

Upregulation of pERK and c-JUN by γ -tocotrienol and not α -tocopherol are essential to the differential effect on apoptosis in prostate cancer cells.

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Abstract

Background: α -tocopherol (AT) and γ -tocotrienol (GT3) are vitamin E isoforms considered to have potential chemopreventive properties. AT has been widely studied in vitro and in clinical trials with mixed results. The latest clinical study (SELECT trial) tested AT in prostate cancer patients, determined that AT provided no benefit, and could promote cancer. Conversely, GT3 has shown antineoplastic properties in several in vitro studies, with no clinical studies published to date. GT3 causes apoptosis via upregulation of the JNK pathway; however, inhibition results in a partial block of cell death. We compared side by side the mechanistic differences in these cells in response to AT and GT3. Methods: The effects of GT3 and AT were studied on androgen sensitive LNCaP and androgen independent PC-3 prostate cancer cells. Their cytotoxic effects were analyzed via MTT and confirmed by metabolic assays. Cellular pathways were studied by immunoblot. Quantitative analysis and the determination of relationships between cell signaling events were analyzed for both agents tested. Non-cancerous prostate RWPE-1 cells were also included as a control. Results: The RAF/RAS/ERK pathway was significantly activated by GT3 in LNCaP and PC-3 cells but not by AT. This activation is essential for the apoptotic effect by GT3 as demonstrated the complete inhibition of apoptosis by MEK1 inhibitor U0126. Phospho-c-Jun was upregulated by GT3 but not AT. No changes were observed on AKT for either agent, and no release of cytochrome c into the cytoplasm was detected. Caspases 9 and 3 were efficiently activated by GT3 on both cell lines irrespective of androgen sensitivity, but not in cells dosed with AT. Cell viability of non-cancerous RWPE-1 cells was affected neither by GT3 nor AT. Conclusions: c-JUN is a recognized master regulator of apoptosis as shown previously in prostate cancer. However, the mechanism of action of GT3 in these cells also include a significant activation of ERK which is essential for the apoptotic effect of GT3. The activation of both, ERK and c-JUN, is required for apoptosis and may suggest a relevant step in ensuring circumvention of mechanisms of resistance related to the constitutive activation of MEK1.

Background

Prostate cancer is the most common malignancy in males, accounting for 19% of new cancer cases in males and 29,430 deaths in 2018 [1]. Most forms are either curable using local interventions, or indolent requiring timely surveillance. However, some forms are aggressive and metastasize to bones and other organs, leading to early morbidity and mortality [2]. Epidemiological studies suggest that disease progression may be associated to environmental factors and lifestyle, with a specific emphasis on diet [3]. Thus, prostate cancer prevalence and behavior makes it an ideal target for chemoprevention, or the use of chemical agents to disrupt progression of the disease to invasive cancer [4]. Among potential antineoplastic agents, antioxidants are known to exert protective mechanisms on healthy cells that may prevent disease progression. Vitamin E is an antioxidant, abundant in certain nuts, whole grains, and vegetables. It is an essential lipid-soluble vitamin composed of eight isoforms, four tocopherols and four tocotrienols (α , β , γ , and δ for each of these). Among these isoforms, α -tocopherol (AT) has the highest bioavailability [5] and its properties have been evaluated in clinical studies. Indeed, AT was the earliest isoform to be considered as a potential agent in prostate cancer prevention in male smokers in a study.

However, AT was used as a synthetic all-racemic-alpha tocopheryl acetate (rac-ATA) form with very promising beneficial results [6] thus gaining the acceptance of this synthetic molecule. Rac-ATA was used recently in the Selenium, Vitamin E and Prostate Cancer Chemoprevention Trial (SELECT), with very different outcomes [7]; it was concluded that this synthetic form of AT alone or in combination with selenium has no chemo preventive properties, and in fact could promote prostate cancer. The SELECT trial was not the first clinical study that showed a lack of antineoplastic effects by AT, in fact it confirmed the results obtained in the Women Health Study, which used a natural source of AT in cancers of the lung, breast and colon [8]. Conversely, an extensive number of research studies *in vitro* and in animal models suggest that tocotrienols have important antineoplastic effects in cancers of the breast, liver, pancreas, and prostate, but no clinical studies have evaluated their potential therapeutic use. Among the tocotrienols, γ -tocotrienol (GT3) has been studied in cancers of the breast, pancreas and prostate [9], where it has shown that it inhibits cell proliferation and cell invasion via chemotherapy sensitization, and by suppressing EGFR, NF- κ B, and Id proteins, and activating the JNK signaling pathway and caspases 9 and 3. While these studies suggest GT3 as a potential chemopreventive agent that may be useful as an adjunctive treatment option for some forms of prostate cancer, the role of the RAF/RAS/ERK MAPK pathway and the relationship with other signaling cascades has not been clearly established. Here we present the specific mechanistic differences between AT and GT3 in both androgen-sensitive and androgen-independent prostate cancer cells and its multipronged effect on crucial cell signaling pathways affecting proliferation and survival.

Methods

Cell lines and culture conditions

The androgen independent PC-3 prostate cancer cell line, derived from a bone metastasis of a grade IV human prostatic adenocarcinoma displaying epithelial morphology, and the androgen sensitive LNCaP prostate cancer cells, derived from a left supraclavicular lymph node of human prostate carcinoma, were purchased from the American Type Culture Collection (Manassas, VA), and maintained as recommended by the supplier. Briefly, the cells were grown in RPMI1640 supplemented with 10% FBS, and penicillin/streptomycin (Life Technologies, Grand Island, NY). RWPE-1 primary prostate epithelial cells were purchased from Lonza Bioscience (Basel, Switzerland) and grown as instructed, in keratinocyte SFM medium supplemented with growth factors (Fisher Scientific, Waltham, MA). For cytotoxicity and metabolism activity assays, the cells were seeded on 96 well plates at a density of 5×10^3 cells/well. For immunoblotting experiments, the cells were seeded on 60 mm plates at a density of at 5×10^5 cells/cm².

Reagents

GT3 (98% pure), was purchased from Cayman Chemical (Ann Arbor, MI). The concentration of GT3 in ethanol was determined using an HP-8542A diode array spectrophotometer with the following maximum wavelengths (λ_{\max}) and molar extinction coefficients (ϵ): GT3 λ_{\max} 298 nm,

$\epsilon = 4230$. AT (97% pure) was purchased from Tama Biochemical (Tokyo, Japan), its concentration was determined in the same fashion as for GT3.

Cytotoxicity assay

LNCaP, PC-3, and RWPE-1 cells were dosed with GT3 or AT at concentrations of 10, 20, 40, 60, and 80 μM . After treatment, 3-(4, 5-methylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide (MTT) was added and incubated for 3 hours (Invitrogen, Carlsbad, CA). Formazan products were solubilized with acidified SDS overnight. Optical density was measured at 570 nm using a Spectramax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). All experiments were done at least three times.

Metabolism activity assay

Cells were treated with either GT3 or AT in concentrations of 10, 20, 40, 60, and 80 μM ; dissolution vehicle was ethanol. Following 6, 12 and 24 hours of treatment, plates were allowed to come to room temperature before 100 μL /well of CellTiter Glo[®] reagent from Promega (Madison, WI, USA) was added. Luminescence indicative of the presence of ATP, was measured on a luminometer (Promega).

Western blot analysis

LNCaP, PC-3 and RWPE-1 cells were treated with indicated concentrations of GT3, AT, or vehicle as a control for 6 or 12 hours. Harvested cells were lysed with RIPA Buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton, 0.1% SDS, 10 mM Tris, 100 μM sodium orthovanadate, 50 mM sodium fluoride) containing phosphatase and protease inhibitors (Sigma, St. Louis, MO). The protein concentrations of the cell lysates were determined by the BCA protein assay (Cytoskeleton, Denver, CO). The samples were run in SDS-PAGE and blotted onto nitrocellulose or PVDF membranes (Pall Life Sciences, Ann Arbor, MI). Blotted membranes were processed according to recommended protocols for each antibody against total and phosphorylated forms of Erk, c-Jun, BAD, (Cell Signaling, Danvers, MA, USA), Akt (EMD Millipore, Billerica, MA), cytochrome C (BD Biosciences, San Jose, CA), and actin with an antibody from Sigma (St. Louis, MO). Caspase activation was analyzed with anti-cleaved caspase 3 from Cell Signaling, Abcam and R&D systems. The signal of primary monoclonal or polyclonal antibodies was detected using affinity-purified secondary antibodies with no cross-reactivity with other species, coupled to peroxidase (Pierce and Promega, Fitchburg, WI, USA) and analyzed by a chemiluminescent system. The intensities of the bands on x-ray film were estimated by digitizing the image using Image J, and were compared against a control.

Statistical analysis

Data are represented as the mean \pm SE. Comparisons were done relative to the control, and analyses were run by Student's t test when comparing against a control, or ANOVA followed by Bonferroni test when comparing two treatment groups. $p < 0.05$, $p < 0.01$ and $p < 0.001$ indicate statistical significance. (GraphPad Prism 7, La Jolla, CA). The data are shown with error bars representing standard deviation.

Results

GT3 inhibits cell viability in a time and dose dependent manner in androgen-sensitive LNCaP and androgen-independent PC-3 prostate cancer cells, but not AT. Recent findings indicate that AT may promote proliferation of prostate cancer cells [7]. Conversely, it has been reported that GT3 may cause apoptosis on prostate cancer cells [9]. To test whether these effects are sustained and time dependent LNCaP and PC-3 prostate cancer cells were dosed with either GT3 or AT at concentrations ranging from 5 - 80 μ M. MTT and metabolic activity assays were run at six and twelve hours after dosing. At six hours, LNCaP (Figure 1A) and PC3 (Figure 1B) cells dosed with AT show a constant trend towards sustaining cell viability and slight increase in proliferation at 80 μ M. The effect of GT3 at lower concentrations is similar to that of AT. However, a downward trend is apparent at concentrations above 40 μ M suggesting loss of cell viability and inhibition of metabolic activity. The MTT and metabolic activity assays at twelve hours after dosing show that the effects observed at six hours continue their trend, with a significantly larger difference in the effect of AT and GT3 on both cell lines at concentrations above 40 μ M (Figure 1C and 1D). Previous studies on prostate, have reported no inhibition of cell viability on normal cells. This observation is confirmed via MTT and metabolic activity assays on non-cancerous prostate cells RWPE-1 after dosing with AT or GT3 (Figure 1E).

Activation of both, ERK and c-JUN are observed in LNCaP and PC3 cells after treatment with GT3 but not AT. It has been reported that GT3 activates the c-Jun N-terminal kinase JNK signaling pathway in prostate cancer cells, with the subsequent phosphorylation of c-JUN [9]. In this study, it was shown that the addition of a specific JNK inhibitor was able to block partially the activity of the protein, suggesting a potential additional source of activation by GT3. Since ERK1/2 can phosphorylate c-JUN[10] we decided to probe the phosphorylation status of these proteins via immunoblot after dosing LNCaP and PC3 cells with GT3 or AT. Analysis of LNCaP cells show a 1.98 fold increase in the levels of the phosphorylated form of ERK after dosing with 80 μ M of GT3 as compared to the control (Figure 2A). Phospho-c-JUN follows a similar trend, and its levels increase 1.45 and 1.32 fold after dosing with 60 and 80 μ M of GT3 (Figure 2C). Conversely, in cells dosed with AT, the phosphorylated form of ERK is significantly lower (-22.68%) at 80 μ M, and a dramatic decrease (-68%) for phospho-c-JUN (Figures 2B and 2D). As in LNCaP cells, a similar trend is observed in PC3 cells. The expression levels of phospho-ERK show a significant fold increase of 3.71, 4.57, and 5.29 after dosing with GT3 at concentrations of 40, 60 and 80 μ M respectively (Figure 2G). The levels of the phosphorylated form of c-JUN show an appreciable fold increase of 1.50 and 1.69 at 60 and 80 μ M, respectively. (Figure 2I). In the case of AT, there is no significant change in these proteins after dosing (Figures 2H and 2J).

Neither GT3 nor AT has an effect on the expression levels of the activated form of AKT on

LNCaP and PC3 cells. To test whether GT3 or AT have an effect on cell survival via the PI3K pathway, we determined the expression levels of the phosphorylated form of AKT at serine 473, a survival marker. LNCaP (Figures 2E and 2F) and PC3 cells (Figures 2K and 2L) dosed with GT3 or AT showed no significant changes on the expression levels of phospho-AKT as compared to the control, within the concentration range tested. In the case of non-cancerous prostate cells RWPE-1, there is no significant change on the activated forms of ERK (Figure 3B), c-JUN (Figures 3C and 3D), or AKT (Figures 3E and 3F) after treatment with GT3 or AT, except for phospho-ERK after treatment with GT3 80 μ M, which shows a fold increase of 3.08 (Figure 3A).

There is no significant change in the activated form of BAD in LNCaP and PC3 cells dosed with GT3 or AT. The loss of phosphorylation of BAD allows it to bind BCL-X_L and BCL2 and exert its apoptotic effect [11]. There are two phosphorylation sites on BAD, serine 136 and serine 112. It has been reported that the phosphorylation of serine 136 is dependent on phospho-AKT [12]. We have shown above that there is no change in this protein, and we were unable to detect phospho BAD serine 136 under the described experimental conditions (results not shown). The phosphorylation of BAD at serine 112 requires the activation of the RAF/RAS/ERK pathway[13] and changes may be detectable if the targeted pathway includes mitochondrial involvement. LNCaP and PC3 cells dosed with GT3 show no significant change on the expression levels of phospho-BAD at serine 112 (Figures 4A and 4E). When LncCaP and PC3 cells are dosed with AT, a downward trend in the expression levels of this activated form is observed. Statistical analysis of at least three assays show significant decrease (\approx 56% and 38%) for PC3 when the cells are dosed with 60 and 80 μ M of AT, respectively (Figure 4F, but not for LNCaP cells (Figure 4B).

There is no evidence of release of cytochrome c into the cytoplasm in LNCaP and PC3 cells

dosed with GT3 or AT. To test whether the intrinsic pathway of apoptosis is activated by GT3 or AT, we analyzed the levels of cytochrome c in the cytoplasm and the mitochondria after dosing LNCaP and PC3 and found no significant change as compared to the control for either GT3 (Figures 4C and 4G) or AT (Figures 4D and 4H).

GT3 activates caspases 9 and 3 in LNCaP and PC3 cells but not AT. The MTT and metabolic assays above had shown the inhibition of cell viability of LNCaP and PC3 cells by GT3. To test whether caspases 9 and 3 were involved in this process, we dosed the cells with GT3 or AT at the indicated concentrations. GT3 caused cleavage of caspases 9 above concentrations of 60 μ M in both LNCaP and PC3 cells (Figures 5A and 5E). Caspase 3 activation is observed above 60 and 80 μ M in LNCaP and PC3 cells respectively (Figures 5C and 5G). Conversely, AT causes no detectable effects on caspase 9 after dosing LNCaP and PC3 cells as shown in figures 5B and 5F. Similarly, cleaved caspase 3 is undetectable in these cells when

dosed with AT (Figures 5D and 5H). The observed results are in agreement with caspase activation by GT3 in prostate cancer cells reported previously [9].

The activation of the RAF/RAS/ERK pathway is essential for the inhibition of cell viability of prostate cells by GT3. To determine whether the observed activation of the ERK MAPK pathway is relevant to the effect on cell viability of prostate cancer cells by GT3, we used the MEK inhibitor U0126. As shown on Figures 6A and 6B, treating LNCaP and PC3 cells with U0126 completely precludes the cytotoxic effect of GT3, suggesting that the phosphorylation of ERK is required for causing apoptosis in these cells. Non-cancerous prostate RWPE-1 cells treated with inhibitor U0126 and GT3 show no difference to cells dosed with GT3 alone (Figure 6C).

Discussion

In vitro studies have shown that inhibition of cell viability of prostate cancer cells by GT3 is the result of a multipronged effect that involves the activation of the JNK pathway and suppression of NF- κ B [9]. Activated c-JUN at serine 73 is one of the components of transcription factor AP-1, which is involved in processes conducive to oncogenesis and cancer progression [14] as well as the initiation of apoptosis. The type of effect observed is cell dependent and is dictated by the characteristics of the molecules activating AP-1. Apoptosis can be initiated via the activation of both, the JNK and ERK pathways, with the subsequent formation AP-1 and caspase cleavage [15]. It has been reported that treatment of prostate cancer LNCaP and PC3 cells causes upregulation of activated JNK and its target c-JUN. However, the use of a SP600125, a specific JNK inhibitor, causes a partial block of the downstream phosphorylation of c-JUN, suggesting an additional source of activation. Here we have shown a significant upregulation of phospho-ERK, reported to be required for triggering apoptosis, thus circumventing mechanisms of resistance associated with constitutive activation of MEK1 [16]. Apoptotic effects involving the activation of both, c-JUN and ERK have been previously observed in LNCaP and PC3 prostate cancer cells treated with isothiocyanates, compounds present in cruciferous vegetables [15]. Our studies with inhibitor U0126, suggest that the phosphorylation of ERK is essential for the activation of caspases 9 and 3 and subsequent apoptosis of LNCaP and PC3 cells treated with GT3. However, apoptosis also requires the presence of activated c-JUN as demonstrated by the effect of AT on these cells, where the sole presence of upregulated phospho-ERK will not initiate apoptosis. Thus, the inhibition of cell viability by GT3 in prostate cancer cells requires the presence of the phosphorylated forms of ERK and c-JUN. Our results also exclude the involvement of the intrinsic pathway of apoptosis and the subsequent release of cytochrome c from the mitochondria. This process is initiated by the loss of phosphorylation of BAD at serine 112 or 136. Phospho-ERK is responsible for the activation of BAD at serine 112, whereas activated AKT at serine 473 is responsible for the activation of serine 136. We observed no change in phospho-AKT at serine 473, and were unable to detect phosphorylated BAD at serine 136. Additionally, there were no changes on the levels of activated BAD at serine 112 or on the levels of cytochrome c in the mitochondria.

Conclusions

The significant upregulation of activated c-JUN in GT3-mediated cell death is consistent with prior studies [9]. However, c-JUN can be phosphorylated by kinases other than JNK, which may allow GT3 to evade prostate cancer cell resistance [17]. Prostate cancer cells treated with GT3 display increased expression of activated ERK which can in turn activate apoptosis regardless of androgen sensitivity. The relevance of the involvement of these two pathways in the apoptotic process has been previously observed before in prostate cancer cells treated with isothiocyanates [15]. Caspase activation by GT3 and subsequent apoptosis is consistent with prior studies [18, 19]. AKT is an important target in prostate cancer therapy and modulates BAD [20, 21], c-JUN [22] and ERK [23]. However, neither the phosphorylation of AKT at serine 473 nor the expression levels of the protein are affected by GT3 under the conditions studied. Sustained activation of the ERK pathway is thought to be one of the mechanisms behind pro-apoptotic effects of cancer drugs [24]. In fact, the phosphorylated forms of ERK and c-JUN can trigger apoptosis without involvement of AKT[15].

Declarations

- Ethics approval and consent to participate

Not applicable (this manuscript does not report or involved the use of any animal or human data or tissue.

- Consent for publication

Not applicable.

- Availability of data and materials

All data analyzed during this study are included in this published article. The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

- Competing interests

The authors declare that they have no competing interests.

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Figures

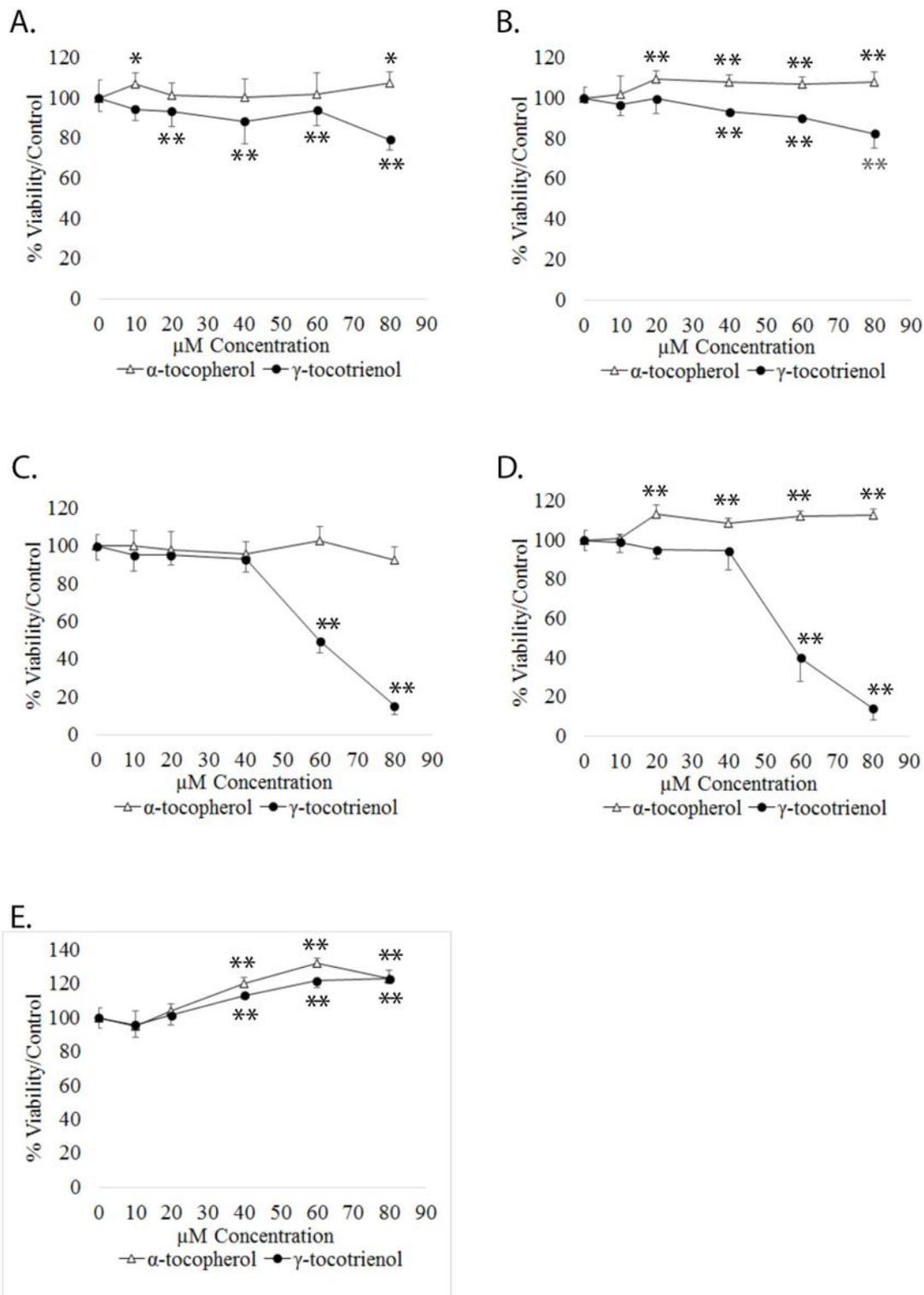


Figure 1

Effect of AT and GT3 on prostate cancer cells. A and B: LNCaP and PC-3 were treated with AT or GT3 at doses ranging from 10 to 80μM. After six hours of treatment, cell viability was determined via MTT and metabolic activity. C, D and E: LNCaP, PC-3, and non-tumorigenic RWPE-1 cells underwent the same treatment as described above for 12 hours. All graphs shown correspond to data obtained by analysis of metabolic activity from at least three independent experiments. For quantification spectrophotometric

data were calculated as percentages of the value for the untreated cells (100%) \pm standard deviations n=3, *p<0.05, **p<0.01.

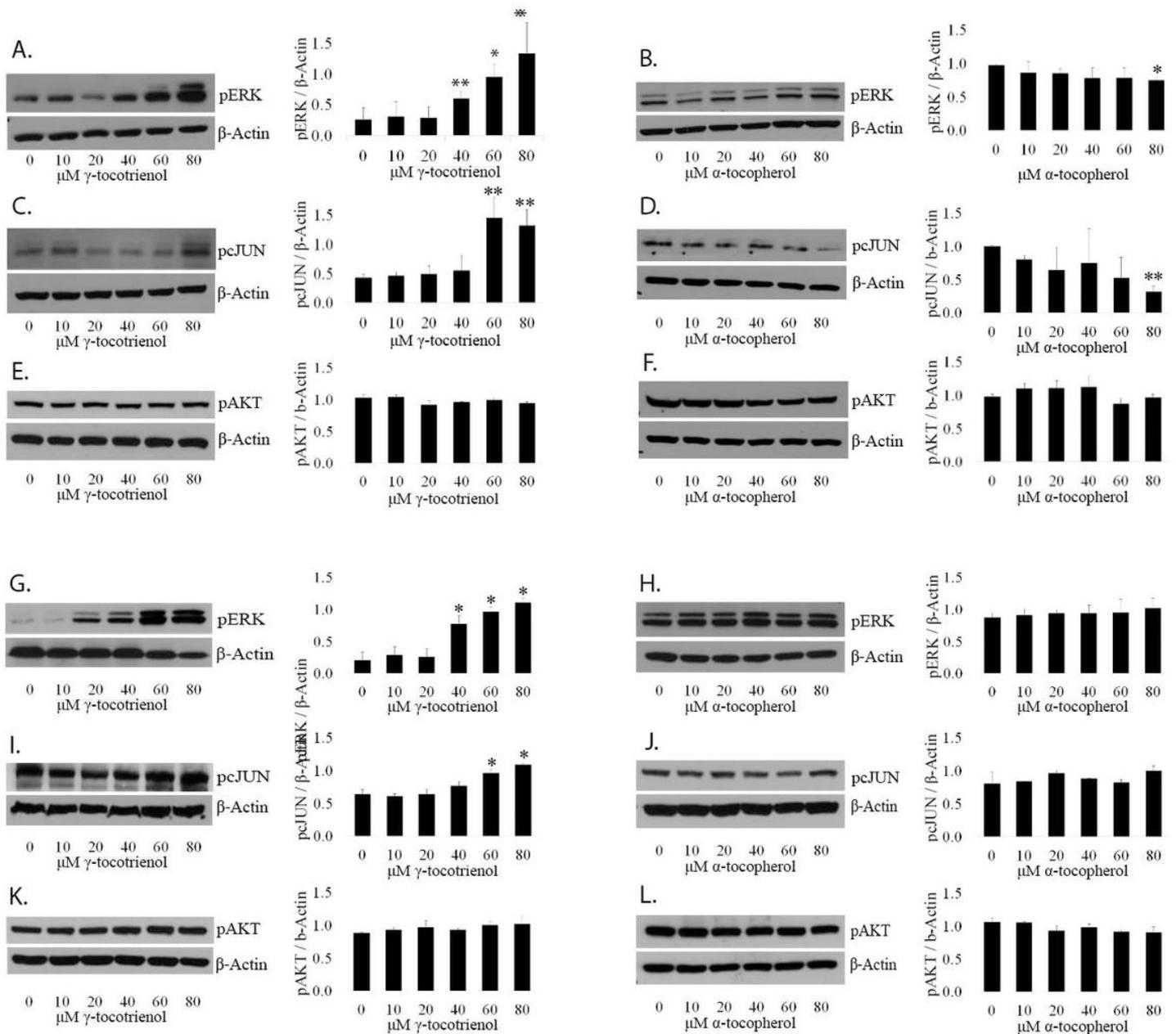


Figure 2

Involvement of the pERK, pc-JUN and pAKT in the response to treatment with GT3 or AT of prostate cancer cells. A, C, and E: LNCaP cells were grown on 6-wellplates and treated with GT3 for six hours at doses ranging from 10 to 80 μ M. Immunoblots from SDS total extracts were obtained using antibodies against (A) pERK, (C) pc-JUN, and (E) pAKT. B, D, and F: Similarly, LNCaP cells treated with AT for six hours under at the same dose range as described above. Immunoblots from total SDS extracts using antibodies against (B) pERK, (D) pc-JUN and (F) pAKT, are shown above. G, I, and K: PC3 cells were grown on 6-wellplates and treated with GT3 in the same manner as described above. Immunoblots were obtained from SDS total extracts by using antibodies against (G) pERK, (I) pc-JUN, and (K) pAKT. H, J,

and L: Immunoblots of SDS total extracts obtained from PC3 cells treated with AT as described above, were obtained using antibodies against (H) pERK, (J) pc-JUN, and (L) pAKT. Cells that were treated with neither GT3 nor AT, only with dissolution vehicle, were run as controls for each experiment. The membranes were reprobbed for actin as a loading control. The results presented are representative of three separate experiments. The values of each analyzed protein were normalized to actin. The values are averages \pm standard deviations from three independent experiments; * $p < 0.05$, ** $p < 0.01$.

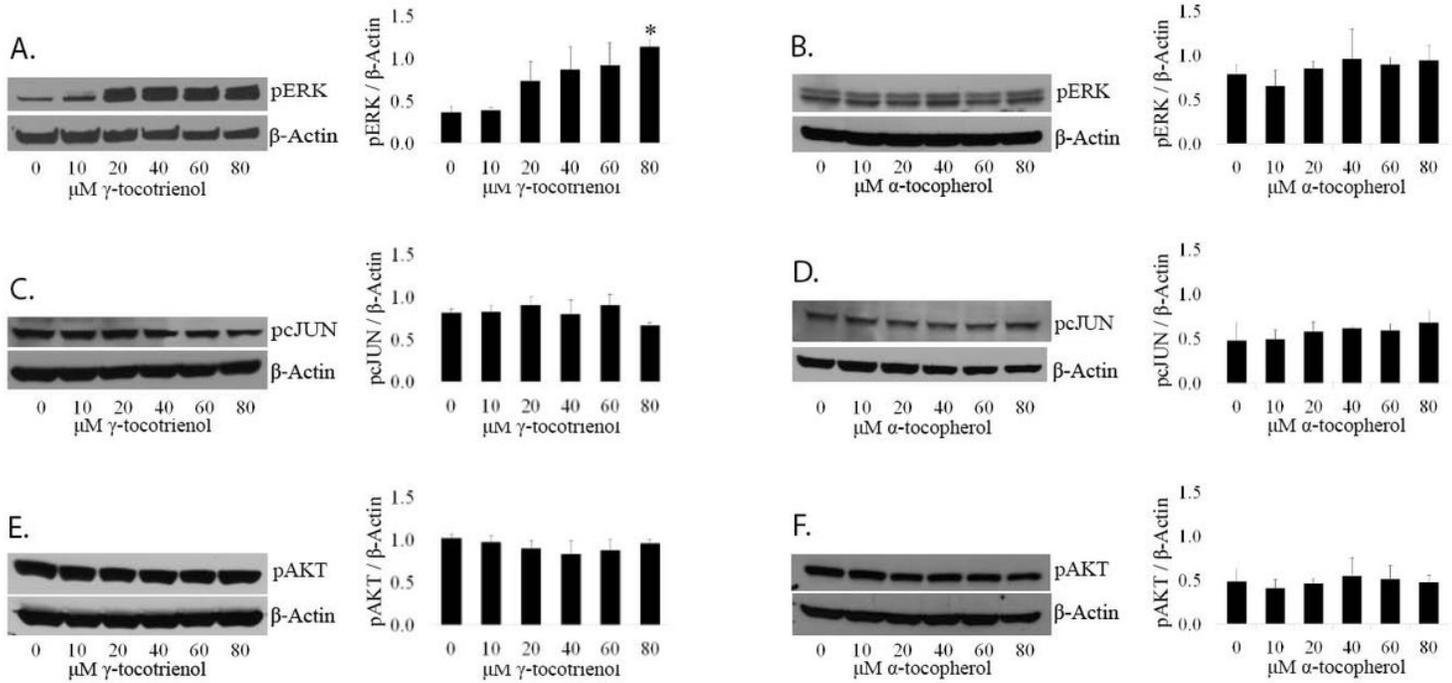


Figure 3

GT3 and AT have a similar effect on non-tumorigenic prostate cells. A and B: RWPE-1 cells grown on 6-wellplates and treated with (A) GT3 or (B) AT for six hours at doses ranging from 10 to 80 μM were analyzed by immunoblot using antibodies against pERK. C and D: RWPE-1 cells were treated as described above, and analyzed via immunoblot. SDS total extracts of (C) GT3 or (D) AT treated cells were probed using antibodies against pc-JUN. E and F: The expression levels of pAKT were analyzed via immunoblot in RWPE-1 cells treated with (E) GT3 or (F) AT. The membranes were reprobbed for actin as a loading control. The values of each analyzed protein were normalized to actin. The values are averages \pm standard deviations from at least three independent experiments; * $p < 0.05$.

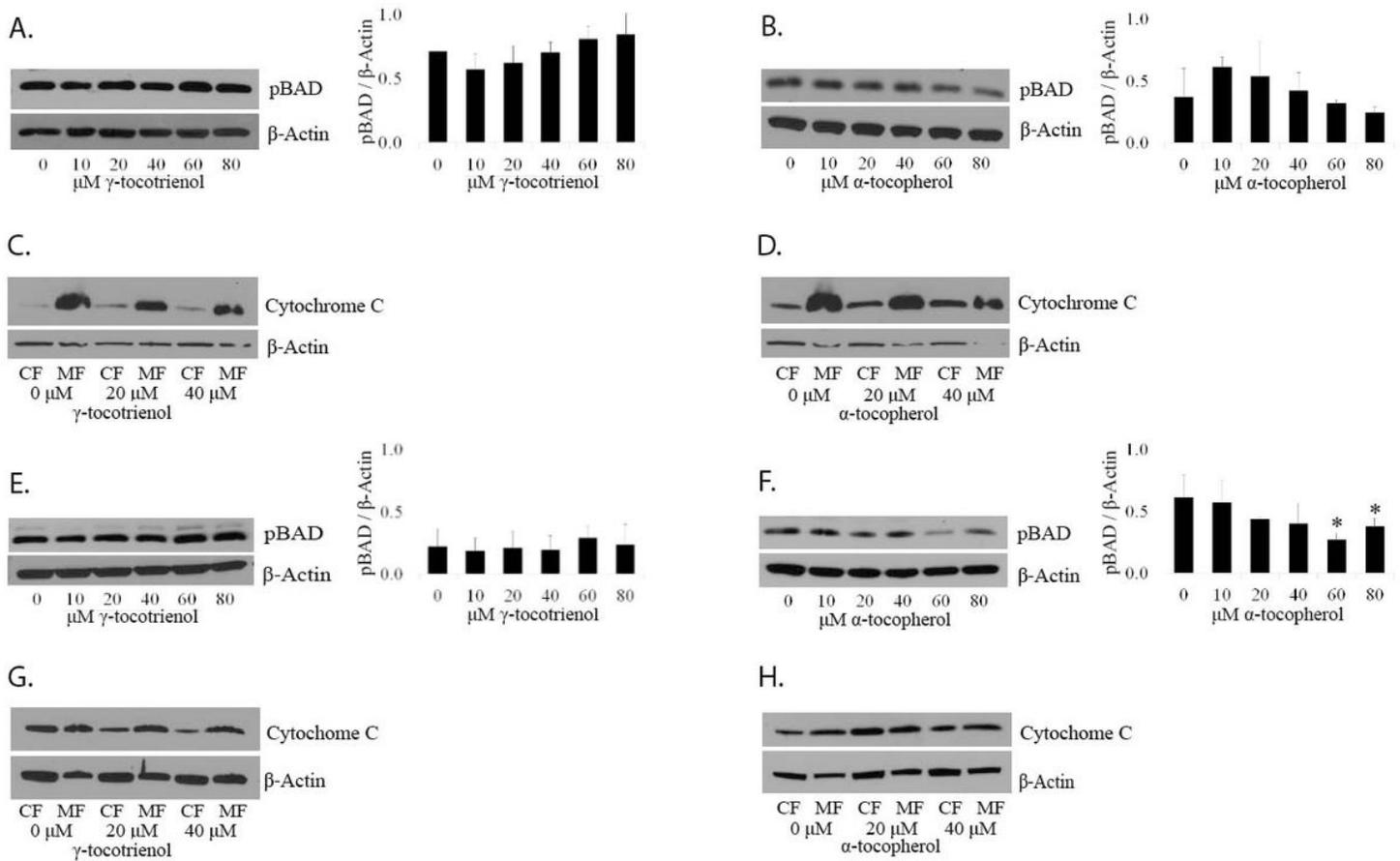


Figure 4

Effect on mitochondrial function of GT3 and AT on prostate cancer cells. A and B: LNCaP cells grown on 6-well plates and treated with (A) GT3 or (B) AT for six hours at doses ranging from 10 to 80 μ M were analyzed by immunoblot of SDS total extracts using antibodies against pBAD serine 112. C and D: Cytoplasmic (CF) and mitochondrial fractions (MF) were obtained from LNCaP cells treated with (C) GT3 or (D) AT as described above, and analyzed for the presence of cytochrome C by immunoblot. E and F: PC3 cells were grown on 6-well plates and dosed as described above with (E) GT3 or (F) AT. The SDS total extracts were analyzed for the presence of pBAD serine 112 via immunoblot. G and H: PC3 cells dosed with (G) GT3 or (H) AT as described above were processed to obtain cytoplasmic (CF) and mitochondrial fractions (MF) and probed for the presence of cytochrome C via immunoblot. The values of each analyzed protein were normalized to actin. The values are averages \pm standard deviations from at least three independent experiments,* $p < 0.05$.

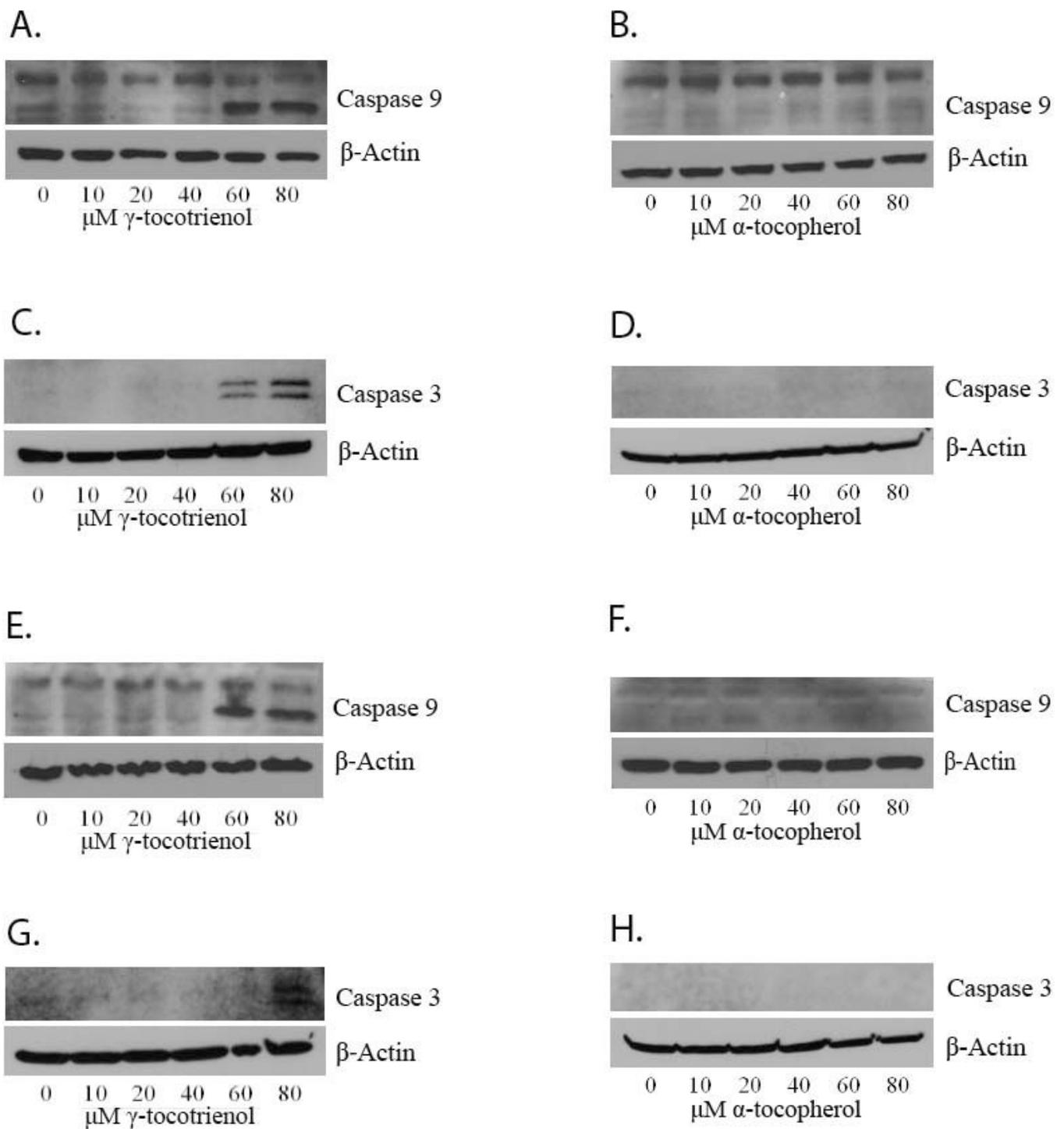


Figure 5

Apoptotic effect of GT3 and AT on prostate cancer cells. A, B, C, and D: SDS total extracts of LNCaP cells treated with (A and C) GT3 or (B and D) AT at doses ranging from 10 to 80 μ M were by immunoblot analyzed for the presence of cleaved caspase 9 (A and B) and caspase 3 (C and D). E, F, G, and H: In the same manner as described above, PC3 cells treated with (E and G) GT3 or (F and H) AT, were processed for immunoblot analysis with antibodies against cleaved caspases 9 and 3. The values of each analyzed

protein were normalized to actin. The results shown are representative of at least three independent experiments.

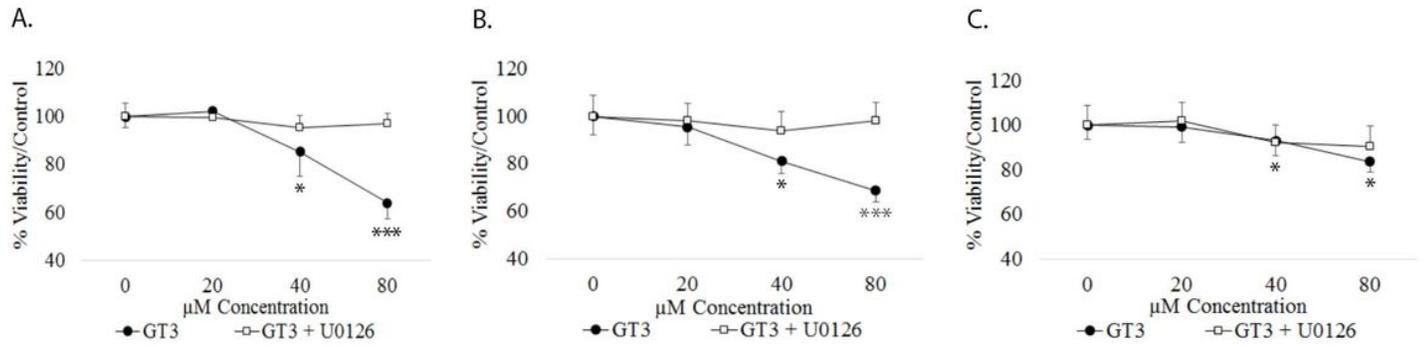


Figure 6

Effect of GT3 and AT on prostate cancer cells in the presence of inhibitor U0126. A, B and C: Prostate cancer cells (A) LNCaP, (B) PC-3, and (C) non-tumorigenic RWPE-1 cells were grown in 96-well plates. Two plates were used per cell line; plate one was pre-treated with inhibitor U0126 for one hour, after which, plates one and two were dosed for 24 hours with GT3 at 0, 20, 40 and 80 μM. Cell viability was analyzed by MTT or metabolic assays. For quantification spectrophotometric data were calculated as percentages of the value for the untreated cells (100%) ± standard deviations from at least three independent experiments, *p < 0.05, ***p < 0.001. Statistical analysis using ANOVA and Bonferroni test indicates a significant difference between cells treated with and without inhibitor.