

In vitro and *In vivo* Anticancer Effects of the Novel Vitamin E Ether Analogue *RRR*- α -Tocopheryloxybutyl Sulfonic Acid in Prostate Cancer

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Abstract Purpose: Among derivatives of α -vitamin E, α -vitamin E succinate (VES), has attracted much attention due to its potent anti-prostate cancer activity *in vitro* and *in vivo*. However, the *in vivo* antitumor activity of VES might be compromised if administered orally due to the VES hydrolysis by esterases in the gastrointestinal tract.

Experimental Design: New nonhydrolyzable VES ether analogues were synthesized and their growth inhibition was screened by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide growth assay. Among them, *RRR*- α -tocopheryloxybutyl sulfonic acid (VEBSA) was further characterized by terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling apoptosis assay, soft agar assay, and *in vivo* tumor formation.

Results: VEBSA has potent antitumor ability, albeit to a lesser extent than VES, in *in vitro* cultured prostate cancer LNCaP and PC3 cells. Like VES, VEBSA induced apoptosis, repressed androgen receptor protein expression, and enhanced vitamin D receptor expression, suggesting that VEBSA can go through mechanisms similar to those used by VES to inhibit the growth of prostate cancer cells *in vitro*. However, 6 weeks of oral consumption of VEBSA, but not of VES, reduced the tumor burden in the xenografted prostate tumors in nude mice. Furthermore, oral intake of VEBSA for 20 weeks inhibited prostate tumor growth and progression more efficiently compared with VES in the prostate cancer tumor model of TRAMP mice.

Conclusion: Oral consumption of VEBSA allows a greater anticancer activity compared with VES. Chemoprevention prefers the oral consumption of agents; the advantage of VEBSA over VES to be administered orally will allow VEBSA to serve as an agent for both preventive and therapeutic purposes for prostate cancer.

Growing lines of evidence suggest that VES is one of the most potent α -vitamin E analogues in terms of antiproliferative activity, and the underlying mechanisms in different cancer cells have been proposed (1–8). Importantly, VES selectively inhibits the growth of cancer cells without affecting normal cells. This has been shown in cultured cells and animal models

(3, 9–11). However, the efficacy of VES in mouse cancer models requires VES to be delivered by i.p. administration, not by oral gavage (12), suggesting that i.v. application is required for VES to inhibit tumor growth in humans. Therefore, VES might be inconvenient for chemopreventive or therapeutic purposes. It is hypothesized that the low efficacy of VES by oral intake might be due to low bioavailability of VES, possibly caused by the presence of esterases in the gastrointestinal tract, which hydrolyze VES to α -vitamin E and succinic acid, which do not have antiproliferative activity on cancer cells. To validate whether low bioavailability is the cause of the low efficacy of VES administered orally, we measured and calculated the tissue concentrations of VES in mice after either oral gavage or i.p. administration. Although twice as much VES was administered by oral gavage compared with i.p. administration, the oral gavage VES resulted in lower VES amounts in serum, liver, prostate, and testis, at least by 5-fold, than those after i.p. administration (data not shown). Consistently, it has been reported that the resistance of ovarian carcinoma cp70 cells to VES-induced apoptosis was due to high levels of cellular esterase. This conclusion is based on the observation that VES did not induce apoptosis in cp70 cells unless the cells were pretreated with the esterase inhibitor bis-(*p*-nitrophenyl) phosphate (13). Thus, the bioavailability of VES is a major concern for its application in chemoprevention.

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Translational Relevance

Vitamin E succinate (VES) has attracted much attention due to its antiproliferative function *in vitro*. However, it only exhibits its antitumor function *in vivo* through i.p. injection, which is not convenient for humans, especially for chemopreventive purpose. The aim of this study was to develop new VES analogues with greater bioavailability *in vivo*. We found that orally administered *RRR*- α -tocopheryloxybutyl sulfonic acid (VEBSA) is better than VES in reducing prostate tumor burden without overt toxicity in preclinical mouse cancer models. In addition, the serum level of VEBSA is substantially higher than that of VES after mice were gavaged with the same amounts of VEBSA and VES. Those studies suggest that VEBSA could be an excellent and better agent for both chemopreventive and chemotherapeutic purposes for prostate cancer. Our study thus provides a strong basis to design protocols for prostate cancer therapy in future clinical trials.

The aim of this study was to develop new VES analogues with greater bioavailability *in vivo*. We developed a number of new nonhydrolyzable ether analogues of VES with sulfonic and phosphonic moieties. After screening their effects on the growth of prostate cancer cells, we selected *RRR*- α -tocopheryloxybutyl sulfonic acid (VEBSA) for further characterization due to its relatively higher antiproliferative activity. VEBSA required twice the concentration relative to VES to reach a similar antiproliferative effect as VES on the growth of *in vitro* cultured prostate cancer cells. However, the same amount of orally administered VEBSA is better than VES in reducing prostate tumor burden in preclinical mouse cancer models. Those results suggest that VEBSA could be an excellent and better agent for both chemopreventive and chemotherapeutic purposes for prostate cancer.

Materials and Methods

Cell culture and culture conditions. The LNCaP and PC3 cells were maintained as described previously (14).

Chemicals and reagents. VES and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma. Phospho-Akt Ser473 antibody was from Cell Signaling Technology. Vitamin D receptor (VDR) and glyceraldehyde-3-phosphate dehydrogenase antibodies were purchased from Santa Cruz Biotechnology. Androgen receptor antibody NH27 was produced as previously reported (15).

MTT cell growth assay. MTT growth assay was done as described previously (14). IC_{50} was calculated by curve fitting (16).

Soft agar colony formation assay. The effects of VES and VEBSA on the survival of prostate cancer cells were determined by soft agar colony forming assay. After 3-d treatment with VES or VEBSA, prostate cancer cells were trypsinized and counted; 5,000 cells were suspended in 0.4% low melting agarose (Cambrex) and then layered on top of 1.5 mL of 0.8% agarose in six-well culture plates. Cells were incubated at 37°C in humidified incubator for 3 to 4 wk. Then the plates were stained with 0.5 mL of 0.005% crystal violet in methanol for 2 h and colonies were counted under a dissecting microscope.

Quantification of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay. Cell apoptosis was detected by

the Fluorescent FragEL DNA fragmentation kit (EMD Bioscience), as instructed by the manufacturer. Briefly, after being treated with VES or VEBSA for 3 d, prostate cancer cells were trypsinized and counted and fixed in 4% paraformaldehyde (in PBS) for 15 min at room temperature, and then stored in 80% ethanol at 4°C overnight. Cells (1×10^6) were washed with PBS and incubated with 20 μ g/mL proteinase K for 5 min at room temperature, and then incubated with Fluorescein-FragEL TdT labeling reaction mix at 37°C for 1.5 h in the dark; apoptotic cells were counted by flow cytometry (FACSCalibur, BD Company).

Western blot analysis. Western blot analysis was done as described previously (14).

In vivo tumor studies. Six-week-old male athymic nude mice (Charles River) were injected s.c. in two flanks with 1×10^6 PC3 cells resuspended in 30% Matrigel (BD). One week after cell implantation, animals were sorted randomly into three groups with six mice for each group. TRAMP mice in a pure C57BL/6 background were maintained and bred, and transgene screening was done as previously described (17). Male TRAMP mice (7-8 wk old) were randomly distributed into three groups ($n = 9$). VEBSA, VES, or vehicle control was then administered by oral gavage at the dosage of 200 μ L of 50 mmol/L in sesame oil containing 10% DMSO. Compounds were orally administered on Monday, Wednesday, and Friday followed by a 2-d rest, for a total of 6 wk for nude mice and 22 wk for TRAMP mice. Mice were monitored weekly for body weight and tumor formation. Xenograft tumor volumes were calculated using the following formula: $(\text{length} \times \text{width}^2)/2$ (18). At necropsy, xenograft tumors were dissected out and weighed. For TRAMP mice, the genitourinary apparatus consisting of bladder, urethra, seminal vesicles, ampullary gland, and the prostate was excised and weighed. The dorsolateral prostate was excised for histologic analysis. Several nude mouse and TRAMP mouse organs, including brain, heart, lungs, liver, kidneys, spleen, gastrointestinal tract, and testis, were collected for H&E staining to assess the drug toxicity effect. All the animal procedures were reviewed and approved by the University Committee on Animal Resources at the University of Rochester (19).

Results

Synthesis of α -vitamin E analogues. All-racemic α -tocopherol (synthetic compound) was purchased from Acros and used to synthesize the all-racemic α -tocopherol analogues (VES, VEBSA, and VEPSA). The *R,R,R*- α -tocopherol, used to synthesize the *R,R,R*- α -tocopherol analogues (*RRR*-VEBSA, *RRR*-VEPSA, *RRR*-VEBPA, and *RRR*-VEPPA), was obtained from the hydrolysis of the succinate ester of (+)- α -tocopherol succinate, which was purchased from Sigma-Aldrich.

All-racemic α -tocopherol succinate (Rac-VES) was obtained in one step via esterification of α -tocopherol with succinic anhydride in the presence of 4-(dimethylamino)pyridine (Fig. 1A, Eq. 1). The sulfonic acid analogues, VEBSA and VEPSA, were synthesized from α -tocopherol by alkylation of the alkoxide of α -tocopherol with 1,3-propane sultone and 1,4-butane sultone, respectively (Fig. 1A, Eq. 2). The phosphonic acid analogues, *RRR*-VEBPA and *RRR*-VEPPA, were synthesized from *R,R,R*- α -tocopherol via five-step sequences involving alkylation with 3-bromopropanol or 4-bromobutanol, respectively, followed by conversion of the terminal alcohol to an iodide. The iodide was displaced with triethylphosphite and the resulting phosphonates were subsequently hydrolyzed to produce the phosphonic acids (Fig. 1A, Eq. 3).

α -Vitamin E ether analogues inhibit prostate cancer cell growth. It has been shown that VES, but not its metabolic products, α -vitamin E or succinic acid, has strong antitumor

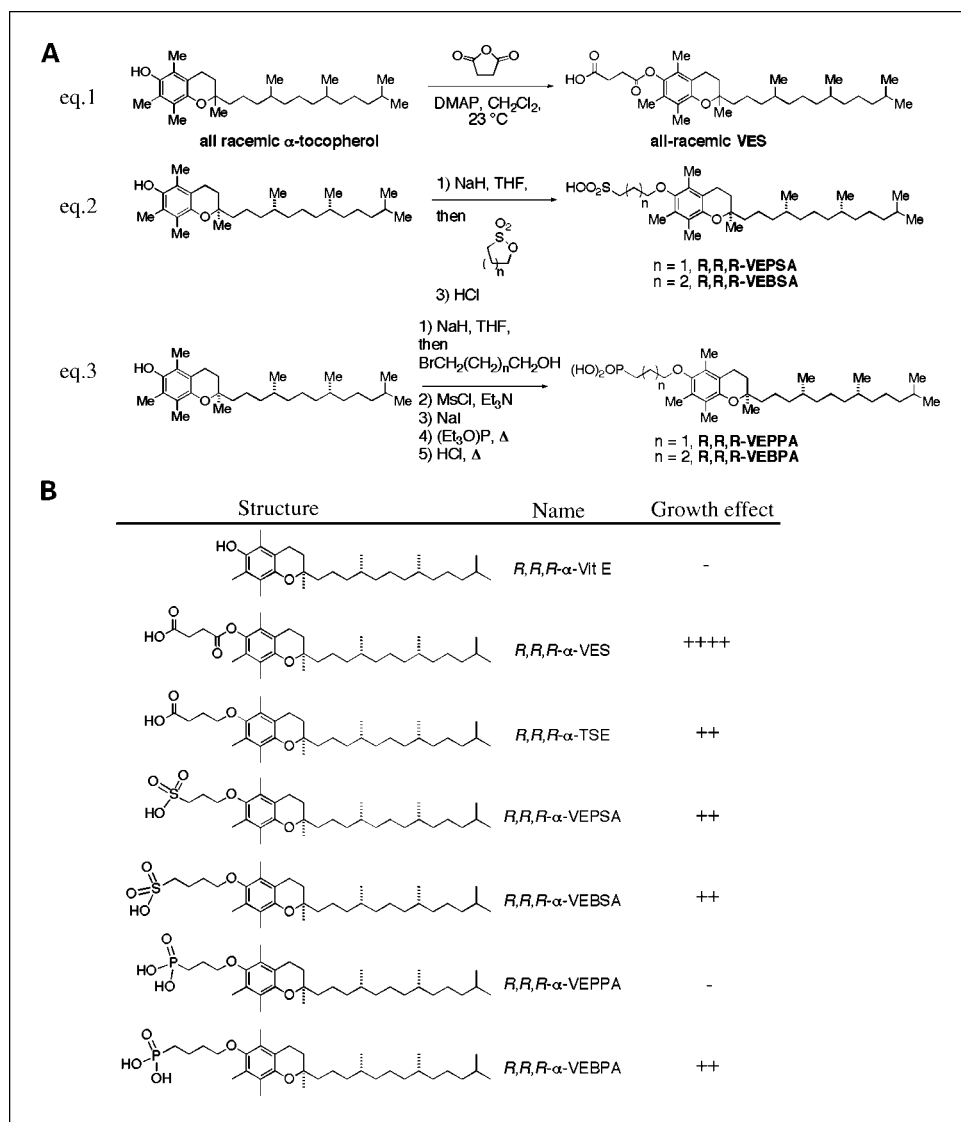


Fig. 1. *A*, synthesis of α -vitamin E analogues. *B*, schematic presentation of structures of α -vitamin E, α -vitamin E ester analogue VES, and ether analogues TSE, VEBSA, VEBPA, VEPPA, and VEBPA and their relative growth effects on prostate cancer cells. After prostate cancer LNCaP cells were seeded, the cells were treated with 50 $\mu\text{mol/L}$ vitamin E ether analogues for 3 d and examined by MTT growth assay; 20 $\mu\text{mol/L}$ VES served as positive control.

activity (2, 3). In addition, the acidic side chain is important for VES antiapoptotic effect (20, 21). Therefore, we expected that novel nonhydrolyzable ether derivatives of VES with other acidic side chains, including the sulfonic moiety (*RRR*-VEBSA and *RRR*-VEPSA) or the phosphonic moiety (VEBPA and VEPPA), would maintain VES antineoplasia activity with less degradation *in vivo*. First, we synthesized several vitamin E ether analogues (for the details, see Supplementary Materials and Methods). Then we screened these ether analogues by examining their growth inhibitory effect on prostate cancer cells via the MTT growth assay using 20 $\mu\text{mol/L}$ VES and 50 $\mu\text{mol/L}$ tocopheryloxybutyric acid (TSE) as comparisons (22). VEBSA, VEPSA, and VEBPA at 50 $\mu\text{mol/L}$ have similar antiproliferative activities as VES at 20 $\mu\text{mol/L}$ and TSE at 50 $\mu\text{mol/L}$ in prostate cancer LNCaP cells and the repression reached ~20% to 30% after 3 days of treatment. However, VEPPA at 50 $\mu\text{mol/L}$ did not significantly inhibit LNCaP cell growth (Fig. 1B).

Racemic forms of α -vitamin E analogues exhibit similar growth inhibitory efficacy as their chiral forms. Most of the α -vitamin E supplements on the market belong to synthetic α -vitamin E.

However, synthetic vitamin E is not identical to natural-source vitamin E, which occurs as a single stereoisomer, *RRR*- α -vitamin E. Synthetic α -vitamin E, known as all-racemic α -vitamin E (Rac- α -vitamin E), contains eight stereoisomers. It would be interesting to know whether the racemic forms of α -vitamin E analogues would have different antiproliferative activities on cancer cells compared with their chiral forms. We synthesized all-racemic VES (Rac-VES), all-racemic VEBSA (Rac-VEBSA), and all-racemic VEPSA (Rac-VEPSA) and investigated their growth effect compared with their counterpart chiral forms. As shown in Fig. 2A, both *RRR*-VES and Rac-VES at 20 $\mu\text{mol/L}$ inhibited LNCaP cell growth by ~25% and the growth inhibition reached ~50% with 30 $\mu\text{mol/L}$ treatment for 3 days. Similarly, there is no significant difference between the effects of *RRR*-VEBSA and Rac-VEBSA, or between *RRR*-VEPSA and Rac-VEPSA, on the growth of prostate cancer LNCaP cells (Fig. 2A). Those data indicated that racemic forms of α -vitamin E analogues have similar growth inhibitory capacity as their chiral forms in cultured cancer cells.

VEBSA reduces the cell viability and colony-forming ability of prostate cancer cells. Due to the relatively strong

antiproliferative activity, we chose *RRR*-VEBSA for further characterization. We calculated the IC_{50} value of VEBSA in 3-day growth inhibition assays of both androgen-responsive prostate cancer LNCaP cells and androgen-independent PC3 cells. As shown in Fig. 2B, VEBSA inhibited the growth of both LNCaP and PC3 cells at an IC_{50} value of $\sim 65 \mu\text{mol/L}$, whereas the IC_{50} value of VES is $\sim 30 \mu\text{mol/L}$ for both cell lines. These data indicated that VEBSA could effectively inhibit the growth of *in vitro* cultured prostate cancer cells; nevertheless, VES inhibits cell growth to a greater degree than VEBSA.

The effects of VEBSA and VES on the survival of prostate cancer cells were compared by soft agar colony forming assay, which mimics the *in vivo* tumor growth. We used the IC_{50} concentrations for VES ($30 \mu\text{mol/L}$) and VEBSA ($65 \mu\text{mol/L}$) to treat prostate cancer LNCaP and PC3 cells for 3 days, and then performed soft agar assay. As shown in Fig. 3A, the surviving fraction of LNCaP cells after VES or VEBSA treatment was reduced to $\sim 40\%$ and $\sim 60\%$ compared with control treatment, respectively. VES and VEBSA can dramatically reduce the colonies by $\sim 90\%$ and $\sim 95\%$ in PC3 cells, respectively. These data indicated that VEBSA, similar to VES, exhibits inhibitory activity on the colony-forming capacity in prostate cancer cells.

VEBSA induces apoptosis in prostate cancer cells. An effective way to control cancer cell viability is by regulating cell apoptosis. VES could induce apoptosis in many types of cancer cells, including prostate (10, 23). We were also interested in determining the VEBSA-induced apoptosis in prostate cancer cells. The prostate cancer LNCaP and PC3 cells were treated for 3 days in normal culture medium (RPMI with 10% fetal

bovine serum) and terminal deoxyribonucleotidyl transferase-mediated dUTP nick end labeling assay was then done. As shown in Fig. 3B, vehicle, VES, and VEBSA treatment induced $\sim 0.27\%$, $\sim 1.2\%$, and $\sim 4.5\%$ LNCaP cells to undergo apoptosis, respectively. The differences between the drug and control treatments are statistically significant. Similar results were obtained from PC3 cells. These data indicated that both VES and VEBSA significantly induced apoptosis in prostate cancer cells. VEBSA seems to have more potential proapoptotic activity compared with VES when using the individual IC_{50} concentration determined by cell viability in prostate cancer cells.

VEBSA regulates the expression of androgen receptor and VDR in prostate cancer cells. We reported earlier that VES inhibits prostate cancer cell growth via multiple mechanisms, including reducing the expression of androgen receptor and prostate-specific antigen and elevating VDR expression (3). Thus, we were interested in determining whether VEBSA has similar mechanisms as VES to inhibit prostate cancer cell growth. The levels of androgen receptor and VDR were examined in LNCaP and PC3 cells after treatment with VES and VEBSA using the IC_{50} concentrations. As shown in Fig. 4, both VES and VEBSA inhibited androgen receptor expression in LNCaP cells and enhanced VDR expression in LNCaP and PC3 cells with no influence on Akt phosphorylation level, indicating that VEBSA, similar to VES, may go through the androgen receptor/PSA and VDR pathways to control cell proliferation.

Oral administration of *RRR*-VEBSA, but not of *RRR*-VES, suppresses prostate cancer tumor growth in nude mice. Next, we asked the question whether *RRR*-VEBSA and/or *RRR*-VES could reduce tumor growth *in vivo*. VEBSA is a new nonhydrolyzable VES ether derivative, which was designed to improve the *in vivo* biological effects of hydrolyzable VES. To confirm that VEBSA has a better *in vivo* biostability than VES via oral administration, we used the same oral dose of VEBSA and VES to treat the animal with prostate cancer.

Prostate cancer PC3 cells were injected s.c. in two flank sites of male nude mice. The mice were given VEBSA or VES by oral gavage on Monday, Wednesday, and Friday followed by a 2-day rest, for a total of 6 weeks. During the experiment, the body weights of mice were monitored weekly and did not differ significantly among the group administrated with vehicle control, VES, or VEBSA (Fig. 5A). In addition, the general appearance, skin, fur, and food consumption did not alter and histologic tissue analyses of liver, kidney, and heart remained normal (data not shown), indicating that VEBSA and VES oral gavage did not have outward toxicity in the mice. The tumor growth rate in the VEBSA treatment group is much slower than in the control group observed after the 3rd week of drug treatment (Fig. 5B). The image in Fig. 5C showed the typical tumor burden following oral administration with control, VES, and VEBSA. The tumor weights at the end of experiment were 0.48 ± 0.13 , 0.51 ± 0.22 , and 0.16 ± 0.05 g from the mice gavaged with control, VES, and VEBSA, respectively. Whereas oral administration of VES showed little effect on the xenograft tumor growth, the statistical calculations suggested that the tumors in the VEBSA-treated group were significantly smaller compared with the tumors in the control group. The slightly yet insignificantly reduced body weight in mice with VEBSA treatment can be attributed to loss in tumor weight. Overall, the differences

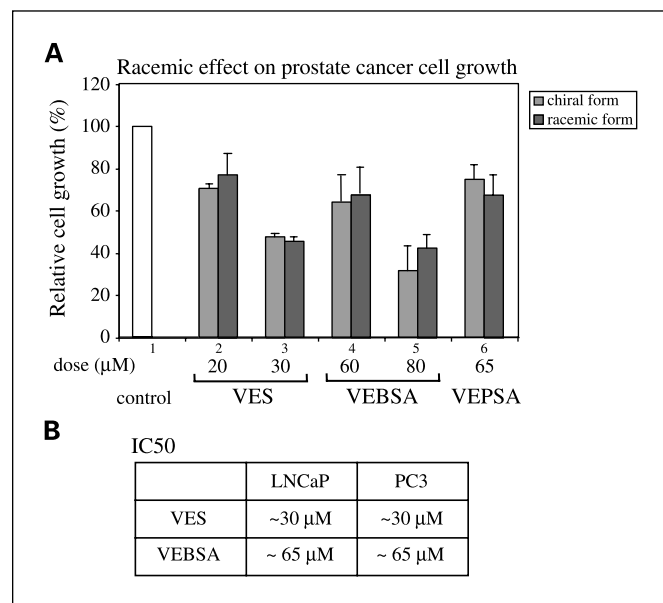


Fig. 2. α -Vitamin E ether analogues inhibit prostate cancer cell growth. **A**, racemic forms of α -vitamin E analogues exhibit similar growth inhibitory efficacy as the chiral forms. LNCaP cells were treated with *RRR*-VES or Rac-VES at 20 or 30 $\mu\text{mol/L}$, *RRR*-VEBSA or Rac-VEBSA at 60 or 80 $\mu\text{mol/L}$, and *RRR*-VEPSA or Rac-VEPSA at 65 $\mu\text{mol/L}$ for 3 d and examined by MTT growth assay. Columns, mean of triplicate samples; bars, SD. Student's *t* test was done to determine the difference of each drug at each dose and the data show no statistical differences in growth effects between racemic and chiral forms. **B**, IC_{50} concentrations of VES and VEBSA in prostate cancer cells. LNCaP and PC3 cells were treated with VES or VEBSA at a series of concentrations for 3 d. IC_{50} values were determined by MTT assay.

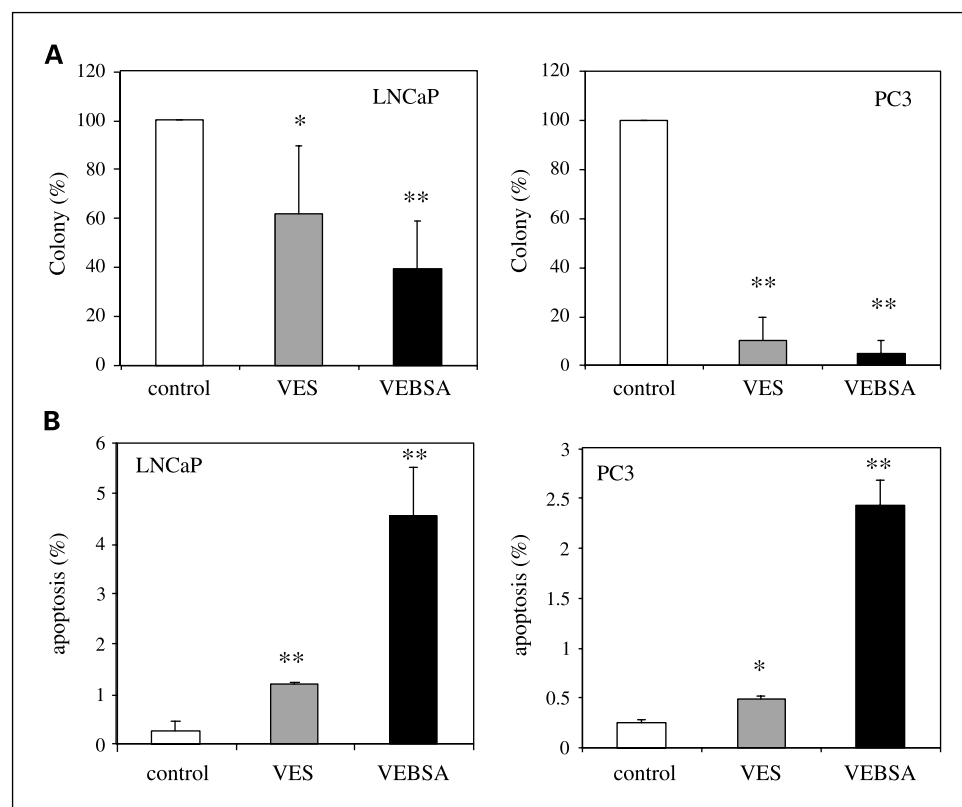


Fig. 3. VEBSA and VES reduce the colony-forming ability of and induce apoptosis in prostate cancer cells. *A*, VEBSA and VES reduce the colony-forming ability of prostate cancer cells. The effects of VES and VEBSA on the survival of prostate cancer LNCaP (*left*) and PC3 (*right*) cells were determined by soft agar colony forming assay. Prostate cancer LNCaP and PC3 cells were treated with 30 $\mu\text{mol/L}$ VES or 65 $\mu\text{mol/L}$ VEBSA for 3 d; 5,000 cells were then suspended in 0.4% low melting agarose (Cambrex) and layered on top of 1.5 mL of 0.8% agarose in six-well culture plates. Cells were incubated at 37°C in humidified incubator for 3 to 4 wk. The plates were stained with 0.5 mL of 0.005% crystal violet for 2 h and colonies were counted under a dissecting microscope. Columns, mean of at least three independent experiments; bars, SD. *B*, VEBSA and VES induce apoptosis in prostate cancer cells. LNCaP (*left*) and PC3 (*right*) cells were treated with VES at 30 $\mu\text{mol/L}$ or VEBSA at 65 $\mu\text{mol/L}$ for 3 d. Apoptotic cells were investigated by terminal deoxynucleotidyl transferase – mediated dUTP nick end labeling assay. Columns, mean of three independent experiments; bars, SD. *, $P < 0.05$; **, $P < 0.01$, compared with the control (one-way ANOVA, Dunnett's test).

in body weights of different treatment groups are not statistically significant, indicating no observed toxicity. Taken together, oral intake of VEBSA, but not of VES, can reduce prostate cancer growth in the xenograft nude mouse cancer model.

Oral administration of RRR-VEBSA has a better efficacy than RRR-VES in reducing tumor burden in TRAMP mouse prostate cancer model. To further examine if oral administration of VEBSA can also prevent prostate cancer occurrence and progression, we applied the TRAMP mouse model, which spontaneously develops tumors with stages mimicking human prostate cancer progression. TRAMP mouse contains a transgene with probasin-promoter to drive SV40 T-antigen special expression in prostate to induce spontaneous prostate cancer with an intact immune system (17). The mice were fed RRR-VES, RRR-VEBSA, and vehicle control by oral gavage for ~22 weeks. As shown in Fig. 6A, the genitourinary tract weight at the end of experiment was 3.23 ± 1.65 , 1.93 ± 0.86 , and 1.44 ± 0.58 g from the mice gavaged with control, VES, and VEBSA, respectively. The statistical calculations suggested that the genitourinary tract weights in the VEBSA- and VES-treated groups were significantly less than those in the control group. Histologic examination revealed that the tumors in control mice were in the transition from the well-differentiated to the poorly differentiated stage. The VES-treated tumors were primarily at the well-differentiated stage, and VEBSA-treated tumors were in less malignant stages containing prostatic intraepithelial neoplasia and well-differentiated tumors. Our immunohistochemical staining results revealed similar levels of the SV40 T-antigen transgenes in the dorsolateral prostates of control and treatment mice, and the results clearly ruled out the possibility that

these tumor burden differences were caused by an inadequate expression of SV40 T-antigen transgenes in the prostate of TRAMP mice (Fig. 6C).

In addition, the body weights were monitored and did not show significant changes between control and treated groups (Fig. 6B). The histopathologic examination of liver, kidney, testis, and heart did not differ significantly between the experimental mice and control mice (data not shown),

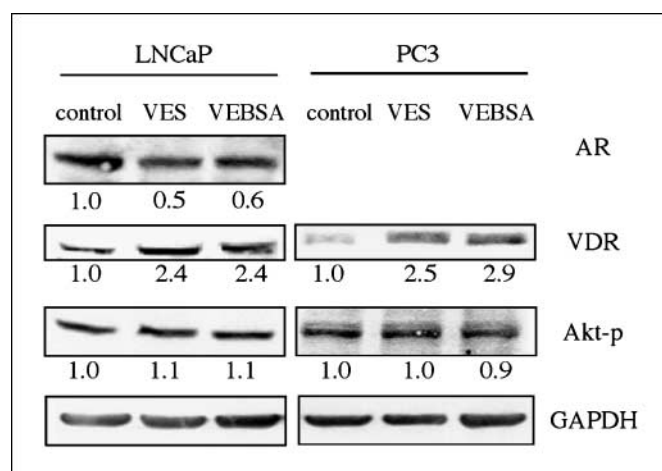


Fig. 4. VEBSA, similar to VES, could differentially regulate the expression of androgen receptor (AR) and VDR in prostate cancer cells. Prostate cancer LNCaP and PC3 cells were treated with 30 $\mu\text{mol/L}$ VES or 65 $\mu\text{mol/L}$ VEBSA for 3 d. The cells were harvested and Western blot analyses done with indicated antibodies. The results represent at least two independent experiments. The quantitative data are presented by normalizing with the loading control.

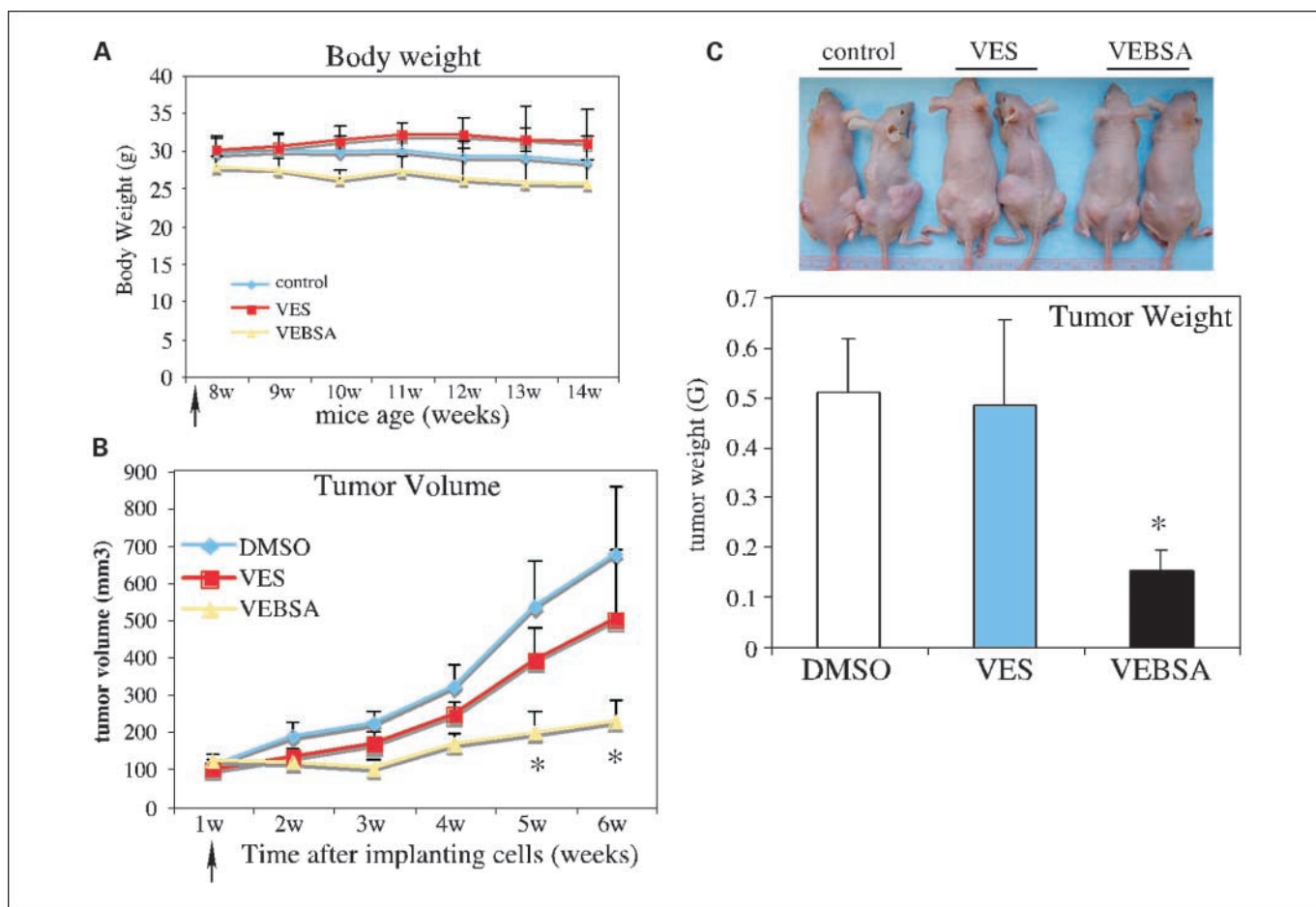


Fig. 5. Oral administration of VEBSA, but not of VES, inhibits the growth of PC3 tumor xenografts in athymic male mice. PC3 cells (1×10^6) mixed with 30% Matrigel were s.c. injected into each of two flanks in nude mice. One week after tumor injection, treatments were initiated. *RRR*-VES, *RRR*-VEBSA, and mock control were delivered by gavage thrice per week for a total of 6 wk. The dose for VES and VEBSA was 200 μ L of 50 mmol/L in sesame oil with 10% DMSO. The control was 200 μ L of sesame oil with 10% DMSO. Body weight and tumor volume were determined weekly. **A**, body weights of treated mice did not change significantly compared with control mice. Points, mean ($n = 6$); bars, SD. **B**, the PC3 xenograft tumors with VEBSA treatment have slower growth rate in nude mice. Tumor volume was assessed with the following formula: tumor volume = (length \times width²)/2. Points, mean tumor volume; bars, SE. *, $P < 0.05$, compared with control at the same time point (two-way ANOVA with Bonferroni posttest). **C**, the PC3 xenograft with VEBSA treatment has a lower tumor weight. Six weeks after tumor implantation, mice were sacrificed and the tumors were isolated and weighed. The image represents the typical tumor formation in the nude mice. Columns, mean weight for each group; bars, SE. *, $P < 0.05$, compared with control (one-way ANOVA followed by Dunnett's test).

suggesting that oral gavage of VEBSA for ~ 22 weeks did not result in toxicity in those animal models.

To further test our hypothesis that VEBSA has a better *in vivo* bioavailability than VES, we have developed a method to monitor the serum levels of VEBSA (the methods are shown in Supplementary data). The results in Fig. 6D showed that the serum level of VEBSA was ~ 15 μ mol/L, whereas that of VES was ~ 2 μ mol/L, after the mice orally received an individual compound for 1 month. Together, our results in Fig. 6 indicate that VEBSA does have a better bioavailability and antitumor effect compared with VES *in vivo*.

Discussion

VES is one of the most potent α -vitamin E derivatives available on the market in term of antiproliferative activity. Several groups have developed new α -vitamin E analogues based on the structure of VES to further improve its antitumor effect, especially its proapoptotic efficacy. For example, Birringer et al. (21) modified VES functional moieties and

found that α -tocopheryl maleate can induce more cells to undergo apoptosis compared with VES in *in vitro* cultured cancer cells. Tomic-Vaic et al. (24) developed new vitamin E amide analogues, and those amide compounds have more apoptosis induction than the ester counterparts. Shiau et al. (23) created new VES analogues with truncated side chains based on the structural model that VES can dock into the binding site of Bcl-xl. Again, these new compounds have stronger ability to induce apoptosis than VES. However, there are no reports to show that those three VES analogues have been tested in any *in vivo* animal prostate cancer models with an intact immune system. In general, cancer chemoprevention and therapy prefers agents that can be given orally. However, the esterase and other endogenous enzymes in the gastrointestinal tract might hydrolyze the ester and amide bonds of those three VES analogues resulting in loss of their efficacy after oral intake, thus comprising their applications for the clinical use.

The goal of this study was to develop VES ether analogues with greater bioavailability *in vivo* after oral intake without

sacrificing its antitumor activity. We replaced the ester bond with an ether bond in the new VES analogues. The ether bond has an advantage over the ester bond because the compounds will not be cleaved by esterases in the gastrointestinal tract and become more stable *in vivo*. Based on previous reports, an acidic side chain seems necessary for the VES antitumor effect (21). VES is a weak acid with a low pK_a (5.64; ref. 25). The pH of most tumor interstitium is typically around 6.2 to 6.5 (26), and this acidic tumor interstitium can facilitate protonation of the charged VES to enhance VES uptake into the cells (25). The concept that tumor pH controls the efficacy of weak acid has been shown *in vivo* for chemotherapeutics (27–29). Because previously investigated VES analogues have been primarily limited to modifying carboxylic acid containing functional arms (12, 30–32), we decided to investigate the ability of other acidic arms to induce antiproliferative effects in cancer cells. In other antineoplastic agents, the sulfate group and the phosphate group can make the compounds become weak acids in low pH milieu and render the compounds with greater anticancer efficacy (33, 34). It was interesting to expand our study to investigate whether α -vitamin E ether analogues with the sulfonic or phosphonic moiety have any effect on the growth of prostate cancer cells. We found that there is little apparent correlation with pK_a and antiproliferative activity of the tested vitamin E analogues. If the correlation with pK_a were dominant, one would expect the antiproliferative effects of phosphonic acids [the first pK_a of $\text{MePO}_2(\text{OH})_2$ is estimated at 1–3 in H_2O ; ref. 35], to be more similar to VES (the pK_a of $\text{CH}_3\text{CO}_2\text{H}$ is ~ 4.8 in H_2O ; ref. 36), and should be better than the sulfonic acids (for example, the pK_a of MeSO_2OH is estimated at -2 in H_2O ; ref. 36). It is possible, however, that the higher acidity of the sulfonic acid analogue, VEBSA, may contribute to its greater degree of apoptosis induction compared with VES.

Most α -vitamin E supplements on the market are synthetic. However, synthetic vitamin E is not identical to natural-source vitamin E. In nature, α -vitamin E occurs as a single stereoisomer, *RRR*- α -tocopherol. Synthetic vitamin E, known as all-racemic α -vitamin E (*Rac*- α -vitamin E), contains eight stereoisomers (*RRR*, *RRS*, *RSS*, *SSS*, *SRR*, *SSR*, *SSS*, and *SRS*), which differ in whether they can be “right” or “left” (R or S) at three different places in the α -vitamin E molecule (37). In this study, we determined whether there was a distinct difference in the antiproliferative activity of the racemic analogues versus the chiral analogues *in vitro*. If S would not be as active as R, the racemic version of α -vitamin E analogues would be less efficient to suppress prostate cancer cell growth *in vitro*. We found that the chiral and racemic forms of VES and VES derivatives did not have substantial differences in suppressing prostate cancer LNCaP cell growth, suggesting that the chiral center is not important to determine antiproliferative activity in the cultured cancer cells.

However, these stereoisomers might have differential absorption and transportation efficiency through oral administration. It is also possible that some isomers might be more potent than others, or those eight isomers compete with each other to have the same antiproliferative activity as the chiral form. If eight isomers have the same activity, our data then suggest that the compounds might exhibit their activity by docking its nonpolar chroman and side chain in an achiral phospholipid membrane (much as α -vitamin E does) and disrupting membrane protein-

protein interaction with its acidic functional domain (carboxylic acid or sulfonic acid) that is present in the cytoplasm of the cell. If it were docking entirely in an enzyme pocket, as Dr. Chen and coworkers proposed (23), the chirality of the side chain would very likely affect the activity of the compound. However, our results challenge this possibility. Furthermore, it remains to be tested whether these stereoisomers might have differential absorption and transportation efficiency through oral administration.

In this study, we found that VEBSA could use similar mechanisms as VES to inhibit the growth of prostate cancer cells. Those mechanisms for the VEBSA to inhibit cell viability include (a) induction of apoptosis (Fig. 3), (b) repression of androgen receptor protein expression, (c) enhancement of VDR expression (Fig. 4), and (d) modulation of cell cycle molecule expression (data not shown). This may explain why we observed that VEBSA induces more apoptosis than does VES at their IC_{50} concentrations. Importantly, as expected, we found that oral intake of VEBSA could reduce the prostate tumor burden both in xenograft tumors in nude mice and in the TRAMP mouse prostate cancer model. We have developed methods to monitor the biodistribution of VEBSA, and the better efficacy of VEBSA, compared with VES, to inhibit *in vivo* prostate tumor burden supports our hypothesis that VEBSA is a nonhydrolyzable compound with greater bioavailability than VES *in vivo*. Interestingly, we also found that oral intake of VES for a longer period (such as 22 weeks in TRAMP) can inhibit prostate tumors in TRAMP mice, although its efficiency is still far less than that of VEBSA. The reasonable explanation is that certain levels of VES might escape from esterase digestion in the gastrointestinal tract. The undigested VES accumulates in prostate to exhibit its antitumor function after long intake of VES (such as 22 weeks for TRAMP). However, a short time (such as 6 weeks for nude mice) does not allow *in vivo* accumulation of intact VES and the execution of its function. Nevertheless, our study supported our hypothesis that oral intake of VEBSA could more effectively reduce the prostate tumor burden *in vivo*. Further studies to investigate whether VEBSA has any effect on metastasis or angiogenesis will be done in the future.

One feature of a good antitumor agent is to exhibit antitumor activity without side effects. VEBSA can inhibit prostate tumor burden *in vivo* without any obvious toxicity in mice. This is supported by the observation that the body weight, general appearance, and histopathologic examination of the liver, kidney, intestine, spinal cord, prostate, testis, and jejunum did not differ significantly between the VEBSA-treated mice and vehicle control-treated mice (data not shown). The toxicity tests have been done in nude mice for 6 weeks, in C57BL/6 mice (the parental strain of TRAMP mice) for 8 weeks, and in TRAMP mice for 22 weeks. Those results from preclinical animal cancer models suggest that oral administration of VEBSA is an effective and better strategy to treat prostate cancer without observable toxicity.

Kline et al. have developed another α -vitamin E ether analogue, TEA (methyl carboxyl moiety), which was shown to inhibit the growth of breast cancer cells *in vitro* and reduce tumor burden and metastasis in the mouse breast tumor model (12, 30). However, it has not been tested in any *in vivo* prostate cancer animal model. We also examined the TEA effect on prostate cancer cells and found that TEA could

potentially inhibit prostate cancer cell growth *in vitro*, similar to VEBSA and VES.⁵ It will be interesting to investigate whether the oral intake of TEA can repress the tumor burden in a prostate tumor animal model in the future. Furthermore, it was reported that VES with a shortened side chain is associated with better activity against prostate cancer cell growth *in vitro* (23). We may develop a new VEBSA analogue with a shorter side chain and/or with replacement of the butyl sulfonate group with a methyl sulfonate group to test whether this new compound has more potent antitumor effects.

Nevertheless, in this study, we examined a group of VES ether analogues with sulfonic moiety or phosphonic moiety and found that the oral administration of VEBSA could effectively inhibit prostate cancer growth without observed toxicity.

Taken together, a newly developed nonhydrolyzable VEBSA could serve as an alternative to VES and could potentially be applied both as a chemopreventive and a chemotherapeutic agent in prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

⁵ Personal communication.

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