

Review

## The Antitumor Efficacy of Calcitriol: Preclinical Studies

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**Abstract.** *Studies in our laboratory demonstrate that vitamin D (1,25 dihydroxycholecalciferol or calcitriol) has significant antitumor activity in vitro and in vivo in murine and human squamous cell, prostate, lung, pancreatic and myeloma model systems. Calcitriol induces G0/G1 arrest, modulates p27 and p21, the cyclin-dependent kinase (cdk) inhibitors implicated in G1 arrest, and induces cleavage of caspase 3, PARP and the mitogen-activated protein kinase (MEK) in a caspase-dependent manner. Calcitriol also decreases phospho-Erk (P-Erk) and phospho-Akt (P-Akt), kinases that regulate cell survival pathways and up-regulate the pro-apoptotic signaling molecule, MEKK-1. Glucocorticoids enhance calcitriol-mediated activities pre-clinically in vitro and in vivo. Dexamethasone (dex) significantly potentiated the antitumor effect of calcitriol and decreased calcitriol-induced hypercalcemia. Both in vitro and in vivo, dex increased vitamin D receptor (VDR) ligand binding in the tumor while decreasing binding in intestinal mucosa, the site of calcium absorption. These studies demonstrated that calcitriol has significant antiproliferative activity in a number of pre-clinical model systems and form the groundwork for on-going clinical studies investigating calcitriol as an anticancer agent.*

Vitamin D is a steroid hormone, which modulates calcium homeostasis through actions on kidney, bone and the intestinal tract (1). Vitamin D is synthesized in the skin from 7-dehydro-cholesterol in response to ultraviolet light, is 25-hydroxylated to 25-hydroxycholecalciferol in the liver and 1-hydroxylated to the active form,

1,25-dihydroxycholecalciferol or calcitriol in the kidney (1, 2). In addition to classic effects on bone and mineral metabolism, calcitriol is also involved in the proliferation and differentiation of a variety of different cell types and tissues (1-21).

Studies in our laboratory demonstrated that vitamin D (1,25 dihydroxycholecalciferol or calcitriol) had significant antitumor activity *in vitro* and *in vivo* in a variety of murine, rat and human tumor model systems (squamous cell carcinoma, prostate, lung, pancreatic and myeloma) (3-6, 18-21). Calcitriol induced significant cell cycle arrest, induced and modulated apoptotic markers and decreased survival signals (4-10) both *in vitro* and *in vivo* in a number of these model systems. Glucocorticoids enhance calcitriol-mediated activities pre-clinically (*in vitro* and *in vivo*) and clinically. We demonstrated that dexamethasone (dex) significantly potentiated the antitumor effect of calcitriol and decreased calcitriol-induced hypercalcemia (16, 17). Both *in vitro* and *in vivo*, dex increased vitamin D receptor (VDR) ligand binding in the tumor while decreasing binding in intestinal mucosa (16), the site of calcium absorption (22). P-Erk and P-Akt were also decreased with calcitriol/dex, as compared to either agent alone (17). When dex was added to calcitriol and a number of cytotoxic drugs, a greater antitumor effect was observed than with each drug alone or any two drug combinations (18-20). Dex enhanced VDRE transcriptional activity (17) through a potential effect on coactivator stimulation of transcription. In a phase II trial in androgen-independent prostate cancer (AIPC), oral calcitriol (12 mg/day QDx3, weekly) and dex (4 mg QDx4, weekly), a 50% reduction in prostate specific antigen (PSA) was seen in 31% of the patients, but no hypercalcemia (22) or molecular changes in peripheral blood monocytes (PBM) similar to those observed in cell lines. Antitumor effects were also noted following calcitriol/dex in men with localized disease with a rising PSA following prostatectomy or irradiation. Therefore,

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glucocorticoids enhance calcitriol antitumor activity with decreased toxicity, which has important therapeutic implications across tumor types.

### VDR and Calcitriol Signaling Pathways

The effects of calcitriol are mediated by binding to a specific intracellular receptor (VDR), a member of the steroid hormone receptor superfamily (24). The VDR is a high affinity, low-capacity receptor protein of 48-55 kDa primarily located in the nucleus, though evidence exists for cytoplasmic receptors (25, 26). The VDR acts as a ligand-dependent transcription factor that binds to vitamin D response element(s) (VDRE) as a VDR: retinoid-X receptor (RXR) heterodimer. This interaction results in ligand-dependent activation or repression of target genes. The identities and function of all these genes, however, are unknown (26). The binding of calcitriol to the VDR induced receptor phosphorylation and the ligand-bound, phosphorylated receptor stimulated transcription (27). Calcitriol may act independently of this genomic pathway (28); it can activate protein kinase C (29), modulate phospholipid metabolism (30), stimulate the formation of cyclic nucleotides (31), trigger calcium transport (32) and activate Raf and MAPK (Erk) activities (33), all in a manner independent of VDR/DNA binding. Transcription activation through calcitriol and VDR is enhanced by nuclear receptor coactivator proteins such as steroid receptor coactivators (SRCs), vitamin D receptor-interacting protein (DRIPs) complexes and the recently described nuclear coactivator-62 kDa/Ski-interacting protein (NCoA62/SKIP) (34-37).

### VDR, Calcitriol and Antitumor Activity

The VDR is found, not only in classic target organs (intestinal mucosa, kidney, bone), but also in many other epithelial and mesenchymal cells as well as leukemic cells, and many malignant cell types (1, 2). Calcitriol inhibits growth *in vitro* and *in vivo* in breast and colon cancer models (38, 39). Calcitriol can induce differentiation, cell cycle arrest and/or apoptosis in leukemic and tumor cells (40, 41). Progression through the cell cycle is regulated by cyclins and their associated cyclin-dependent kinases (cdk). The cdk inhibitors p21<sup>Waf1/Cip1</sup> and p27<sup>Kip</sup> are implicated in G1-phase arrest (42). In HL-60 cells calcitriol arrests cells in G1; this effect is mediated through an increase in p27 (40). Calcitriol-mediated arrest in G0/G1 is also observed in breast cancer lines (43). In U937, a human myelomonocytic cell line, a functional VDRE was identified in the p21 promoter region and transcriptional activation of p21 by the VDR induced differentiation in this cell line (41).

### Calcitriol and Apoptosis

In multiple model systems (murine syngeneic SCC VII/SF, metastatic Dunning rat prostate adenocarcinoma (MLL) and the human xenograft PC-3 and LNCaP prostate, MV522 lung, Capan-1 pancreatic, NCI H929 and RPMI 8226 myeloma), calcitriol had significant antiproliferative effects *in vitro* and *in vivo* (3-6, 18-21). Calcitriol also caused arrest of tumor cells in G0/G1 and was associated with decreased expression of p21 mRNA and protein, and increased expression of p27 mRNA and protein and Rb dephosphorylation (8). *In vivo*, a decrease in tumor volume induced by calcitriol correlated with a significant decrease in the intratumoral p21 expression. Apoptosis was mediated by caspases activation (44). The bcl-2 protein, which is overexpressed in many tumors, suppressed apoptosis and the bax protein promoted apoptotic cell death (45). Calcitriol induced apoptosis in MCF-7 breast cancer cells as well as in HL-60 leukemic cells and the expression of bcl-2 was down-regulated by calcitriol in HL-60 (46). Apoptotic cell death also resulted in specific cleavage of key cellular proteins including poly (ADP-ribose) polymerase or PARP (47). We demonstrated by Western blot that calcitriol induced 90-100% PARP cleavage in MLL prostate cancer cells (5). The caspase inhibitors, ZVAD-fmk and DEVD-fmk, had no direct effect on cells but significantly inhibited the calcitriol-mediated increase in annexin binding. In addition, bax was unchanged and bcl-2 was decreased with calcitriol, resulting in an increased bax/bcl-2 ratio, which favors cell death.

*In vitro*, exponentially-growing tumor cells express significant levels of P-Erk. Erk1/2 are known to transduce mitogenic and survival signals to the nucleus in response to a number of extracellular stimuli (48). Treatment with calcitriol alone demonstrated strong inhibition of P-Erk (9). An increase in VDR expression accompanied the loss of P-Erk in calcitriol-treated cells. Importantly, Erk protein levels were unchanged in any of the treatment groups. Upstream of Erk, the growth-promoting/pro-survival signaling molecule MEK was cleaved in a caspase-dependent manner in cells induced to undergo apoptosis with calcitriol. Cleavage resulted in nearly complete loss of full-length MEK and P-Erk. The phosphorylation and expression of Akt, a kinase regulating a second cell survival pathway, was also inhibited with calcitriol. The pro-apoptotic signaling molecule MEKK-1, a stress-activated 195 kDa serine/threonine protein kinase, was also up-regulated and proteolytically processed at the N-terminus in cells induced to undergo apoptosis after treatment with calcitriol (Figure 1A). Using subcellular fractionation, MEKK-1 was localized nearly exclusively in the cytosolic fraction. These results suggested that calcitriol induces apoptosis *via* the up-regulation and aberrant cytosolic expression of MEKK-1. SCC was also examined for changes

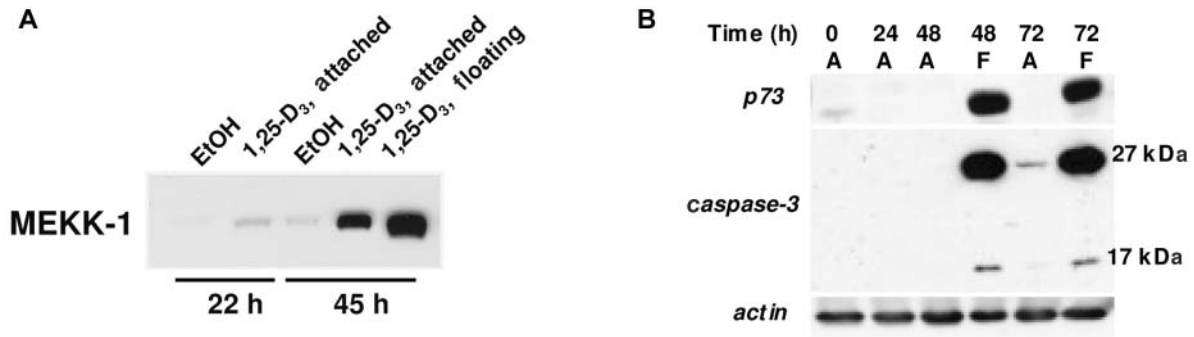


Figure 1. Effect of calcitriol on MEKK-1 (A), caspase-3 and p73 (B). SCC cells were treated with 10 nM calcitriol at the IC<sub>50</sub> and after 48 h analyzed by Western blot.

in p73, the p53 homolog with reportedly pro-apoptotic activity (49), following treatment with calcitriol. SCC cells were treated as described above and cells separated into attached (A) and floating (F) cell populations. As shown in Figure 1B, p73 was strongly induced by calcitriol in the floating population. p73 was strongly induced only in those cells expressing cleaved, activated caspase-3 that had detached and expressed apoptosis markers. These data suggest that calcitriol may induce apoptosis uniquely through the p73 pathway. Inhibition of p73 function impacts cellular sensitivity to calcitriol; SCC cells, transiently transfected with a plasmid encoding dominant negative p73, were not as sensitive to calcitriol as the controls. Studies using human lung MV522 and human pancreatic Capan-1 models resulted in similar effects (20, 21).

### Calcitriol Enhanced Taxane- or Platinum-mediated Antitumor Activity

*In vitro* and *in vivo*, pretreatment with calcitriol or the analog, 1,25dihydroxy16ene-23yne-cholecalciferol significantly enhanced cisplatin-, carboplatin-, docetaxel- or paclitaxel-mediated antitumor activity (6, 7, 11, 12, 18). As described below, clinical trials were initiated based on these pre-clinical data to examine the combination of calcitriol and either carboplatin or paclitaxel. Enhanced antitumor activity with calcitriol and paclitaxel/cisplatin was associated with a synergistic increase in caspase-3 cleavage, generation of p27/p17 cleavage fragments, loss of full-length PARP and a decrease in MEK, P-MEK, P-Erk and P-Akt expressions as compared to calcitriol or cytotoxic drug alone. In addition, it was observed that p73 was strongly induced by the combination of calcitriol and drug, with little induction by either agent alone. The expression of MEKK-1 was up-regulated by the combination. Cytotoxic drugs that were not synergistic with calcitriol (*e.g.*, carmustine, BCNU) (18) did not result in the modulation of these survival and apoptotic

signals. These data suggest that the enhanced antitumor activity was mediated by up-regulation of MEKK-1 and/or p73 and loss of pro-survival signals through Akt/ Erk.

### Glucocorticoids and Calcitriol

Treatment of cells with calcitriol, glucocorticoids, estrogens, retinoic acid and parathyroid hormone influences the cellular content of the VDR (50-54). While glucocorticoids do not bind the VDR (55), they influenced calcitriol-ligand binding to the VDR in normal cells and tissues (51, 56). Glucocorticoids modulate calcitriol effects on Ca<sup>2+</sup> transport and may alter the metabolism of calcitriol (57, 58). In addition, glucocorticoids are utilized clinically to ameliorate hypercalcemia in a number of clinical indications, including calcitriol intoxication (58). Although known for their anti-inflammatory activity, glucocorticoids are often used to manage patients with multiple myeloma, leukemia, lymphoma and progressive prostate and breast cancer (59, 60). The role of glucocorticoids as anti-neoplastic agents in epithelial tumors is less well defined; their use in these tumors is clearly palliative. Glucocorticoids do not induce apoptosis consistently in prostate cancer cells, yet growth inhibitory effects are well documented (6). In a number of large randomized clinical trials in prostate cancer, glucocorticoids alone yielded PSA response rates (>50% decrease, > 1 month) from 10-15% (62-64). We demonstrated a significant response with calcitriol and dex (23); this dose/schedule of dex has not been previously studied in patients with prostate cancer.

We have demonstrated that dex significantly enhanced calcitriol antitumor efficacy, *in vitro* and *in vivo* (16, 17). In SCC and PC-3, dex was able to markedly enhance *in vitro* and *in vivo* clonogenic cell kill as compared to either agent alone. This combination induced significant tumor regression in these model systems. In addition, administration of dex inhibited calcitriol-induced hypercalcemia that was observed

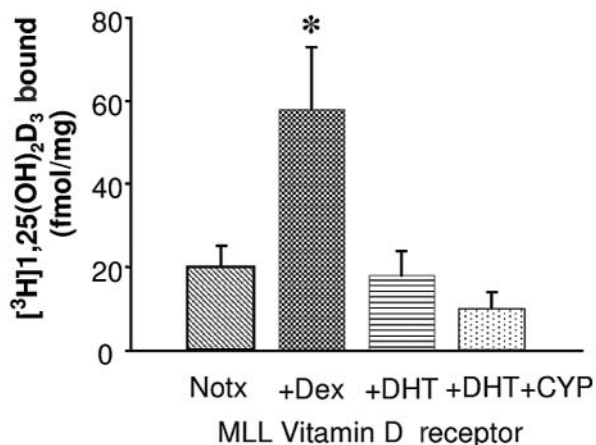


Figure 2. VDR in rat prostate MLL tumor cells treated for 24 h with dex, DHT, or DHT and cyproterone. Bars represent the mean ± SD of calcitriol bound (fmol/mg) \*Significantly different than control,  $p < 0.001$  (ANOVA).

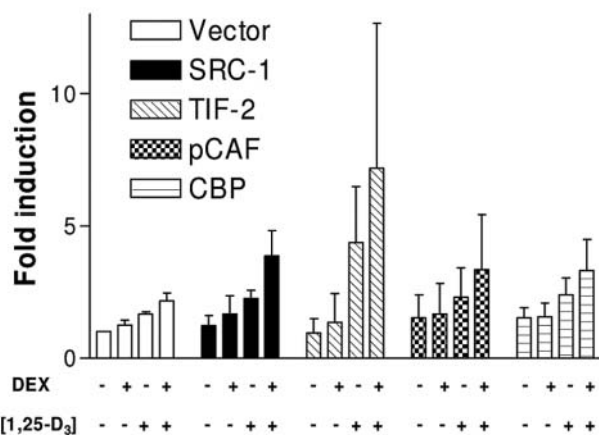


Figure 3. VDRE-luciferase reporter assay to examine the effect of dex (500 nM) on calcitriol (10 nM) transcriptional activity using the DR-3 VDRE.

with chronic administration. To further examine the effects of calcitriol and dex, the antiproliferative, cell cycle and apoptotic effects of this combination were investigated in SCC. The glucocorticoid antagonist, RU486, was able to block the dex-induced enhancement of calcitriol antiproliferative activity. Calcitriol induced cell cycle arrest (4, 7, 8) and dex plus calcitriol resulted in a higher percentage of cells in G0/G1-phase as compared to either calcitriol or dex alone with significant inhibition with RU486, the GR antagonist. The combination of calcitriol/dex led to an increase in the cleaved, active form of caspase-3 and a further reduction in full length PARP as compared to calcitriol alone, and RU486 blocked this effect. In addition, the levels of P-Erk and P-Akt were reduced in cells treated with calcitriol and a further reduction was observed in combination with dex, suggesting that dex enhances calcitriol pro-apoptotic signaling. RU486 inhibited the effects of dex on both Erk and Akt, suggesting that the glucocorticoid receptor (GR) may also be required for these activities and that they may be important targets for antitumor activity. The heterodimeric partner of VDR, RXR, did not to play a role in the ability of dex to increase calcitriol-mediated antitumor activity either *in vitro* or *in vivo* (17). In addition, glucocorticoids had no effect on VDR mRNA levels using Northern blot analysis. In PC-3, calcitriol did not induce apoptosis but induced cell cycle arrest and a decrease in p21 *in vitro* (6), however, with the addition of dex, a significant increase in antitumor activity *in vivo* was observed, as measured by the excision clonogenic assay and tumor re-growth delay.

Dex significantly increased the VDR receptor content (number) without changing the affinity for the ligand (Kd) (16). In addition, the combination of calcitriol and dex resulted in a significant increase in VDR protein as

compared to calcitriol alone or dex alone. It was also examined whether changes could be observed *in vivo* in animals treated with calcitriol. In tumor-bearing mice treated for 3 days with calcitriol, VDR protein expression was induced in the tumors from animals treated with calcitriol which correlated with an increase in antitumor effect (6, 12, 18, 20). Changes in VDR mRNA expression, however, were not observed *in vitro* or *in vivo* by treatment with dex. The increase in VDR by calcitriol was only observed in the tumor and kidney of animals treated with dex, not in other tissues except in the intestinal mucosa, the site of calcium absorption, where a significant decrease was observed in number and binding of VDR. These data suggest that the ability of dex to enhance antitumor activity and decrease toxicity is mediated through the VDR. GR protein levels were increased by calcitriol with no effect observed with the combination, suggesting cross-talk between these two steroid receptors. To examine the effect of dihydroxytestosterone (DHT) on the ability of calcitriol to bind to the VDR, MLL rat prostate tumor cells were treated with dex, DHT or DHT and cyproterone, a steroidal anti-androgen, and assayed for effects on the VDR by saturation equilibrium binding. As shown in Figure 2, dex enhanced the VDR receptor content as previously demonstrated in SCC (16), whereas DHT alone or in combination with cyproterone had no enhancing effect on the VDR number.

### Effect of Dexamethasone on Calcitriol-induced Transcriptional Activity

The ability of dex to modulate VDR-mediated transcription regulation on target genes was assessed using the DR3 VDRE luciferase reporter construct. SCC cells were transfected with

a luciferase reporter construct containing a DR-3 type VDRE and then treated with various concentrations of calcitriol, either in the absence or presence of dex for 48 h. As shown in Figure 3, dex enhanced the activity of calcitriol from the DR-3 reporter at 10 nM final concentration of calcitriol. When other VDRE constructs were tested (IP-9, DR-4, DR-6/DR-3), no significant difference was observed between any of the treatment groups, indicating that the VDR response to dex in SCC cells was DR-3 specific.

### Dexamethasone Enhancement of Chemotherapy and Calcitriol Antitumor Activity

While dex enhances the antiproliferative activities of calcitriol, a significant increase in antitumor activity was also observed when dex was added to chemotherapeutic drugs in combination with calcitriol in a number of model systems *in vitro* and *in vivo* (19-21). SCC tumor-bearing mice treated with calcitriol, dex and cisplatin resulted in a significantly greater antitumor effect than any agent alone or two-drug combination (Figure 4). Similar enhancement with dex was observed *in vivo* with docetaxel/calcitriol/dex in PC-3, paclitaxel/carboplatin/calcitriol/dex in the xenograft MV-522 lung model and mitoxantrone/dex in PC-3 (18-20), as compared to any single agent alone or two-drug combination (data not shown). *In vitro*, a significant increase in the expression of apoptotic markers and decrease in survival signals were observed when dex was added to combinations of calcitriol and paclitaxel, docetaxel or cisplatin.

### Conclusion

We have pre-clinical evidence that calcitriol and dex resulted in significant antitumor effects *in vitro* and *in vivo* in a number of tumor model systems and that dex could prevent calcitriol-induced hypercalcemia. Clinically, calcitriol and dex resulted in an antitumor effect in men with AIPC. It was demonstrated that glucocorticoid enhancement of calcitriol's antitumor activity appeared to be steroid receptor-mediated, involved modulation of the cell's survival pathways and could significantly enhance the antitumor activity of chemotherapeutic agents. Dex enhanced VDRE transcriptional activity and may play a role in coactivator stimulation of transcription. Glucocorticoids play a significant role in the management of the cancer patient; the exact mechanism(s) of effect are unclear. Glucocorticoids modulate the activities of other steroid hormones and steroid hormone receptors. Therefore, we have defined and characterized the mechanisms of calcitriol's antitumor activity with the ultimate goal of exploiting these therapeutically. We have presented a series of well-characterized tumor models where the response to calcitriol was documented and in which we have extended mechanistic questions from *in vitro* to *in vivo*

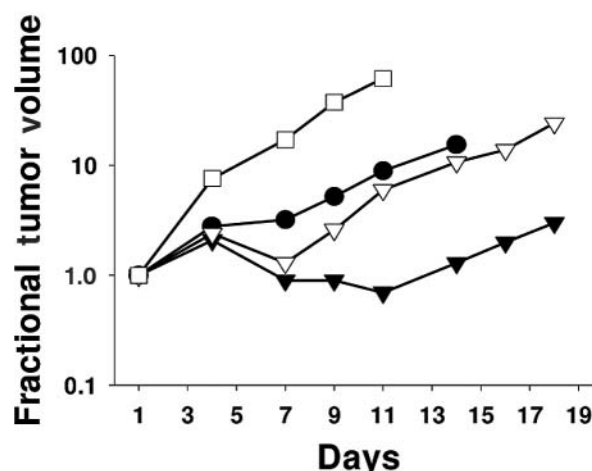


Figure 4. Effect of dex on calcitriol enhancement of chemotherapeutic efficacy. Tumor-bearing mice (10-15/group) were treated with either vehicle alone (□), cisplatin/dex (●), calcitriol/dex (▽) or calcitriol/dex/cisplatin (▼) in the model system of SCC. To simplify the figure, the other treatment groups of single and two drug combinations were omitted. (calcitriol 0.625 µg; dex 9 µg/mouse; cisplatin 6 mg/kg).

systems. In addition, we propose to conduct clinical trials that parallel our pre-clinical work to determine whether correlations can be made between model systems and man. Future studies will examine the mechanisms of calcitriol-mediated activities and form a unique background for the potential use of calcitriol with glucocorticoids for the therapy of solid tumors. It is anticipated that the information gained through these studies will permit the design of more effective clinical therapies.

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