

·Original Article·

α -Vitamin E derivative, RRR- α -tocopheryloxybutyric acid inhibits the proliferation of prostate cancer cells

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Abstract

Aim: To investigate the activity of RRR- α -tocopheryloxybutyric acid (TOB), an ether analog of RRR- α -tocopheryl succinate (VES), in prostate cancer cells. **Methods:** VES and TOB were used to treat prostate cancer LNCaP, PC3, and 22Rv1 cells and primary-cultured prostate fibroblasts. The proliferation rates were determined by MTT assay, the cell viabilities were determined by trypan blue exclusion assay, and the cell deaths were evaluated by using Cell Death Detection ELISA kit. The protein expression levels were determined by Western blot analysis. **Results:** The MTT growth assay demonstrated that TOB could effectively suppress the proliferation of prostate cancer cells, but not normal prostate fibroblasts. Mechanism dissections revealed that TOB reduced cell viability and induced apoptosis in prostate cancer cells similar to VES. In addition, both TOB and VES suppressed prostate-specific antigen (PSA) at the transcriptional level leading to reduced PSA protein expression. Furthermore, vitamin D receptor (VDR) expression increased after the addition of TOB. **Conclusion:** Our data suggests that the VES derivative, TOB, is effective in inhibiting prostate cancer cell proliferation, suggesting that TOB could be used for both chemopreventive and chemotherapeutic purposes in the future. (*Asian J Androl* 2007 Jan; 9: 31–39)

Keywords: α -tocopheryloxybutyric acid; α -vitamin E succinate; prostate cancer; prostate-specific antigen; vitamin D receptor; LNCaP; PC3, 22Rv1

1 Introduction

In the USA, prostate cancer is the leading cause of new cancer cases, accounting for approximately thirty-three percent of all new cases in males and is the third

leading cause of cancer-related death of men [1]. Factors such as diet, exercise and environment are all related in the subsequent development of prostate cancer. For example, studies from the American Cancer Society showed that in China the death rate from prostate cancer was nearly 16 times less than that of the USA [1]. This discrepancy could be attributed to numerous environmental and lifestyle factors. Epidemiological studies have indicated the protective role of certain vitamins and minerals in the prevention of prostate cancer. In particular, α -vitamin E analogs have been demonstrated to be potent anticancer agents [2–4].

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One of the most effective derivatives of α -vitamin E is the esterified analog RRR- α -tocopheryl succinate (VES). Studies from our lab and others have demonstrated the success of this compound in inhibiting malignant cells through multiple mechanisms [5–8]. However, VES can be hydrolyzed by esterase in the gastrointestinal tract, which impacts its efficacy and potency by oral intake. In this study, we overcame this limitation with the use of RRR- α -tocopheryloxybutyric acid (TOB), a non-hydrolyzable ether form of VES (Figure 1). These two vitamin E analogs are virtually identical except that the ester group of VES is replaced with an ether group for TOB. Therefore, TOB may be resistant to enzymatic hydrolysis during digestion, allowing the intact TOB to be orally taken, absorbed through the digestive tract, and subsequently delivered to the peripheral tissues.

While there have not been many studies on TOB, previous *in vitro* studies show that this compound is able to enhance necrotic-like cell death in breast cancer cells with its inactivation of the human epidermal growth factor receptor 2 (HER-2) and the induction of apoptosis [9, 10]. Research *in vivo* has demonstrated the efficacy of TOB in the suppression of cell proliferation in the mouse lung tumorigenesis model through the suppression of the Erk cascade [11]. These studies are important because they show that this inhibition of cell proliferation is independent of TOB antioxidative effect. Also, the method of treatment in these studies shows the effectiveness of oral administration of the compound, which highlights the clinical potential of TOB. While VES can be hydrolyzed in the

digestive tract with oral administration, TOB cannot be hydrolyzed and this provides an easy mechanism to deliver TOB throughout the body.

In this study, we extended the TOB studies into prostate cancer cells. Our studies indicated that TOB, like its ester analog VES, targeted the AR/PSA and VDR pathways to inhibit the proliferation of prostate cancer cells *in vitro*.

2 Materials and methods

2.1 Chemicals and reagents

TOB (RRR- α -TOB) was synthesized from RRR- α -vitamin E according to the method described by Fariss *et al.* [12]. VES (RRR- α -VES), succinic acid (Suc), silibinin and 5-dihydrotestosterone (DHT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The antibodies used for PSA, VDR, GAPDH and β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

2.2 Cell culture and treatment

Prostate cancer LNCaP, PC3 and 22Rv1 cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI medium 1640 with 10% fetal bovine serum (FBS). The primary-cultured fibroblasts were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS. The cells were treated with ethanol or Suc as controls, and TOB, VES, silibinin or DHT. During the treatment, the medium and

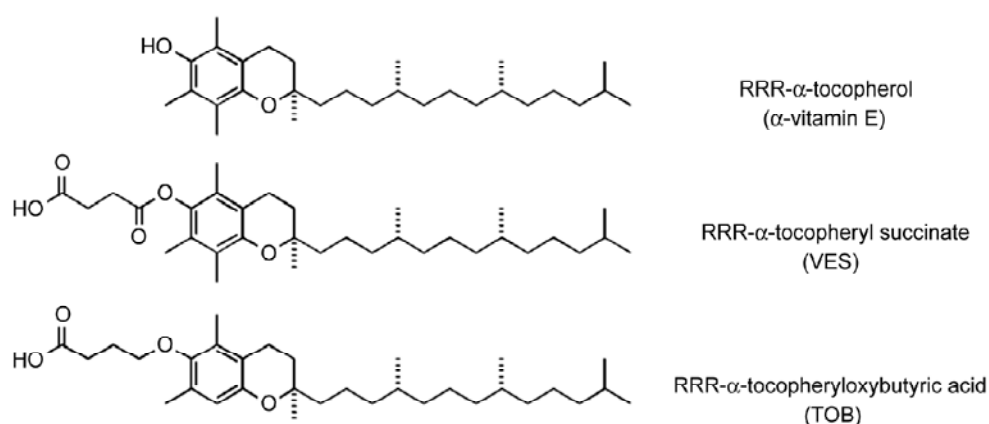


Figure 1. Schematic presentation of structures of RRR- α -vitamin E (α -Vit E), RRR- α -vitamin E succinate (VES) and RRR- α -tocopheryloxybutyric acid (TOB).

fresh drug treatments were applied every two days.

2.3 Establishment of primary-cultured prostate fibroblast

The prostate specimen was obtained from a prostate cancer patient undergoing radical prostatectomy at the University of Rochester Medical Center (NY, USA) under the guidelines of the Institutional Review Board. Prostate cancer sections were processed by routine histological technique by two independent pathologists. Portions of benign prostate tissues were cut into small pieces (~1–2 mm³), washed with culture medium, then plated on cell culture dishes with DMEM with 10% FBS. After 1–2 passages, the majority of cells (> 95%) were fibroblasts. All the experiments were performed within 10 passages.

2.4 Thiazolyl blue (MTT) growth assay

Briefly, cells were seeded on a 12-well plate or a 24-well plate. After approximately one day, cells were then treated with VES or TOB for 0 to 6 days. At the indicated time points, 0.5 mg/mL of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added into each well. After 3-hour incubation at 37°C, 1 mL of 0.04 mol/L HCl in isopropyl alcohol was added into each well. The absorbance was read at a wavelength of 595 nm. The MTT assay was used as a quantitative colorimetric assay to measure cell survival and proliferation and was performed as previously reported [5, 13].

2.5 Cell viability determined by trypan blue exclusion assay

LNCaP cells (2×10^4) were seeded in each well of 24-well plates. After approximately 1 day, the cells were then treated with VES or TOB. At the indicated time period, the cells were trypsinized, neutralized by medium, stained with 0.4% trypan blue solution, and then counted using a hemocytometer.

2.6 Cell death (apoptosis) assay

LNCaP cells were treated with 20 µmol/L VES or 50 µmol/L TOB for 4 days, then apoptotic cells were evaluated by using the Cell Death Detection ELISA^{plus} kit (Roche Molecular Biochemicals, Indianapolis, IN, USA). The preparation of cell lysates and the assay procedures were performed according to the manufacturer's protocol. Absorbance at 405 nm was measured as the indicator of apoptotic cells. The reference wavelength was 490 nm.

2.7 Cell transfection

For the luciferase reporter assay, LNCaP cells were seeded in 12-well plates at a concentration of 2×10^5 cells/well in RPMI 1640 medium with 10% charcoal-stripped fetal bovine serum (CSFBS) for approximately one day to allow cells to attach to the dish. Then the cells were transfected with the 6.0 kb PSA promoter-linked luciferase reporter (PSA6.0-Luc) by using Superfect (Qiagen, Valencia, CA, USA). After approximately one-day transfection, the cells were treated with several fresh drugs and allowed to grow for an additional one day. Every well, except for the ethanol control, was treated with DHT (5×10^{-9} mol/L) in addition to the drug. The plasmid pRL-TK was used as an internal control to monitor the transfection efficiency.

2.8 Western blot analysis

LNCaP cells were cultured on 100-mm dishes and grown to approximately 40% confluence. The cells were then treated with drugs and harvested either on day 2 or 4. Fifty microgram of protein from total cell lysates was resolved by 10% SDS-PAGE gel, and transferred to nitrocellulose membrane. After being blocked with blocking buffer (phosphate buffered saline [PBS] containing 0.1% Tween 20 and 10% FBS) for 2 hours, the membrane was incubated with primary antibody for 2 hours at room temperature. The membrane was then incubated with AP-conjugated secondary antibodies for another 2 hours at room temperature. The protein was detected by alkaline phosphate reagents (Santa Cruz Biotechnology, CA, USA).

2.9 Statistical analysis

The unpaired *t*-test was used to determine statistical differences between treatment and control. $P < 0.05$ was considered significant and $P < 0.01$ was considered highly significant.

3 Results

3.1 TOB inhibits cell proliferation of prostate cancer cells

We previously reported that VES has a strong anti-tumor activity but its metabolic products, α -vitamin E and Suc, do not [5]. Therefore, we expected that TOB, a non-hydrolyzable ether derivative of VES, could reproduce VES antineoplasia activity with a greater bioavailability *in vivo*. First, we examined TOB antiproliferative activity on differ-

ent prostate cancer cells by MTT assay. As shown in Figure 2, TOB dose-dependently suppressed the cell proliferation of prostate cancer LNCaP, 22Rv1 and PC3 cells. TOB at 50 $\mu\text{mol/L}$ has a similar suppressive activity to

20 $\mu\text{mol/L}$ VES, suggesting that TOB can effectively inhibit the proliferation of prostate cancer cells, but to a lesser extent than VES. In the following experiments we fixed the dose of TOB at 50 $\mu\text{mol/L}$ and VES at 20 $\mu\text{mol/L}$ because of their similar anti-proliferative efficacy in prostate cancer cells.

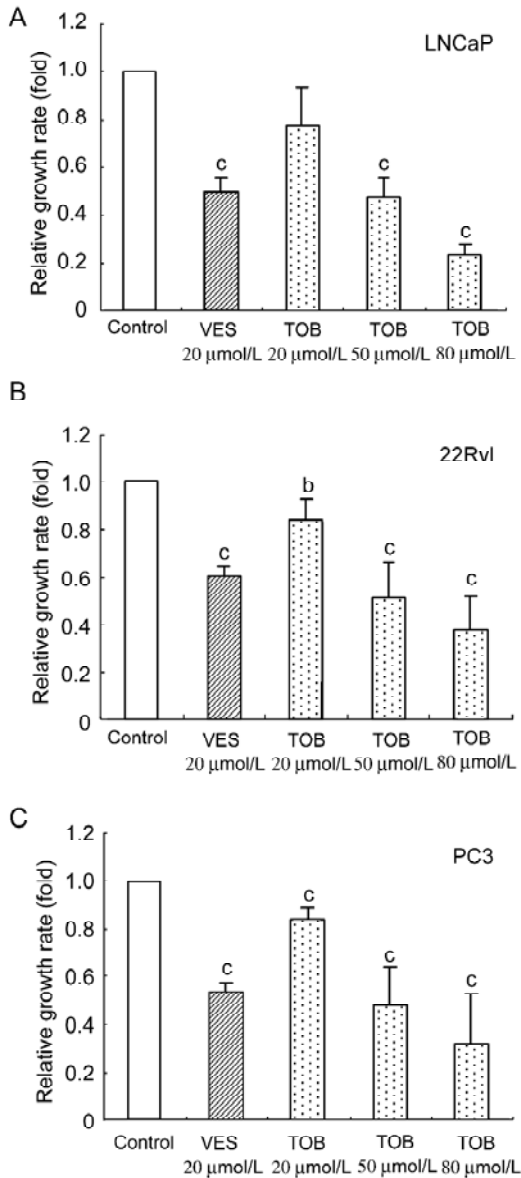


Figure 2. RRR- α -tocopheryloxybutyric acid (TOB) inhibits the growth of different prostate cancer cells. Prostate cancer LNCaP (A), 22Rv1 (B) and PC3 cells (C) were treated with VES at 20 $\mu\text{mol/L}$, or TOB at 20 $\mu\text{mol/L}$, 50 $\mu\text{mol/L}$ or 80 $\mu\text{mol/L}$ for 6 days. Cell proliferation was determined by MTT assay. The results of control are set as 1.0. The data are represented as mean \pm SD ($n = 3$). ^b $P < 0.05$, ^c $P < 0.01$, compared with the control.

3.2 TOB preferentially suppresses the proliferation of prostate cancer cells compared to non-malignant prostate fibroblasts

To determine TOB's effect on prostate cancer cell proliferation, we investigated TOB's antiproliferative effect on prostate cancer LNCaP cells and on primary-cultured prostate fibroblasts. As shown in Figure 3A, TOB at 50 $\mu\text{mol/L}$ exhibited a similar antiproliferative activity to VES at 20 $\mu\text{mol/L}$ in a time-dependent manner in prostate cancer LNCaP cells. The effectiveness could be observed within 2 days of treatment and showed the greatest effect over 4–6 days of treatment with an approximate 50% reduction in the presence of 50 $\mu\text{mol/L}$ TOB and 20 $\mu\text{mol/L}$ VES. In contrast, 50 $\mu\text{mol/L}$ TOB and 20 $\mu\text{mol/L}$ VES had little impact on the proliferation of prostate fibroblasts (Figure 3B). We next calculated the IC₅₀ of VES and TOB over a 4-day interval. The IC₅₀ values of VES and TOB in fibroblasts were $29.3 \pm 1.5 \mu\text{mol/L}$ and $83.7 \pm 3.8 \mu\text{mol/L}$, respectively, while they were $19.3 \pm 1.5 \mu\text{mol/L}$ and $47.3 \pm 3.5 \mu\text{mol/L}$ in LNCaP cells, respectively. These data suggested that TOB has a strong ability to reduce the proliferation of cancer cells compared to benign cells and that TOB can inhibit the cancer cell growth, although to a lesser extent than VES.

Furthermore, the anti-proliferative activity of TOB was confirmed by direct cell number counting using a hemocytometer after staining LNCaP cells with trypan blue (Figure 3C). In addition, both VES and TOB could induce apoptosis by approximately 6–7 fold compared to the control in LNCaP cells (Figure 3D). Therefore, TOB exhibited anti-tumor activity through a reduction of cell viability and the induction of apoptosis in cancer cells.

3.3 TOB inhibits the expression of PSA, a diagnostic marker for prostate cancer

PSA is a marker that can be used to screen and monitor the progression of prostate cancer [14]. We have previously reported that VES could suppress the expression of PSA [5]. To determine whether TOB has a similar effect on PSA expression, we performed western blot analysis and our results revealed that both VES and TOB

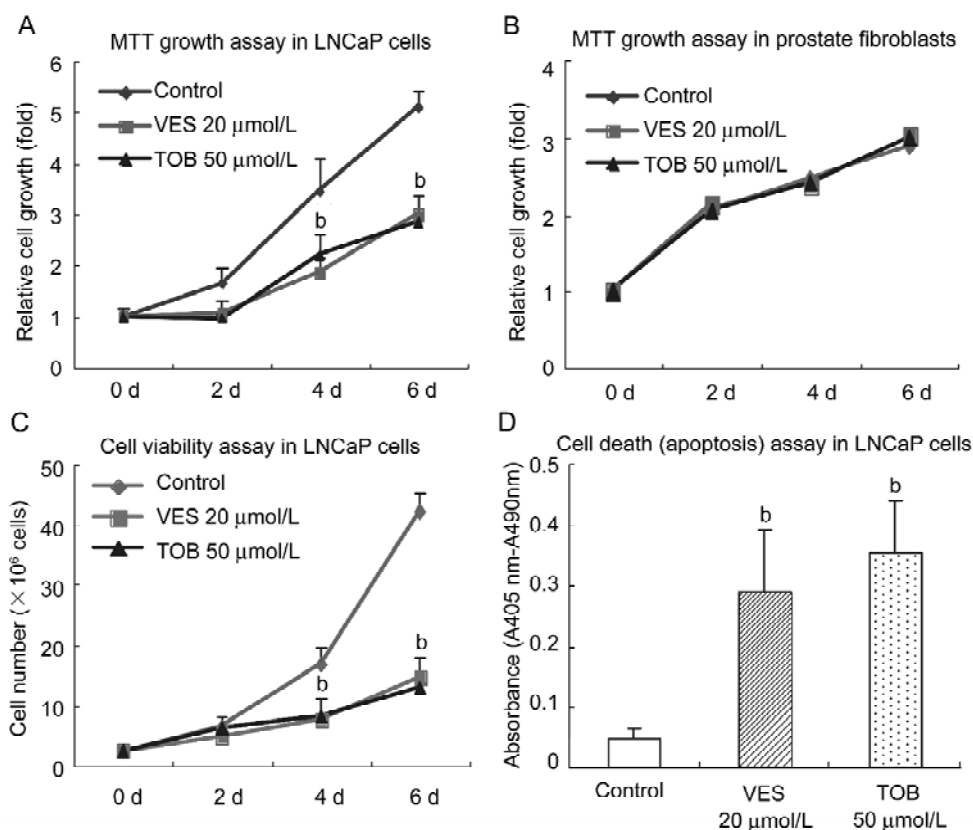


Figure 3. RRR- α -tocopheryloxybutyric acid (TOB) preferentially inhibits the proliferation of prostate cancer LNCaP cells as compared to normal prostate fibroblast cells. MTT assay was performed to measure cell proliferation with 20 μ mol/L RRR- α -vitamin E succinate (VES) or 50 μ mol/L TOB treatment for the indicated times in LNCaP cells (A) and non-malignant prostate fibroblasts (B). The results of day 0 are set as 1. The data are represented as mean \pm SD of triplicate samples. ^b $P < 0.05$, ^c $P < 0.01$, compared to the control at each time point. (C): TOB reduces the viability of prostate cancer LNCaP cells. LNCaP cells were seeded on 24-well plates and treated with 20 μ mol/L VES or 50 μ mol/L TOB for the indicated time periods, then the cells were stained with trypan blue and counted using a hemacytometer. The data are represented as mean \pm SD of triplicate samples. ^b $P < 0.05$, ^c $P < 0.01$, compared to the control at each time point. (D): TOB induces apoptosis in prostate cancer LNCaP cells. LNCaP cells were seeded and treated with 20 μ mol/L VES or 50 μ mol/L TOB for 4 days. The apoptosis was evaluated by the determination of cytoplasmic histone-associated-DNA fragments using Cell Death ELISA kit. The data are represented as mean \pm SD. ^b $P < 0.05$, ^c $P < 0.01$, compared to the control.

were able to inhibit PSA at the protein level (Figure 4A).

Knowing that TOB could inhibit prostate cancer cell proliferation and suppress the expression of PSA, we were interested in determining whether the reduction of PSA expression is the consequence of general growth inhibition. Our results indicated that 10 μ g/L silibinin had a similar antiproliferative activity to VES and TOB. The growth inhibition reached up to approximately 50% compared to the control after a 4-day treatment (Figure 4B, right panel). However, 10 μ g/L silibinin could not reduce PSA protein expression while 20 μ mol/L VES and 50 μ mol/L TOB reduced PSA protein expressions by

40%–60% (Figure 4B, left panel).

To further determine the mechanism by which TOB inhibited PSA protein expression, we used a luciferase reporter driven by the 6.0 kb of PSA 5'-promoter (PSA6.0-Luc) to investigate TOB effects. As shown in Figure 4C, both VES and TOB could suppress DHT-induced PSA6.0-Luc activity by 60%–70%, suggesting that TOB could repress PSA expression at the transcriptional level. Collectively, the results in Figure 4 show that TOB could repress PSA expression in prostate cancer cells.

3.4 TOB increases the expression of VDR

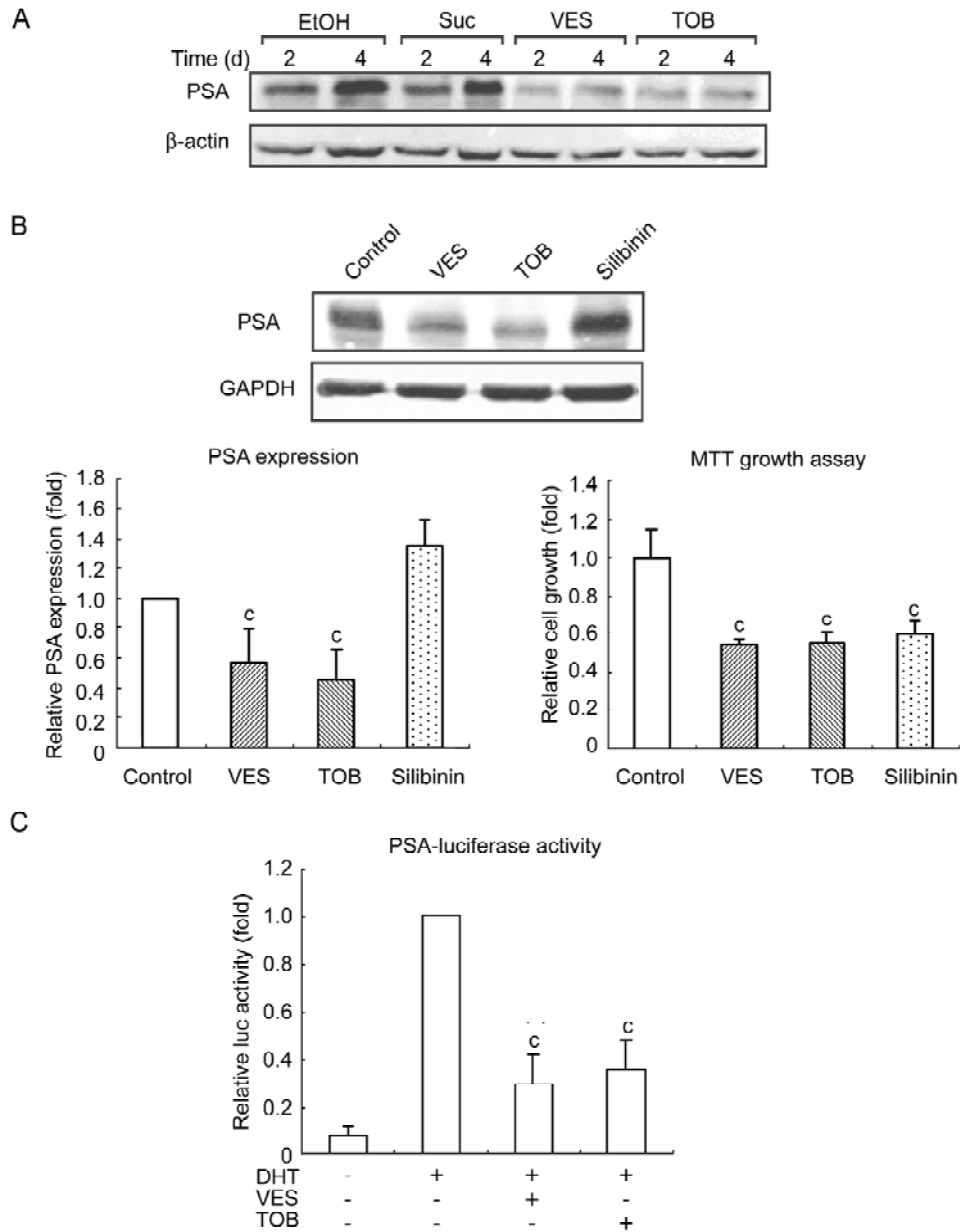


Figure 4. RRR- α -tocopheryloxybutyric acid (TOB) inhibits the expression of prostate specific antigen (PSA). (A): TOB inhibits PSA at protein level. LNCaP cells were treated with 20 μ mol/L RRR- α -vitamin E succinate (VES), 50 μ mol/L TOB or 20 μ mol/L succinic acid (Suc) for the indicated time period. The cells were harvested and Western blot analyses were performed with anti-PSA antibody. β -actin was used as the loading control. (B): The reduction of PSA protein levels is not the consequence of the growth inhibition. LNCaP cells were treated with 20 μ mol/L VES, 50 μ mol/L TOB or 10 μ g/L silibinin for 4 days. The expressions of PSA were revealed by Western blot analyses. GAPDH was used as protein loading control. The image data represent one of three experiments. ImageJ software (<http://rsb.info.nih.gov/ij/>) was used to quantify the PSA levels. The data of control are set as 1.0. The data are represented as mean \pm SD of three to six independent experiments. $^{\circ}P < 0.01$, compared to control (left panel). The anti-proliferative effects of those agents in LNCaP cells were determined by MTT assay (right panel). The data of control are set as 1.0. The data are represented as mean \pm SD. $^{\circ}P < 0.01$, compared to control. (C): TOB inhibits PSA at the transcriptional level. LNCaP cells were transiently transfected with PSA6.0-Luc plasmids with treatment of 20 μ mol/L VES or 50 μ mol/L TOB in the presence of 5 nmol/L 5-dihydrotestosterone (DHT) for one day, and then luciferase activity was assayed. pRL-TK served as the internal control for transfection efficiency. The effect of DHT treatment is set as 1.0. The data are represented as mean \pm SD of three independent experiments. $^{\circ}P < 0.01$, compared to the DHT treatment only.

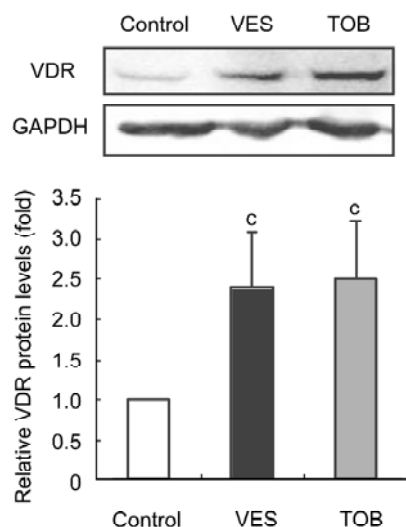


Figure 5. RRR- α -tocopheryloxybutyric acid (TOB) enhances the expression of vitamin D receptor (VDR). LNCaP cells were treated with 20 μ mol/L VES or 50 μ mol/L TOB for 4 days. The cells were harvested and Western blot analyses were performed with anti-VDR antibody. GAPDH was used as the loading control. The image data represent one of three experiments. ImageJ software (<http://rsb.info.nih.gov/ij/>) was used to quantify the VDR protein levels. VDR level of control treatment is set as 1.0. The data are represented as mean \pm SD of at least three independent experiments. ^c $P < 0.01$, compared to the control.

The VDR is a member of the nuclear receptor superfamily and it mediates vitamin D antiproliferative activity in various cancers, including prostate cancer [15]. In our previous study, we found that one potential mechanism by which VES suppressed LNCaP cell proliferation could be through the increase of VDR expression [5]. However, it is unclear whether TOB may also promote the expression of VDR protein. Therefore, we tested if TOB could exhibit a similar effect like VS to enhance VDR expression. The data in Figure 5 shows that the addition of either VES or TOB can increase the expression of VDR in LNCaP cells. The induction was approximately 2.5 fold compared to the control treatment. Clearly, our result indicates that TOB can also increase the expression of VDR in prostate cancer cells.

4 Discussion

4.1 TOB differentially inhibits the proliferation of prostate cancer cells as compared to primary cultured fibroblasts

The cell proliferation data in this study showed the efficacy of TOB in inhibiting the proliferation of LNCaP cells, and other prostate cancer PC3 and 22Rv1 cells. However, 50 μ mol/L TOB did not affect non-malignant fibroblast proliferation, suggesting that it might have selectively inhibitory effects on the proliferation of prostate cancer cells. The further analysis showed that the IC₅₀ of TOB is approximately 48 μ mol/L in LNCaP cells, while it is \sim 83 μ mol/L in normal fibroblast cells. This may provide a safety dose threshold for potential pre-clinical and clinical application of TOB.

4.2 TOB inhibits PSA protein expression

In this study, we found that TOB and VES could inhibit the protein expression levels of PSA. It is unlikely that this inhibition is the consequence of general antiproliferative activity. This conclusion is driven from the evidence that silibinin at 10 μ g/L could inhibit the proliferation of prostate cancer cells, but it could not inhibit PSA protein expression (Figure 4B). We did not rule out the possibility that a high dose of silibinin could also reduce PSA expression. Previously, Zi *et al.* [16] reported that silibinin at 50 μ g/L inhibited the growth of LNCaP cells by approximately 90% and significantly reduced PSA expression, yet a high dose of silibinin could be toxic to cells. Importantly, our results clearly indicate that 10 μ g/mL silibinin can effectively inhibit the cell viability without altering the PSA expression. Therefore, reduction of PSA is not a robust consequence triggered by growth inhibition.

4.3 TOB increases VDR expression

The use of vitamin D and targeting the VDR in the anticancer pathway of prostate cancer has been of great interest. However, high doses of vitamin D can induce numerous side effects, including hypercalcemia, which may complicate the therapeutic effects of vitamin D in prostate cancer treatment [17]. Therefore, it is of great interest to determine how to sensitize low-dose-vitamin D-mediated anti-tumor activity for clinical application.

In our study, we found that TOB could induce VDR expression. Thus, it is possible that TOB can enhance vitamin D-mediated anti-tumor activity in prostate cancer cells. TOB might sensitize prostate cancer cells to the effects induced by vitamin D at lower doses that otherwise would not exhibit antiproliferative activity in prostate cancer cells. The data from this study built a base to further test whether the combined treatment of TOB and

vitamin D has a better control for prostate cancer.

4.4 VES versus TOB in anticancer effect

Previous studies have reported that VES has antiproliferative activity in cultured cancer cells, but not in normal cells [5]. VES has also been reported as able to inhibit the growth of mammalian tumor in mice. However, such anti-tumor activity can only be exhibited by intraperitoneal injection and not by oral administration [18] (unpublished data, 2006). The main reason could be that VES is hydrolyzed to Suc and α -vitamin E, which do not have strong anti-tumor activities. To compensate this defect, TOB, the ether analog of VES, has been created [12]. In this study, we further characterized TOB in prostate cancer cells and our results indicated that TOB can inhibit the proliferation of prostate cancer cells, although to a slightly lesser extent compared to VES. It seems that the mechanisms by which TOB exhibits anti-tumor activity are similar to those of VES in cultured prostate cancer cells. Both can reduce cell viability and induce apoptosis in prostate cancer cells. In addition, TOB and VES can modulate the expression of PSA and VDR. These studies suggest that TOB has a similar effect to VES in cultured prostate cancer cells. Supporting our results, Wu *et al.* [19] also reported that TOB can act through mechanisms similar to VES to inhibit the proliferation of prostate cancer cells *in vitro*. An important point to consider clinically is that TOB has an ether bond instead of an ester bond in VES, therefore, TOB might be resistant to digestive esterase hydrolysis. As a consequence of this, the half-life of TOB will increase and the bioavailability of TOB will be greater than that of VES *in vivo* after oral intake. An investigation into this possibility will be performed in the future. If the results are positive, TOB unique *in vivo* properties might confer to it a greater clinical advantage than VES, allowing TOB to serve as a better chemopreventive and chemotherapeutic drug in prostate cancer treatment.

4.5 Potential clinical application of TOB in prostate cancer

Although there have been many advances in radiotherapy, chemotherapy and surgery, the use of complementary therapies for prostate cancer remains of key interest [20]. The use of vitamin E in complementary therapy could provide many benefits to cancer patients through its complementary or synergistic effects.

TOB has a bright future in clinical application. Its

ether side group provides great clinical promise as it can be orally administered to cancer patients. Another benefit would be to potentially treat androgen-independent prostate cancer patients as current anti-androgen therapies are ineffective [20]. The next step is to pursue the studies of TOB by oral administration in preclinical animal models. If the results are positive, it might allow us to develop a new potential therapeutic approach to battle against prostate cancer.

5 Conclusion

In conclusion, we report that TOB, the ether analog of VES, significantly and differentially inhibits the growth of prostate cancer cells compared to non-malignant prostate fibroblasts. This inhibition is associated with the reduction of cell viability and induction of apoptosis. In addition, TOB can decrease the expression of PSA at both the protein and transcriptional levels and increase VDR expression. Because of its ether bond, TOB might resist esterase hydrolysis in the gastrointestinal tract and may have a greater clinical advantage than VES. Therefore, TOB can serve as a better chemopreventive and chemotherapeutic drug in prostate cancer treatment. The further identification of new VES ether analogs with higher anticancer efficacy will have a significant clinical ramification.

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Erratum

Efficacy and safety of on demand tadalafil in the treatment of East and Southeast Asian men with erectile dysfunction: a randomized double-blind, parallel, placebo-controlled clinical study

Wai Chun Andrew Yip, Han-Sun Chiang, Jesus Benjamin Mendoza, Hui-Meng Tan, Man-Kay Li, Wei Christine Wang, Vladimir Kopernicky

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