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The Effects of the Vitamin E Isomers Gamma Tocopherol and Gamma Tocotrienol on the NFkB Pathway in the PC-3 Cell Line.

Brittney Rudder
East Tennessee State University

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By

Brittney Rudder

The Honors College

University Honors Scholars Program

East Tennessee State University

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Research Advisor: Dr. Sharon Campbell

Faculty Reader: Dr. Charlotte Laraine Powers

Faculty Reader: Dr. Foster Levy

Abstract

Regions along the Mediterranean and Southern Asia have lower prostate cancer incidence compared to the rest of the world. It has been hypothesized that one of the potential contributing factors for this low incidence includes a higher intake of vitamin E (tocopherols and tocotrienols). This study examines the potential of gamma tocopherol (GT) and gamma tocotrienol (GT3) to reduce prostate cancer proliferation by examining their effects on the NFκB pathway. NFκB is known to inhibit apoptosis in cancer cells. Our data shows that both GT and GT3 are capable of down regulation of NFκB precursors and up regulation of Caspase 8, indicating an induction of apoptosis.

Background

Prostate cancer is the second leading cause of cancer deaths in and the most common non-cutaneous malignancy in U.S. men (1, 2). Prostate cancer growth and progression is initially dependent on male hormones (androgens), and is thus frequently treated using chemical and/or surgical androgen ablation therapy. This treatment works for a time, but in most cases the cancer recurs in an untreatable, androgen-independent form (2).

The occurrence of prostate cancer varies greatly from country to country. It is far more frequent in North America, particularly the United States, and Western Europe than the Mediterranean or Asia. Interestingly, men who relocate from countries with a low incidence of prostate cancer to countries with a high incidence often develop prostate cancer at a rate intermediate of that of their native population and their new home. This implicates diet and nutrition as significant factors in the development and progression of prostate cancer, and dietary fat has been repeatedly linked to an increase in prostate cancer risk (1).

As a dietary antioxidant, vitamin E can bind free radicals and may help to prevent oxidative damage to prostate epithelium. Vitamin E naturally occurs as four tocopherol isoforms, alpha tocopherol (AT), beta tocopherol (BT), delta tocopherol (DT), and gamma tocopherol (GT) as well as four tocotrienol isoforms, alpha tocotrienol (AT3), beta tocotrienol (BT3), delta tocotrienol (DT3), and gamma tocotrienol (GT3). These isoforms can most commonly be found in vegetable oils, nuts, and whole grains (3, 4). In the Mediterranean, there is not only a balance of tocopherols but a higher intake than in America. Similarly, a substantial amount of tocotrienols (mostly GT3) is found in Palm and Rice Bran Oil, which are common in the Asian

diet where prostate cancer incidence is lowest. This indicates that vitamin E may play some role in the prevention of prostate cancer.

While the chroman head group is common in both tocopherols and tocotrienols, the former have three chiral carbons on its phytyl tail and the latter have a farnesyl tail with three unsaturated bonds. The number and position of the methyl groups determine the specific tocopherol (AT, BT, GT, or DT) or tocotrienol compound (AT3, BT3, GT3, or DT3) (5).

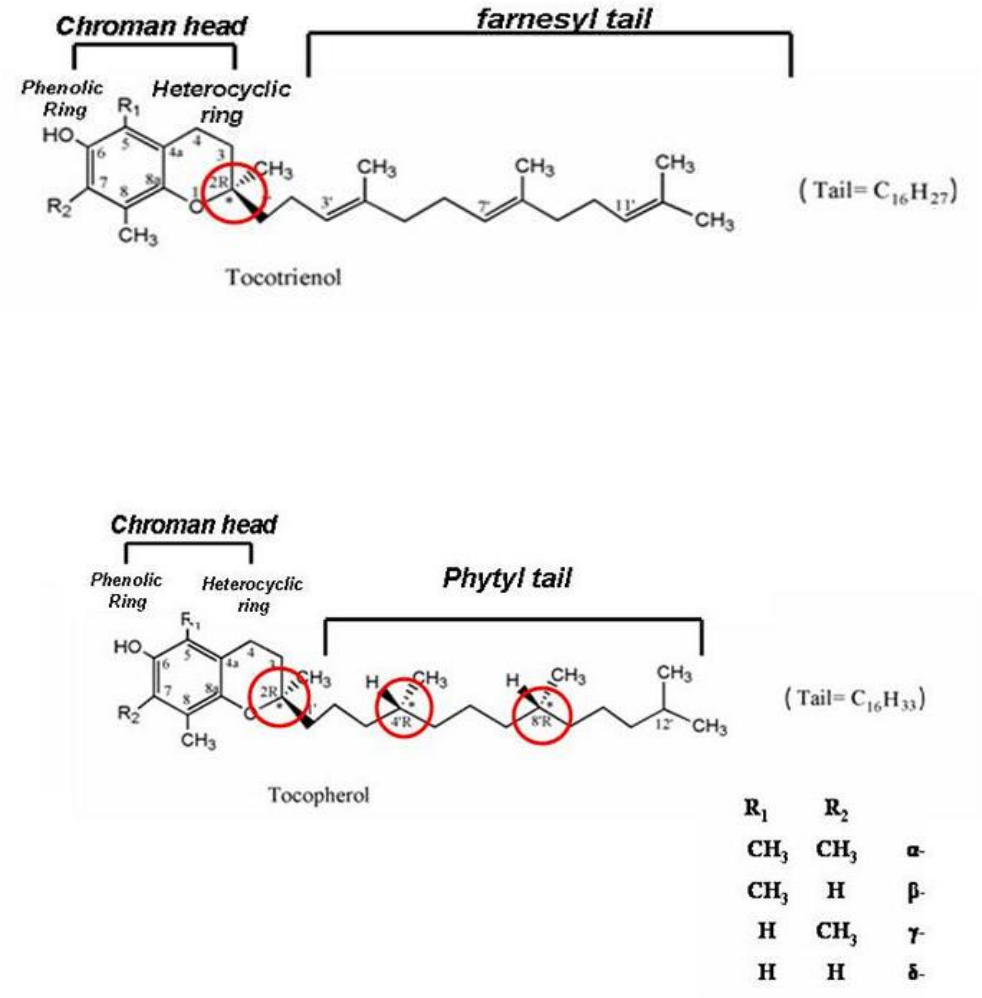

Figure 1. Vitamin E isomers (5)

Vitamin E's role in cancer prevention has become something of a controversial topic in recent years. The results of the widely publicized SELECT trial indicated that vitamin E has no preventative value in prostate cancer. It is important to note, however, that the select trial used only AT, completely ignoring the seven other vitamin E forms. Furthermore, an exceedingly high dosage (400 IU daily) was provided to participants (6). Conversely the Alpha-Tocopherol, Beta-Carotene study (ATBC) showed a significantly decreased incidence of prostate cancer with vitamin E intake while examining a much lower dosage of 50 IU AT (7). The current recommendation for daily vitamin E intake in adults is only 22 IU (8). It is also noteworthy that alpha tocopherol is found four fold less in the North American diet compared with gamma tocopherol (9).

Despite this, alpha-tocopherol has been the principal focus of the majority of studies examining the relationship between vitamin E and cancer. As it has become increasingly apparent that the different vitamin E isoforms act through different mechanisms, it is clear that this narrow focus on alpha tocopherol is not an adequate investigation of the vitamin's chemopreventive effects. In fact, when compared in a large scale study, dietary gamma tocopherol intake was found to have an inverse, significant relationship to prostate cancer risk, while supplemental alpha tocopherol did not have an affect (10). Furthermore, a systematic review of studies concerning vitamin E and prostate cancer showed that lower doses (<300 IU) of vitamin E had a chemopreventive effect while higher doses (especially ≥ 400 IU) had harmful effects (11).

The purpose of this study was to examine the effects of GT and GT3 on the NFκB pathway in the PC-3 cell line. The PC-3 cell line was first isolated and characterized in 1978. It

is derived from a human prostatic adenocarcinoma that metastasized to bone. It is a poorly differentiated, highly aggressive, and androgen-independent cell line (12). Results were obtained by treating PC-3 cells at various time points and concentrations of GT and GT3, then examining proteins of interest through Western Blot analysis. Efforts were made to obtain data for the effects of both GT and GT3 on all proteins, but technical difficulties and time constraints prevented this.

Experimental Procedure

Materials. The GT 99% pure was a generous donation by the Cognis Corporation, LaGrange, IL. The GT3 was generously donated by Carotech Corporation with a purity of 98%, (Kuala Lumpur, Malaysia).

Cell Culture. The PC-3 prostate cancer cell line, CRL-1435 (derived from a bone metastasis of a grade IV of a human prostatic adenocarcinoma displaying epithelial morphology) was purchased from American Type Culture Collection (Manassas, VA). PC-3 cells were maintained as a monolayer culture in RPMI 1640 media (Gibco BRL, Rockville, MD) supplemented with 10% FBS and 50 IU penicillin/streptomycin in a humidified atmosphere of 5% CO2 at 37º C and were subcultured at 75% confluence

Enrichment of Vitamin E Media. Concentrations of Vitamin E (tocopherols or tocotrienols) were determined in ethanol using a HP-8542A diode array spectrophotometer with the following maximum 5 wavelengths (λmax) and molar extinction coefficients(Є): GT λmax = 298 nm Є = 3810, GT3 λmax = 298 nm Є = 4230. Prior to treatment, the cell culture medium was enriched with tocopherol or tocotrienol by adding the appropriate amount of tocopherol in ethanol (ethanol concentrations never exceeded 50 μL ethanol per mL of media). In the vehicle-

treated cells, ethanol is added to the complete culture medium at the same concentration that is added to the treatments.

Western Blot Analysis. The protein concentration of the cell lysates was determined by the BCA protein assay (Pierce Biotechnology, Rockford, IL). Cell lysates were separated by electrophoresis on a 10% SDS polyacrylamide gel in Tris HCl buffer and electro transferred onto Hybond-ECL nitrocellulose membrane as using a Biorad electroblotter at 100 V for 1 hour. Blotted membranes were blocked overnight with 5% BSA or 5% skimmed milk and incubated with the primary antibodies. Following primary antibody incubation the blots were probed with the appropriate secondary antibody conjugated with horseradish peroxidase. The signal was measured using Super Signal West Pico Chemiluminescent Substrate (Pierce Biotechnology, Inc, Rockford, IL).

Results

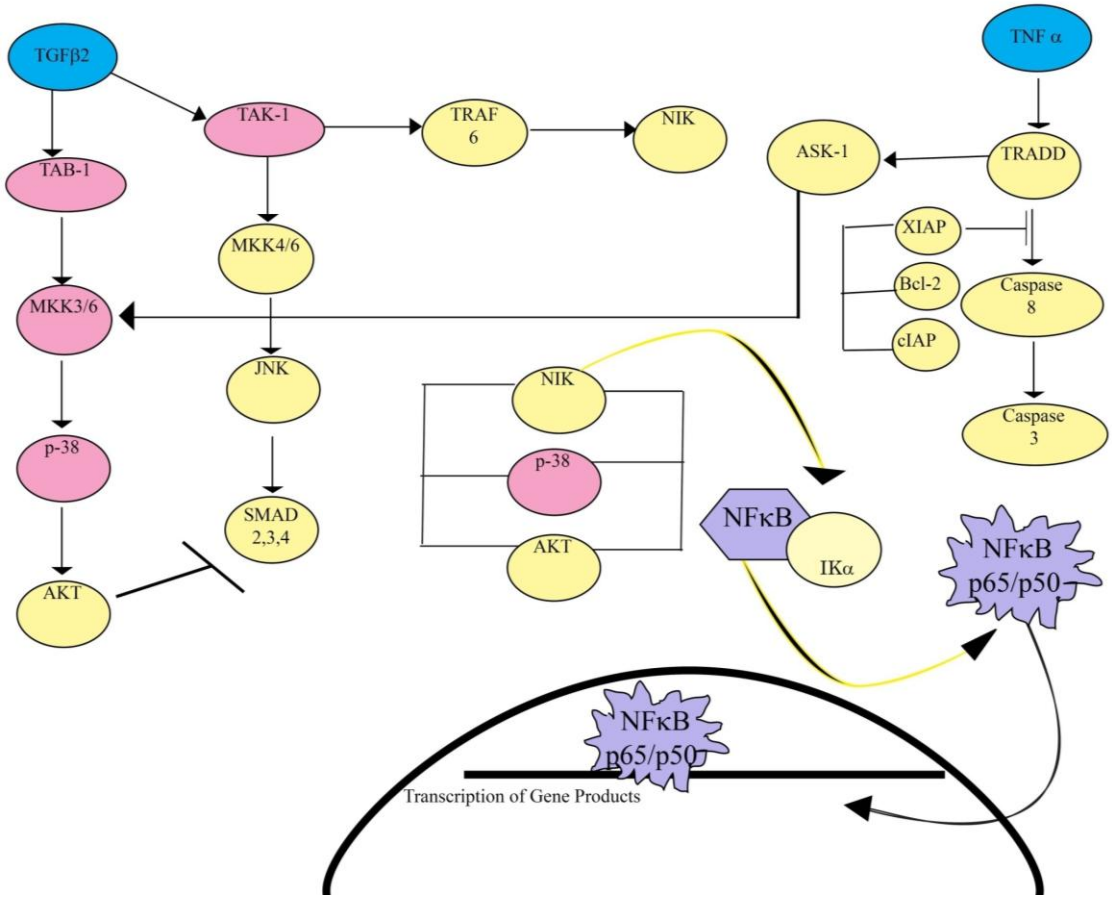

Figure 2: The NFκB pathway

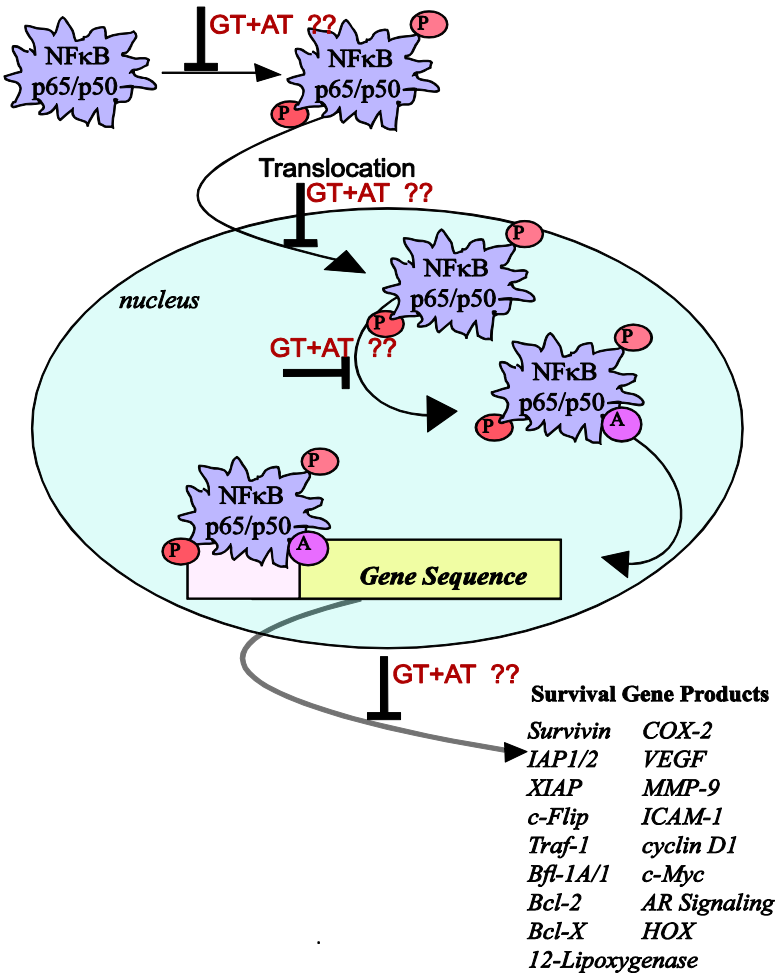

Figure 3: Activation of NFkB as a transcription factor

TGFβ2 has been previously demonstrated to play a role in the cell survival of PC-3 cells, showing that the knock out of TGFβ2 results in down-regulation of NFκB, inhibiting proliferation of PC-3 cells (13). We therefore chose to examine the effects of GT3 on TGFβ2 expression. We found that TGFβ2 is down regulated in cells treated with 20 μM GT3 for 9 hours (Figure 2).

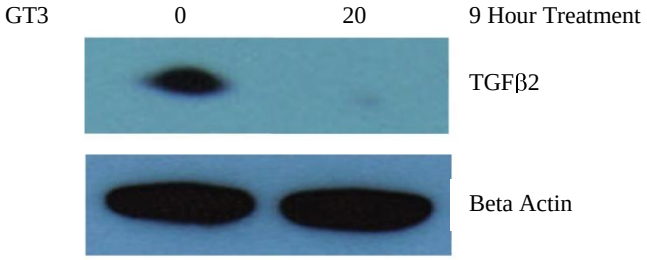

Figure 4: TGFβ2 is down regulated by GT3

We next looked at NFκB itself to determine if our results would be consistent with the previous indications that TGFβ2 is involved in up regulation of NFκB. We found that NFκB was down regulated in cells treated with 5 μM GT3 for 24 hours (Figure 3).

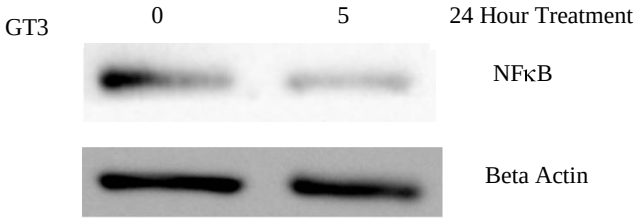

Figure 5: NFκB is down regulated by GT3

As we did find that GT3 modulated NFκB, we decided to further examine its effects on this pathway by looking at proteins known to have an effect on NFκB phosphorylation (p38, MKK 3/6, and TAK1) as well as targets of NFκB's activity as a transcription factor (XIAP and Caspase 8). Down regulation of p38 was found with both GT and GT3 after treatment for 6 hours (Figures 4 and 5). Both MKK 3/6 and TAK1 were down regulated by GT3 (Figures 6 and 7). XIAP was down regulated by GT at 5 μM and 10 μM after 3 hours of treatment and by GT3 at 5

μM after 24 hours of treatment (Figures 8 and 9). Caspase 8 is cleaved by GT at 6 μM and GT3 at 10 nM both after 24 hours of treatment (Figure 10).

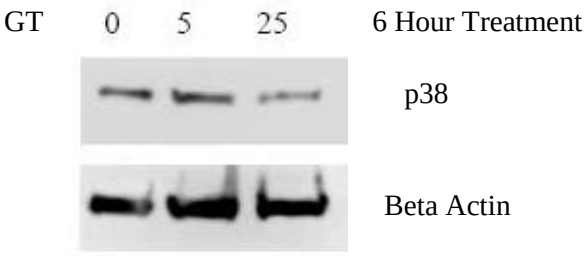

Figure 6: GT down regulates p38

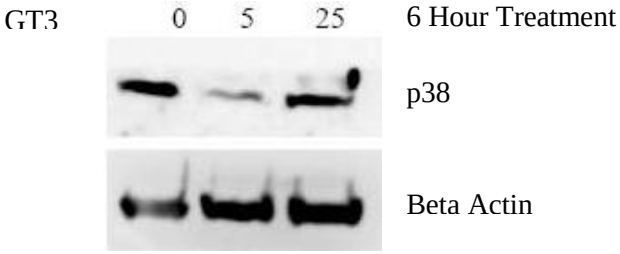

Figure 7: GT3 down regulates p38

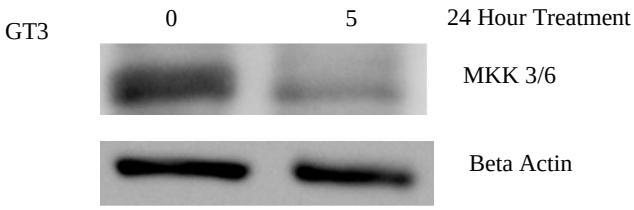

Figure 8: MKK 3/6 is down regulated by GT3

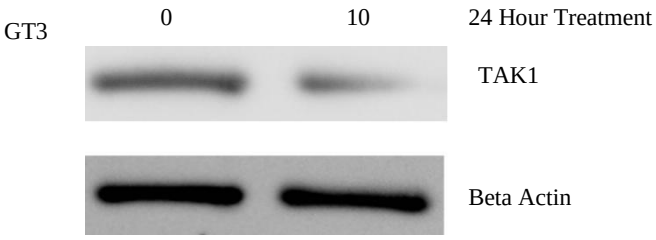

Figure 9: TAK1 is down regulated by GT3

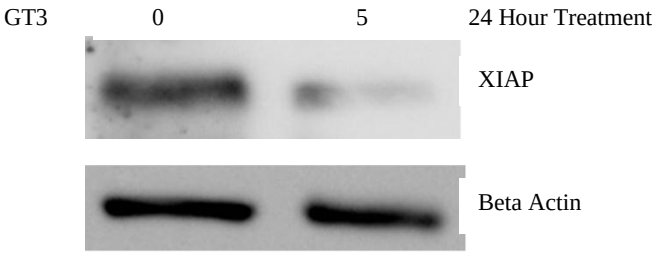

Figure 10: XIAP is down regulated by GT3

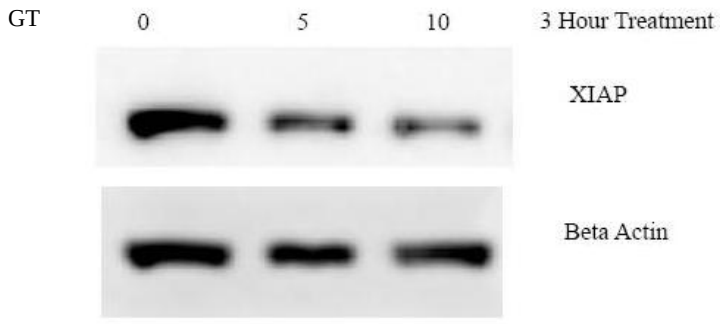

Figure 11: XIAP is down regulated by GT

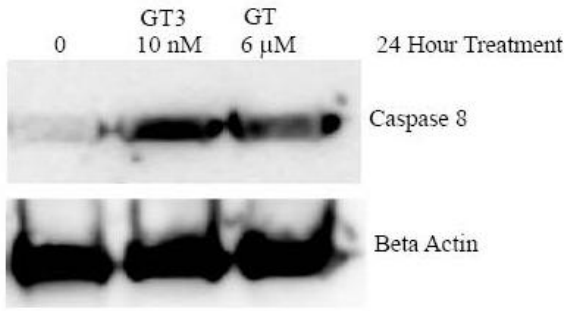

Figure 12: Increased cleavage of Caspase 8 following treatment with both GT and GT3

Discussion

NFκB must be phosphorylated to enter the nucleus and upregulate the target genes. We used an antibody to phophsorylated NFκB which targets the Serine 536 phosphorylation site on NFκB. When we saw downregulation of this site which is specific for p38 phosphorylation, we surmised that p38 was involved. MKK 3/6 activates p38 through phosphorylation. MKK 3/6 is in turn phosphorylated by TAK1, with the overall result being activation of NFκB. TGFβ2 activates TAK1 and is therefore the source of NFκB activation. Our results showed down regulation of all of these proteins, leaving NFκB in its inactive form. Thus, NFκB is unable to act as a transcription factor. Consistent with the inhibition of NFκB activation, XIAP, a target of NFκB as a transcription factor which is up regulated by NFκB is also down regulated by both GT and GT3. XIAP serves as an apoptosis inhibitor, by blocking the caspase pathways. The cleavage of Caspase 8, therefore, is consistent with our results and demonstrates that both GT and GT3 induce apoptosis. The results show that the positive effects of GT and GT3 treatment occur at low concentrations comparable to those found in the diet. Previously determined levels show that circulating Vitamin E concentrations range from 3 to 20 μM in the human body (14-16), making our treatment concentrations physiologically relevant. In conclusion, GT and GT3 are effective inhibitors of the androgen-independent prostate cancer cells of the PC-3 cell line.

References

1. Fair, W.R., Fleshner, N.E., Heston, W. (1997) Cancer of the Prostate: A Nutritional Disease? *Urology* 50 (6), 840-848

2. Jung, C., Kim, R., Zhang, H., Lee, S., Jeng, M. (2004) HOXB13 Induces Growth Suppression of Prostate Cancer Cells as a Repressor of Hormone-Activated Androgen Receptor Signaling. *J Cancer Research* 65, 9185-9192

3. Sylvester, P.W. and Shah, S.J. (2005) *Front Biosci* 10, 699-709

4. Aguilar, F. Autrup, H., Barlow, S., Castle, L., Crebelli, R., Dekant, W., Engel, K., Gontard, N., Gott, D., Grilli, S., Gurtler, R., Larsen, J.C., Leclercq, C., Leblanc, J.C., Malcata, X., Mennes, W., Milana, M., Pratt, I., Rietjens, I., Tobback, P., Toldra, F. (2008) *The EFSA Journal* 640, 1-34

5. Campbell, S. (n.d.). *Sharon Campbell, ph.d* . Retrieved from http://www.etsu.edu/com/biochem/faculty/campbells.aspx

6. Lippman, S.M., Klein, E.A., Goodman, P.J., Lucia, M.S., Thompson, I.M., et. al (2005) Designing the Selenium and Vitamin E Cancer Prevention Trial (SELECT). *JAMA* 301, 39-51

7. Albanes, D., Heinonen, O.P., Huttunen, J.K., Taylor, P.R., Virtamo, J.E., et. al (1995) Effects of alpha-tocopherol and beta-carotene supplements on cancer incidence in the Alpha-Tocopherol Beta-Carotene Cancer Prevention Study. *J Natl Cancer Inst* 97, 94-102

8. Institute of Medicine. Food and Nutrition Board. Dietary Reference Intakes: Vitamin C, Vitamin E, Selenium, and Carotenoids. Washington, DC: National Academy Press, 2000.

9. Stone, W.L. and Papas, A.M. (1997) Tocopherols and the etiology of colon cancer. *J Natl Cancer Inst* 89, 1006-1014

10. Wright, M.E., Weinstein, S.J., Lawson, K.A., Albanes, D., Subar, A.F., Dixon, L.B., Mouw, T., Schatzkin, A., Leitzmann, M.F. (2007) Supplemental and Dietary Vitamin E Intakes and Risk

of Prostate Cancer in a Large Prospective Study. *Cancer Epidemiol Biomarkers Prev* 16 (6), 1128-1135

11. Ma, R.W.L and Chapman, K. (2009) A systematic review of the effect of diet in prostate cancer prevention and treatment. *J of Human Nutr and Dietetics* 22, 187-199

12. Kaighn, M.E., Narayan, K.S., Ohnuki, Y., Lechner, J.F., Jones, L.W. (1979) Establishment and Characterization of a Human Prostatic Carcinoma Cell Line (PC-3). *Invest Urol* 17(1), 16-23

13. Lu , T.; Burdelya, L. G.; Swiatkowski, S. M.; Boiko, A. D.; Howe, P. H.; Stark, G. R.; Gudkov, A. V. (2004) Secreted transforming growth factor beta-2 activates NF-kappaB, blocks apoptosis, and is essential for the survival of some tumor cells. *Proc Natl Acad Sci U S A* 101, 7112-7117.

14. Khosla, P.; Patel, V.; Whinter, J. M.; Khanna, S.; Rakhkovskaya, M.; Roy, S.; Sen, C. K. (2006) Postprandial levels of the natural vitamin E tocotrienol in human circulation. *Antioxid. Redox. Signal* 8, 1059-1068.

15. Ford, E. S.; Schleicher, R. L.; Mokdad, A. H.; Ajani, U. A.; Liu, S. (2006).Distribution of serum concentrations of {alpha}-tocopherol and {gamma}-tocopherol in the US population. *Am. J Clin Nutr.* 84, 375-383.

16. Kelly, F. J.; Lee, R. O. S. A.; Mudway, I. S. (2004) Inter- and Intra-Individual Vitamin E Uptake in Healthy Subjects Is Highly Repeatable across a Wide Supplementation Dose Range. *Ann NY Acad Sci* 1031, 22-39.