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Vitamin E (Tocotrienols) and Prostate Cancer:  
A Proteomics Approach

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

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

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by  
Christian Mbangha Muenyi  
August 2007

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Keywords: vitamin E, gamma-tocotrienol, delta-tocotrienol, apoptosis, necrosis, proteomics

## ABSTRACT

Vitamin E (Tocotrienols) and Prostate Cancer:

A Proteomics Approach

by

Christian Mbangha Muenyi

Proteomics is the large scale study of proteins in cells or organisms. The purpose of this study was to characterize the proteomic alterations occurring in a prostate cancer (LNCaP) cell line after treatment with delta-tocotrienol (a form of vitamin E not very prevalent from most dietary sources). We found that both gamma- and delta-tocotrienols induced time and concentration dependent growth inhibition and programmed cell death (apoptosis) in LNCaP cells. Secondly, we used two-dimensional gel electrophoresis (2-DE) to characterize changes in protein expression levels associated with this treatment. Our results show that a specific set of proteins are regulated at both early and late times following treatment with delta-tocotrienol and these proteins have been characterized by their apparent molecular weights and isoelectric points. The alteration observed at early time points are particularly interesting because these changes are likely to reflect the underlying molecular mechanisms for triggering cancer cell death.

## ACKNOWLEDGEMENTS

I wish to express sincere appreciation to Dr. William L. Stone for his excellent mentorship. My gratitude to him cannot all be expressed in words! I wish to also thank him for giving me the opportunity to go through all the experiences I have had in his lab. Dr. Kasmai might not know the impact he created in my life when he agreed to be my co-advisor, but I am saying it here that it was a life-changing decision. I thank him so much for all his kindness. I also extend sincere gratitude to Dr. Ho for readily accepting to be a member of my thesis committee. I will be doing myself a disfavor if I do not say here how appreciative I am to Dr. Thomas T-S. Huang. He ‘adopted’ me as one of his children when I got to the US and played a very vital role in facilitating my admission into ETSU. I can never thank him enough for that. I also wish to mention those who made life easy for me in Dr. Stone’s lab. First, I wish to say my heart goes to Hongsong for always being there and willing to assist me. Qui Min also did a wonderful job in helping me out in the lab. My appreciation too goes to all the members of the Internal Medicine Journal Club for helping me cope with public speaking. The staff and students of the department of chemistry at ETSU really provided a friendly academic environment for me and I sincerely thank them for that. My heart also goes to my family back at home and to all my friends who have made my life here worth living. I also wish to acknowledge in a very special way the efforts of my wife in the realization of this project. To crown it, I wish to say a big thank you to Christine Hendershott for her role in correcting my manuscript.

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## CHAPTER 1

### INTRODUCTION

The various forms of vitamin E have been under intensive study as chemopreventive and chemotherapeutic agents for various types of cancer (1). Many *in vitro*, animal, and epidemiological studies have presented strong evidence of an anticancer activity for vitamin E, but there are few studies of vitamin E in prostate cancer (2). Gamma-tocopherol is the predominant form of vitamin E found in the U.S. diet, and alpha-tocopherol is the form most often found in dietary supplements and in the body (3). Tocotrienols have, however, been found to be more effective than tocopherols in inhibiting cell growth and inducing apoptosis in cancer cells (4, 5). Differences in the cellular uptake between tocopherols and tocotrienols could be a factor explaining these differences (5, 6). Also, a non-antioxidant mechanism has been suggested to explain the antitumorigenic effects of vitamin E. Gamma-tocopherol, for example, has a weaker antioxidant capacity than alpha-tocopherol but has a more significant inhibition on cancer cell proliferation than alpha-tocopherol. Also, prevailing evidence that these antioxidant vitamins appear to have some protective effects against diseases associated with reactive free radicals, such as coronary heart disease and cancer, means that diets rich in vitamin E, such as whole grains and vegetables, can reduce the risk of these diseases (7). But the mechanisms by which the various forms of vitamin E induce apoptosis in cancer cells remain largely unknown (8). Proteomics, which is the large scale study of proteins expressed by a cell or an organism, may assist in understanding the cellular and molecular events associated with the cancer chemopreventive and chemotherapeutic effects of vitamin E.

## Chemical Structure and Properties of Vitamin E Family

Vitamin E is composed of a family of at least eight different organic compounds. There are four tocopherols (alpha-T, beta-T, gamma-T, and delta-T) and four tocotrienols (alpha-T3, beta-T3, gamma-T3, and delta-T3). They are fat-soluble vitamins and function as antioxidants by preventing the propagation of free radicals. They also have non-antioxidant functions such as regulating cellular signal transduction pathways and preventing infertility (9-11). They all have a chroman ring (head). They are distinguished by the phytyl tail of the tocopherols and the farnesyl tail of the tocotrienols. Tocopherols have three chiral carbons. One of the chiral centers is located at the carbon 2 (C2) of the heterocyclic ring, while the other two are located on the 4<sup>th</sup> and 8<sup>th</sup> carbon of the phytyl tail (Figure 1). Each chiral center has two possible configurations, R or S (i.e. right or left configuration, respectively). Therefore, tocopherols can have 8 ( $2^3$ ) stereoisomers, but naturally occurring tocopherol exists in the RRR configuration at all 3 chiral carbons. Synthetic tocopherol is a mixture of almost equal amounts of all the possible stereoisomers (RRR, RRS, RSS, SSS, SSR, SRR, SRS, and RSR) and is usually identified as all-rac (all-racemic) or as dl-tocopherol. Tocotrienols have a chiral center at the C2 carbon in the heterocyclic ring, which can have two possible stereoisomers (i.e. R or S configuration) and three double bonds located at the 3', 7', and 11' positions of the farnesyl group. The double bonds at the 3' and 7' positions can generate geometric isomers (E or Z isomers). Natural tocotrienols are EE in the 3' and 7' positions. The structures of the tocopherols and tocotrienols are shown in Figure 1.



## Prostate Cancer Prevalence

Prostate cancer is a disease in which cells in the prostate gland become abnormal and start to grow uncontrollably, forming tumors. Prostate cancer is second only to lung cancer as the most frequently diagnosed and leading cause of cancer death for men in the United States (12). On average, one man in six will be diagnosed with prostate cancer during his lifetime, and more than 200,000 men in the U.S. are diagnosed with prostate cancer every year.

## Chemotherapy and Chemoprevention

Perhaps the areas of cancer research with the greatest potential for reducing cancer mortality are chemotherapy and chemoprevention. Chemotherapy is the treatment of existing cancer using specific chemical agents or drugs that are selectively destructive to malignant cells and tissues while chemoprevention is the use of chemical agents, drugs, or food supplements to prevent the future development of cancer. Epidemiological observations, experimental evidence from animal carcinogenesis models, knock-out models, *in vitro* studies with cancer cell lines, and clinical trials have shown the efficacy of this approach.

One of the principal goals of cancer chemotherapeutic or chemopreventive agents is the elimination of damaged or malignant cells through cell cycle inhibition or induction of programmed cell death (apoptosis), leaving normal cells unaffected (13). Knowing the molecular mechanisms involved in inducing cancer cell death is of vital importance because it gives us an idea of the specific proteins regulated and this can lead to the design of more specific drugs that can selectively target cancer cells. A major goal of chemotherapy and chemoprevention is to design agents with a very high level of specificity. Proteomics provides the optimal tool for the study of these molecular mechanisms.

## Vitamin E as a Chemotherapeutic and Chemopreventive Agent

Vitamin E has been under intensive study as a chemotherapeutic and/or chemopreventive agent for many types of cancers (1). Multiple primary and secondary intervention trials of vitamin E for the prevention of various cancers are currently underway; these studies should define the role vitamin E plays in the prevention and treatment of various cancers (e.g. oral cavity, pharynx, oesophagus, prostate, colon, lung, breast, skin, cervix, and bladder) (14). Various chemical modifications of vitamin E have also been made and used as chemopreventive agents for various types of cancers. There is increasing evidence that vitamin E succinate is the most effective antitumor analogue of vitamin E (1).

### Cell Culture Studies

Cell culture studies show that antioxidant vitamins (like vitamin E) and some phytochemicals selectively induce apoptosis in cancer cells but not in normal cells and also prevent angiogenesis and metastasis (15). Collectively, these studies suggest a potential role for antioxidants like vitamin E as supplements in cancer therapy. While tocopherols have been found in many different plant species and tissues, and their biosynthesis, physiology, and distribution have been studied in detail, little is known about the physiology and distribution of tocotrienols (9). Although chemically very similar, tocotrienols have been shown to display significantly more potent apoptotic activities than tocopherols in preneoplastic, neoplastic, and highly malignant cells grown in culture at treatment doses that show no adverse effects on normal cell growth or function (10). The inhibitory effects of the various forms of vitamin E on cell growth follow the order:  $\alpha$ -T <  $\alpha$ -T3 <  $\gamma$ -T <  $\gamma$ -T3 <  $\delta$ -T3 (11). The difference in terms of apoptosis induction by the different homologues of vitamin E seems to be

related to their different rates of cellular incorporation (5) and does not depend on their antioxidant properties as mentioned earlier (7). However exciting these initial findings might be, the challenge is that the exact intracellular mechanisms that mediate vitamin E-induced apoptosis is still not understood.

### Animal Studies

Daily injection of vitamin E succinate has been shown to significantly suppress tumor growth as well as lung metastases in mouse models (1). Also, experiments have shown that vitamin E potentiated the antitumorigenic effects lycopene in a prostate cancer rat model (16).

### Epidemiological and Clinical Studies

Many epidemiological studies suggest that a high intake of vitamin E rich foods specifically lowers cancer risk, but to date mechanistic studies are scarce (10). Also, epidemiological and clinical studies provide evidence that antioxidants, anti-inflammatory agents, some polyunsaturated fatty acids, and several other phytochemicals possess unique modes of action against cancer growth. However, the mode of action of several of these agents at the gene transcription level is not completely understood.

Other studies suggest that environmental and diet related factors may mediate the transformation of latent prostate cancer into clinically apparent tumors. Selenium and vitamin E have been shown to decrease the risk of prostate cancer. Some human trials using vitamin E alone or in combination with other nutrients are in progress, but to date there has been little *in vivo* evidence supporting their preventive capabilities. The development of chemopreventive agents against prostate cancer would benefit from conclusive evidence of their efficacy in animal models that emulate human disease (17).



Despite confounding factors present in clinical studies assessing the effect of diet on cancer risk, the data remain compelling that a variety of nutrients may prevent the development and progression of prostate cancer (18). But at this time, nothing has been proven effective as a chemoprevention agent against prostate cancer (19). Ongoing studies on nutrition and prostate cancer may bring the required evidence to support what is still only a hypothesis at present. However, absolute recommendations will have to await the results of long-term prospective clinical trials (20).

Treatment of cancer using radiation and anticancer drugs reduces inherent antioxidants and induces oxidative stress. Vitamins E and C have been shown to improve adverse side effects associated with free radical damage to normal cells in cancer therapy and to reduce the recurrence of some cancers.

### Rationale of Our Research

Our research is a follow up on a previous finding in our lab that showed gamma-T3 and delta-T3 have a greater cytotoxic effect on the androgen-dependent prostate cancer (LNCaP) cell line than their tocopherol counterparts and concentrations of less than or equal to 40  $\mu$ M inhibited the growth of prostate cancer cells but had no significant effect on non-tumorigenic prostate epithelial cells. In the work performed here, we determined the minimum effective concentration of gamma- or delta-T3 to inhibit cell growth using a cell viability assay. We also used the propidium iodide assay to study cell death and a caspase 3 enzymatic assay to determine the molecular mechanism of cell death. This was followed by the use of two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (2-DE) to investigate the proteomic alteration patterns in androgen dependent prostate cancer (LNCaP) cells in response to delta-T3

treatment. This is in an effort to understand the underlying molecular mechanism by which this isoform of vitamin E can induce apoptosis in LNCaP cells. It is hoped that this knowledge will help in the development of more specific and efficient therapies for the treatment of prostate cancer. For example, if a certain protein is implicated in the disease treatment, the 3D structure of the protein may provide the information a computer program needs to design drugs to alter the action of the protein. A molecule that binds the active site of an enzyme can alter the activity of the enzyme. This is the basis of new drug-discovery tools that can help design new drugs to alter the functioning of proteins involved in diseases. Researchers can also use these same techniques to develop personalized drugs that are optimally effective for individuals based on specific genetic traits.

## Proteomics

### Definition

Proteomics is the large-scale study of proteins in cells or organisms and is aimed at identifying and characterizing protein expression, function, post-translational modifications, regulation, trafficking, interactions, and structure and their perturbations by disease and drug action. The classical proteomic approach is based on protein separation using two-dimensional gel electrophoresis (2-DE) followed by protein identification and characterization by mass spectrometry (MS) analysis (21). The word proteome is derived from PROTEins and genOME, because proteins are expressed by the genome. Thus, the proteome refers to all the proteins produced by an organism, much like the genome is the entire set of genes. The term proteomics was formulated to make an analogy with genomics (the study of an organism's entire genome). Therefore, proteomics is the study of the composition, structure, function, and interaction of the

proteins directing the activities of each living cell. As the main components of the physiological pathways of the cells, proteins perform the vital functions of the body. While proteomics is often viewed as the "next step", it is much more complicated than genomics. The human body may contain more than 2 million different proteins, each having different functions. Most importantly, while the genome is a rather constant entity, the proteome differs from cell to cell and is constantly changing through biochemical interactions with the genome and the environment. One organism has very different protein expression in different parts of its body, in different stages of its life cycle, and in different environmental conditions. The proteome is therefore dynamic and in constant flux. Proteomics uses a combination of sophisticated techniques including 2-DE, MS, and bioinformatics to characterize, quantify, and analyze cellular proteins in a global way. The application of proteomics provides major opportunities to elucidate disease mechanisms and to identify new therapeutic targets (22).

Many proteins experience post-translational modifications that have a profound effect on their activities. Some proteins, for example, are not active until they become phosphorylated. Specialized methods, such as phosphoproteomics and glycoproteomics, have been developed to study proteins with post-translational modifications. Furthermore, many transcripts give rise to more than one protein through alternative splicing or alternative post-translational modifications. Finally, many proteins form complexes with other proteins or RNA molecules, and they only function in the presence of these other molecules. These complexities suggest that protein diversity cannot be fully characterized by gene expression analysis alone, making proteomics a useful tool for characterizing cells and tissues that complement the information obtained from genomic studies.

## Proteomics Data Management

To catalog all human proteins and ascertain their functions and interactions present a daunting challenge for scientists. Consequently, to continue with the current growth rate of proteomics, new approaches must be developed to insure accurate, consistent, and transparent data management and mining that are critical to the success of proteomic workflow and for biomarker discovery in particular. There is an international collaboration to achieve these goals, coordinated by the Human Proteome Organization (HUPO). Initiatives have been launched to develop standard data formats for exchanging mass spectrometry proteomic data, including the Proteomics Standards Initiative formed by the HUPO. Definition of common standards for data representation and analysis and the creation of data repositories are essential to compare, exchange, and share data within the proteomic community. There are some publicly available proteomic database repositories such as SwissProt and Uniprot (23) for protein sequences annotated for function, subcellular location, and known potential post-translational modifications. The availability of bioinformatics solutions is crucial for proteomics technologies to fulfill their promise of adding further definition to the functional output of the human genome (24, 25).

## Branches of Proteomics

The field of proteomics can be broadly divided into three major areas: expression proteomics, functional proteomics, and proteome related bioinformatics. Our research focuses primarily on expression proteomics.

Expression Proteomics. Expression proteomics deals with global quantitative analysis and identification of proteins encoded in genomes and expressed in different tissues and cell populations. Current research in expression proteomics requires that proteins be resolved on a

massive scale. The 2-DE remains the main technique used for protein separation. In 2-DE, proteins are separated first by isoelectric point and then by molecular weight. Protein spots in a gel can be visualized using a variety of chemical or fluorescent stains. The protein spots can often be quantified by the intensity of their stain. Once proteins are separated and quantified, they have to be identified. Individual spots can be cut out of the gel and cleaved into peptides with proteolytic enzymes. These peptides can then be identified by MS.

Protein mixtures can also be analyzed without prior separation. These procedures begin with proteolytic digestion of the proteins in a complex mixture. The resulting peptides are then injected onto a liquid chromatography column (LC) consisting of strong cation exchange (SCX) material back-to-back with reversed phase (RP) material inside fused silica capillaries that separates peptides first by anion exchange and then by hydrophobicity. LC can be coupled directly to a mass spectrometer. This more complicated method is known as multidimensional protein identification technology (MUDPIT).

### Mass Spectrometry

Once the 2-DE separation has been completed and protein spots selected for identification, gel plugs containing the proteins of interest are removed from the gel. Each plug is then processed to extract the protein from the gel and to remove gel components. The protein(s) in a plug are digested with trypsin and the resulting peptides are analyzed by mass spectrometry to obtain their MS data. Generally, there are three different types of MS data that can be used for database search. They are molecular weights of peptides that can be used for peptide mass mapping, combination of mass data and partial amino acid sequence that can be

used for sequence tag, and tandem mass spectrometry data that are used for MS/MS fragmentation ion search.

## CHAPTER 2 METHODOLOGY

### Materials

#### Cell Culture and Reagents

RPMI 1640 medium without phenol red, fetal bovine serum (FBS), penicillin (10,000 units/mL), streptomycin (10,000 µg/mL), phosphate buffer saline (PBS, pH 7.4), and 0.4% trypan blue were purchased from Invitrogen Corporation (Grand Island, NY), propidium iodide, MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide), dimethylsulfoxide (DMSO), phenylmethylsulphonyl fluoride (PMSF 100 mM), and staurosporine were purchased from SIGMA Chemical Co. (St. Louis, MO). Ethanol was purchased from Aaper Alcohol and Chemical Co. (Shelbyville, KY), gamma-T3 and delta-T3 were purchased from United States Biochemical Corporation (Cleveland, OH). The bicinochoninic acid (BCA) protein assay reagent kit was purchased from PIERCE (Rockford, IL). The Caspase kit was purchased from Molecular Probes (Eugene, OR). Corning 25 cm<sup>2</sup> and 75 cm<sup>2</sup> tissue culture flasks (sterile), corning centrifuge tube (15.0 mL, 50.0 mL), and cell culture plates (sterile) were purchased from Corning Co. (Corning, NY). Cell scrapers (25.0 cm, sterile) were purchased from Fisher Scientific (Fair Lawn, NJ). Syringe filters (0.2 µm and 0.45 µm) were purchased from Gellman Science (Ann; Arbor, MI).

#### 2-DE Products and Reagents

The following products and reagents were from Invitrogen Corporation (Grand Island, NY): ZOOM 2D Protein Solubilizer, XCell *SureLock* Mini-Cell, ZOOM IPGRunner Cassettes ZOOM Strips (pH 3-10), electrode wicks, sealing tapes, 4X NuPAGE LDS Sample Buffer,

NuPAGE Sample Reducing Agent, ZOOM Dual power supply, XCell6 MultiGel unit, NuPAGE antioxidant, ZOOM Carrier ampholytes, ZOOM equilibration tray, Novex 4-20% gel, running buffer, agarose, protein molecular weight markers, Molecular Imager FX and Quantity One software were from Bio Rad (Hercules, CA). Iodoacetamide was from Acros Organics (Geel, Belgium), SilverSnap Stain kit II from PIERCE (Rockford, IL), Dymensions gel analysis software from SynGene (Frederick, MD), dithiothreitol (DTT), N,N-dimethylacrylamide (DMA), bromophenol blue, protease inhibitor cocktail, and tris base were from Sigma-Aldrich (St. Louis, MO).

#### Preparation of Reagents

Distilled Water. Deionized water was distilled and filtered through a Sep-Pak Cartridge (Atlanta, GA) and stored at 4 °C.

Propidium Iodide (PI, 1 mg/mL). Propidium iodide (10 mg) was dissolved in 10 mL of dimethyl sulfoxide in a light protected Eppendorf vial and stored refrigerated at 4 °C.

Staurosporine (50 µM). A 50 µL aliquot of 1 mM stock staurosporine was diluted to 1000 µL with dimethylsulfoxide .

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (0.5 mg/mL). MTT (2.5 mg) was dissolved in 5 mL of RPMI 1640 medium without phenol red, enriched with 10% FBS and 50 IU penicillin/streptomycin.

Trypan Blue (0.04%). A 1.0 mL aliquot of 0.4% trypan blue in 0.85% saline was diluted to 10.0 mL with distilled water.

Culture Medium Enriched With 10% FBS and 50 IU Penicillin and Streptomycin. RPMI 1640 (450.0 mL) without phenol red was supplemented with 50.0 mL fetal bovine serum (FBS) and 2.5 mL penicillin (10,000 units/mL) / streptomycin (10,000 µg/mL).



Gamma or Delta-T3. The desired mass of gamma or delta-T3 was dissolved in ethanol to give the required concentration of the stock solution. The solution was stored at 4 °C until used.

Ac-DEVD-AFC (5 Mm) Substrate Solution. Acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (25 mg) was dissolved in 260 µL of DMSO. The substrate was heated gently (approximately 50 °C) for complete dissolution. The solution was stored desiccated at -20 °C and protected from light.

Dithiothreitol (DTT) (1 mM) Solution. DTT (100 mg) was dissolved in 650 µL of distilled water and stored desiccated at -20 °C.

2 X Ac-DEVD-AFC Substrate Working Solution. A 15 µL aliquot of 5 mM Ac-DEVD-AFC substrate was mixed with 1480 µL of 2 x reaction buffer.

Bicinochonic Acid (BCA) Working Solution. BCA protein assay reagent A (containing sodium carbonate, sodium bicarbonate, bicinochonic acid reagent, and sodium tartrate in 0.1 N sodium hydroxide) and BCA protein assay reagent B (4% cupric sulfate) were mixed in the ratio of 50:1, prepared freshly as required.

Bovine Serum Albumin (BSA) (1 mg/mL). A 1 mL aliquot of 2 mg/mL BSA (in 0.9% NaCl solution containing sodium azide) was mixed with 1 mL of 2 x cell lysis buffer.

950 µL of Lysis Buffer.

This buffer is prepared fresh, just prior to use.

1X ZOOM 2D Protein Solubilizer 1 or 2 -----	909 µL
1 M Tris Base-----	3 µL
100X Protease Inhibitor Cocktail -----	10 µL
2 M DTT-----	10 µL
Distilled water-----	18 µL

Total volume-----950  $\mu$ L

This was mixed well and stored on ice until use.

Quant-It Working Solution. This solution was made by diluting Quant-iT protein reagent 1:200 in Quant-iT protein buffer. For example, for about 100 assays a 100  $\mu$ L aliquot of Quant-iT protein reagent (Component A) and a 20 mL aliquot of Quant-iT protein buffer (Component B) were put in a disposable plastic container and mixed well.

Diluting The Lysate For Each ZOOM Strip.

A 140  $\mu$ L of diluted sample was needed per strip and a master mix of common components was prepared for multiple samples as follows:

1X ZOOM 2D Protein Solubilizer 1 or 2 -----128  $\mu$ L

Lysate -----10–12  $\mu$ L

2 M DTT -----0.7  $\mu$ L

Appropriate ZOOM Carrier Ampholytes (v/v) ----- 0.4–1.6  $\mu$ L

Bromophenol Blue -----Trace

Deionized water-----to 140  $\mu$ L

1X NuPAGE LDS Sample Buffer. This was prepared by diluting 10 mL of 4X NuPAGE LDS Sample Buffer with 30 mL of deionized water.

Sample Reducing Solution. This was prepared by adding 1.0 mL NuPAGE sample Reducing Agent to 9.0 mL of 1X NuPAGE LDS Sample Buffer in a 15 mL conical tube.

125 mM Sample Alkylating Solution. Prepared by dissolving 232 mg of fresh iodoacetamide in 10 mL of 1X NuPAGE LDS Sample in a 15 mL conical tube.

1x Tris Glycine SDS Running Buffer. This running buffer was made by weighing 10 g of SDS into a 1000 mL container of 10x Tris Glycine buffer. A 100 mL aliquot was then taken and brought to 1000 mL working solution using distilled water.

0.5% Agarose Solution. This solution was prepared by weighing 0.5 g of agarose into a 250 mL flask containing 100 mL of 1x Tris Glycine SDS running buffer and microwaving for about 2 minutes to dissolve the agarose. The solution was kept in a water bath maintained at 55-65 °C until needed.

Gel-Fixing Solution (30% Ethanol:10% Acetic Acid). The fixing solution was prepared by adding 300 mL of USP-grade 95% (v/v) ethanol to 500 mL of water followed by 100 mL of acetic acid and adjusting the total volume to 1000 mL with water.

Gel-Washing Solution (10% Ethanol). The washing solution was prepared by adding 100 mL of USP-grade 95% (v/v) ethanol to 700 mL of water and adjusting the total volume to 1000 mL with distilled water.

Sensitizer Working Solution. This was prepared by mixing 1 part SilverSNAP Sensitizer with 500 parts distilled water (e.g., mix 50 µL Sensitizer with 25 mL water).

Stain Working Solution. This was prepared by mixing 1 part SilverSNAP Enhancer with 50 parts SilverSNAP Stain (e.g., 0.5 mL of Enhancer with 25 mL Stain).

Developer Working Solution. This was prepared by mixing 1 part SilverSNAP Enhancer with 50 parts SilverSNAP Developer (e.g., mix 0.5 mL of Enhancer with 25 mL Developer).

Stopping Solution (5% Acetic Acid). This was prepared by adding 50 mL of acetic acid to 900 mL of water and adjusting the total volume to 1000 mL.

## Experimental Design

### Part I: Cell Culture Studies

The prostate cancer cell line (LNCaP) was purchased from the American Type Culture Collection (ATCC; Manassas, VA). LNCaP cells were cultured at 37 °C in a humidified incubator (95% air and 5% CO<sub>2</sub>). Confluent cells were incubated with gamma-T3 or delta-T3 enriched cell culture medium and the following measurements were made:

- The MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide) assay to measure metabolically active cells. The concentration of gamma-T3 or delta-T3 was varied to determine the minimum effective concentration.
- Time-dependent propidium iodide (PI) assay to measure dead cells.
- Time-dependent caspase 3 enzymatic assay to measure apoptosis.
- BCA protein assay.

All fluorescence measurements were done using a FLUOstar\*

GALAXYBMGTECHNOLOGY microplate reader (Durham, NC). BCA protein assay and MTT assay measurements were carried out using a UVmax kinetic microplate reader (Molecular Devices; Sunnyvale, CA). A diagram of experimental design is shown in Figure 2 below.

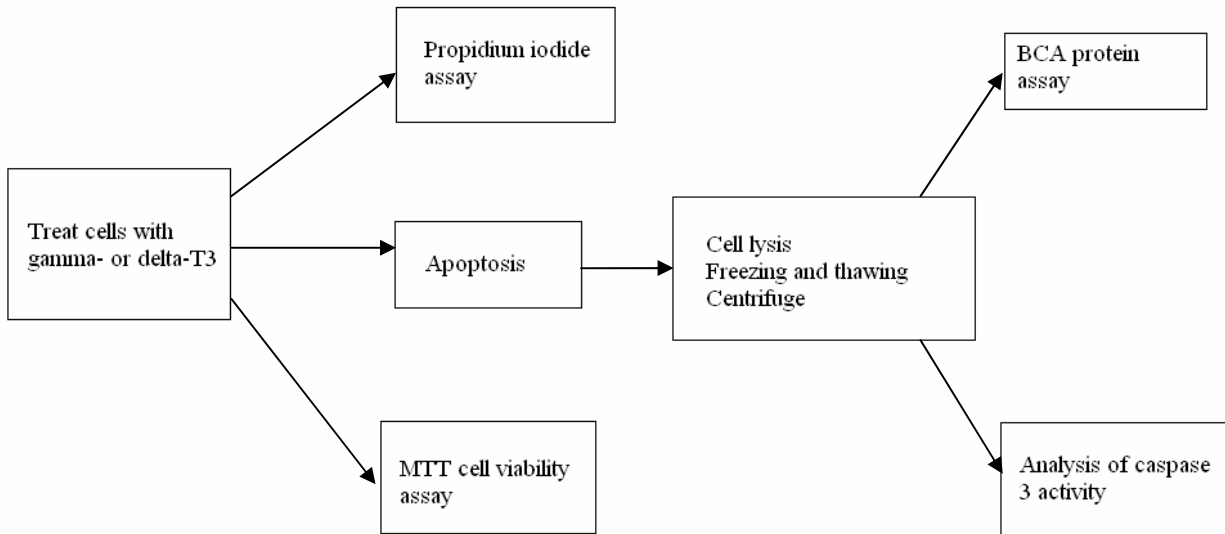


Figure 2. Diagram of experimental design for cell culture studies

### Description of Cell Line

#### LNCaP Cell Line (ATCC CRL-1740)

This is a human prostate carcinoma cell line that was established in 1977 from the left supraclavicular lymph node metastasis from a 50-year-old Caucasian man with prostate carcinoma. LNCaP cells are androgen-dependent, i.e., their continued growth depends upon the presence of androgens.

### Cell Counting

LNCaP cells were suspended in culture medium; a 100  $\mu$ L aliquot of the suspension was mixed with 100  $\mu$ L of 0.04% trypan blue. An aliquot was taken for cell counting using a Nikon Inverted Phase Contrast Microscope (American Optical Corporation; Buffalo, NY) and a hemocytometer.

## Cytotoxicity

Cytotoxicity is the ability of a chemical compound to kill cells. An objective of this research was to study the cytotoxicity of gamma- and delta-T3. Various assays have been designed to measure cytotoxicity. In our research, we used the propidium iodide assay to measure dead cells in a population of cells and to measure total number of cells. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used to measure metabolically active cells, the caspase-3 enzymatic activity was used to assay for apoptosis.

The MTT assay measures metabolically active cells. The uptake of tetrazolium salt (MTT) by cells leads to the cleavage of the yellow tetrazolium salt MTT by the succinate-tetrazolium reductase system to form purple formazan crystals in the respiratory chain of mitochondria. The formazan crystals are membrane impermeable and are insoluble in aqueous solution but become soluble and are liberated into the assay buffer upon addition of solubilizing agents like DMSO.

The propidium iodide assay measures dead cells. Propidium iodide is a nucleic acid-binding fluorophore. It is excluded from viable cells but is membrane permeable to dying or dead cells. Propidium iodide intercalates into double stranded nucleic acid by interacting with four or five of its base pairs. This binding greatly enhances its fluorescence.

The caspase 3 assay is used to measure apoptosis (programmed cell death) in cells. Caspases are a family of cysteine proteases that become activated when a cell is undergoing apoptosis. There are more than 10 caspases. Caspase 2, 8, 9, and 10 are involved in initiation of apoptosis, while caspase 3, 6, and 7 execute the death order by destroying essential proteins in the cell. Before an apoptosis-triggered event, caspases exist as inactive proenzymes, which become activated during apoptosis.

## Apoptosis and Necrosis

Apoptosis and necrosis are two possible mechanisms through which cytotoxic drugs can induce cell death. When cells are exposed to cytotoxic drugs, they lose the integrity of their plasma membrane and become permeable irrespective of whether necrosis or apoptosis is the form of cell death induced. The caspase assay distinguishes between necrosis and apoptosis.

Necrosis is accidental cell death caused by adverse conditions in the environment such as burns and exposure to a very toxic agent. It is characterized by the swelling of the cell and its organelles and a subsequent rupture and release of intracellular contents, causing damage to surrounding cells and leading to inflammation.

Apoptosis (an active process of programmed cell death) (26) plays an important role both in human embryonic development and in adult tissue homeostasis as the most common mechanism by which the body eliminates damaged or unneeded cells without local inflammation from leakage of cell contents (27, 28). Cells undergoing apoptosis exhibit a characteristic pattern of morphologic changes, including early activation of caspases (a family of cysteine specific proteases that become activated when a cell undergoes apoptosis), cell shrinkage and bubbling of the plasma membrane (blebbing) (29). The hallmark of apoptosis is the fragmentation of DNA. Apoptotic bodies formed during apoptosis are engulfed by phagocytic cells like the macrophages and dendritic cells. Phagocytes secrete cytokines that regulate inflammation. Apoptosis is very important for growth and development.

### Principle of the MTT Assay

The MTT assay is used to measure metabolically active or live cells (30). When cells take up MTT, the yellow tetrazolium salt (MTT) is reduced in metabolically active cells by mitochondrial reductase to form insoluble purple formazan crystals. These crystals are solubilized by the addition of a solubilizing agent such as DMSO, followed by incubation of the plate for 30 minutes at 37 °C to dissolve the crystals. The soluble formazan product is quantified colorimetrically by measuring the absorbance at 570 nm, which directly corresponds to the number of viable cells. A schematic diagram of the MTT assay is shown in Figure 3.

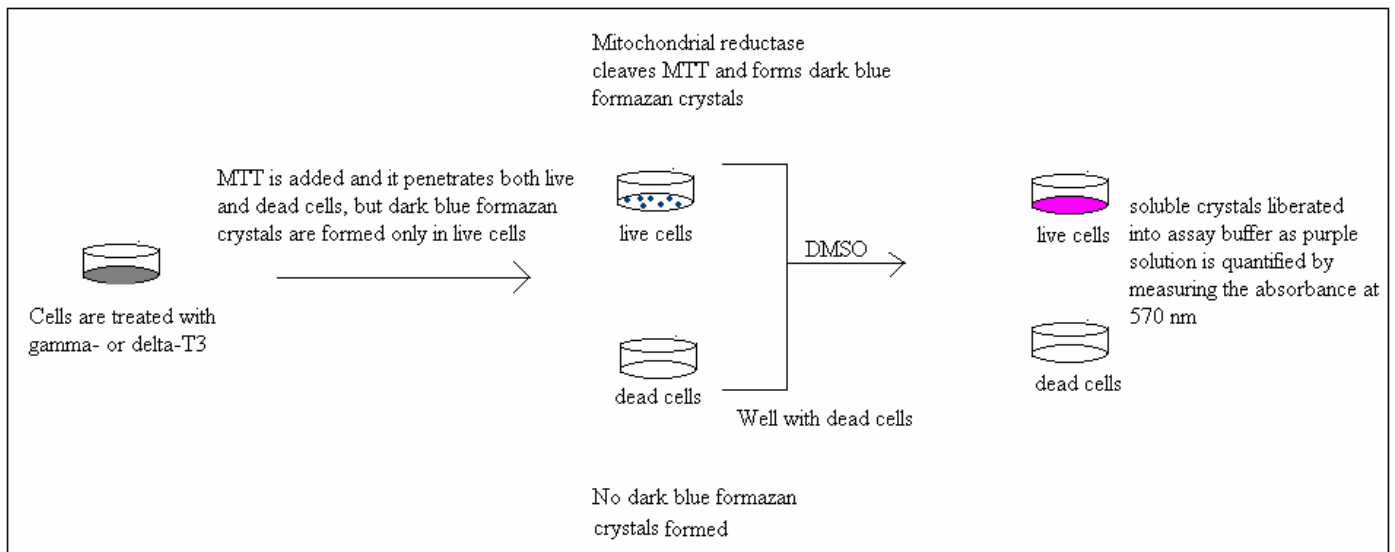


Figure 3. Diagrammatic representation of the rationale of the MTT assay

### MTT Cell Proliferation Assay

LNCaP cells ( $1 \times 10^5$  cells per well) were subcultured in a 96 well Costar tissue culture plate for 24 hours. Cells were then treated with gamma-T3 or delta-T3 enriched medium and incubated for the required time point at 37 °C in humidified incubator (95% air and 5% CO<sub>2</sub>). Positive and negative controls were 0.5 μM staurosporine and vehicle treated cells (the vehicle is the same volume of absolute-200 proof ethanol as the gamma- or delta-T3 added to the medium),



respectively. At the end of the incubation period, the treated medium was removed and 200  $\mu\text{L}$  of 0.5 mg/mL MTT solution was added to each well and the plate was incubated for 30 minutes. Then the MTT medium was replaced with 200  $\mu\text{L}$  of DMSO and the plate incubated for 30 minutes to solubilize the purple formazan crystals and absorbance measured at 570 nm wavelength with a UVmax kinetic microplate reader (Molecular Devices; Sunnyvale, CA).

#### Principle of the Propidium Iodide (PI) Assay

The propidium iodide assay measures live cells (31). Propidium iodide is a nucleic acid-binding fluorophore. It is membrane permeable to dying or dead cells but excluded by viable cells. Propidium iodide intercalates into double stranded nucleic acid by interacting with its base pairs. The binding occurs stoichiometrically with one propidium iodide molecule binding with 4 to 5 base pairs of DNA or double stranded RNA, enhancing its fluorescence about 20 to 30 fold. Excitation of the propidium iodide-nucleic acid complex at 485 nm gives rise to maximum emission at 650 nm. The increase in fluorescence intensity of treated cells with respect to the control (vehicle treated cells) represents an increase in the quantity of dead cells. Figure 4 illustrates the PI assay.

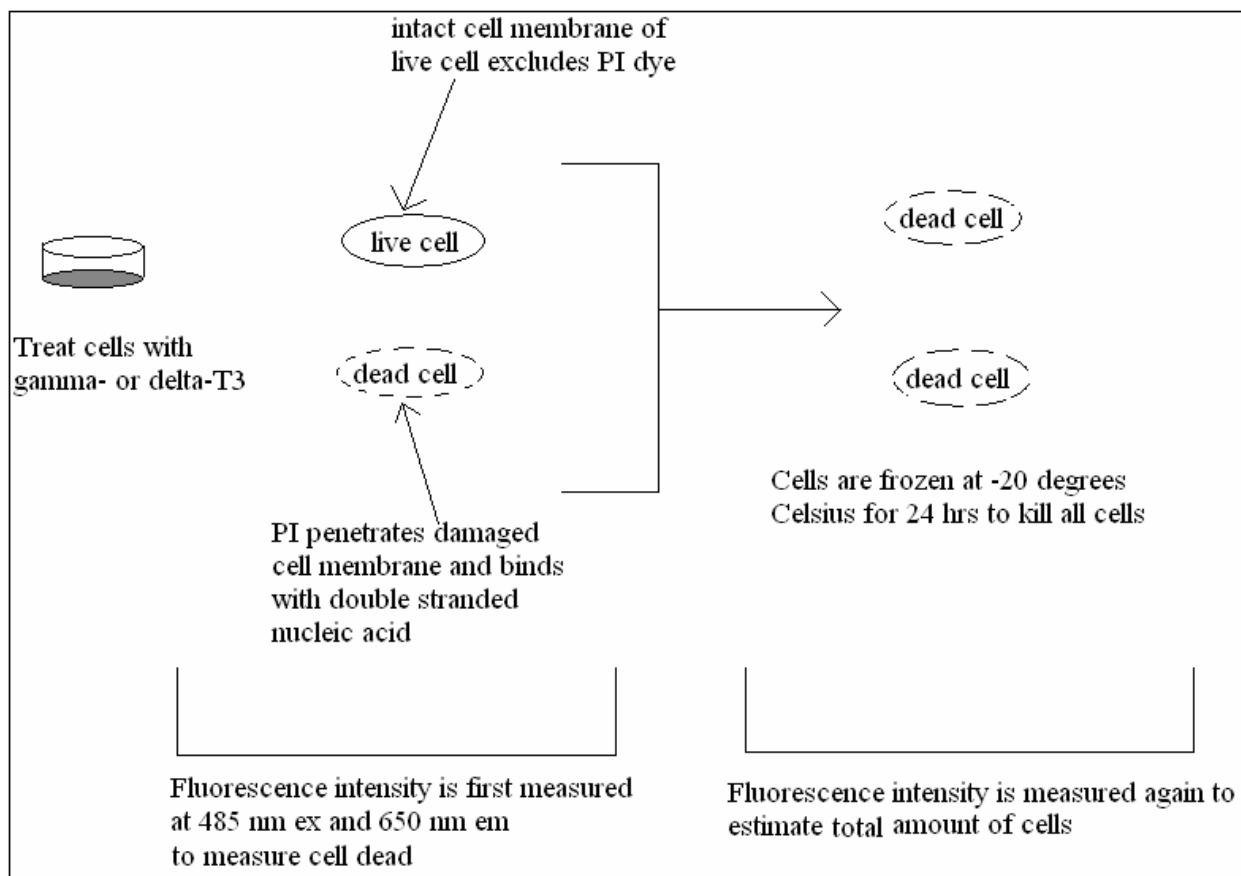


Figure 4. Diagrammatic representation of the rationale of propidium iodide assay

### Cell Viability Determination by Propidium Iodide (PI) Assay

Adherent cells ( $1 \times 10^5$  cells per well) were subcultured in a 96 well Costar tissue culture plate for 24 hours to let cells adhere to the bottom of the wells. Cells were treated with gamma-T3 or delta-T3 enriched culture medium and incubated at  $37^\circ\text{C}$  in humidified incubator (95% air and 5%  $\text{CO}_2$ ) for the required time point. The positive control was  $0.5 \mu\text{M}$  staurosporine and negative control was vehicle treated cells (empty medium + ethanol). After the required time point of incubation, the cells were stained with  $50 \mu\text{g/mL}$  PI in  $25 \mu\text{L}$  for 10 minutes. The fluorescent blank was cell culture medium in each case. The plate was incubated for 5 minutes at  $37^\circ\text{C}$  and the initial fluorescence intensity from the dead cells was measured using a

fluorescence microplate reader (FLUOstar \* Galaxy, BMG Technology) with an excitation wavelength of 485 nm and an emission wavelength of 650 nm (first measurement). After freezing the cells at -20 °C for 24 hrs, PI had access to total DNA, leading to total cell population counts (second measurement).

### Principle of Caspase 3 Enzymatic Assay

The caspase 3 assay is used to detect apoptotic cells (32). Caspases are a family of cysteine proteases that become activated when a cell undergoes apoptosis. Caspase substrates are synthesized such that they mimic the cleavage site of the various caspase substrates *in vivo* like poly (ADP-ribose) polymerase (PARP). In our assays we used acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC for caspase 3). When a caspase enzyme is activated during apoptosis, it cleaves its specific substrate at the aspartate residue to release the fluorogenic substance, 7-amino-4-trifluoromethyl coumarin (AFC), which can be detected fluorimetrically. When conjugated, AFC emits blue light at 390 nm, but in the free form AFC emits a yellow-green fluorescence at 500 nm. A schematic diagram of the caspase assays is shown in Figure 5.

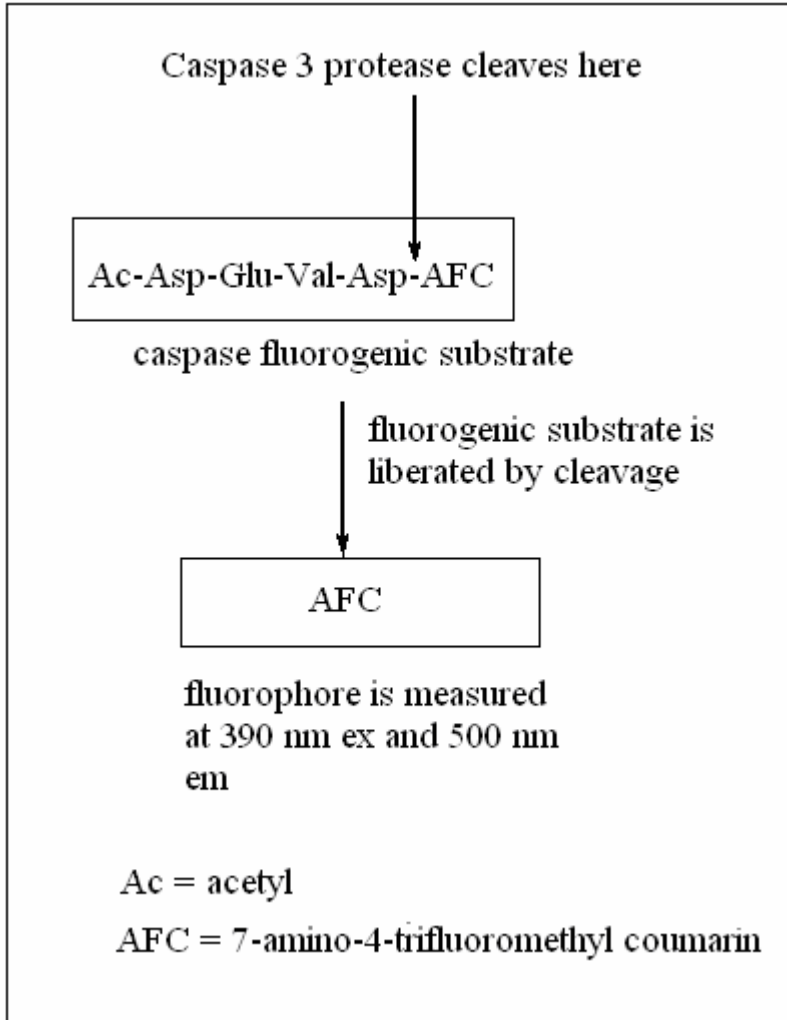


Figure 5. Diagrammatic representation of the rationale of caspase 3 activity.

### Measurement of Caspase 3 Activity

We measured caspase 3 activity in the LNCaP cell line fluorimetrically. A 2.5 mL aliquot of cells ( $1 \times 10^5$  cells) was cultured in a Falcon 6 well plate for 24 hours and incubated with 2.5 mL gamma-T3 or delta-T3 enriched medium for various time periods. The negative control was untreated cells and the positive control was 0.5  $\mu$ M staurosporine. At the end of the incubation period, the cells were collected, resuspended in lysis buffer, and frozen for 30 minutes at -80 °C. Two cycles of freezing and thawing were carried out. The samples were then

centrifuged at 2000 x g for 5 minutes. A 10  $\mu$ L aliquot of cell lysate from each sample was reserved for the BCA protein assay. For caspase 3 assay, a 40  $\mu$ L aliquot of fluorogenic substrate Ac-DEVD-AFC in 5x reaction buffer (10 mM HEPES pH 7.5, containing 50 mM NaCl and 2.5 mM dithiothreitol, respectively) was added to 40  $\mu$ L cell lysate and incubated in the FLUOstar\* Galaxy microplate reader at 37 °C while measurements were made. The released AFC (7-amino-4-trifluormethyl coumarin) was measured at 390 nm for excitation and 500 nm for emission.

#### The principle of BCA Protein Assay

Bicinchoninic acid (BCA) is a sensitive, stable, and highly specific reagent for the detection of cuprous ion ( $\text{Cu}^+$ ) (33). When incubated with protein, a  $\text{Cu}^{2+}$ -protein complex is formed under alkaline conditions. The presence of cysteine, cystine, tryptophan, tyrosine, and the peptide bond in protein are thought to reduce  $\text{Cu}^{2+}$  to  $\text{Cu}^+$ . This reaction depends on the amount of protein present. More protein results in a greater amount of  $\text{Cu}^+$  produced. Chelation of two BCA molecules with one  $\text{Cu}^+$  ion gives purple coloration in a basic medium. This complex is water-soluble and exhibits a strong absorbance at 562 nm.

An alternative to the BCA assay is the Quant-iT protein assay that measures protein amounts fluorometrically. This is a recommended assay for our proteomics study because the protein solubilizer we are using interferes with the BCA assay. The Quant-iT assay is also easy and accurate. The assay is highly tolerant to common contaminants such as salts, solvents, or DNA, but not detergents. Unlike the BCA assay, the Quant-iT kit comes with pre-diluted BSA standards.

Measurement of Protein Concentration by the BCA Protein Assay

Protein levels were measured by mixing 10 µL aliquot of cell lysate (from caspase assay) with 200 µL of BCA working solution (made freshly), and after incubating for 30 minutes, the absorbance was measured at 562 nm using an UVmax kinetic microplate reader (Molecular Devices; Sunnyvale, CA). A 10 µL aliquot of 1x cell lysis buffer was used as a blank.

A Bovine Serum Albumin (BSA) Standard Curve for BCA protein assay was constructed by mixing 10 µL of 1x cell lysis buffer, 0.4 mg/mL, 0.8 mg/mL, 1.2 mg/mL, 1.6 mg/mL, and 2.0 mg/mL BSA with 200 µL of BCA working solution. The plate was incubated for 30 minutes and the absorbance measured at 562 nm using a UVmax kinetic microplate reader (Molecular Devices; Sunnyvale, CA).

Part II: Proteomics Studies

Two-dimensional Polyacrylamide Gel Electrophoresis (2-DE)

Theory of Electrophoresis.

- If a macromolecule has a net charge  $q$ , then application of an electric field  $E$  will result in an applied force  $F$ :

$$F=qE..... (1)$$

- The macromolecule will accelerate until a steady-state velocity  $v$  is reached:

$$v=qE/f..... (2)$$

Where  $f$  is the frictional force that is equal and opposite to the applied electric forces  $F$ .

- The mobility of a molecule,  $\mu$ , is equal to  $v/E$ , which can be combined with the previous equation to give:

$$\mu = q/f \dots\dots\dots (3)$$

- Thus, the charge, shape, and size of the molecule influences mobility in the absence of a matrix.

Two dimensional gel electrophoresis (2-DE) is a form of gel electrophoresis used commonly to separate proteins in two dimensions at right angles to each other. This involves a number of steps. First, a sample containing a protein mixture is prepared for isoelectric focusing. The sample is then applied to a gel where an electric current is used to separate the proteins by isoelectric point (pI). This is the pH at which the protein has a net charge of zero. After further processing (equilibration), this gel is applied to the top of a second gel, a traditional sodium dodecyl sulfate polyacrylamide gel, where application of an electric current will force the migration of the proteins from the first to the second dimension gel based on their apparent molecular masses. This technique allows the separation of proteins into distinct spots within a gel. Depending on the size of the gels used, some overlap of proteins may occur, but most often each spot will represent a single protein (34).

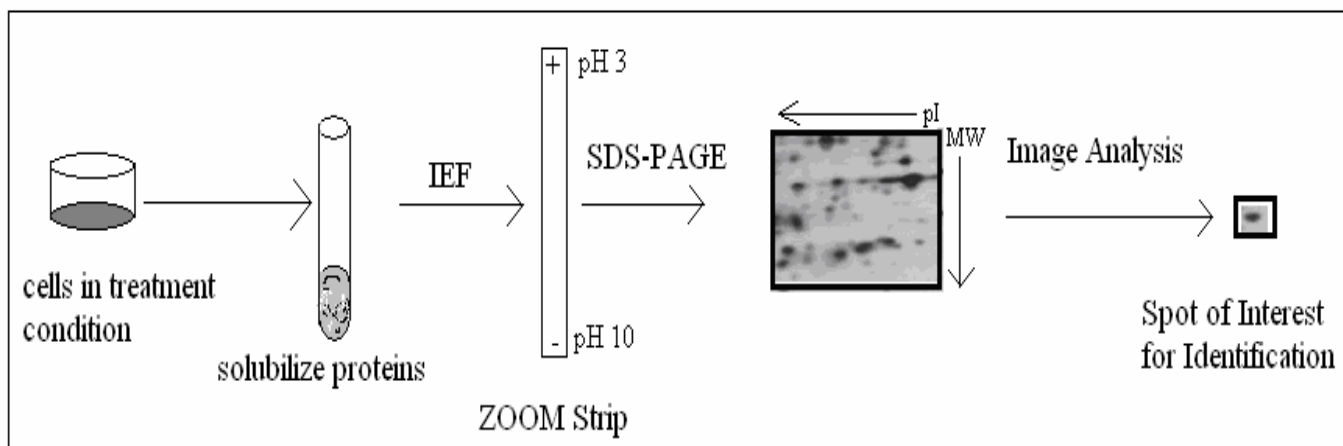


Figure 6. Flow Diagram for 2-DE

### Sample Preparation

Sample preparation is the key to success in 2-DE for adequate results whether the approach is protein separation by 2-DE or liquid chromatography (LC). Sample preparation depends on the protein solubility, size, charge, the isoelectric point, and whether electrophoresis is being done under native or denaturing conditions. Typically, membrane proteins are the most difficult proteins to solubilize and release into an aqueous environment. Sample preparation methods can range from simple solubilization into buffer to complex extractions using chaotropic and reducing agents as well as detergents. Anionic or cationic detergents such as sodium dodecyl sulfate are not appropriate for the isoelectric-focusing step because they result in a charge shift. So they are not used to solubilize membrane proteins for 1-DE separations by IEF (35).

The major objectives of good sample preparation method are:

1. Reproducibly solubilize all proteins;
2. Prevent protein aggregation during focusing;
3. Prevent chemical modifications of proteins;



4. Remove nucleic acids and other interfering molecules;
5. Yield proteins of interest at detectable levels, which may involve the removal of abundant proteins or non-relevant classes.

Sample preparation is an art requiring much experimentation before the correct conditions for a particular sample are found. Slight changes in the concentrations of the solubilizing agents can have a major effect on the final appearance of the gels.

### Solubilization

This is achieved by the use of chaotropic agents (such as urea), neutral detergents (such as CHAPS), reducing agents (such as dithiothreitol), buffers, and ampholytes (the charged species responsible for the pH gradient in isoelectric focusing). There are only a small number of chemicals that can satisfy these criteria along with other important requirements such as minimizing ionic strength. A low ionic strength allows high voltages to be applied during isoelectric focusing, allowing for a faster and more efficient focusing (36, 37).

Chaotropic agents disrupt hydrogen bonds. This disruption occurs both in the proteins and the aqueous phase of the solubilizing buffer. Chaotropic agents prevent unwanted protein aggregation and the formation of secondary structures that can alter protein mobility. The most commonly used chaotropes are 6-8M urea and 6M guanidinium hydrochloride, with urea being an uncharged molecule and guanidinium hydrochloride being charged. Protein solubilization solutions that contain both urea and thiourea have improved solubilization of integral membrane proteins compared with standard solubilization solutions without thiourea (38). Neutral surfactants are also used in solubilization solutions because they act synergistically with the chaotropes to solubilize membrane proteins. The surfactant binds to the hydrophobic domains exposed by chaotropic agents as they unfold the proteins keeping them solubilized. These

hydrophobic domains can cause aggregation, precipitation, or adsorption to the immobilized pH gradient (IPG) strips during IEF.

Detergents disrupt hydrophobic interactions between species as well as promoting solubility. Detergents must be non-ionic or zwitterionic to prevent them migrating during the isoelectric focusing step. Reducing agents disrupt disulfide bonds between cysteine residues. This allows analysis of single subunits of protein. The most commonly used reducing agents are dithiothreitol (DTT) and dithioerythritol (DTE). Ampholytes are low molecular weight molecules having a zwitterionic character. They are derived synthetically and comprise a multitude of varying pI values. In polyacrylamide gels containing these ampholytes, a linear pH gradient is built up when an electric field is applied.

The ampholyte molecules carry a net charge and thus migrate in the electric field between the electrodes. They stop moving as soon as they reach the position corresponding to their pI and form small plateaus (stationary stacks). This prevents precipitation and ensures good separation of protein bands at their pI. Adding salt to the protein sample can also prevent protein precipitation. However, as soon as one performs isoelectric focusing, the salt will migrate away from the protein, thereby promoting precipitation. Carrier ampholytes are often included in the buffer (39).

In addition to these solubilizing agents, the anionic dye bromophenol blue and protease inhibitors are also added. The dye is applied in very low amounts and is very useful to monitor the running conditions. The protease inhibitors can inactivate most of the proteolytic enzymes in a cell or tissue extract. Usually a “cocktail” of proteases is used.

### Preparing LNCaP Cells Protein Sample for Isoelectric Focusing

A 2.5 mL aliquot of LNCaP cells ( $1 \times 10^5$  cells) was cultured in a Falcon 6 well plate for 24 hrs and incubated with 2.5 mL of 20  $\mu$ M delta-T3 enriched medium for 3 hr or 6 hr time periods. The negative control was untreated cells. At the end of the incubation period, the cells were harvested by scraping and transferred into a 15 mL Falcon centrifuge tubes. The cells were harvested by centrifugation at 300 x g for 10 minutes. The cells were washed twice with cold PBS to remove any residual medium components and the supernatant discarded. During the washing steps, fresh cell lysis buffer was prepared as described in the preparation of reagents section. The cell pellet was resuspended in the lysis buffer containing the protease inhibitor cocktail and incubated on ice for 30 minutes with intermittent vortexing at 10 minute intervals. The sample was then centrifuged at 13,000 x g for 20 minutes at 4 °C to remove any particulate material. The supernatants were transferred and aliquoted to sterile microcentrifuge tubes.

### Estimating Protein Amount Using the Quant-iT Assay

Protein concentrations were measured by mixing 10  $\mu$ L of each unknown protein sample into separate wells of a 96 well plate containing 200  $\mu$ L of Quant-iT working solution (made freshly) in triplicates and mixed well. Triplicates of 10  $\mu$ L of each BSA standard were also added to separate wells and mixed well. The plate was incubated at room temperature for 30 minutes and the fluorescence measured at 485 nm excitation and 590 nm emission using the FLUOstar\* Galaxy microplate reader. A standard curve was used to determine the protein amounts.

### Rehydrating ZOOM Strips

Rehydration was performed according to the manufacturer's manual. A 140  $\mu$ L of the sample rehydration buffer containing the appropriate amount of the protein sample was loaded

into each sample loading well of the ZOOM IPGRunner Cassette. The Strips were each gently slid into the enclosed channels of the cassette using the fingers to guide the strip. The sample loading wells were sealed and the cassette with strips was incubated for 1 hr at room temperature to rehydrate the strips.

#### Principle of Isoelectric Focusing (IEF)

This is a technique for separating proteins and other molecules by their electric charge differences. It takes advantage of the fact that a protein's molecular charge changes with the pH of its surroundings due to protonation and deprotonation of functional groups. Protein molecules are distributed over the medium on an IPG strip with a pH gradient (usually created by aliphatic ampholytes) during the rehydration step. In this step, the strip is soaked in the rehydration solution containing the protein sample. When an electric current is passed through the medium, a "positive" anode end and a "negative" cathode end are created. Negatively charged particles are forced to migrate through the pH gradient toward the "positive" end while positively charged particles move toward the "negative" end. As a protein molecule moves towards the pole opposite of its charge, it moves through the changing pH gradient until its pI is reached and no further migration will occur within the gel.

Performing IEF. IEF was performed following the manufacturer's manual. The ZOOM IPGRunner core and cassette with the rehydrated strips were assembled and the lid of the mini-cell was placed on the core. With the power supply turned off, the electrode cords were connected to the power supply. The power supply was set to the following running conditions and the power turned on.

#### Current

50 mA per strip

### Power

0.1 W per strip

### Voltage

200 V for 20 minutes

450 V for 15 minutes

750 V for 15 minutes

2000 V for 60 minutes

The power was turned off at the end of the run and the cables were disconnected from the power supply. The lid was removed and the water poured off from the mini-cell chamber. The cassettes were prepared for equilibration.

### Equilibration

Equilibration prepares proteins from isoelectric focusing for the second dimension. The process reduces disulfide bonds and alkylates the resultant sulfurs of thiol groups. It also coats the proteins with SDS which is necessary for the final separation. The reducing and alkylating agents used here include DTT or DTE and N, N-dimethyl amide or iodoacetamide respectively.

Performing Equilibration of the Zoom Strips. Equilibration was also performed according to the manufacturer's manual. A 5-15 mL of the sample reducing buffer was added through the spout on the equilibration tray and incubated for 15 minutes at room temperature. The reducing solution was removed and a 5–15 mL of the alkylating solution was added. This was incubated for another 15 minutes at room temperature. The spent alkylating solution was also removed. The strips were then removed from the equilibration tray in preparation for SDS-PAGE.

## SDS-PAGE

SDS-PAGE stands for sodium dodecyl sulfate polyacrylamide gel electrophoresis. SDS-PAGE is the next step after IEF. Here, polypeptides are separated based on their “apparent” molecular weights, using discontinuous polyacrylamide gel as a support medium and SDS to denature the proteins. SDS, which is a negatively charged detergent, forms a complex with the polypeptide molecules at approximately 1.4 g SDS/g protein and masks the charge of the polypeptide molecules. An anionic complex with roughly the same net negative charge per unit mass is formed. This makes the proteins, regardless of their native pI's, migrate towards the anode with the application of an electric current. SDS-PAGE is based solely on molecular weight because the charge-to-mass ratio is nearly the same among all SDS-denatured polypeptides and the polyacrylamide matrix serves a molecular sieve for the separation of the SDS-protein complex by size.

The use of gradient gels in which the percentage of polyacrylamide increases from the cathodic to the anodic ends of the gel has greatly enhanced the scope of separation using SDS-PAGE. The separation interval is wider and the spots are sharper. The type of gradient gel used depends on the molecular weight range of the proteins in the sample. As the SDS-protein complex migrates towards the anode, a certain polyacrylamide percentage is reached at which migration is increasingly slowed. Polyacrylamide gels restrain larger molecules from migrating as fast as smaller molecules. Larger molecules will remain near the top of the gel while smaller molecules will migrate towards the bottom. Standards with known molecular weights can be used to estimate the molecular weights of the sample proteins. Therefore, protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance

of major proteins in a sample after staining, and to determine the distribution of proteins among fractions (40).

Performing SDS-PAGE. SDS-PAGE was performed following the manufacturer's manual. The strips were placed into the gel wells of the cassettes. About 400  $\mu\text{L}$  of 0.5% agarose solution was added in each gel well containing the strips, avoiding any overflow of the agarose solution into the molecular weight marker wells. The agarose solution was allowed to solidify. The molecular weight marker wells were filled with about 100  $\mu\text{L}$  of the running solution, followed by about 1-2  $\mu\text{L}$  of the molecular weight marker.

The gels were then assembled into the XCell6 MultiGel unit after the agarose solution had solidified. The upper buffer chamber (the void formed between the two gel cassettes on each side of the buffer core), which is also the cathode, was filled with 250 mL of the 1x running buffer. A 667  $\mu\text{L}$  volume of NuPAGE antioxidant was added to each of the upper buffer chambers. The lower buffer chamber (anode) was filled with 670 mL of 1x running buffer. The lid was placed on the assembled unit and with the power turned off, the electrode codes were connected to the power supply. The power supply was set to the following electrophoresis conditions and the power was turned on.

Voltage

125 V (Constant)

Current

Start: 30-40 mA per gel. End: 8-12 mA per gel.

Power

200 watts (maximum)

## Time

100-130 minutes.

At the end of the electrophoresis run, the gels were removed from the cassette plate for staining.

## Staining, Scanning, and Gel Analysis

The result of the second dimension is a gel in which proteins are spread out on the surface of the gel. In order to visualize proteins they must be stained. The technique used depends on the desired sensitivity, dynamic range, ease of use, and compatibility with MS. The most widely applied staining techniques in proteomics are coomassie brilliant blue, silver, and fluorescence staining. In the case of silver staining, a silver colloid is applied to the gel. The silver binds to cysteine groups within the protein. The silver is darkened by exposure to ultra-violet light. The darkness of the silver can be related to the amount of silver and therefore to the amount of protein at a given location on the gel. This measurement can only give approximate amounts but is adequate for most purposes. Gel plugs of these spots can be removed and further analyzed by MS to determine the identity of each individual protein spot. For the gels to be analyzed for differential expression of protein spots, they must be converted to digital images. Depending on the software that is to be used, the image format can either be JPEG or TIFF.

## Gel Staining Using SilverSnap Stain Solution

Staining was performed following the manufacturer's manual. All steps were performed in a single clean staining tray (plastic or glass) with constant gentle shaking. The gels were subjected to two washings with generous volumes of distilled water for 5 minutes each. The gels were then fixed for at least 15 minutes. The fixing solution was replaced and fixed again for another 15 minutes. This was followed by two 5 minute washings using the gel washing solution



and another two 5 minute washings with distilled water. The gels were then incubated in Sensitizer Working Solution for exactly 1 minute followed with two changes of distilled water for 1 minute each. The gels were incubated in Stain Working Solution for 30 minutes and then quickly washed with two changes of ultrapure water for 20 seconds each. Developer Working Solution was added immediately and incubated until protein bands appeared. Developer Working Solution was replaced with stop solution when the desired band intensity was reached.

Gel Scanning. The stained gels were scanned using the Molecular Imager FX scanner at 50 micrometer resolution and saved as a TIFF image.

Gel Analysis. The gels were analyzed using Dymensions software following the steps in the user's manual. Registered spots between control and drug induced appeared dark, while spots that were present in one image only appeared green or magenta. This view allowed us to assess the differences between the samples. Normalized volume ratios and height ratios were used to compare the consensus spots from both samples.

## CHAPTER 3

### RESULTS

#### Effects of Gamma-T3 and Delta-T3 on the Viability of LNCaP Cells

Previous results obtained in our lab showed that gamma-T3 and delta-T3 kill LNCaP cells. This result was confirmed and extended in this study. In particular, we determined the minimum concentration of gamma-T3 or delta-T3 required to significantly reduce the viability of LNCaP cells. Figure 7 shows the viability of LNCaP cells treated for 24 hours with different concentrations of gamma-T3 or delta-T3 enriched cell culture medium. In these experiments, cell viability was measured using the MTT assay. Staurosporine (STAUR), a compound known to induce apoptotic cell death, was used as a positive control in all the cytotoxic experiments. Compared with vehicle treated cells, the viability of LNCaP cells was significantly suppressed at concentrations of gamma-T3 or delta-T3  $\geq 15 \mu\text{M}$  (Figure 7). This suppression increased at 20  $\mu\text{M}$  but did not change significantly even at 40  $\mu\text{M}$  of either gamma-T3 or delta-T3.

We also carried out time-dependent cell viability assay to determine the time point at which a decreased viability was significant. This is important because our proteomic studies are focused on investigating both the early and the late term molecular events during prostate cancer cell death. Figure 8 shows the time dependent cell viability assay of LNCaP cells treated with 20  $\mu\text{M}$  of gamma- or delta-T3 for 3, 6, 12, and 24 hrs. Significant viability decrease started at 6 hrs for delta-T3 and became more apparent at 12 and 24 hrs. At 6 hrs, gamma-T3 showed no significant cell viability decrease. It only showed significant growth inhibition starting at 12 hrs (even though it might have started immediately after 6 hrs).

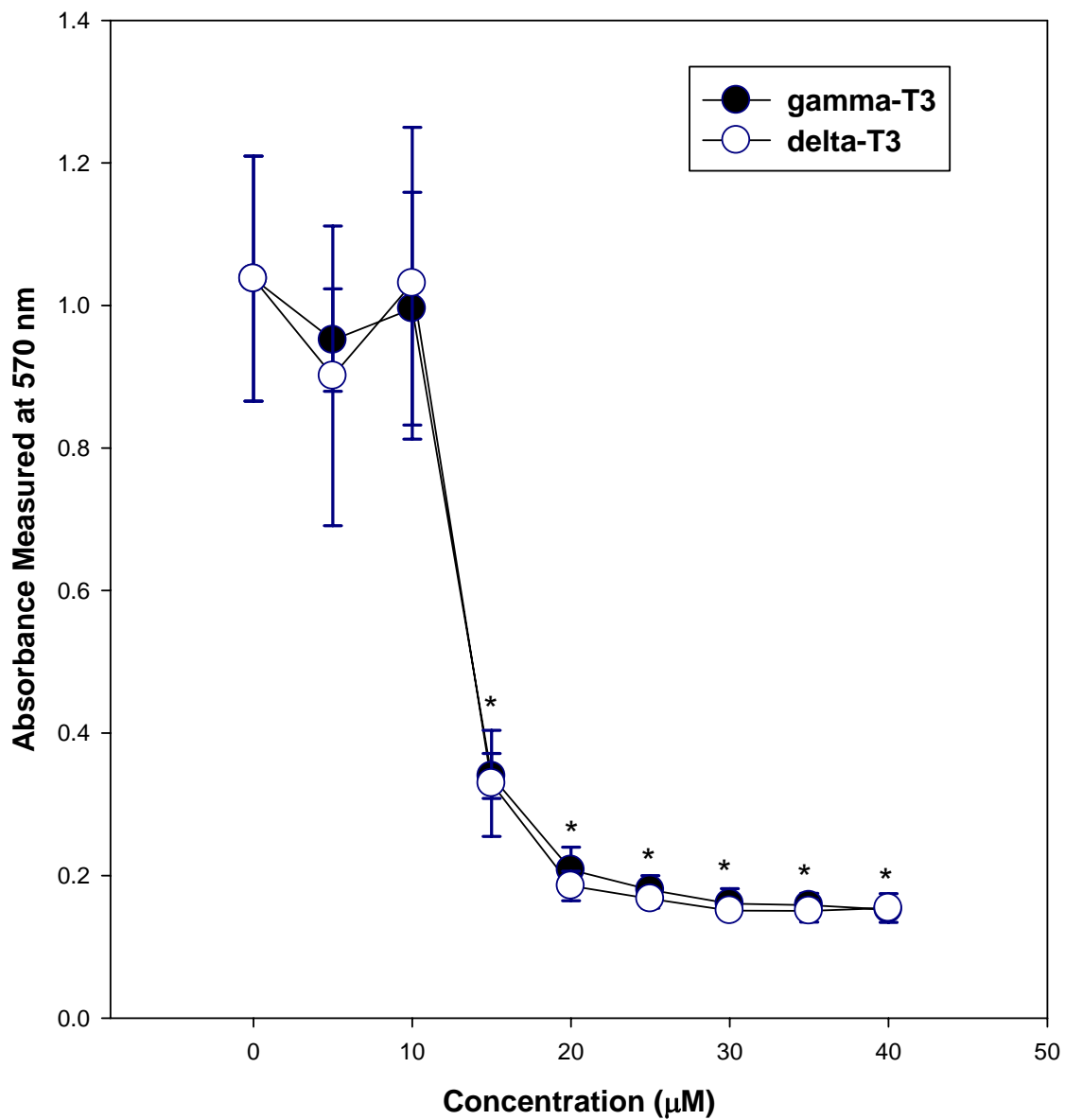


Figure 7. Effects of gamma-T3 or delta-T3 on LNCaP cell viability. Cells were exposed to various concentrations of gamma-T3 or delta-T3 for 24 hours at 37°C. Viability was analyzed by the MTT assay as described in Material and Method Section. Data are expressed as means of eight experiments  $\pm$  SEM. \*  $p < 0.05$  compared with vehicle treated cells (0  $\mu\text{M}$ ).

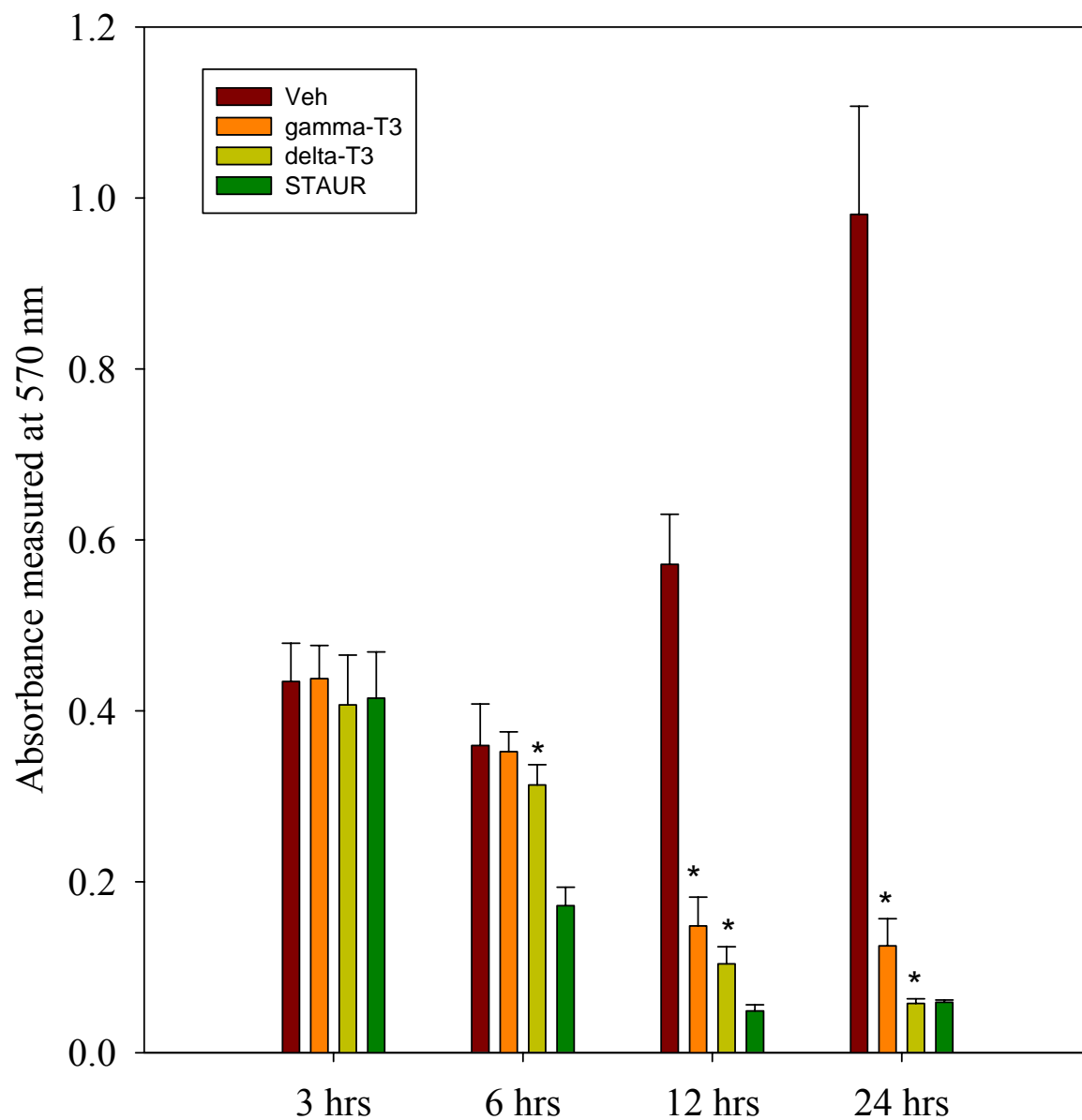


Figure 8. Time dependent MTT cell viability assay in LNCaP cells. Cells were incubated with 20  $\mu$ M of gamma-T3 or delta-T3 for the indicated time periods. Values are the mean  $\pm$  SEM of eight experiments. \*  $p < 0.05$  compared with the vehicle treated cells.

## Evaluation of Dead LNCaP Cells Treated with Gamma-T3 or Delta-T3 Using Propidium Iodide Staining Assay

In order to find out whether the above MTT cell viability result was due to cell death or growth inhibition, LNCaP cell death was assayed using propidium iodide after 3, 6, 12, and 24 hrs after treating the cells with 20  $\mu$ M of gamma-T3 or delta-T3. To estimate the total number of cells per well, the 96 well plate was frozen at -20  $^{\circ}$ C for 24 hrs after the initial PI reading to kill all remaining live cells. The fluorescence intensity of PI was re-measured and this reading corresponded to the total number of cells per well. The fluorescence intensity of PI increased in a time dependent manner for each treatment as shown in Figure 9. The number of dead cells increased gradually with a significant increase occurring at 6 hrs compared with vehicle treated cells. The increase continues through to 24 hrs for each treatment. This result agrees very closely with the time dependent MTT assay above. Significant cell death started at 6 hrs for both gamma- and delta-T3, with delta-T3 showing a more significant cell death than gamma-T3 at all time points.

The fluorescence intensity of PI after permeabilizing live cells by freezing at -20  $^{\circ}$ C for 24 hrs is shown in Figure 10. The fluorescence intensity increased gradually with time for the vehicle treated cells showing that the total number of cells (live plus dead cells) is increasing with time. With the exception of vehicle treatment, the total number of cells for each time point is fairly constant. Figure 11 shows the percentage of dead cells after treatment of LNCaP cells with 20  $\mu$ M of gamma- or delta-T3 for the indicated time points. The result indicates that the percentage of dead cells increases with time for the various treatments. The percentage cell death is calculated as follows:

$$\text{percentage of dead cells} = \frac{\text{fluorescence intensity of the PI assay}}{\text{fluorescence intensity after freezing the cells at } -20^{\circ}\text{C for 24 hrs}} \times 100$$

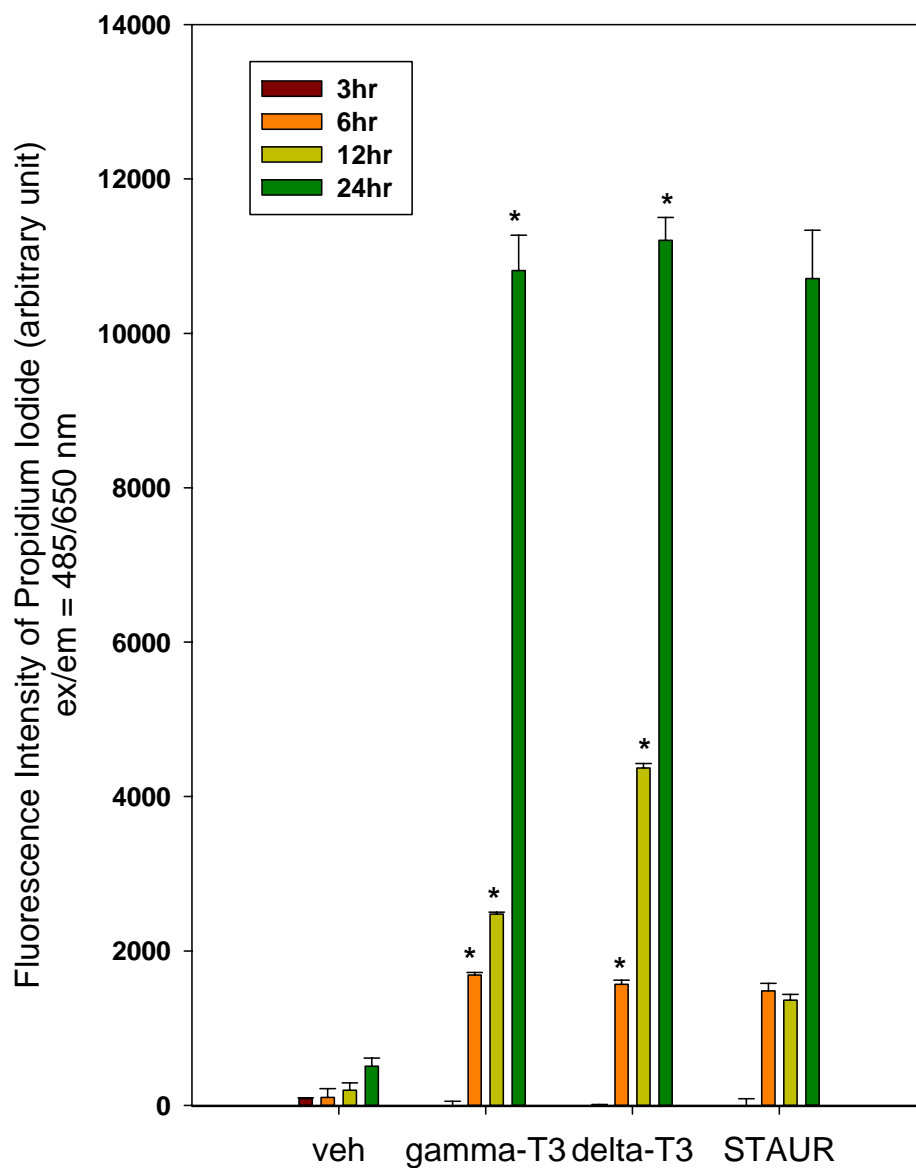


Figure 9. Measurement of cell death using the PI assay after exposing LNCaP cells to gamma-T3 or delta-T3. Cells were incubated with 20  $\mu$ M of gamma-T3 or delta-T3 for the various times at 37°C. Propidium iodide (50  $\mu$ g/mL PI in 25  $\mu$ L) was added into each well after drug treatment and the initial fluorescence intensities measured using a fluorescence microplate reader. Data are expressed as means of eight experiments  $\pm$  SEM. ‘\*’ P< 0.05 compared with vehicle.

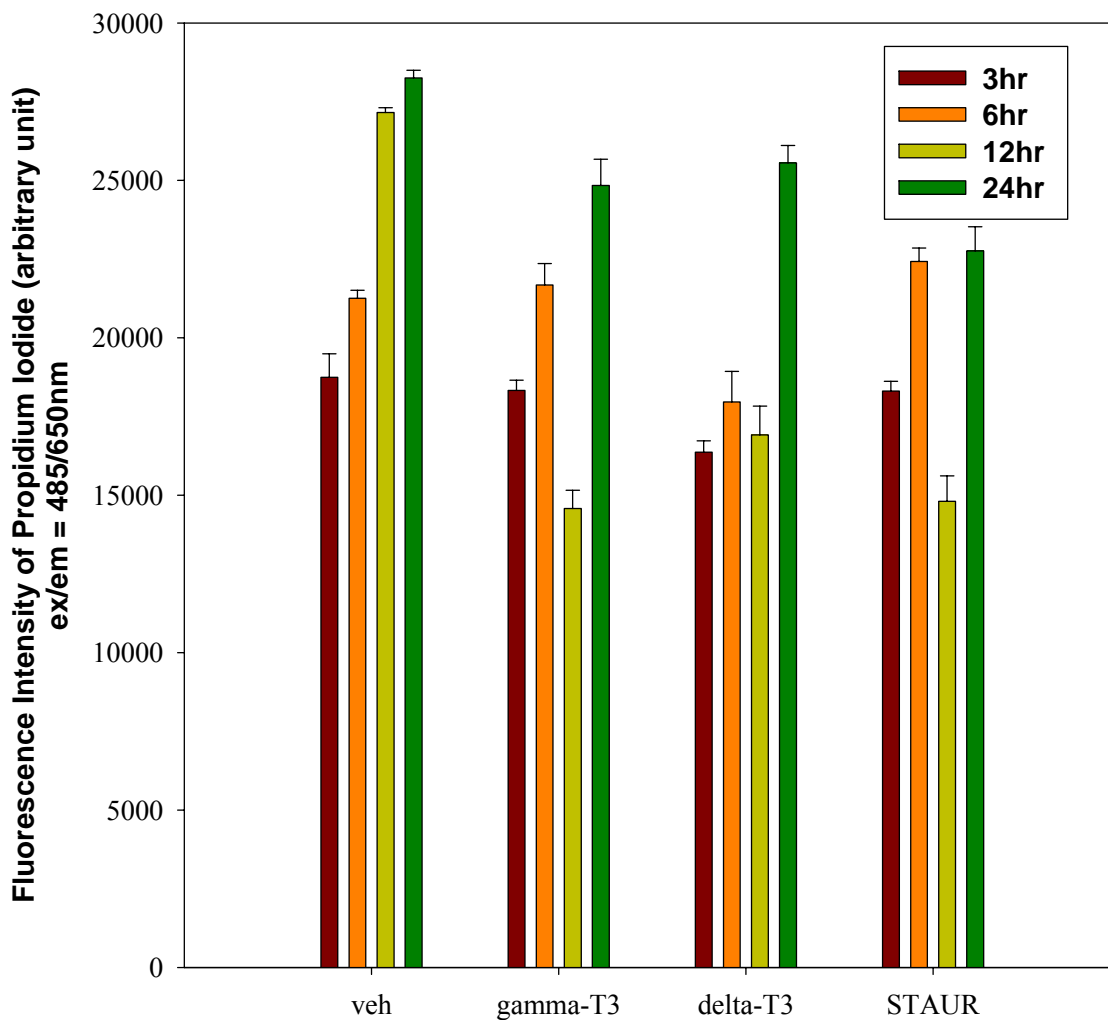


Figure 10. Fluorescence from total number of dead cells determined by PI assay. Cells were permeabilized after the first PI reading by freezing at -20 °C for 24 hrs. Fluorescence intensity of PI corresponding to total number of cells was remeasured. This represents a typical result of three independent experiments. Data are expressed as mean of eight experiments  $\pm$  SEM.

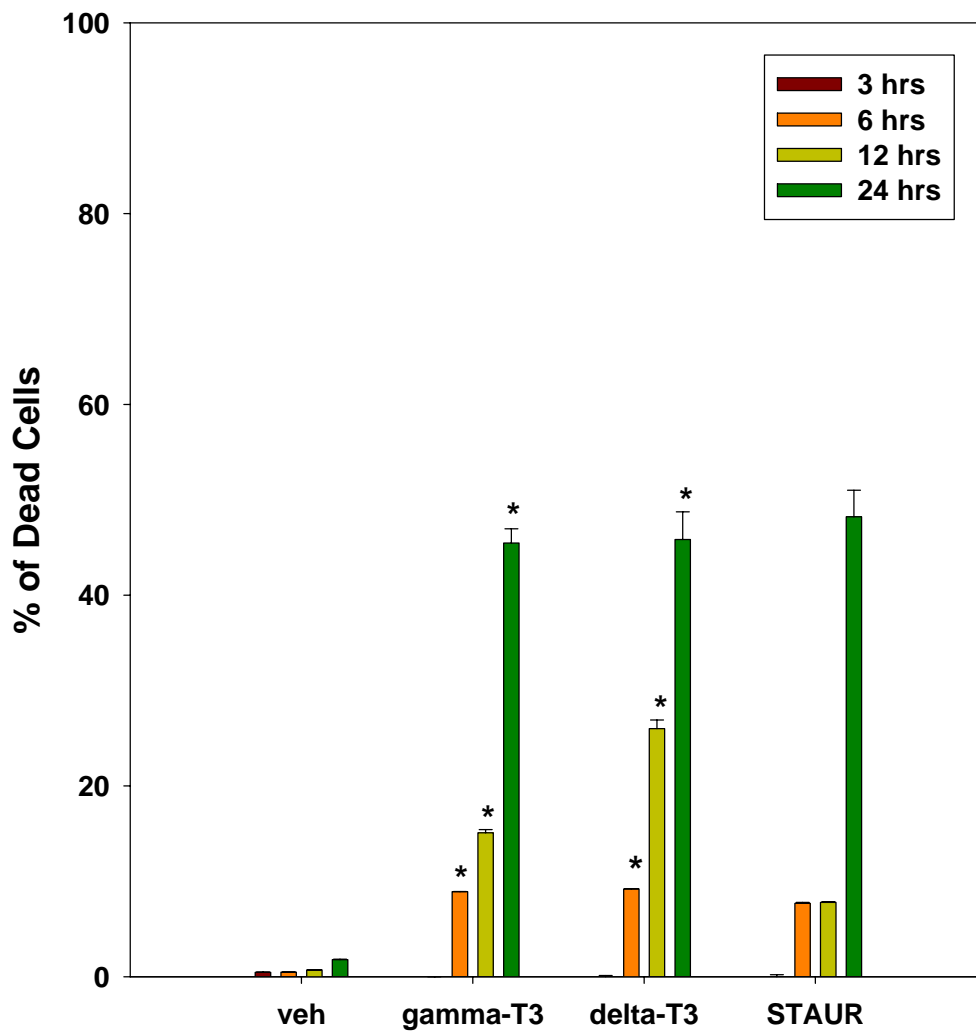


Figure 11. Percentage of dead cells after exposure of LNCaP cells to gamma-T3 or delta-T3. Cells were incubated with 20  $\mu$ M of gamma-T3 or delta-T3 for the indicated time points. ‘\*’ ( $p < 0.05$ ), means statistically different from vehicle treated cells. Each value is a mean of eight experiments  $\pm$  SEM.

Collectively, the data presented above show that the decreased cell number caused by gamma-T3 or delta-T3 is primarily the result of cell death rather than growth inhibition.



### Evaluation of Cell Death Caused by Gamma-T3 or Delta-T3 in LNCaP Cells Using Caspase 3 Enzymatic Assay

Caspases accompany apoptosis. Caspase 3 was used to discriminate between apoptotic and necrotic cell death. The involvement of caspase 3 was evaluated because it is the most important executioner of apoptosis. Treatment of LNCaP cells for 3, 6, or 12 hours with 20  $\mu$ M of gamma-T3 or delta-T3 enriched cell culture medium led to a significant increase in caspase 3 enzymatic activity beginning at 6 hrs (see Figure 12). Delta-T3 shows greater caspase 3 activity than gamma-T3. The caspase 3 activity becomes very significant at the 12 hr treatment. These results indicate that cell death induced by these two isoforms of vitamin E is apoptotic and not necrotic in nature.

It is important to state here that no significant caspase 3 activity was found before 3 hrs and, hence, protein changes at 3 hrs would likely reflect signal transduction events rather than the protein changes induced by caspases, which would begin at 6 hrs.

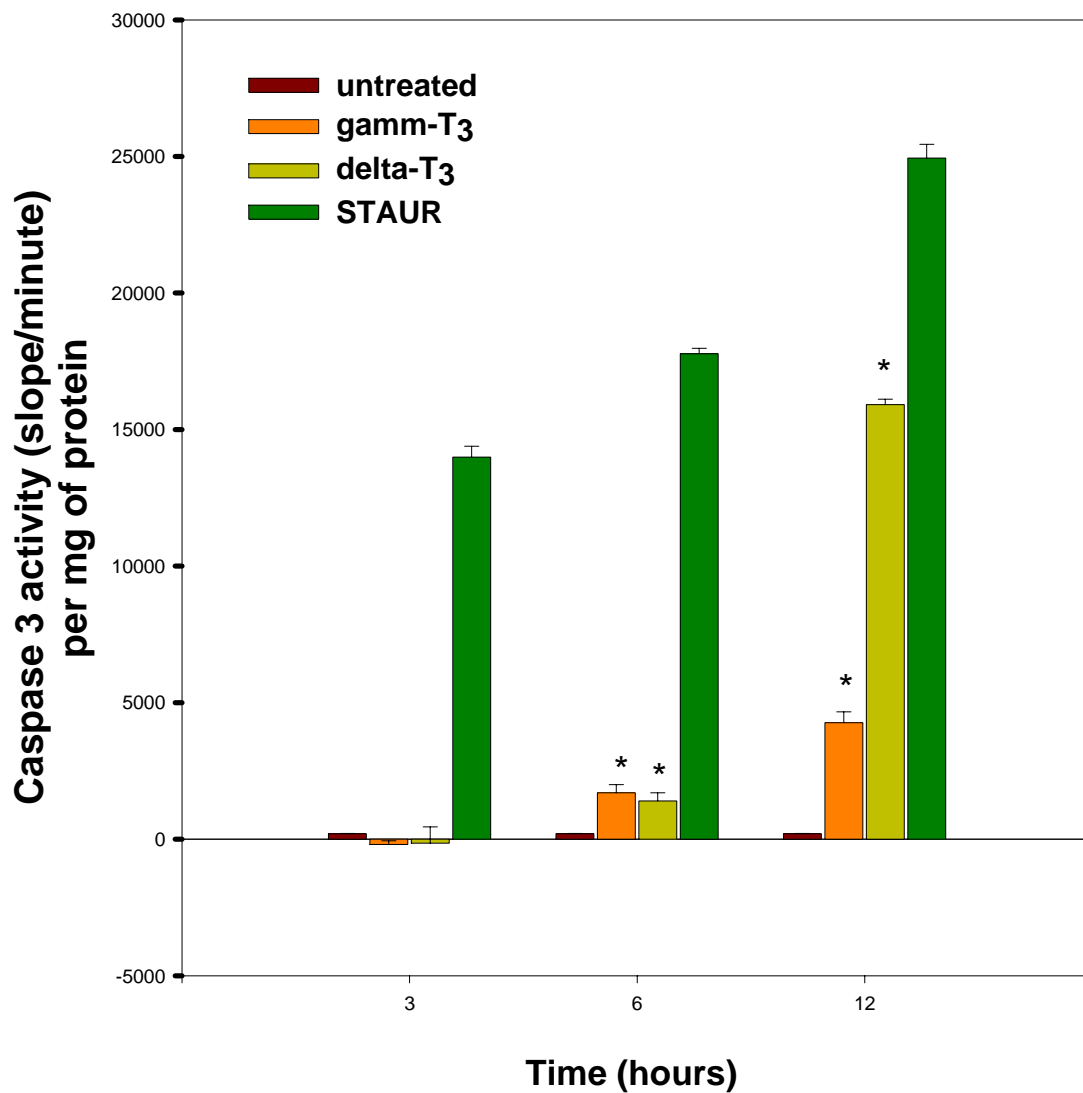


Figure 12. Apoptosis in LNCaP cells induced by gamma-T3 or delta-T3 involves caspase 3 activation. Cells were treated with 20  $\mu$ M of gamma-T3 or delta-T for the various time points. The activation of caspase 3 was measured as the increase in activity with respect to the control per mg of protein. \* $p < 0.05$  compared with vehicle treated cells. The data shown are the mean of eight experiments  $\pm$  SEM.

### Quant-iT Protein Assay

The Quant-iT protein assay was used to estimate the concentration of protein samples prior to loading onto Zoom Strips for rehydration so that exactly the same amount of different protein samples is loaded per gel. Plotting the fluorescence intensity values versus the protein concentration for the standard albumin solutions produces the plot shown in Figure 13.

SigmaPlot generates the predicted or best fit plot (Figure 14) from which the concentrations of the unknown protein samples can be extrapolated. SigmaPlot can also use the predicted results to generate a table of fluorescence values against protein concentration within the limit of the standard albumin plot. Protein concentrations of the unknown samples can be directly read off this table.

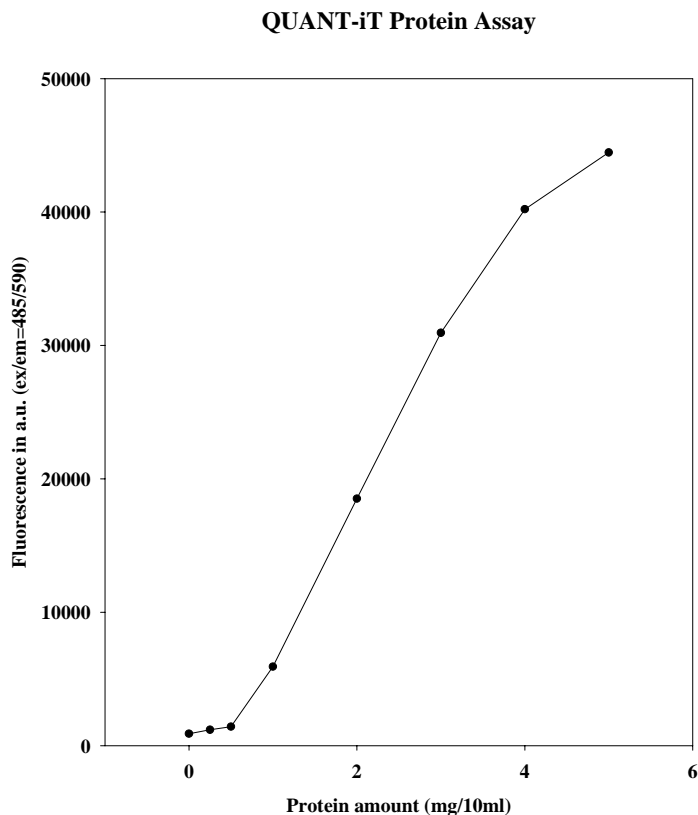


Figure 13. Raw plot of fluorescence intensity versus protein concentration for a standard albumin solution.

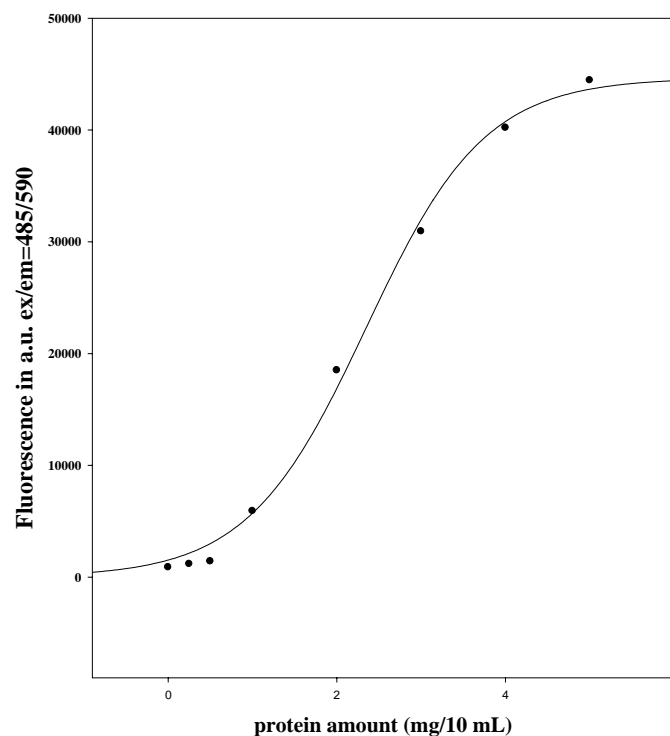


Figure 14. Predicted sigmoid curve for the calculation of protein concentration. This curve is computer generated from the raw curve in Figure 13. Protein concentrations of unknown samples can be read off this standard curve.

### Effects of 3 and 6-hour Delta-T3 Treatment on the Proteome of LNCaP Cells

We performed broad range 2-DE with IEF Zoom strips ranging from pI 3 to 10 without prefractionation of the samples as described in the material and method section. Figure 15 is the superimposed image of the silver stained gels of 3 hr vehicle and 3 hr delta-T3 treated samples, while Figure 16 is the superimposed image of the silver stained gels of 6 hr vehicle and 6 hr delta-T3 treated samples. Results from the gel analysis software indicated that 21 and 22 proteins were regulated for the 3 hr and 6 hr treatment samples respectively, but all these spots could not be represented on the gel image because not all the spots are true spots, and some of

the spots were not discernible. It was for this reason that only three proteins were singled out for the 3 hr treatment and five for the 6 hr treatment.

We also characterized some of the proteins by their isoelectric point and molecular weights. Five proteins from the 3 hr treatment and seven from the 6 hr treatment were characterized by their apparent isoelectric points (pI) and molecular weight (MW) in kilo Daltons (kDA) (Tables 1 and 2). These were protein spots that could be clearly seen on the gels. All the discernible proteins were not singled out on the gel images, but they can be located on the polyacrylamide gels.

The green and magenta colors on the gel images are computer generated colors aimed at distinguishing spots of one treatment sample gels from the other. Spots that appear green are spots of the delta-T3 treatment and are thus upregulated relative to the vehicle treatment, and spots that appear magenta are spots of the vehicle treatment and are thus downregulated. Proteins that are equally abundant in both samples appear dark.

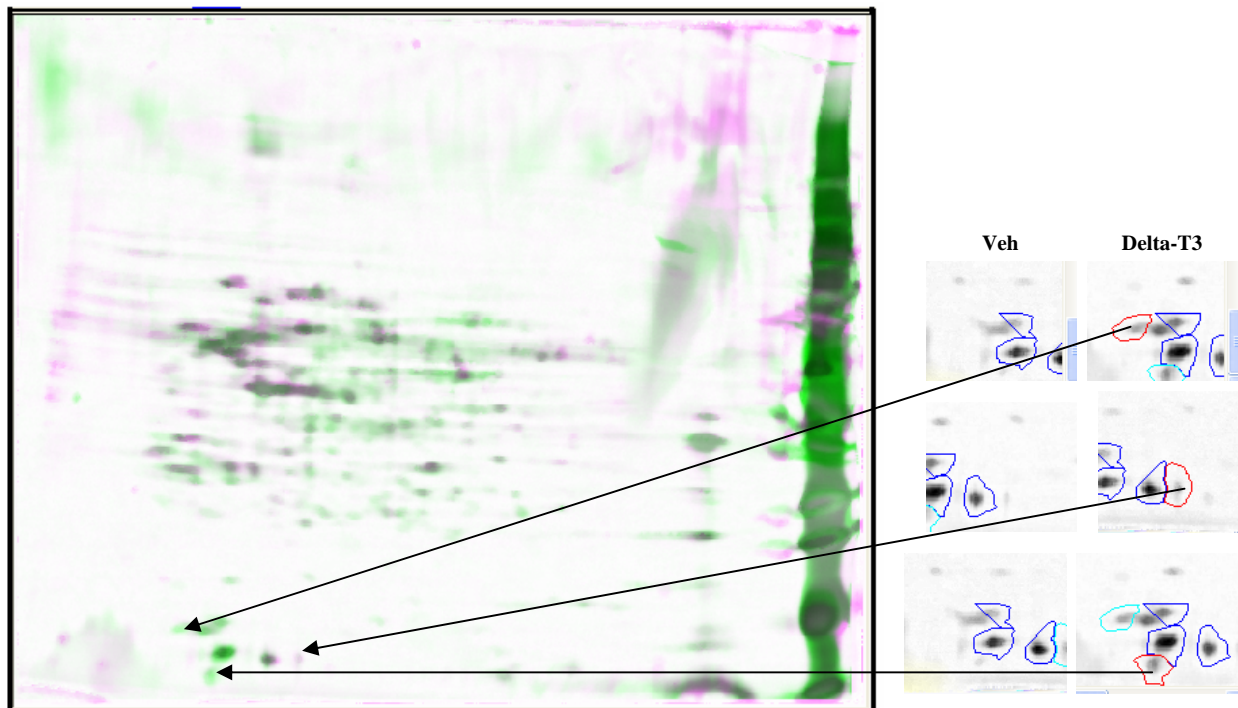


Figure 15. Three-hour treated superimposed gel images from duplicates of vehicle and delta-T3 treated samples. On the right of the gel image are some spots of interest. Spots that appear green are up regulated and spots that appear magenta are down regulated, whereas proteins that are equally abundant in both samples appear dark. Range of horizontal axis is from 3 to 10 pH units (left to right), while full range of vertical axis is from 10 to 250 kDA (bottom to top).

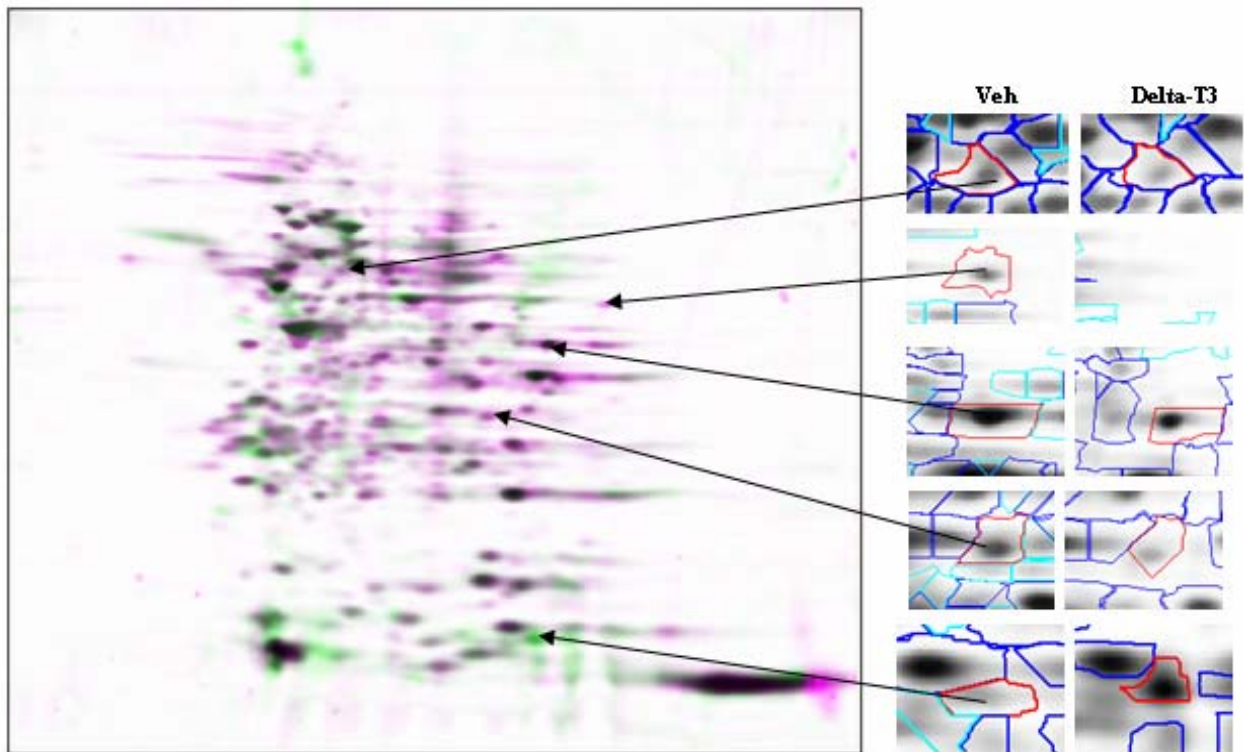


Figure 16. Six-hour treated Superimposed gel images from duplicates of vehicle and delta-T3 treated samples. On the right of the gel image are some spots of interest. Spots that appear green are up regulated and spots that appear magenta are down regulated, whereas proteins that are equally abundant in both samples appear dark. Range of horizontal axis is from 3 to 10 pH units (left to right), while full range of vertical axis is from 10 to 250 kDA (bottom to top).

Table 1

Spots of Interest for 3-hr Treatment

Spot #	NVR	HR	pI	MW/KD
1	3.005	2.215	4.8	87.5
2	-5.377	-1.972	6.3	65
3	3.466	2.011	7.5	50
4	3.321	3.349	6.8	30
5	3.147	2.617	6.4	30

NVR=Normalized Volume Ratio of vehicle treated sample compared to delta-T3 treated sample. Negative values represent down regulated spots while positive vales represent up regulated spots  
HR =Height Ratio of vehicle treated sample compared to delta-T3 treated sample. Negative values represent down regulated spots while positive vales represent up regulated spots



Table 2

Spots of Interest for 6-hr Treatment

Spot #	NVR	HR	pl	MW/kD
1	-3.035	-1.495	4.5	50-60
2	-4.661	-4.335	3.8	50-60
3	-2.866	-1.953	6.6	50
4	-4.333	-2.669	7.7	37-50
5	-2.269	-2.221	6.9	25-37
6	5.9	2.052	7.1	10-15
7	3.231	1.896	7.8	10-12

NVR=Normalized Volume Ratio of vehicle treated sample compared to delta-T3 treated sample. Negative values represent down regulated spots while positive vales represent up regulated spots  
HR =Height Ratio of vehicle treated sample compared to delta-T3 treated sample. Negative values represent down regulated spots while positive vales represent up regulated spots

## CHAPTER 4

### DISCUSSION

This research was aimed at studying the protein changes occurring during prostate cancer (LNCaP) cell death induced by isoforms of vitamin E. The initial part of our investigation was focused on the cytotoxicity of gamma- and delta-T3 in LNCaP cells because previous results in our lab showed that these isoforms of vitamin E were more cytotoxic to cancer cells compared to normal cells. Both apoptotic cell death and decreased cell viability were observed in LNCaP cells exposed to gamma- and delta-T3 with delta-T3 showing a statistically insignificant greater cytotoxicity than gamma-T3. Cell viability assays were performed that demonstrated a dose dependent and time dependent suppression of cell viability by gamma- and delta-T3 in LNCaP cells. Cell viability was significantly suppressed starting at concentrations of gamma- or delta-T3 greater than or equal to 15  $\mu\text{M}$  (Figure 7) and the time dependent assay showed that cell growth inhibition only started after 6 hrs of treatment (Figure 8).

The above findings show that gamma-T3 and delta-T3 independently have an anti-proliferation effect on LNCaP cells. To be able to distinguish between cell growth inhibition and cell death, we performed an assay to measure dead cells. Dead cells were evaluated using the PI assay, and total number of cells per well was estimated by freezing the cells at  $-20\text{ }^{\circ}\text{C}$  for 24 hrs after the initial PI measurement to kill all remaining live cells. The percentage of dead cells was obtained as the proportion of the fluorescence intensity of dead cells to that of total cells. The data in Figure 9 revealed that gamma- and delta-T3 kill LNCaP cells in a time dependent manner, while the data in Figure 10 indicated that the total number of cells increased with time for the vehicle treated cells, thereby showing that these cells were growing with time. The

results of the percentage of dead cells (Figure 11) illustrated the time dependent cell killing property of gamma- and delta-T3 in LNCaP cells. At the 3-hr treatment, there was no detectable cell death. At 6 hrs, about 8% of cells had been killed by gamma- and delta-T3. At 12 hrs, about 15% and 25% of cells had been killed by gamma- and delta-T3 respectively, while at 24 hrs, about 45% of cells had been killed by gamma- and delta-T3. This confirms the earlier assertion that delta-T3 is slightly more cytotoxic than gamma-T3.

Cells die when exposed to cytotoxic drugs irrespective of whether the form of cell death is necrotic or apoptotic. The driving force behind most cancer studies is to induce apoptosis in cancer cells. In order to distinguish between the forms of cell death triggered by gamma- and delta-T3 in LNCaP cells, a caspase 3 enzymatic assay was performed and the results (Figure 12) indicated that cell death induced by gamma- and delta-T3 was apoptotic and not necrotic. Caspase 3 was activated in a time dependent manner with significant activation occurring at 6 hrs for cells treated with 20  $\mu$ M of gamma- or delta-T3.

Our cell culture studies demonstrated the inhibition of cellular proliferation and killing of LNCaP cells by gamma- or delta-T3 occurs through apoptosis, but the molecular mechanisms responsible for this cytotoxicity are still largely unknown. The second part of our investigation was focused on the proteomics aspect. The above findings suggest that gamma- and delta-T3 can serve as very effective chemotherapeutic or chemopreventive agents for the treatment of prostate cancer. Knowledge of the proteomic events taking place during LNCaP cell death will be vital in designing more effective drugs to treat androgen-dependent prostate cancer.

The above results showed that the minimum effective concentration of gamma- and delta-T3 required to kill LNCaP cells was 20  $\mu$ M, and that delta-T3 was more effective than gamma-T3. We decided to use the more effective delta-T3 for our proteomics study. In an effort to find

out which proteins were regulated during LNCaP cell death, we did 2-DE with LNCaP protein samples treated with delta-T3 for 3 hrs (Figure 13) and 6 hrs (Figure 14). Our results show that different groups of proteins were regulated in early and late term treatments. Results from the gel analysis software indicated that 21 proteins were apparently regulated during the 3-hr treatment while 22 different proteins were apparently regulated during the 6-hr treatment. All these proteins could not be represented on the gel images, but they can be traced on the polyacrylamide gels. We have characterized some of the proteins by isoelectric point and molecular weight. Five proteins from the 3-hr treatment and seven from the 6-hr treatment were characterized by their apparent isoelectric points (pI) and molecular weight (MW) in kilo Daltons (kDA) (Tables I and II). The next task is to confirm these protein spots using other protein separation techniques such as difference in gel electrophoresis (DIGE) and then use mass spectroscopy and protein database search engines to identify the proteins and their functions.

## CHAPTER 5

### CONCLUSION

Gamma-T3 and delta-T3 forms of vitamin E were shown to induce programmed cell death (apoptosis) and decrease cell viability in LNCaP cells, with delta-T3 displaying a greater apoptotic activity than gamma-T3. Apoptotic cell death occurred in a time-dependent manner in LNCaP cells treated with gamma- or delta-T3. Previous results from our lab show that concentrations of gamma- or delta-T3 that kill LNCaP cells have no significant effect on non-tumorigenic prostate epithelial cells. Because gamma-T3 and delta-T3 could selectively kill LNCaP cells, they may be useful in treating prostate cancer either as chemopreventive or chemotherapeutic agents.

We have used proteomics to identify certain proteins which were differentially expressed in early and/or late term treatment periods with delta-T3. If these proteins are confirmed and identified by mass spectroscopy and database searches, it might present a critical piece of information for the design of more effective drugs for the treatment and prevention of prostate cancer.

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