

GAMMA TOCOTRIENOL AND PROSTATE CANCER: THE REGULATION OF TWO INDEPENDENT PATHWAYS TO POTENTIATE CELL GROWTH INHIBITION AND APOPTOSIS

CAMPBELL, S E*; WHALEY, S G*; PHILLIPS, R*; AGGARWAL, B B*; STIMMEL, J B**;
LEESNITZER, L**; BLANCHARD, S G**; STONE, W L**; MUENYI, CHRISTIAN** and KRISHNAN, K*

ABSTRACT

Dietary vitamin E, highly expressed in palm oil, exists as either tocopherols or tocotrienols. Evidence indicates that vitamin Es may be potent cancer preventive agents. In this study, the γ - and δ - isoforms of vitamin E were found to be the most effective at cancer cell growth inhibition, with the tocotrienols being more effective than the tocopherols in androgen-independent PC-3 prostate cancer cells. To assure that these compounds were selective toward cancer cells, the growth arrest of PrEC normal prostate cells was compared to PC-3 cells. At concentrations of $\leq 30 \mu\text{M}$ dietary, γ -vitamin Es showed no significant growth arrest on PrEC cell growth, but selectively inhibited growth in the PC-3 cancer cells. Moreover γ -tocotrienol demonstrated a greater potential to inhibit growth in cancer cells at these lower concentrations than did γ -tocopherol. Two independent pathways important in carcinogenesis were tested: PPAR γ and NF κ B. The PPAR γ was up regulated by both dietary γ -vitamin Es by the modulation of the endogenous ligand 15-S-HETE, while NF κ B was only regulated by γ -tocotrienol. The modulation of NF κ B was confirmed by the down regulation of the pro-apoptotic proteins cIAP, xIAP, and BcL-2 which potentiate apoptosis and are down stream effectors of NF κ B.

Keywords: PPAR gamma, NF κ B, TNF alpha, gamma-tocopherol, gamma-tocotrienol.

Date received: 15 April 2008; **Sent for revision:** 17 April 2008; **Received in final form:** 6 June 2008; **Accepted:** 2 July 2008.

INTRODUCTION

Prostate cancer is the most prevalent cancer in American men and the second leading cause of

cancer deaths. The probability of developing prostate cancer in the United States is one in six. Prostate cancer has the greatest country-to-country variation in incidence of any reportable cancer, suggesting the importance of nutrition. Current treatment for metastatic prostate cancer involves androgen ablation, resulting in reduced cellular proliferation and induction of programmed cell death (apoptosis). A fraction of the prostate cancer cells continue to grow even in the absence of androgen. These androgen-independent metastatic prostate cells are unresponsive to conventional anti-proliferative agents and usually result in a fatal outcome. Preventable risk factors for prostate cancer include diet and chronic inflammation. Animal experiments show that a combination of alpha-tocopherol (AT), selenium and lycopene dramatically block prostate cancer (Venkateswaran *et al.*, 2004). The alpha-tocopherol, beta carotene (ATBC) study, a randomized, placebo controlled clinical study looking at the influence of AT and/or beta-carotene

* Departments of Internal Medicine,
East Tennessee State University,
Johnson City,
TN 37614, USA.
E-mail: campbese@etsu.edu

** Departments of Internal Medicine and Pediatrics
East Tennessee State University,
Johnson City,
TN 37614, USA.

+ The U.T. M.D. Anderson Cancer Centre,
Houston, TX 77030, USA.

** Glaxo-Smith Kline Inc.,
Research Triangle Park, NC 27709,
USA.

on the prevention of lung cancer in male smokers, had a side arm study which indicated substantially fewer prostate cancers in the participants taking AT (50 IU day⁻¹) compared to those not taking this supplement. A subsequent study of supplemental vitamin E intake in a large cohort of men in the United States showed that high plasma levels of gamma-tocopherol (GT) appeared to enhance the ability of AT and selenium to protect against prostate cancer. Men with the highest levels of GT had a five-fold reduction in their risk of developing prostate cancer compared with men with the lowest levels of GT (Helzlsouer *et al.*, 2000).

These data demonstrated the need to look at the activity of other vitamin E isoforms in the carcinogenic pathways of prostate cancer. The term vitamin E refers to four tocopherol isoforms (a, b, g, d) and four corresponding tocotrienol isoforms, which can be found in vegetable oils, nuts and whole grains. The difference between the two families exist in the 13-carbon-side chain. Tocopherols have phytyl tail, while tocotrienols have a farnesyl tail. Much of the previous research implicating vitamin E as a chemopreventive has focused on a-tocopherol while other isoforms of vitamin E have been largely ignored (Stone and Papas, 1997). With the emerging about gamma tocopherol's ability to modulate pathways of risk in colorectal cancer such as cyclooxygenase-2 (Beharka *et al.*, 2002; Jiang *et al.*, 2000; Sakamoto *et al.*, 1996), we began to explore the possibility that gamma-tocopherol could be an effective chemopreventive for prostate cancer. In the meantime, tocotrienols have begun to demonstrate potential health benefits, particularly with respect to cancer. For example, Kashiwagi demonstrated a redox-silent analogue of tocotrienol can inhibit the cell survival and invasion in A549 human lung cancer cells (Nesaretnam, 2008). This was achieved by a inhibition of Akt activation through Src-kinase which reduced fibrinolytic factors such as plasminogen activator-1 (PAI-1).

Nesaretnam demonstrated that there was a significant difference in the total tocotrienol levels between malignant and benign breast adipose tissues in Malaysian patients (Matringe *et al.*, 2008). The concentrations of tocotrienols were lower in malignant tumors than in benign tissue (Matringe *et al.*, 2008). This supports the hypothesis that tocotrienols may be protective against breast cancer. No data has been reported examining the potential of tocotrienols against prostate cancer. With these data in mind, we decided to compare the potential chemopreventive ability of gamma-tocopherol to gamma-tocotrienol in the aggressive androgen-independent prostate cancer cell line PC-3. Our study evaluated the potential of tocotrienols to prevent prostate cancer and determined which molecular pathways in prostate cancer cell lines are modulated with treatment.

EXPERIMENTAL

Materials

The following chemicals were obtained from the indicated source: RRR- α -tocopherol (AT) (Eastman Chemical, Kingsport, TN, 99% pure), RRR- γ -tocopherol (GT) (Tama Biochemical, Tokyo, Japan, 97% pure), RRR- δ -tocopherol (DT) (Tama Biochemical, Tokyo, Japan, 97% pure) 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 R- α -tocotrienol (AT3), R- γ -tocotrienol (GT3), R- δ -tocotrienol (DT3) (Carotech, Inc., Edison, NJ), and 15-deoxy- $\Delta^{12,14}$ -PGJ₂ (15-dPGJ₂) (BioMol Research Lab, Plymouth Meeting, PA).

Cell Culture

The PC-3 cell line was purchased from American Type Culture Collection, ATCC (Manassas, VA). The PC-3 prostate cancer cell line was maintained as a monolayer culture in RPMI 1640 media (Gibco BRL, Rockville, MD) supplemented with 10% FBS and 100 U ml⁻¹ penicillin and 100 μ g ml⁻¹ streptomycin in a humidified atmosphere of 5% CO₂ at 37°C and were subcultured at 75% confluence. The PrEC primary prostate epithelial cells were purchased from Lonza Bioscience and maintained by strict adherence to manufacturer's instructions in atmosphere of 5% CO₂ at 37°C.

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 wavelengths (λ_{max}) and molar extinction coefficients (C): AT λ_{max} = 292 nm ϵ = 3270, GT λ_{max} = 298 nm ϵ = 3810, DT λ_{max} = 298 nm ϵ = 3520, AT3 λ_{max} = 292 nm ϵ = 3870, GT3 λ_{max} = 298 nm ϵ = 4230, DT3 λ_{max} = 292 nm ϵ = 3300. 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 μ l ethanol per ml of media) to five volumes of 7.5% bovine serum albumin (BSA). The BSA/tocopherol mixture was added to complete culture medium. In the vehicle-treated cells, BSA/ethanol is added to the complete culture medium at the same concentration that is added to the treatments.

MTT Assay

The PC-3 cells were plated at a concentration of 1 x 10⁵ cells/well (for up to 72 hr treatments) in a

96-well flat bottom plate and allowed to adhere for 24 hr prior to treatment. Vitamin E supplemented media was added to concentrations of 5, 10, 20, 30, 40, 50, 60, 80, or 100 μM and incubated for 24 or 72 hr. After treatment, cells were incubated in 10 μl of 0.5 mg ml^{-1} MTT solution until crystals are observed, but not longer than 4 hr. Acid-SDS reagent was added to each well and mixed by shaking for 5 min followed by 30 min incubation time at 37°C. The absorbance at 570 nm was monitored using a Spectramax plus 190 UV-Vis spectrophotometer (Molecular Devices, Sunnyvale, CA).

Treatment of Cells for RNA/Protein Extraction

The PC-3 human prostate cancer cells or PrEC cells were seeded at 5×10^6 cells/plate in 100 x 20 mm plates 24 hr before treatment. Then cells were treated with either AT, GT, DT, AT3, GT3, DT3, vehicle (BSA + ethanol) or troglitazone or 15-deoxy- $\Delta^{12,14}$ dPGJ2 (positive controls for PPAR γ up regulation) for 24 hr.

Western Blot Analysis

Treated cells were lysed with lysis buffer (PBS, 0.1% Triton, 0.1% SDS) in the presence of protease inhibitor cocktail (Sigma Chemical, St. Louis, MO) to obtain total cell lysates. Lysates were subjected to a freeze/thaw cycle at -80°C then centrifuged at 10 000 g for 10 min to sediment the particulate material. To obtain cytosolic or nuclear lysates, the treated cells were lysed according to manufacturers' instructions using the Panomics Nuclear Extraction Kit (Panomics, Fremont, CA). Protein concentration was measured using the BCA protein assay (Pierce Biotechnology, Rockford, IL). Total cellular protein was separated by SDS polyacrylamide gel electrophoresis and electrotransferred onto Hybond-ECL nitrocellulose membrane. Blotted membranes were incubated with the PPAR γ , B-actin, cyclin D1, cyclin D3, cIAP-2, xIAP-2, p-NF κ B, or Bcl-2 primary antibodies and 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).

Quantitative PCR

Quantitative real time PCR was performed as previously described (Campbell *et al.*, 2003). In brief, the primer pair used to amplify PPAR γ 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 PPAR γ Ligand Binding Domain (LBD)

Storage buffer [50 mM Tris pH 8, 50 mM KCl (100 ml)] was added to 500 mg of streptavidin-coated LEADseeker SPA beads (Amersham). After 30 min of mixing on a Nutator platform, the beads were centrifuged at 1000 \times g for 10 min, and the supernatant discarded. The bead pellet was resuspended in 100 ml of storage buffer at 5 mg ml^{-1} . 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^{-1} BSA, 10 mM DTT) and 120-150 nM of biotinylated PPAR γ LBD (Nichols *et al.*, 1998) was added to a final volume of 12.5 ml. The slurry was incubated for at least 1 hr with gentle agitation followed by centrifugation at 1000 \times 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 supernatant. 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 ^3H -BRL49653 was diluted to 150 nM using assay buffer and sonicated for 5 s (Nichols *et al.*, 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^{-1} PPAR γ LBD-coated beads. Vitamin E isoforms were diluted in DMSO with using concentrations from 1 nM to 100 μM . Following a 2-15 hr 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 unlabelled BRL49653.

Determination of 15-S-HETE in Prostate Cells and Tissue Culture Medium by ELISA Assay

The PC-3 cells were seeded at 3×10^6 cells in a 100-mm tissue culture dish. Following 24 hr of incubation with vitamin E, culture medium was collected and the cells were harvested by trypsinization. Intracellular and extracellular 15-S-HETE were extracted using the method of Subbarayan *et al.* (2005). For the ELISA Assay the extracted samples were reconstituted in 300 μl Assay buffer from the 15-S-HETE ELISA kit (Assay Designs, Ann Arbor, Michigan). The 15-S-HETE was then measured according to the manufacturer's protocol.

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).

RESULTS AND DISCUSSION

Comparison of Percent Growth Arrest Following Treatment of Prostate Cancer Cells with α -, γ -, and δ -Isoforms of Vitamin E Demonstrates that Vitamin E-mediated Cell Growth Arrest is Time and Concentration Dependent and GT3 and DT3 are More Potent Growth Inhibitors

The treatment of androgen-independent PC-3 prostate cancer cells at varying concentrations demonstrates the growth arrest of the vitamin E isoforms to be both concentration and time dependent (*Tables 1 and 2*). The GT3 and DT3 isoforms are the most potent of all the isoforms tested. They significantly inhibit cell growth as early as 24 hr with 20 μ M. None of the other vitamin E

isoforms significantly reduce cell proliferation at this time point with treatments less than 80 μ M. Once all isoforms achieve statistical significance with respect to growth inhibition, it is clear that the gamma and delta isoforms are more effective than the alpha isoforms and the tocotrienols are more effective than the tocopherols.

Comparison of Growth Arrest Induced by GT and GT3 in Normal Prostate Cells Versus Prostate Cancer Cells Demonstrates that the γ -Vitamin Es are Selective toward the Cancer Cells, Particularly at Concentrations < 40 μ M

The PrEC cells are primary prostate cells derived from normal prostate epithelia. The profile of markers in PrEC cells is consistent with that expected for pubertal prostate epithelial cells. The PC-3 cells were derived from a 62-year-old male with grade IV adenocarcinoma. They are representative of androgen-independent prostate cancer that occurs after androgen-ablation. Comparison of the PrEC cell growth inhibition with the PC-3 growth inhibition with both γ -vitamin Es show the cancer cells are more sensitive to lower concentrations than are the normal pubertal prostate epithelia (*Figure 1*). In addition, when comparing GT- (*Figure 1a*) with GT3- (*Figure*

TABLE 1. GROWTH INHIBITION VALUES FOR TREATMENTS AT 24 hr

24 hr	20 μ M		40 μ M		80 μ M	
Vitamin E isoform	Mean % growth inhibition	p-value	Mean % growth inhibition	p-value	Mean % growth inhibition	p-value
AT	0	NS	0	NS	15.9	p<0.00001
GT	0	NS	0	NS	25.9	p<0.0001
DT	0	NS	0	NS	43.9	p<0.00001
AT3	0	NS	0	NS	63.8	p<0.00001
GT3	15.2	p<0.00001	58.3	p<0.00001	79.4	p<0.00001
DT3	43.0	p<0.00001	89.0	p<0.00001	89.1	p<0.0001

Notes: *The p-value is comparison of Raw MTT absorbance to vehicle-treated controls. **Mean % growth inhibition = 100 - [(MTT_t / MTT_v)*100], where MTT_t = MTT absorbance for specific treatment and MTT_v = MTT absorbance for vehicle-treated cells.

TABLE 2. GROWTH INHIBITION VALUES FOR TREATMENTS AT 72 hr

72 hr	20 μ M		40 μ M		80 μ M	
Vitamin E isoform	Mean % growth inhibition	p-value	Mean % growth inhibition	p-value	Mean % growth inhibition	p-value
AT	0	NS	0	NS	47.6	p<0.00001
GT	15.6	p<0.01	28.6	p<0.01	68.7	p<0.00001
DT	23.1	p<0.00001	47.5	p<0.00001	83.1	p<0.00001
AT3	0	NS	55.2	p<0.00001	63.8	p<0.00001
GT3	20.5	p<0.01	60.2	p<0.00001	87.1	p<0.00001
DT3	28.9	p<0.00001	82.0	p<0.00001	94.6	p<0.00001

Notes: *The p-value is comparison of Raw MTT absorbance to vehicle-treated controls. **Mean % growth inhibition = 100 - [(MTT_t / MTT_v)*100], where MTT_t = MTT absorbance for specific treatment and MTT_v = MTT absorbance for vehicle-treated cells.

1b) treated cells, a higher percentage of growth arrest is achieved with the GT3 at concentrations lower than 40 μM .

GT3 and GT Both Up Regulate the Production of PPAR γ Protein and mRNA

Peroxisome proliferator activated receptors (PPARs) are ligand-activated transcription factors.

They function to modulate gene expression by binding to peroxisome proliferator response elements (PPREs) in the DNA. The 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 retinoic acid receptor (R α R) (Figure 2). Ligand activation of PPAR γ results in growth inhibition, differentiation, and apoptosis in a number

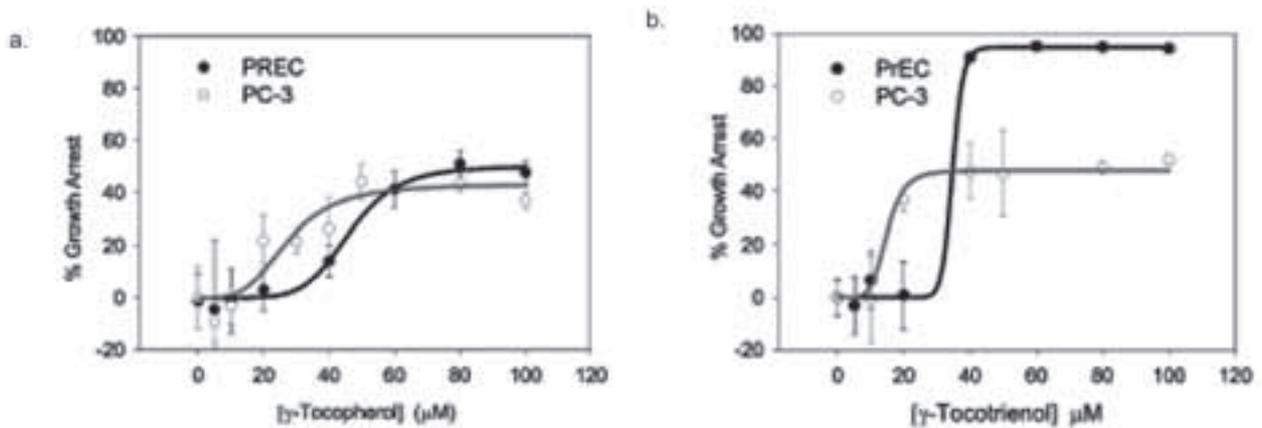


Figure 1. Vitamin E isoforms exhibit differential growth inhibitory effects on normal prostate cells (PrEC) cells compared with prostate cancer cells (PC-3). Sigmoidal concentration curves of MTT assay data in normal (PrEC)-closed circles or prostate cancer cells (PC-3)-open circles treated with varying concentrations of GT (A) or GT3 (B). Data are presented as the mean \pm SD.

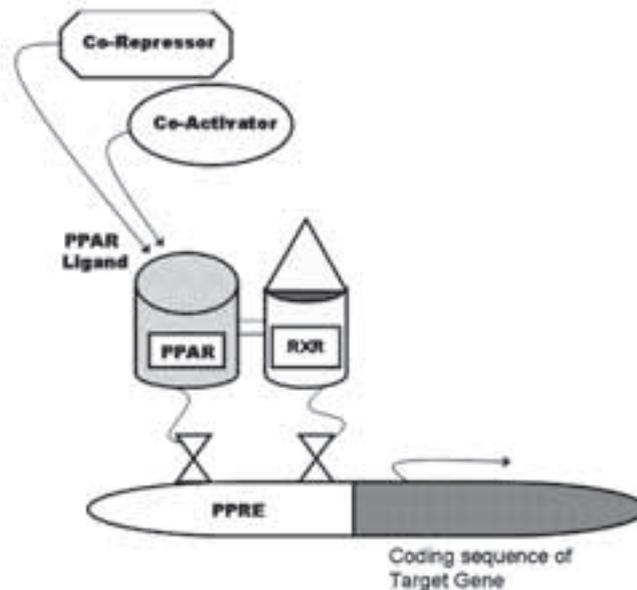


Figure 2. The PPAR γ is a protein that acts as a nuclear transcription factor binding as a heterodimer with the RXR receptor. Nuclear transcription factors have a DNA binding domain and a ligand binding domain (LBD). The DNA binding domain binds to the response element in the DNA up stream of a coding sequence to influence gene expression. The LBD binds molecules that can bring either a co-activator of gene expression or a co-repressor of gene expression depending upon the ligand recruited to the LBD. The PPAR γ ligands down regulated the gene expression of proteins involved in carcinogenesis and cancer progression such as cell proliferation proteins, cell cycle proteins, and matrix metalloproteinases involved in angiogenesis and metastasis. Further PPAR γ activity can up regulate pathways involved in cell death, such as TNF α .

of different cancer cells including: breast, prostate, colon and pancreatic cancers (Murphy and Holder, 2000), (Kitamura *et al.*, 1999), (Eibl *et al.*, 2002), (Itami *et al.*, 2001), (Mueller *et al.*, 2000). Endogenous ligands are metabolites of arachidonic acid and linoleic acid such as prostaglandins D1 and D2, 15-S-hydroxyeicosatetraenoic acid (HETE), and 12-S-HETE (Yoshimatsu *et al.*, 2001), (Yu *et al.*, 1995). Synthetic ligands of PPAR γ include family members of the thiazolidinediones: troglitazone, pioglitazone, and rosiglitazone (Baek *et al.*, 2003), (Davies *et al.*, 1999), (Hishinuma, *et al.*, 2002), (Itami *et al.*, 2001). Previous data from our lab demonstrated that GT could up regulate PPAR γ mRNA and protein expression in human colon cancer cell lines (Campbell *et al.*, 2003). *Figure 3a* shows the expression of PPAR γ mRNA following treatment with either 5 μ M GT3 or GT for 24 hr. The expression of PPAR γ in GT3-treated cells is significantly higher than that of either GT- or the vehicle-treated cells at the concentration and time point tested. *Figure 3b* shows the expression of PPAR γ protein following treatment with either GT3 or GT at various concentrations for 24 hr. The data reveal that GT begins to up regulate PPAR γ protein at 10 μ M, however it does not persist with the up regulation throughout all the concentrations tested. On the other hand, GT3 is able to up regulate the protein expression of PPAR γ at 5 μ M and PPAR γ is consistently up regulated compared to the vehicle (0 μ M) with all concentrations tested.

The γ -vitamin E Isoforms are Not Direct PPAR γ Ligands

A scintillation proximity assay was employed to determine if GT or GT3 are direct PPAR γ ligands (Nichols *et al.*, 1998). Neither GT (*Figure 3c*) or GT3 (*Figure 3d*) could displace the 3H-labelled BRL49653 bound to the PPAR γ ligand binding domain through competitive displacement, indicating that these vitamin E isoforms are not direct PPAR γ ligands. The inability of the γ -vitamin Es to directly bind the PPAR γ ligand binding domain indicates that there must be an endogenous ligand up regulated through treatment with GT or GT3.

GT and GT3 Up Regulate the Production of the PPAR γ Ligand, 15-S-HETE

The 15-S-HETE is a known PPAR γ ligand that has demonstrated the ability to regulate PPAR γ and inhibit cellular proliferation in prostate cancer cells (Shappell *et al.*, 2001). We used an ELISA assay to test for the production of 15-S-HETE in the PC-3 prostate cancer cells following treatment with 40 and 60 μ M GT or GT3 at 24 hr (*Figures 3e* and *3f*). We found that GT3 significantly up regulates the

production of 15-S-HETE at 24 hr in PC-3 cells treated with 40 and 60 μ M. The production of 15-S-HETE in PC-3 cells treated with 60 μ M GT3 at 24 hr was so high that residual 15-S-HETE was secreted into the extracellular matrix or media (*Figure 3f*).

GT3 But Not GT Inhibits the Translocation of Phosphorylated NF κ B to the Nucleus in PC-3 Cancer Cells

The NF κ B is a transcription factor that is modulated by a number of signaling molecules. The signaling molecules can activate receptors on the cell surface. The receptors send signals in the form of a phosphorylation cascade that activates NF κ B. The NF κ B activation results in the activation of gene targets involved in the inflammatory response and anti-apoptosis pathways. *Figure 4* illustrates the different cell surface receptors capable of activating NF κ B. Constitutive activation of NF κ B occurs in many cancer cells. The result is constant expression of proteins that bind to TRAFs and caspases to prevent apoptosis. These proteins are called 'inhibitor of apoptosis Proteins' (IAP). The constitutive expression of IAPs results in resistance to TNF α -mediated cell death (*Figure 5*). In addition, cell cycle proteins and other inhibitors of apoptosis such as Bcl-2 cause cancer cells to proliferate out of control. Ahn *et al.* (2007) in the model system KBM-5 cells (containing both TNF alpha receptors) demonstrated that NF κ B translocation to the nucleus was prevented by GT3 (Ahn *et al.*, 2007). The prostate cancer cell line PC-3 was shown to have over expression of NF κ B and be dependent upon its expression for continued growth (Lu *et al.*, 2004). The PC-3 cell lysates treated with the γ -vitamin Es were assayed to determine if NF κ B translocation was disrupted (*Figure 6*). Western blot analysis of the p65 subunit of NF κ B demonstrates that nuclear and cytosolic phosphorylated NF κ B (pNF κ B) is down regulated by treatment with both γ -vitamin Es. However, GT3 is better at down regulating pNF κ B than GT.

GT3 Down Regulates Genes that are Modulated by NF κ B to Potentiate Apoptosis and Inhibition of Cell Proliferation in PC-3 Prostate Cancer Cells

The PC-3 prostate cancer cells were also assayed for the regulation of down stream genes in the NF κ B pathway such as the cyclins, IAP and Bcl-2. Cyclin D1 and cyclin D3 were both down regulated to a much higher extent with GT3 than with GT. The inhibitors of apoptosis from the surviving family such as cIAP and xIAP were down regulated with GT3 treatment as well. Further, GT3 also down regulates the antiapoptotic protein Bcl-2.

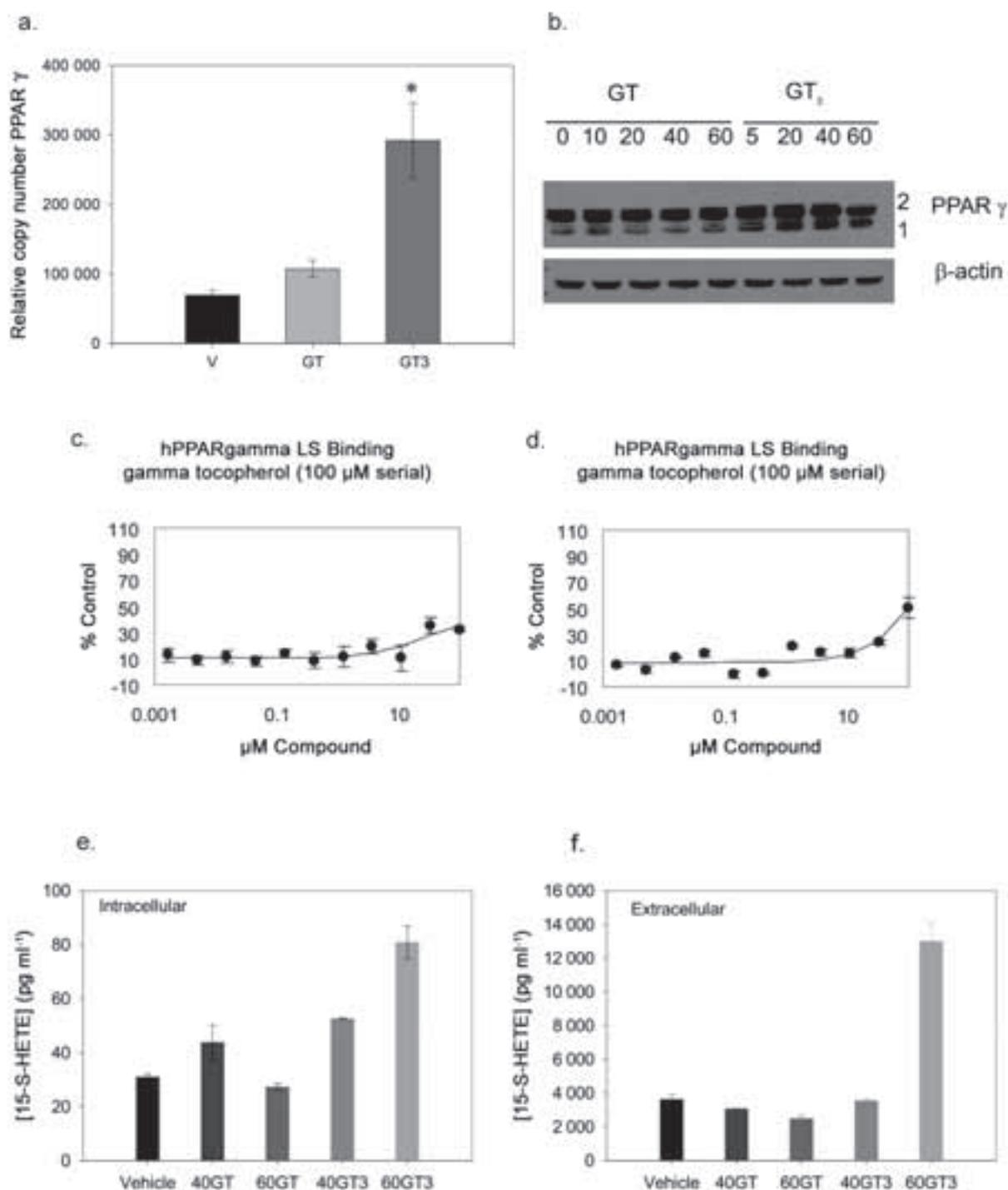


Figure 3. The γ -vitamin Es up regulate the expression of PPAR γ . The GT and GT3 are not PPAR γ ligands, but rather up regulate the expression of the endogenous PPAR γ ligand, 15-S-HETE. a. The QPCR analysis of mRNA expression of PPAR γ following treatment in PC-3 cells at 5 μ M GT or GT3 for 24 hr. b. Western Blot analysis of PPAR γ and β -actin (loading control) following treatment with GT or GT3 for 24 hr at indicated concentrations. Blot analysis detected both isoforms of PPAR γ (1 and 2) as indicated. c. Scintillation proximity assay measuring the displacement of rosiglitazone by GT at indicated concentrations. d. Scintillation proximity assay measuring the displacement of rosiglitazone by GT3 at indicated concentrations. e. ELISA analysis of intracellular concentrations of 15-S-HETE (pg ml⁻¹) following treatments at 40 and 60 μ M concentrations of GT or GT3 for 24 hr. f. ELISA analysis of extracellular (media) concentrations of 15-S-HETE (pg ml⁻¹) following treatments at 40 and 60 μ M concentrations of GT or GT3 for 24 hr.

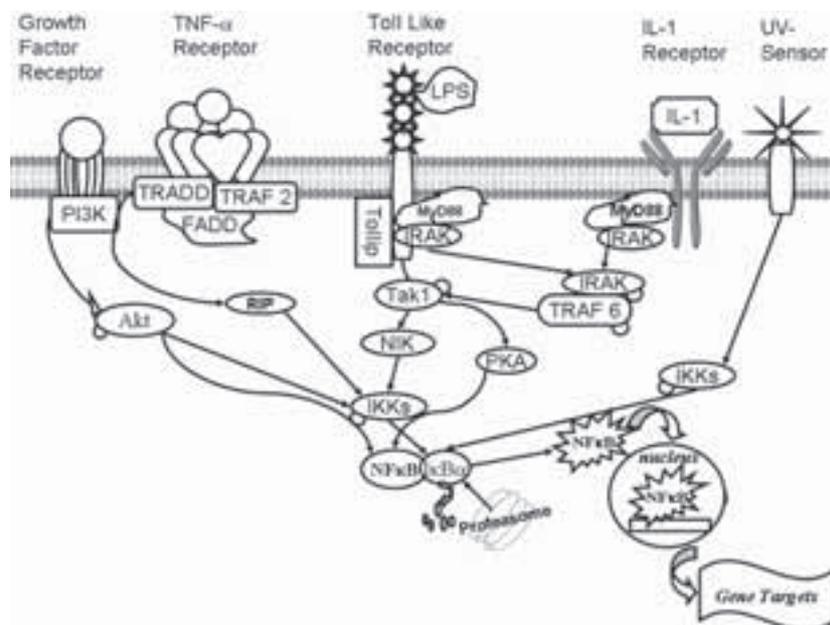


Figure 4. The activation of NFκB can occur through numerous receptors by various signaling mechanisms. Growth factors can stimulate NFκB activation through the PI3Kinase pathway and Akt signaling. The TNF α receptor can stimulate activation of NFκB through RIP-kinase. Toll-like receptors can be activated by the presence of bacteria, LPS or viral factors to stimulate the activation of NFκB. The activation of numerous cytokines such as IL-1 can stimulate the activation of NFκB by binding to cytokine-specific receptors. Exposure to ultraviolet light can also stimulate the activation of NFκB through an unknown pathway.

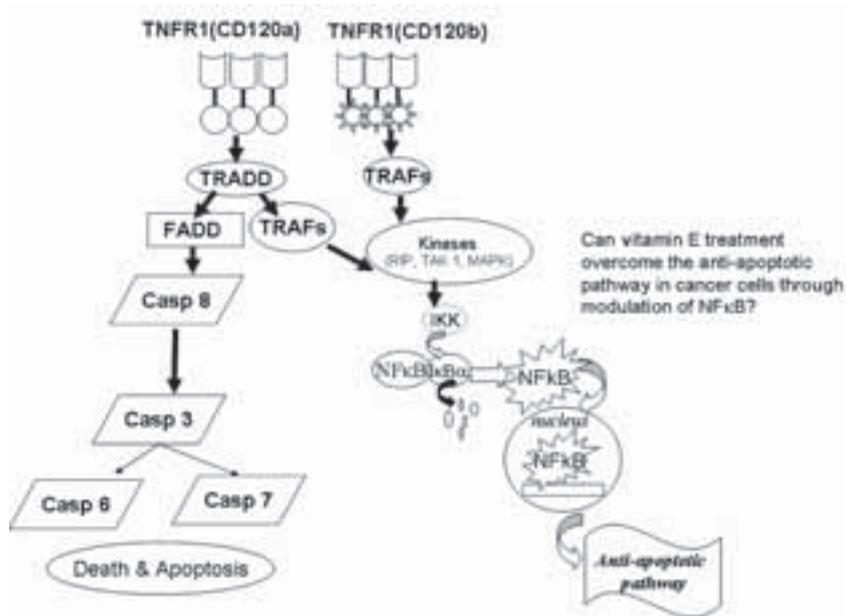


Figure 5. The TNF α pathway has two different responses that respond to invasion, injury or trauma to the cell. In non-carcinogenic cells TNF α is activated in response to bacterial invasion, injury or trauma to a cell. The binding of TNF α to the TNF α receptor results in the activation of the death response and the inflammatory response. The death response is activated independently of the inflammatory response, the NFκB pathway. The determination of which response is activated by TNF α (cell death or inflammation) is dependent on the cellular factors active in the cell. When NFκB is activated it enters the nucleus and activates cell cycle genes and antiapoptotic genes, in order to reduce the trauma or remove the invader and proliferate new cells. Cell signals (accumulation of NFκB and IκBα in the nucleus) will reduce the activation when the tissue repair is complete. In the many types of cancer, NFκB is constitutively up regulated or turned on. The constitutive up regulation of NFκB results in resistance to apoptosis and cancer cell survival, through the constant expression of antiapoptotic factors such as cIAP-2, x-IAP, Bcl2 and cell cycle proteins such as cyclin D1 and cyclin D3.

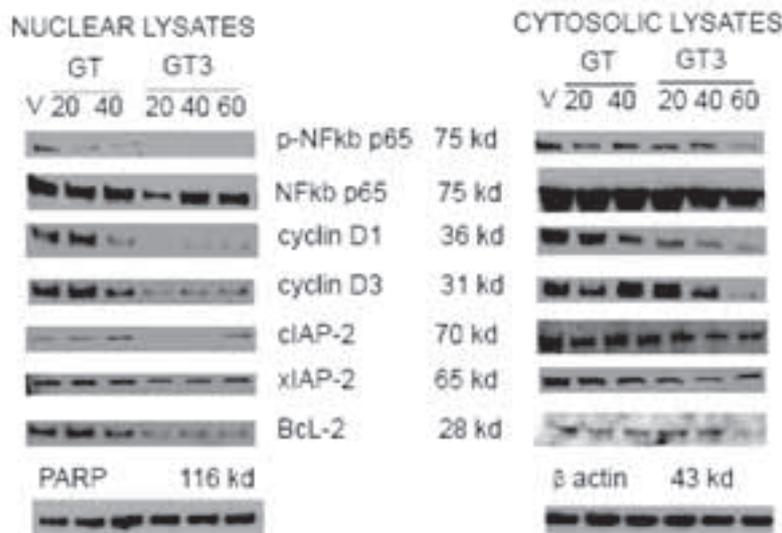


Figure 6. The GT3 results in decreased p-NFκB transported to the nucleus, lower concentrations of p-NFκB in the cytoplasm and reduces the expression of the NFκB-modulated antiapoptotic genes cIAP-2, x-IAP, Bcl2 and cell cycle protein cyclin D1 and cyclin D3. Nuclear and cell lysates were analysed with western blotting following treatment with 20 and 40 μM GT or 20, 40 or 60 μM GT3.

CONCLUSION

Our data demonstrates that *R*-γ-tocotrienol (GT3) modulates several key pathways involved in prostate cancer risk including: dietary fat and inflammation. First, GT3 up regulates the PPAR γ pathway. The PPAR γ is a ligand activated nuclear receptor that modulates fat metabolism. More importantly ligand activation of PPAR γ results in growth inhibition, differentiation, and apoptosis in a number of different cancer cells including prostate. The PPAR γ activation results in: (1) the shut down of carcinogenic pathways such as cell cycle progression and matrix metalloproteinases (involved in angiogenesis) and; (2) the up regulation of pathways such as apoptosis and the TNF α pathway. Second, GT3 modulates the 15-lipoxygenase metabolite of arachidonic acid, 15-HETE. Arachidonic acid stimulates prostate cancer cell growth and plays a role in inflammation. Further, arachidonic acid is hypothesized to play a role in prostate carcinogenesis. Third, GT3 modulates the NFκB pathway. The NFκB is a key modulator of inflammatory processes and cell cycle progression. We propose that GT3 could have a superior preventive growth effect on prostate tumours than α-tocopherol or γ-tocopherol and should be considered in the next generation of prostate cancer prevention trials.

ACKNOWLEDGEMENT

Many special thanks to Mr W L Leong at Carotech, Inc. for the generous supply of tocotrienols. We

would also like to thank the Malaysian Palm Oil Board for the opportunity to present this data.

REFERENCES

- AHN, K S; SETHI, G; KRISHNAN, K and AGGARWAL, B B (2007). Gamma-tocotrienol inhibits nuclear factor-kappa B signaling pathway through inhibition of receptor-interacting protein and TAK1 leading to suppression of antiapoptotic gene products and potentiation of apoptosis. *J. Biological Chemistry* Vol. 282 No. 1: 809-820.
- BAEK, S J; WILSON, L C; HSI, L C and ELING, T E (2003). Troglitazone, a peroxisome proliferator-activated receptor gamma (PPARgamma) ligand, selectively induces the early growth response-1 gene independently of PPARgamma. A novel mechanism for its anti-tumorigenic activity. *J. Biological Chemistry* Vol. 278 No. 8: 5845-5853.
- BEHARKA, A A; WU, D; SERAFINI, M and MEYDANI, S N (2002). Mechanism of vitamin E inhibition of cyclooxygenase activity in macrophages from old mice: role of peroxynitrite. *Free Radic. Biol. Med.* Vol. 32 No. 6: 503-511.
- CAMPBELL, S E; STONE, W L; WHALEY, S G; QUI, M and KRISHNAN, K (2003). Gamma tocopherol upregulates peroxisome proliferator activated receptor (PPAR) gamma expression in SW 480 human colon cancer cell lines. *BMC Cancer* Vol. 3 No. 25.

DAVIES, G F; KHANDELWAL, R L and ROESLER, W J (1999). Troglitazone induces expression of PPAR γ in liver. *Mol Cell Biol. Res Commun. Vol. 2 No. 3:* 202-208.

EIB, L G; WENTE, M N; REBER, H A and HINES, O J (2002). Peroxisome proliferator-activated receptor gamma induces pancreatic cancer cell apoptosis. *Biochem Biophys Res Commun. Vol. 287:* 522-529.

HELZLSOUER, K J; HUANG, H Y; ALBERG, A J; HOFFMAN, S; BURKE, A; NORKUS, E P; MORRIS, J S and COMSTOCK, G W (2000). Association between alpha-tocopherol, gamma-tocopherol, selenium and subsequent prostate cancer. *J. Natl. Cancer Inst. Vol. 92 No. 24:* 2018-2022.

HISHINUMA, T; YAMAZAKI, T and MIZUGAKI, M (2002). Troglitazone has a reducing effect on thromboxane production. *Prostaglandins and Other Lipid Mediators Vol. 62:* 135-143.

ITAMI, A; WATANABE, G; SHIMADA, Y; HASHIMOTO, Y; KAWAMURA, J; KATO, M; HOSOTANI, R and IMAMURA, M (2001). Ligands for peroxisome proliferator-activated receptor gamma inhibit growth of pancreatic cancers both *in vitro* and *in vivo*. *Int. J. Cancer Vol. 94:* 370-376.

JIANG, Q; ELSON-SCHWAB, I; COURTEMANCHE, C and AMES, B N (2000). Gamma-tocopherol and its major metabolite, in contrast to alpha-tocopherol, inhibit cyclooxygenase activity in macrophages and epithelial cells. *Proc. Natl. Acad. Sci. USA Vol. 97:* 11494-11499.

KITAMURA, S; MIYAZAKI, Y; SHINOMURA, Y; KONDO, S; KANAYAMA, S and MATSUZAWA, Y (1999). Peroxisome proliferator-activated receptor gamma induces growth arrest and differentiation markers of human colon cancer cells. *Jpn J. Cancer Res. Vol. 90:* 75-80.

LU, T; BURDELYA, L G; SWIATKOWSKI, S M; BOIKO, A D; HOWE, P H; STARK, G R and 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. USA Vol. 101:* 7112-7117.

MATRINCE, M; KSAS, B; REY, P and HAVAUX, M (2008). Tocotrienols, the unsaturated forms of vitamin E, can function as antioxidants and lipid protectors in tobacco leaves. *Plant Physiol.*

MUELLER, E; SMITH, M; SARRAF, P; KROLL, T; AIYER, A; KAUFMAN, D S; OH, W; DEMETRI, G; FIGG, W D; ZHOU, X P; ENG, C; SPIEGELMAN, B

M and KANTOFF, P W (2000). Effects of ligand activation of peroxisome proliferator-activated receptor gamma in human prostate cancer. *Proc. Natl Acad. Sci. USA Vol. 97 No. 20:* 10990-10995.

MURPHY, G J and HOLDER, J C (2000). PPAR gamma agonists: therapeutic role in diabetes, inflammation and cancer. *Trends in Pharma. Sci. Vol. 21:* 469-474.

NESARETNAM, K (2008). Multitargeted therapy of cancer by tocotrienols. *Cancer Lett.*

NICHOLS, J S; PARKS, D J; CONSLER, T G and BLANCHARD, S G (1998). Development of a scintillation proximity assay for peroxisome proliferator-activated receptor ligand binding domain. *Analytical Biochemistry Vol. 257:* 112-119.

SAKAMOTO, W; FUJIE, K; NISHIHARA, J; HANDA, H; UEDA, N and YAMAMOTO, S (1996). Effect of vitamin E on expression of cyclooxygenase-2 in lipopolysaccharide-stimulated rat macrophages. *Biochim. Biophys. Acta Vol. 1304 No. 2:* 139-144.

SHAPPELL, S B; GUPTA, R A; MANNING, S; WHITEHEAD, R; BOEGLIN, W E; SCHNEIDER, C; CASE, T; PRICE, J; JACK, G S; WHEELER, T M; MATUSIK, R J; BRASH, A R and DUBOIS, R N (2001). 15S-Hydroxyeicosatetraenoic acid activates peroxisome proliferator-activated receptor gamma and inhibits proliferation in PC3 prostate carcinoma cells. *Cancer Res. Vol. 61 No. 2:* 497-503.

STONE, W L and PAPAS, A M (1997). Tocopherols and the etiology of colon cancer. *J. Natl Cancer Inst. Vol. 89 No. 14:* 1006-1014.

SUBBARAYAN, V; XU, X C; KIM, J; YANG, P; HOQUE, A; SABICHI, A L; LLANSA, N; MENDOZA, G; LOGOTHETIS, C J; NEWMAN, R A; LIPPMAN, S M and MENTER, D G (2005). Inverse relationship between 15-lipoxygenase-2 and PPAR-gamma gene expression in normal epithelia compared with tumor epithelia. *Neoplasia. Vol. 7 No. 3:* 280-293.

VENKATESWARAN, V; FLESHNER, N E; SUGAR, L M and KLOTZ, L H (2004). Antioxidants block prostate cancer in Lady Transgenic Mice. *Cancer Research Vol. 64 No. 16:* 5891-5896.

YOSHIMATSU, K; ALTORKI, N K; GOLIJANIN, D; ZHANG, F; JAKOBSSON, P J; DANNENBERG, A J and SUBBARAMAIAH, K (2001). Inducible prostaglandin E synthase is overexpressed in non-small cell lung cancer. *Clinical Cancer Research Vol. 7 No. 9:* 2669-2674.

YU, K; BAYONA, W; KALLEN, C B; HARDING, H P; RAVERA, C P; MCMAHON, G; BROWN, M and LAZAR, M A (1995). Differential activation of

peroxisome proliferator-activated receptors by eicosanoids. *J. Biological Chemistry* Vol. 270 No. 41: 23975-23983.

NEW COLUMNS

The *Journal of Oil Palm Research* (JOPR) is introducing two new columns in the June 2009 issue.

Interested parties are invited to contribute ideas and comments related to the published articles or on how to improve the overall quality of the journal, so as to make it more presentable and useful to the readers.

Contributors are encouraged to abide by the guidelines stated below:

Guidelines for LETTERS TO EDITOR

A 'Letter to Editor' should be concise. Please include your full name (add a pseudonym, if you like), address, gender and phone number (for reference only). You can send by e-mail, regular mail or fax (603-8922 3564). Personal information of sender will be kept confidential. Send your letter to: Malaysian Palm Oil Board, P.O. 10620, 50720 Kuala Lumpur (Attn: Publication Section) or e-mail us at: pub@mpob.gov.my

Some common guidelines are:

- There will be a very short word limit, which is strictly enforced. A general guideline would be 200-250 words.
- You must not submit the same letter to more than one publication at the same time.
- The letter should refer to a specific article recently published by JOPR.
- There is a strict deadline for responding to a given article. The sooner you respond to specific article, the more likely it is that your letter will be published.

Guidelines for SHORT COMMUNICATIONS

- Short Communications are original short articles which are published with the objective of disseminating technical ideas of the originator without losing time. This will provide researchers with a venue where they can share their most current results and developments in the shortest possible time. The Short Communications, like regular papers will be reviewed by expert reviewers and evaluated by editor. Unlike regular papers, Short Communications will be published within six months of submission.
- Short communications should be prepared in a camera-ready format and limited to 2000 words and not more than four illustrations (*i.e.* Figures and Tables).
- Format: Abstract (~80 words), Keywords, Introduction, Materials and Methods, Results and Discussion, Conclusion and References.
- In order to help expedite the reviewing process, authors are advised to suggest a list of two unbiased potential reviewers to the Editor. Please include their names and e-mail addresses in the submission e-mail. These reviewers should not be related to the author, nor should they be associates or collaborators.