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**14. ABSTRACT**

In this study we demonstrated that vitamin E isoforms, tocopherols and tocotrienols, have variable growth inhibitory effects on both types of prostate cancer cell line models. The gamma isoforms are more effective than the alpha isoforms and the tocotrienols are more effective than the tocopherols. This study further showed that the vitamin E-mediated inhibition of cell proliferation is preferential for cancer cells at concentrations of about 40 µM or lower. Delta-tocotrienol (DT3), in particular, is infective against normal prostate epithelial cells but highly effective against LNCaP cancer cells. Collectively, our data supports the view that tocotrienols, particularly DT3 may prove very useful as chemotherapeutic or chemopreventive agents for treating prostate cancer. Our next will be to initiate experiments in animal models and then to initiate clinical studies

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prostate cancer, tocopherols, tocotrienols, chemoprevention, chemotherapy, apoptosis

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## INTRODUCTION:

Prostate cancer has the greatest country-to-country variation in incidence of any reportable cancer suggesting the importance of nutrition and environmental factors [1-3]. Some evidence supports the protective effects of tomato products (lycopene), soy products (isoflavonoids) and fruits. Secondary endpoint analysis of recent intervention trials found inverse associations of vitamin E and selenium with prostate cancer. Nutritional antioxidants such as vitamin E may help prevent prostate cancer by reducing oxidative damage to DNA, slowing the growth of prostate tumors or by promoting the programmed cell death (apoptosis) of prostate tumors. Most studies relating cancer to vitamin E nutriture have, however, paid little attention to the different forms of vitamin E. Vitamin E is not a single compound and includes four tocopherols and four tocotrienols. Our long-term hypothesis is that tocotrienols will be superior to tocopherols in preventing prostate cancer. In the studies described below, we will use the PC-3 (androgen-independent) and LNCaP (androgen-dependent) cell lines. Many human prostate cancers arise because these cancerous cells have escaped apoptotic cell death. A key strategy in cancer prevention is, therefore, to promote apoptotic cell death in cancerous cells. There is considerable evidence that vitamin E plays a role in the induction of apoptosis but there are no systematic studies comparing tocopherols to tocotrienols in prostate cancer cell lines. Our specific aims are to determine if tocotrienols are superior to tocopherols in: (1) inhibiting prostate cell line growth by altering apoptosis; (2) inhibiting the expression and activity of COX-2; (3) enhancing the expression and activation of PPAR-gamma; (4) increasing intracellular levels of vitamin E.

## BODY:

**Task 1**: To determine if tocotrienols are superior to tocopherols in inhibiting prostate cell line growth by altering apoptosis.

When this research was initiated there was evidence that alpha-tocopherol could induce apoptosis in prostate cell lines but tocotrienols had not yet been tested. Our planed experiments included measuring the activation of caspases as an early marker for apoptosis and DNA fragmentation as a marker for the later stages of apoptosis. Task 1 represents the most important series of experiments for this proposal and our results are very positive and were somewhat expanded beyond the initial experimental design. A major publication has been submitted and a copy attached to the appendix which provides detailed experimental methodology. In addition, we have extended some experiments to colon cancer cell lines since our prostate cancer cell lines were found to have some methodological limitations. We have been invited to present this work (oral presentation) at the 2006 International Workshop on Micronutrients, Oxidative Stress and the Environment in Kuching, Malaysia.

### Tocotrienols are more Potent than Tocopherols at Growth Inhibition of Human Prostate Cancer Cell Lines, LNCaP and PC-3

In this study LNCaP cells were used as a model for hormone-dependent prostate cancer and PC-3 cells as a model for the more aggressive hormone-independent prostate cancer. Figure 1 shows the influence of alpha-tocopherol (AT), alpha-tocotrienol (AT3), gamma-tocopherol (GT) and gamma-tocotrienol on cell proliferation as function of time using the MTT assay (see attached paper for Methods). Distinct differences in the abilities of tocopherols and tocotrienols to inhibit cell proliferation were observed at concentrations of 40 and 80 µM. Within 48 hours of the 40 µM vitamin E treatments, effective reduction of cell proliferation in PC-3 cells was detected in the tocotrienol-treated group (See Figure 1A). Neither alpha-tocopherol (AT) nor gamma-tocopherol (GT) was effective at 40 µM for as long as 72

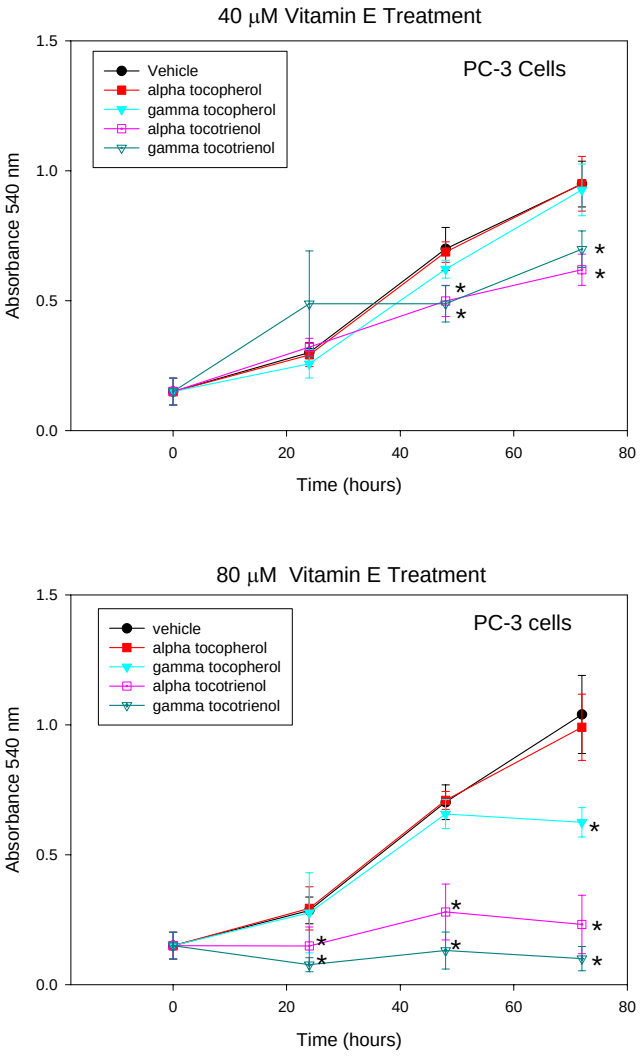



**Figure 1 A and 1 B**

hours. The 80 µM treatment in PC-3 cells resulted in reduced cell proliferation over the vehicle-treated cells as early as 24 hours with the tocotrienols, but not the tocopherols (See Figure 1B). GT displays a growth inhibitory effect at 80 µM but only after 72 hours: AT was not statistically different from the vehicle treated PC-3 cells at any concentration tested over the 72 hour time-course.

Since the LNCaP cells are androgen-dependent, we expected them to be more sensitive to the reduction in cell proliferation induced by vitamin E than the more resistant androgen-independent PC-3 cell line. As anticipated, the LNCaP cells were more sensitive than PC-3 cells as shown by the significant reduction in LNCaP proliferation with 40 µM treatment with all isoforms of vitamin E at 72 hours (see Figure 2A), while the same treatment in the PC-3 cells resulted in significant reduction in cell proliferation only with alpha-tocotrienol (AT3) and gamma-tocotrienol (GT3) and not with the AT and GT. Significant reduction in cell proliferation occurred in the LNCaP cell line with the GT and GT3 within 24 hours following the 40 µM treatment (see Figure 2A). Camptothecin at 10 µM was used as a positive control and demonstrated a reduction in cell proliferation at every time tested (data not shown).

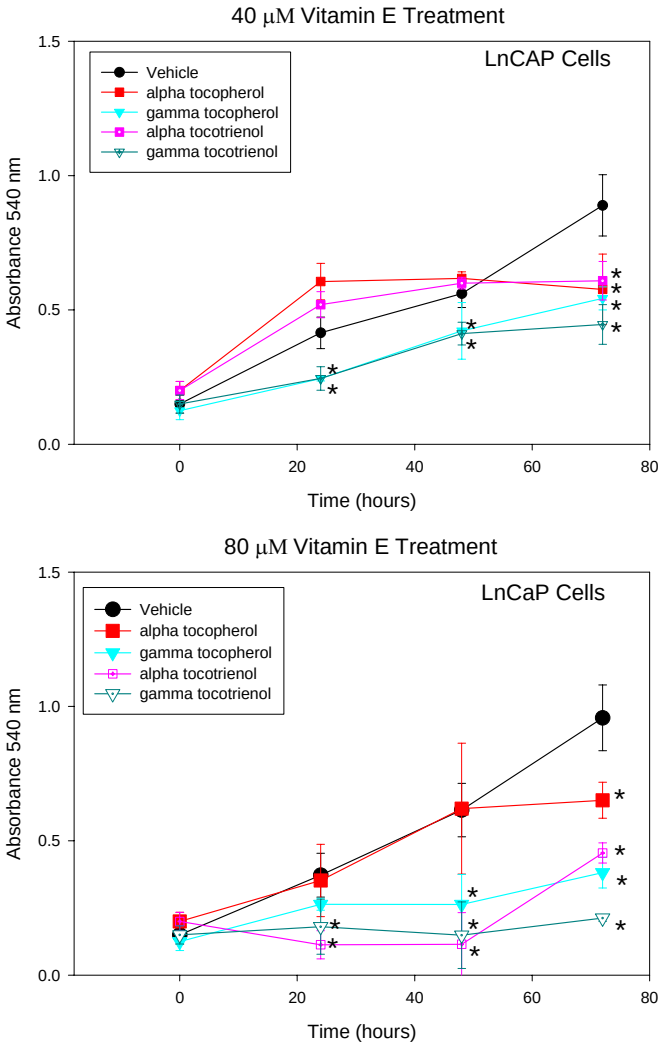



**Figure 2 A and 2 B**

### Tocotrienols Preferentially Cause Growth Inhibition in Human Prostate Cancer Cells compared to Normal Prostate Epithelial Cells

We next determined whether the reduction in cell proliferation observed with tocopherol and tocotrienol treatment was preferential for prostate cancer cell lines Concentrations of 0, 40 and 100 µM AT, GT, AT3 and GT3 were tested for 24 hours in non-cancerous prostate epithelial cells RWPE (Figure 3A) as well as the androgen-sensitive LNCaP cells (Figure 3B). At concentrations of 40 µM, all vitamin E isoforms tested, except GT3 have an insignificant

proliferative effect on non-cancerous RWPE prostate epithelia, whereas treatment of the LNCaP cancer cells at this concentration resulted in a profound reduction in cell proliferation with all the tested isoforms expect AT. AT and GT did not inhibit cell proliferation in the RWPE non-cancerous cell line at 100 µM, while the AT3 caused a small reduction in cell viability and GT3 caused a marked reduction. At 100 µM AT treatment of LNCaP cells showed no inhibition of cell proliferation but all other isoforms showed a statistically significant decrease from the vehicle-treated cells. Additional data (not shown) show that delta-tocotrienol (DT3) was also not effective at inhibiting RWPE-1 cell growth at 40 µM but was very effective at this concentration with LNCaP cells. These data show that at concentrations less than 40 µM (for 24 hours) the tocotrienol forms of vitamin E show a preferential growth inhibition for the LNCaP prostate cancer cells.

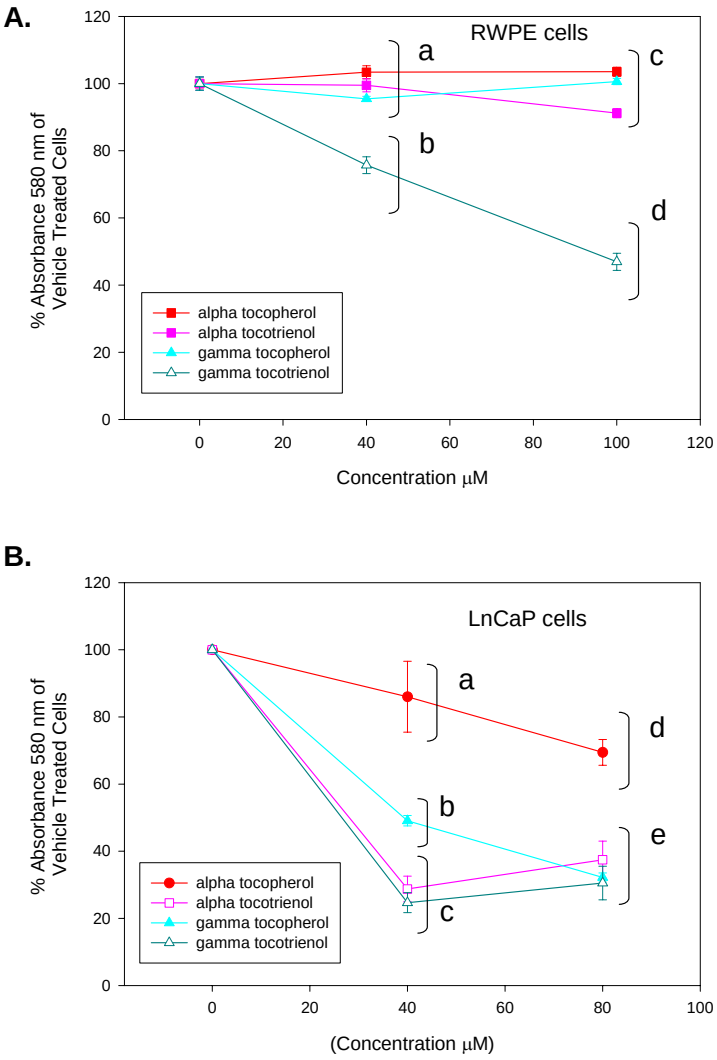



**Figure 3 A and B**

### Gamma-Tocotrienol Induces Apoptosis at Physiologically Achievable Concentrations

While vitamin E concentrations of 40 and 100 µM may be achievable with intravenous antioxidant liposomes [4], these concentrations are most likely not achievable by dietary means. Therefore, we decided to study the effects of the most potent of the vitamin E isoforms, i.e. GT3 in PC-3 cells at lower concentrations using a more sensitive assay to determine the lowest concentration by which we could achieve sustainable cell death.

The PC-3 cells were treated with 0, 0.1, 1, 5, 10, 20 and 40 µM GT3 for 6, 24, and 72 hours (Figures 4, 5, and 6 respectively in the attached paper). Following treatment, the cells were stained simultaneously with three fluorescent dyes and observed by fluorescent microscopy. Calcien/AM penetrates the plasma membrane and is hydrolyzed to its fluorescent from by intracellular esterases and stains the cytoplasm green and is retained by an intact plasma membrane. Mitotracker red stains the actively respiring mitochondria orange. These stains are

used to detect healthy cells. DAPI is a fluorescent stain that has enhanced blue fluorescence when binding to DNA. DAPI staining occurs when the plasma and nuclear membranes lose integrity allowing the stain to access the DNA. DAPI stained nuclei are a result of apoptosis.

The DAPI staining in Figure 4 (see attached paper for these pictures) demonstrated that 1 µM GT3 can result in cellular apoptosis over the control in as little as 6 hours which increases at 24 hours (Figure 5 of appendix), but as demonstrated in Figure 6 (see appendix), the 1 µM GT3 effect does not persist in apoptosis assays at 72 hours. In fact, with 1 µM GT3 treatment at 72 hours results in a higher number of healthy cells compared to apoptotic cells. The density and staining of the cells treated with 1 µM for 72 hours is similar to that of the control (untreated cells). This demonstrates that the PC-3 cells are able to continue proliferation at 1 µM even though apoptosis occurs in a small population of the cells (15% as confirmed by the MTT Assay). Increasing number of apoptotic cells are observed at 6 hours with increasing concentrations of GT3. As with the 1 µM treatment, the 5 µM treatment results in increased % DAPI apoptotic staining at 6 and 24 hours, but by 72 hours the cell density and staining are similar to that of the control cells. Upon treatment with 10 µM and 20 µM, there is increased % DAPI staining at 6 hours that persists through the 72 hour time. In addition, at these concentrations, the cell density with respect to the control cell density has changed. The control cells are at 90-95% confluent, while treatment with GT3 at 10 and 20 µM resulted in large regions on the culture plate where there living cells are no longer present.

**Gamma-Tocotrienol Induces Apoptosis by Activation of Caspases 3, 8 And 9**

We evaluated apoptotic markers in both cell lines after tocopherol and tocotrienol treatment using varying concentrations of tocopherols and tocotrienols (Figure 7) to confirm that the DAPI staining seen Figures 4, 5, and 6 were a result of apoptotic induction and not necrosis and to determine if there were differences in apoptotic induction among the vitamin E isoforms. First, we tested varying concentrations of GT for 24 hours in the LNCaP cell line then monitored differences in cleavage of caspase 3 by Western blot analysis (Figure 7A). Caspase 3 cleavage was observed with 80 µM GT treatment but not with 20 or 40 µM GT.

The vitamin E isoforms were then tested at 40 µM for 6 hours in the LNCaP cell line (Figure 7B). These data show that GT3, but not AT, GT or GT3, induced cleavage of caspase 3. Higher concentrations of GT (80 and 100 µM) do result in caspase 3 cleavage (data not shown). Also, cleavage of caspase 9 was greater with the GT3 isoform than the other isoforms tested. These data demonstrate that GT3 is more potent at apoptotic induction than its tocopherol counterpart. To verify this, we tested GT and GT3 treated LNCaP cells at several concentrations for 24 hours and monitored the activity of caspase 3 (Figure 7C). Caspase 3 was activated by both GT and GT3 at 40 and 80µM but was more effectively activated by GT3 than GT.

We measured the activation of caspase 3, 8 and 9 in LnCaP cells over time following the treatment with 80 µM GT3 (Figure 7D). The fluorescent units were normalized to the maximum fluorescence observed for the purpose of determining the order of caspase activation. The

results demonstrate that caspase 3, 8, and 9 are simultaneously activated by 6 hours with 80 µM GT3 treatment.

To confirm apoptosis is occurring with GT and GT3 treated LNCaP cells at 24 hours, we performed a DNA laddering analysis (Figure 7E). These data mirror those found in Figure 8A; GT is more effective at apoptotic induction at 80 and 100 µM, whereas GT3 is effective even at 40 µM.

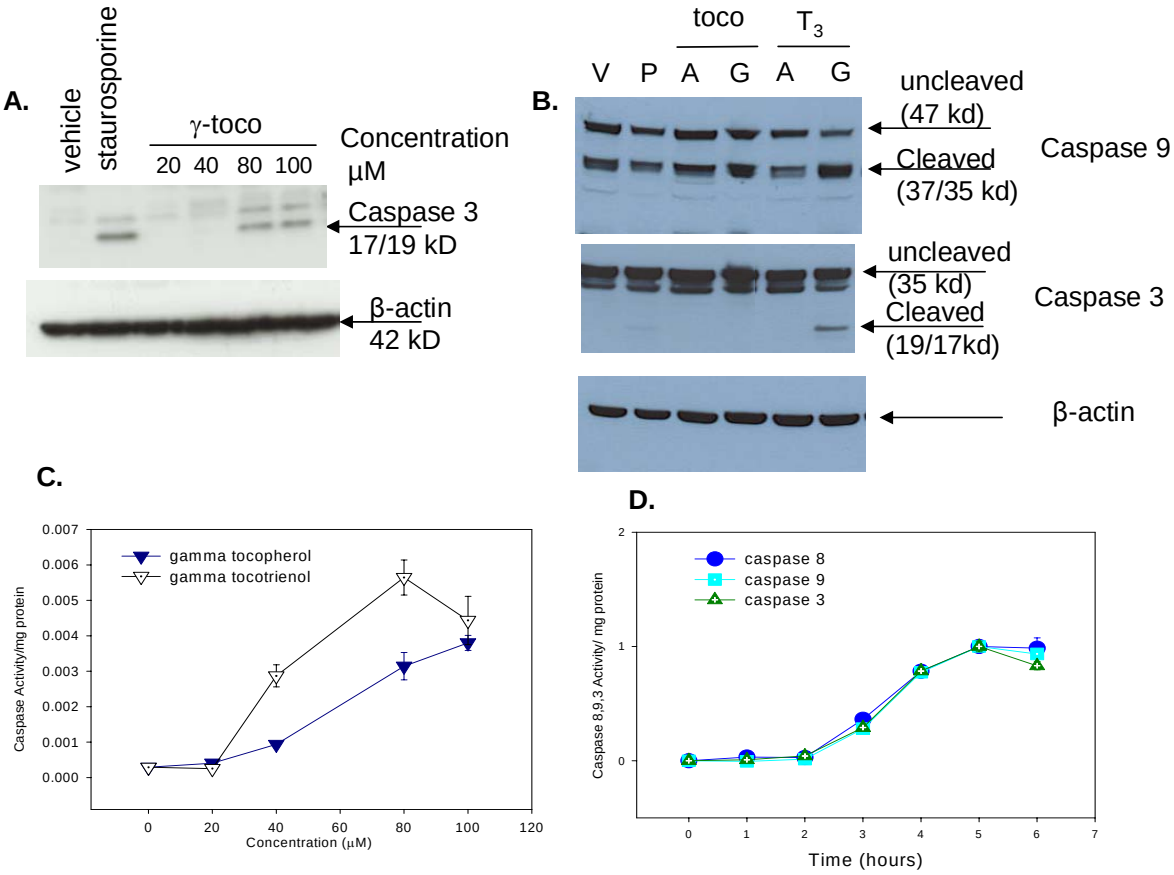

A.
vehicle
staurosporine
γ-toco
20 40 80 100
Concentration μM
Caspase 3
17/19 kD
β-actin
42 kD
B.
toco
T3
V P A G A G
uncleaved
(47 kd)
Cleaved
(37/35 kd)
Caspase 9
uncleaved
(35 kd)
Cleaved
(19/17kd)
Caspase 3
β-actin
C.
gamma tocopherol
gamma tocotrienol
Caspase Activity/mg protein
Concentration (μM)
D.
caspase 8
caspase 9
caspase 3
Caspase 8,9,3 Activity/ mg protein
Time (hours)
E.
Vehicle
S
g-toco
40 80 100
g-T3
40 80 μM

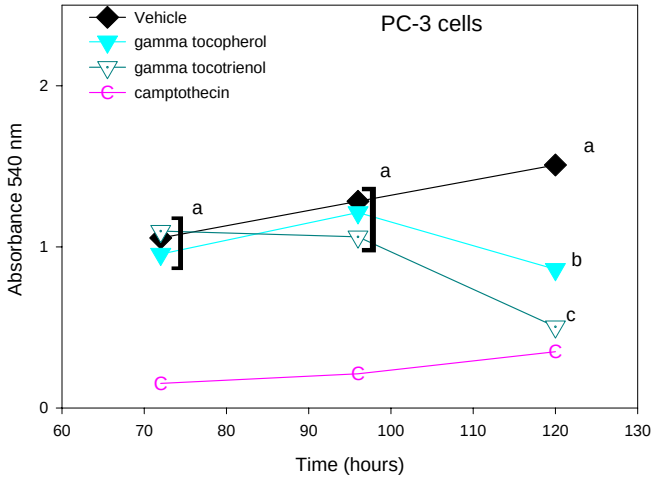
Figure 7

**The Potential Chemopreventive Effects of Tocopherols and Tocotrienols**

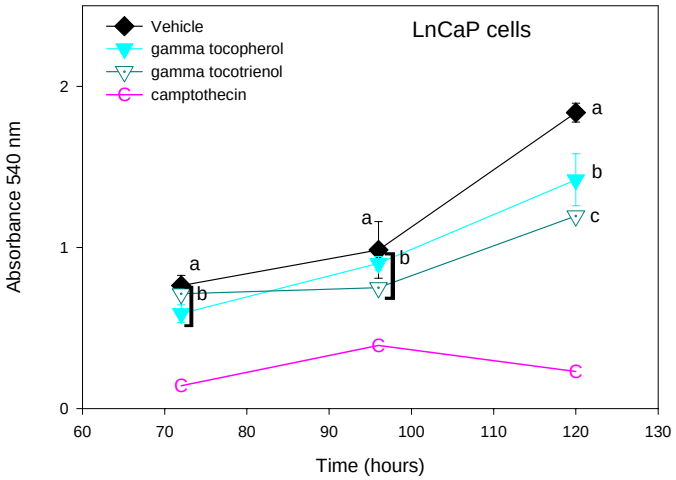
We then determined if the gamma-vitamin E isoforms could serve as potential chemopreventive agents by maintaining physiologically obtainable concentrations (by dietary supplementation) of over an extended time period. Further, we were interested in testing whether there were differences between the tocopherol and tocotrienol isoforms at these later time points. Figure 8 displays the results of the 10 µM treatment with GT or GT3 for 3, 4 and 5 days in the PC-3 (Figure 8A) or the LnCaP (Figure 8B) cell lines. Data for each time interval were analyzed by one-way ANOVA and Tukey's test. Within time intervals, statistical differences between means are represented by a different letter. Consistent with the data shown in Figure 2, the PC-3 cells were slightly resistant to the vitamin E treatment and required a 5-day exposure before differences in means compared with vehicle-treated cells were demonstrated. In addition, the 5-day exposure in PC-3 cells showed GT3 to be more effective at inhibiting cell proliferation than GT. The 10 µM GT and GT3 treatment in LNCaP cells demonstrated a statistical difference with both isoforms compared with the vehicle at 3 days. At day 5, GT3 was found to be the most effective isoform for reducing cell viability in both cell lines.

**Figure 8**

A.




B.




**Task 2:** Determine if tocotrienols are superior to tocopherols in inhibiting the expression and activity of COX-2.

Considerable evidence suggests that COX-2 inhibitors are excellent candidates for prostate cancer chemopreventive agents. While it is known that gamma-tocopherol (but not alpha-tocopherol) is an inhibitor of COX-2, there is no information about the potentially inhibitory roles of tocotrienols. This task was unsuccessful in the prostate cancer cell lines since they had extremely low levels of COX-2 activity. Our results are consistent with reported by Wagner et al. [5] who also found that very low levels of COX-2 in PC-3 and LNCaP cell lines. This work,

however, is now being pursed in colon cancer cell lines and our ongoing results are encouraging.

### Tocotrienols and tocopherols activate the nuclear transcription factor, PPAR gamma, but are not direct PPAR gamma ligands

**Task 3:** To determine if tocotrienols are superior to tocopherols in enhancing the expression and activation of PPAR-gamma.

The rationale for this task rests on the assertions that an increase in the activation or expression of PPAR-gamma could cause prostate cancer cell lines to differentiate, to undergo nonapoptotic death or to undergo apoptosis. Although alpha-tocopherol is known to induce the expression of PPAR-gamma (in some cell lines) there has been no similar information published to-date on tocotrienols [6]. Troglitazone, an oral antidiabetic agent, is an activator of PPAR-gamma and this drug has an alpha-tocopherol moiety in its chemical structure [7].

The results from this task have also been very positive with the prostate cell lines and the work has also been extended to colon cancer cells (see [8].

### Tocotrienols and Tocopherols Activate the Nuclear Transcription Factor, PPAR Gamma, but are not Direct PPAR Gamma Ligands

Upon finding that the gamma isoforms of vitamin E (tocopherol and tocotrienol) could effectively promote apoptosis in prostate cancer cell lines we became interested in locating a potential molecular target for the effects of vitamin E isoforms on prostate cancer cells. Since PPAR γ is a molecular target for chemoprevention and, since we had shown that PPAR gamma is activated by tocopherols (GT>AT) in colon cancer cells [8], we studied the effects of tocopherol and tocotrienol treatment on PPAR γ mRNA in PC3 and LNCaP cells using real-time PCR . Figure 9 shows that 5 μM tocopherol or tocotrienol treatments can up regulate PPAR γ mRNA in both LNCaP and PC-3 by 24 hours with varying efficiency depending upon the cell line and vitamin E isoforms tested.

In the PC-3 cells, for example, GT3 was most effective in stimulating PPAR γ mRNA expression, while in the LNCaP cell line GT3 was the least effective while and GT was the most effective. Further, in the LNCaP cell line GT mediated PPAR γ mRNA expression was greater than that of AT but this was not the case for the PC-3 cell line. Figure 9 also demonstrates that vitamin E isoforms not only up regulate the mRNA expression but also the protein expression. Significant differences in PPAR γ protein expression among the vitamin E isoforms were not, however, detected in these prostate cell lines.

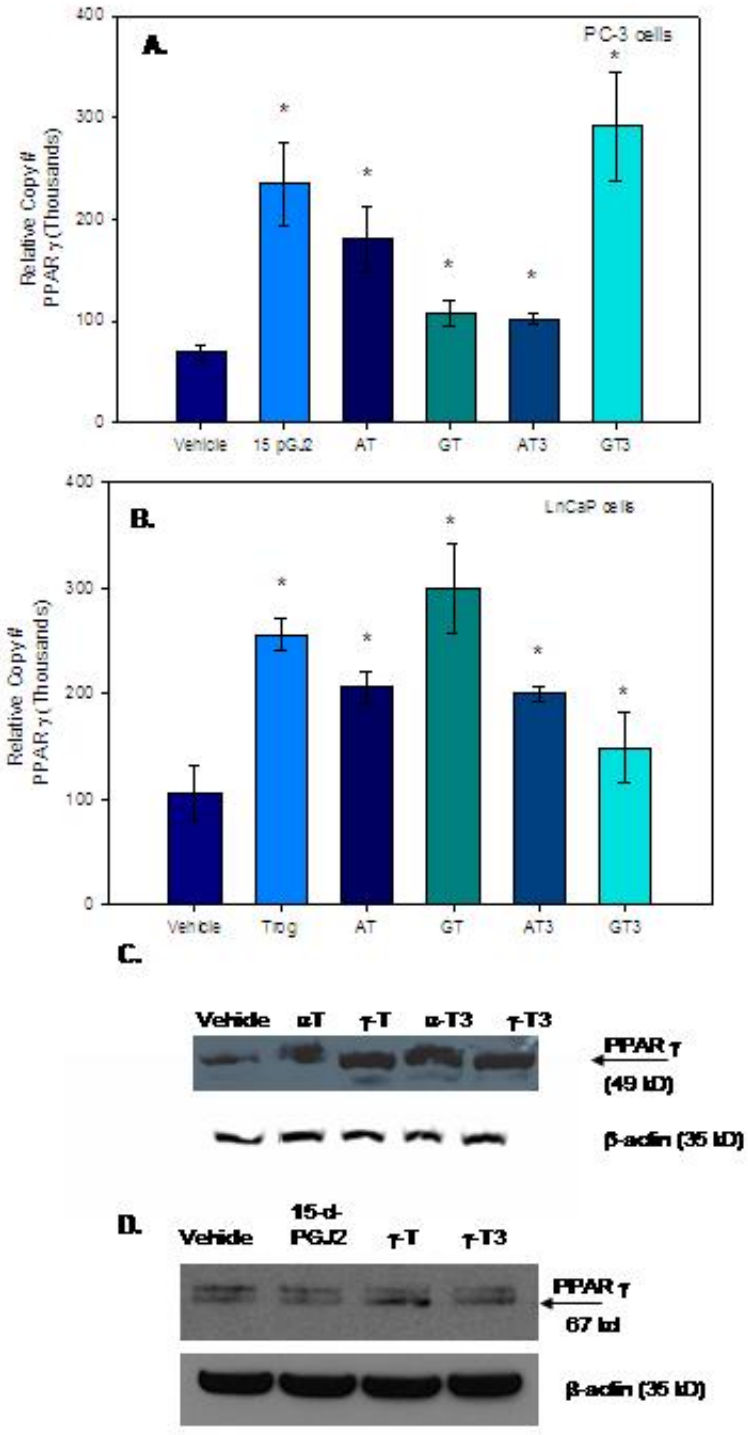

A.
PC-3 cells
Relative Copy # PPAR γ (Thousands)
400
300
200
100
0
Vehicle
15 pGJ2
AT
GT
AT3
GT3
B.
LnCaP cells
Relative Copy # PPAR γ (Thousands)
Vehicle
Trog
AT
GT
AT3
GT3
C.
Vehicle
αT
γ-T
α-T3
γ-T3
PPAR γ
(49 kD)
β-actin (35 kD)
D.
Vehicle
15-d-PGJ2
γ-T
γ-T3
PPAR γ
67 kd
β-actin (35 kD)

**Figure 9**

Upon determining that tocopherols and tocotrienols could activate PPAR γ, we performed radioactive competitive binding assays to determine if vitamin E isoforms are PPAR γ ligands. Tocopherols and tocotrienols were tested for competitive displacement of radiolabeled PPAR γ agonist, rosiglitazone, in the scintillation proximity assay from concentrations of 1 nM to 100 µM. Using this assay, a test agent is judged a PPAR γ ligand when it can displace at least 50% of the bound ligand (rosiglitazone). These data indicted that none of the vitamin E isoforms achieved 50% displacement (see attached paper for detailed data) at 100 µM concentration of vitamin E isoform. These data suggests that both tocopherols and tocotrienols are not direct PPAR γ ligands and may act through indirect pathways that lead to PPAR γ activation.

**Task 4:** To determine if tocotrienols are superior to tocopherols in increasing intracellular levels of vitamin E

We (as well as other investigators) have found that the cellular uptake of various forms of vitamin E differ quite dramatically [9]. In order to properly interpret the data obtained from Specific Aims 1 to 3, it is essential to know the intracellular levels of vitamin E after incubation with the various forms (tocotrienols and tocopherols). The data reported by McIntyre et al. [10] for mammary epithelial cells suggest that tocotrienols accumulate to higher levels than tocopherols. In the research reported here we determined if this was also the case for PC-3 and LNCaP prostate cancer cell lines.

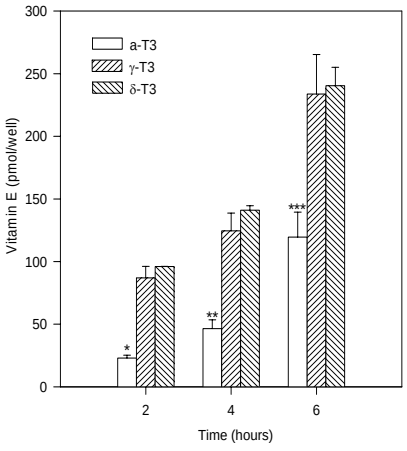
**The Uptake of alpha-, gamma-, delta-Tocotrienols by PC-3 Cells**

Tocopherols and tocotrienols in cells were measured by a highly sensitive HPLC technique using an electrochemical detector [9]. We also developed a method to increase the intracellular levels of vitamin E without adding ethanol or dimethylsulfoxide to cells [9].

**The Uptake of alpha-, gamma-, delta-Tocotrienols by PC-3 Cells**

In these experiments, the uptake of alpha-, gamma- and –delta -tocotrienols (AT3, GT3, DT3, respectively) by PC-3 cells was measured as a function of time and at different concentrations in the medium. Confluent ($4\times10^6$cells/well) PC-3 cells were placed in cell culture plate overnight to let the cells attach the bottom of wells. The medium was changed and the cells incubated with 1µM of tocotrienols in

Figure 10. Uptake of α-, γ- or δ-Tocotrienols by PC-3 Cells



medium for 2 hours, 4 hours, 6 hours. In the second experiment, the cells were incubated with 0.25 µM, 0.50 µM, or 1.0 µM tocotrienols for 4 hours to test uptake at different concentrations of tocotrienols.

Figure 10 (also reproduced in the appendix at larger magnification since this may be difficult to read) shows the uptake 1µM of AT3, GT3 and DT3 by PC-3 cells as a function of time. From these data is clear that uptake of AT3 is much slower than that of GT3 or DT3. The uptake GT3 and DT3 were not statistically different. Additional analytical data (not shown) suggests that the decreases of tocotrienols (picomoles/well) in medium are much larger than the increases of tocotrienols (picomoles/well) in the cells. Clearly, most tocotrienols were lost by *in vitro* oxidation and metabolism rather than by PC-3 cell uptake.

Figure 11 shows the uptake of AT3, GT3 and DT3 by PC-3 cells at different concentrations in the medium. With increasing concentrations, the uptake amount of GT3 and DT3 are markedly increased, while the uptake amount of AT3 increased only slightly. The uptakes of all three tocotrienols were all dose-dependent.

## Figure 11 Uptake at Different Tocotrienol Concentrations

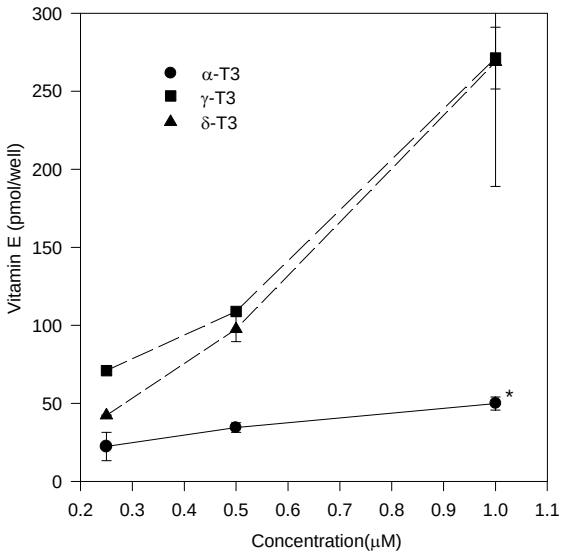



Figure 12 shows the uptake of tocotrienols per mg of protein of PC3 cells. The protein normalized levels of DT3 in PC-3 cells are much higher than that of AT3 or DT3 ($p<0.05$).

In order to compare the uptake between tocotrienols and tocopherols, we also measured the uptake of GT and GT3. Confluent PC-3 cells were incubated with GT or GT3 enriched medium for 4 hours. Figure 13 shows the uptake of GT3 is much higher than that of GT.

**Figure 12. Protein Normalized Concentrations of Tocotrienols**

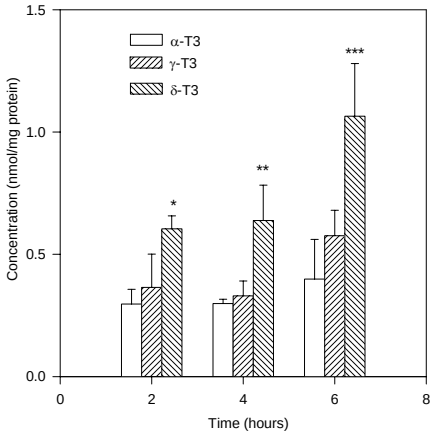
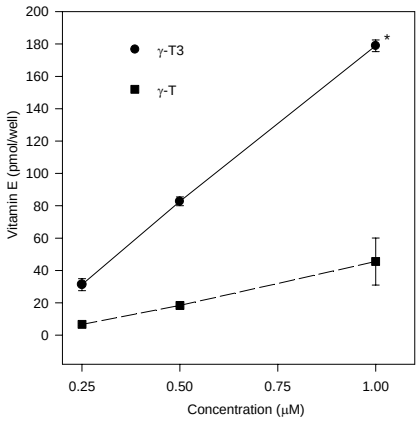


**Figure 13. Comparative Uptake of γ-Tocotrienol and γ-Tocopherol**




## KEY RESEARCH ACCOMPLISHMENTS:

- Gamma-tocotrienol and delta-tocotrienol, vitamin E isoforms with an isoprenoid tail, induces growth arrest in both androgen-dependent, LNCaP and androgen-independent, PC-3 human prostate cancer cells more effectively than the vitamin E isoforms, alpha-tocopherol, gamma-tocopherol and delta-tocopherol (with phytyl tails).
- Gamma-tocotrienol-induced growth arrest results in caspase 3, 8 and 9 activation, PPAR gamma activation, and apoptotic cell death.
- The effects of tocotrienols on growth of human prostate cancer cells are concentration and time dependent and occur at physiological concentrations without affecting growth of normal prostate epithelial cells.
- Ligand-binding assays indicate that neither gamma-tocotrienol nor the other vitamin E isoforms are direct PPAR gamma ligands.

- In the cellular study our results suggested that position 5 of chroman head and side-chain are very important in determining the uptake of tocotrienols and growth inhibition of PC-3 cells.
- The uptake and growth inhibition by different forms of vitamin E are not necessarily related to the antioxidant properties of tocotrienols.

## REPORTABLE OUTCOMES:

Manuscripts:

Campbell, S.E., Stone, W.L., Whaley, S.G, Qui, M., Krishnan, K.: Gamma-Tocopherol Upregulates Peroxisome Proliferator Activated Receptor (PPAR) Gamma Expression in SW 480 Human Colon Cancer Cell Lines, 3:23, BMC Cancer (2003).

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Stone, W.L., Krishnan, K., Papas, A.M., Oxidants and Antioxidants in the Pathology of Colon Cancer, in Redox-Genome Interactions in Health and Disease, ISBN 0-8247-4048-3, Marcel Dekker, Inc, NY (2004).

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Stone, W.L., Antioxidant Liposomes, invited oral presentation at “Advances in Molecular Medicine Conference, East Tennessee State University, Oct 15, 2004, Johnson City, TN.

Stone, W.L., Vitamin E and Cancer, invited presentation at the Internal Medicine Research Seminar Series at East Tennessee State University, Oct 26th, 2005, Johnson City, TN

Degrees obtained that are supported by this award:

Tianming Zuo, M.S., 2003, Chemistry Department, “The In Vitro Cellular Uptake and Physiochemical Properties of Tocotrienols”

Clarisse Sornsay Muenyi, M.S., 2005, Chemistry Department, “The Cytotoxicity of Tocopheryl d-Alpha-Tocopheryl Polyethylene Glycol 1000 Succinate.”

Christelle Kuyeb, M.S., 2005, Chemistry Department, “The Cellular Uptake of Tocopheryl d-Alpha-Tocopheryl Polyethylene Glycol 1000 Succinate.”

Christian M. Muenyi, M.S., 2007, Chemistry Department. Vitamin E and Prostate Cancer: A Proteomic Approach

Qian Li, PhD, The Use of Vitamin E Containing Liposomes to Treat Prostate Cancer

Funding applied for based on work supported by this award:

Comparative Abilities of Vitamin E Isoforms to Prevent Prostate Cancer submitted to the DOD Idea Development Award ($505,301): This proposal received a "very good" score but was not funded (see attached review in Appendix). This proposal will be resubmitted since considerable new preliminary data now supports this proposal.

Employment or research opportunities applied for and/or received based on experience/training supported by this award:

Two MS level graduate students completed research with support from this grant and both have been accepted in excellent PhD programs. Dr. Hongsong Yang (A Visiting Scholar from China) also worked on this project and she has subsequently applied and been awarded permanent residency to continue research in this area. She is currently employed on a funded DOD grant.

Invited Presentations at International Meetings

Stone, et al., have been invited to present the results of this study at the 6$^{th}$ International Society for Free Radical Research Workshop to be held in Kuching, Malaysia (June 29$^{th}$ to July 2$^{nd}$, 2006)

**CONCLUSION:**

In this study we demonstrated that vitamin E isoforms, tocopherols and tocotrienols, have variable growth inhibitory effects on both types of prostate cancer cell line models. The gamma isoforms are more effective than the alpha isoforms and the tocotrienols are more effective than the tocopherols. This study further showed that the vitamin E-mediated inhibition of cell proliferation is preferential for cancer cells at concentrations of about 40 µM or lower. DT3, in particular, is infective against normal prostate epithelial cells but highly effective against LNCaP cancer cells. GT3, AT3 and DT3 demonstrate a profound effect on the cell proliferation in LNCaP and PC-3 cells at 80 µM.

This study also demonstrates the ability of the GT3 isoform to inhibit cell proliferation within 6 hours of treatment with dietary concentrations (5 and 10 µM). These data demonstrate that 10 µM is required to maintain continued cell death through 72 hours. The concentration required to maintain cell death may be significantly less under physiological conditions. To maintain tissue culture cell lines oxygen concentrations are significantly higher than the oxygen concentrations maintained physiologically in most human tissues. Low concentrations of an antioxidant in such a high oxygen environment may lead to auto oxidation of the vitamin E.

Dietary concentrations of the gamma isoforms (10 µM) maintain the reduction of cell proliferation in both prostate cancer cell lines out to five days. The most interesting aspect of these data is that there is a more profound result at 5 days in the androgen-independent PC-3 cells than the androgen-dependent LnCaP cells. This is demonstrated in figure 9 by the negative slope in the growth curve of the PC-3 cells. Further testing is required, but these data suggest that the gamma isoforms of vitamin E maybe effective at treating the most resistant forms of prostate cancer at dietary concentrations.

Collectively, our data supports the view that tocotrienols, particularly DT3 may prove very useful as chemotherapeutic or chemopreventive agents for treating prostate cancer. Our next will be to initiate experiments in animal models and then to initiate clinical studies.

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# Tocotrienols are superior to tocopherols at growth inhibition and apoptosis of human prostate cancer cells

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**Abstract:** Gamma-tocotrienol, a vitamin E isoform with an isoprenoid tail, induces growth arrest in both androgen-dependent, LNCaP and androgen-independent, PC-3 human prostate cancer cells more effectively than the vitamin E isoforms, alpha-tocopherol and gamma-tocopherol with a phytyl tail. Gamma-tocotrienol-induced growth arrest results in caspase 3, 8 and 9 activation, PPAR gamma activation, and apoptotic cell death. The effects of tocotrienols on growth of human prostate cancer cells are concentration and time dependent and occur at physiological concentrations without affecting growth of normal prostate epithelial cells. Gamma-tocotrienol is more potent than the tocopherols at induction of growth arrest and apoptosis. Ligand-binding assays indicate that neither gamma-tocotrienol nor the other vitamin E isoforms are direct PPAR gamma ligands. Alternate mechanisms of PPAR gamma activation have to be evaluated.

*Key words*: Prostate cancer, Gamma-tocotrienol, Growth arrest, Apoptosis, PPAR-gamma

## Introduction

Prostate cancer has the greatest country-to-country variation in incidence of any reportable cancer suggesting the importance of nutritional and environmental factors [Fair, Fleshner, and Heston, 1997]. Micronutrient antioxidants like vitamin E neutralize free radicals and may play a role in preventing oxidative damage to prostate epithelium. Vitamin E consists of a mixture of tocopherols and tocotrienols and is present in vegetable oils, nuts and whole grains. Much of the previous research connecting vitamin E to cancer has been focused on alpha-tocopherol and other isoforms of vitamin E have been largely ignored (Stone and Papas, JNCI). Secondary endpoint analyses of intervention trials have found inverse associations between vitamin E, selenium, and prostate cancer. Experiments with Lady transgenic mice show that dietary

antioxidants (a combination of alpha-tocopherol, selenium and lycopene) dramatically block prostate cancer {2358}. Dietary antioxidants such as vitamin E may help prevent prostate cancer by reducing oxidative damage to DNA, slowing the growth of prostate tumors or by promoting the programmed cell death (apoptosis) of prostate tumors. The ability of vitamin E isoforms and analogues to induce apoptosis in cancer cells vary depending upon the isoforms and may be unrelated to their antioxidant potencies. Vitamin E isoforms have gene regulatory functions unrelated to their antioxidant properties [Stauble, Boscoboinik, Tasinato, and Azzi, 1994], [Azzi, Gysin, Kempna, Ricciarelli, Villacorta, Visarius, and Zingg, 2003], [Azzi, Gysin, Kempna, Munteanu, Negis, Villacorta, Visarius, and Zingg, 2004]. For example, gamma-tocopherol has demonstrated the ability to down regulate cyclin $D_1$ and cyclin E levels in cancer cell lines [Gysin, Azzi, and Visarius, 2002]. Similarly, alpha- and beta-tocopherols have demonstrated differential inhibition of integrins [Breyer and Azzi, 2001]. Tocotrienols, which have an isoprenoid tail, inhibit 3-hydroxy-3-methylglutaryl-coenzyme A (HMG Co A) reductase pathway leading to mevalonate depletion and inhibition of prenylation of critical signaling protein. Tocotrienols regulate cholesterol production in mammalian cells by post-transcriptional suppression of HMG Co A reductase [Parker, Pearce, Clark, Gordon, and Wright, 1993]. Targeting the mevalonate pathway for cancer therapy and prevention is currently under investigation [Swanson and Hohl, 2006] [Fritz and Kaina, 2006] [Jacobs, Rodriguez, Brady, Connell, Thun, and Calle, 2006]. Further, our laboratories have demonstrated that RRR-alpha and RRR-gamma tocopherol have the ability to up regulate the expression of peroxisome proliferator activated receptor (PPAR) γ protein and mRNA in colon cancer cell lines with gamma-tocopherol being more effective than alpha-tocopherol [Campbell SE, Stone WL, Whaley SG, Qui M, and Krishnan K, 2003]. PPARs are ligand-activated nuclear proteins and members of the nuclear receptor super family that regulate gene expression by binding to DNA as heterodimers with the 9-cis retinoic acid receptor. Ligand activation of PPAR γ results in growth inhibition, differentiation, and apoptosis in a number of different cancer cells including: breast, prostate, colon and pancreatic cancers [Murphy and Holder, 2000;Kitamura S, Miyazaki Y, Shinomura Y, Kondo S, Kanayama S, and Matsuzawa Y, 1999;Eibl G, Wente MN, Reber HA, and Hines OJ., 2002;Itami A, Watanabe G, Shimada Y, Hashimoto Y, Kawamura J, Kato M, Hosotani R, and Imamura M, 2001;Mueller, Smith, Sarraf, Kroll, Aiyer, Kaufman, Oh, Demetri, Figg, Zhou, Eng, Spiegelman, and Kantoff, 2000]. These studies support the view that different isoforms of vitamin E could exert anticancer effects by mechanisms unrelated to their antioxidant activity.

In this study, we compared the effects of the RRR-alpha-tocopherol (AT) and RRR-gamma-tocopherol (GT) to that of the alpha-tocotrienol (AT3) and gamma-tocotrienol (GT3) on cell proliferation using both androgen-dependent (LnCaP) and independent (PC-3) cell lines. This study demonstrates that the tocotrienol isoforms tested are more effective at inhibiting prostate cancer cell proliferation and inducing apoptosis than the corresponding tocopherols. The inhibition of cell proliferation by the tocotrienols is selective to cancer cells. We demonstrated that the vitamin E isoforms (AT, GT, AT3, and GT3) have little or no effect on noncancerous prostate cells. Both GT and GT3 were able to significantly inhibit cancer cell proliferation and induce apoptosis over a 5 day time-course and at, a physiological concentration of 10 μM. In addition, we have shown that GT3 can reduce cell proliferation and induce apoptosis at concentrations as low as 1 μM. Apoptosis assays indicate involvement of caspase 3, 8, and 9 with GT3 being more effective at apoptotic induction than the other vitamin E isoforms tested. Previously, we had demonstrated that treatment of colon cancer cell lines with AT and GT result in activation of PPAR γ [Campbell SE, Stone WL, Whaley SG, Qui M, and Krishnan K, 2003]. This study has confirmed that PPAR γ activation also occurs in tocopherol and tocotrienol treated prostate cancer cells. Further, we have demonstrated that the activation of PPAR γ by vitamin E isoforms does not occur through direct ligand activation.

## Materials and Methods

**Chemicals**. The following chemicals were obtained: α-tocopherol (Eastman Chemical, Kingsport, TN, 99% pure RRR-α-tocopherol), γ-tocopherol (Tama Biochemical, Tokyo, Japan, 97% pure RRR-γ-tocopherol), troglitazone (BioMol Research Lab, Plymouth Meeting, PA), bovine serum albumin (Gibco BRL, Gaithersburg, MD), proteinase K (Sigma Chemical, St. Louis,MO), RNAse A (Sigma Chemical), camptothecin (Sigma Chemical) and tocotrienols (Carotech, Inc., Edison, NJ), 15-deoxy-Δ-12,14-$PGJ_2$ (BioMol Research Lab, Plymouth Meeting, PA), calcein acetoxymethyl (AM) ester, MitoTracker Red CM-H2XRos, and 4'-6-diamidino-2-phenylindole (DAPI) (Molecular Probes-Invitrogen Corporation, Carlsbad, CA).

**Cell Culture.** PC-3, LNCaP and RWPE cell lines were purchased from American Type Culture Collection, ATCC (Manasssas, VA). PC-3 and LnCaP prostate cancer cell lines were maintained as a monolayer culture in RPMI 1680 media (Gibco BRL, Rockville, MD) supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere of 5% $CO_2$ at 37º C. RWPE cells were maintained in keratinocyte SFM media supplemented with 5 ng/mL human recombinant EGF (Gibco), 50 ng/mL bovine pituitary extract (Gibco) and 50 IU penicillin/streptomycin streptomycin in a humidified atmosphere of 5% $CO_2$ at 37º C. All cells were subcultured when they had grown to 75% confluence.

**Enrichment of Vitamin E Media.** Prior to treatment, the appropriate cell culture medium or PBS was enriched with tocopherol by adding the appropriate amount of tocopherol in ethanol (ethanol concentrations never exceeded 50 µL ethanol per mL of media) to five volumes of 7% bovine serum albumin (BSA). The BSA/tocopherol mixture was vortexed and added to culture medium supplemented containing 10% fetal bovine serum (FBS) and 50 IU penicillin/streptomycin or PBS. In the vehicle-treated cells, the tocopherol was omitted from the BSA/ethanol that is added to the culture medium containing 10% FBS.

**MTT Assay.** PC-3 and LNCaP cells were plated at a concentration of 1 X $10^5$ cells/well (for up to 1-3 day treatments) or 1000 cells/well for (3-5 day treatments) in a 96-well flat bottom plate and allowed to adhere for 24 hours prior to treatment. Vitamin E supplemented media was added to a concentration of 10, 20, 25 40, 50, 80 or 100 µM and incubated for 1,2,3,4 or 5 days. After the treatment, the cells were incubated in 200 µL of 0.5 mg/mL MTT solution for 30 mins. DMSO reagent was added to each well and mixed by shaking for 5 minutes followed by a 30-min incubation time at 37 °C. The absorbance at 540 nm was monitored using a Spectramax plus 384 UV-Vis spectrophotometer (Molecular Devices, Sunnyvale, CA).

**Analysis of Cell viability, Apoptosis and Mitochondrial Distribution by Fluorescence Microscopy and MTT Assay**

The cells were plated in Costar black plastic 96-well plates with clear bottoms and treated for 6, 24, and 72 hours with 0, 0.1, 1,5, 10, 20 and 40 µM gamma tocotrienol. Following the treatment, cells were triple stained with calcein AM (2µM), DAPI (1ng/ml), and MitoTracker Red CM-H2XRos (1µM) in phenol red free and serum free Delbucco's minimal essential medium (DMEM), (Invitrogen Inc., Carlsbad, CA). Apoptosis and nuclear morphology, DNA dye uptake, and cellular staining were assessed by fluorescence microscopy. Fluorescence microscopy was performed using an Olympus IX-70 inverted microscope. Image acquisition was achieved using a Quantix charged coupled device camera and IP Labs software (Scanalytics, Inc., Fairfax, VA) on a Macintosh computer (Apple Computer Corporation, Cupertino, CA). Ninety six-well plates were then analyzed by MTT (3-(4,5-dimethylthiazol-,2-yl)-2,5-diphenyltetrazolium bromide) assay, solubilized in dimethylsulfoxide and quantified by measuring absorbance at a wavelength of 540nm on an M-5 multiwell plate reader (Molecular Devices, Downingtown, PA).

**Caspase Activity Assay.** Cells were plated at 2 X$10^6$ cells/well in 6 well plates twenty-four hours prior to treatment. Cells were treated with tocopherol or tocotrienol-enriched media for varying times (0-24 hours). After treatment, the cells were collected and washed twice with PBS. For optimal lysis, the cells were incubated in lysis buffer (10 mM Tris HCl, 0.01% Triton X-100, 1 mM EDTA, 100 mM NaCl, pH 7.5) and subjected to two freeze/thaw cycles at -80 ºC. Cell lysates, in 40 µL aliquots, were added to a microtiter plate well followed by addition of working solution which included reaction

buffer (10 mM PIPES, 2 mM EDTA, 0.1% CHAPS, pH 7.4) and 50 mM substrate. Caspase 3, 8 or 9 activation was measured using the Caspase-3 substrate, Ac-DEVD-AFC (Biosource, Camarillo, CA), Caspase 8 substrate, Ac-IETD-AFC, (Biosource, Camarillo, CA) or Caspase 9 substrate, Ac-LEHD-AFC (Kamiya Biomedical, Seattle, WA) using a Fluorostar Galaxy fluorimeter (BMG Labtechnologies, Offenburg, Germany) ($\lambda_{ex}$ = 390 nm $\lambda_{em}$ = 500 nm).

**Western Blot Analysis.** Treated cells were lysed with lysis buffer (140 mM NaCl, 10 mM $Na_2HPO_4$, 10 mM $KH_2PO_4$ 0.1% Triton, 0.1% SDS) in the presence of protease inhibitor cocktail (Sigma Chemical, St. Louis, MO). Lysates were subjected to a freeze/thaw cycle at –80°C then centrifuged at 10,000 g for 10 minutes to sediment the particulate material. Protein concentration in the supernatant was measured using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Total cellular protein was separated SDS polyacrylamide gel electrophoresis and electrotransferred onto Hybond-ECL nitrocellulose membrane using an X cell II Mini Cell Blot module (San Diego, CA). Blotted membranes were incubated with the following primary antibodies: caspase 3, 8, 9, (Cell Signaling Technologies, Beverly, MA) or PPAR γ (Santa Cruz Biotechnology, San Diego, CA) and probed with the described antibody conjugated with horseradish peroxidase. The signal was measured using ECL Western blotting detection reagents 1 & 2 (1mL each) (Amersham Biosciences, Arlington Heights, IL).

**Treatment of Cells for RNA/protein extraction.** PC-3 and LNCaP human prostate cancer cells were seeded at $5 \times 10^6$ cells/plate in 100 × 20 mm plates 24 hours before treatment with either 5 µM or 10 µM AT, GT, AT3, GT3, vehicle (BSA + ethanol) or 100 µM troglitazone or 15-deoxy-Δ12,14 PGJ2 (positive control) for 24 hours or 48 hours.

**Quantitative PCR.** Quantitative real time PCR was performed as previously described [Campbell SE, Stone WL, Whaley SG, Qui M, and Krishnan K, 2003]. In brief, the primer pair used to amplify PPARγ was upper 5’ AAG CCC TTC ACT ACT GTT GAC T 3’ and lower 5’ CAG GCT CCA CTT TGA TTG 3’. The PCR system used a fluorescent dye (SYBR Green) for transcript detection.

**LEADseeker Scintillation Proximity Assay of tocopherol and tocotrienol binding to PPARγ LBD.** Storage buffer (50mM Tris pH 8, 50 mM KCl (100 mL)) was added to 500 mg of streptavidin-coated LEADseeker SPA beads (Amersham). After 30 minutes of mixing on a Nutator platform, the beads were centrifuged at 1000 *x g* for 10 min, and the supernatant discarded. The bead pellet was resuspended in 100 mL of storage buffer at 5 mg/mL. At room temperature, the bead stock (5 mL) was diluted with 7.5 mL of assay buffer (50 mM Tris pH 8, 50 mM KCl, 2 mM EDTA, 5 mM CHAPS, 0.1 mg/mL BSA, 10 mM DTT) and 120-150 nM of biotinylated PPAR γ LBD [Nichols, Parks, Consler, and Blanchard, 1998] was added to a final volume of 12.5 mL. The slurry was incubated for at least one hour with gentle agitation followed by centrifugation at 1000 *x g* for 10 min, and the supernatant discarded. The bead pellet was gently washed with 12-15 mL of assay buffer and centrifuged as described previously, discarding the supernatent. The bead pellet was resuspended in 45 mL of assay buffer and 5 mL of 1 mM biotin and incubated for 1.5 hr with gentle agitation. A stock solution of radioligand $^{3}$H-BRL49653 was diluted to 150 nM using assay buffer and sonicated for 5 sec [Nichols, Parks, Consler, and Blanchard, 1998]. The radioligand was mixed with an equal volume of receptor-coated beads and incubated for 15 min. This mixture was added to 384-well assay plates (NUNC, 264675) containing 0.5 µL compound as a one-step addition of 25 µL. Final assay concentrations were 75 nM radioligand and 0.25 mg/mL PPARγ LBD-coated beads. Tocopherols were diluted in DMSO with a starting concentration of 100 µM. Following a 2-15 hour incubation at room temperature, covered and in the dark, the signal at 613 nM was determined using a Viewlux plate imager (Perkin Elmer). Non-specific binding was determined by 20 µM unlabeled BRL49653.

**Statistical Analysis.** One-way analysis of variance (ANOVA) followed by a post hoc analysis with Tukey's test were used to compare multiple means. Probability levels (p-values) of $< 0.05$ indicate statistical significance. The data are shown as means with error bars representing standard deviation (SD). For western blot analyses and Real Time PCR assays, results were assessed by student's t-test or one-way ANOVA.

**Results**

*Tocotrienols are more potent than tocopherols at growth inhibition of human prostate cancer cell lines, LNCaP and PC-3*

In this study we used the LnCaP cell line as a model for hormone-dependent prostate cancer and the PC-3 cell line as a model for the more aggressive hormone-independent prostate cancer. We initially measured the effects of 20, 40, 80 and 100 μM tocopherol and tocotrienol on cell proliferation using the MTT assay in both the LnCaP and the PC-3 prostate cancer cells. When treating either the LnCaP or PC-3 cells with 100 μM vitamin E (data not shown), cell death statistically different from the 80 μM treatment was not demonstrated. The growth curves were similar. At doses of 20 μM, there were no detectable differences between controls and the vitamin E isoforms using the MTT assay (data not shown). Distinct differences in the abilities of tocopherols and tocotrienols to inhibit cell proliferation were observed at concentrations of 40 and 80 μM in PC-3 cells (Figure 1) and LnCaP cells (Figure 2). The lowest effective concentration tested over the 72-hour time course using this assay in either cell line was 40 μM. Within 48 hours of the 40 μM vitamin E treatments, effective reduction of cell proliferation in PC-3 cells was detected in the tocotrienol-treated samples (See Figure 1A). Neither AT nor GT were effective at 40 μM at times up to 72 hours. The 80 μM tocopherol or tocotrienol treatment in PC-3 cells resulted in reduced cell proliferation over the vehicle-treated cells as early as 24 hours with the tocotrienols, but not the tocopherols (See Figure 1B). GT displays a reductive effect at the 80 μM concentration only after 72 hours, but AT was not statistically different from the vehicle treated PC-3 cells at any concentration tested over the 72 hour time-course.

Since the LNCaP cells are a model for the androgen-dependent prostate cancer that occur in early stages when there is less resistant to chemotherapy treatment we would expect them to be more sensitive to the reduction in cell proliferation induced by vitamin E than the PC-3 cell line which is a model for the more resistant, later stage prostate cancer. As anticipated, the LNCaP cells were more sensitive to the reduction of cell proliferation by the vitamin E isoforms than PC-3 cells as demonstrated by the significant reduction in LNCaP proliferation with 40 μM treatment with all isoforms of vitamin E (see Figure 2), while the same treatment in the PC-3 cells resulted in significant reduction in cell proliferation with AT3 and GT3 only and not with the AT and GT. Significant reduction in cell proliferation occurred in the LNCaP cell line with the GT and GT3 within 24 hours following the 40 μM treatment (See Figure 2A). Camptothecin at 10 μM was used as a positive control and demonstrated a reduction in cell proliferation at every time tested (data not shown).

*Tocotrienols selectively cause growth inhibition in human prostate cancer cells and spare normal prostate epithelial cells*

We next determined whether the reduction in cell proliferation observed with tocopherol and tocotrienol treatment was selective for prostate cancer cell lines Concentrations of 0, 40 and 100 µM AT, GT, AT3 and GT3 were tested for 24 hours in non-cancerous prostate epithelial cells RWPE (Figure 3A) as well as the androgen-sensitive LNCaP cells (Figure 3B). At concentrations of 40 µM, all vitamin E isoforms tested have an insignificant proliferative effect on non-cancerous RWPE prostate epithelia, whereas treatment of the LNCaP cancer cells at this concentration resulted in a profound reduction in cell proliferation with all the tested isoforms except AT. AT and GT did not inhibit cell proliferation in the RWPE non-cancerous cell line at 100 µM, while the AT3 caused a small reduction in cell viability and GT3 caused a marked reduction. At 100 µM AT treatment of LNCaP cells showed no inhibition of cell proliferation but all other isoforms showed a statistically significant decrease from the vehicle-treated cells. Collectively, these data show that at concentrations less than 40 mM (for 24 hours) the tocotrienol forms of vitamin E are selectively very effective at inhibiting the growth of LNCaP prostate cancer cells.

## *Gamma-tocotrienol induces apoptosis at physiologically achievable concentrations*

While vitamin E concentrations of 40 and 100 µM may be achievable with intravenous antioxidant liposomes [Stone WL and Smith M, 2004], these concentrations are most likely not achievable by dietary means. Therefore, we decided to study the effects of the most potent of the vitamin E isoforms, i.e. GT3 in PC-3 cells at lower concentrations using a more sensitive assay to determine the lowest concentration by which we could achieve sustainable cell death. The PC-3 cells were treated with 0, 0.1, 1, 5, 10, 20 and 40 µM GT3 for 6, 24, and 72 hours (Figures 4, 5, and 6 respectively). Following treatment, the cells were stained simultaneously with three fluorescent dyes and observed by fluorescent microscopy. Calcien penetrates the plasma membrane and is activated to its fluorescent from by intracellular esterases and stains the cytoplasm green and is retained by an intact plasma membrane. Mitotracker red stains the actively respiring mitochondria orange. These stains are used to detect healthy cells. DAPI is a fluorescent stain that has enhanced blue fluorescence when binding to DNA. DAPI staining occurs when

the plasma and nuclear membranes lose integrity allowing the stain to access the DNA. DAPI stained nuclei are a result of apoptosis. DAPI staining in Figure 4 demonstrates that 1 μM GT3 can result in cellular apoptosis over the control in as little as 6 hours which increases at 24 hours (Figure 5), but as demonstrated in Figure 6, the 1 μM GT3 effect does not persist in apoptosis assays at 72 hours. In fact, with 1 μM GT3 treatment at 72 hours results in a higher number of healthy cells compared to apoptotic cells. The density and staining of the cells treated with 1 μM for 72 hours is similar to that of the control (untreated cells). This demonstrates that the PC-3 cells are able to continue proliferation at 1 μM even though apoptosis occurs in a small population of the cells (15% as confirmed by the MTT Assay). Increasing number of apoptotic cells are observed at 6 hours with increasing concentrations of GT3. As with the 1 μM treatment, the 5 μM treatment results in increased % DAPI apoptotic staining at 6 and 24 hours, but by 72 hours the cell density and staining are similar to that of the control cells. Upon treatment with 10 μM and 20 μM, there is increased % DAPI staining at 6 hours that persists through the 72 hour time. In addition, at these concentrations, the cell density with respect to the control cell density has changed. The control cells are at 90-95% confluent, while treatment with GT3 at 10 and 20 μM resulted in large regions on the culture plate where there living cells are no longer present.

### *Gamma-tocotrienol induces apoptosis by activation of caspases 3, 8 and 9*

We have evaluated apoptotic markers in both cell lines after tocopherol and tocotrienol treatment using varying concentrations of tocopherols and tocotrienols (Figure 8) to confirm that the DAPI staining seen Figures 4, 5, and 6 are a result of apoptotic induction and not necrosis as well as to determine if there are differences in apoptotic induction among the vitamin E isoforms. First, we tested varying concentrations of GT for 24 hours in the LNCaP cell line then monitored differences in cleavage of caspase 3 by Western blot analysis (Figure 8A). We found that caspase 3 cleavage does occur as a result of GT treatment, however a concentration 80 μM concentration for 24 hours is required. Cleavage of a caspase 3 by GT is not detected with a 20 or 40 μM concentration. The vitamin E isoforms were then tested at 40 μM for 6 hours in the LNCaP cell line (Figure 8B). These data show that GT3 induced cleavage of caspase 3. This cleavage is not seen with any other form of vitamin E at this concentration and time point. Also, cleavage of caspase 9 is greater with the GT3 isoform than the other isoforms

tested. As with the MTT cell proliferation data, we did not see an effect when using 20 μM GT (Figure 8A) or GT3 (data not shown). The 40 μM gamma-tocopherol treatment did not demonstrate cleavage of caspase 3, however with the 40 μM GT3 treatment caspase 3 was cleaved. Higher concentrations of GT (80 and 100 μM) do result in caspase 3 cleavage. These data demonstrate that GT3 is more potent at apoptotic induction than its tocopherol counterpart. To verify this, we tested GT and GT3 at several concentrations for 24 hours and monitored the activity of caspase 3 (Figure 8C). Caspase 3 is activated by both GT and GT3 at 40 and 80 μM, but is more effectively activated by GT3 than GT. We measured the activation of caspase 3, 8 and 9 in LNCaP cells over time following the treatment with 80 μM GT3 (Figure 8D). The fluorescent units were normalized to the maximum fluorescence observed for the purpose of determining the order of caspase activation. The results demonstrate that caspase 3, 8, and 9 are simultaneously activated by 6 hours with 80 μM GT3 treatment. To confirm apoptosis is occurring with GT and GT3 treatment at 24 hours, we performed a DNA laddering analysis (Figure 8E). These data mirror those found in Figure 8A; GT is more effective at apoptotic induction at 80 and 100 μM, whereas GT3 is effective even at 40 μM.

We then determined if the gamma vitamin E isoforms could serve as chemopreventives by maintaining dietary concentrations of vitamin over times extended beyond 72 hours. Further, we were interested in testing whether there were differences between the tocopherol and tocotrienol isoforms at these later time points. Figure 9 displays the results of the 10 μM treatment with GT or GT3 for 3, 4 and 5 days in the PC-3 (Figure 9A) or the LnCaP (Figure 9B) cell lines. Data for each time interval were analyzed by one-way Anova and Tukey's test. Within time intervals, statistical differences between means are represented by a different letter. Consistent with the data shown in Figure 2, the PC-3 cells were slightly resistant to the vitamin E treatment and required a 5-day exposure before differences in means compared with vehicle-treated cells were demonstrated. In addition, the 5-day exposure in PC-3 cells showed that GT3 is statistically more effective at inhibiting cell proliferation than GT. The 10 μM GT and GT3 treatment in LnCaP cells demonstrated a statistical difference with both isoforms compared with the vehicle at 3 days. Statistical difference between the tocopherol and tocotrienol was observed at the 5-day time point with GT3 being more effective at reducing cell viability in both cell lines. As in the PC-3 cells, the GT3 was statistically more significant at inhibiting cell proliferation than GT in the LnCaP cells.

*Tocotrienols and tocopherols activate the nuclear transcription factor, PPAR gamma, but are not direct PPAR gamma ligands*

Upon finding that the gamma isoforms of vitamin E (tocopherol and tocotrienol) could effectively result in apoptosis of prostate cancer cell lines we became interested in locating a potential molecular target for the effects of vitamin E isoforms on prostate cancer cells. Since PPAR γ is a molecular target for chemoprevention and since we had shown that PPAR gamma is activated by tocopherols (GT>AT) in colon cancer cells [Campbell SE, Stone WL, Whaley SG, Qui M, and Krishnan K, 2003], we studied the effects of tocopherol and tocotrienol treatment on PPAR γ mRNA in PC3 and LnCaP cells using real-time PCR. Figure 10 shows that 5 µM tocopherol or tocotrienol treatment can up regulate PPAR γ mRNA in both LnCaP and PC-3 by 24 hours with varying efficiency depending upon the cell line and vitamin E isoforms tested. For example, in the PC-3 cells GT3 stimulated the most PPAR γ mRNA expression, while in the LnCaP cell line GT3 stimulated the least PPAR γ mRNA expression with GT stimulated the most PPAR γ mRNA expresssion. Further, in the LnCaP cell line GT-mediated PPAR γ expression is greater than that of AT, but this is not the case in the PC-3 cell line. Figure 10 also demonstrates that vitamin E isoforms not only up regulate the mRNA expression but also the protein expression. Significant differences in PPAR γ protein expression among the vitamin E isoforms were not detected in these prostate cell lines.

Upon determining that tocopherols and tocotrienols could activate PPAR γ, we performed radioactive competitive binding assays to determine if vitamin E isoforms are PPAR γ ligands. Tocopherols and tocotrienols were tested for competitive displacement of radiolabeled PPAR γ agonist, rosiglitazone, in the scintillation proximity assay from concentrations of 1 nM to 100 µM. Using this assay, a test agent is judged a PPAR γ ligand when it can displace at least 50% of the bound ligand (rosiglitazone). These data show that none of the vitamin E isoforms achieved 50% displacement (Table I) at 100 µM concentration of vitamin E isoform. This suggests that both tocopherols and tocotrienols are not direct PPAR γ ligands and may act through an indirect pathway that leads to PPAR γ activation.

**Discussion**

In this study we demonstrated that vitamin E isoforms, tocopherols and tocotrienols, have variable growth inhibitory effects on both types of prostate cancer cell line models. The gamma isoforms are more effective than the alpha isoforms and the tocotrienols are more effective than the tocopherols. This study further showed that the vitamin E-mediated inhibition of cell proliferation is cancer cell selective at concentrations of about 40 µM or lower. GT3 and AT3 demonstrate a profound effect on the cell proliferation in LNCaP and PC-3 cells at 80 µM. This study also demonstrates the ability of the GT3 isoform to inhibit cell proliferation within 6 hours of treatment with dietary concentrations (5 and 10 µM). These data demonstrate that 10 µM is required to maintain continued cell death through 72 hours. The concentration required to maintain cell death may be significantly less under physiological conditions. To maintain tissue culture cell lines oxygen concentrations are significantly higher than the oxygen concentrations maintained physiologically in most human tissues. Low concentrations of an antioxidant in such a high oxygen environment may lead to auto oxidation of the vitamin E. So, in fact. the intracellular concentration of vitamin E may have actually been significantly lower than the vitamin E added to the media. Due to the nature of the fluorescent staining assay, intracellular concentrations of vitamin E were not determined.

Dietary concentrations of the gamma isoforms (10 µM) maintain the reduction of cell proliferation in both prostate cancer cell lines out to five days. The most interesting aspect of these data is that there is a more profound result at 5 days in the androgen-independent PC-3 cells than the androgen-dependent LnCaP cells. This is demonstrated in figure 9 by the negative slope in the growth curve of the PC-3 cells. Further testing is required, but these data suggest that the gamma isoforms of vitamin E maybe effective at treating the most resistant forms of prostate cancer at dietary concentrations.

PPAR γ activation results in growth inhibition, differentiation, and apoptosis in a number of different cancer cells including: breast, prostate, colon and pancreatic cancers [Murphy and Holder, 2000;Kitamura S, Miyazaki Y, Shinomura Y, Kondo S, Kanayama S, and Matsuzawa Y, 1999;Eibl G, Wente MN, Reber HA, and Hines OJ., 2002;Itami A, Watanabe G, Shimada Y, Hashimoto Y, Kawamura J, Kato M, Hosotani R, and Imamura M, 2001;Mueller, Smith, Sarraf, Kroll, Aiyer, Kaufman, Oh, Demetri, Figg, Zhou, Eng, Spiegelman, and Kantoff, 2000] [Sarraf P, Mueller E, Jones D, King FJ, DeAngelo DJ, Partridge JB, Holden SA, Chen LB, Singer S, Fletcher C, and Spiegelman BM., 1998]. Our data indicates

PPAR γ mRNA and protein is up regulated in both PC-3 and LNCaP cell lines with all of the vitamin E isoforms tested (Figure 10). GT is more efficient at up regulating PPAR γ in LNCaP cell lines, while GT3 is more efficient at the up regulation of PPAR γ in PC-3 cell lines. Further, we have demonstrated that while PPAR γ is modulated by vitamin E, it is not a direct ligand for PPAR activation. Hence vitamin E may modulate endogenous PPAR ligands which result in PPAR γ activation. It is also possible that the water soluble vitamin E metabolites may be PPAR ligands. This is under investigation in our laboratories. Our laboratory is also currently investigating the possibility that vitamin E modulates lipid pathways involving cyclooxygenase and lipoxygenase that produce endogenous ligands for PPAR γ or inhibits chemicals that repress PPAR $\gamma$ activity [Zuo, Wu, Morris, Stimmel, Leesnitzer, Fischer, Lippman, and Shureiqi, 2005].

These data have demonstrated that GT3 is more effective at inhibiting cell growth and inducing apoptosis in prostate cancer lines than AT and GT. This may be due to their ability to activate PPAR γ (Figure 10) and the intrinsic and extrinsic pathways to apoptosis (Figure 8D). Figure 8D demonstrates that 80 μM GT3 can activate caspase 3, 8, and 9 within 6 hours of treatment, activating apoptosis through the fas ligand as well as the mitochondrial-mediated apoptotic pathway.

The data demonstrating the association of vitamin E with cancer outcomes is mixed [Vena, Graham, Freudenheim, Marshall, Zielezny, Swanson, and Sufrin, 1992;Riboli, Gonzalez, Lopez-Abente, Errezola, Izarzugaza, Escolar, Nebot, Hemon, and Agudo, 1991;Hu, La Vecchia, Negri, Chatenoud, Bosetti, Jia, Liu, Huang, Bi, and Wang, 1999] [Ronco, De Stefani, Boffetta, Deneo-Pellegrini, Mendilaharsu, and Leborgne, 1999;Yuan, Wang, Ross, Henderson, and Yu, 1995]. In the Prostate, Lung, Colorectal, and Ovarian (PLCO) Cancer Screening Trial, it was shown that vitamin E supplementation conferred no protection for healthy non-smokers but had a marginal protective benefit against prostate cancer in male smokers [Kirsh, Hayes, Mayne, Chatterjee, Subar, Dixon, Albanes, Andriole, Urban, and Peters, 2006]. The limitations to this study, by the author's own admission, may be that the food frequency questionnaire did not capture nut or type of vegetable oil consumption, both of which are major sources of Vitamin E, particularly $\gamma$-tocopherol. In addition, their food frequency questionnaire had limited

ability to quantify vitamin E isoforms other than AT. Our data suggests that AT has the lowest impact on cancer cells and attempts to overcome these limitations should be made in future trials. Further, it has been demonstrated that the use of AT supplements decreases the circulating levels of other vitamin E isoforms [Huang and Appel, 2003]. The National Cancer Institute sponsored large randomized study for prostate cancer prevention (SELECT) has completed its enrollment [Lippman, Goodman, Klein, Parnes, Thompson, Kristal, Santella, Probstfield, Moinpour, Albanes, Taylor, Minasian, Hoque, Thomas, Crowley, Gaziano, Stanford, Cook, Fleshner, Lieber, Walther, Khuri, Karp, Schwartz, Ford, and Coltman, 2005]. This study uses a four by four factorial design and is testing selenium and AT. Tocotrienols or tocotrienol-containing vitamin E mixtures may be have a role in prostate cancer prevention as a dietary recommendation and for testing in prostate cancer chemoprevention trials. In addition tocotrienols may have a role as an adjunct in treatment of prostate cancer [Kumar, Raghavan, Hieber, Ege, Mog, Parra, Hildabrand, Singh, Srinivasan, Toles, Karikari, Petrovics, Srivastava, and Papas, 6 A.D.] [Jiang, Wong, Fyrst, Saba, and Ames, 2004]. If the SELECT trial does not show a protective effect for AT, tocotrienols should be considered for prostate cancer prevention trials in the future.

**Figure Legends**:

**Figure 1**. Vitamin E isoform treatment results in significant reduction in cell proliferation compared with the vehicle in PC-3 prostate cancer cell lines. A. MTT cell proliferation assay of the PC-3 cell line treated with 40 µM vitamin E isoforms over time. B. MTT cell proliferation assay of PC-3 cell lines treated with 80 µM vitamin E isoforms over time. Camptothecin was used as a positive control cell death, but were omitted from the graph for the sake of simplification. The asterisks demonstrate statistical significant differences from the vehicle-treated cells ($p < 0.05$). The data reported are averages of at least two independent trials performed in triplicate.

**Figure 2**. Vitamin E isoform treatment results in significant reduction in cell proliferation compared with the vehicle in LNCaP prostate cancer cell lines. A. MTT cell proliferation assay of the LNCaP cell line treated with 40 µM vitamin E isoforms over time. B. MTT cell proliferation assay of the LNCaP cell line treated with 80 µM vitamin E isoforms over time. Camptothecin was used as a positive control cell death, but were omitted from the graph for the sake of simplification. The asterisks demonstrate statistical significant differences from the vehicle-treated cells ($p < 0.05$). The data reported are averages of at least two independent trials performed in triplicate.

**Figure 3**. Tocopherol and tocotrienol treatment demonstrate a protective effect in non cancerous tissue, but gamma tocopherol and tocotrienol treatment results in significant cell death in the LNCaP cell line. MTT Assay at varying concentrations after 24 hour treatment using RWPE (A) non-cancerous prostate cells and LNCaP (B) androgen-dependent prostate cancer cell lines. Data points within time intervals containing different letters are statistically different from each other ($p<0.05$).

**Figure 4**. PC-3 cells treated with the indicated concentration (0.1, 1, 5, 10, 20 and 40 µM) of gamma tocotrienol for 6 hours and stained for fluorescent microscopy.

**Figure 5**. PC-3 cells treated with the indicated concentration (0.1, 1, 5, 10, 20 and 40 µM) concentration of gamma tocotrienol for 24 hours and stained for fluorescent microscopy.

**Figure 6**. PC-3 cells treated with the indicated concentration (0.1, 1, 5, 10, 20 and 40 µM) of gamma tocotrienol for 72 hours and stained for fluorescent microscopy.

**Figure 7**. MTT Assay of PC-3 cells treated with gamma tocotrienol concentrations that correspond with the fluorescent microscopy stains.

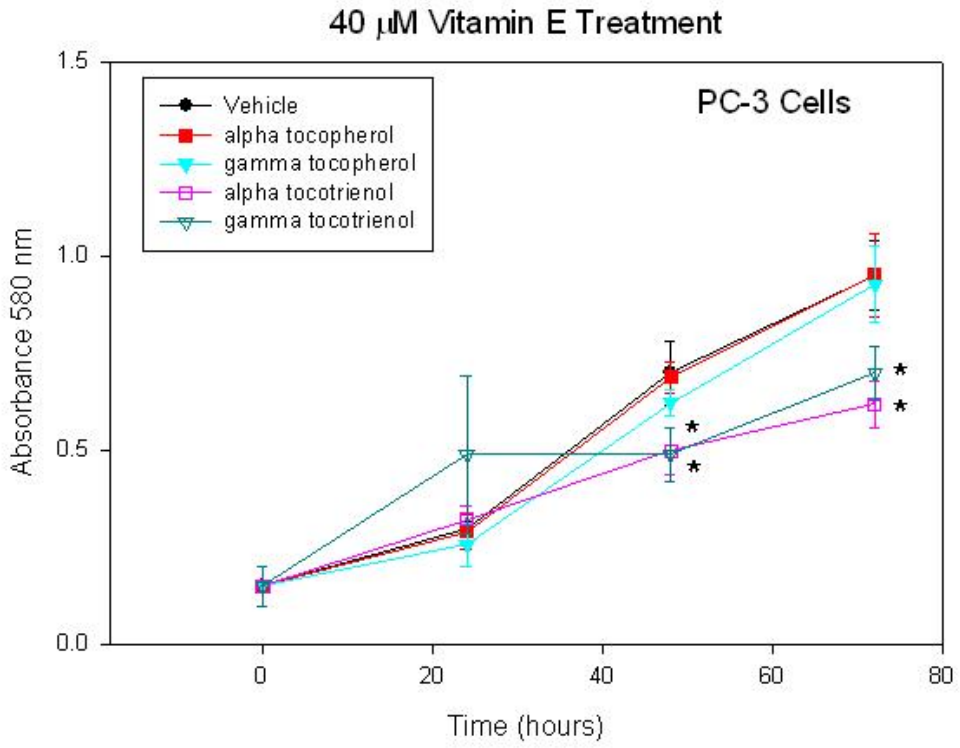
**Figure 8**. Vitamin E isoforms induce apoptosis. A. Western Blot analysis of LnCaP cell lysates after 24-hour treatment with varying concentrations of γ-tocopherol. B. Western Blot analysis of LNCaP cell lysates following 40 µM tocopherol/tocotrienol treatment for

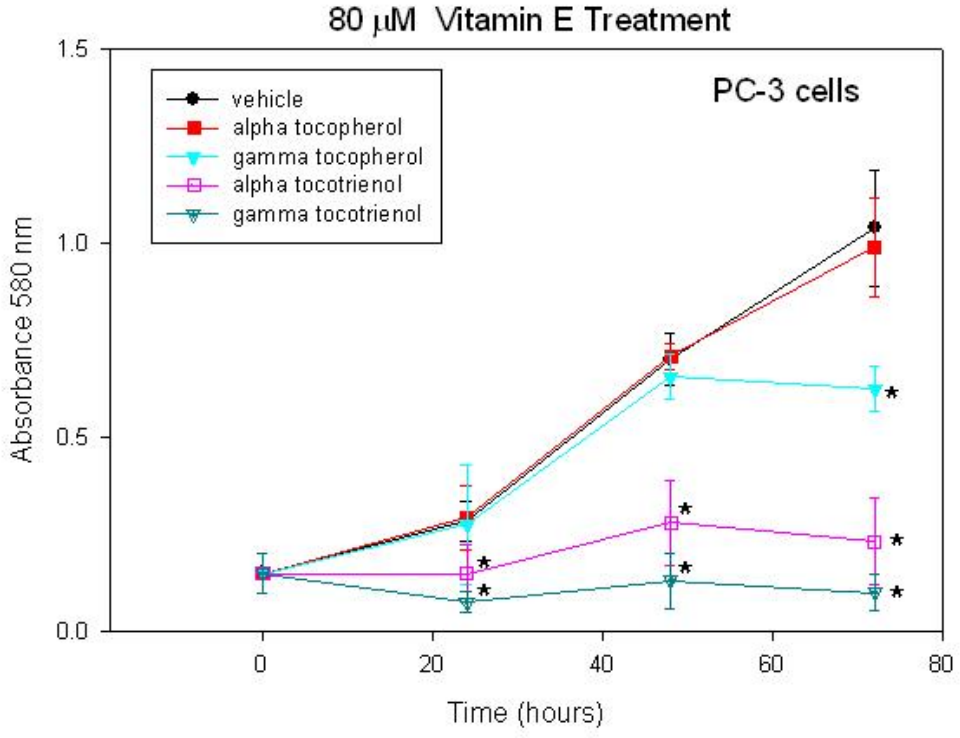
6 hours. C. Analysis of LNCaP cell lysates for Caspase 3 activity after 24-hour treatment with varying concentrations of γ-tocopherol or γ-tocotrienol. D. Analysis of LNCaP cell lysates for Caspase 3, 8 and 9 activity after 24-hour treatment with 80 µM γ-tocotrienol. E. DNA laddering assay performed on LNCaP cells following treatment with gamma tocopherol or gamma tocotrienol for 24 hours at varying concentrations.

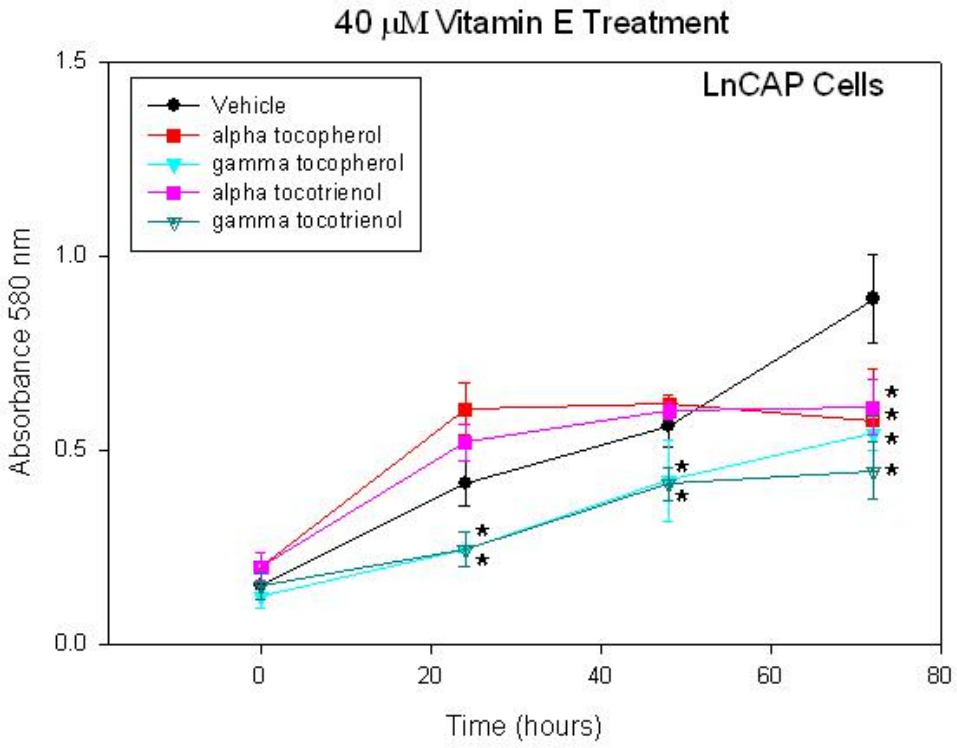
**Figure 9**. MTT Assay using at physiological concentrations of gamma tocopherol and gamma tocotrienol (10 µM) for 72, 96 and 120 hours in A) PC-3 cells and B) LNCaP cells. Camptothecin was used as a positive control cell death in both cell lines.

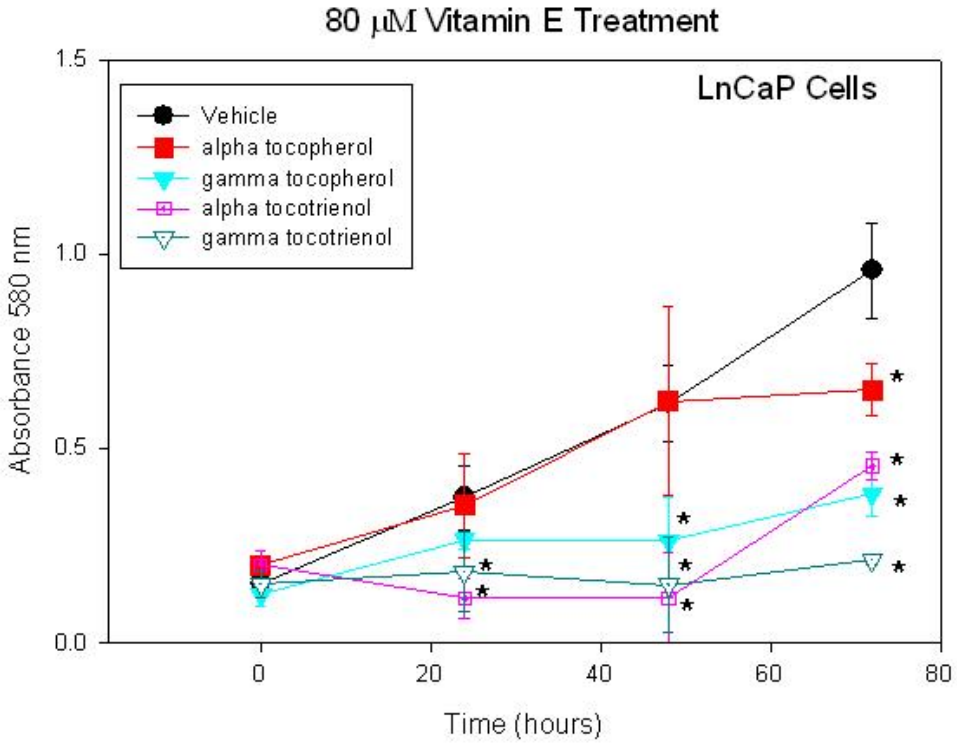
**Figure 10**. Vitamin E isoforms up regulate PPAR γ protein and mRNA expression in prostate cancer cell lines. A. Real-time PCR analysis of PC-3 mRNA after 24-hour treatment with 5 µM tocopherol/tocotrienol. B. Real-time PCR analysis of LNCaP mRNA after 24-hour treatment with 5 µM tocopherol/tocotrienol. C. Western blot analysis of PC-3 cell lysates after 48-hour treatment with 5 µM tocopherol/tocotrienol. Three independent assays performed in triplicate. *$p<0.05$ show statistically differential expression from vehicle.

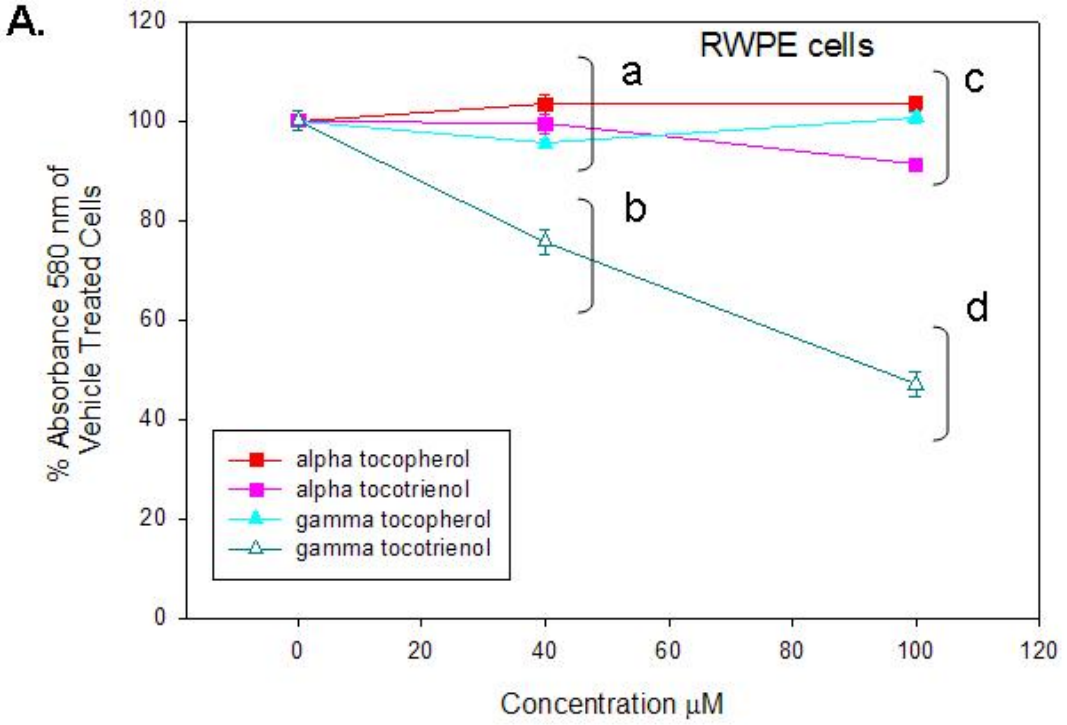
**Figure 11**. Vitamin E isoforms are not PPAR γ ligands. The results of the scintillation proximity assay using the human PPAR γ ligand binding domain bound to the radiolabeled ligand, rosiglitazone using 1 nm to 100 µM concentrations of A. alpha tocopherol, B. alpha tocotrienol, C. gamma tocopherol, D. gamma tocotrienol,

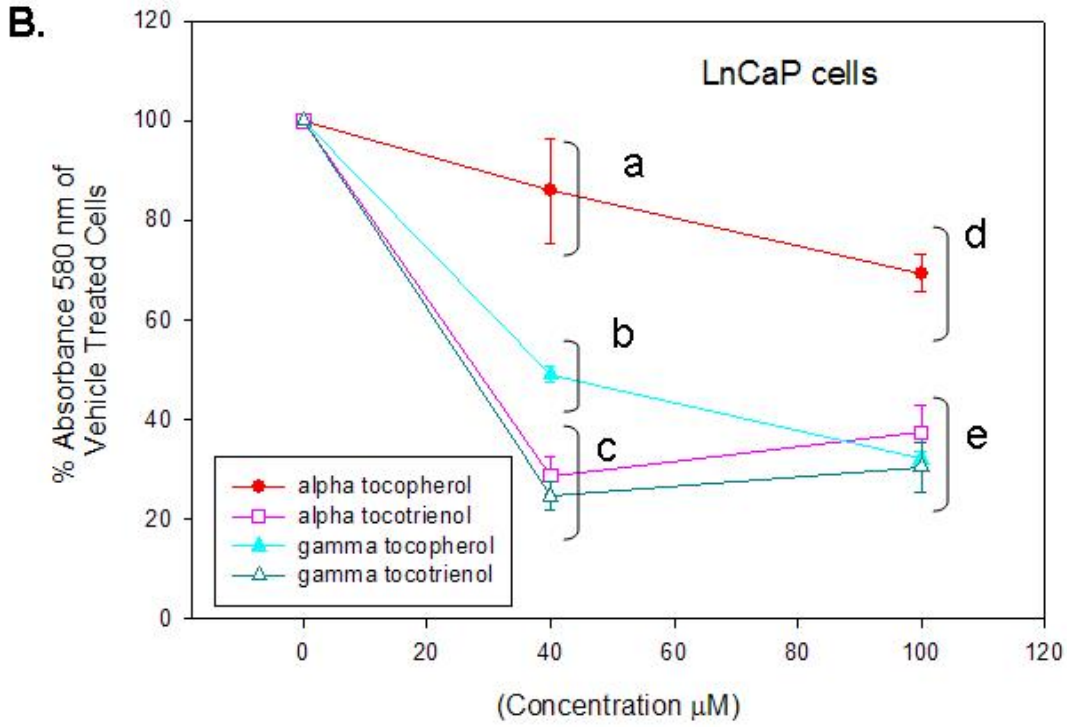

40 μM Vitamin E Treatment
PC-3 Cells
Vehicle
alpha tocopherol
gamma tocopherol
alpha tocotrienol
gamma tocotrienol
Absorbance 580 nm
Time (hours)
0.0
0.5
1.0
1.5
0
20
40
60
80
*

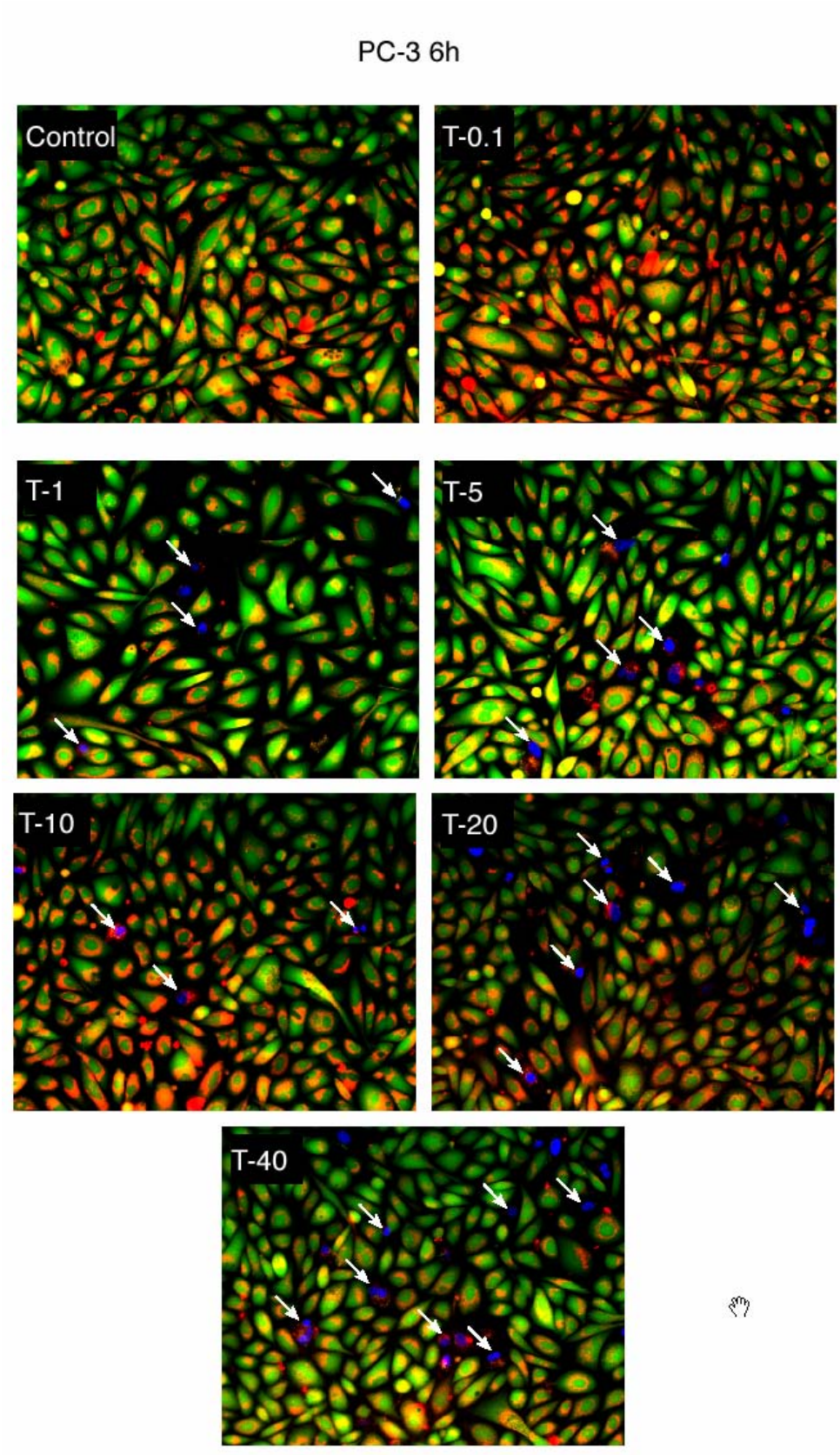

80 μM Vitamin E Treatment
PC-3 cells
vehicle
alpha tocopherol
gamma tocopherol
alpha tocotrienol
gamma tocotrienol
Absorbance 580 nm
Time (hours)
0.0
0.5
1.0
1.5
0
20
40
60
80
*


40 μM Vitamin E Treatment
LnCAP Cells
Vehicle
alpha tocopherol
gamma tocopherol
alpha tocotrienol
gamma tocotrienol
Absorbance 580 nm
Time (hours)
0.0
0.5
1.0
1.5
0
20
40
60
80

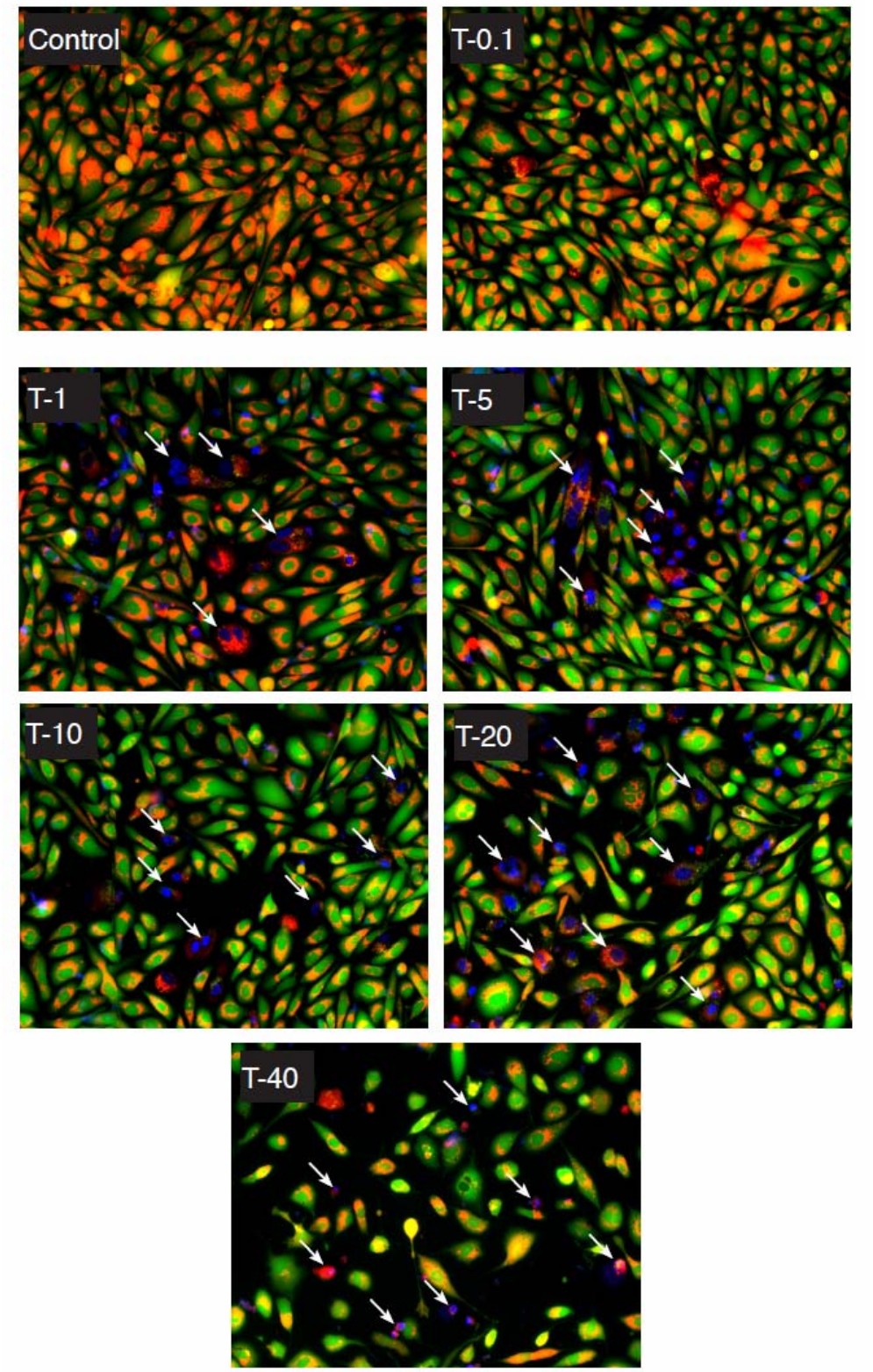

80 μM Vitamin E Treatment
LnCaP Cells
Vehicle
alpha tocopherol
gamma tocopherol
alpha tocotrienol
gamma tocotrienol
Absorbance 580 nm
Time (hours)
0.0
0.5
1.0
1.5
0
20
40
60
80


A.
RWPE cells
% Absorbance 580 nm of Vehicle Treated Cells
Concentration µM
alpha tocopherol
alpha tocotrienol
gamma tocopherol
gamma tocotrienol
a
b
c
d
0
20
40
60
80
100
120

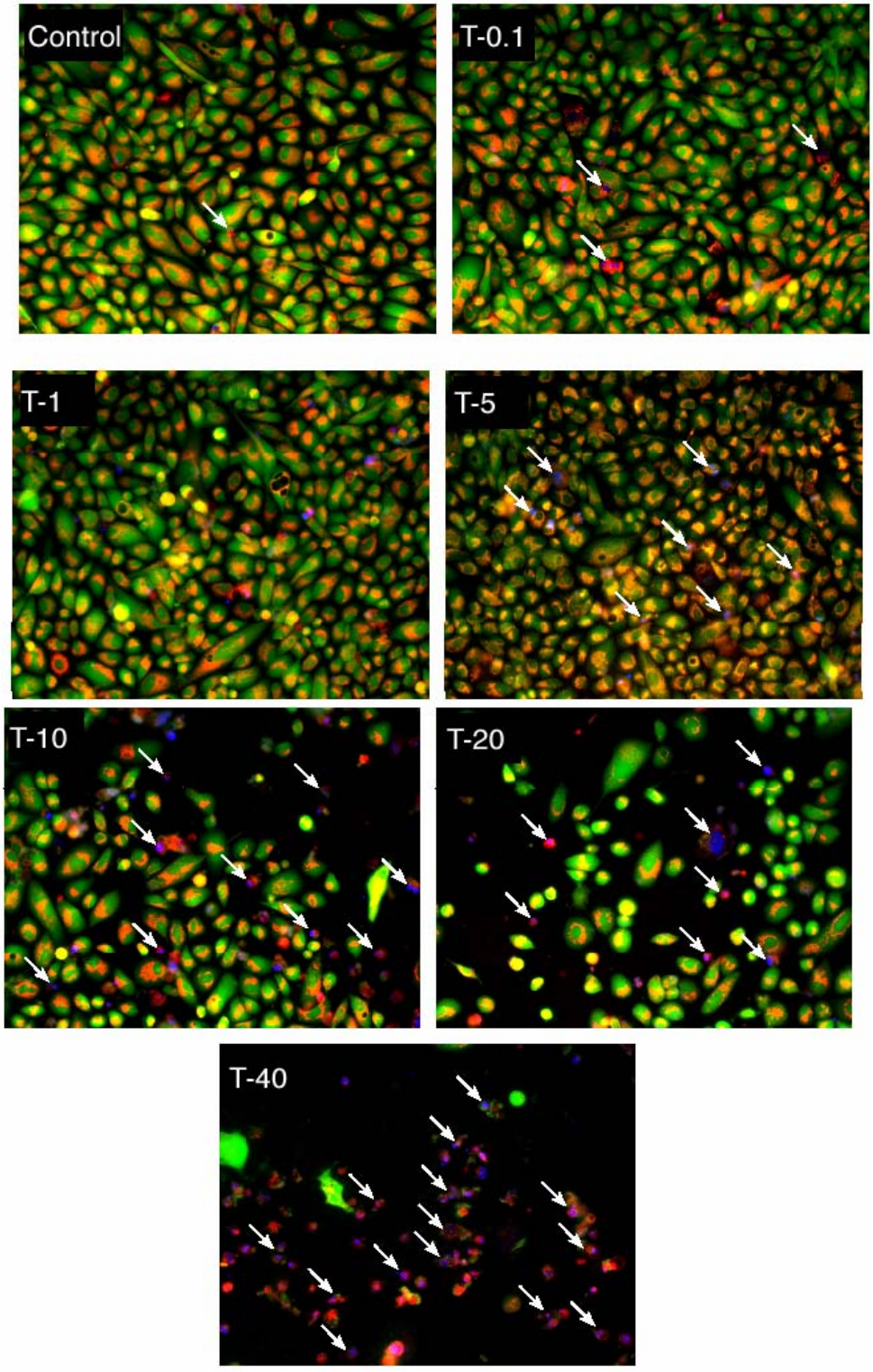

B.
LnCaP cells
% Absorbance 580 nm of Vehicle Treated Cells
(Concentration µM)
alpha tocopherol
alpha tocotrienol
gamma tocopherol
gamma tocotrienol
a
b
c
d
e
0
20
40
60
80
100
120

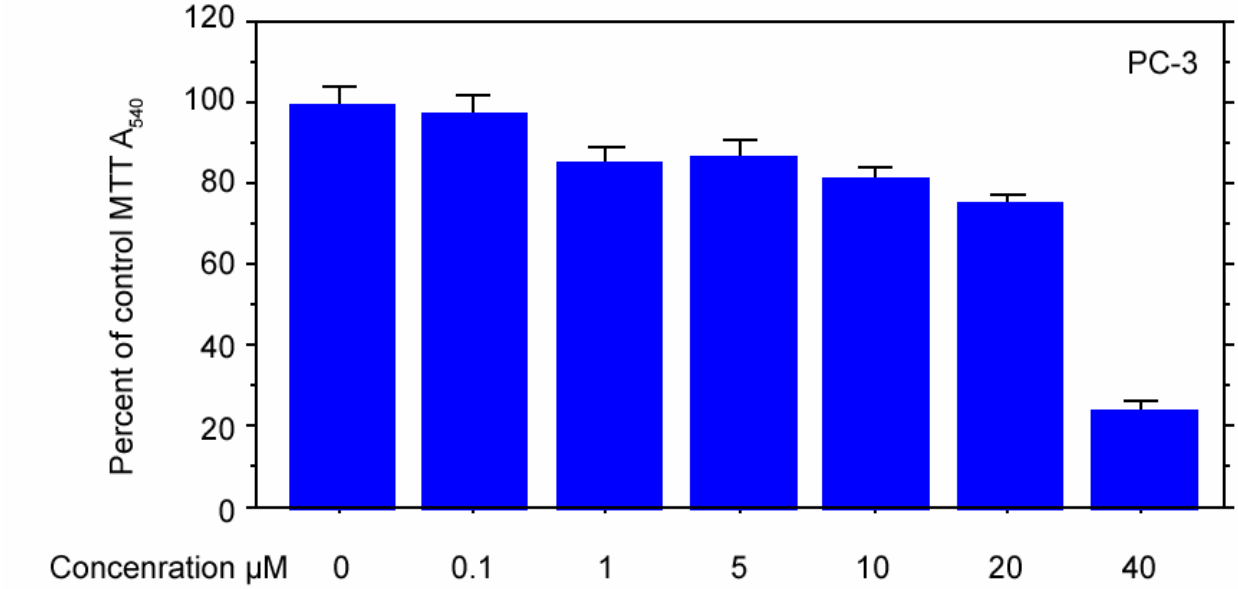

PC-3 6h
Control
T-0.1
T-1
T-5
T-10
T-20
T-40

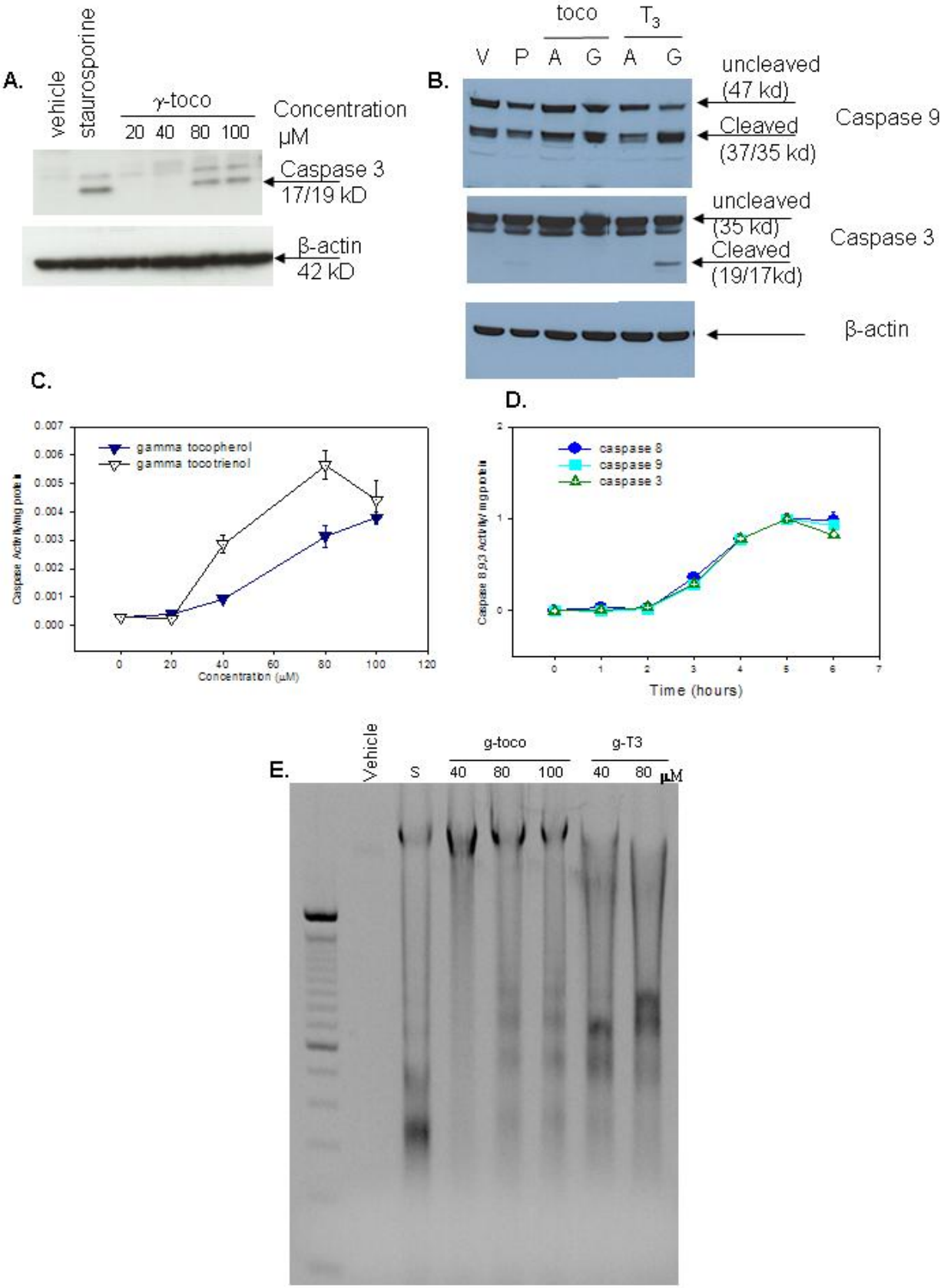
## PC-3 -24 hours



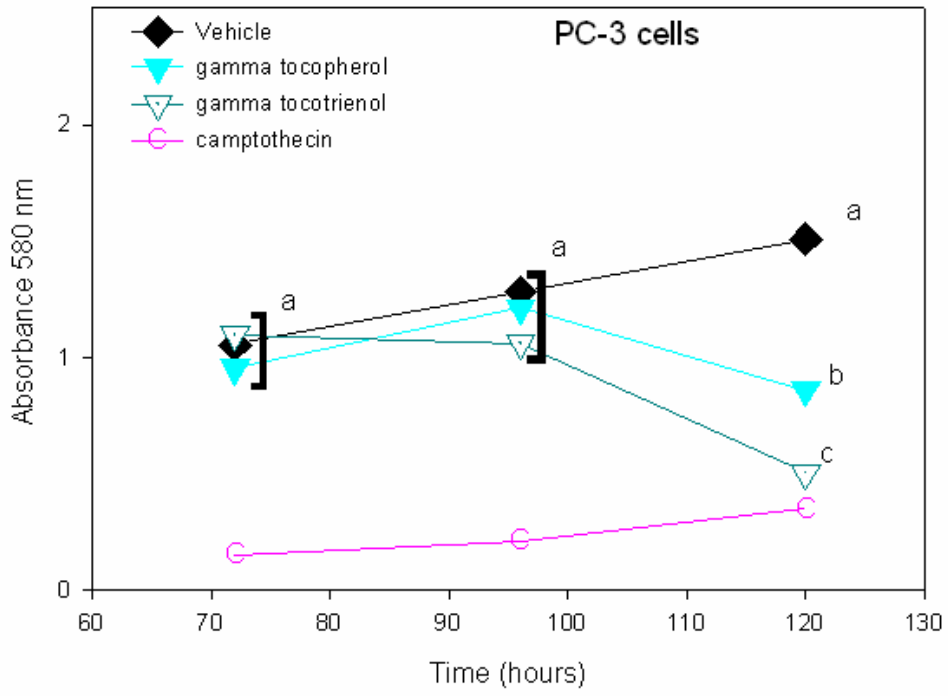
PC-3 72h



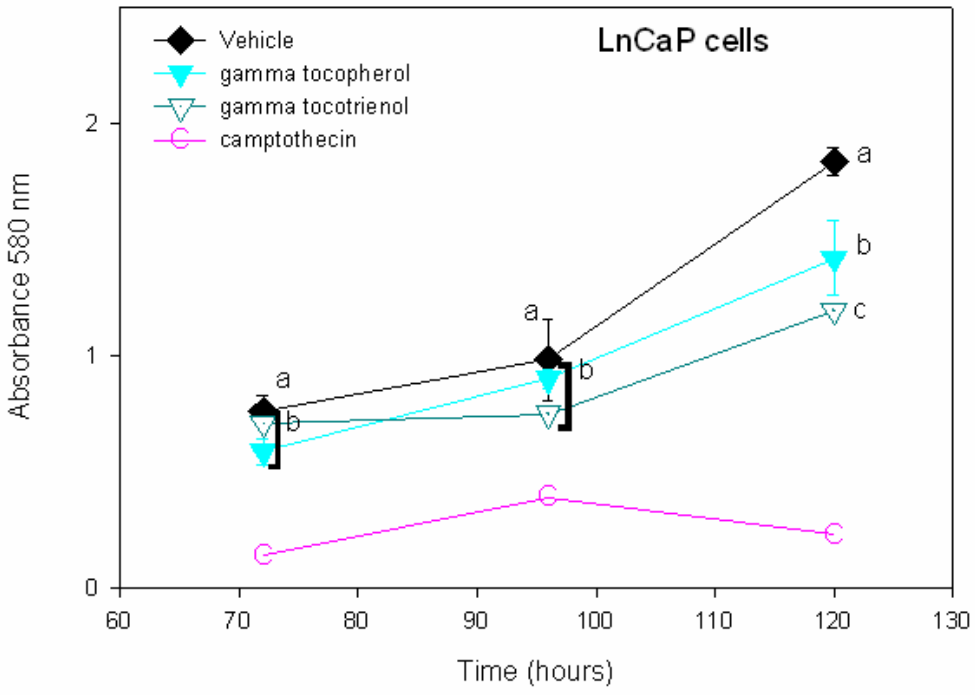

PC-3
Percent of control MTT $A_{540}$
120
100
80
60
40
20
0
Concenration μM
0
0.1
1
5
10
20
40

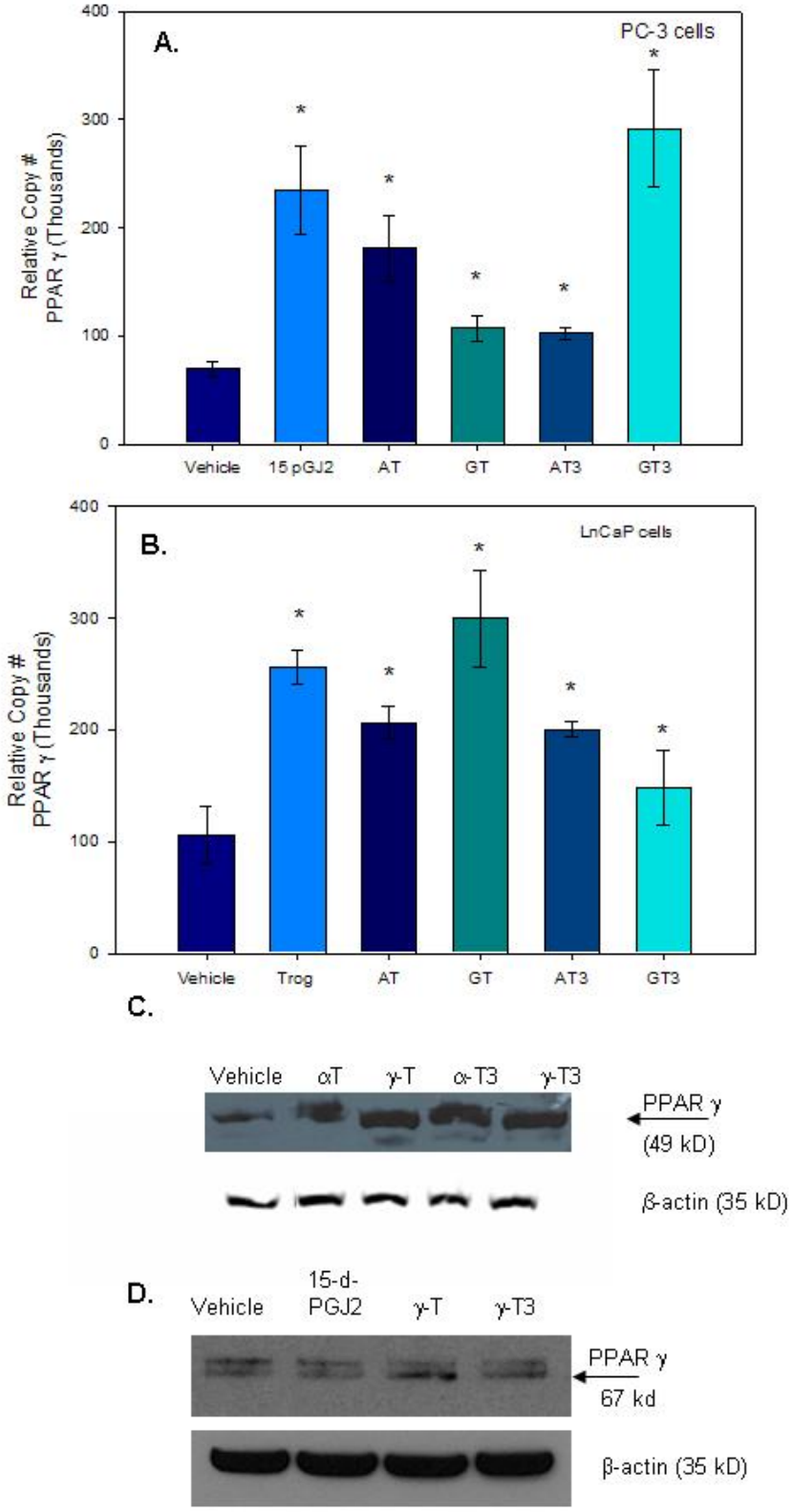

A.
vehicle
staurosporine
γ-toco
20 40 80 100
Concentration μM
Caspase 3 17/19 kD
β-actin 42 kD
B.
toco
T3
V P A G A G
uncleaved (47 kd)
Cleaved (37/35 kd)
Caspase 9
uncleaved (35 kd)
Cleaved (19/17kd)
Caspase 3
β-actin
C.
gamma tocopherol
gamma tocotrienol
Concentration (μM)
D.
caspase 8
caspase 9
caspase 3
Time (hours)
E.
Vehicle
S
g-toco
40 80 100
g-T3
40 80 μM

A.


PC-3 cells
Vehicle
gamma tocopherol
gamma tocotrienol
camptothecin
Absorbance 580 nm
Time (hours)
0
1
2
60
70
80
90
100
110
120
130
a
a
a
b
c

B.


LnCaP cells
Vehicle
gamma tocopherol
gamma tocotrienol
camptothecin
Absorbance 580 nm
Time (hours)
0
1
2
60
70
80
90
100
110
120
130
a
a
a
b
b
b
c


A.
PC-3 cells
Relative Copy # PPAR γ (Thousands)
400
300
200
100
0
Vehicle
15 pGJ2
AT
GT
AT3
GT3
B.
LnCaP cells
Relative Copy # PPAR γ (Thousands)
Vehicle
Trog
AT
GT
AT3
GT3
C.
Vehicle
αT
γ-T
α-T3
γ-T3
PPAR γ
(49 kD)
β-actin (35 kD)
D.
Vehicle
15-d-PGJ2
γ-T
γ-T3
PPAR γ
67 kd
β-actin (35 kD)

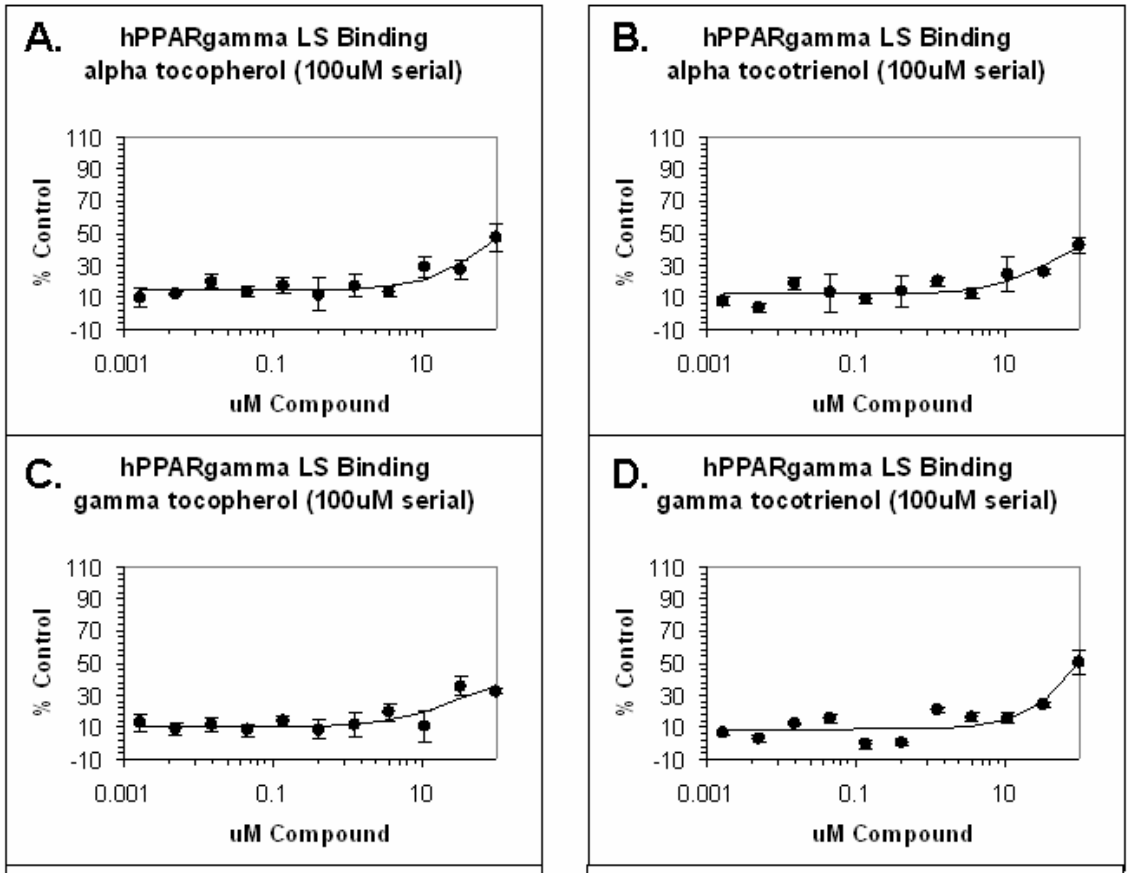

A.
hPPARgamma LS Binding
alpha tocopherol (100uM serial)
% Control
110
90
70
50
30
10
-10
0.001
0.1
10
uM Compound
B.
hPPARgamma LS Binding
alpha tocotrienol (100uM serial)
% Control
110
90
70
50
30
10
-10
0.001
0.1
10
uM Compound
C.
hPPARgamma LS Binding
gamma tocopherol (100uM serial)
% Control
110
90
70
50
30
10
-10
0.001
0.1
10
uM Compound
D.
hPPARgamma LS Binding
gamma tocotrienol (100uM serial)
% Control
110
90
70
50
30
10
-10
0.001
0.1
10
uM Compound

Table I. Percent Displacement of PPAR γ Ligand, Rosiglitazone by 100 μM Vitamin E isoforms

| **Vitamin E Isoform** | **% Displacement of PPAR Ligand, Rosiglitazone** |
|---|---|
| Alpha tocopherol | 30.43% |
| Gamma tocopherol | 24.73% |
| Alpha tocotrienol | 29.7% |
| Gamma tocotrienol | 33.4% |

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