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The Potential Therapeutic Benefits of Vitamin D in the Treatment of Estrogen Receptor Positive Breast Cancer

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Abstract

Calcitriol (1,25-dihydroxyvitamin D₃), the hormonally active form of vitamin D, inhibits the growth of many malignant cells including breast cancer (BCa) cells. The mechanisms of calcitriol anticancer actions include cell cycle arrest, stimulation of apoptosis and inhibition of invasion, metastasis and angiogenesis. In addition we have discovered new pathways of calcitriol action that are especially relevant in inhibiting the growth of estrogen receptor positive (ER+) BCa cells. Calcitriol suppresses COX-2 expression and increases that of 15-PGDH thereby reducing the levels of inflammatory prostaglandins (PGs). Our *in vitro* and *in vivo* studies show that calcitriol decreases the expression of aromatase, the enzyme that catalyzes estrogen synthesis selectively in BCa cells and in the mammary adipose tissue surrounding BCa, by a direct repression of aromatase transcription *via* promoter II as well as an indirect effect due to the reduction in the levels of PGs, which are major stimulator of aromatase transcription through promoter II. Calcitriol down-regulates the expression of ER α and thereby attenuates estrogen signaling in BCa cells including the proliferative stimulus provided by estrogens. Thus the inhibition of estrogen synthesis and signaling by calcitriol and its anti-inflammatory actions will play an important role in inhibiting ER+ BCa. We hypothesize that dietary vitamin D would exhibit similar anticancer activity due to the presence of the enzyme 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) in breast cells ensuring conversion of circulating 25-hydroxyvitamin D to calcitriol locally within the breast micro-environment where it can act in a paracrine manner to inhibit BCa growth. Cell culture and *in vivo* data in mice strongly suggest that calcitriol and dietary vitamin D would play a beneficial role in the prevention and/or treatment of ER+ BCa in women.

Keywords

Calcitriol; breast cancer; anti-proliferative effects; anti-inflammatory effects; aromatase; prostaglandins; aromatase inhibitors; estrogen receptor; dietary vitamin D

Introduction

Breast cancer (BCa) is the most common cancer in women [1]. Estrogens drive the proliferation of mammary epithelial cells and therefore promote the growth of estrogen receptor positive (ER+) BCa. Approximately 70% of BCa are ER+ and are responsive to

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endocrine therapy. The hormonal drugs used to treat ER+ BCa are designed to antagonize the mitogenic effects of estrogens and include: selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene that bind to the ER and act as antagonists in the breast; selective estrogen receptor down-regulators (SERDs) such as fulvestrant that bind to and target ER for degradation; and aromatase inhibitors (AIs) that inhibit the activity of aromatase (CYP19A1), the enzyme that catalyzes the synthesis of estrogens from androgenic precursors [2]. Currently AIs are the first line therapy to prevent BCa progression in postmenopausal women following primary therapy [2–4]. In spite of the available treatments, the incidence of BCa continues to rise and increasing emphasis is being placed on BCa chemoprevention, including approaches to reduce exposure to carcinogens and the use of nutritional agents to prevent and/or delay the development of BCa. This review explores the pathways in cultured cells and animal models that provide strong evidence for the chemopreventive and therapeutic activity of vitamin D in ER+ BCa.

Calcitriol, the hormonally active form of vitamin D, (1,25-dihydroxyvitamin D₃), plays an important role in calcium homeostasis through its actions in intestine, kidney, parathyroid glands and bone [5]. In recent years it has been recognized that in addition to its actions on calcium and bone homeostasis, calcitriol also exhibits anti-proliferative and pro-differentiation activities indicating its potential use in the prevention and treatment of multiple cancers including BCa [6–13].

Vitamin D and BCa

Dietary vitamin D₃ is not merely a vitamin but the essential precursor to the potent steroid hormone 1,25(OH)₂D₃ or calcitriol [5], which has anti-proliferative, anti-inflammatory, pro-differentiating and pro-apoptotic activities in many cancers including BCa [8,10]. Epidemiological studies indicate that vitamin D (no subscript denotes either vitamin D₂ or D₃) exhibits anticancer activity against BCa and other cancers [8,10,14–16]. Sunlight exposure, which promotes synthesis of vitamin D₃ in the skin and dietary intake of vitamin D₃ are inversely associated with BCa risk and mortality [16–20]. An inverse association has also been reported between BCa risk and the levels of serum 25-hydroxyvitamin D [25(OH)D], the circulating prohormone, which reflects both sun exposure and dietary vitamin D intake [21]. A serum 25(OH)D level of approximately 52 ng/ml has been shown to be associated with a reduction by 50% in the incidence of BCa [15]. However, the epidemiologic studies are not always consistent. Studies in some cancer cohorts do not show a protective effect of serum 25(OH)D levels [22]. A study in pancreatic cancer patients report an increased risk at very high concentrations (> or = 100 nmol/L) of serum 25(OH)D [23] suggesting a “J” or “U” shaped dose response curve. However, others have expressed the opinion that there is limited data to support the U-shaped dose-response relationship of serum 25(OH)D level to disease [24].

Vitamin D signaling plays an important role in the development of the normal mammary gland [14,25]. Studies in vitamin D receptor (VDR) knock out mice provide evidence that calcitriol signaling through the VDR opposes estrogen driven proliferation of mammary epithelial cells and maintains normal differentiation [25]. Calcitriol exerts antiproliferative, pro-apoptotic and pro-differentiation actions in several malignant cells including BCa cells. As discussed below, the effects of calcitriol to limit estrogen synthesis and signaling would be particularly beneficial in the treatment of ER+ BCa.

Mechanisms of the anticancer actions of calcitriol

Regulation of proliferation, apoptosis, invasion and metastasis

Calcitriol inhibits the growth of both ER+ and ER-negative human BCa cell lines [reviewed in [7,14,26]]. In general ER+ BCa cell lines appear to be more sensitive to the growth inhibitory effects of calcitriol than the ER-negative cell lines [27]. In most BCa cells calcitriol induces cell cycle arrest by increasing the expression of cyclin-dependent kinase inhibitors and decreasing cyclin-dependent kinase activity [28–30]. In some cells calcitriol promotes apoptosis by modulating the expression of the *bcl-2* family of genes [7,27] and in other BCa cells potentiates the induction of apoptosis through the death receptor pathway [7,31,32]. Calcitriol and its analogs also inhibit the growth of BCa cells by regulating the expression of oncogenes such as *c-myc* and *c-fos* and modulating the actions of the genes that encode several growth factors including epidermal growth factor (EGF), transforming growth factor β (TGF β) and insulin-like growth factor-I (IGF-I) [reviewed in [7,14]]. Further, calcitriol and its analogs induce a more differentiated phenotype in some BCa cells reversing the myoepithelial features associated with more aggressive forms of BCa [33,34].

Calcitriol reduces the invasive and metastatic potential of several BCa cells [35–37] by stimulating the expression of E-cadherin [34], decreasing the activities of matrix metalloproteinases (MMPs), urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator and increasing the expression of plasminogen activator inhibitor 1 (PAI1) and MMP inhibitor 1 [37]. Calcitriol also exhibits potent anti-angiogenic activity that could contribute to its actions to inhibit invasion and metastasis *in vivo* [7,14].

Anti-inflammatory effects

A variety of stimuli acting either systemically or locally within the breast, the prostate or other sites trigger chronic inflammation that has been recognized as a risk factor for cancer development [38,39]. Calcitriol has been shown to exhibit significant anti-inflammatory actions in several malignant cells including BCa cells [10,11,40,41]. Prostaglandins (PGs) are pro-inflammatory molecules that play an important role in the development and progression of BCa [42]. PGs released from BCa cells or from surrounding breast adipose stromal cells mediate autocrine/paracrine stimulation of tumor progression by promoting cell proliferation, resistance to apoptosis and stimulating tumor cell migration, metastasis and angiogenesis [43]. Elevated expression of COX-2, the rate-limiting enzyme catalyzing PG synthesis, is associated with larger tumor size, higher histological grade and poorer prognosis in BCa patients [44]. COX-2 over-expression may be an important factor in promoting tumor progression in ER-negative tumors and COX-2 is a potential drug target in BCa therapy [43]. 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which catalyzes the conversion of PGs to biologically inactive keto-derivatives, exhibits a tumor suppressive role in BCa [45]. In both ER+ and ER-negative human BCa cells, calcitriol down-regulates the expression of COX-2 and increases that of 15-PGDH thereby limiting the synthesis and biological actions of pro-inflammatory PGs [46]. The calcitriol-mediated decrease in COX-2 expression in BCa cells is especially interesting, because it has been shown that there is a tight coupling between the expression levels of COX-2 and aromatase in tumor samples from BCa patients [47,48].

Inhibition of estrogen synthesis and signaling

Our studies in experimental models of BCa have revealed that, in addition to acting through the multiple molecular pathways discussed above, calcitriol also mediates actions that would be especially effective in ER+ BCa. These actions, to be discussed below, include the inhibition of both the synthesis and the biological actions of estrogens, the major stimulators of BCa growth [46,49]. Calcitriol represses the expression of the gene encoding aromatase

(*CYP19A1*), the enzyme that catalyzes estrogen synthesis from androgenic precursors. Calcitriol also down-regulates ER α [50,51], the nuclear receptor that mediates estrogen actions. These combined actions reduce both the levels of the estrogenic hormones and their biological activities within the breast [41] (Fig 1).

Regulation of aromatase by calcitriol—The ovaries are the principal source of circulating estrogens in premenopausal women. In humans a number of other tissues including the breast express aromatase and hence have the capacity to synthesize estrogens locally. Importantly, estrogen synthesized locally becomes the major source of the hormone in the breast after menopause when circulating estrogen levels from the ovaries dramatically decline. This is why the class of drugs known as aromatase inhibitors (AIs) has become the primary therapy after surgery for ER+ BCa.

Aromatase expression is higher in human BCa than in normal breast tissue [52]. In postmenopausal women with BCa, estrogen levels within the breast tissue are elevated severalfold higher than the serum levels indicating tumor accumulation or local synthesis of estrogens that can drive BCa growth [53]. Therefore aromatase is critical for the progression of ER+ BCa in postmenopausal women. While BCa cells express aromatase and have the capacity to synthesize estrogens, in the normal breast, aromatase is primarily expressed in the stromal mesenchymal cells of the breast adipose tissue referred to as breast adipose fibroblasts (BAFs) or adipose stromal cells (ASCs) [54]. Aromatase levels are higher in the undifferentiated preadipocytes compared to the more mature and differentiated lipid laden adipocytes [54]. Aromatase transcription is primarily driven by its tissue specific promoter I. 4 in normal breast adipose tissue and bone [54]. However, in the presence of BCa, the transcription switches from promoter I.4 to predominantly promoter I.3 and promoter II both in the cancerous epithelial cells and the surrounding BAFs/ASCs [54].

Calcitriol is a known regulator of aromatase expression in bone and it causes the up-regulation of aromatase mRNA which results in increased activity in osteoblasts and fibroblasts [55,56]. This is a protective action in bone preserving local estrogen production. Other studies in keratinocytes [57] and prostate cancer cells [58] found no effect of calcitriol on aromatase expression. Interestingly, recent *in vitro* and *in vivo* studies from our laboratory demonstrate that calcitriol regulates the expression of aromatase in a tissue-selective manner [46,49]. This differential regulation of aromatase in various tissues has been referred to as selective aromatase modulator or SAM activity [54]. Our findings reveal that calcitriol significantly decreases aromatase expression in both ER+ and ER-negative human BCa cells and a cell culture model of preadipocytes [46]. The mechanism of aromatase down-regulation in BCa cells appears to be a direct repression by calcitriol of aromatase transcription via promoter II through the vitamin D response elements (VDREs) that we have identified in this promoter [46]. In contrast, as reported previously [55,56] calcitriol substantially increases aromatase expression in human osteosarcoma cells with osteoblastic features while causing a modest increase or no change in ovarian aromatase [46,49]. These differential actions demonstrate the tissue selectivity of aromatase regulation by calcitriol. Further, as discussed above, calcitriol also significantly reduces the levels of PGs through the suppression of COX-2 and induction of 15-PGDH in BCa cells and mammary adipose tissue (Fig. 1). Both aromatase promoters I.3 and II, predominantly used in malignant breast epithelial cells and the BAFs/ASCs surrounding a breast tumor, are responsive to cAMP [59] and are significantly stimulated by PGE₂ [60,61]. Thus the calcitriol-mediated reduction of biologically active PG levels provides an important second and indirect mechanism for its down-regulatory effect on aromatase expression in BCa cells and the tumor adjacent BAFs/ASCs (Fig 1, [46]).

Our recent studies in immunocompromised mice, bearing MCF-7 xenografts in their mammary fat pads, validate the cell culture data and demonstrate tissue selective regulation of aromatase *in vivo* by calcitriol [49]. Administration of calcitriol to the mice decreased aromatase expression in MCF-7 xenograft tumors. Importantly, calcitriol acted as a SAM in the mouse, decreasing aromatase expression in the mammary adipose tissue, while increasing it in bone marrow cells and not changing it in the ovaries and uteri. As a result calcitriol treatment caused significant reductions in estrogen levels in the xenograft tumors and surrounding breast adipose tissue [49].

Combination of calcitriol and aromatase inhibitors as a therapeutic approach in ER+ BCa—Since AIs inhibit the enzymatic activity of aromatase, while calcitriol reduces aromatase expression through transcriptional repression of the aromatase gene, we hypothesized that the combination of these agents might exhibit cooperative anticancer activity. When BCa cell cultures were treated with combinations of calcitriol and AIs, we observed enhanced growth inhibitory effects [46]. The different mechanisms of aromatase regulation due to calcitriol and the AIs were also complementary when tested *in vivo*. In nude mice, administration of combinations of calcitriol and AIs caused maximal inhibition of estrogen synthesis in the tumor microenvironment as reflected by the estradiol and estrone levels measured in the xenograft tumors and surrounding mammary fat [49]. At the doses tested, the calcitriol-AI combinations exhibited statistically significant increases in tumor shrinkage compared to the individual agents. Although a clear cut synergy due to the combinations could not be demonstrated in terms of tumor retardation, the combinations exhibited additive and synergistic effects to regulate tumor gene expression reflecting cooperative anticancer activity to suppress proliferation and inflammation [49].

AIs have become the major therapeutic agents to prevent ER+ BCa progression or recurrence in postmenopausal women after primary therapy [3,62,63]. However, AIs inhibit estrogen synthesis globally and therefore have a detrimental effect at sites such as bone [64,65] where adequate estrogen synthesis is required for the maintenance of normal bone homeostasis. The development of SAMs that inhibit aromatase expression in breast, but allow unimpaired estrogen synthesis at other desirable sites such as bone, would have great utility in BCa therapy [54]. Based on our *in vitro* and *in vivo* studies [46,49], we postulate that calcitriol acts as a SAM, decreasing aromatase expression in BCa cells and the breast adipose/stromal tissue surrounding BCa, while increasing aromatase expression in bone cells. Thus calcitriol has the potential to ameliorate the AI-induced side effect of osteoporosis when administered in combination with an AI in BCa patients.

Down-regulation of ER α by calcitriol—The growth stimulating actions of estrogen require the presence of ER α , the specific nuclear receptor that mediates the proliferative response to estrogens [66]. We [51] and others [30,50,67] have shown that calcitriol down-regulates ER α expression in BCa cells. As a result calcitriol and its analogs suppress estrogenic bioresponses in BCa cells including the induction of the expression of estrogen responsive genes such as the progesterone receptor and pS2 and attenuates the stimulation of BCa cell growth by estradiol [51,67]. The mechanism of ER down-regulation appears to be a direct transcriptional repression of the *ER α* gene by calcitriol [50,51]. Further characterization of the *ER α* promoter by us (unpublished observations) and others [50] has identified negative vitamin D response elements (nVDREs) in the *ER α* promoter that mediate the repressive effects of calcitriol on the *ER α* gene. Combinations of calcitriol or its analogs with ER antagonists such as tamoxifen or ICI 182, 780 also exhibit enhanced inhibition of the growth of BCa cells [67–69].

We postulate that the cumulative actions of calcitriol cause a decrease both in the level of locally produced estrogens by the BCa epithelial cells and the surrounding breast adipose/

stromal tissue and in the levels of ER α , the receptor through which they act in BCa cells. Thus, the down-regulation of both the hormone synthesis and receptor expression levels significantly reduces the important proliferative stimulus of estrogens on ER+ BCa cells [41] (Fig. 1).

Dietary vitamin D in BCa therapy and chemoprevention

Studies have demonstrated the presence of the enzyme 25-hydroxyvitamin D-1 α -hydroxylase (CYP27B1) in normal and malignant mammary epithelial cells [70–72], which converts the circulating prohormone 25(OH)D₃ to the active hormone calcitriol [1,25(OH)₂D₃]. These observations raise the possibility that dietary vitamin D [the precursor to 25(OH)D] rather than the active hormone calcitriol could be used in BCa therapy. Raising the blood levels of 25(OH)D through dietary vitamin D supplementation, provides increased levels of the substrate for 1 α -hydroxylase in target tissues like breast for the local production of elevated levels of calcitriol, which could then exert autocrine/paracrine actions to inhibit BCa growth.

This therapeutic approach avoids the systemic side effect of hypercalcemia, which may be associated with high dose calcitriol [6,8]. The ability of 25(OH)D to cause hypercalcemia is very much reduced because of its significantly lower affinity for the VDR [73,74] unless extremely elevated serum levels are achieved. Vitamin D related hypercalcemia usually results from elevated circulating levels of the active hormone 1,25(OH)₂D that cause increased intestinal calcium absorption and increased bone resorption. The expression of renal 1 α -hydroxylase is tightly controlled by parathyroid hormone (PTH) [5]. Following a rise in serum calcium concentration, PTH levels would be suppressed leading to a decrease in 1 α -hydroxylase expression and the restriction of the conversion of 25(OH)D to calcitriol thereby limiting the ability of vitamin D supplementation to cause hypercalcemia [5]. Treatment with calcitriol bypasses this checkpoint thereby increasing the risk of hypercalcemia [6,8]. Since extra-renal 1 α -hydroxylase within cancer cells does not appear to be regulated by PTH [75] the extent of 25(OH)D conversion to calcitriol in the extra-renal sites is not restricted by the normal feedback system for renal 1 α -hydroxylase. Therefore local production of calcitriol within the BCa seems to be predominantly dependent on the levels of the circulating substrate 25(OH)D, although other regulators may eventually be identified. Thus dietary vitamin D₃ ingestion will retain the beneficial paracrine effects of calcitriol within the breast, while avoiding systemic hypercalcemia that is much more likely to be associated with high dose calcitriol administration [6,8].

Ongoing studies in our laboratory are evaluating the therapeutic potential of dietary vitamin D in mouse models of BCa. One of our studies evaluated the efficacy of dietary vitamin D to inhibit the growth of human BCa xenograft tumors in nude mice. The data revealed an appreciable tumor inhibitory effect of dietary vitamin D, which was equivalent to that elicited by calcitriol at the doses tested without causing significant elevations in serum calcium levels [76]. These data are supportive of the hypothesis that dietary vitamin D may potentially be useful in the chemoprevention and treatment of BCa since it is a very safe, economical and easily available nutritional agent. Further, current data suggest that adequate vitamin D nutrition and avoidance of vitamin D deficiency is an important factor in reducing BCa risk [77–79].

Summary and Conclusions

Calcitriol exhibits antiproliferative effects in BCa cell cultures and retards tumor growth in animal models of BCa through a variety of mechanisms. In addition to the many well-known anticancer pathways active in multiple malignant cells, we propose several new mechanisms that are specifically effective in ER+ BCa cells. We have identified new calcitriol target

genes revealing additional molecular pathways of calcitriol action in BCa. By suppressing the expression of COX-2 and increasing that of 15-PGDH calcitriol reduces the levels of biologically active PGs in BCa cells and thereby exerts significant anti-inflammatory activity. Acting as a SAM, calcitriol decreases aromatase expression in BCa cells and the breast adipose/stromal tissue surrounding BCa, while increasing aromatase expression in bone cells. The mechanism of the down-regulatory effect of calcitriol on BCa aromatase expression is two fold: a direct repression of aromatase transcription *via* promoter II through the VDREs present in this promoter and an indirect effect due to the reduction in the levels of PGs, which are major stimulators of aromatase transcription through promoter II in BCa. Further, in addition to suppressing estrogen synthesis, calcitriol inhibits estrogen actions by down-regulating ER α expression. We hypothesize that due to its suppressive effects on estrogen synthesis and signaling calcitriol will have enhanced therapeutic utility in ER+ BCa. Calcitriol could be used as a therapy in the neo-adjuvant setting or it could be administered in combination with existing drugs for ER+ BCa such as the AIs or SERMs to enhance their potencies. We believe that current data support the hypothesis that dietary vitamin D supplementation is potentially useful in the chemoprevention and treatment of BCa due, in part, to the presence of 1 α -hydroxylase in the breast tissue ensuring local synthesis of calcitriol. Our findings, as well as a large body of preclinical data, indicate that adequate vitamin D nutrition and avoidance of vitamin D deficiency are important in reducing BCa risk. Clinical prospective trials in women remain to be done to demonstrate the beneficial actions of calcitriol and vitamin D predicted from preclinical studies.

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Highlights

- Calcitriol, (active form of vitamin D) exerts anticancer effects in breast cancer models
- Calcitriol inhibits prostaglandin synthesis and exerts anti-inflammatory effects
- Calcitriol inhibits estrogen synthesis and signaling and may be beneficial in ER + breast cancer
- Dietary vitamin D can be converted to calcitriol in the breast and can exert anticancer effects
- Calcitriol and dietary vitamin D may be useful in breast cancer treatment and chemoprevention

Fig 1. Inhibition of estrogen synthesis and signaling by calcitriol

Panel A. Cox-2, prostaglandin and aromatase regulation of estrogen synthesis and signaling in the breast microenvironment leading to increased BCa proliferation. Panel B. The ability of calcitriol to inhibit the prostaglandin and estrogen signaling pathways and thereby inhibit BCa proliferation. Calcitriol decreases the expression of aromatase, the enzyme that converts androgenic precursors to estrogens both in the cancerous breast epithelial cells (breast cancer, BCa cell) and in the breast adipose fibroblasts (BAF) in the stroma surrounding the tumor by a direct transcriptional repression of the aromatase promoter II. Calcitriol also suppresses the expression of COX-2 in the BCa cells and BAFs, thereby reducing the levels of PGE2. PGE2 stimulates proliferation, angiogenesis and other pro-carcinogenic pathways and inhibits apoptosis. PGE2 is also a major stimulator of aromatase transcription via promoter II. The decrease in PGE2 therefore provides a second, indirect mechanism for aromatase repression by calcitriol both in the BCa cells and the surrounding BAFs leading to a decrease in estrogen synthesis in the BCa microenvironment. Calcitriol also down-regulates ER α levels by the direct transcriptional repression of the ER α promoter. The down-regulation of both the hormone (E2) and receptor (ER α) levels by calcitriol thus significantly reduces the important proliferative stimulus of estrogens on ER+ BCa cells. Calcitriol also inhibits BCa proliferation by additional pathways including cell

cycle arrest, proapoptotic and pro-differentiation actions, anti-inflammation, etc. which are active in both ER+ and ER-negative BCa cells.

AA = arachidonic acid, E2 = estradiol, ER α = estrogen receptor α , PGE2 = prostaglandin E2, T = testosterone.

(The figure has been adapted from a figure in [41] and used with permission).