

## Molecular Mechanism(s) Involved in 25-Hydroxyvitamin D's Antiproliferative Effects in *CYP27B1*-transfected LNCaP Cells

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**Abstract.** *Background/Aim:*  $1\alpha,25(\text{OH})_2\text{D}$  has been shown to induce cell-cycle regulation, apoptosis and differentiation in prostate cancer cells. Previous studies have demonstrated that prostate and some prostate cancer cells have the ability to convert  $25(\text{OH})\text{D}_3$  to  $1\alpha,25(\text{OH})_2\text{D}_3$ . The aim of the present study was to elucidate the role of  $1\alpha,25(\text{OH})_2\text{D}_3$  production by 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (*CYP27B1*) on prostate cancer cell growth. *Materials and Methods:* LNCaP cells were stably transfected with *CYP27B*. *Results:* Stably-transfected 1 $\alpha$ -OHase LNCaP cells converted  $25(\text{OH})\text{D}_3$  to  $1\alpha,25(\text{OH})_2\text{D}_3$  unlike untransfected LNCaP cells. There was a dose-dependent decrease in  $^3\text{H}$ -thymidine incorporation in  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated LNCaP cells, not seen with  $25(\text{OH})\text{D}_3$  treatment, and in stably transfected 1 $\alpha$ -OHase LNCaP cells treated with  $25(\text{OH})\text{D}_3$ .  $1\alpha,25(\text{OH})_2\text{D}_3$ -treated LNCaP cells and  $25(\text{OH})\text{D}_3$ -treated stably-transfected 1 $\alpha$ -OHase LNCaP cells demonstrated an increased  $G_1$  phase accumulation and apoptosis, while  $25(\text{OH})\text{D}_3$  treatment had no effect in LNCaP cells. *Conclusion:* The present study supports the hypothesis that local production of  $1\alpha,25(\text{OH})_2\text{D}$  is important in inhibiting prostate cancer development and growth.

Prostate cancer is the most prevalent cancer among men and the second leading cause of cancer-related death among men in the US. It is estimated that 217,730 men were diagnosed with and 32,050 men died of prostate cancer in 2010 (1). Prostate cancer occurs more often in elderly men and as the

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average life expectancy continues to increase and the elderly population grows there is a greater need for effective preventative strategies and therapeutics for prostate cancer.

Garland *et al.* first determined in the Johns Hopkins University Operation Clue Cohort, following an eight-year study, serum  $25(\text{OH})\text{D}$  levels as a biomarker for cancer risk (2). Schwartz proposed that vitamin D deficiency underlies the major risks for prostate cancer, including age, Black race and Northern latitudes (3). Hanchette and Schwartz examined the geographic distributions of UV radiation and prostate cancer mortality in 3,073 counties of the contiguous United States and found that they were inversely correlated (4). Ahonen *et al.*, in a nested case-control study, concluded that low serum  $25(\text{OH})\text{D}$  levels were associated with an increased risk for earlier progressive and more aggressive development of prostate cancer (5). There is a relationship between vitamin D status and prostate cancer. However, the exact mechanism(s) of vitamins D's effects on prostate cancer growth and progression remain elusive.

$25(\text{OH})\text{D}$  is the major circulating form of vitamin D due to its high binding affinity to the vitamin D binding protein resulting in reduced clearance and an increased half-life. Bioactivation of  $25(\text{OH})\text{D}$  to  $1\alpha,25(\text{OH})_2\text{D}$  requires a hydroxylation at the 1 $\alpha$  position. The enzyme responsible for this conversion is 25-hydroxyvitamin D-1 $\alpha$ -hydroxylase (1 $\alpha$ -OHase; *CYP27B1*). Both prostate primary and cancer cell lines were shown to have 1 $\alpha$ -OHase activity suggesting that vitamin D autocrine and paracrine functions were important in modulating prostate cell growth (6, 7). Hsu *et al.* demonstrated a marked decline in 1 $\alpha$ -OHase activity in benign prostate hyperplasia and prostate cancer cells compared to normal prostate cells in specimens from men of similar ages (8). Ma *et al.* concluded that the reduced 1 $\alpha$ -OHase in prostate cancer cell lines was due to decreased gene expression, while in primary cultures and tissues the cause was post-translational (9). These findings indicate that the loss of 1 $\alpha$ -OHase, an enzyme that synthesizes the growth-inhibitory hormone,  $1\alpha,25(\text{OH})_2\text{D}$ , may be associated with development and progression of prostate cancer.

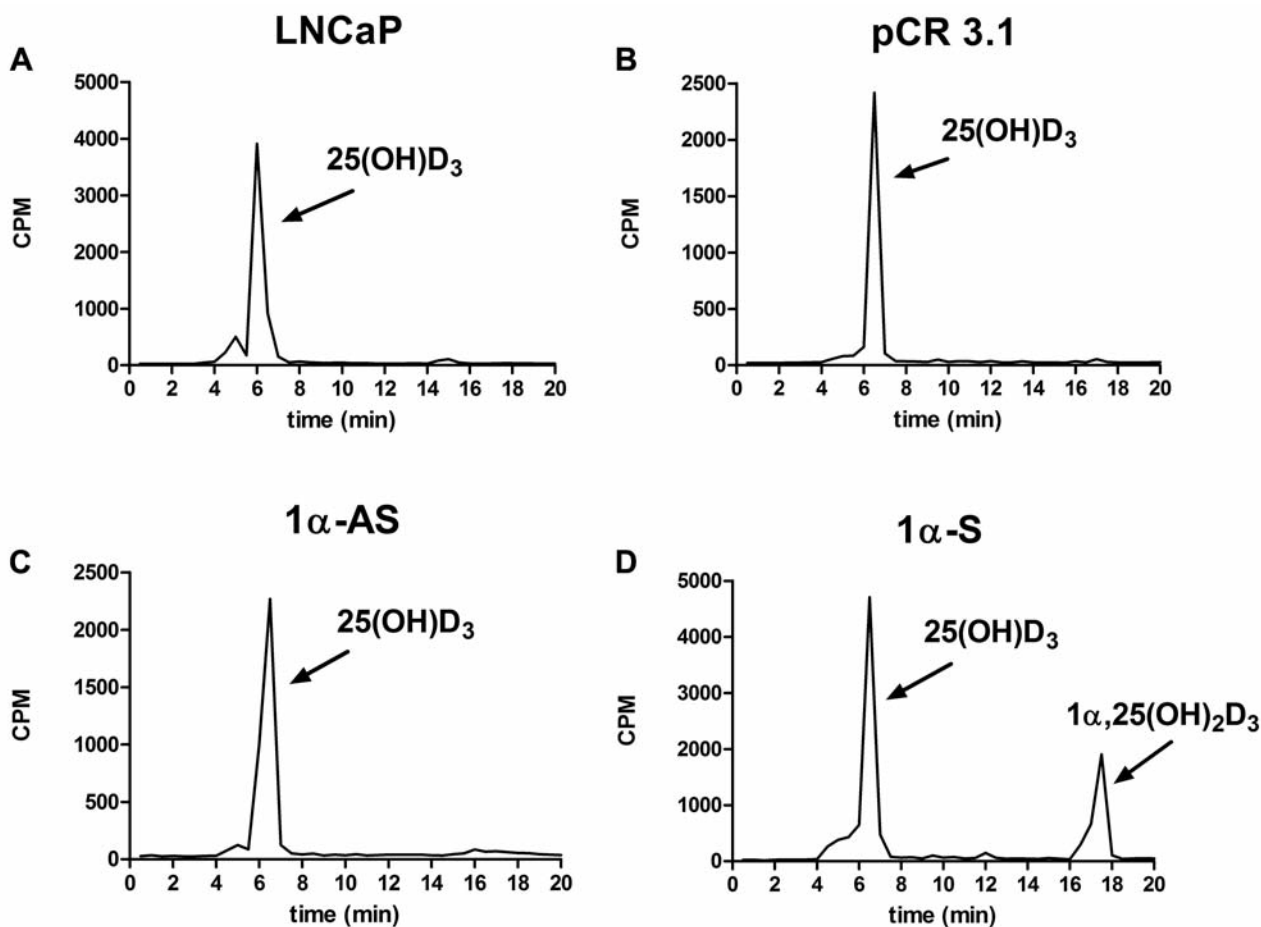


Figure 1.  $^3\text{H}$ - $25(\text{OH})\text{D}_3$  conversion to  $3\text{H}$ - $1\alpha,25(\text{OH})_2\text{D}_3$ . A. Untransfected LNCaP. B. pCR3.1, stably-transfected empty plasmid vector. C.  $1\alpha$ -AS, stably-transfected CYP27B1 antisense.  $1\alpha$ -S, stably-transfected CYP27B1 sense strand LNCaP cells.

$1\alpha,25(\text{OH})_2\text{D}_3$  has numerous physiological actions, including calcium homeostasis, immune response, cellular proliferation and differentiation and apoptosis. Prostate-specific antigen, a differentiation marker, can be increased with  $1\alpha,25(\text{OH})_2\text{D}_3$ . Additionally,  $1\alpha,25(\text{OH})_2\text{D}_3$  has been shown to inhibit the invasiveness of prostate cancer cells (10).  $1\alpha,25(\text{OH})_2\text{D}_3$  up-regulated cell-cycle regulatory genes and caused cellular arrest at the  $G_1$ -S phase transition in cancer cells (11-13).

We choose to use LNCaP cells as an *in vitro* model because it is an androgen-sensitive prostate cancer cell line that has a functional VDR and P53. These cells respond to  $1\alpha,25(\text{OH})_2\text{D}_3$  but not  $25(\text{OH})\text{D}_3$  and have been shown to lack  $1\alpha$ -OHase activity (7). As LNCaP cells do not have  $1\alpha$ -OHase activity, we wanted to determine if restoration of  $1\alpha$ -OHase would have similar anti-cancer effects with  $25(\text{OH})\text{D}_3$  as is seen with  $1\alpha,25(\text{OH})_2\text{D}_3$ .

## Materials and Methods

**Stable transfection of LNCaP cells with  $1\alpha$ -OHase cDNA.** LNCaP cells were obtained from American Type Culture Collection (Rockville, MD, USA) and transfected using Lipofectamine (Invitrogen, Carlsbad, CA, USA) with either  $1\alpha$ -OHase sense vectors,  $1\alpha$ -OHase antisense vectors or empty vectors (pCR 3.1; 6  $\mu\text{g}$  per 60mm dish) and then cultured in RPMI 1640 medium supplemented with 10% FBS, 100 IU/ml penicillin, 100  $\mu\text{g}/\text{ml}$  streptomycin and 2 mM L-glutamine as described (12). The cells were maintained on RPMI 1640 with 10% FBS and Geneticin (G418 at 350  $\mu\text{g}/\text{ml}$ ). After transfection, cells were passaged twice for expansion, then aliquots were made. From each aliquot, cells were cultured between 5-10 times before starting with a new aliquot. LNCaP cells stably transfected with the sense strand of  $1\alpha$ -OHase were designated  $1\alpha$ -S; antisense  $1\alpha$ -OHase as  $1\alpha$ -AS and pCR3.1 vector as pCR3.1.

**Confocal microscopy.** LNCaP and  $1\alpha$ -S cells were grown on 4-chamber slides (Thermo Scientific, Carlsbad, CA, USA). Cells

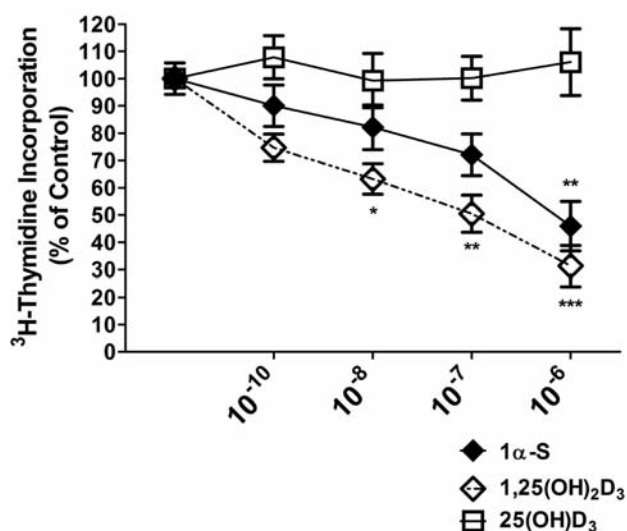


Figure 2. Dose response effect of 25(OH)D<sub>3</sub> and 1α,25(OH)<sub>2</sub>D<sub>3</sub> on <sup>3</sup>H-thymidine incorporation into DNA in LNCaP and stably transfected 1α-OHase LNCaP cells. Filled diamond-25(OH)D<sub>3</sub> treatment in stably-transfected 1α-OHase LNCaP cells. Open diamond 1α,25(OH)<sub>2</sub>D<sub>3</sub> treated LNCaP cells. Open square, 25(OH)D<sub>3</sub>-treated LNCaP cells. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

were stained using CellMask Orange (Invitrogen), Mitotracker Deep Red (Invitrogen) and DAPI (Invitrogen) per manufacturer's protocols, then fixed using Slow Fade Gold. Imaging was performed on a Zeiss LSM 510 confocal microscope (Zeiss; Thornwood, NY, USA) as described previously (14). Images were taken using a ×63 objective with an optical slice of 3 μm. Z-stacks were taken with an optical slice of 1 μm at an interval of 0.5 μm. Images were collected with ×4 averaging. Detector gain and amplitude offset were determined for each experiment to maximize the linear range without saturation and were kept consistent for comparable experiments. Average fluorescence intensities were measured in individual cells using Zeiss LSM software (Zeiss; Thornwood, NY, USA) for region of interest and graphed throughout time.

**<sup>3</sup>H-25(OH)D<sub>3</sub> metabolism to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub>.** Assessment of 1α-OHase activity was performed in LNCaP, 1α-S, 1α-AS and pCR3.1 cells in the presence of 50 nM of <sup>3</sup>H-25(OH)D<sub>3</sub> and 10 μM 1,2-dianiloethane (DPPD) as previously described (7). DPPD is an antioxidant used to prevent the auto-oxidation of 25(OH)D<sub>3</sub> to 1α,25(OH)<sub>2</sub>D<sub>3</sub>. Cells were incubated for 3 hours at 37°C then placed on ice to remove tritiated media.

**Methanol extract 25(OH)D<sub>3</sub> and metabolites.** Extraction occurred at room temperature for 15 minutes with vigorous shaking and repeated twice. Extracts were combined, dried under nitrogen gas then re-suspended in methylene chloride:isopropanol (19:1). High performance liquid chromatography (HPLC) on a Waters Associates model 501 was used to separate <sup>3</sup>H-25(OH)D<sub>3</sub> from its metabolites. The column was Zorbax silica (5 μm, 250×4.6mm). The mobile phase was methylene chloride:isopropanol (19:1) with

a flow rate of 1.2 ml/minute. Fractions were collected every 30 seconds, dried under nitrogen gas, scintillation fluid was added and radioactivity was assessed by β-liquid scintillation counter (Microbeta Trilux 1450 LSC; Perkin-Elmer, Waltham, MA, USA).

**<sup>3</sup>H-Thymidine incorporation.** Tritiated thymidine incorporation was used to measure the amount of DNA synthesis in LNCaP cells as previously described (15). Briefly, cells were plated in 24-well plates at a concentration of 30,000 cells/well. Once the wells were 50% confluent, media with FBS were replaced with fresh media without FBS and the cells were grown for an additional 24 h. Cells were then treated with 25(OH)D<sub>3</sub> and 1α,25(OH)<sub>2</sub>D<sub>3</sub> at 10<sup>-10</sup> to 10<sup>-6</sup> M concentrations. Eighteen hours later, cells were treated with 10 μCi <sup>3</sup>H-thymidine and incubated at 37°C for 3 h. The plate was then placed on ice and the <sup>3</sup>H-thymidine was removed with three ice-cold PBS washings. Perchloric acid (5%) was added to the wells and the plate was incubated on ice for 20 min to remove externally [<sup>3</sup>H]-thymidine-labeled DNA and other macromolecules. More perchloric acid was added and the plate was incubated at 70°C for 30 min to extract intracellular [<sup>3</sup>H]-thymidine-labeled DNA. The radioactivity in the extracts was determined by a liquid scintillation counter.

**Caspase 3 activity.** Caspase 3 activity was assessed using the Caspase 3 Assay kit (Sigma-Aldrich, St. Louis, MI, USA) to determine any apoptotic effects 25(OH)D<sub>3</sub> and 1α,25(OH)<sub>2</sub>D<sub>3</sub> had on prostate cancer cells. LNCaP, 1α-AS, pCR3.1 and stably transfected 1α-OHase LNCaP cells were either treated with 25(OH)D<sub>3</sub> and 1α,25(OH)<sub>2</sub>D<sub>3</sub> at 10<sup>-10</sup> to 10<sup>-6</sup> M concentrations. In addition to untreated, nontransfected cells as controls, there was a caspase 3 positive control, a reagent blank negative control, a negative siRNA control and an inhibitor-treated cell lysate control in these experiments. Absorbance was read on a microplate reader (Varioskan; Thermo Scientific, MA, Waltham, USA) at 405 nm and results were calculated using a p-nitroaniline calibration curve.

**Cell-cycle progression.** To assess changes in cell-cycle progression due to 25(OH)D and 1α,25(OH)<sub>2</sub>D treatment, propidium iodide (PI) staining and fluorescence-activated cell sorting (FACS) were performed. LNCaP, stably transfected 1α-OHase LNCaP and DU-145 cells were treated with 25(OH)D and 1α,25(OH)<sub>2</sub>D at 10<sup>-10</sup> to 10<sup>-6</sup> M concentrations. After treatment, media was removed; cells were washed with 1× PBS 3 times, trypsinized, centrifuged at 500×g for 2 min and collected at 1×10<sup>6</sup> cells/ml concentration in 15 ml centrifuge tubes. Cell pellets were washed once with 1× PBS. Cells were re-suspended and fixed in 5 ml of ice cold ethanol, then stored at -20°C overnight. Samples were centrifuged at 500×g for 10 minutes to pellet fixed cells. The resulting pellets were incubated in a PI staining solution (1× PBS+50 μg/ml PI+100 μg/ml RNase A) for 30 minutes in the dark at 4°C. After incubation, cells were transferred to fresh tubes and then analyzed using FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). At least 10,000 forward scatter gated events were collected per specimen. PI fluorescence was collected using linear amplification with doublet discrimination. PI emissions were collected using a 575 band pass filter. Analysis was done using CellQuest Pro v5.2 (Becton Dickinson, Franklin Lakes, NJ, USA).

**Statistical analysis.** All experiments were performed in triplicate, unless otherwise indicated, and mean values were presented

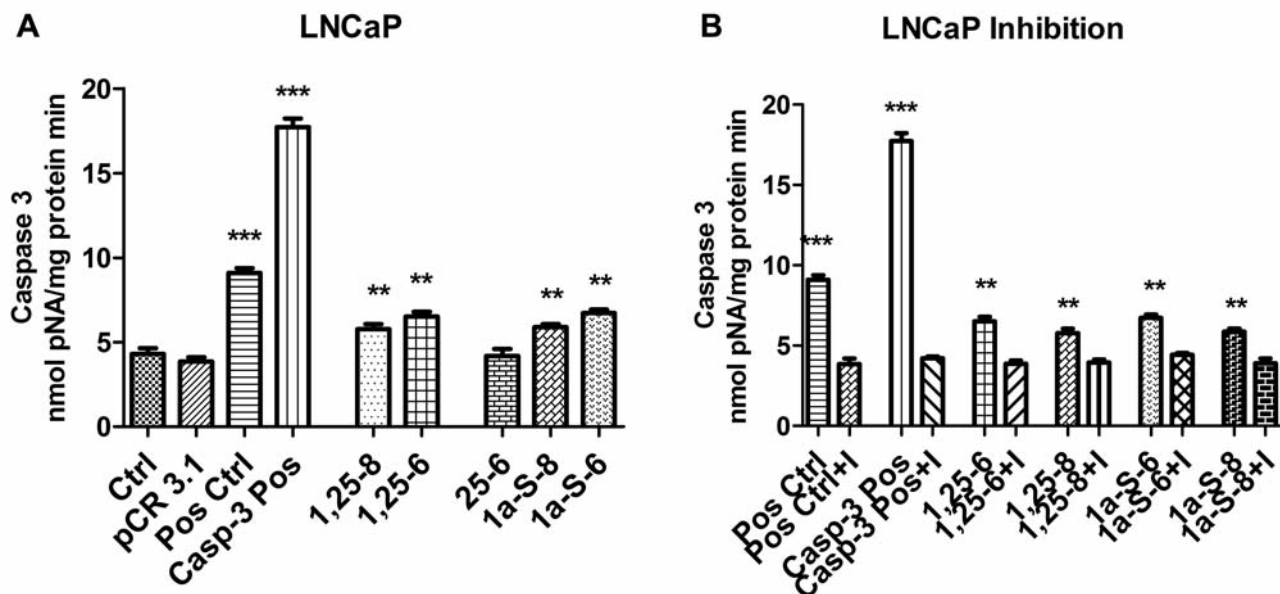


Figure 3 A and B. Caspase 3 activity in LNCaP and stably transfected 1α-OHase LNCaP cells. Pos Ctrl-(1μg/mL) staurosporine treated LNCaP cells, Casp-3 Pos-caspase 3 purified protein, which served as a positive control. Bars represent means±SEM of 3 experiments. \*, p<0.05, \*\*, p<0.01, \*\*\*, p<0.001 versus control, +, p<0.05 versus 1,25(OH)D at 10<sup>-6</sup>M.

as±standard error of mean (SEM). A one-way ANOVA was used to statistically analyze the recorded data. The software used for these analyses was GraphPad PRISM v5 (La Jolla, CA, USA). For all statistical tests, p<0.05 was considered to be significant.

**Results**

*CYP27B1 gene expression and localization within LNCaP cells.* In stably-transfected 1α-OHase cells, 1α-S, mRNA expression for *CYP27B1* was shown to be significantly abundant (4.1±0.5-fold) compared to LNCaP cells. The transfected 1α-OHase had a green fluorescent protein GFP tag that was used to detect its localization within the cell. In stably-transfected 1α-OHase LNCaP cells, in addition to the 1α-OHase –GFP, the nucleus was stained blue and the mitochondria were stained red. The presence of the 1α-OHase protein was perinuclear and punctuate in appearance. When both the 1α-OHase and the mitochondria images were superimposed, the resulting image showed a co-localization of the 1α-OHase (green fluorescence) with the mitochondria (red fluorescence) that appeared yellowish-green, consistent with 1α-OHase localization in the mitochondria.

*<sup>3</sup>H-25(OH)D<sub>3</sub> conversion to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub>.* To determine whether the transfected 1α-OHase was functional, the efficiency of <sup>3</sup>H-25(OH)D<sub>3</sub> conversion to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub> in stably transfected 1α-OHase LNCaP cells was evaluated.

HPLC analysis revealed that a significant increase in the conversion of <sup>3</sup>H-25(OH)D<sub>3</sub> to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub> in stably-transfected 1α-OHase LNCaP cells compared to an undetectable amount of conversion in pCR3.1 (vector), 1α-AS (antisense) or LNCaP cells. In stably-transfected 1α-OHase LNCaP cells, an average of 15.8±4.5% of <sup>3</sup>H-25(OH)D<sub>3</sub> was converted to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub> compared to no conversion by LNCaP cells. The HPLC chromatogram illustrated that the 1α,25(OH)<sub>2</sub>D<sub>3</sub> fraction was well-separated from 25(OH)D<sub>3</sub> (Figure 1 panel C).

To determine the importance of intracellular 1α,25(OH)<sub>2</sub>D<sub>3</sub> production on prostate cancer proliferation, LNCaP cells were stably transfected with *CYP27B1*. The conversion of <sup>3</sup>H-25(OH)D<sub>3</sub> to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub> was determined using HPLC to separate <sup>3</sup>H-25(OH)D<sub>3</sub> from its metabolites, including <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub>. There was no detectable conversion of <sup>3</sup>H-25(OH)D to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub> in LNCaP, pCR3.1 and 1α-AS cells. The newly stably-transfected 1α-OHase LNCaP cells had an initial 40% conversion of <sup>3</sup>H-25(OH)D to <sup>3</sup>H-1α,25(OH)<sub>2</sub>D<sub>3</sub> (Figure 1). *<sup>3</sup>H-Thymidine incorporation.* Cellular proliferation for LNCaP and stably-transfected 1α-OHase LNCaP cells was assessed by the direct measurement of <sup>3</sup>H-thymidine incorporation. Thymidine incorporation was significantly decreased in LNCaP cells treated with 1α,25(OH)<sub>2</sub>D<sub>3</sub> at 10<sup>-8</sup>M, 10<sup>-7</sup>M, 10<sup>-6</sup>M by 37.8±5.6%, 50.5±6.8% and 68.7±7.5%, respectively (Figure 2). Treatment with 25(OH)D<sub>3</sub> resulted in no significant decrease in <sup>3</sup>H-



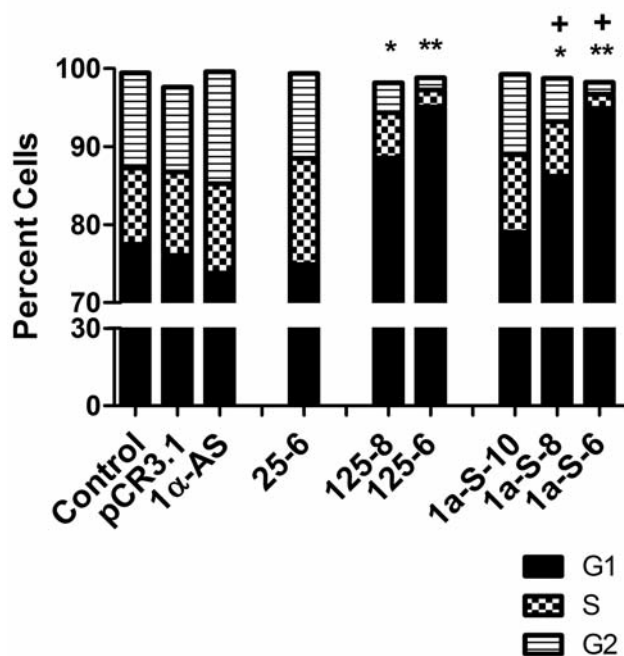


Figure 4. Effect of 25(OH)D<sub>3</sub> and 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> on cell cycle progression in LNCaP and stably transfected 1 $\alpha$ -OHase LNCaP cells. \* $p < 0.05$ , \*\* $p < 0.01$ , + $p < 0.05$  compared to 25(OH)D<sub>3</sub> at 10<sup>-6</sup>M

thymidine incorporation. With 25(OH)D<sub>3</sub> treatment, there was a downward trend in the inhibition of thymidine incorporation that was significant at 10<sup>-6</sup>M (54.1 $\pm$ 9.0%) in stably-transfected 1 $\alpha$ -OHase LNCaP cells.

**Caspase 3 Activity.** To verify that the DNA fragmentation seen with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and 25(OH)D<sub>3</sub> treatments was the result of cells undergoing apoptosis, caspase 3 activity was assessed by cleavage of acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) resulting in the release of the p-nitroaniline (pNA). Non-transfected, untreated LNCaP cells, which served as control, produced 4.3 $\pm$ 0.3 nmol pNA/mg protein<sup>-1</sup> min<sup>-1</sup>. Neither pCR3.1, 1 $\alpha$ -AS nor LNCaP cells treated with 25(OH)D<sub>3</sub> at 10<sup>-6</sup>M had increased caspase 3 activity. 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> at 10<sup>-8</sup>M and 10<sup>-6</sup>M produced 5.8 $\pm$ 0.3 and 6.5 $\pm$ 0.3 nmol pNA/mg protein<sup>-1</sup> min<sup>-1</sup>, respectively (Figure 3A). In stably-transfected 1 $\alpha$ -OHase LNCaP cells, 25(OH)D<sub>3</sub> treatment at 10<sup>-8</sup>M and 10<sup>-6</sup>M produced 5.9 $\pm$ 0.2 and 6.7 $\pm$ 0.2 nmol pNA/mg protein<sup>-1</sup> min<sup>-1</sup>, respectively (Figure 3A). Additionally, there was a siRNA negative control and two positive controls; one induced by 1  $\mu$ g/ml staurosporine and purified caspase 3 enzyme that was provided in the kit. This increased activity was inhibited with the addition of Ac-DEVD-CHO, a reversible caspase 3 inhibitor (Figure 3B). In DU-145 cells, treatment with 25(OH)D<sub>3</sub> at 10<sup>-6</sup>M with or

without *CYP27B1* suppression had no effect on Caspase 3 activity compared to control (3.4 $\pm$ 0.04 vs. 3.2 $\pm$ 0.2 nmol pNA/mg protein<sup>-1</sup> min<sup>-1</sup>).

**Cell-cycle analysis.** Several studies in a variety of cell types have demonstrated that 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> inhibits the G<sub>1</sub>-S phase transition in the cell cycle (11-13). To determine if 25(OH)D<sub>3</sub> treatment would change cell-cycle progression in stably transfected 1 $\alpha$ -OHase LNCaP cells, cells were PI stained and FACScanned. LNCaP cells treated with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> at 10<sup>-8</sup>M had 88.5% and 10<sup>-6</sup>M had 95.1% of cells in the G<sub>1</sub> phase compared to control (77.5 $\pm$ 3.6%). With 25(OH)D<sub>3</sub> treatment at 10<sup>-8</sup>M and 10<sup>-6</sup>M, the percentage of stably transfected 1 $\alpha$ -OHase LNCaP cells in the G<sub>1</sub> phase was 86.2% and 94.9%, respectively. LNCaP cells treated with 25(OH)D<sub>3</sub> at 10<sup>-6</sup>M had no significant change in the percentage of cells in the G<sub>1</sub> phase compared to control (Figure 4).

## Discussion

LNCaP cells have undetectable 1 $\alpha$ -OHase activity, which may be responsible for the lack of growth inhibition with 25(OH)D<sub>3</sub> treatment. However, these cells do respond to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>. LNCaP cells were stably transfected with the *CYP27B1* gene. Stably transfected 1 $\alpha$ -OHase LNCaP cells were assessed for 1 $\alpha$ -OHase activity and demonstrated conversion of <sup>3</sup>H-25(OH)D<sub>3</sub> to 3H-1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, whereas no conversion was observed in the vehicle control, pCR3.1, antisense strand transfected, 1 $\alpha$ -AS, nor untransfected LNCaP cells.

Earlier work by Whitlatch *et al.* demonstrated that prostate cancer cells have less 1 $\alpha$ -OHase activity compared to normal prostate cells and with enhanced gene expression of 1 $\alpha$ -OHase in LNCaP cells there was increased conversion of 25(OH)D<sub>3</sub> to 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> and increased 25(OH)D<sub>3</sub>-induced growth inhibition (7). These results suggest that the loss of 1 $\alpha$ -OHase may be associated with prostate cancer. We have shown that with reinstated 1 $\alpha$ -OHase activity, LNCaP cells respond to 25(OH)D<sub>3</sub> resulting in increased apoptotic activity, inhibition of cell-cycle progression and decreased proliferation.

In LNCaP cells, 25(OH)D<sub>3</sub> had no inhibitory effect on proliferation even at 10<sup>-6</sup>M. However, with 1 $\alpha$ -OHase activity restored, 25(OH)D<sub>3</sub> inhibited proliferation in a dose-dependent manner in stably-transfected 1 $\alpha$ -OHase LNCaP cells. <sup>3</sup>H-thymidine incorporation results for LNCaP cells support the hypothesis that 1 $\alpha$ -OHase is important for controlling prostate cancer cell proliferation. These results, in addition to 25(OH)D<sub>3</sub>'s antiproliferative effect in stably transfected 1 $\alpha$ -OHase LNCaP cells, suggest that (i) local intracellular 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> production may be important for inhibiting prostate cancer proliferation and (ii) 1 $\alpha$ -OHase is

necessary for the local production of  $1\alpha,25(\text{OH})_2\text{D}$ . As prostate cancer cells have been shown to have less  $1\alpha\text{-OHase}$  than normal prostate cells and the lack of  $1\alpha\text{-OHase}$  activity in LNCaP results in little to no suppression of proliferation with  $25(\text{OH})\text{D}_3$  treatment, prostate cancer cells may evade autocrine and paracrine regulation by  $1\alpha,25(\text{OH})_2\text{D}$  by inactivating *CYP27B1*.  $^3\text{H}$ -thymidine incorporation is a measurement of cells going through the synthesis phase of the cell cycle and is an incomplete picture of proliferation because it does not account for cells undergoing apoptosis.

Apoptosis was assessed using caspase 3 activity measurements. In LNCaP cells,  $1\alpha,25(\text{OH})_2\text{D}_3$  induced apoptosis. Guzey *et al.* assessed caspase 3 activity in LNCaP cells but did not report any increase in activity with  $1\alpha,25(\text{OH})_2\text{D}_3$  treatment (16). While  $25(\text{OH})\text{D}_3$  did not induce apoptosis in untransfected LNCaP cells, in stably-transfected  $1\alpha\text{-OHase}$  LNCaP cells,  $25(\text{OH})\text{D}_3$  induced apoptosis at  $10^{-8}\text{M}$ . This data, along with the  $1\alpha,25(\text{OH})_2\text{D}_3$ 's inhibition of DNA synthesis, suggest that  $1\alpha,25(\text{OH})_2\text{D}$  has multiple mechanisms in inhibiting prostate cancer cellular proliferation.

The decrease in proliferation seen with  $1\alpha,25(\text{OH})_2\text{D}_3$  or  $25(\text{OH})\text{D}_3$  treatment may be caused by inhibition of cell cycle progression. Cell-cycle analysis demonstrated that  $1\alpha,25(\text{OH})_2\text{D}_3$  in LNCaP cells and  $25(\text{OH})\text{D}_3$  treatment in stably transfected  $1\alpha\text{-OHase}$  LNCaP cells resulted a significant  $\text{G}_0/\text{G}_1$  phase accumulation. Increased P21, P27 and P53 can result in cellular senescence with the cells becoming stationary in one cell-cycle phase ( $\text{G}_0$ ) (17). The increased expression of P21 and P27 by  $1\alpha,25(\text{OH})_2\text{D}_3$  in prostate cancer cells has been demonstrated by other groups (11, 12, 18). For LNCaP cells,  $1\alpha,25(\text{OH})_2\text{D}_3$  increased *CDKN1A* and *CDKN1B* but not *TP53* mRNA expression. With  $25(\text{OH})\text{D}_3$  treatment, *CDKN1A*, *CDKN1B* and *TP53* mRNA were induced in stably transfected  $1\alpha\text{-OHase}$  LNCaP cells. The increased *TP53* expression with  $25(\text{OH})\text{D}_3$  treatment in stably-transfected  $1\alpha\text{-OHase}$  LNCaP cells was interesting. Several groups have speculated about VDR and P53 interaction because *CDKN1A* expression is transcriptionally activated by both. Saramaki *et al.* demonstrated multiple VDREs in the *CDKN1A* promoter and in 2009 the same group showed that these VDREs are in close proximity to P53-responsive elements (19). However, the group did not determine a physical interaction between VDR and P53.

In conclusion,  $25(\text{OH})\text{D}_3$  treatment in stably transfected  $1\alpha\text{-OHase}$  LNCaP transfects had antiproliferative effects that were similar to LNCaP cells treated with  $1\alpha,25(\text{OH})_2\text{D}_3$ . While the conversion of  $25(\text{OH})\text{D}_3$  to  $1\alpha,25(\text{OH})_2\text{D}_3$  in stably transfected  $1\alpha\text{-OHase}$  LNCaP was small, the amount of  $1\alpha,25(\text{OH})_2\text{D}_3$  produced appeared to be effective in decreasing DNA synthesis, suppressing  $\text{G}_1\text{-S}$  phase transition and increasing mRNA expression in genes associated with cell-cycle regulation and apoptosis.

This evidence, in addition to the fact that extra-renal  $1\alpha\text{-OHase}$  is not regulated like renal  $1\alpha\text{-OHase}$ , provides more support to epidemiological studies that correlate lower serum  $25(\text{OH})\text{D}$  levels and increased prostate cancer risk. While the present study supports the hypothesis that it is the local intracellular conversion to  $1\alpha,25(\text{OH})_2\text{D}$  that is important to prostate cancer regulation, the question of whether  $1\alpha\text{-OHase}$  loss is a proto-oncogenic or carcinogenic result remains unanswered.

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