Institute of Biochemistry Kiev/Ukraine. The molecule, whose structure is

demonstrated in Figure 5, is synthesized by a side chain modification of natural alpha tocopherol. Experiments performed on human epithelial carcinoma cell line HeLa-S3K, human cervical carcinoma cell line C-4-1, human acute leukemia cell cultures CEM-C-1 and CEM-C-7 and then on animal model have clearly shown that this compound drastically decreased tumor size and level of metastasis as demonstrated in Figure 6. So, the vitamin E derivative has an antitumor, as well as anti-metastatic activity on mice under conditions of tumor growth as shown in our previous experiments.

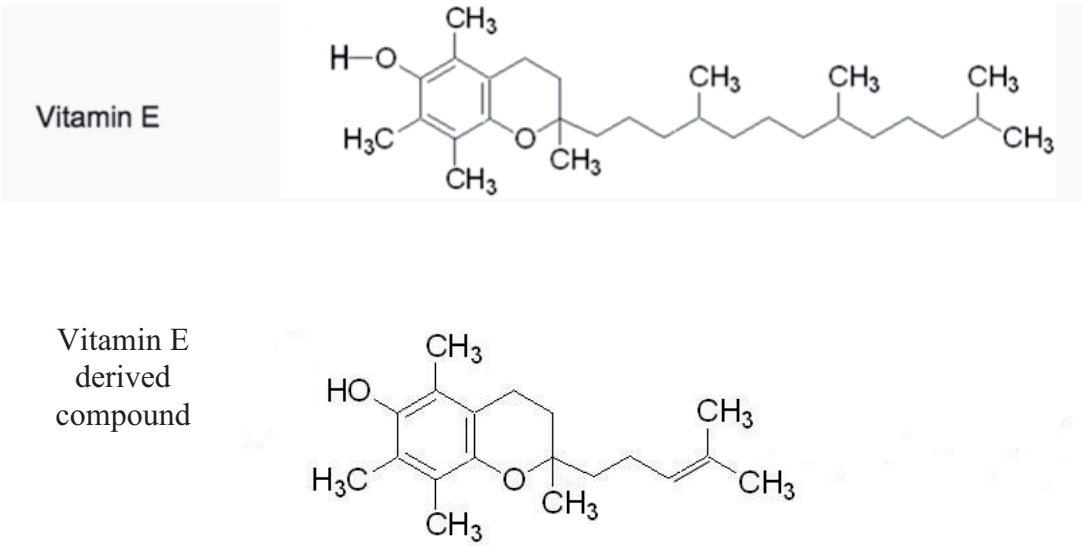


Figure 5: Chemical structure of Vitamin E and Vitamin E derived compound

A

B

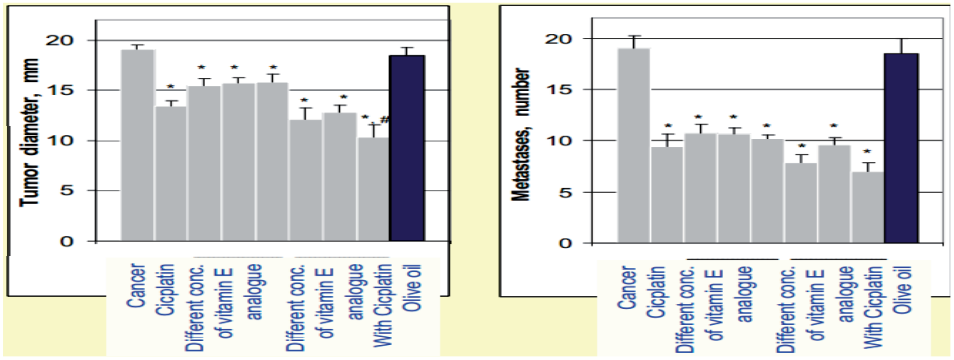


Figure 6: Antitumor effect of compound derived from vitamin E with shortened side chains on Lewis lung carcinoma model in mice C57BL/6. From left to right: intact control, mouse with tumor and mouse with tumor treated by oral administration of vitamin E derived compound, respectively. (**A**) Comparison of the influences of vitamin E derived compound on tumor size (left panel) and metastasis (right panel) of lung cancer mouse models. Vitamin E derived compound applied in different concentrations either with chemotherapeutic agent cisplatin or alone. (**B**)

1.7. Aim of the Study

Cancer is a multi-factorial disease characterized by uncontrolled cell proliferation, invasion and metastasis with accumulated mutations in tumor suppressor and/or proto-onco genes. It has been known that vitamins that have high antioxidant capacity (like vitamin A, C and E) play important roles in adjuvant therapy and as cancer protective agents (Mamede et al., 2011). Vitamin E is a group of fat-soluble antioxidant nutrients consisting of tocopherols and tocotrienols. Various research has been carried out on the utility of Vitamin E derivatives on cancer treatment (Wada, 2012). Energy metabolism of cells alters during cancer progression. Especially, anaerobic glucose degradation and lipid biosynthesis increase due to high energy need of cells (Santos and Schulze, 2012). Additionally, elevation in saturated and unsaturated lipid, phospholipid, sphingolipid and triglyceride amounts in cancer cells was reported in previous research (Gillies and Morse, 2005; Glunde et al., 2010). Prostate cancer cells are regulated by the lipid derivative molecules like androgen. Therefore, it is expected that there might be significant alteration in the lipid metabolism of prostate tissues during cancer development. As Vitamin E is a lipid-soluble molecule, it has been considered that vitamin E and vitamin E analogues should be effective on lipid metabolism of cancer cells. Although there is some research related with the general effects of vitamin E on cancer progression, there is no comprehensive report so far about its effects on lipid metabolism. A novel vitamin E derived compound, which has a modification on the side chain, was synthesized by our Ukrainian partner. In lung cancer mouse model study, a significant decrease in the tumor mass was observed in response to treatment with this novel vitamin E derived molecule (Figure 6). Based on the considerations stated above, it is thought that, the vitamin E derived compound

could be effective in prostate cancer therapy as a prospective novel therapeutic molecule.

In this thesis study, it is aimed to investigate the influences of the mentioned compound on prostate cancer cells as a pre-clinical in vitro study for the first time. With this motivation we aimed to;

1) Determine the effective dose of vitamin E-derived compound on prostate cells by performing toxicity studies and detect the effect of the molecule on general cancer properties
2) Reveal the effects on lipid metabolism of cancer cells in response to Vitamin E derived compound treatment by using biophysical and molecular biology techniques.
3) Analyze the biochemical pathways that play roles in cancer development, by protein microarray technology.

Outputs of this study will not only show the effect of the compound on prostate cancer cells, but also reveal the changes in lipid metabolism and other biochemical pathways during cancer progression. The findings will also be the basis of further animal model and clinical phase studies and finally could contribute to the shaping of new treatment options in prostate cancer therapy in the near future.

CHAPTER 2

MATERIALS AND METHODS

2.1. Cell Culture and Treatments

Androgen sensitive, metastatic LNCaP prostate cancer cell line was a kind gift from Assoc.Prof.Dr. Lokman Varışlı (Harran University), and the normal prostate epithelium cell line PNT2 was purchased from E.C.A.C.C.(The European Collection of Authenticated Cell Culture). They were grown in RPMI medium (Biowest) supplemented with 2 mM L-Glutamine, 10% FBS (Biowest) and 1% penicillin/streptomycin/amphotericin (Lonza). The cells were grown in a humidified incubator at 37°C containing 5% CO2. Cell culture media was bought from Biowest. For the treatments, alpha-tocopherol derived compound (synthesized by our Ukranian collaborator) was applied to the cells as follows: Molecules were solved in absolute ethanol, then 1mM stock solutions were prepared with the appropriate cell culture medium by reducing the final EtOH concentration to 0.5%. The treatments were performed after the cells reached to 70-80% confluency. The final EtOH concentration did not exceed to 0.1% for treated cells. All dilutions were carried out in cell culture medium.

2.2. Cell Toxicity Studies, XTT Viability Assay

Cell viability and toxicity were detected by XTT assay kit (Roche) according to the manufacturer's instructions. A total of $1x10^4$ cells were inoculated in 96-well plate (Greiner) for 24h and then the culture medium was removed. Freshly prepared 100 µl treatment medium was added to each well. Due to the fact that 0-200 µM interval was used in most alpha-tocopherol studies (Jiang et al., 2004; Shah et al., 2003), we firstly decided to try 0, 25, 50, 100 and 200 µM concentrations for 24, 48 and 72 hours separately. Because we were not able to calculate IC_{50} value precisely, we

decided to use narrow treatment interval as 100-200 μM and 100, 125, 150, 175, 200 μM concentrations were applied for 48 hours. Following each incubation, 50 μl XTT reagent was added to each well and absorbance values were recorded at 475 nm with Multiskan microplate reader (Thermo Scientific). The background absorbances were recorded at 675 nm and subtracted from the absorbances of 475 nm values.

Cell viability curves were drawn for each plate and absorbance of untreated group was considered as 100% viability. IC_{50} values for each plate was calculated by linear regression analysis.

2.3. BrdU Cell Proliferation Assay

Cellular proliferation level was determined by using BrdU incorporation assay kit (Roche). In proliferating cells, BrdU incorporates instead of thymidine in newly synthesized DNA. Assay principle depends on the spectroscopic detection of BrdU level. Firstly, cells were allowed to grow for 48 hours with respective treatment culture mediums. Then, 175 μM was applied as optimum dose and 100 μM and 200 μM treatments were applied as lower and upper doses respectively for each experimental group. After treatments, cells were labeled with BrdU for 4 hours and absorbances were measured at 492nm at ELISA reader (Thermo Scientific).

2.4. Apoptosis Assays

In order to test apoptotic properties of alpha tocopherol and alpha-tocopherol derived compound, Annexin V and JC-1 tests were performed. Annexin V test determines apoptotic status of cells, on the other hand, JC-1 assay detects mitochondrial depolarization.

2.4.1. Annexin V Assay

For Annexin V assay, BD Bioscience's kit (catalog number 556547) was used. According to manufacturer's instructions, cells were both stained with Annexin V and PI. After 48 hours of 175 μM alpha-tocopherol derived compound treatment, the cells were washed twice with cold PBS and then resuspended in 1X Binding Buffer at a concentration of 1 x 10^6 cells/ml. Then 100 μl of the solution containing 1 x 10^5 cells was transferred to 5ml tube and 5μl Annexin V and PI were added. After

staining, cells incubated for 15 min at dark and 400 µl binding buffer were added. Finally, cells were analyzed at flow cytometry (BD Biosciences) within 1 hour.

2.4.2. JC-1 Assay

For JC-1 assay, BD Bioscience's kit (catalog number 551302) was used. 5 x 10^5 cells were grown and following treatments, the assay was carried out according to manufacturer's instructions. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) is a lipophilic fluorochrome that is used to evaluate the status of the mitochondrial membrane potential. JC-1 does not accumulate in mitochondria with depolarized potential and remains in the cytoplasm as monomers. These monomers do not have the red spectral shift, and therefore have lowered fluorescence in the FL-2 channel. Thus, in mitochondria having a transition from polarized to depolarized state due to apoptosis, JC-1 leaks out of the mitochondria into the cytoplasm as monomers resulting in a decrease of red fluorescence. The fluorescence signal came from each cell was analyzed by flow cytometry (BD Biosciences).

2.5. In vitro Scratch Wound Healing Assay

The influence of alpha-tocopherol derived compound treatment on cellular motility was determined by an in vitro scratch wound healing assay. Cells were seeded in 6-well plate and allowed to grow until they become confluent. Then each well was scratched with a sterile 1000 µl pipette tip. Cells were washed 2 times with PBS in order to remove debris then incubated in 175 µM treatment medium for 48h. Images were captured at 0, 24 and 48 hours with inverted light microscope (Bel Engineering) and analyzed with ImageJ 1.42 (http://rsbweb.nih.gov/ij/) program to measure the wound closure.

2.6. Boyden Chamber Migration Assay

The migration abilities of LNCaP cells treated with alpha tocopherol derived compound were detected by an in vitro Boyden chamber assay. A total of 5 × 10^4 cells in 0.5 mL serum-free RPMI-1640 medium were added to the upper wells of Boyden chambers containing membranes with 8 µm pores (Corning). Cells were allowed to migrate for 48 h after treated with alpha tocopherol derived compound. Cells that were not migrated were removed by wiping with cotton swabs and then, the chambers were fixed in 100% methanol for 10 min, stained with modified

Giemsa staining solution for 2 min and washed twice with distilled water. The migration of LNCaP prostate cancer cells was measured under a light microscope with 4X and 10X objective and photos were taken by camera.

2.7. RNA Isolation and RT-PCR Array

Total RNA was isolated from each group by using RNeasy Mini Kit (Qiagen), the quality of RNAs was checked with agarose gel electrophoresis, then by starting with 1 μg RNA, cDNA was synthesized by using RT2 First Strand Kit (Qiagen) according to manufacturer's instructions. In order to check expression levels of genes involved in lipid metabolism, human fatty acid metabolism kit (Qiagen, catalog number: PAHS-007Z) was used. The experiments were performed according to manufacturer's instructions. Array data was analyzed by using PCR array data analysis software (Qiagen).

2.8. Protein Isolation and Western Blot

Total protein from cells was isolated by using ReadyPrep Protein Extraction Kit (Biorad) containing protease inhibitors (Roche). The total protein content was determined by RC DC Protein Assay Kit II (Biorad). The total cell proteins (40 μg) were run on 10% polyacrylamide gel for 1 hour at 100V and transferred to a PVDF membrane by Trans-Blot Turbo Transfer system (Biorad) at 12 minutes. Following transfer, membrane was blocked with 5% BSA and incubated overnight with respective antibodies (LIPE primary antibody- 1:100 dilution, catalog number: ab103281,Abcam; SREBP1 primary antibody- 1:500 dilution, catalog number: ab28481,Abcam; FASN primary antibody- 1:1000 dilution, catalog number: ab128856, Abcam; Vimentin primary antibody- 1:1000 dilution, catalog number: 5741,Cell Signaling; p-Erk-Thr202/Tyr204 primary antibody- 1:1000 dilution, catalog number: 4376,Cell Signaling; p-Akt-Ser473 primary antibody- 1:1000 dilution, catalog number: 4058,Cell Signaling; Cleaved caspase-3 primary antibody- 1:1000 dilution, catalog number: 9664,Cell Signaling) and washed with TBS-T for 3 times, then it was incubated for 1 hour with anti-rabbit HRP conjugated secondary antibody (1:2000 dilution, Abcam) and washed again with TBS-T. After washing steps, the bands were visualized by applying Clarity ECL Western Blotting Substrate

(Biorad). Actin (1:2000 dilution catalog number: sc-47778 HRP, Santa Cruz) was used as loading control.

2.9. Protein Array

In order to check expression levels of cancer associated proteins, Human Oncology Proteome Profiler Array Kit (R&D systems catalog number:ARY026) was used. The experiments were carried out according to manufacturer's instructions. 100 μg protein sample was used for each group. Data was analyzed by using ImageJ software.

2.10. Lipid Peroxidation Assays

In order to test lipid peroxidation levels upon alpha-tocopherol derived compound treatment, we measured some specific molecules namely Malondialdehyde (MDA) and 8-isoprostane. which are formed during lipid peroxidation. MDA and 8-isoprostane levels were measured by using assay kits (abcam, ab118970 and ab175819 respectively). The experiments were performed according to the manufacturer's instructions. Standart curves were drawn for each molecule and the level of peroxidation was calculated.

2.11. ESR Spectroscopy

5X106 LNCaP cells were treated with 175 μM alpha-tocopherol derived compound in T75 (Corning) flasks for 48 hours. After treatment, cells were trypsinized and labeled with 16-DSA (16-doxylstearic acid) as described previously (Ozek et al., 2010).

The labeling process was as follows, firstly, a stock solution of 16-DSA (10^{-2} M) was prepared by dissolving in ethanol, then the cells were spin labelled by incubating a suspension of cells in PBS (5X106 cells/ml) for 60 min at 37◦C with gentle shaking to a final concentration of 10^{-4} M 16-DSA. In order to get rid of unbound spin labels, the labeled cells were washed with PBS and centrifuged at 14000 rpm for 5 min.

For ESR measurements, the cell pellet was transferred to a glass capillary and ESR spectra were obtained at X-band, at 9.85 GHz, with 100 G sweep width, 2 Gauss modulation amplitude and at 10 mW microwave power by using a Bruker EMX X-

band (9–10 GHz). The fluidity of membranes of each group was determined by calculating the rotational correlation time (τc) as described previously (Severcan and Cannistraro, 1989). The formula used for calculations and representative spectrum (Figure 7) is given below.

$$\tau c = 6.5 \times 10^{-10} W_0 \left[\left(\frac{h_0}{h_{-1}} \right)^{1/2} - 1 \right]$$

K=6.5 X 10^{-10} s G^{-1} is a constant that depends on microwave frequency and the magnetic anisotropy of the spin label, W_0 is the peak-to-peak width of the central line, and h_0 / h_1 is the ratio of the heights of the central and high field lines, respectively.

In order to have information about membrane lipid order 5- doxyl stearic acid is generally used (Yonar and Sunnetcioglu, 2014). The formula for calculating lipid order is given below and a representative spectrum of 5-DSA labeleled cell is given in Figure 8. The same experimental procedure was used for 5-DSA labeling.

$$S = \frac{1}{7}(A_{max} + 2A_{min}) - \left\{ \left[\frac{1}{7}(A_{max} + 2A_{min}) \right]^2 - 0.46(A_{max} - A_{min}) + 0.6 \right\}^{\frac{1}{2}}$$

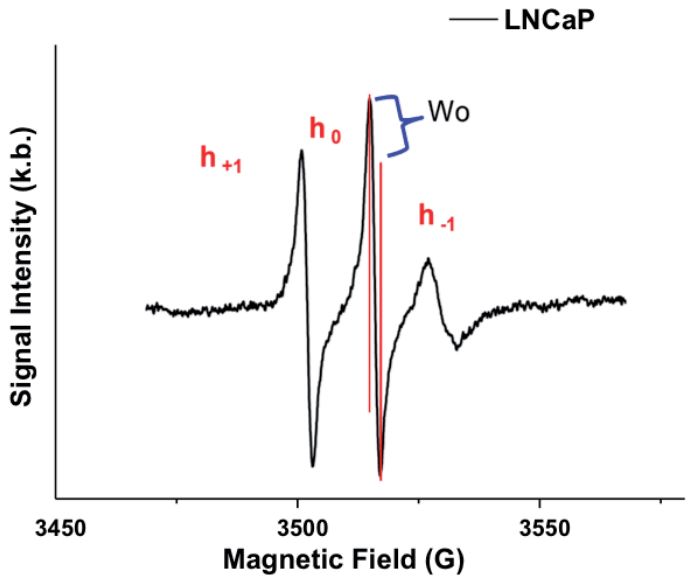


Figure 7: Representative ESR spectrum of 16-DSA labelled cells

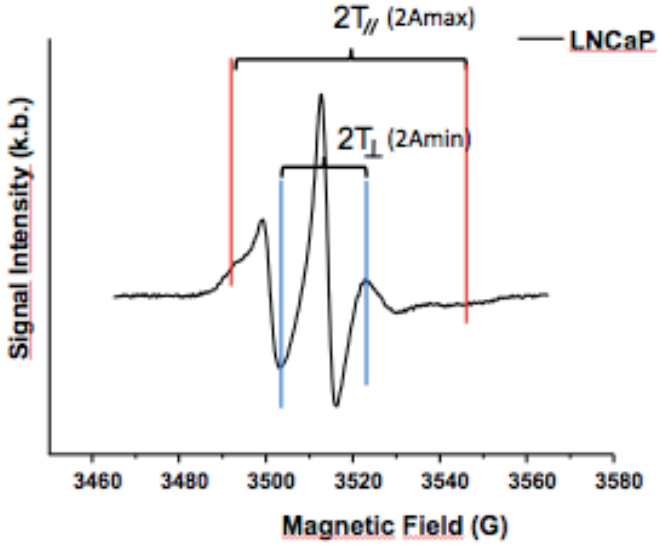


Figure 8: Representative ESR spectrum of 5-DSA labelled cells

2.12. ATR-FTIR Spectroscopy

The cells were treated with 175 µM alpha-tocopherol derived compound and grown in 6 separate T75 flasks (Corning) for 48 h. Also, control cells were allowed to grow in 6 separate flasks for 48 hours. After appropriate incubation times, the cells were trypsinized and counted with Scepter 2.0 cell counter (Milipore) and 5X106 LNCaP cells were pelleted and resuspended in 10 ul PBS and kept on ice during measurements.

2 µl cell suspension was placed on ZnSe ATR crystal (Perkin-Elmer Inc., equipped with ATR accessory). The solution on crystal was gently dried by mild N2 flow for 5 minutes to remove water. The samples were scanned 100 times between 4000 and 650 cm−1 at 4 cm −1 resolution. Each independent sample was scanned in 3 replicates and averages were taken for detailed analysis.

For spectral data analyses, second derivative raw spectra were used to increase band resolution. Band frequency and bandwidth values were determined by using OPUS 5.5 software (Bruker Optics) and band areas were calculated by Spectrum 100 software. Normalized and baseline corrected spectra were used for visual demonstration of the spectral variations in the figures.

2.13. Chemometric Analysis

Chemometric analyses were carried out with Unscrambler X 10.3 (Camo Software) multivariate analysis (MVA) software. Second derivative vector normalized spectra were used for PCA and HCA analyses. Mean centered PCA was applied over the range of whole IR (4000-650 cm^{-1}) and C-H Lipid (3050-2800 cm^{-1}) regions. Results were shown as loading and score plots.

HCA analyses were performed over the range of 3050-2800 cm^{-1}. Ward's algorithm calculated the squared Euclidiean distance between the samples. Clustering depends on the relative distance among the spectra. Results were demonstrated as dendrograms.

2.14. Statistics

All the results in figures were shown as mean± standart error of mean. Analysis of the data and drawing of graphs were carried out by using GraphPad Prism 6.0 software. In order to examine the statistical significance of data for control versus treated cells, One-way ANOVA was conducted. p values less than 0.05 were accepted as significantly different from the control group.

CHAPTER 3

RESULTS

3.1. Vitamin E Derived Compound Decreases Viability and Proliferation of LNCaP Prostate Cancer Cells

XTT assay was carried out in order to determine the cell viability.
First of all, the IC50 values were calculated (Figure 9,10,11,12 and 13). At the beginning, the studies were performed at 0-200 μM doses (Figure 9; *** $p<0,001$; * $p<0,05$ and Figure 10), then 100-200 μM doses were applied to detect IC50 value (Figure 11; *** $p<0,001$; * $p<0,05$ and Figure 12). The optimum dose and treatment time were chosen as 175 μM and 48h, respectively after calculating IC50 value. Furthermore, XTT assay was conducted by using PNT2 prostate normal cell line in order to test toxicity of the molecule on healthy cells. It was concluded that vitamin E derived compound has no toxic effect on normal cells, which suggests less side effect, as can be seen from the graphic (Figure 13). Moreover, vitamin E derived compound treatment in LNCaP cells induced a significant reduction in proliferation rate when analyzed by the colorimetric BrdU assay (Figure 14; ** $p<0,01$).

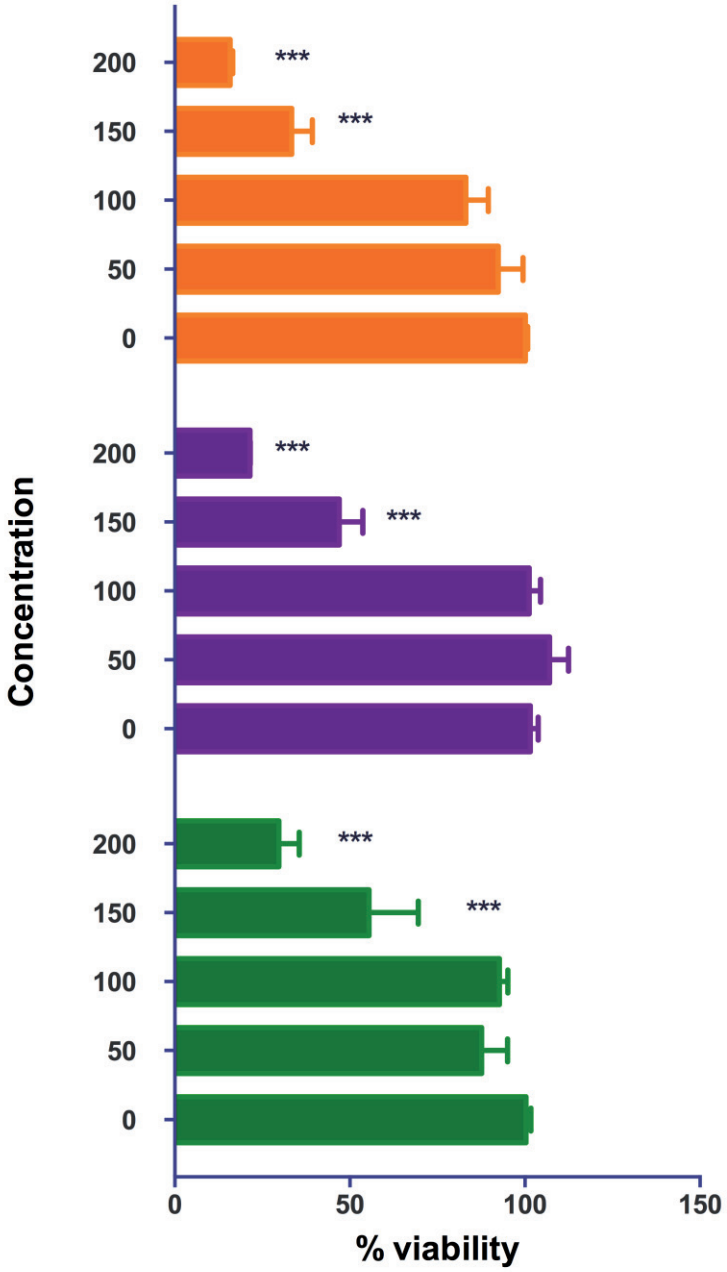


Figure 9: LNCaP cells were placed in 96-well plates and treated with 50,100,150 and 200 µM alpha tocopherol derived compound for 24h (green), 48h (purple) and 72 h (orange). The cellular viability was determined for each time point by an XTT assay. Each point represents the means +− S.E.M. (n = 12; ***p<0,001).

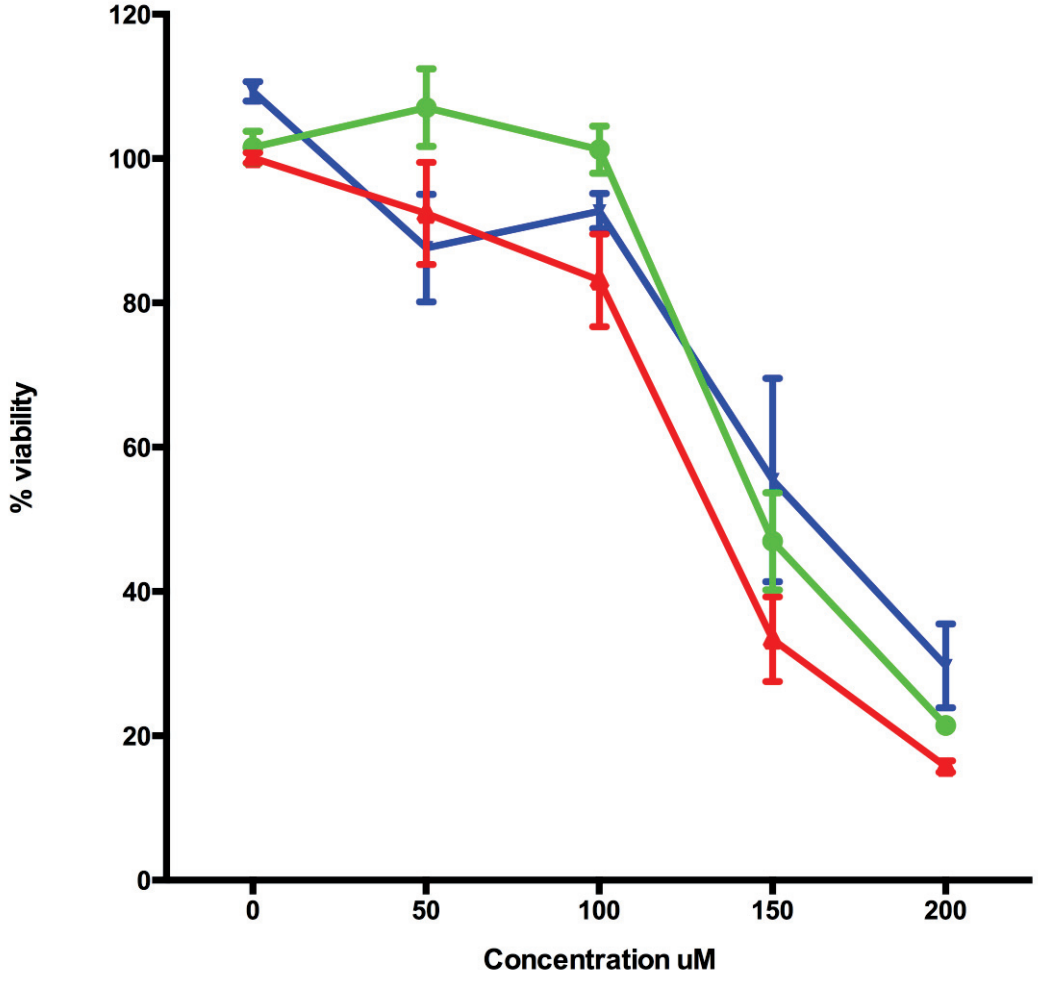


Figure 10: Dose response curve of 24h (blue), 48h (green) and 72h (red) different concentration treated LNCaP cells (n=12).

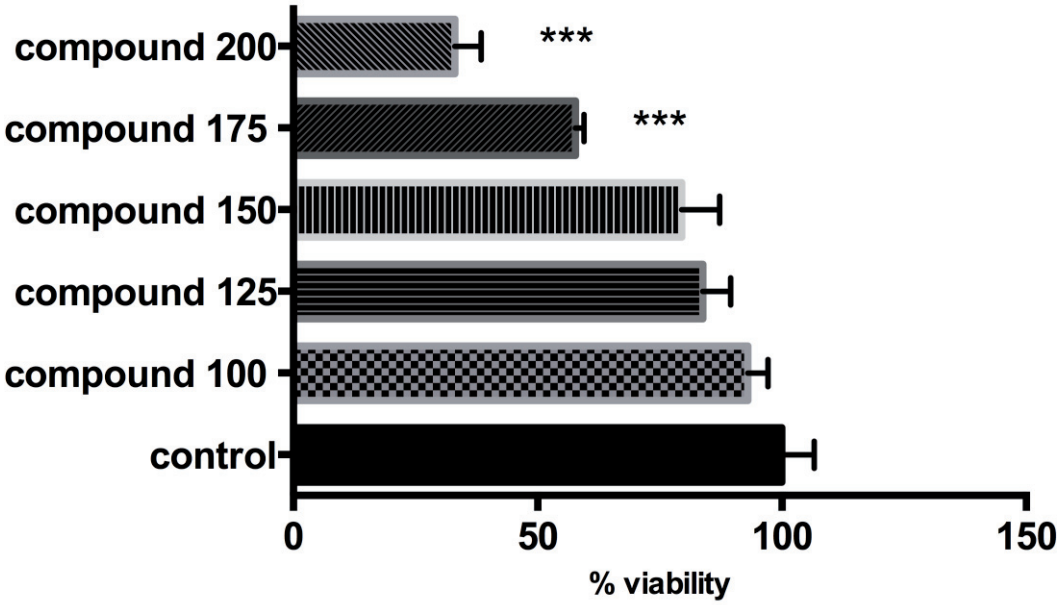


Figure 11: LNCaP cells were treated with 100,125,150,175,200 µM alpha tocopherol derived compound for 48h (n=6; ***p<0,001)

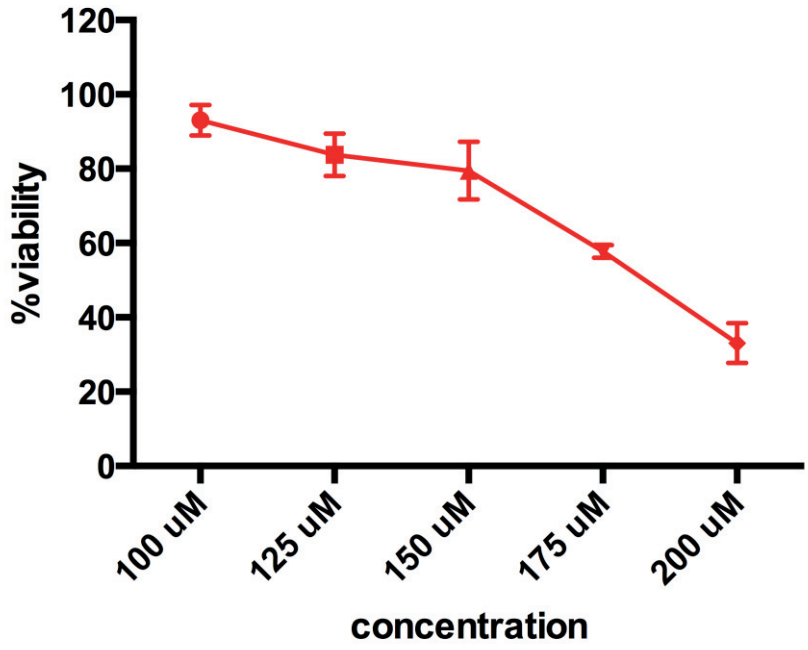


Figure 12 : Dose response curve of LNCaP cells treated with 100,125,150,175,200 µM alpha tocopherol derived compound for 48h (n=6)

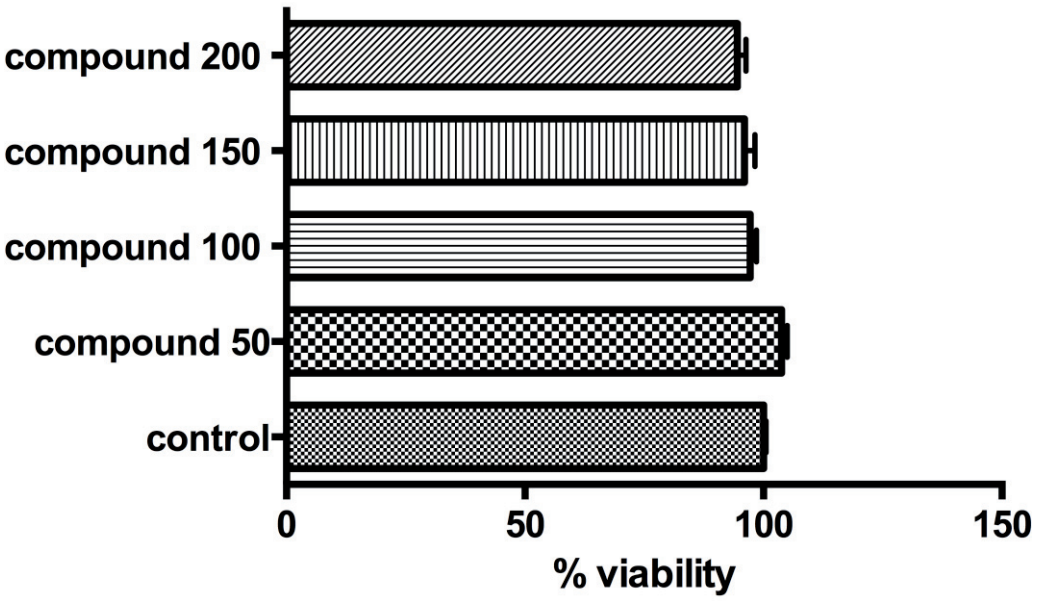


Figure 13: PNT2 normal prostate cell line were treated wih 50,100,150 and 200 μM alpha tocopherol derived compound for 48h (n=6).

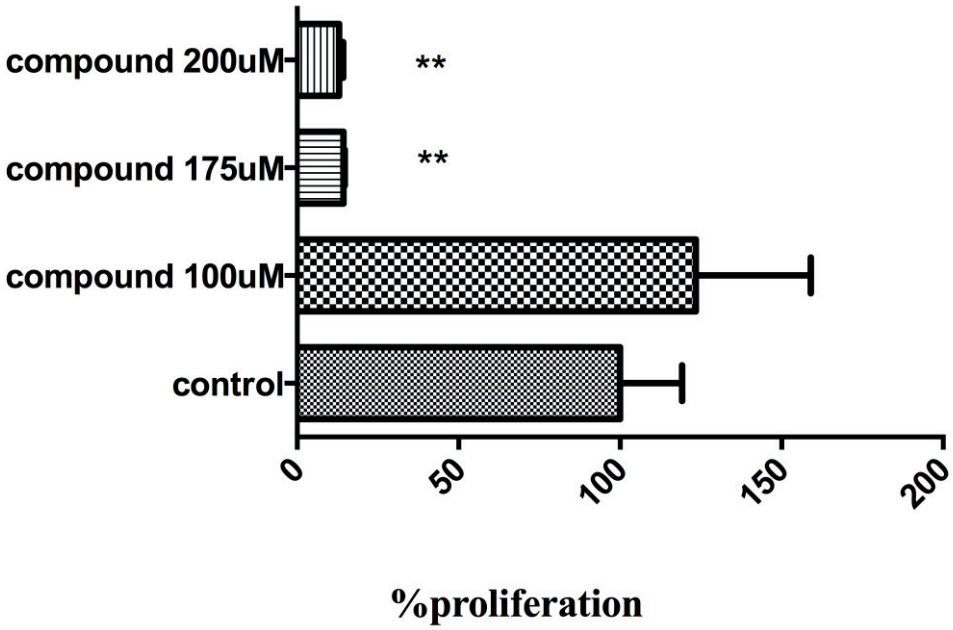


Figure 14: BrdU cellular proliferation test results of alpha tocopherol derived compound (n=6; **p<0,01).

3.2. Vitamin E Derived Compound Induces Apoptosis of LNCaP Prostate Cancer Cells

In order to detect the apoptotic effect of alpha tocopherol derived compound, Annexin V and JC-1 assays were carried out. Vitamin E derived compound(analog) promotes apoptosis by causing mitochondrial membrane depolarization as shown in Figure 15A and 17A. According to Annexin V test results, 10.5% increase in the late apoptosis of LNCaP cells was observed in vitamin E derived compound treated group, but no remarkable change was detected in untreated control samples (Figure 15A and C). Moreover, based on JC-1 results, 29.5% rise was observed in mitochondrial membrane depolarization of vitamin E derived compound treated cells (Figure 17A and C). Percentages of late apoptosis and mitochondrial depolarization are given as graphs in Figure 16 and 18 respectively. DMSO was used as apoptosis inducer positive control in both cases.

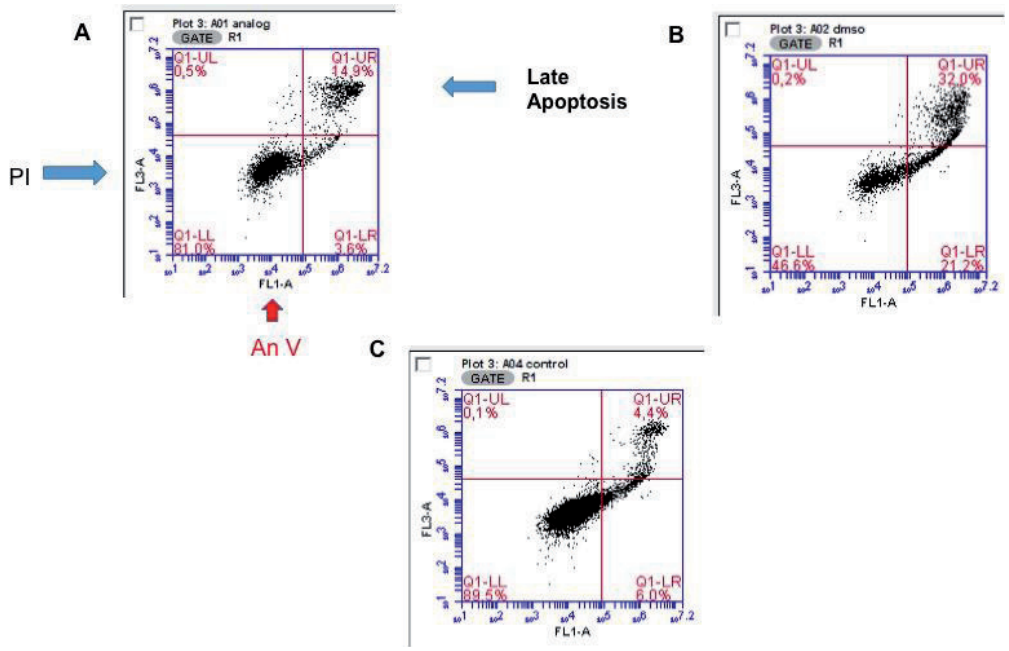


Figure 15: Flow cytometric analysis of (A) Alpha tocopherol derived compound (B) DMSO apoptosis inducer positive control (C) Control LNCaP cells (n=4 for each group). Y axis represents PI staining, X axis represents Annexin V staining. Late apoptotic cells were accumulated at upper right corner of the quadrant which is indicated by an arrow.

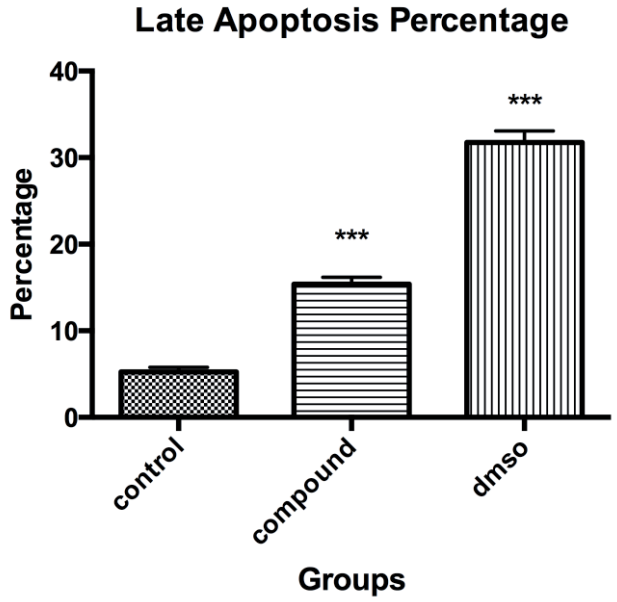


Figure 16: Late apoptosis percentage of different groups (n=4 ***; p<0,001)

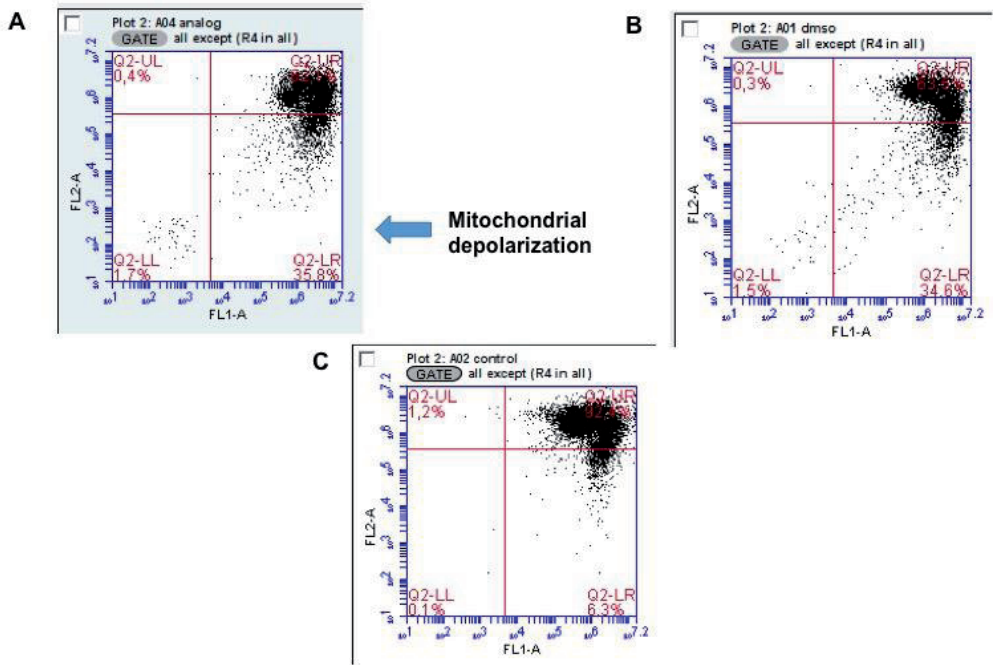


Figure 17: Mitochondrial membrane potential analysis of (A) Alpha tocopherol derived compound (B) DMSO apoptosis inducer positive control (C) Control LNCaP cells (n=4 for each group). Upon mitochondrial depolarization, the signal migrates to the lower right corner of the quadrant which is indicated by an arrow.

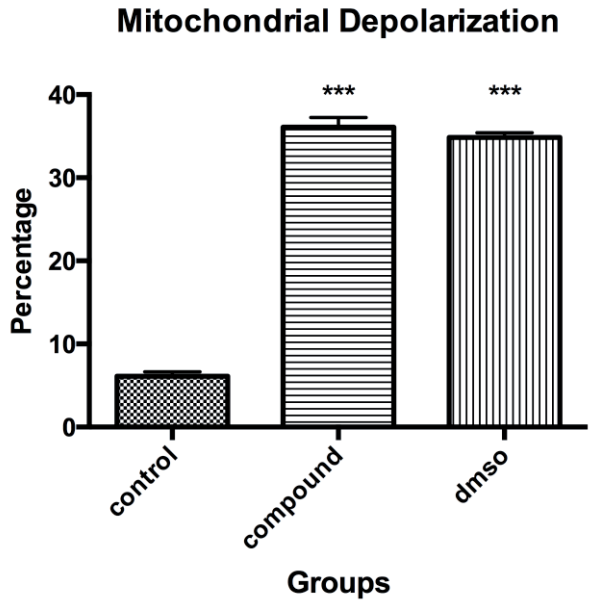


Figure 18: Mitochondrial depolarization percentage of different groups (n=4; *** p<0,001)

3.3. Vitamin E Derived Compound Alters Motility, Migration Abilities of Prostate Cancer Cells and Induces Changes in Biochemical Pathways

In order to reveal the role of alpha tocopherol derived compound on the motility of LNCaP cells, in vitro scratch-wound healing assay was performed. After 48 h of incubation with alpha tocopherol derived compound, the result of wound closure is demonstrated in Figure 19. It was shown on the graph that alpha tocopherol derived compound treatment significantly decreased the percentage of wound closure representing a loss of motility (Figure 20).

To clarify the effect of alpha tocopherol derived compound on the migratory ability of LNCaP cells, migration assay was performed. As it can be seen from images, alpha tocopherol derived compound reduced migration of LNCaP prostate cancer cells (Figure 21 and 22). These results suggest the anti-metastatic role of alpha tocopherol derived molecule.

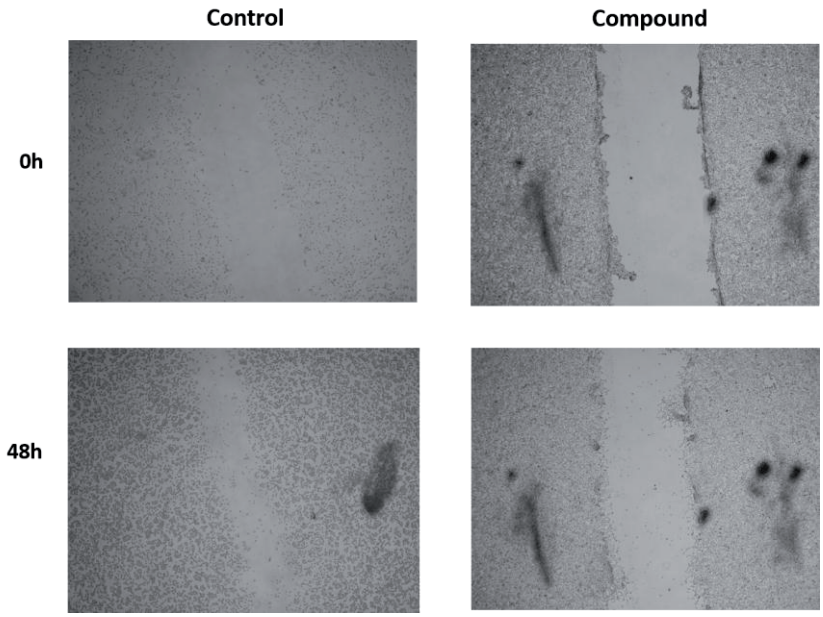


Figure 19: Scratch-wound healing assay inverted microscope images for 48 h 175μM compound treatment is given. Cells treated with alpha tocopherol derived compound were not able to close the wound in the confluent culture, when compared with untreated control cells.

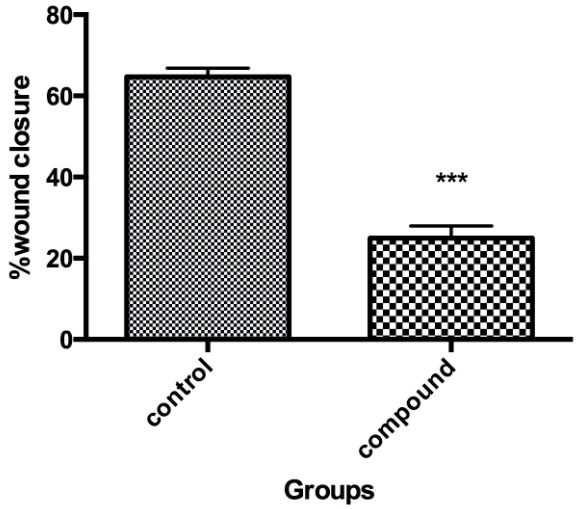


Figure 20: Wound closure percentages, the columns demonstrate that compound treated cells significantly reduced motility (***$p<0.001$) in LNCaP cells with 25% wound closure for treated cells compared with 64% for untreated cells. (The results are the means +− S.E.M; n=6 for each group).

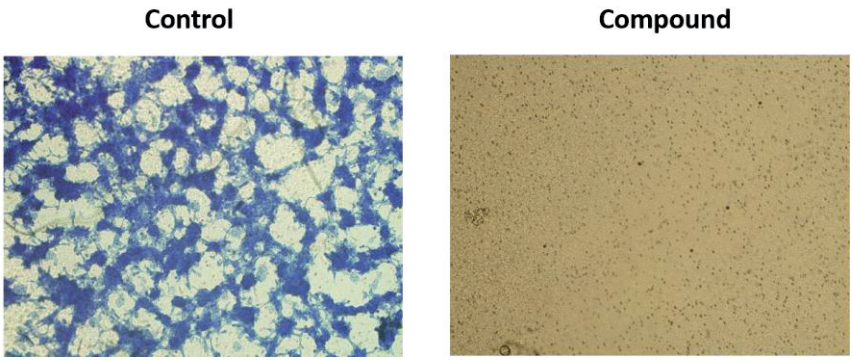


Figure 21: Transwell migration assay was performed and no migrated cell was observed in compound treated group (n=4 for each group).

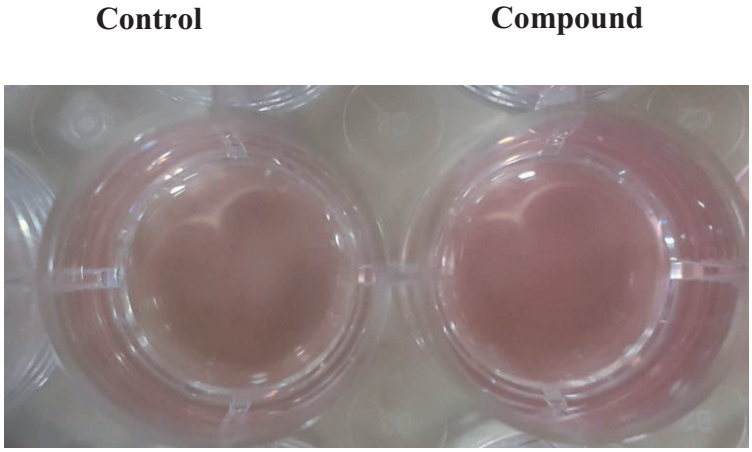


Figure 22: Images of the lower chamber of migration assay. In compound treated group, the color of the medium is darker than other groups, which indicates less migration.

Furthermore, alterations on cancer associated biochemical pathways were determined by protein array technique. After isolating proteins, their concentrations were determined based on standard graphic which is shown in Figure 23. Proteins present in this array with their specific positions are given in Table 5 and Figure 24. After analyzing each spot with the help of Image J software, significant upregulations in EpCAM/TROP1, Serpin B5, p53 and significant downregulations in progranulin, vimentin and SNAIL proteins were observed (Figure 25,26 and 27).

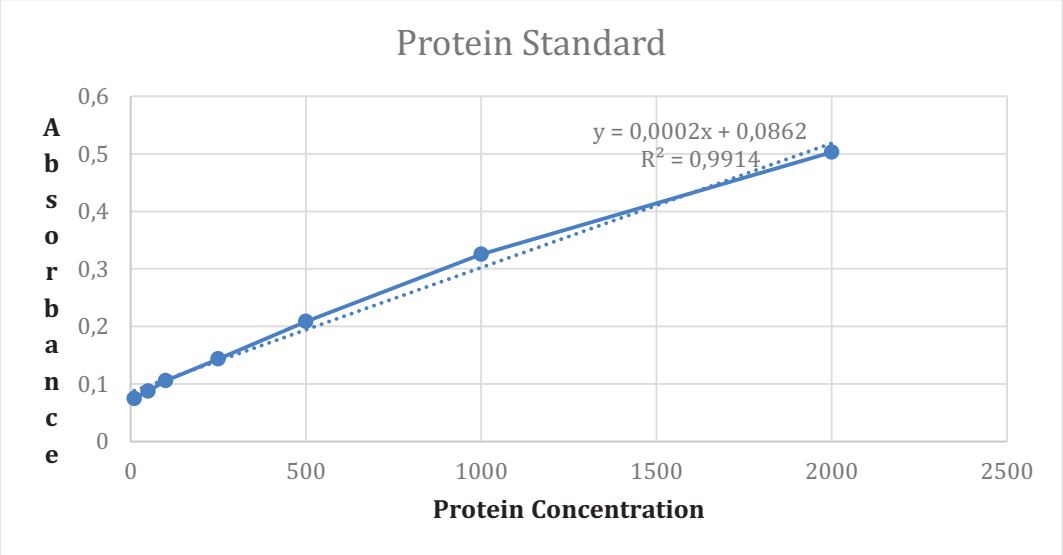


Figure 23: Protein standard graphic

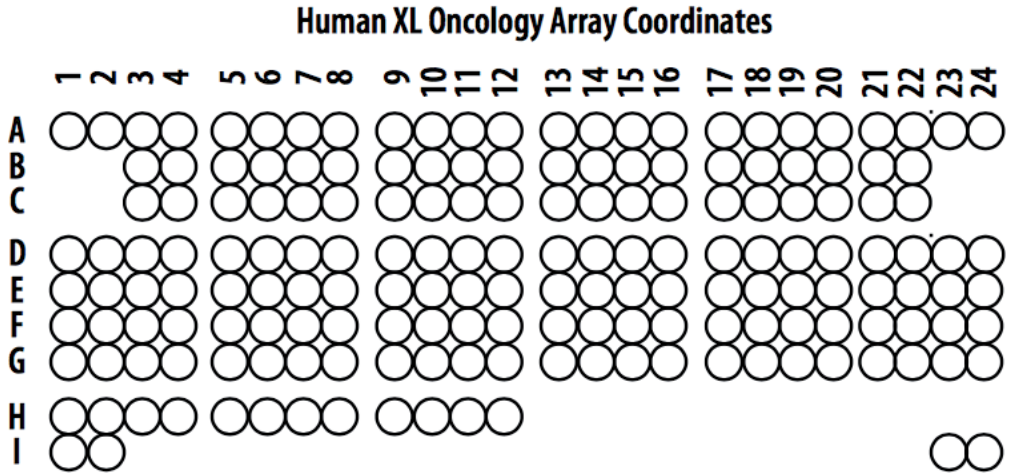


Figure 24: Human Oncology Array coordinates. Each spot represent a specific protein associated with cancer (R&D Systems, USA).

Table 5: Proteins present in Human XL Oncology Array Kit (R&D Systems, USA).

Coordinate	Protein	Alternative Name
A1, A2	Reference Spots	_____
A3, A4	α-Fetoprotein	AFP, DSCAM2
A5, A6	Amphiregulin	AREG
A7, A8	Angiopoietin-1	ANGPT1
A9, A10	Angiopoietin-like 4	ANGPTL4
A11, A12	ENPP-2/Autotaxin	ATX, Lysophosphatidic Acid, NPP2, PDNP2
A13, A14	Axl	Ark, Ufo
A15, A16	BCL-x	BCL2L1
A17, A18	CA125/MUC16	MUC16
A19, A20	E-Cadherin	Arc-1, CAD1, Cadherin-1, CD324, CDH1, Cell-CAM120/80, ECAD, L-CAM
A21, A22	VE-Cadherin	Cadherin-5, CD144, CDH5
A23, A24	Reference Spots	_____
B3, B4	CapG	AFCP
B5, B6	Carbonic Anhydrase	CA9, G250, MN, RCC
B7, B8	Cathepsin B	CTSB
B9, B10	Cathepsin D	CTSD
B11, B12	Cathepsin S	CTSS
B13, B14	CEACAM-5	CD66e, CEA
B15, B16	Decorin	DCN, DSPG2, PG-II, PSG2, SLRR1B
B17, B18	Dkk-1	Dickkopf-1
B19, B20	DLL1	Delta 1

B21, B22	EGF R/ErbB1	ErbB, ErbB1, HER-1
C3, C4	Endoglin/CD105	CD105, ENG
C5, C6	Endostatin	COL18A1
C7, C8	Enolase 2	ENO2; γ-Enolase; NSE
C9, C10	eNOS	NOS3
C11, C12	EpCAM/TROP1	17-1A, CD326, GA733-2, gp40, KS1/4, M4S1, TACSTD1
C13, C14	ERα/NR3A1	ESR1, NR3A1
C15, C16	ErbB2	CD340, HER2, Neu Oncogene, NGL, TKR1
C17, C18	ErbB3/Her3	HER3
C19, C20	ErbB4	HER4
C21, C22	FGF basic	FGF2, FGF-2, FGF2AS, GFG1, HBGH-2, NUDT6, Prostatropin
D1, D2	FoxC2	Fkh14, LD, MFH1
D3, D4	FoxO1/FKHR	FKH1, FKHR
D5, D6	Galectin-3	AGE-R3, CBP35, GAL3, L29, LGALS3, Mac-2
D7, D8	GM-CSF	CSF2
D9, D10	CG α/β (HCG)	CGB, CGB3, Choriogonadotropin
D11, D12	HGF R/c-Met	MET

Coordinate	Protein	Alternative Name
D13, D14	HIF-1α	HIF1A
D15, D16	HNF-3β	FoxA2
D17, D18	HO-1/HMOX1	HSP32
D19, D20	ICAM-1/CD54	_____
D21, D22	IL-2 Rα	CD25, IL2RA
D23, D24	IL-6	BSF-2, IFN-β2, MGI-2A
E1, E2	CXCL8/IL-8	GCP1, IL8, LAI, MDNCF, NAP1, NCF, TCF, TSG1
E3, E4	IL-18 BPa	IL18BP
E5, E6	Kallikrein 3/PSA	KLK3
E7, E8	Kallikrein 5	KLK5, KLK-L2, SCTE
E9, E10	Kallikrien 6	KLK6, Neurosin, Protease M, PRSS18, PRSS9, SP59, Zyme
E11, E12	Leptin	LEP, OB
E13, E14	Lumican	LDC, LUM, SLRR2D
E15, E16	CCL2/MCP-1	MCAF
E17, E18	CCL8/MCP-2	_____
E19, E20	CCL7/MCP-3	MARC
E21, E22	M-CSF	CSF1, CSF-1

E23, E24	Mesothelin	CAK1, MPF, MSLN, SMR
F1, F2	CCL3/MIP-1α	LD78a; MIP-1 alpha
F3, F4	CCL20/MIP-3α	exodus-1; LARC; MIP-3 alpha
F5, F6	MMP-2	Gelatinase A
F7, F8	MMP-3	Stromelysin-1
F9, F10	MMP-9	CLG4B, Gelatinase B, GELB
F11, F12	MSP/MST1	HGFL, MST1, SF2
F13, F14	MUC-1	CD227, Episialin, H23AG, KL-6, Mucin-1, PEM, PEMT
F15, F16	Nectin-4	LNIR, PRR4, PVRL4
F17, F18	Osteopontin (OPN)	Eta-1, Spp1
F19, F20	p27/Kip1	CDKN1B
F21, F22	p53	BCC7, LFS1, TP53, TRP53
F23, F24	PDGF-AA	_____
G1, G2	CD31/PECAM-1	PECAM1
G3, G4	Progesterone R/NR3C3	_____
G5, G6	Progranulin	Acrogranin, GEP, GP88, GRN, PCDGF, PEPI, PGRN, Proepithelin
G7, G8	Prolactin	PRL
G9, G10	Prostasin/Prss8	_____
G11, G12	E-Selectin/CD62E	ELAM1, LECAM2, SELE
G13, G14	Serpin B5/Maspin	PI5
G15, G16	Serpin E1/PAI-1	Nexin, PLANH1

Coordinate	Protein	Alternative Name
G17, G18	Snail	SLUGH2, SNAH, SNAI1
G19, G20	SPARC	BM-40, Osteonectin
G21, G22	Survivin	API4, BIRC5
G23, G24	Tenascin C	Cytotactin, HXB, Tenascin J1, TNC
H1, H2	Thrombospondin-1	THBS1, TSP-1
H3, H4	Tie-2	_____
H5, H6	u-Plasminogen Activator/Urokinase	PLAU, uPA
H7, H8	VCAM-1/CD106	_____
H9, H10	VEGF	VAS, Vasculotropin, VEGFA, VPF
H11, H12	Vimentin	VIM
I1, I2	Reference Spots	_____
I23, I24	Negative Control	_____

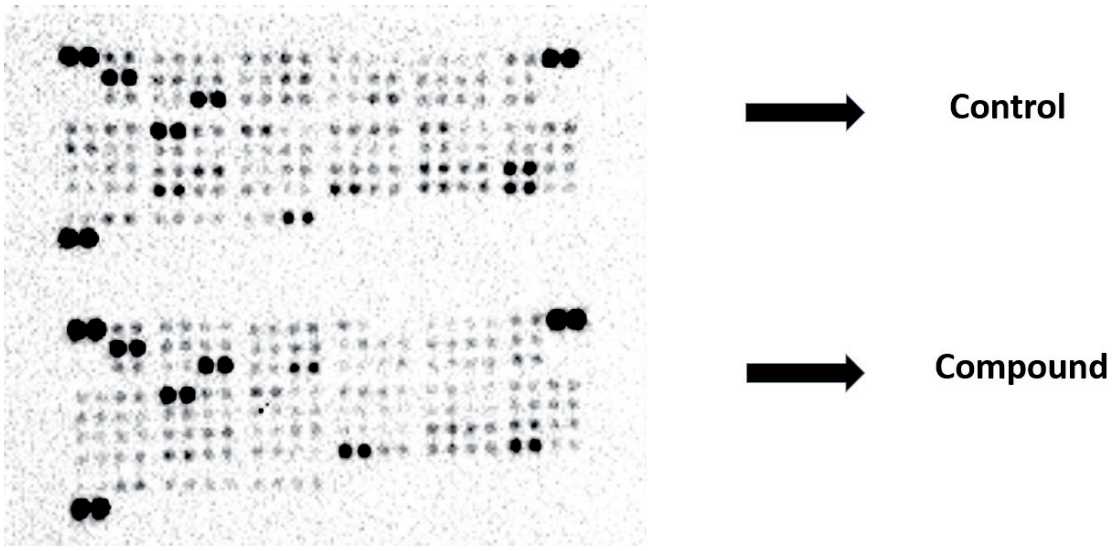


Figure 25: Protein array results of each group (First replicate) Each spot represents each protein. Untreated control and novel compound treated groups are shown.

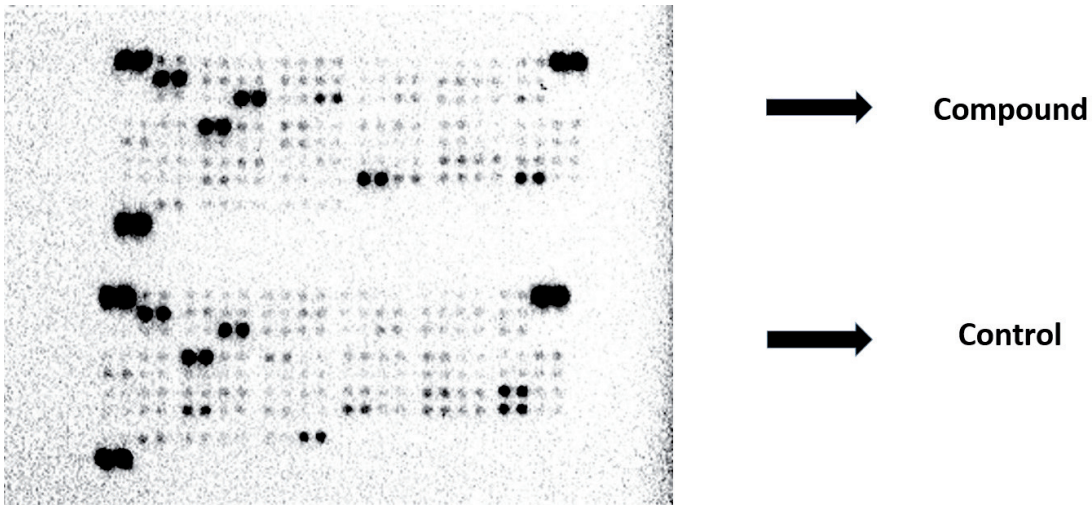
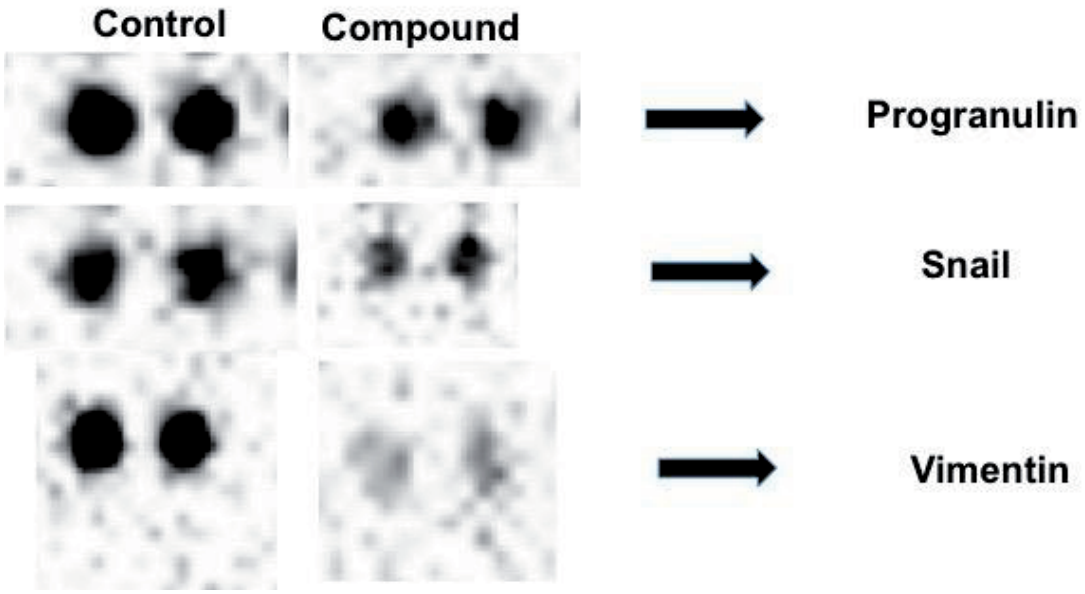


Figure 26: Protein array results of each group (Second replicate)

A



B

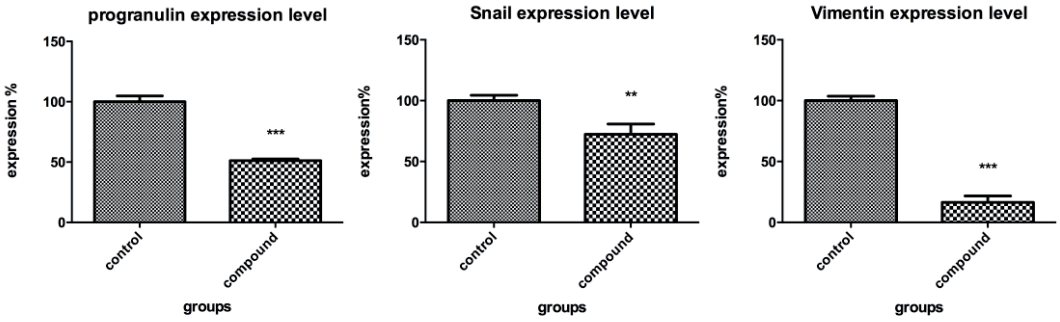
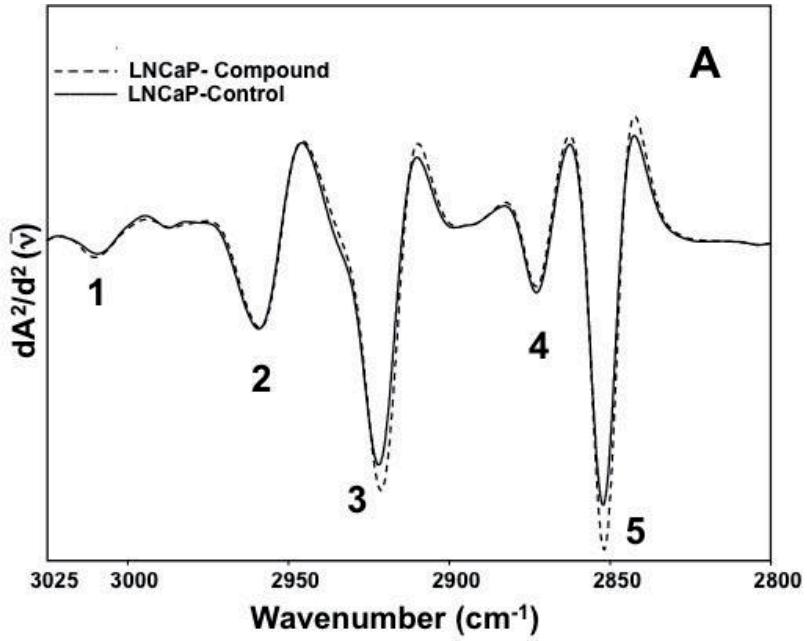


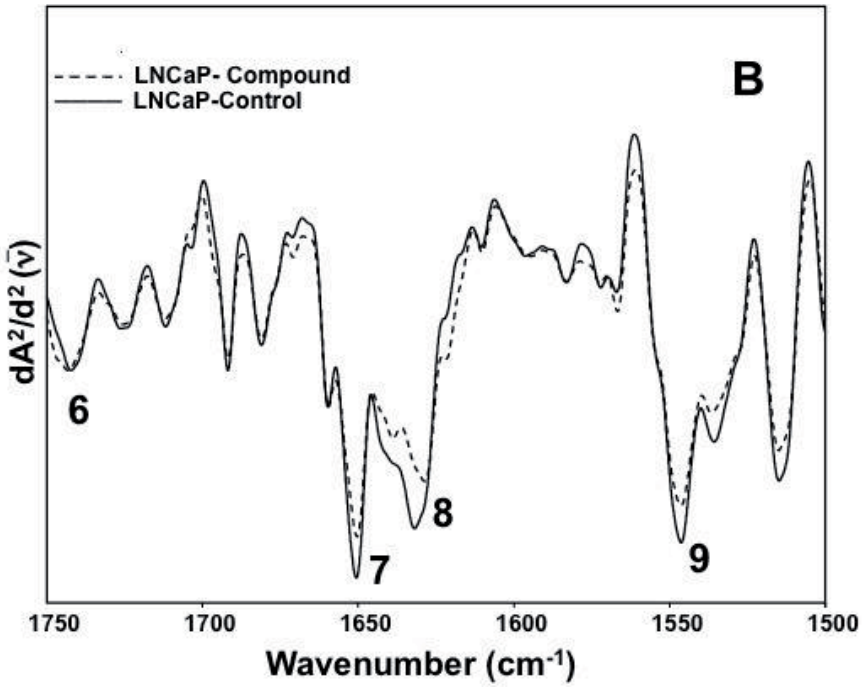
Figure 27 (A) The results of Progranulin, Snail and Vimentin proteins that are present in Human Oncology Antibody Array (n=4 for each group) **(B)** Expression level percentages of metastasis associated proteins: Progranulin, Snail and Vimentin (n=4; ***p<0,001; **p<0,01).

Expression levels of metastasis associated proteins: Progranulin, Snail and Vimentin are given in Figure 27A. Compound treatment decreased expression levels 49%, 28%, 84% respectively as demonstrated in Figure 27B. Downregulation of vimentin was validated by western blot as shown in Figure 44 and 46. Downregulations in these metastasis associated proteins support the migration assay results and suggest anti- metastatic role of alpha tocopherol derived molecule.

3.4. Determination of Antioxidant Capacity, Changes in Membrane Lipid Dynamics and Alterations in Lipid Metabolism

Lipid peroxidation levels were determined by the kits specific to lipid peroxidation end products: Malondialdehyde (MDA) and 8-Isoprostane molecules. These experiments were also supported with ATR-FTIR spectroscopy studies. Furthermore, membrane lipid dynamics were analyzed with the help of ESR spectroscopy and ATR-FTIR spectroscopy. On the other hand, alterations in lipid metabolism were detected by performing lipid metabolism related PCR array, western blot and ATR-FTIR spectroscopy by analyzing unsaturated, saturated lipid, triglyceride and cholesterol ester bands. Band assignments of major absorptions in IR spectra of prostate cancer cell in 3025–900 cm^{-1} region based on literature are presented in Table 6 (Aksoy et al., 2012; Cakmak et al., 2011; Ozek et al., 2010). Also, representative second derivative spectra specific to control and alpha tocopherol derived compound treated LNCaP cells are given in Figure 28 A, B, C.


LNCaP- Compound
LNCaP-Control
A
dA²/d²(ν̄)
1
2
3
4
5
3025
3000
2950
2900
2850
2800
Wavenumber (cm⁻¹)


LNCaP- Compound
LNCaP-Control
B
dA²/d²(ν̄)
6
7
8
9
1750
1700
1650
1600
1550
1500
Wavenumber (cm⁻¹)

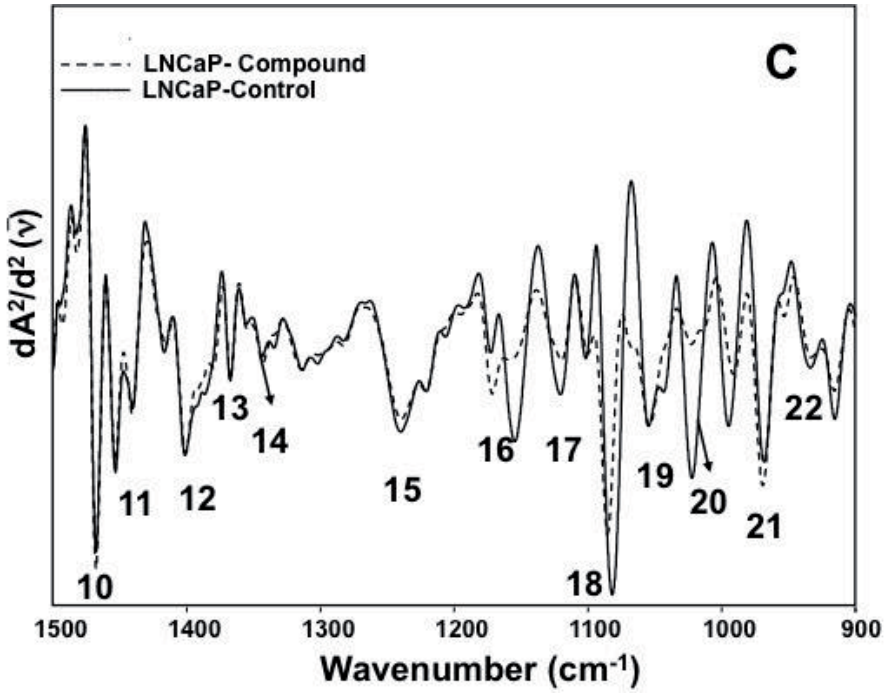


Figure 28: Representative second derivative spectra belonging to control and alpha tocopherol derived compound treated LNCaP prostate cancer cells in the (A) 3025-2800 cm^{-1} (B) 1750-1500 cm^{-1} (C) 1500-900 cm^{-1} region

Table 6: Band assignments of major absorptions in IR spectra of prostate cancer cell in 3025–900 cm^{-1} region based on literature(Aksoy et al., 2012; Cakmak et al., 2011; Ozek et al., 2010).

Peak no	Wavenumber (cm^{-1})	Definition of the spectral assignment
1	3008	Olefinic, =CH stretching,unsaturated lipids
2	2958	CH_3 asymmetric stretching: lipids, protein side chains, with some contribution from carbohydrates and nucleic acids
3	2921	CH_2 asymmetric stretching: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
4	2872	CH_3 symmetric stretching: protein side chains, lipids, with some contribution from carbohydrates and nucleic acids
5	2851	CH_2 symmetric stretching: mainly lipids, with the little contribution from proteins, carbohydrates, nucleic acids
6	1740	Ester C-O stretching:triglyceride, cholesterol ester
7	1651	Amide I: mainly protein C=O stretching, alpha helical structure
8	1631	Amide I: protein beta sheet
9	1546	Amide II: protein N—H bending, C—N stretching
10	1468	CH_2 scissoring: lipids
11	1454	CH_2 bending: mainly lipids, with little contribution from proteins
12	1401	COO^- symmetric strecthing: fatty acids
13	1367	CH_3 symmetric bending: lipids
14	1343	CH_2 wagging: phospholipid, fatty acid, triglyceride, amino acid side chains
15	1238	PO_2^- asymmetric stretching, fully hydrogen-bonded: mainly nucleic acids with the little contribution from phospholipids
16	1172	CO-O-C antisymmetric stretching: ester bonds in cholesteryl esters
17	1118	C–O stretching: ribose
18	1085	PO_2^- symmetric stretching: nucleic acids and phospholipids
19	1040	PO_2^- asymmetric stretching: nucleic acids and phospholipids
20	1022	DNA
21	968	C-N-C stretching: ribose phosphate main chain vibrations of RNA, DNA
22	934	Z-type DNA

3.4.1. Antioxidant capacity of Vitamin E and Vitamin E derived compound

The level of lipid peroxidation was revealed via MDA and 8-Isoprostane kits. Peroxidation percentages were calculated and 33% significant reduction in MDA test, 25% significant reduction in 8-isoprostane test were observed in alpha tocopherol derived compound treated cells (Figure 29, 30). Furthermore, these results were supported by ATR-FTIR spectroscopy. Band intensity ratio of CH_2 asymmetric stretching/ CH_3 asymmetric stretching bands are used to get information about chain length of fatty acids. The higher ratio of these bands indicates the presence of longer chain fatty acids and higher lipid content, while lower ratio suggests shorter fatty acids (Wang et al., 2005). According to band area ratio, longer fatty acid chains were observed in alpha tocopherol derived compound treated samples (Figure 31) suggesting either attenuated level of degradation of lipids due to less lipid oxidation by free radicals or synthesis of longer chain fatty acids (Wang et al., 2005).

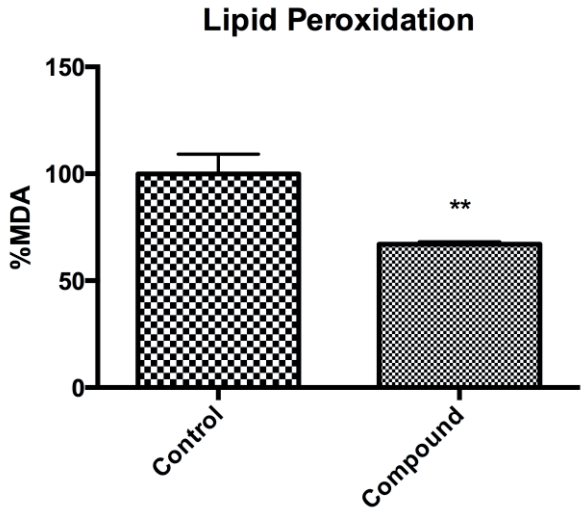


Figure 29: Lipid peroxidation percentage, MDA test results (The average of the control group was considered as 100% in each experiment) (The results are the means +− S.E.M; n=6; **p<0,01)

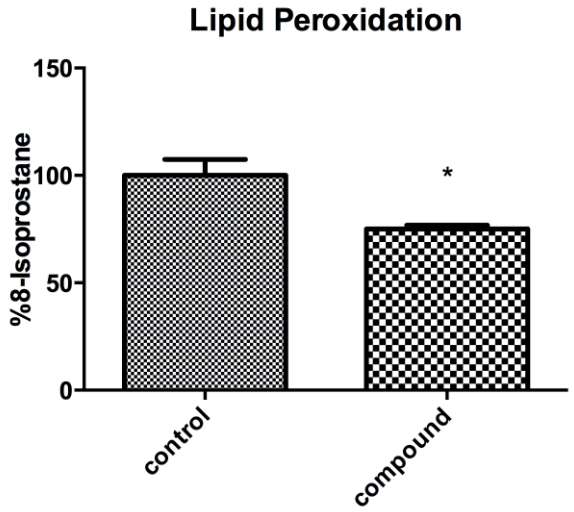


Figure 30: Lipid peroxidation percentage, 8-Isoprostane test results (The average of the control group was considered as 100% in each experiment) (The results are the means +− S.E.M; n=6; *p<0,05)

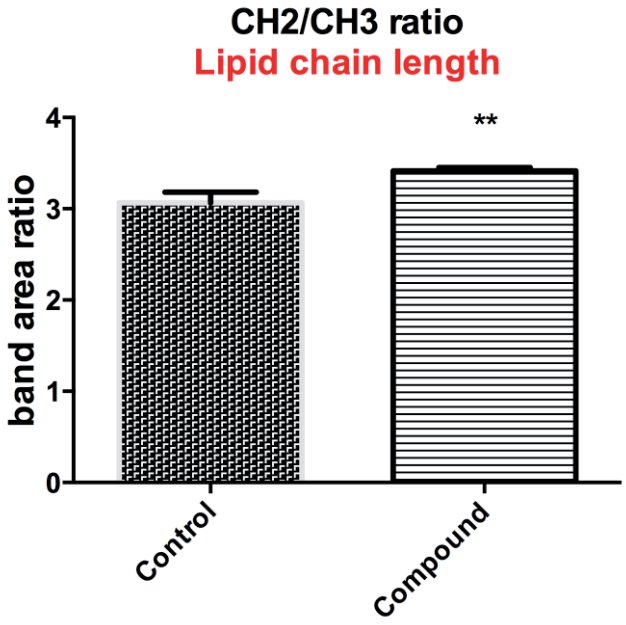


Figure 31: The intensity ratio of CH2 asymmetric/ CH3 asymmetric stretching bands (The results are the means +− S.E.M; n=6; **p<0,01)

3.4.2. Lipid Metabolism Alterations

For monitoring the roles of alpha tocopherol derived compound on lipid metabolism of prostate cancer cells, ATR-FTIR, lipid metabolism RT-PCR Array and western blot techniques were used.

ATR-FTIR spectroscopy was used to detect the alterations in the cellular lipid metabolism by analyzing the band area of the spectral bands corresponding to lipids. As the band area gives information about the amount of respective molecules, we analyzed lipid related regions. Firstly, the band position at 3008 cm^{-1} in a typical IR spectrum of a biological sample corresponds to the olefinic groups (=CH stretching) In order to detect the alterations in unsaturated lipids and unsaturation index, the olefinic band (3008 cm^{-1}) is generally used (Gasper et al., 2009; Krishnakumar et al., 2009; Severcan et al., 2005). As it can be seen from Figure 32 no significant change in unsaturated lipids was found in alpha tocopherol derived compound treated group. Next, CH_2 symmetric stretching band was analyzed due to the fact that this band is arisen from lipid acyl chains (Gasper et al., 2009; Ozek et al., 2014; Severcan et al.,

2005). An elevation was observed in saturated lipids in compound treated cells as demonstrated in Figure 33. Also, triglyceride levels are usually analyzed by examining the band at 1740 cm^{-1} Ester C=O stretching (Nara et al., 2002). In compound treated cells, significant increase was detected as shown in Figure 34. As it was shown before, the amount of cholesterol was determined by analyzing band at 1172 cm^{-1} CO-O-C antisymmetric stretching (Ozek et al., 2010). As can be seen from Figure 35, the level of cholesterol ester significantly elevated in compound treated group.

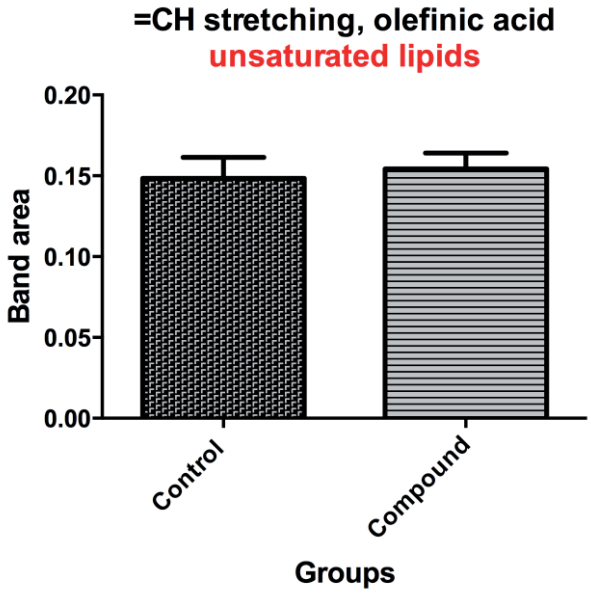
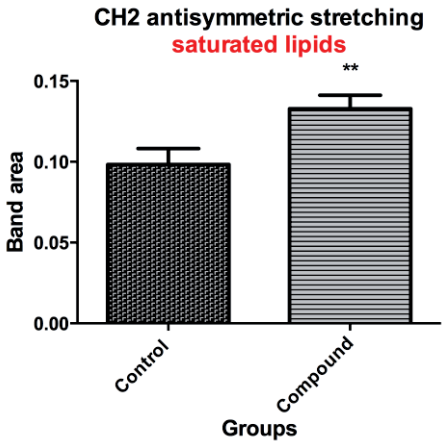
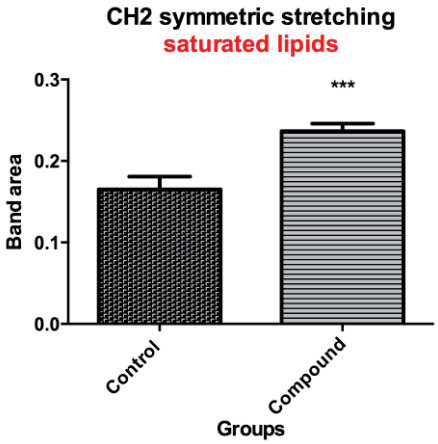


Figure 32 Average band area changes of 3008cm^{-1}, unsaturated lipids (The results are the means +− S.E.M; n=6) No significant change was observed.

A



B



C

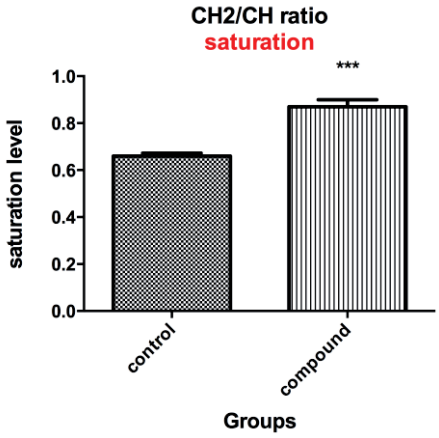


Figure 33: Average band area changes of (A) 2921cm^{-1} , saturated lipids ; (B) 2851cm^{-1}, saturated lipids ; (C) saturation level of fatty acids (The results are the means +− S.E.M; n=6; ***p<0,001; **p<0,01)

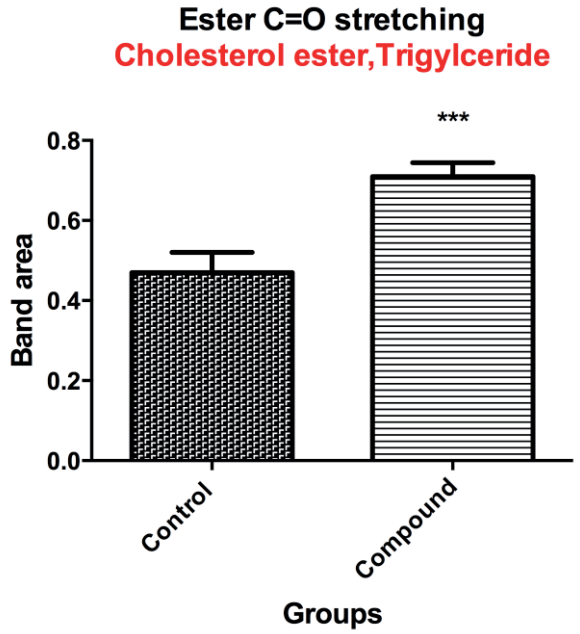


Figure 34: Average band area changes of 1740 cm^{-1} region, cholesterol ester-triglyceride (The results are the means +− S.E.M; n=6; ***p<0,001)

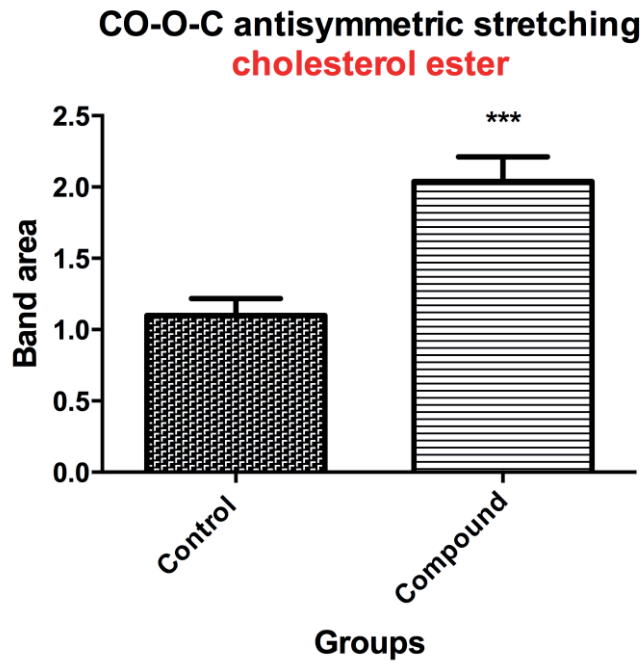


Figure 35: Average band area changes of 1172 cm^{-1} region, cholesterol ester (The results are the means +− S.E.M; n=6; ***p<0,001)

Moreover, hierarchical clustering and principal component analysis were carried out at 3025-2800 cm^{-1} lipid region and remarkable separation of the compound treated cells from control group was obtained (Figure 40). The principal component analysis was also applied to the whole region and striking separation was observed in both cases (Figure 36 and 37). These strong discriminations prove the activity of alpha tocopherol derived molecule.

In PCA analyis, to determine the spectral origins of the segregation of the groups, loading plots are generally analyzed since these plots enables the spectral origin of the variations which differentiate the data groupings according to the wavenumbers. The positive and negative score values in this plot indicate increased and decreased contribution of IR of the respective signals in the measured IR spectra respectively. The loading plots obtained in the whole and lipid regions are shown in Figures 38 and 39, respectively. As can be inferred from Figure 38, the spectral variations between analyzed groups are observed mainly into two spectral regions, C-H stretching (lipid) and fingerprint (1800-650 cm^{-1}) regions which have high positive score values. The detailed analysis of the loading graph for lipid region shows that the high positive values are obtained in two main lipid associated bands: 2921 cm^{-1} (CH_2 asymmetric stretching) and 2854 cm^{-1} (CH_2 symmetric stretching) (Figure 39). These results imply an increase in the contribution of saturated lipid contents to the successful discrimination between the studied groups and also supports the increased saturated lipid content in the compound treated group with respect to the control one.

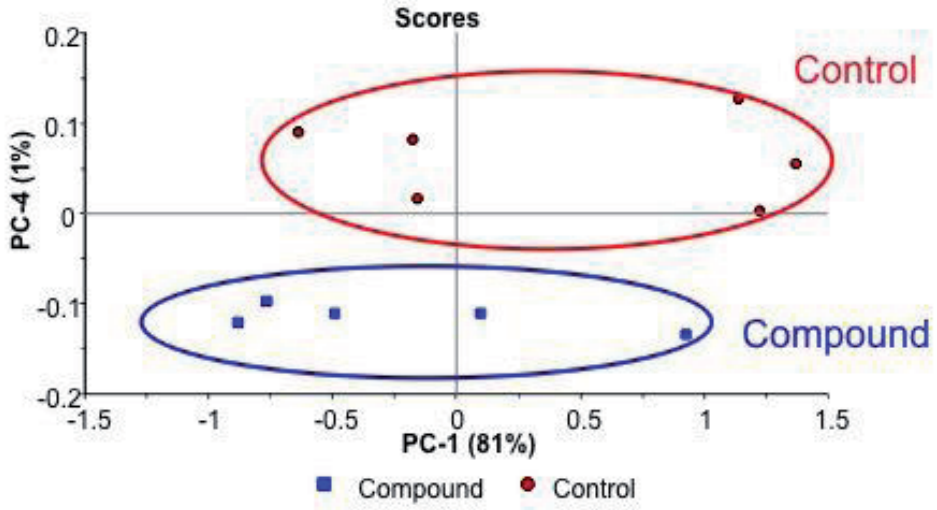


Figure 36: Control(Red), Analog(Blue). Principal component analysis was performed with second derivative spectra in the whole spectral region (4000- 650 cm^{-1}).

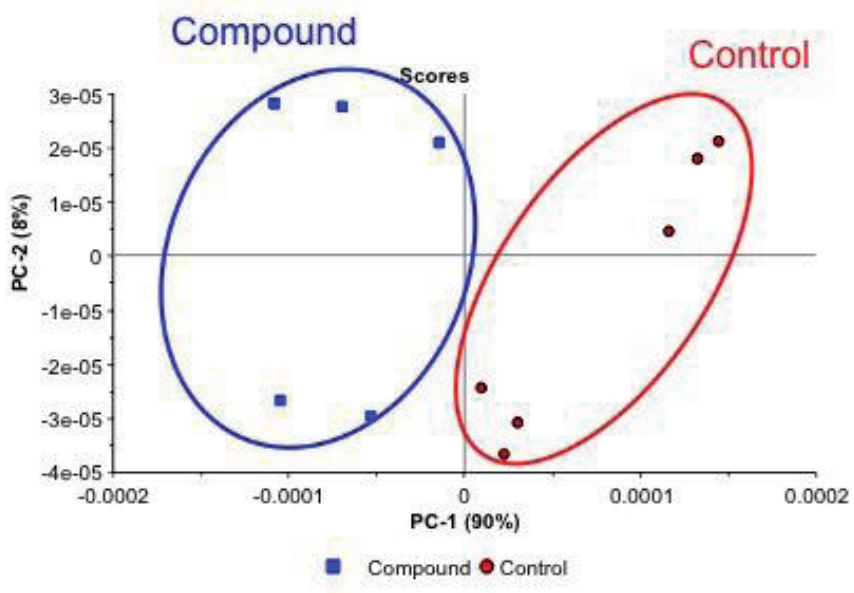


Figure 37: Control(Red), Analog(Blue). Principal component analysis was performed with second derivative spectra in the spectral range of 3025-2800 cm^{-1} (Lipid region).

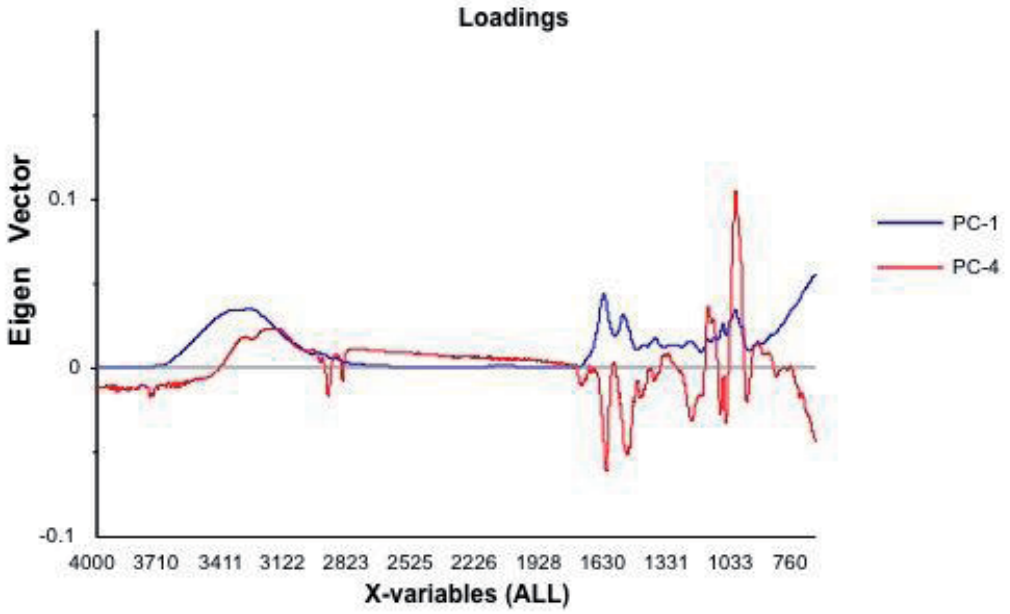


Figure 38: PCA loading plots in the 4000-650 cm^{-1} whole spectral region .

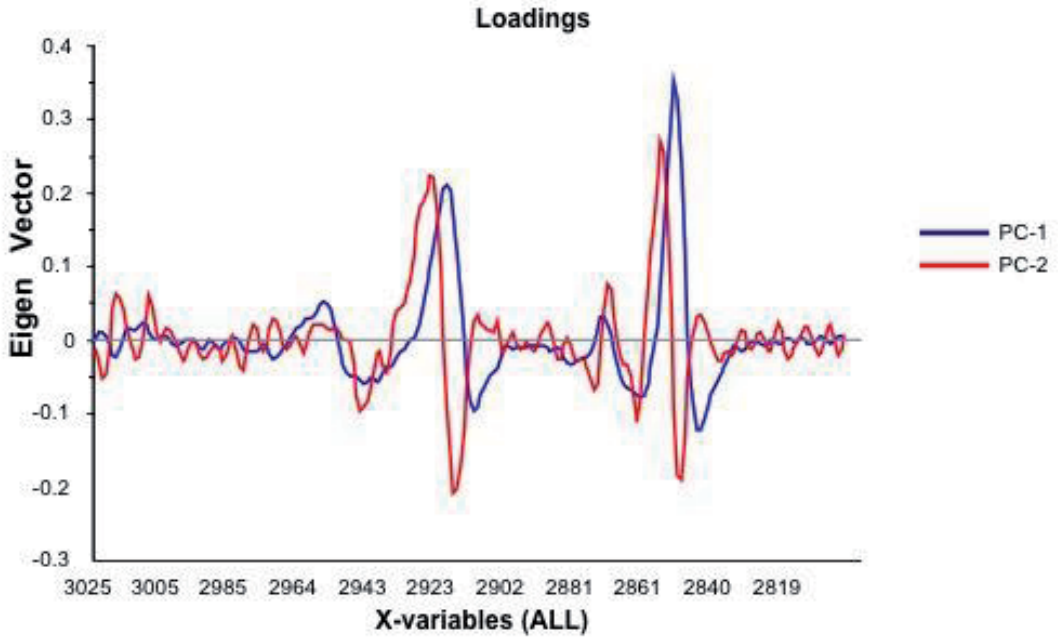


Figure 39: PCA loading plots in the 3025-2800 cm^{-1} spectral region for lipids.

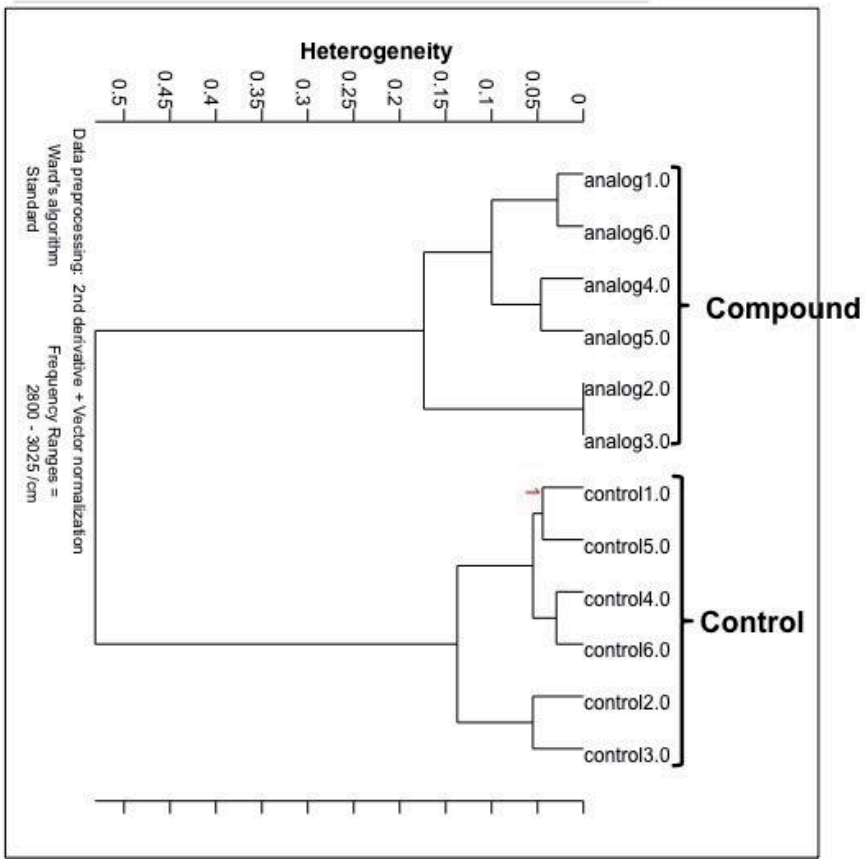


Figure 40: Hierarchical clustering of compound and control group of LNCaP cells; Clustering was performed by using Ward's algorithm and second derivative spectra in the spectral range of 3025-2800 cm^{-1} (Lipid region).

In order to better understand the lipid metabolism changes at molecular level, RT-PCR array and western blot assays were performed. Isolated intact RNAs before cDNA synthesis is demonstrated on the gel in Figure 41. According to RT-PCR array results, alterations on genes related to beta oxidation, fatty acid synthesis, triglyceride breakdown, fatty acid binding and fatty acid uptake were shown in Table 7 (compound). Also, expression differences for alpha tocopherol derived compound group was shown as a graph in Figure 42.

Moreover, expression levels of lipid metabolism associated proteins such as LIPE, FASN and SREBP1 were determined with western blot as demonstrated in Figure 43. Protein levels of metastasis associated protein vimentin, cellular proliferation related p-Akt(Ser473), p-Erk(Thr202/Tyr204) and apoptosis associated cleaved caspase-3 were detected as well (Figure 43). Expression percentage levels of respective proteins were also presented in Figure 44 and 45. While there was upregulation in apoptosis related cleaved caspase-3 protein, downregulations were observed in lipid metabolism, cellular proliferation and metastasis associated proteins.

In case of compound treatment, hormone sensitive lipase (LIPE) downregulated both at mRNA and protein levels, this downregulation is in accordance with the elevated levels of triglycerides. Accumulated triglycerides indicate possible lipotoxicity mechanism. Sterol regulatory element binding protein 1 (SREBP1) was shown to prevent lipotoxicity in cancer cells and loss of its activity caused an imbalance between unsaturated and saturated lipids due to decreased levels of unsaturated fatty acids (Williams et al., 2013). Upon compound treatment, reduction in SREBP1 levels at protein level and increase in the saturation level of fatty acids were observed in FTIR spectroscopy. These results imply imbalance between saturated and unsaturated fatty acid levels and possible lipotoxicity event. Also, Akt is known to play role in prostate cancer progression. Upon phosphorylation at Ser 473, it becomes enzymatically active and induces cellular events leading to proliferation. Therefore, downregulation of p-Akt caused a decrease in proliferation, which was also detected by BrdU assay. Similarly, phosphorylation of Erk at Thr202/Tyr204 triggers signaling events that eventually leads to cellular division. Thus, the observed

downregulation in p-Erk disrupted the cell proliferation process. In addition, apoptosis was clarified at molecular level by performing cleaved caspase-3 western blot. Caspase-3 is considered as the performer of apoptosis and cleavage at Asp175 is required for its activation. Upregulation of cleaved caspase-3 proved the apoptotic role of vitamin E derived compound.

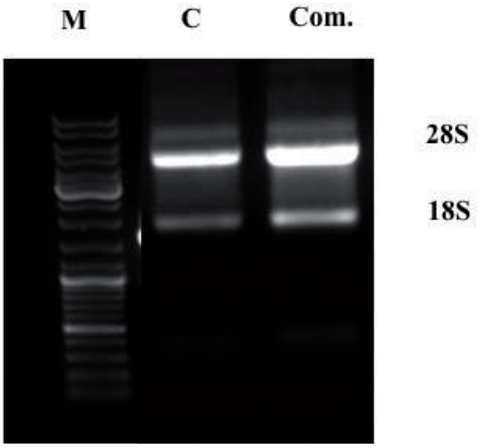


Figure 41: RNA Isolation gel running. This image demonstrates that the RNA loaded to PCR array was intact. M: Marker; C: Control; Com: Compound

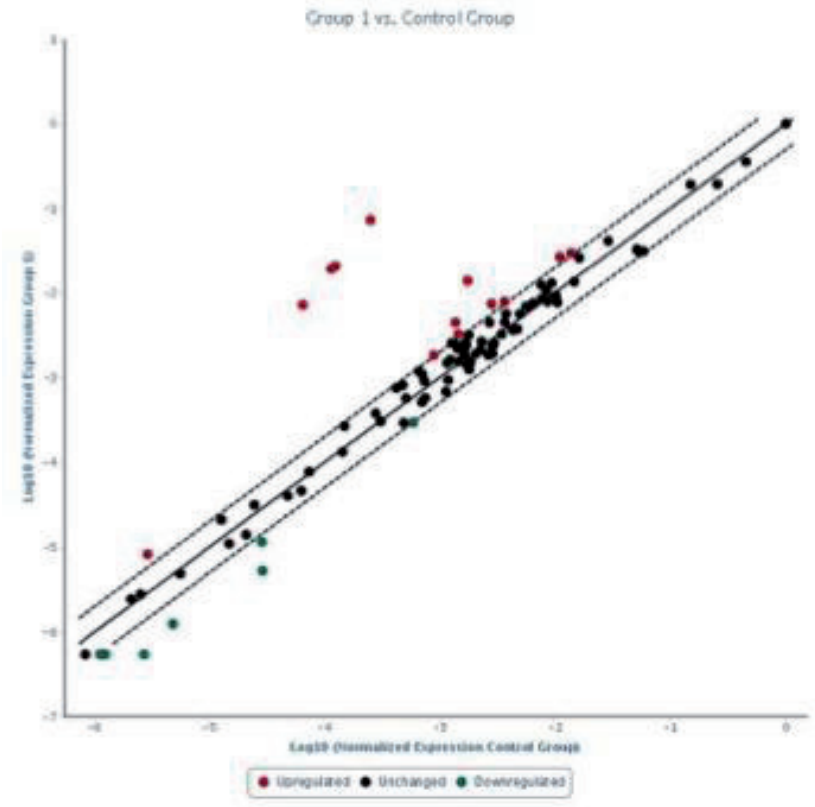


Figure 42: Expression differences of Compound vs. Control group. Red dots indicate upregulated, green dots indicate downregulated lipid metabolism associated genes.

Table 7 Alpha-tocopherol derived molecule treatment alters expression of various lipid metabolism related genes. The results of Lipid Metabolism RT-PCR Array. Fold regulation values represent ratios of the compound treated versus untreated control group of LNCaP cells (n=2).

Upregulated Genes		
Gene Name	**Gene Function**	**Fold Regulation**
ACAT2	Beta Oxidation	7,86
HMGCS1	Cholesterol Synthesis	6,67
HMGCL	Ketogenesis	5,6
SLC27A6	Fatty Acid Uptake	4,5
SLC27A1	Fatty Acid Uptake	3,5
ACADVL	Beta Oxidation	2,41
FASN	Fatty Acid Synthesis	2,23
PRKAA1	Inhibiting Fatty Acid Synthesis	2,14
MUT	TCA Cycle	2,13
GPD2	Fatty Acid Metabolism	2,06
Downregulated Genes		
FABP4	Fatty Acid Binding	4,54
HMGCS2	Cholesterol Synthesis	3,99
LIPE	Triglyceride Breakdown	3,88
ACAD11	Beta Oxidation	3,23
FABP2	Fatty Acid Binding	2,81
FABP7	Fatty Acid Binding	2,29
FABP1	Fatty Acid Binding	2,2

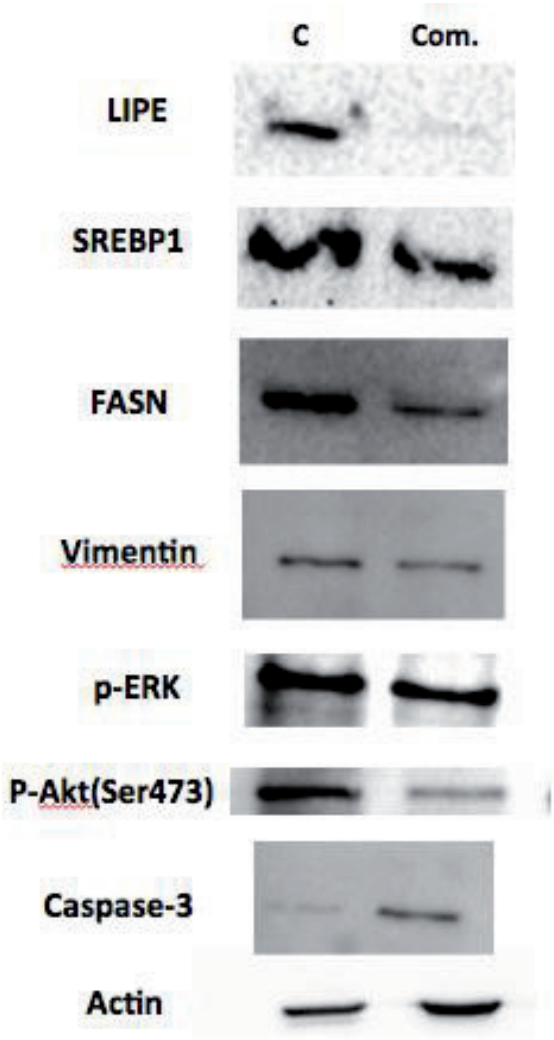


Figure 43: Western blot analysis of lipid metabolism, apoptosis, cellular proliferation and metastasis associated proteins. Total protein lysates were isolated from both 175μM, 48h compound treated and untreated control cells and probed with respective antibodies. Images were analyzed by using ImageJ image processing program. Downregulations were observed in compound treated groups compared to untreated controls in lipid metabolism associated LIPE,SREBP1 and FASN, cellular proliferation associated p-Akt and p-Erk and metastasis associated vimentin proteins. While upregulation was observed in apoptosis related cleaved caspase-3 protein. 40μg protein was loaded and Actin was used for equal protein loading. Lane 1: Untreated control ; Lane 2: Compound treated (n=4)

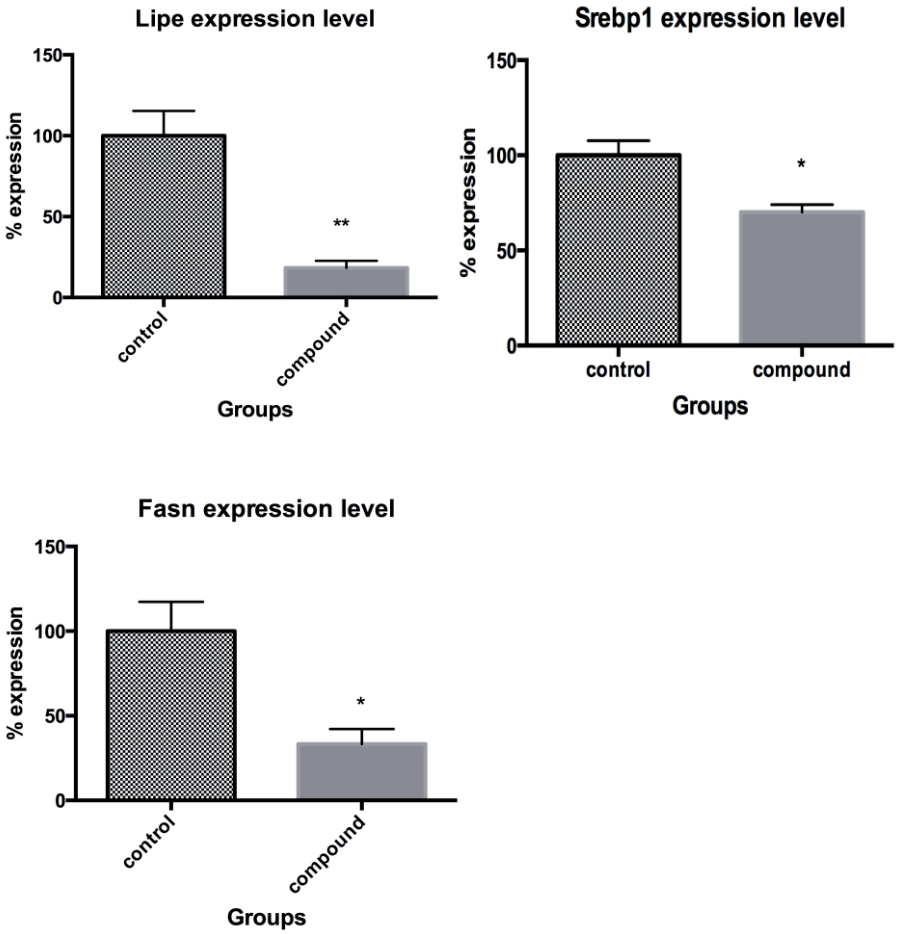


Figure 44: Expression percentage levels of lipid metabolism associated proteins: Lipe (n=4; ** p<0,01), Srebp1 (n=4;* p<0,05), Fasn (n=4; * p<0,05) Untreated groups were presented as control and the average expression levels of untreated control groups were considered as 100. Cells were treated with 175 μM novel compound for 48 h. Relative expression percentages were %18.3 for Lipe, %70 for Srebp1 and %33 for Fasn. Error bars represent SEM of 4 biological replicates. Asterisks denote significance.

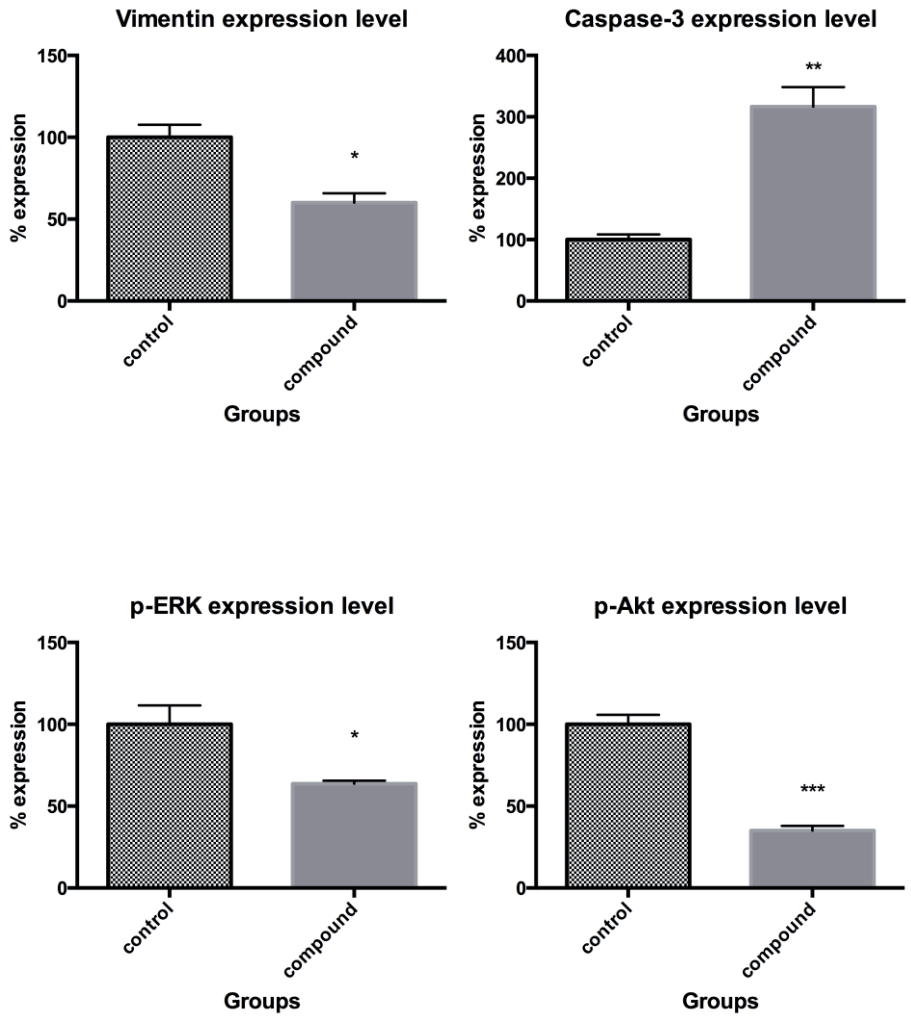


Figure 45: Expression percentage levels of metastasis associated Vimentin (n=4; * p<0,05), cellular proliferation associated p-Erk (Thr202/Tyr204, n=4; * p<0,05), p-Akt (Ser473, n=4; *** p<0,001) and apoptosis associated Cleaved Caspase-3 (n=4;** p<0,01) proteins. Untreated groups were presented as control and the average expression level of untreated control group was considered as 100. Cells were treated with 175 μM novel compound for 48 h. Relative expression percentages were %60 for Vimentin, %63,7 for p-Erk, %35 for p-Akt and %316,6 for cleaved caspase-3. Error bars represent SEM of 4 biological replicates. Asterisks denote significance.

3.4.3. Changes in Membrane Dynamics

In ESR spectroscopy, 16-DSA and 5-DSA spin labels were used to determine membrane lipid fluidity and order respectively. In order to get information regarding membrane fluidity, 16- doxyl stearic acid was used to label prostate cancer cells. This labeling provides information on the motion of the lower portion of the chain towards the center of the membrane (Ozek et al., 2010). The rotational correlation time of the spin label was calculated by using ESR spectra. As can be seen from Figure 46, no significant change was observed in correlation time of any group. This result indicates no significant change in membrane fluidity due to alpha tocopherol derived compound treatment.

On the other hand, order parameter was calculated to detect lipid order in LNCaP prostate cancer cells. In order to have information about membrane lipid order 5-doxyl stearic acid is generally used (Yonar and Sunnetcioglu, 2014). By using this label, it was concluded that there was an increase in lipid order of compound treated cells (Figure 47). Besides ESR spectroscopy, wavenumbers of CH_2 asymmetric stretching were calculated in ATR-FTIR. The shift in the wavenumber of CH_2 asymmetric band to lower value demonstrate an increase in the number of trans conformers of lipid molecules which indicate an increase in lipid order (Ozek et al., 2010; Turker et al., 2014). Shifts to low frequencies were detected in the compound treated prostate cancer cells as shown in Figure 48.

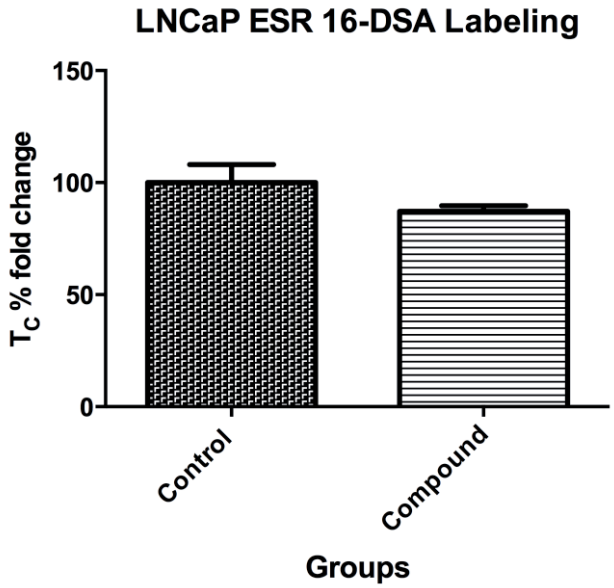


Figure 46: Rotational correlation time indicating membrane fluidity obtained from the ESR spectra of the 16-DSA labelled treated and untreated LNCaP cells (The results are the means +− S.E.M; n=3)

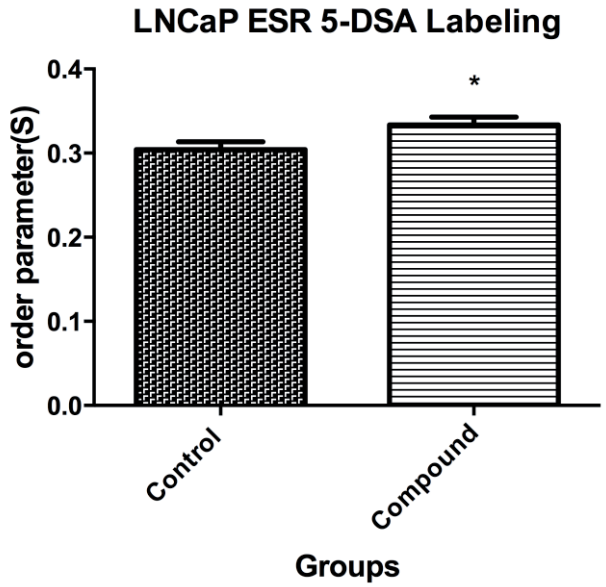


Figure 47: Order parameter obtained from the ESR spectra of the 5-DSA labelled treated and untreated LNCaP cells (The results are the means +− S.E.M; n=3; *p<0,05)

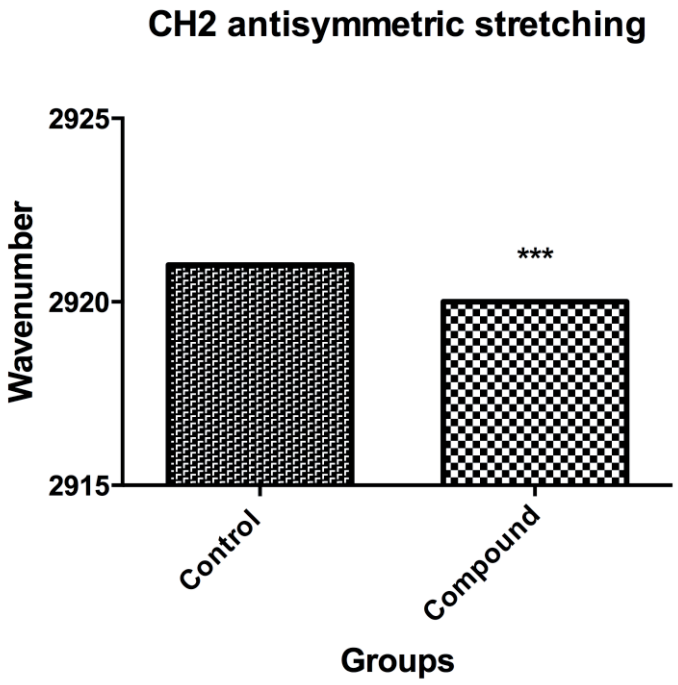


Figure 48: Wavenumber changes of CH_2 asymmetric stretching band indicating membrane lipid order (The results are the means +− S.E.M; n=6; ***p<0,001)

CHAPTER 4

DISCUSSION

In the current study, the influences alpha tocopherol derived compound on general cancer characteristics and on the biophysical and biochemical properties of the cellular lipids were investigated in metastatic prostate cancer cell line LNCaP. Also, cancer related biochemical pathways were analyzed in order to further clarify the underlying mechanisms.

Firstly, we examined the effects of alpha tocopherol derived compound on viability of both prostate cancer LNCaP and normal prostate epithelium PNT2 cell line. XTT assay was performed for this purpose. Different time points and concentrations were applied as can be seen in Figure 9. According to XTT results, it was concluded that LNCaP cells reached IC_{50} value at 175 μM concentration upon alpha tocopherol derived compound treatment. Despite the fact that compound treatment was influential both at 48h and 72h; 48h treatment was chosen for the following experiments, because alpha tocopherol was generally applied at 48h in most of the cancer cell lines (Ju et al., 2010; Wada, 2012). On the other hand, no significant change was detected on the viability of normal prostate epithelium PNT2 cells on treated groups. This result is consistent with the previous studies carried out with different alpha tocopherol analogs like alpha tocopherol succinate which showed less toxic effect to normal cells (Neuzil et al., 2007; Zhao et al., 2009). Therefore, this results indicates that alpha tocopherol derived compound has less side effect to healthy cells.

We then examined the effect of the molecule on cellular proliferation and apoptosis. Our data demonstrated that alpha tocopherol derivative decreased proliferation rate at both 175 μM and 200 μM concentrations. This results proved the anti-proliferative roles of alpha tocopherol derived molecule and suggesting a G1 arrest in cell cycle. Additionally, apoptosis inducing roles of novel compound was proved by Annexin V and JC-1 assays. It was previously shown that apoptosis triggered by different alpha tocopherol analogs by various mechanisms (Zhao et al., 2009).

It was induced by either intrinsic or extrinsic apoptotic pathway, but in most cases it was induced by intrinsic pathway which is characterized by mitochondrial outer membrane permeabilization (Neuzil et al., 1999; Weber et al., 2003; Zu et al., 2005). Mitochondrial destabilization which was experimentally proved by JC-1 assay in this study, causes subsequent events such as cytochrome c release and caspase activation to initiate apoptosis. Moreover, metabolic changes toward accumulation of lipids or cholesterol may stimulate apoptosis in cancer cells (Beloribi-Djefaflia et al., 2016; Huang and Freter, 2015). For instance, saturated fatty acid accumulation caused apoptosis in MDA-MB-231 breast cancer cells (Hardy et al., 2003). Also, cholesterol overload was shown to induce cell cycle arrest in glioblastoma cells (Rios-Marco et al., 2013). Furthermore, increased levels of ceramide, which is composed of sphingosine and fatty acid, can also promote cell death in pancreatic adenocarcinoma and glioma (Carracedo et al., 2006; Ponnusamy et al., 2010). Lipids regulating apoptosis signaling in cells are demonstrated in Figure 49.

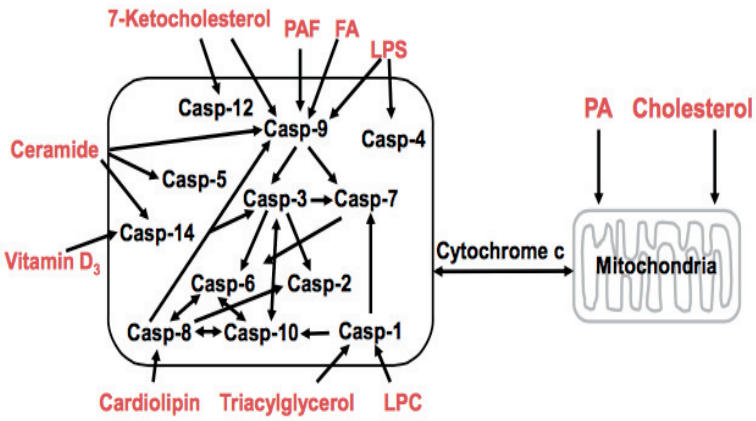


Figure 49: Lipids regulating apoptosis signaling in cells (Huang and Freter, 2015)

In this study, apoptosis was triggered either by mitochondrial membrane permeabilization determined by JC-1 assay or high amount of lipids detected by ATR-FTIR spectroscopy. Although it was not experimentally proved, apoptosis might be stimulated by extrinsic apoptotic pathway as it was previously shown with other alpha tocopherol analog molecules (Turley et al., 1997; Wu et al., 2002).

The crucial steps in metastasis are adhesion and migration of the primary tumors to a distant site. According to motility and migration assay results, compound decreased the mobility of prostate cancer cells. It was also found from the protein array that downregulations were present in some metastasis associated proteins such as progranulin, vimentin and SNAIL. Downregulation in vimentin was validated with western blot as well. These results imply anti-metastatic capacity of alpha tocopherol derived molecule.

Next, we examined the antioxidant capacity of the molecule. It was found that the levels of lipid peroxidation specific products, Malondialdehyde (MDA) and 8-Isoprostane decreased significantly. Besides examining lipid peroxidation specific products, the chain length of fatty acids were measured with ATR-FTIR spectroscopy by taking CH_2/CH_3 ratio. Band intensity ratio of CH_2 asymmetric stretching/ CH_3 asymmetric stretching bands are used to get information about chain length of fatty acids. The higher ratio of these bands indicates the presence of longer chain fatty acids and higher lipid content, while lower ratio suggests shorter fatty acids (Wang et al., 2005). According to band area ratio, longer fatty acid chains were observed in novel compound treated samples (Figure 31) suggesting either attenuated level of degradation of lipids due to less lipid oxidation by free radicals or synthesis of longer chain fatty acids.

Also, compound caused lipid metabolism changes, which were detected by ATR-FTIR spectroscopy, RT-PCR Array and Western Blot techniques. According to ATR-FTIR studies, compound treatment led to elevation at cholesterol, triglyceride and saturated fatty acid levels as shown in Figures 35, 34 and 33 respectively. Moreover, RT-PCR array results shows alterations on genes related to beta oxidation, fatty acid synthesis, triglyceride breakdown, fatty acid binding and fatty

acid uptake were shown in Table 7 (compound). In case of compound treatment, hormone sensitive lipase (LIPE) downregulated both at mRNA and protein levels, this downregulation is in accordance with the elevated levels of triglycerides. Accumulated triglycerides indicate possible lipotoxicity mechanism. Sterol regulatory element binding protein 1 (SREBP1) was shown to prevent lipotoxicity in cancer cells and loss of its activity caused an imbalance between unsaturated and saturated lipids due to decreased levels of unsaturated fatty acids (Williams et al., 2013). Upon compound treatment, reduction in SREBP1 levels at protein level and increase in the saturation level of fatty acids were observed in FTIR spectroscopy. These results imply imbalance between saturated and unsaturated fatty acid levels and possible lipotoxicity event. Furthermore, although upregulation was observed at mRNA level, fatty acid synthase (FASN) enzyme is downregulated at protein level in the compound treated group. This result indicates possible miRNA regulation of FASN. Based on SREBP1 and FASN downregulations, de novo synthesis of fatty acids are thought to be inhibited by compound treatment. The elevation of saturated fatty acids can be explained with overexpression of lipid uptaking genes such as SLC27A1 and SLC27A6, which were detected at mRNA level.

Moreover, expression level changes of apoptosis related cleaved caspase-3 and cellular proliferation related p-Akt(Ser473), p-Erk(Thr202/Tyr204) proteins were detected with western blot. Akt is known to play role in prostate cancer progression. Upon phosphorylation at Ser 473, it becomes enzymatically active and induces cellular events leading to proliferation. Therefore, downregulation of p-Akt caused a decrease in proliferation, which was also detected by BrdU assay. Similarly, phosphorylation of Erk at Thr202/Tyr204 triggers signaling events that eventually leads to cellular division. Thus, the observed downregulation in p-Erk disrupted the cell proliferation process. In addition, apoptosis was clarified at molecular level by performing cleaved caspase-3 western blot. Caspase-3 is considered as the performer of apoptosis and cleavage at Asp175 is required for its activation. Upregulation of cleaved caspase-3 proved the apoptotic role of vitamin E derived compound.

Furthermore, alterations in membrane dynamics were determined by ESR and ATR-FTIR spectroscopy techniques. No significant changes were observed in the fluidity of prostate cancer cells. On the other hand, an increase in the lipid order of cells was detected in compound treated group. It has already been shown that alterations in lipid order influences cellular shape and eventually these changes affects uptake of drugs into the cell (Moore et al., 1997). Thus, compound treatment may cause changes in cell functions due to the alterations occurred in cell shape and thickness of the plasma membrane.

CHAPTER 5

CONCLUSION

As a conclusion, we have demonstrated in this study that:

(1) alpha tocopherol derived compound had less toxicity to normal prostate epithelium cell line PNT2;

(2) compound treatment caused reduction in proliferation and increase in apoptosis of the prostate cancer cell line LNCaP by causing mitochondrial membrane depolarization;

(3) cells treated with the novel compound had a remarkable reduction in their motility and migration capacity implying anti metastatic activity ;

(4) compound treatment led to alterations on lipid metabolism which were detected at both spectroscopic and protein, mRNA levels ; and

(5) cells treated with compound show decreased lipid peroxidation and beta oxidation.

(6) cells treated with compound show increased lipid order that leads to changes in cellular membrane structure.

Besides these promising results, compound's similar molecular structure to alpha tocopherol gives advantage to our molecule to be easily transported and metabolized throughout the human body.

These properties of alpha tocopherol derived compound emphasize the importance of this molecule as a possible therapeutic option in prostate carcinogenesis.

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CURRICULUM VITAE

Personal

Surname, Name: Ergen, Nuri

Date of Birth: 13.05.1987

E-mail address:nergenetik21@gmail.com

Education

2001-2004 Ankara ARI Private Science High School (With Scholarship) GPA:5,00 out of 5,00

2004-2008 BSc- ISTANBUL University, Department of Molecular Biology and Genetics GPA: 3,02 out of 4,00

2009- 2012 MSc- ANKARA University, Biotechnology Institute

GPA: 3,59 out of 4,00

2012- 2016 PhD- Middle East Technical University, Biological Sciences

GPA: 3,64 out of 4,00

Seminars,Workshops and Congresses

27-29 November 2015, 4th International Congress of the Molecular Biology Association of Turkey

24-25 June 2011,Turkish Academy of Sciences VI. Stem Cell Course and Symposium

6-7 June 2011, RNA Interference Course organized by Ankara University

8-10 November 2010, Biotechnology Politics and Biosafety in Turkey organized by Ankara University

28-31 October 2009, Medicinal Genetics Congress organized by Turkish Academy of Sciences

25 May 2009, Ankara Biotechnology Workshops organized by Biotechnology Institute

June 2006, Sabancı Universtiy Istanbul

21st Century: Century of Biotechnology Seminars

March 2006,Istanbul University

Molecular Biology and Genetics Congress

Congress Abstracts

Effects of aqueous extracts of Urtica diocia L. leaves on lifespan of Caenorhabditis elegans, 59th International Congress and Annual Meeting of the Society for Medicinal Plant and Natural Product Research

Research Interests

Cancer Genetics, Targeted Therapies of Cancer,
Biotechnological Drug Production, Aging, Stem Cell

Research Projects I'm Involved in

Investigation of the effects of Turkish folk medicine on C.elegans lifespan (TÜBİTAK Project Supervised by Assistant Prof.Dr. Arzu ATALAY)

Effect of 15-Lipoxygenase-1 (15-LOX-1) expression on Angiogenesis in Colorectal Cancer HCT-116 and Prostate cancer PC3 cells (TÜBİTAK Project supervised by Associate Prof.Dr.Sreeparna BANERJEE)

PhD Thesis: Synthesis of a Novel Vitamin-E Analogue and Investigation of its Anticancer Properties in Breast and Prostate Cancer Cells by Biochemical, Biophysical and Molecular Biological Techniques (A Joint TÜBİTAK Project with Ukranian Academy of Sciences Supervised by Prof.Feride SEVERCAN)

Publications

Ergen N., Hosbas S., Deliorman Orhan D., Aslan M., Sezik E., Atalay A. 2016 "Investigation of the effects of Turkish folk medicine on *Caenorhabditis elegans* lifespan" Pharmaceutical Biology (Sent to journal for publication, under revision)

Techniques I'm Familiar with

C.elegans Techniques, RNAi, Cell Culture Techniques, PCR, Western Blot, FTIR

Career Goal

Working on drug R&D (biotechnological or biosimilar products)

Personal Interests

History, Literature, Art, Basketball, Football, Motorsports