Antiproliferative effects of 1,25-dihydroxyvitamin D$_3$ on breast cells - A mini review

Abstract

The hormone 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$), the active form of vitamin D$_3$, is an important regulator of calcium homeostasis, exerts antiproliferative effects on various cell systems and can induce differentiation in some kinds of hematopoietic cells. These effects are triggered by its receptor, vitamin D receptor (VDR), a phosphoprotein member of the nuclear receptor superfamily, which functions as a transcriptional factor. VDR binds as a heterodimer with retinoid X receptor (RXR) to hexameric repeats, characterized as vitamin D-responsive elements present in the regulatory region of target genes such as osteocalcin, osteopontin, calbindin-D$_{28K}$, calbindin-D$_{9K}$, p21$^{\text{WAF}}$/CIP1, TGF-$\beta$2 and vitamin D 24-hydroxylase. Many factors such as glucocorticoids, estrogens, retinoids, proliferation rate and cell transformation can modulate VDR levels. VDR is expressed in mammary tissue and breast cancer cells, which are potential targets to hormone action. Besides having antiproliferative properties, vitamin D might also reduce the invasiveness of cancer cells and act as an antiangiogenesis agent. All of these antitumoral features suggest that the properties of vitamin D could be explored for chemopreventive and therapeutic purposes in cancer. However, hypercalcemia is an undesirable side effect associated with pharmacological doses of 1,25-(OH)$_2$D$_3$. Some promising 1,25-(OH)$_2$D$_3$ analogs have been developed, which are less hypercalcemic in spite of being potent antiproliferative agents. They represent a new field of investigation.

Mechanism of action of vitamin D

Vitamin D is in fact a secosteroid hormone which in its active form, 1,25-dihydroxyvitamin D$_3$ (1,25-(OH)$_2$D$_3$), is an important regulator of bone development and metabolism and calcium homeostasis. Besides these well-known functions on classical target tissues (bone, kidneys, intestine, parathyroids), 1,25-(OH)$_2$D$_3$ plays an important role in the regulation of cell growth and differentiation in cells other than its classical targets.

Vitamin D can be obtained in two distinct ways, i.e., through dietary intake (fatty fish, fish oil, mushrooms, vitamin D-fortified food such as milk) and/or the endogenous pathway, in which the precursor 7-dehydrocholesterol, present in the skin, upon the action of sunlight becomes pre-vitamin D, the latter...
being subsequently hydroxylated in the liver and kidneys to 25-(OH)D₃ and 1,25-(OH)₂D₃, the active form (1).

The hormone exerts its effects via genomic and non-genomic mechanisms. In the first case, the response is triggered by its nuclear receptor - the vitamin D receptor or VDR, which is a *trans*-acting transcriptional factor and a member of the nuclear hormone receptor superfamily. The N-terminal domain of VDR is configured into two zinc-coordinated fingers responsible for DNA recognition and binding, whereas the C-terminal domain binds the 1,25-(OH)₂D₃, VDR binds selectively to DNA primarily as a heterodimer with retinoid X receptor (RXR). The binding of the hormone and the receptor causes a conformational alteration in the linkage domain of the latter with consequent dissociation of co-repressors, facilitating the interactions between VDR and co-activator proteins such as the members of the p160, SRC-1, 2 and 3 families, and the protein complex named DRIP. These co-activators modulate the chromatin structure and the contact with the basal transcriptional factors (2). The receptor/1,25-(OH)₂D₃ complex regulates gene transcription both positively and negatively through binding motifs in the promoter regions of target genes, designated vitamin D response elements, or VDREs. Several VDREs have been characterized and they generally consist of two direct repeats of six nucleotides (AGGTCA) separated by three aleatory nucleotides; however, neither the sequence of half-sites nor the spacing between them is well conserved. VDREs have been identified in genes such as calbindin-D₂₈K (3) and calbindin-D₉K, which are calcium-binding proteins mainly present in mammalian kidney and intestine, respectively; osteocalcin (4) and osteopontin (5), which are bone matrix proteins produced by osteoblasts; vitamin D 24-hydroxylase (6), an enzyme that inactivates 1,25-(OH)₂D₃; p21⁠<sup>WAF1/CIP1</sup> (7), a cyclin-dependent kinase inhibitor; c-fos, an early response gene, and transforming growth factor β2 (TGF-β2) (8).

VDR has been detected in numerous classical and nonclassical target tissues of 1,25-(OH)₂D₃, in tumors of various origins and cell lines such as NIH-3T3 mouse fibroblasts and MCF-7 human breast cancer cells (9,10). The responsiveness of target cells to the hormone depends on the amount of VDR and many factors can modulate VDR levels. Previous studies by our group and others have shown, for example, that glucocorticoids, prolactin, estrogens, retinoids and growth factors might influence VDR expression in mammary and leukemic cells (9,11-14). In HL-60 myeloblastic cells, VDR content correlated indirectly with the proliferation rate expressed by the fraction of cells in the G0/G1 phase (13). However, in other leukemic cell lines such as U937 and K562, phorbol ester treatment caused growth arrest, which was not accompanied by VDR down-regulation (14). Our data for leukemic cells suggest that VDR expression is not consistently changed upon inhibition of cell proliferation.

In addition to this genomic pathway, vitamin D has also been reported to induce nontranscriptional responses involving activation of transmembrane signal transduction pathways. Moreover, 1,25-(OH)₂D₃ modulates voltage-dependent Ca<sup>2+</sup> channel-mediated Ca<sup>2+</sup> influx in cultured chick muscle cells by a non-genomic pathway involving G protein-dependent stimulation of both the adenylyl cyclase/cyclic AMP/PKA messenger system and a phosphoinositide-specific phospholipase C (PLC). The rapid activation of PLC, in turn, generates diacylglycerol and inositol-1,4,5-triphosphate, promoting the activation of protein kinase C and rapid release of Ca<sup>2+</sup> from endogenous stores (15). These effects could be mediated by a putative but as yet unidentified membrane receptor (16). Other findings support evidence that some actions of 1,25-(OH)₂D₃ such as monocyte differentiation are mediated by activation of phosphatidylinositol 3-

kinase, a lipid kinase, which forms a complex with VDR, suggesting that non-genomic and genomic mechanisms could take place in concert (17).

**Vitamin D and breast cancer**

Epidemiological studies have shown that death rate and incidence of breast cancer tend to increase with increasing latitude, suggesting that solar radiation might play a protective role in breast cancer development. These studies also provide provocative data indicating an inverse relationship between decreased sunlight exposure and diminished vitamin D production on the skin and higher breast cancer incidence and mortality (18,19). According to this hypothesis, it was reported that white women affected by breast cancer show lower 1,25-(OH)_{2}D_{3} blood levels than unaffected ones (20). In addition, Mawer et al. (21), in a study on breast cancer patients, found the highest serum 1,25-(OH)_{2}D_{3} levels in the early stage as compared to more advanced bone metastatic disease.

Another possible link between the vitamin D pathway and breast cancer was recently reported as an amplification of CYP24, located in a region of recurrent aberration at 20q13.2 in breast cancer. This gene encodes vitamin D 24-hydroxylase, an enzyme responsible for 1,25-(OH)_{2}D_{3} degradation, and its overexpression could lead to abrogation of growth control mediated by vitamin D (22). On the other hand, 25-(OH)D_{3}-1α-hydroxylase, responsible for 25-(OH)D_{3} activation, was detected in normal human breast as well as in breast carcinoma samples, indicating that both normal and cancerous tissues could be capable of 25-(OH)D_{3}-1α-hydroxylation (23) and local synthesis of 1,25-(OH)_{2}D_{3}.

Receptors for 1,25-(OH)_{2}D_{3} in MCF-7, a cultured breast cancer cell line, were first shown by Eisman et al. (10) in 1979 and later VDR expression was reported in carcinogen-induced rat mammary tumors as well (24). VDR was also detected in some normal breast tissues such as the mammary gland of pregnant and lactating rabbits but not in virgin rabbits (25).

The antiproliferative effect of 1,25-(OH)_{2}D_{3} was demonstrated *in vitro* in MCF-7 cells and other estrogen receptor-positive as well as -negative breast cancer cell lines (11,26). The *in vivo* antitumor effect of 1,25-(OH)_{2}D_{3} or its analogs on rat mammary carcinogen (7,12-dimethylbenzanthracene, or N-methyl-N-nitrosourea)-induced tumors was observed as reduced total tumor burden or extended tumor latency and lessened tumor incidence (27,28).

The presence of VDR was also demonstrated in normal human breast tissue (29) and in a large proportion, ranging from 75 to 93%, of breast tumor biopsy specimens, as assessed by specific 1,25-(OH)_{2}D_{3} binding in tumor extracts (25,30) or positive immunostaining using antibodies to VDR (29,31). In some small series of patients, correlations of breast cancer VDR status with prognosis were conflicting. Freake et al. (30) examined breast cancer samples from 56 patients using a hormone-binding assay, and VDR content (less or more than 8 fmol/mg protein) could not predict a difference in probability of survival. In contrast, Colston et al. (31) found a longer disease-free survival among patients with VDR-positive breast tumors as evaluated by immunocytochemistry.

We determined VDR expression in breast cancer or adjacent normal tissue from 50 Brazilian patients. VDR mRNA was detected in almost all samples examined (96%), i.e., tumoral or non-tumoral adjacent tissues (Figure 1) (32). In immunohistochemical assays, strong VDR staining was observed in the nuclei of breast cancer epithelial cells, and it was less intense in infiltrating fibroblasts (Figure 2). Although 54% of the tumors expressed higher VDR mRNA levels as compared to normal breast tissue, no significant difference was detected. Moreover, we could not establish any correlation between VDR mRNA expression in breast tumor tissue and
disease outcome as evaluated by axillary node status (data not shown).

Our next step was to test the action of vitamin D on normal and transformed mammary cells. HC11 is a spontaneously immortalized lineage derived from the mammary gland of midpregnant BALB/c mice. These cells retain characteristics of normal cells such as growth inhibition by cell contact and are able to differentiate in vitro and synthesize milk proteins (β-casein) following stimulation with lactogenic hormones. HC11 cells do not express estrogen receptors and lack wild-type p53 (33). On the other hand, HC11 cells transformed with the oncogene Ha-ras (HC11ras) are no longer growth inhibited upon cell contact, do not respond to lactogenic hormones, and are tumorigenic when injected into nude mice (34). Our studies have demonstrated that only HC11 parental cells are growth inhibited upon 1,25-(OH)$_2$D$_3$ treatment, whereas HC11ras cells respond modestly to the hormone. This differential sensitivity seems to reflect the decreased VDR mRNA content of transformed cells as compared to parental cells (35). Our recent data indicate that even though both cell lines present a similar VDR mRNA transcription rate as evaluated by run off assays, VDR mRNA seems to be less stable in HC11ras than in parental cells (36).

We have also determined if less hypercalcemic 1,25-(OH)$_2$D$_3$ analogs, EB1089 (seocalcitol), which presents a double bond in the C-17 side chain, and KH1060, a C-20 epimeric compound (both donated by Dr. Lise Binderup, Leo Pharmaceutical Products, Ballerup, Denmark), exerted antiproliferative effects on HC11 and HC11ras cells.

![Figure 1](image1.png)  
**Figure 1.** Vitamin D receptor (VDR) mRNA expression in breast cancer or normal adjacent mammary tissue from Brazilian patients as evaluated by Northern blot assays. Total mRNA was separated electrophoretically and hybridization was performed with $^{32}$P-labeled specific probes. VDR mRNA was detected in normal (N) and tumoral (T) samples as well as in MDA-MB231 (breast cancer) and HB4A (normal breast) human cell lines. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as mRNA load control.

![Figure 2](image2.png)  
**Figure 2.** Vitamin D receptor (VDR) expression in breast cancer tissue as determined by immunohistochemistry assay. Strong VDR staining can be observed in the nuclei of epithelial cells. Magnification 600X.
HC11 cells were growth inhibited by both analogs, in contrast to HC11ras cells, as evidenced by the growth curves presented in Figure 3. A lower concentration of KH1060 (1 nM) as compared to 1,25-(OH)$_2$D$_3$ (10 nM) was able to double the duplication time of HC11 cells in a similar way to the parent compound (37).

The inhibitory effects of 1,25-(OH)$_2$D$_3$ on breast cancer cell growth could be mediated by inducing the expression of cyclin-dependent kinase inhibitors such as p21$^{WAF1/CIP1}$ (38) and p27$^{KIP1}$ (39). A functional vitamin D response element was described in the promoter region of the p21$^{WAF1/CIP1}$ gene (7). On the other hand, 1,25-(OH)$_2$D$_3$ positive regulation of the p27$^{KIP1}$ gene does not directly involve VDR but is mediated by the transcription factors Sp1 and NF-Y (40). Other potential molecular effectors of the antiproliferative actions of 1,25-(OH)$_2$D$_3$ could be TGF-ß1 (41), which exerts antiproliferative actions on epithelial cells and its receptor type II (Tß-RII) (42). In addition, it was shown that the c-myc protooncogene could be down-regulated by 1,25-(OH)$_2$D$_3$.

Figure 3. Growth curves of HC11 parental and Ha-ras transformed cells (HC11ras). Cells (2 x 10$^4$) were seeded onto 8.8-cm$^2$ plates and maintained in the absence (control) or presence of 1, 10 or 100 nM EB1089 or 10 pm, 0.1 nM or 1 nM KH1060 and harvested at 24-h intervals. Two independent assays were performed in triplicate and mean cell number was plotted on monolog graph paper.
in breast cancer cells (43). The hormone enhances HOXB4 (a homeobox gene product) that binds to MIE1 sites located at intron 1 of the c-myc gene and as a result a transcriptional elongation block takes place (44). It was also demonstrated that 1,25-(OH)₂D₃ could inhibit the mitogenic activity of insulin and insulin growth factor I-stimulated growth of MCF-7 cells (45) which may be related to insulin growth factor binding protein (IGFBP)-5 (46) and IGFBP-3 (47) up-regulation. Furthermore, the antiproliferative effect of 1,25-(OH)₂D₃ could be modulated by induction of BRCA1 gene expression, as recently reported (48). On the other hand, 1,25-(OH)₂D₃-induced growth inhibition may involve activation of apoptosis in vitro with up-regulation of genes associated with mammary gland apoptosis such as TRPM-2/clusterin and cathepsin B as well as with down-regulation of antiapoptotic genes such as bcl-2 (38). Vitamin D-stimulated apoptotic regression in mice bearing MCF-7 xenografts was also described (49).

The underlying mechanism of the antiproliferative effects of 1,25-(OH)₂D₃ on HC11 cells has yet to be clarified. Our data suggest that it does not involve c-myc down-regulation (Figure 4) (50) or TGF-β1 up-regulation (51). Cell cycle regulators such as cyclins D1 and D3 and p27⁰⁰⁷¹ determined by Western blot were also not involved. We have observed a slight increase of CDKI p21⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰⁰ and cyclin E expression (data not shown).

Vitamin D analogs have already been employed in some clinical studies and topical treatment of patients with locally advanced or cutaneous metastatic breast cancer with calcipotriol resulted in very few responses (52), whereas a phase I study in which patients with advanced breast and colorectal cancer received EB1089 showed a few cases of disease stabilization (53).

In addition to having effects on cell proliferation, 1,25-(OH)₂D₃ has been shown to inhibit the invasive potential of breast cancer cells in vitro (54) and its analog EB1089 can prevent skeletal metastasis development in vivo (55) and angiogenesis in vitro and in vivo (56) in human breast carcinoma cells transplanted into nude mice. Furthermore, an interaction between 1,25-(OH)₂D₃ or analogs with chemotherapeutic drugs active on breast cancer, such as doxorubicin (57) and paclitaxel (58), was also demonstrated, resulting in potentiation of cytotoxicity in MCF-7 cell cultures. In addition, topical 1,25-(OH)₂D₃ increased the antitumor effect of cyclophosphamide in female mice inoculated with murine mammary tumor (59) and additive effects were observed when a vitamin D analog, CB1093, was administered together with paclitaxel to MCF-7 growing in immunodeficient mice (60).

Taken together, these studies indicate that vitamin D and its analogs have important antitumoral properties, which might be explored in chemopreventive as well as in therapeutic cancer approaches.

Concluding remarks

The involvement of the vitamin D₃ pathway in breast carcinogenesis and cancer progression has not been fully clarified. Besides the tumor growth-suppressive activity of vitamin D₃ compounds in vitro and in vivo, additional aspects may be involved in the
antitumoral properties of this vitamin. Vitamin D3 seems also to induce apoptosis and to inhibit the processes of angiogenesis, invasion and metastasis. Interactions between vitamin D3 and chemotherapeutic drugs have been reported and represent another field to be explored. On the other hand, the pharmacological doses of vitamin D3 necessary to induce antiproliferative effects are associated with hypercalcemia in vivo. New vitamin D3 analogs, which are less hypercalcemic but are potent growth inhibitory agents, might be an option to fight cancer development.

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References

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