RRR-α-Vitamin E Succinate Potentiates the Antitumor Effect of Calcitriol in Prostate Cancer without Overt Side Effects

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Abstract

Purpose: To determine the antitumor efficacy of using calcitriol combined with RRR-α-vitamin E succinate (VES) on prostate cancer.

Experimental Design: The effects of VES or VES in combination with calcitriol on the calcitriol target genes were evaluated by Western blot and real-time PCR. The antiproliferation effect of the combination in prostate cancer cells was evaluated by the combination index method. The role of the vitamin D_3 receptor (VDR) in the enhanced antitumor effects of the combination was confirmed by small interfering RNA knockdown strategy. Xenograft-bearing mice were used to reaffirm the antitumor efficacy of this combination. Pathohistology analyses and expressions of VDR and its target genes were analyzed in untreated and treated tumors.

Results: VES selectively increased VDR protein in different prostate cancer cells. Low doses of calcitriol combined with VES were significantly superior to the additive effect of individual treatments against prostate cancer cell proliferation. The expression of VDR target genes involved in antiproliferation were further sensitized in the presence of VES. Knockdown of VDR expression abolished the combination benefits in LNCaP and PC3 cells. Consistently, in prostate cancer xenograft models, VES enhanced the therapeutic efficacy of a tolerated dose of calcitriol yet without overt evidence of systemic toxicity and hypercalcemia. This notable *in vivo* effect was also accompanied by up-regulation of VDR target genes.

Conclusions: Low-dose calcitriol combined with vitamin E analogue could be a solution to the calcemic side effect. The demonstration of superior antitumor activity of low-dose calcitriol plus VES provides the preclinical basis for developing a useful therapeutic strategy for prostate cancer.

Prostate cancer remains a major source of cancer morbidity and mortality in man. Nearly 200,000 new prostate cancer cases will be diagnosed in the United States, and close to 30,000 men will succumb to the disease in 2008 (1). Most prostate tumors initially respond to androgen depletion therapy but ultimately progress to androgen-independent disease. Despite aggressive approaches made in the treatment of prostate cancer in the past few years, limited therapeutic options are available at the advanced stage of disease. This underscores the need for new strategies for the prevention and/or treatment of prostate cancer. Several vitamin E compounds and their derivatives have shown antineoplastic functions in a variety of malignant cell lines with varying activity and mechanism profiles relevant to cancer prevention and treatment (2, 3). RRR-α-vitamin E succinate (VES), one of the most effective forms of vitamin E, exhibits multiple mechanisms in cancer prevention and treatment (4–6). We previously showed that VES can effectively prevent and reduce the growth of prostate cancer with no significant biological toxicity (6, 7). The mechanisms seem to be via (*a*) modulation of the cell cycle, (*b*) inhibiting invasive ability, and (*c*) down-regulation of the nuclear androgen receptor (7–9). Although several nuclear receptors play important roles in controlling cell proliferation of prostate cancer (10–12), little is known about the effect of VES on other nuclear receptor pathways and the efficacy of VES combined with drugs targeting nuclear receptors.

Epidemiologic and dietary studies suggest calcitriol has preventative and therapeutic effects on prostate cancer (13). The antineoplastic effects of calcitriol involve multiple and diverse mechanisms, including induction of growth arrest and apoptosis, and inhibition of metastasis and angiogenesis (14). The classic antiproliferative functions of calcitriol are mediated mainly via the vitamin D_3 receptor (VDR), which forms heterodimers with the retinoid X receptor (RXR) and binds to the VDR response element, resulting in activation of downstream target genes. However, the limitation of clinical application is the side effect of hypercalcemia and hypercalciuria at the pharmacologic doses (15). One alternative strategy for developing a calcitriol-based therapy is to design new calcitriol analogues that have better antiproliferative efficacy and reduced calcemic side effects.

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Translational Relevance

Because vitamin E succinate and calcitriol have been applied in different clinical trials in combination with other agents for prostate cancer therapy, it is a relatively small and close step to initiate clinical trials of vitamin E succinate plus calcitriol to treat prostate cancer. What is important is showing persuasive evidence to prove that combined vitamin E succinate and calcitriol to treat prostate cancer is effective. Our data indeed provide solid and supportive evidence both in prostate cancer cells and in preclinical animal cancer models. Thus, the potential impact of this study could be profound for developing an alternative and improved strategy to treat prostate cancer.

Although numerous calcitriol analogues have been screened to eliminate side effects, clinical trials have yet to show satisfactory results (16, 17). Therefore, a low dose of calcitriol combined with other anticancer reagents is still an alternative and promising strategy (18, 19).

Despite increasing interest in the antineoplastic efficacy of VES and calcitriol, the efficacy of the combination in prostate cancer is still unknown. In the present study, our results indicate that VES can selectively enhance the expression of VDR but not RXRα and estrogen receptor-β (ERβ) in different prostate cancer cells. Treatment with low-dose calcitriol combined with VES was significantly superior to the additive effect of combining individual treatment in *in vitro* cancer cells and in preclinical animal prostate cancer models. The markedly enhanced antineoplastic effect by VES is partially mediated by the expression of VDR and its target genes in cancer cells, not in nonmalignant cells. Together, our findings suggest that a low dose of calcitriol combined with VES may gain a dramatically enhanced anticancer effect without overt toxicity, supporting the possibility of a clinical trial involving this novel combination regimen.

Materials and Methods

Cell culture and reagents. The prostate cancer cell lines LNCaP, 22RV1, and PC3 were propagated at 37°C with 5% CO2 in complete RPMI 1640 medium supplemented with 10% fetal bovine serum. Calcitriol and cycloheximide were dissolved in absolute ethanol as a stock solution of 1 mg/mL and 100 mg/mL, respectively. The antibodies of VDR, RXRα, ERβ, p21, insulin-like growth factor binding protein 3 (IGFBP-3), proliferating cell nuclear antigen, and β-actin were purchased from Santa Cruz Biotechnology. All other reagents, except where indicated, were purchased from Sigma.

Protein sample preparation and Western blot analysis. The protein sample lysates were prepared as described previously (6). Equal amounts of denatured proteins were electrophoresed on a 10% SDS-polyacrylamide gel and transferred to Immobilon membranes (Millipore). The membranes were incubated with primary antibodies followed by alkaline phosphatase-conjugated secondary antibodies for 1 hr at room temperature. Immunoreactive bands were detected by alkaline phosphatase reagents (Bio-Rad).

Reverse transcription and quantitative real-time PCR. Total RNA was extracted and reverse transcribed as described previously (6). Real-time PCR was done with SYBR Green PCR Master Mix (Bio-Rad) at 95°C for 3 min, and 40 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s on an iCycler iQ multicolor real-time PCR system (Bio-Rad). All samples were run in triplicate, and data were analyzed with the use of an iCycler iQ software (Bio-Rad). The primers used in real-time PCR were: CYP24: (F) 5′-CAGCAGCATCTCATCTACC-3′, (R)5′-GACAGCCTCAGAGCATTG-3′; IGFBP-3: (F) 5′-CGAGTCTCAGAGGACAGATACC-3′, (R) 5′- GCGGCAGGCGTCTACTTG-3′; P21: (F) 5′-CCGTGAGCGATGGAACTTC-3′, (R) 5′-AGCAGAGCAGGTGAGGTG-3′; and β-actin: (F)5′-TGTGCCCATCTACGAGGGGTATGC-3′, (R) 5′-GGTACATGGTGGTGCCGCCAGACA-3′.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide cell growth assay and combination index value analysis. Cells ($2\text{-}5 \times 10^3$) were seeded into 24-well plates for 36 hr and treated with the VES, calcitriol, or in combination. Cell growth was evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays as previously described (6). Combination index (CI) values were analyzed with the use of the CalcuSyn version 2.0 (Biosoft). The CI value of nonexclusive drugs is calculated with the formula: CI = (Da + Db)/(Dxa + Dxb) + DaDb/DxaDxb. Da and Db are the doses of drugs A and B to inhibit X% of cell proliferation as single drugs, and Dxa and Dxb are the doses of drugs A and B to inhibit X% of cell growth in a combination regimen. When CI = 1 (represents an additive effect of the two drugs), the CI formula is in the same form as a traditional isobologram equation. Synergism is defined as more than the expected additive effect with CI < 1, and antagonism is defined as CI > 1.

Small interfering RNA. pSUPER-VDR small interfering RNA [siRNA (pSR-VDRi); Dr. Guan Chen, Medical College of Wisconsin, Milwaukee, WI] and scramble siRNA were transfected into Phoenix-Ampo retrovirus packing cells with the use of the SuperFect reagent according to the manufacturer's instructions (Qiagen). Two days after transfection, the supernatants containing retroviruses were used to infect LNCaP and PC3 cells. After 36 hr, infected cells were selected with puromycin (1 μg/mL) and pooled after 2 wk of selection.

Animal study. Six-week-old male athymic mice (Charles River) were inoculated s.c. into each flank with 1×10^6 LNCaP cells in 0.1 mL 100% Matrigel or 7.5×10^5 PC3 cells in 0.1 mL serum-free medium containing 50% Matrigel (BD Biosciences). Once tumors became palpable (>50 mm^3), mice were randomly divided into four groups (n = 7) and injected i.p. with vehicle (DMSO), VES (100 mg/kg), calcitriol (0.0625 μg/mice), and the combination (VES 100 mg/kg and calcitriol 0.0625 μg/mice) twice weekly. Doses of VES and calcitriol were determined by previous studies (5, 20). After the initiation of growth, the body weight and xenograft dimensions were measured weekly. Tumor volume was calculated as 0.52 × length × biggest $width^2$. Animals were sacrificed 24 hr after the last treatment, serum was collected and stored in -80°C, and tumor weights were measured. The xenograft tissues and certain mouse organs (brain, heart, lungs, liver, kidneys, spleen, spinal cord, and gastrointestinal and genitourinary tract) were excised and either fixed for 24 hr in 10% neutral buffered formaldehyde and embedded for H&E staining, or stored in liquid nitrogen for further analysis. The femoral bones were collected to detect bone density by X-ray analysis. Serum calcium levels were measured with the Calcium CPC LiquiColor test kit (Stanbio Laboratory). All of the *in vivo* experiments had two independent sets.

Statistical analysis. Measurements of tumor volume and body weight among the four groups were analyzed through one-way ANOVA coupled with the Student-Newman-Keuls test. Other statistical analyses were done with the Student's t test. P values < 0.05 were considered to be statistically significant.

Results

VES selectively enhances the expression of VDR, but not RXRα and ERβ, in different prostate cancer cells. Our previous publication indicated that VES reduced the expression and activity of androgen receptor (7). We were interested in testing whether VES-mediated growth inhibition may function via regulating the expression of other nuclear receptors, RXRα (10),

ERβ (21), and VDR (22), whose functions are associated with the growth and progression of prostate cancer. Our results indicated that VES can selectively augment VDR protein abundance but has no significant effect on the expression of RXRα and ERβ in LNCaP prostate cancer cells (Fig. 1). To determine whether the selective induction of VDR protein by VES exists in other prostate cancer cell lines, we carried out Western blot analysis to examine the expression levels of VDR, RXRα, and ERβ in androgen-sensitive (22RV1) and androgen-independent prostate cancer cells (PC3) after treatment with VES (20 μmol/L) for 2 and 4 days. Consistently, we found that the expression levels of VDR protein could also be induced in 22RV1 and PC3 cells, and the expression levels of RXRα had no significant changes in those cell lines. The ERβ expression levels also exhibited no significant change in PC3 cells but were undetectable in 22RV1 cells (Fig. 1).

Calcitriol combined with VES is superior to the additive effect of the individual treatments in inhibiting prostate cancer cells. VDR mediates the genomic effect of calcitriol through its ability to modulate the target gene expression and plays a dominant role in the antiproliferation effects of calcitriol. Thus, induction of VDR could lead to an enhanced efficacy of calcitriol. The effect of VES (1, 5, 10, 15, 20, 25, 30, 40, 50, and 70 μmol/L) and calcitriol (0.1, 1, 10, 20, 50, 100, and 200 nmol/L) treatment alone on the viability of LNCaP and PC3 cells was determined by MTT cell growth assay. Our results indicated that the IC50 after 6 days of VES treatment is 18.3 ± 4.2 μmol/L for LNCaP and 21.3 ± 0.6 μmol/L for PC3 cells. The 6-day 100-nmol/L calcitriol exposure resulted in ~50% and ~40% growth inhibition in LNCaP and PC3 cells, respectively (data not shown).

Submaximal doses of VES (1, 5, and 10 μmol/L) combined with different doses of calcitriol (1, 10, and 20 nmol/L) were further evaluated for their growth inhibition effect on prostate cancer cells. We found that VES at all tested dosages had the capacity to enhance the growth inhibition of calcitriol on prostate cancer cells (Fig. 2A and B) compared with the control set at 100%. VES at the concentration of 10 μmol/L combined with calcitriol at 10 or 20 nmol/L seems to be the most efficient combination regimen.

Based on the isobolar method, the Chou and Talalay CI analysis has been used to determine the pharmacologic interaction of two drugs (23, 24). To assess whether these combined effects of VES and calcitriol were additive or synergistic, we used the well-established CalcuSyn software to determine the CI value (25). The CI value of the cotreatment 1 μmol/L VES with 10 nmol/L calcitriol in LNCaP cell was 1.014, which indicated an additive effect. Importantly, the CI values of other 5 sets of combinations were <1, which indicated the synergy effect of the combined treatment (Fig. 2C). Together, our data indicate that a superior effect, either synergistic or at least additive, could be achieved by the combination treatments in LNCaP and PC3 cells.

Effect of calcitriol combined with VES on the expression of VDR downstream target genes. Upon ligand binding, VDR will form a heterodimer with RXR and bind to the vitamin D-responsive element (VDRE) in the promoter and trigger the transcription of the target genes. We sought to determine whether VDR target genes are involved in the superior antiproliferative effect induced by calcitriol combined with VES. Upon establishing that 10 nmol/L calcitriol combined with 10 μmol/L VES exhibited significant growth inhibition, the following VDR target genes were analyzed under the same experimental conditions. CYP24, containing two VDREs in the promoter, is one of the well-studied calcitriol target genes and is highly inducible by calcitriol in prostate cancer cells (26). It has been shown that induction of CYP24 mRNA can serve as an indicator of calcitriol-mediated genomic activity (27). As shown in Fig. 3A, CYP24 mRNA increased about 600-fold after LNCaP cells were treated with calcitriol (10 nmol/L) for 24 hours compared with control-treated LNCaP cells. VES alone cannot induce the CYP24 expression; however, when the cells were exposed to calcitriol (10 nmol/L) combined with VES (10 μmol/L), the mRNA level of CYP24 increased even more (1,600-fold compared with control). Because recent findings show that both IGFBP-3 and cyclin-dependent kinase inhibitor p21 have been implicated as downstream mediators of the growth inhibitory actions of calcitriol (28, 29) and there is a functional VDRE in their promoters (30, 31), we hypothesized that VES may cross-talk with the calcitriol pathway, resulting in an increased antiproliferative function via up-regulating the expression of these target genes. We also examined the mRNA (Fig. 3A) of IGFBP-3 and p21 by real-time PCR in the various treatment groups. Exposure to either VES or calcitriol modulated the mRNA level of IGFBP-3 by 3-fold and 5-fold, and p21 mRNA by 1-fold and 2.4-fold compared with control cells, respectively. The combination therapy illustrates a substantial increase in IGFBP-3 or p21 mRNA compared with the single treatment. The concurrent treatment increased IGFBP-3 and p21 expression to 20-fold and 4.3-fold compared with the control. These results suggest the expression of multiple calcitriol target genes involved in proliferation were being

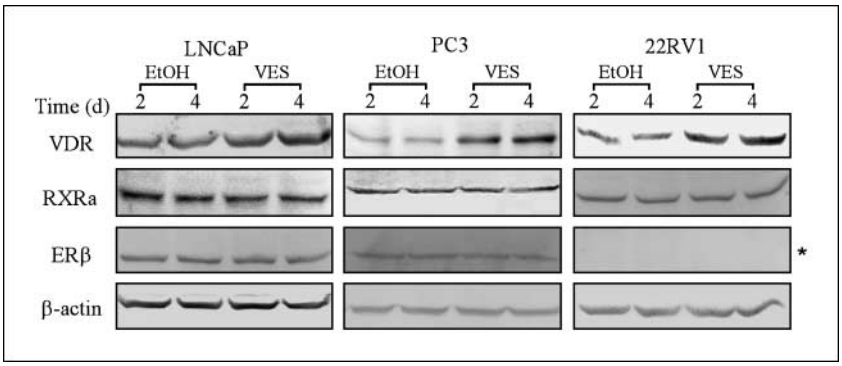

Fig. 1. VES selectively increases VDR protein. LNCaP, PC3, and 22RV1 cells were treated with ethanol or VES (20 μmol/L) for 2 and 4 d. Protein samples of cell lysates were collected, and VDR, RXRα, ERβ, and β-actin protein levels were analyzed by Western blotting.

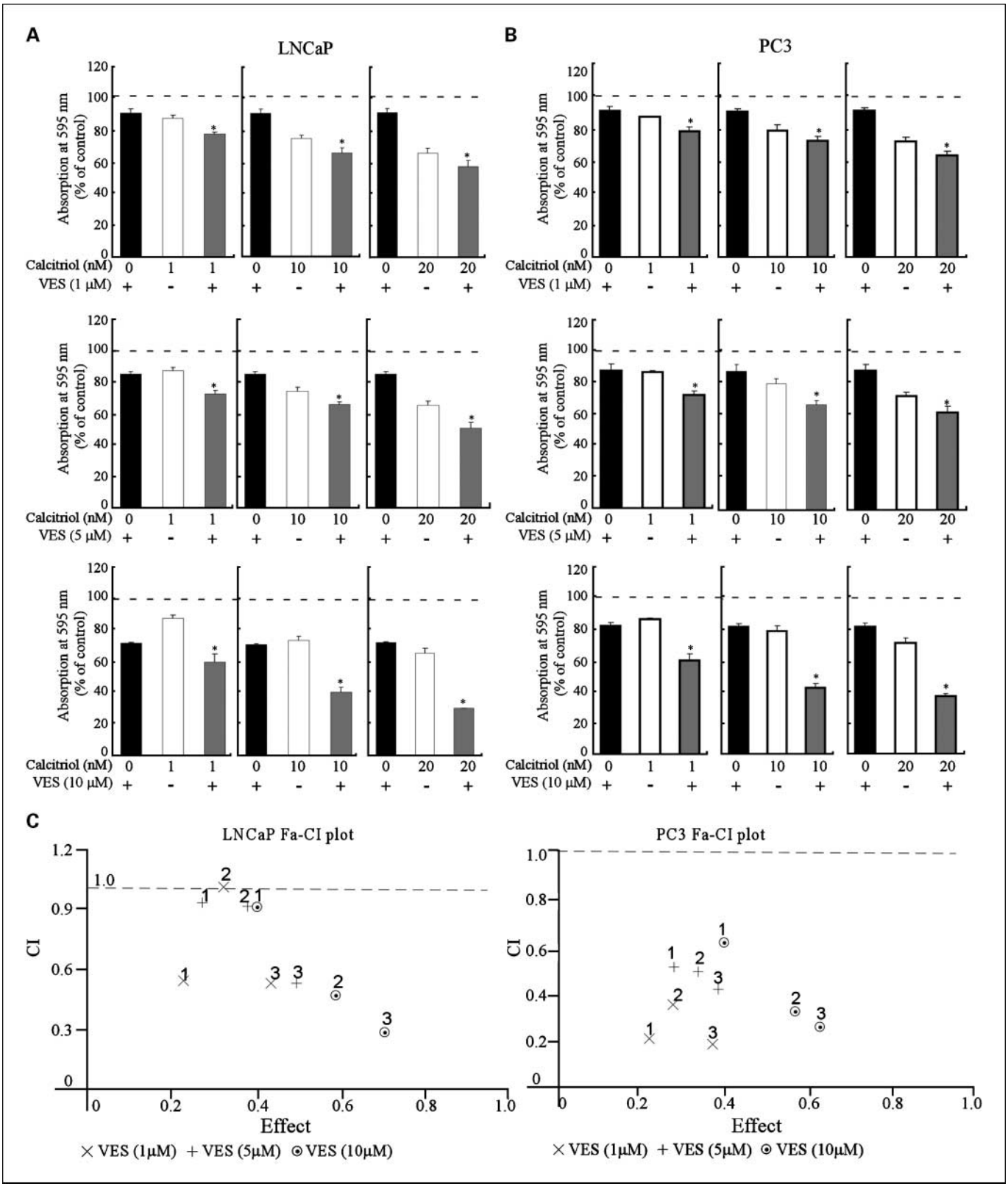

Fig. 2. The effect of combining calcitriol and VES is significantly superior to the sum effect of individual treatments in controlling prostate cancer cells. LNCaP (*A*) and PC3 (*B*) cells were used to test the growth inhibition effects. Cells were plated in 24-well plates ($2\text{-}5 \times 10^3$/well) and pretreated with ethanol and VES (1, 5, and 10 μmol/L) for 24 h and then treated with calcitriol (1, 10, and 20 nmol/L) or in combination. MTT growth assay was done after 6 d of treatment. The results were reported as absorption at 595 nm, considering control cells as 100%. Data, mean ± SD ($n = 3$). *, statistically different ($P < 0.05$). *C,* CI values for combination treatment of calcitriol and VES in LNCaP and PC3 cells were calculated. Effect-CI plots were generated by CalcuSyn 2.0 software. 1, 2, and 3 represent 1, 10, and 20 nmol/L calcitriol combination, respectively.

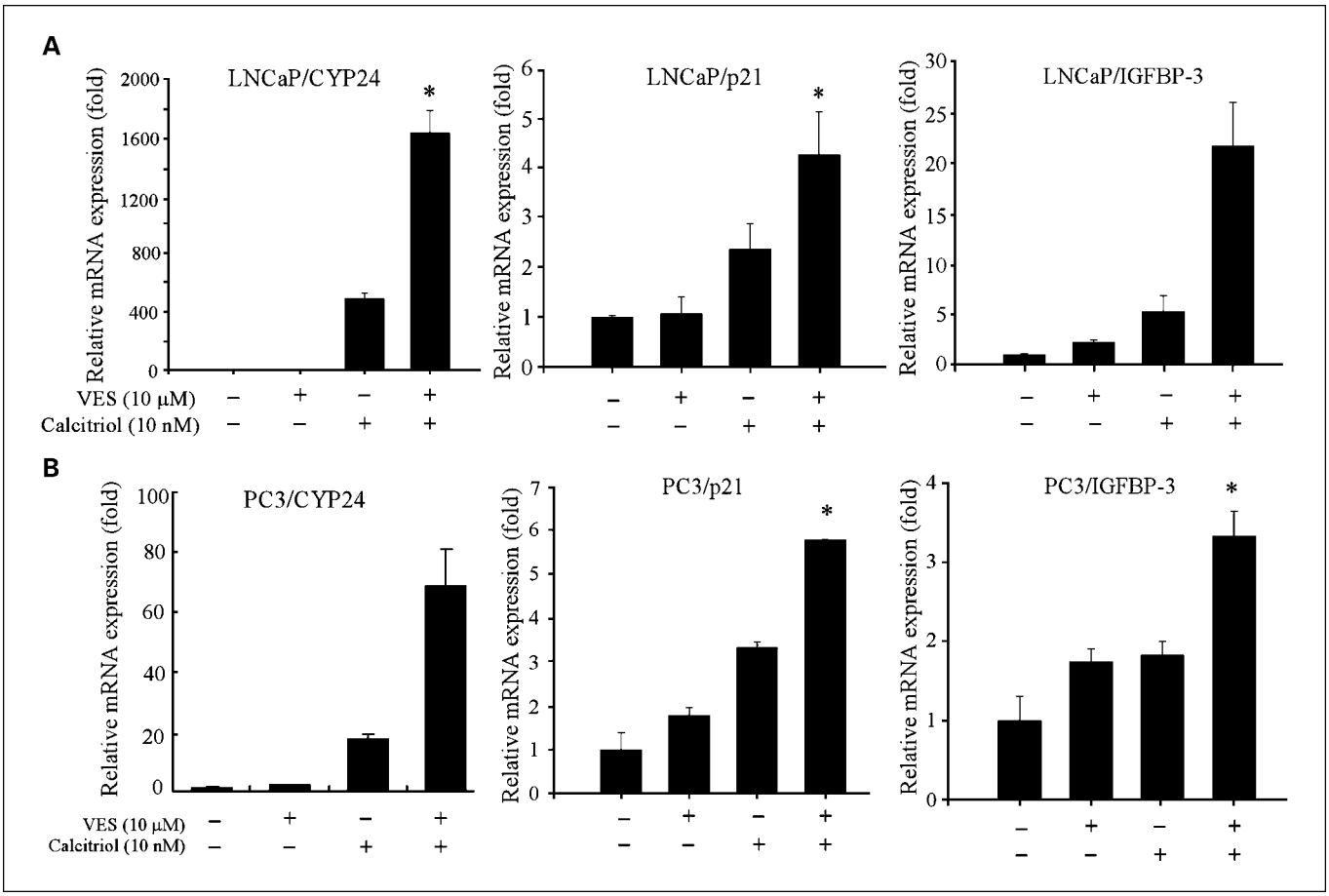

Fig. 3. VES sensitizes calcitriol-induced expression of VDR target genes in prostate cancer cells. After treatment of VES (10 μmol/L) or ethanol for 24 h the cells were exposed to calcitriol (10 nmol/L) for an additional 24 h *A,* CYP24, IGFBP-3, and p21 mRNA levels in LNCaP cells were evaluated by real-time RT-PCR. *B,* CYP24, IGFBP-3, and p21 mRNA levels were analyzed in PC3 cells. Data, mean ± SD from three independent experiments; each sample was evaluated in triplicate in each experiment. *, statistically different ($P < 0.05$) versus VES and calcitriol single treatment. RT-PCR, reverse transcription-PCR.

further up-regulated in the presence of VES. Consistently, similar tendencies were observed in PC3 cells (Fig. 3B). Taken together, these data suggest that multiple VDR-mediated antiproliferation signaling pathways can be sensitized by VES in different prostate cancer cells, which could contribute to the additive and synergistic growth-inhibition effect of VES and calcitriol combination.

VDR is required for the VES-sensitized antiproliferation effect of calcitriol. To determine whether VDR expression level could affect the superior growth-inhibition effect by combining calcitriol and VES, we knocked down endogenous VDR expression in LNCaP and PC3 cells using siRNA. After selection with puromycin for 2 weeks, cells stably transfected with VDR siRNA or control scramble siRNA were harvested and subjected to Western blotting. VDR siRNA led to ~80% depletion of VDR protein in comparison with the scramble control siRNA in LNCaP and PC3 cells (Fig. 4A).

Furthermore, we detected the expression of VDR target genes in the VDR siRNA stable and scramble siRNA stable cells exposed to VES, calcitriol, or in combination with the same condition compared with parental LNCaP and PC3 cells. Real-time PCR data showed VES dramatically augmented calcitriol-induced expression of target genes, including CYP24, IGFBP-3, and p21, in scramble siRNA stably transfected LNCaP and PC3 cells. However, these three target genes have dramatically decreased responsiveness in VDR siRNA stably transfected LNCaP and PC3 cells in the presence of calcitriol only and combination treatments (Fig. 4B and C).

We next tested the growth responsiveness of VDR siRNA and scramble siRNA stable cells treated with VES (10 μmol/L) and calcitriol (10 nmol/L) alone or in combination. After 6 days of treatment, VES can still augment the calcitriol antiproliferation effect in both LNCaP and PC3 scramble siRNA stable cells. The VDR siRNA cells dramatically decreased their response to calcitriol alone as well as to the combination treatment (Fig. 4D). In LNCaP VDR siRNA cells treated with the combination of VES and calcitriol, only 19% of cells showed growth inhibition compared with 63% of scramble siRNA stable cells. However, VES alone was able to maintain its growth inhibitory effect on both the control and VDR-reduced cell lines. The VDR siRNA stably transfected cells can still be inhibited by VES treatment alone. In PC3 VDR siRNA stable cells treated with VES and calcitriol combination, only 20% of cells showed growth inhibition compared with 57% of scramble siRNA stable cells. These data imply that VDR is not only essential for the antiproliferation effect of calcitriol but is also responsible for the superior antiproliferation effect of the VES and calcitriol combination in both LNCaP and PC3 cells.

VES enhanced calcitriol-mediated anticancer activity* in vivo *without causing hypercalcemia. The above *in vitro* mechanistic results strongly support more efficient inhibition of prostate cancer cell viability by the novel combinatorial regimen of VES and cacitriol. We next determined whether VES sensitized the antineoplastic efficacy of calcitriol in preclinical animal studies. To evaluate the singular and combined effects of VES and calcitriol *in vivo*, animals bearing androgen-responsive LNCaP cell xenografts were used. Once tumors became palpable (>50 mm^3), mice were randomly divided into four groups and treated with vehicle, VES, calcitriol, or a combination of both VES and calcitriol. The tumor volumes were monitored weekly. At sacrifice, tumor volumes were increased by 14.35-fold, 8.25-fold, and 7.37-fold in mice treated with DMSO, calcitriol, and

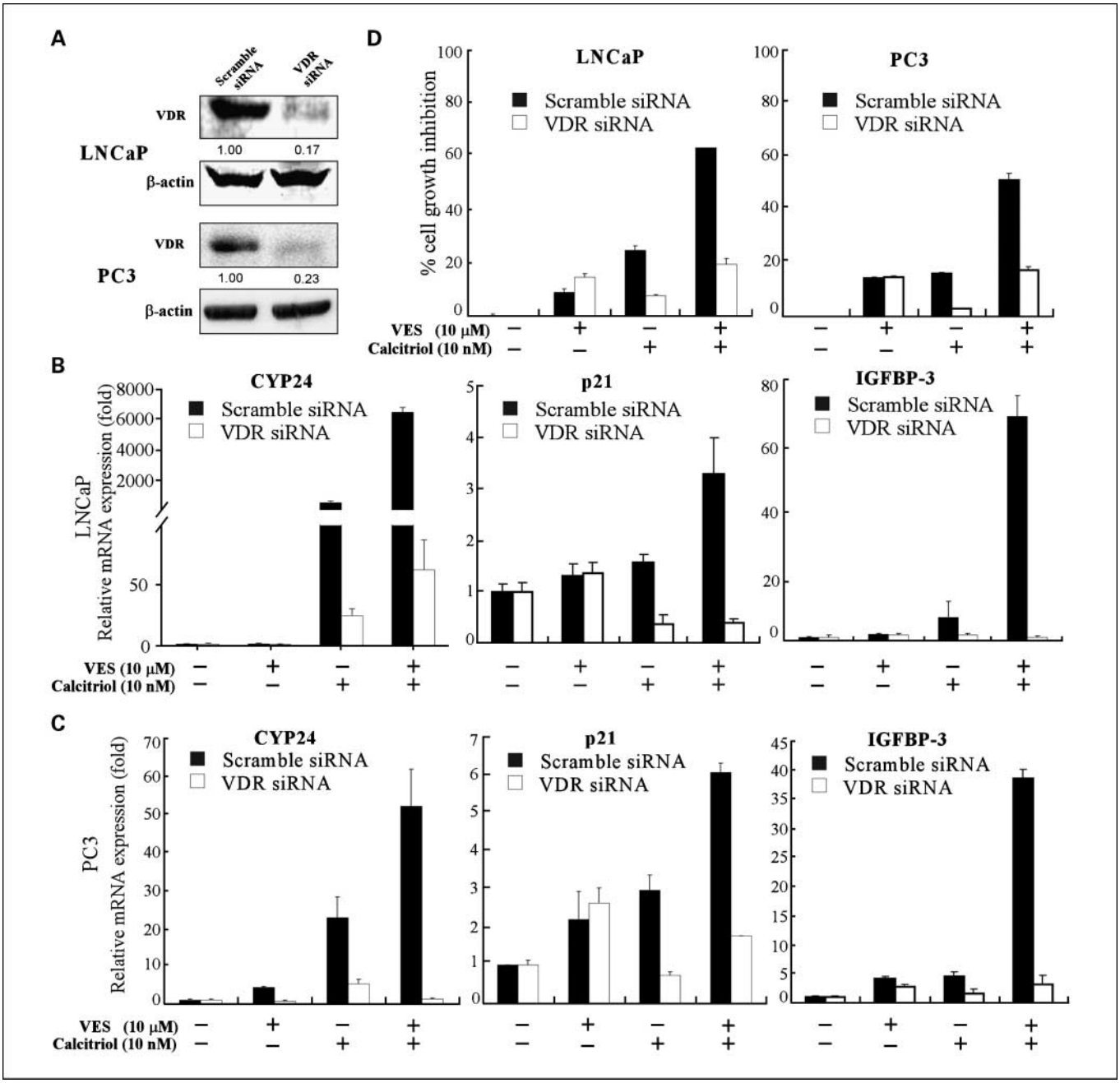

Fig. 4. VDR is required for the VES-sensitized antiproliferation effect of calcitriol. *A,* VDR expression was decreased by retrovirus-based VDR siRNA in LNCaP and PC3 cells. Scramble and VDR siRNA stably transfected cells were generated as described in Materials and Methods. Protein lysates of scramble and VDR siRNA stable cells were collected for VDR Western blotting. Number under panel, percentage of VDR protein in comparison with the scramble control as measured by the Quantity One software. Ratio of VDR/β-actin in scramble siRNA stable cells as 1.00. *B* and *C,* LNCaP and PC3 VDR siRNA or scramble siRNA stable cells were treated with VES (10 μmol/L) for 24 h then exposed to calcitriol (10 nmol/L) for an additional 24 h. RNA was isolated, and CYP24, IGFBP-3, and p21 mRNA levels were analyzed by real-time RT-PCR. *D,* scramble and VDR siRNA stably transfected LNCaP and PC3 cells were treated with vehicle control, VES (10 μmol/L), calcitriol (10 nmol/L), or the combination therapy. MTT growth assay was done after 6 d of treatment. The results are reported as the percentage of inhibition, considering control cells as 0% inhibition. Point, mean ± SD (n = 3).

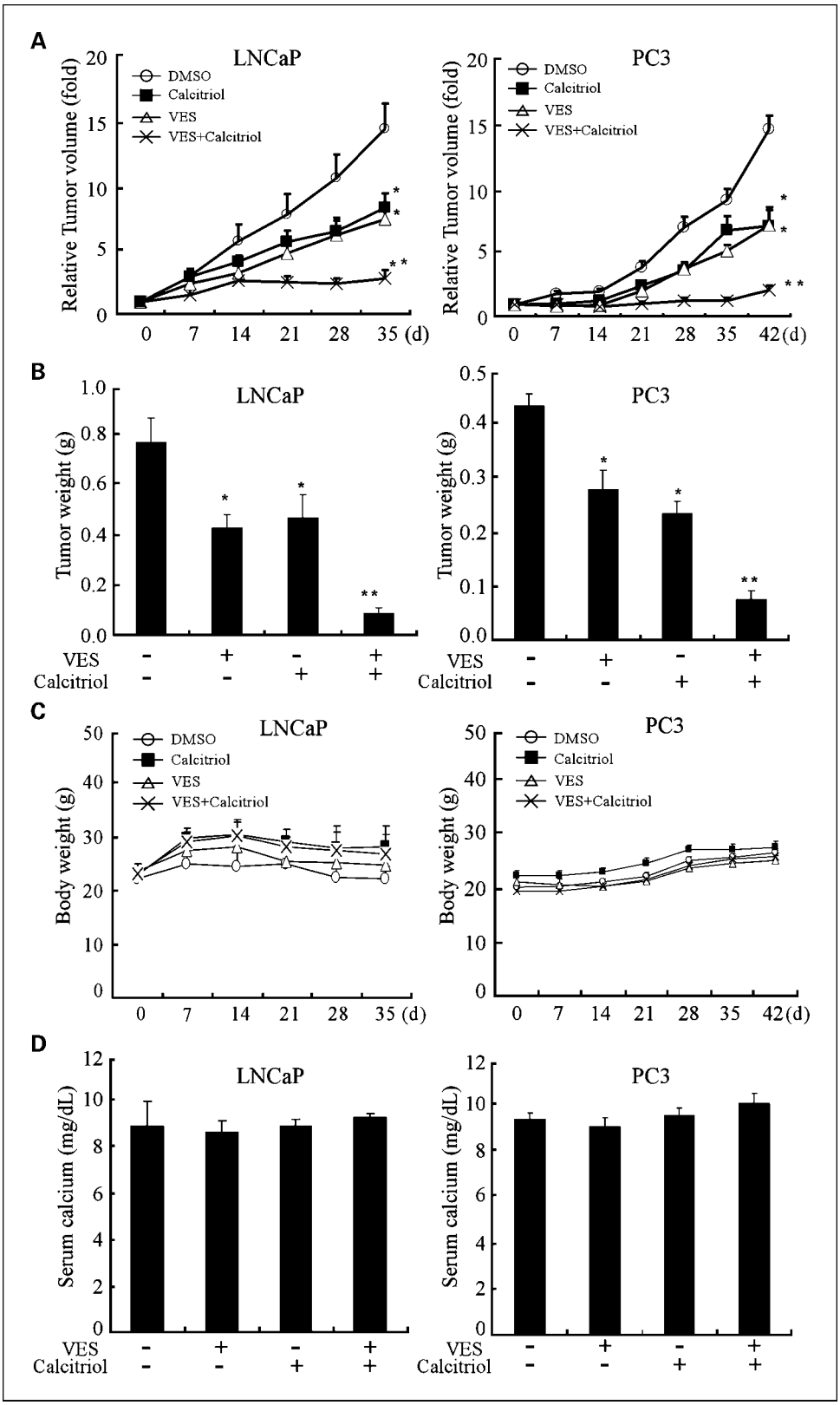

Fig. 5. VES enhances therapeutic effect of calcitriol on prostate cancer in the *in vivo* mouse cancer model. Animals were randomly divided into control and experimental groups after LNCaP or PC3 cells were injected in the dorsolateral flanks on both sides of each mouse. After tumors established to the size of 50 mm^3 (1-fold), mice were i.p. injected with vehicle (DMSO), VES (100 mg/kg), calcitriol (0.0625 μg/mice), or in combination twice weekly. *A,* tumor volumes in different treatment groups. Changes in tumor volume were measured once weekly. Relative changes in tumor volume were calculated by dividing tumor volumes measured during the course of therapy by the initial tumor volume (i.e., volume at day 0). Point, mean ± SE of 10 to 14 tumors. *B,* tumor weights in different treatment group. One day after the last treatment, tumors were harvested and weighed. Point, mean ± SE (n = 10-14 for each treatment group). *C,* no significant body weight changes in different treatment group. Body weights were monitored once a week. Point, mean ± SD (n = 7). *D,* no alteration of serum calcium in different treatment group. One day after the last treatment, mice were euthanized and sera were collected for calcium measurement. Point, mean ± SD of the 5 to 7 mice serum calcium value. *, $P < 0.05$ versus untreated controls. **, $P < 0.05$ versus single-agent VES or calcitriol. The animal experiments were repeated twice with similar results, and one set of results is presented.

VES alone, respectively. In contrast, the tumor volume was increased only 2.81-fold in the combination treatment group ($P < 0.05$; Fig. 5A).

Considering that clinical prostate cancer will progress from an androgen-dependent stage to an androgen-independent stage, a human androgen-independent PC3 xenograft model was also established s.c. to evaluate the *in vivo* therapeutic efficacy of calcitriol combining VES. At sacrifice, tumor volumes were increased by 14.54-fold, 7.06-fold, and 7.14-fold in mice treated with DMSO, calcitriol, and VES alone, respectively.

Notably, the tumor volume was increased only 2.11-fold in the combination-treatment group (Fig. 5A). The tumor weights also support the superior efficacy of the combination treatment (Fig. 5B).

It was shown that the dose of calcitriol we chose did not produce hypercalcemia or other notable toxicities in mice when injected i.p. 3 times weekly for a total of 12 weeks (20). During these experiments, it was found that calcitriol combined with VES did not impair the general health of the treated animals in comparison with treatment of either VES or calcitriol alone. The mean weights of each experimental group showed no statistically significant change compared with the control mice (Fig. 5C). More importantly, none of the mice displayed hypercalcemia as determined by serum calcium measurement (Fig. 5D). Furthermore, there was no appreciable difference among the treatment groups in terms of femoral bone density as determined by X-ray analysis. Histologic analysis of the brain, heart, lungs, liver, kidneys, spleen, spinal cord, and gastrointestinal and genitourinary tracts by H&E staining also produced results illustrating no appreciable difference among the treatment groups (data not shown). Ultimately, our results indicate that the combination treatment has improved anti-neoplastic effects without overt signs of side effects by examining serum calcium level, bone density, and multiple organ histology.

Histologic and molecular analysis of xenograft tumor from animals. The tissue samples obtained in these experiments were examined by H&E staining. In mock control mice, most of the Matrigel was replaced by tumor cells with the typical histologic appearance of poorly differentiated prostate adenocarcinomas without significant necrosis or fibrotic areas. In comparison, increased proportions of noncellular stroma components and scattered necrotic lesions were observed in the mice that received VES or calcitriol alone and in combination. The number of tumor cells was reduced dramatically in the mice that received the combination of

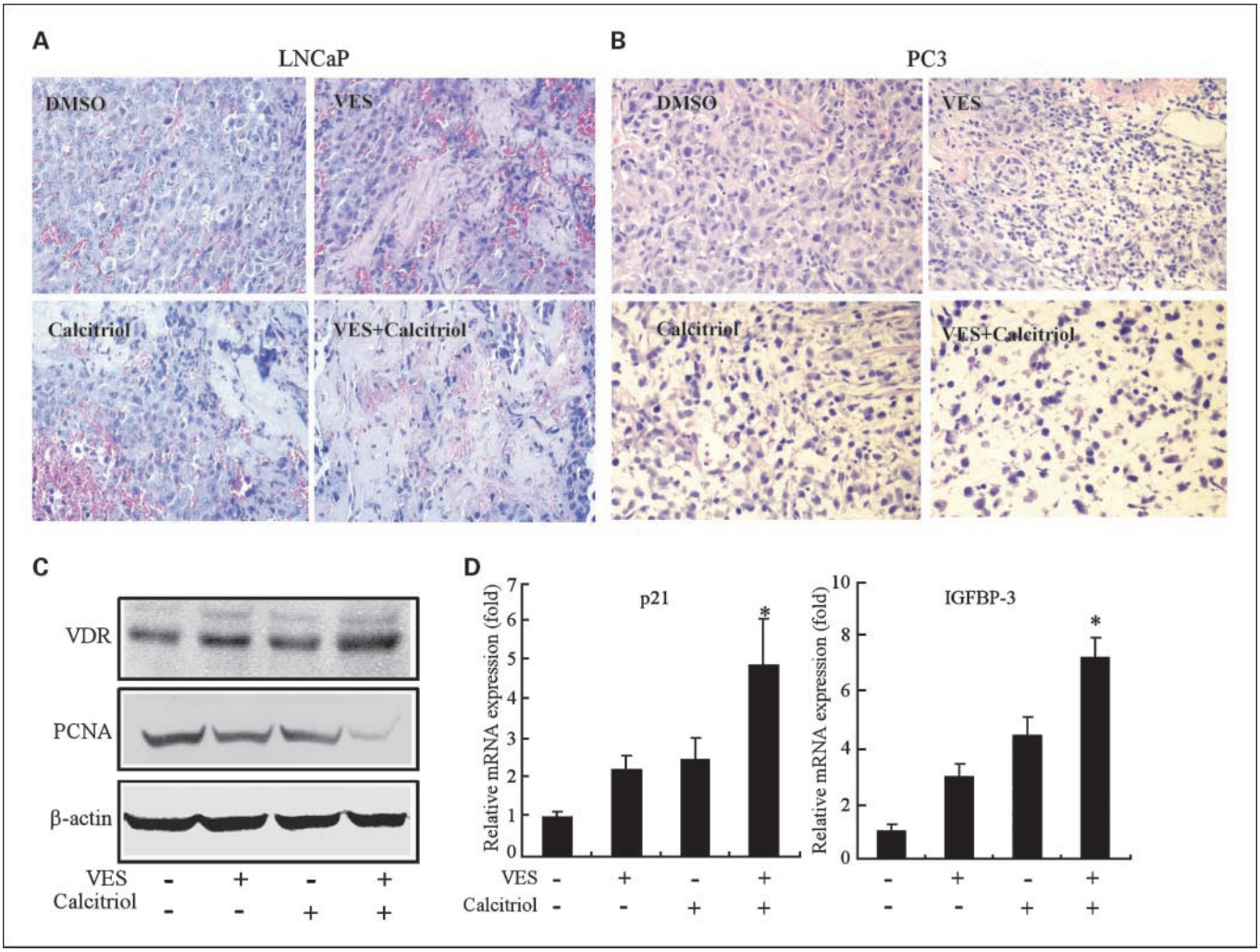

Fig. 6. VES increased calcitriol-mediated antiproliferative activity and the expression of VDR target genes in *in vivo* prostate cancer model. *A* and *B,* LNCaP and PC3 control xenografts have poorly differentiated carcinoma cells without necrosis (original ×400). Xenografts from mice that received VES alone showed an increased proportion of noncellular stroma components and scattered necrotic lesions. Xenografts from mice that were treated with calcitriol alone have a similar histologic appearance as VES single-treatment xenografts (original ×400). Xenografts from mice treated with the combination of VES and calcitriol show less cell density and extensive tumor necrosis and fibrotic changes (original ×400). *C,* extracts from tumor homogenates were subjected to Western blotting for expression levels of VDR, PCNA, and β-actin. *D,* IGFBP-3 and p21 mRNA changes in different group xenografts were detected by real-time RT-PCR. *, statistically different ($P < 0.05$) versus VES tumor lysate. Lysate of each treatment group was a mixture of five individual tumors. PCNA, proliferating cell nuclear antigen.

VES and calcitriol. Numerous areas of tumor were composed of fibrotic and necrotic tissue (Fig. 6A and B).

We subsequently evaluated three randomly selected tumor specimens per group to test whether VES and/or calcitriol combinations alter molecules that were observed during our *in vitro* studies. We measured the expression of VDR and two of its downstream target genes, IGFBP-3 and p21, within tumor tissues. Our results clearly show that the expression of VDR and its downstream effector genes, IGFBP-3 and p21, were significantly up-regulated in specimens obtained from the combination group (Fig. 6D). These *in vivo* results were similar to our *in vitro* findings, suggesting that the activation of VDR is, at least, one of the molecular events by which the drug combination potentiates antitumor activity in our experimental model. Xenograft lysate proteins were further analyzed by Western blotting (Fig. 6C). Consistent with the decreased proliferation of tumors undergoing concurrent treatment, our Western blotting results indicated that tumor cells had decreased expression of the proliferation specific antigen, proliferating cell nuclear antigen, in the combination treatment as compared with single treatment. VDR protein level was elevated in VES-treated xenografts and dramatically increased by the concurrent treatment, yet there was no significant increase in the expression of VDR in calcitriol-treated xenografts. Furthermore, mRNA expression levels of IGFBP-3 and p21 extracted from frozen tissues were also analyzed by real-time PCR. Consistent with the *in vitro* results, both IGFBP-3 and p21 can be further induced by combination treatment compared with the single-treatment tumor xenografts (Fig. 6D).

Collectively, our results indicated VES and calcitriol both have therapeutic effects. The VES also serves as a sensitizer that enhances VDR protein levels in prostate cancer and sensitizes calcitriol antineoplastic function. The antineoplastic effect of the combination is significantly superior to the sum of the individual treatment effects *in vitro* and *in vivo*. These *in vitro* and *in vivo* data suggest that low-dose calcitriol combined with the vitamin E analogue could be a solution to overcome the calcemic side effects and a useful therapeutic strategy for prostate cancer. Clinical testing of low-dose calcitriol in combination with VES agents is promising.

Discussion

In the current study, we have identified a novel calcitriol-based combination strategy targeting prostate cancer. The synergic interactions of combined drug treatments are classically defined using isobolographic analysis, and this method has been used by numerous studies (23, 25, 32). Using isobolographic analysis, our data indicated that a combination of calcitriol and VES was more effective than a single agent in the inhibition of androgen-dependent and androgen-independent prostate cancer *in vitro* and *in vivo*. The rationale for the combination of calcitriol and VES stems from augmenting VDR protein abundance by VES treatment in prostate cancer cells. Previous studies have shown that these individual agents exhibit an antineoplastic effect on prostate cancer, but this is the first time data showing that a combination of calcitriol and VES results in an antitumor effect that was significantly superior to the additive effect of individual treatments on prostate cancer cell growth *in vitro* and *in vivo*.

Both calcitriol and vitamin E compounds were shown to exert potent antineoplastic activity in a broad range of tumor models (3, 33). The clinical applications of calcitriol have been substantially limited by its calcemic actions. Daily doses of 1.5 to 2.5 μg calcitriol have been shown to cause the dose-dependent hypercalcemia and hypercalciura side effects (15), which are often the primary reason for early discontinuation of the chemotherapy and, thus, clinical failure (11). Alteration of calcium homeostasis by calcitriol can be dissociated from its anticancer pathways by antioxidation (34). It has been reported that combinations of antioxidants and calcitriol potentiate the prodifferentiation effects of calcitriol (35) but do not elevate the basal levels of intracellular calcium (36). VES not only has anticancer properties (3) but also has a general antioxidant function (37), which may be due to esterases converting VES into vitamin E to obtain antioxidant activity (38). The dosage of calcitriol used in our animal study was previously shown to not cause significant calcium-related side effects. After calcitriol (0.0625 μg/mice) treatment for 12 weeks, the serum calcium level was determined to be <11 mg/dL (20). However, this low dose of calcitriol did not have the dramatic antiproliferation effect previously witnessed in the LNCaP xenograft model (20). Interestingly, after combination with VES *in vivo*, the antiproliferation effect of low-dose calcitriol was dramatically increased. No overt evidence of systemic toxicity and hypercalcemia was observed. Hence, the combination of low-dosage calcitriol and VES may potentially exert the same or better chemotherapeutic efficiency, without the overt side effects, as a high dosage of calcitriol treatment. Although we have proven the beneficial therapy effect by combination of VES and calcitriol, one limitation of our animal study is the lack of enough combination dosages to show whether a VES and calcitriol combination has an additive or synergistic effect *in vivo*.

Understanding the molecular mechanisms of how calcitriol combined with VES induces VDR expression could provide a foundation for developing new strategies involving calcitriol and VES combination therapy. The detailed mechanisms of how calcitriol combined with VES influences the VDR protein remains to be elucidated. Calcitriol exerts antineoplastic activity through genomic and nongenomic pathways. The calcitriol-mediated genomic effect functioning through VDR plays a very important role in calcitriol-induced growth inhibition. The calcitriol-mediated nongenomic mechanisms are not required for growth inhibition by calcitriol in certain prostate cancer cell lines, such as ALVA-31 (39) and JCA-1 (40). It has also been shown that increased VDR can enhance the cellular sensitivity to calcitriol (41). However, the sensitivity to the antiproliferation function of calcitriol in tumor tissues depends not only on VDR receptor activity but also on the changes in the VDR target gene expression unique to the individual tumors (14). Although LNCaP cells are more sensitive than ALVA-31cells in response to calcitriol treatment, VDR levels are lower in LNCaP cells. Furthermore, DU145 cells have a functional VDR yet only have a marginal response to calcitriol. This may be due to the differences in the activation of the VDR target genes in those prostate cancer cells.

CYP24 is a calcitriol/VDR target gene and can function as an enzyme to initiate the first step catabolism of calcitriol to its less active metabolites; thus, CYP24 is a negative feedback target gene for VDR function. CYP24 inhibitor can increase the

calcitriol half-life in DU145 cells. Although calcitriol-induced levels of CYP24 mRNA are very dramatic compared with its basal levels, the CYP24 activity is low in LNCaP and PC3 cells, only 0.001% of DU145, and may not be sufficient to cause a significant change in the catabolism of calcitriol (26). Several calcitriol-based combination therapies also showed that CYP24 mRNA abundance is not always associated with the CYP24 activity (42). In this study, we used CYP24 as an indicator for the calcitriol genomic effect rather than an antiproliferative-related target gene.

IGFBP-3 and p21 both play important roles in growth inhibitory effects on prostate cancer cells. Several independent reports showed that IGFBP-3 has potent antitumor activities *in vitro* and *in vivo* (43, 44). A prospective study also showed that IGFBP-3 was inversely associated with prostate cancer risk (45). The growth regulatory effect of IGFBP-3 can go through IGF-dependent and IGF-independent pathways to induce cell cycle arrest or apoptosis. Recently, there have been reports suggesting that some potential antiproliferative factors, including VES (6), calcitriol (29), tumor necrosis factor-α (46), transforming growth factor-β (47), and retinoic acid (48), can induce IGFBP-3 expression. Both IGFBP-3-specific antisense oligonucleotides and IGFBP-3-neutralizing antibodies can partially block the antiproliferative effects. These phenomena suggest that IGFBP-3 acts as a key molecule for tumor therapy. Through regulating cell cycle arrest, cyclin-dependent kinase inhibitor p21 also mediates calcitriol antiproliferative effects in ALVA-31 and LNCaP cells (28, 49). VES has been reported to induce p21 expression in some cell lines. The combination of VES and calcitriol has a further induction of multiple VDR-downstream cell cycle arrest and apoptosis relative genes, including IGFBP-3 and p21, which may contribute to the superior antineoplastic effect of combining VES and calcitriol. We also note that there is a disconnection between the magnitude of VDR amplification and the increased expression of VDR target genes when the cells were exposed to VES. It has been reported that cellular CYP24 activity will affect the VDR ligand stability, and the higher cellular CYP24 could reduce the calcitriol responsiveness. Therefore, the reason that calcitriol target gene expression in PC3 cells cannot be induced as well as in LNCaP cells could be due to the differential expression and activity of endogenous CYP24 in different human prostate cancer cell lines (50).

In summary, this report provides evidence that VES sensitizes calcitriol antineoplastic activity by increasing the VDR protein stability and synthesis, and then cross-talks with the calcitriol pathway to enhance calcitriol-mediated antiproliferation activity. The combination of VES with calcitriol has the better therapeutic effect, significantly superior to the additive effect of the individual treatments at inhibiting prostate cancer growth *in vitro* and *in vivo* yet without hypercalcemic side effects (Fig. 5). The VDR siRNA technique further confirms the requirement of VDR expression in mediating the superior anticancer effect of the combining treatment. The *in vitro* and *in vivo* results showed that VES and calcitriol combination therapy is promising. Our data support the clinical application of combining low-dose calcitriol and VES to treat prostate cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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