*Review*

# Mechanisms of Vitamin D-mediated Growth Inhibition in Prostate Cancer Cells: Inhibition of the Prostaglandin Pathway

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**Abstract.** *Calcitriol (1,25-dihydroxyvitamin D₃), the active form of vitamin D, promotes growth inhibition and differentiation in prostate cancer (PCa) cells. To unravel the molecular pathways of calcitriol actions, cDNA microarray analysis was used to identify novel calcitriol target genes including two that play key roles in the metabolism of prostaglandins (PGs), known stimulators of PCa growth and progression. Calcitriol significantly decreases the expression of the PG synthesizing cyclooxygenase-2 (COX-2) gene, while increasing that of PG inactivating 15-prostaglandin dehydrogenase (15-PGDH). Calcitriol also inhibits the expression of the PG receptors EP2 and FP. It reduces the levels of biologically active PGs and inhibits PG actions in PCa cells, thereby decreasing the proliferative stimulus of PGs. We postulate that the regulation of the PG pathway contributes to the growth inhibitory actions of calcitriol. We also propose that calcitriol can be combined with non-steroidal anti-inflammatory drugs (NSAIDs) that inhibit COX enzyme activity, as a potential therapeutic strategy in PCa.*

Prostate cancer (PCa) is the most commonly diagnosed malignancy and the second leading cause of cancer death in North American men (1). According to the American Cancer Society, more than 232,000 men will be diagnosed with PCa in 2005 and approximately 10% of these will die of the disease (1). Primary therapy for PCa involves the removal of the prostate by surgery or radiation therapy. Unfortunately, after initial treatment PCa often recurs. Androgens regulate normal prostate development and growth. Surgical or medical androgen deprivation has been used as the standard treatment for PCa which fails primary

therapy (2). Although there is a good initial response to androgen ablation in most men, the tumors will progress to androgen independence resulting in death (3) since there is currently no adequate treatment for this advanced disease.

Our research was aimed at the development of new therapies for PCa. 1α, 25-Dihydroxyvitamin D₃ (calcitriol), the hormonally-active form of vitamin D, is a promising new therapeutic agent for PCa, which has been shown to exhibit growth inhibitory effects in cell culture and animal models of PCa as well as in clinical settings (4-15). Our goal was to understand the molecular mechanisms mediating the anticancer actions of calcitriol.

## Calcitriol and PCa

Calcitriol is a steroid hormone, well known as a major regulator of calcium homeostasis and bone mineralization (16). However, data accumulated over the past 25 years have indicated that calcitriol and its analogs have potent antiproliferative and pro-differentiation actions in a number of malignancies including PCa (4-15, 17, 18). The anti-proliferative action of calcitriol has been documented in several PCa cell lines (6, 8, 17, 18), as well as in primary cultures of normal and cancer cells derived from surgical specimens of prostate obtained from PCa patients (19, 20). The inhibition of PCa cell growth is seen in both androgen-dependent and androgen-independent PCa cells (18, 21). Similarly, calcitriol and its analogs have been shown to inhibit the growth of PCa in animal models of PCa (8, 11, 12). A pilot clinical study by our group provided evidence that calcitriol effectively slowed the rate of serum PSA rise in PCa patients with early recurrent PCa (22). Recent clinical trials, using high doses of calcitriol in combination with chemotherapy, have shown great promise in prolonging survival and delaying the time to progression in men with androgen-independent PCa (10, 12). The only side-effect of calcitriol therapy appeared to be the development of hypercalcemia. Many pharmaceutical companies and academic centers are attempting to design calcitriol analogs that exhibit increased anticancer potency but

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with a reduced tendency to cause hypercalcemia (23). We believe that calcitriol or a new analog will prove very useful as an adjunct for the therapy of both androgen-dependent and androgen-independent PCa.

Given the potential usefulness of calcitriol in treating and/or preventing PCa, understanding the molecular basis of calcitriol-mediated growth inhibition and the signaling pathways involved in its anticancer effects will more fully define its therapeutic potential, as well as allow the development of better therapeutic approaches to treat PCa and will provide an insight into how calcitriol acts and interacts with other agents in exerting its regulatory actions. This understanding would enable an improved rationale for when and how to implement calcitriol therapy and perhaps how to make calcitriol therapy more effective by combining with other drugs that exhibit synergistic molecular actions.

## Mechanisms of Calcitriol Actions in PCa

Calcitriol exerts its actions by binding to its nuclear receptor, the vitamin D receptor (VDR). After hormone binding, VDR dimerizes with the retinoid X receptor (RXR). The VDR-RXR heterodimer binds to DNA sequences known as vitamin D response elements (VDREs) in the promoter regions of target genes. This calcitriol-VDR complex then recruits co-activator proteins that stimulate the transcriptional apparatus to induce the expression of the target gene. A number of important pathways have been shown to play key roles in calcitriol-mediated growth inhibition. One primary mechanism of calcitriol action is to induce cell cycle arrest in the G1/G0-phase by increasing the expression of genes like the cyclin-dependent protein kinase inhibitors p21 and p27 (8, 13, 24), and to induce apoptosis by down-regulating the expression of anti-apoptotic genes, such as bcl-2 (8, 11, 13). Calcitriol has also been shown to regulate growth factor action through modulation of the expression of genes such as insulin-like growth factor binding protein-3 (IGFBP-3) (8, 13) and transforming growth factor β (TGF-β) (11, 13). In addition, calcitriol also exerts inhibitory effects on tumor cell migration and metastasis, as well as tumor angiogenesis (8, 10, 11, 13).

## Novel Targets of Calcitriol in PCa

Using cDNA microarray analysis to study the regulation of gene expression by calcitriol, we have recently identified 28 genes regulated by calcitriol in LNCaP human PCa cells (25). Among the up-regulated genes is one that encodes $NAD^+$-dependent 15-hydroxyprostaglandin dehydrogenase (15-PGDH). The expression of 15-PGDH is also induced by calcitriol in primary cultures of normal prostatic epithelial cells (26). We found the down-regulation of various genes on our microarrays, including the prostaglandin-endoperoxide

synthase-2, or cyclooxygenase-2 (COX-2) gene (25). COX-2 is the rate-limiting enzyme involved in prostaglandin (PG) synthesis, while 15-PGDH is the primary enzyme responsible for PG catabolism.

## COX-2 and PCa

PGs are long-chain oxygenated polyunsaturated fatty acids derived from arachidonic acid (AA). COX or cyclooxygenases are responsible for the synthesis of the PG precursor $PGH_2$ from AA (27). $PGH_2$ is then converted to the various PGs by specific synthases. PGs have been shown to stimulate the proliferation of many cancers including PCa (28). Many, yet not all, studies have concluded that the expression of COX-2 is elevated in PCa when compared with normal prostate (29-32). In vitro studies using androgen-dependent and androgen-independent PCa cell lines showed that both expressed detectable amounts of COX-2 and secreted $PGE_2$ (29, 33). It has been proposed that COX-2 induces tumorigenesis by various mechanisms including: (i) induction of cell proliferation (34); (ii) decreased apoptosis (35); (iii) increased angiogenesis (36); (iv) increased tumor invasiveness (37); and (v) decreased immune surveillance (33). Non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit COX enzymatic activity and therefore PG synthesis, have been shown to decrease PCa growth in in vitro PCa cell cultures and in vivo in animals bearing PCa tumor xenografts (28, 35, 38). Existing evidence suggests that $PGE_2$ has a specific role in the maintenance of human cancer cell growth and that the activation of COX-2 expression depends primarily upon newly synthesized $PGE_2$ through a positive feedback mechanism (39). Taken together, these data indicate that COX-2 and/or their prostaglandin products play a role in the malignant transformation of the prostate.

## 15-PGDH and Cancer

15-PGDH is the key enzyme initiating the catabolism of biologically-active PGs converting them into inactive keto-derivatives (40), thereby acting as a functional antagonist to COX-2. Three pieces of evidence indicate that the concomitant overexpression of COX-2 and underexpression of 15-PGDH have a role in tumor progression. First, microarray data analysis indicated a down-regulation of 15-PGDH in colon (41) and lung (42) cancers when compared to normal tissues. Second, when colon epithelial cells were chronically treated with the tumor suppressor TGF-β, 15-PGDH gene expression was induced (41). Thirdly, 15-PGDH by itself seemed to have tumor suppressor effects (42). When lung cancer cells transiently overexpressing 15-PGDH were injected into athymic nude mice, there was a substantial decrease in tumor induction and growth when compared to mice implanted with wild-type cancer cells (42).
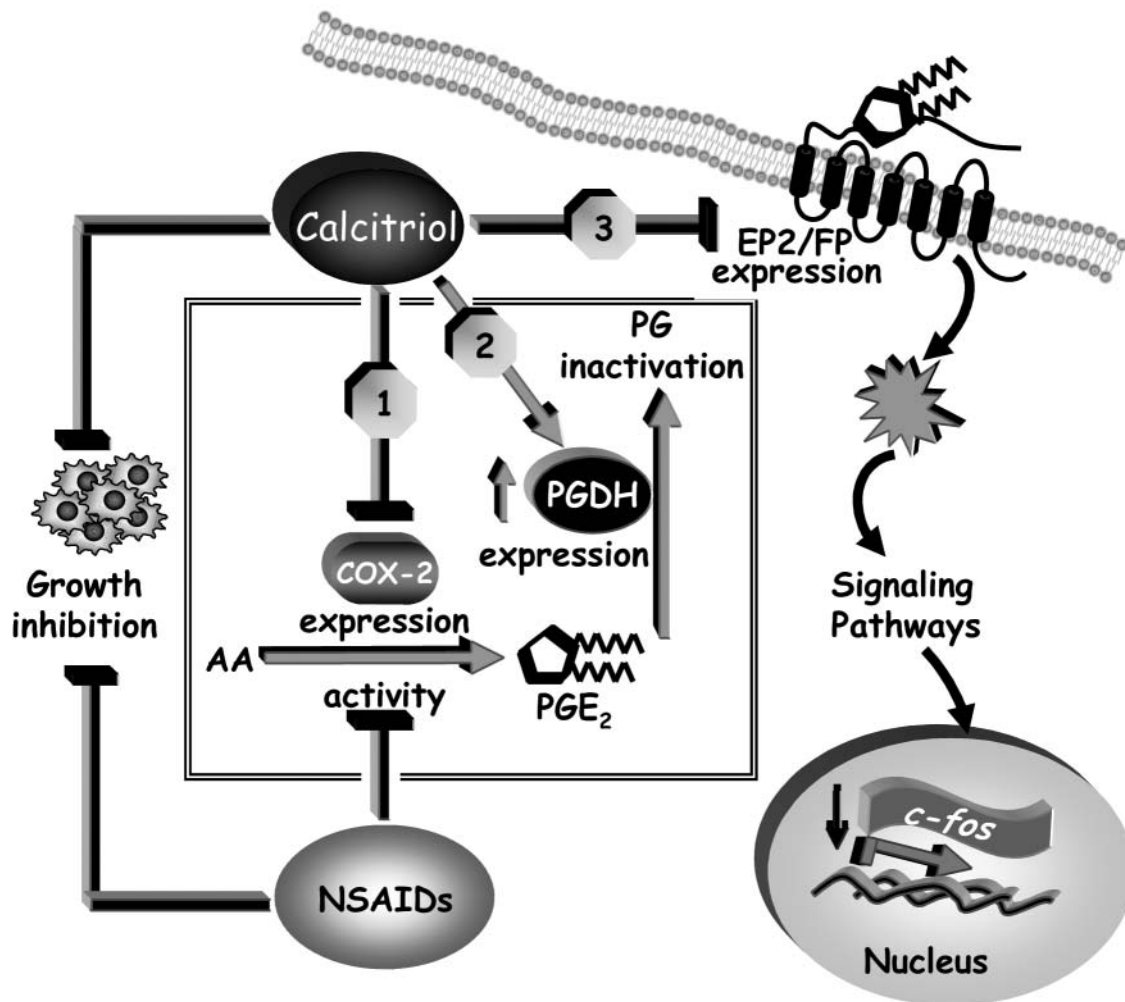
Figure 1. *Calcitriol inhibits prostaglandin actions in prostate cancer cells by three mechanisms: i) decreasing the expression of the prostaglandin synthesizing enzyme cyclooxygenase-2 (COX-2); ii) stimulating the expression of the prostaglandin catabolizing enzyme 15-prostaglandin dehydrogenase (PGDH); and iii) inhibiting EP2 and FP prostaglandin receptor expression. These three actions combined may be involved in blocking PG-mediated actions such as c-fos induction and cell growth. Furthermore, the combination of calcitriol and various non-steroidal anti-inflammatory drugs (NSAIDs) produced synergistic inhibition of prostate cancer cell growth.*

## Calcitriol Actions on the Prostaglandin Pathway in PCa cells

Our microarray data indicated that calcitriol increased the expression of 15-PGDH and significantly decreased the expression of COX-2 in LNCaP human PCa cells (25). Based on these initial results, we further investigated the effect of calcitriol on PG metabolism and PG actions in several established PCa cell lines, as well as primary cultures of prostatic epithelial cells derived from normal prostate and adenocarcinoma specimens removed at surgery. Significant increases were found in 15-PGDH expression and down-regulation of COX-2 expression in both the PCa cell lines and primary prostatic cells (43). This dual action was associated with decreased $PGE_2$ secretion into the conditioned media of PCa cells exposed to calcitriol (43).

PGs exert their biological effects through G-protein coupled membrane receptors which activate different signal transduction pathways. Interestingly, our study showed that calcitriol decreased the mRNA expression of the $PGE_2$ and $PGF_{2\alpha}$ receptor sub-types EP2 and FP, providing yet a third mechanism for the suppression of the biological activity of PGs by calcitriol (43). Thus, as illustrated in Figure 1, calcitriol exerts multiple actions on the PG pathway: the suppression of PG synthesis (due to COX-2 down-regulation), increase of PG catabolism (due to 15-PGDH up-regulation) and the inhibition of PG actions (due to PG receptor down-regulation). We hypothesize that, as a result of these regulatory actions, calcitriol attenuated PG-mediated functional responses in PCa cells such as the induction of the immediate-early gene *c-fos*
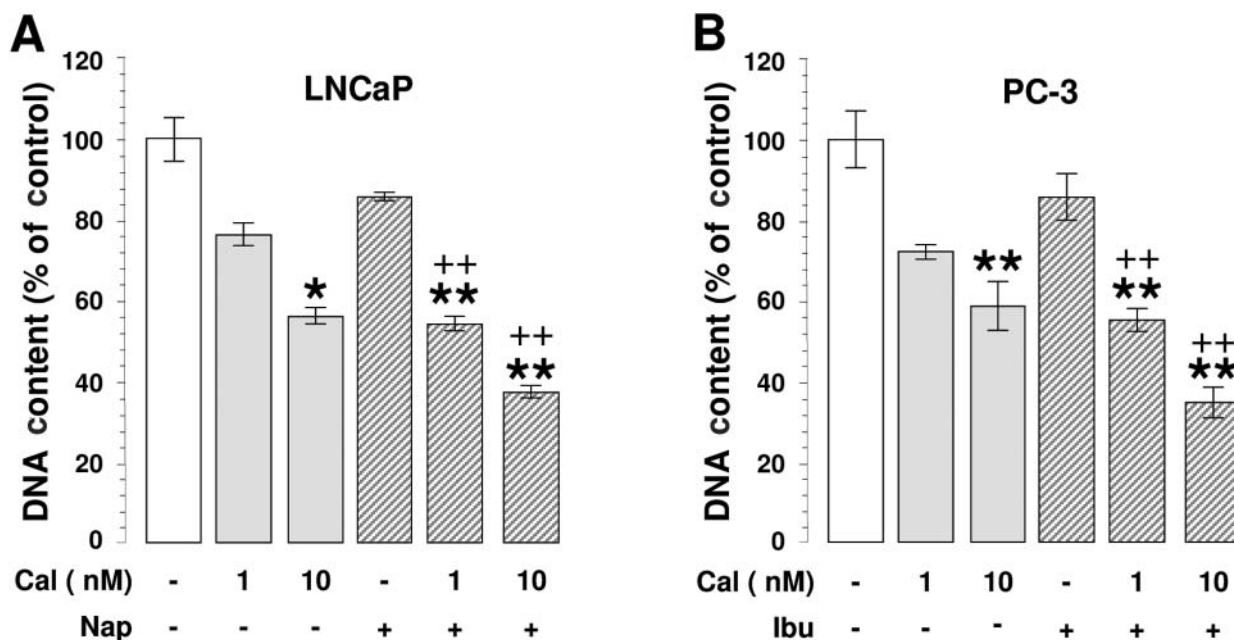
Figure 2. Effects of calcitriol and NSAIDs on LNCaP and PC-3 cell growth inhibition. A) Effects of 1 or 10 nM calcitriol, 200 μmol/L of naproxen (Nap) or the combination of both on LNCaP. B) Effects of 1 or 10 nM calcitriol, 150 μmol/L of ibuprofen (Ibu) or the combination of both on PC-3 cell growth. Cell growth was measured over a time-course of 6 days with fresh medium containing the drugs replenished every 2 days and evaluated by measuring the DNA content. The data are expressed as the percentage of the control set at 100%, columns represent means±SD of three experiments. *p<0.05, **p<0.01 compared with vehicle-treated control group. +p<0.05, ++p<0.01 compared with Nap or Ibu alone. Reproduced with permission from (43).

and the stimulation of PCa cell growth by PGs (43). Since PGs have been shown to promote prostate cell growth, inhibit apoptosis and stimulate PCa progression (28, 29, 33), we postulate that these effects of calcitriol to reduce PG synthesis and its actions significantly contribute to the anticancer effects of calcitriol in PCa.

## Non-steroidal Anti-inflammatory Drugs (NSAIDs) and PCa

Part of the interest in the role of COX enzymes in the development of cancer came from the observation that patients taking NSAIDs had a lower risk of developing cancer (33, 44). NSAIDs decreased PG synthesis by inhibiting COX enzyme activity (27). Inhibition of COX-2 by NSAIDs appeared to play a beneficial role in cancer chemoprevention (33). Interestingly, NSAIDs also regulated 15-PGDH levels in some cells (45, 46). Based on our results, we hypothesize that the action of calcitriol at the genomic level to reduce COX-2 expression decreases the levels of COX-2 protein and will allow the use of lower concentrations of NSAIDs to inhibit COX-2 enzyme activity. Therefore, we expect a combination of calcitriol and an NSAID to exhibit an additive/synergistic effect to inhibit PCa proliferation. Indeed the combination of calcitriol and various NSAIDs produced synergistic inhibition of PCa cell

growth at 2- to 10-fold lower concentrations of the drugs needed to achieve the same effect when used individually (43). As shown in Figure 2, the combination of calcitriol with the non-selective NSAIDs naproxen and ibuprofen resulted in a synergistic enhancement of the inhibition of LNCaP and PC-3 PCa cell growth, respectively. These findings suggest that calcitriol and NSAIDs may be a useful combination for PCa therapy (43).

## Conclusion

Prolonged use of NSAIDs, especially the COX-2 selective drugs, has recently been shown to have some adverse side-effects including increased risk of heart attacks, stroke, sudden death, blood clots, stomach and intestinal bleeding, and kidney failure (47). In comparison, the only known side-effect of calcitriol use in patients is hypercalcemia. However, hypercalcemia can be diminished by intermittent administration of calcitriol (14) or with the use of the new analogs of calcitriol with reduced calcemic effects (23). Our results predict that the combination therapy using calcitriol and an NSAID would allow the use of lower doses of both drugs, thereby reducing their individual side-effects while maintaining enhanced growth-inhibitory effects. Thus the calcitriol-NSAID combination offers a valuable therapeutic approach to PCa treatment.

In summary, our research was directed at understanding the molecular mechanisms of the antiproliferative activity of calcitriol in prostate cells with the goal of developing new strategies to improve PCa treatment. Our investigations revealed that calcitriol acts by three separate mechanisms on the PG pathway in PCa cells: decreasing COX-2 expression, increasing 15-PGDH expression and reducing PG receptor mRNA levels. We believe that these effects may contribute to the suppression of the proliferative stimulus of PGs by calcitriol in PCa cells. The regulation of PG metabolism and biological actions constitutes an additional novel pathway of calcitriol action contributing to its antiproliferative effects in prostate cells. We propose that a combination of calcitriol and a non-selective NSAID, such as naproxen, might be a useful therapeutic and/or chemo-preventive strategy in PCa, as it would achieve greater efficacy and allow the use of lower concentrations of both drugs, thereby reducing their toxic side-effects. The combination approach is an attractive therapeutic strategy that can be swiftly translated to clinical trials since calcitriol and NSAIDs are FDA approved drugs.

## Acknowledgements

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