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## Vitamin D

Biochem Cell Biol. 1994 Nov-Dec; 72(11-12): 537-45.

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### Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens.

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1,25-Dihydroxycholecalciferol D3 (1,25(OH)2D3), the active metabolite of vitamin D, is a potent inhibitor of breast cancer cell growth both in vivo and in vitro. We have shown that MCF-7 cells treated with 100 nM 1,25(OH)2D3 exhibit characteristic apoptotic morphology (pyknotic nuclei, chromatin and cytoplasmic condensation, nuclear matrix protein reorganization) within 48 h. In the experiments reported here, we examined the interactions between 1,25(OH)2D3 and the antiestrogen 4-hydroxytamoxifen (TAM), which also induces apoptosis in MCF-7 cells. Our data suggest that TAM significantly potentiates the reduction in cell number induced by 1,25(OH)2D3 alone. Combined treatment with 1,25(OH)2D3 and TAM enhances the degree of apoptosis assessed using morphological markers that identify chromatin and nuclear matrix protein condensation. We have selected a subclone of MCF-7 cells resistant to 1,25(OH)2D3 (MCF-7D3Res). These cells express the vitamin D receptor and exhibit doubling times comparable to the parental MCF-7 cells, even when grown in 100 nM 1,25(OH)2D3. Treatment of both parental and resistant MCF-7 cells with TAM induces apoptosis and clusterin. These data emphasize that apoptosis can be induced in MCF-7 cells either by activation of vitamin-D-mediated signalling or disruption of estrogen-dependent signalling.

Breast Cancer Res Treat. 1997 Jan; 42(1): 31-41.

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### Comparative effects of 1,25(OH)2D3 and EB1089 on cell cycle kinetics and apoptosis in MCF-7 breast cancer cells.

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1,25-dihydroxyvitamin D3 [1,25(OH)2D3], the active metabolite of vitamin D2 inhibits breast cancer cell growth both in vivo and in vitro. In addition to its anti-proliferative effects, 1,25(OH)2D3 induces morphological and biochemical markers of apoptosis in MCF-7 cells. In the studies reported here, we compared the effects of 1,25(OH)2D3 and EB1089, a low calcemic vitamin D analog, on cell cycle kinetics and apoptosis in MCF-7 cells. Both vitamin D compounds reduced viable MCF-7 cell number in a time and dose dependent manner, with EB1089 approximately 50 fold more potent than 1,25(OH)2D3. Flow cytometric analysis indicated that both agents induced cell cycle arrest in G<sub>1</sub>, which was associated with accumulation of the hypophosphorylated form of the retinoblastoma (Rb) protein. MCF-7 cells treated with either 1,25(OH)2D3 or EB1089 for 48 h exhibited characteristics of apoptosis, including cytoplasmic condensation, pyknotic nuclei, condensed chromatin and DNA fragmentation. Cells treated with either agent exhibited up regulation of proteins associated with mammary gland regression (clusterin and cathepsin B) and down regulation of the anti-apoptotic protein bcl-2. These studies demonstrate that, despite its lower calcemic activity in vivo, the vitamin D analog EB1089 induces effects that are indistinguishable from those of 1,25(OH)2D3 on cell number, cell cycle and indices of apoptosis in MCF-7 cells in vitro. In addition, since both agents rapidly down regulate estrogen receptor, disruption of estrogen dependent signalling may play a role in the induction of apoptosis by vitamin D compounds in MCF-7 cells.

Cancer Res. 1995 Jul 1;55(13):2822-30.

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## 20-epi-vitamin D3 analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines.

Elstner E, Linker-Israeli M, Said J, Umiel T, de Vos S, Shintaku IP, Heber D, Binderup L, Uskokovic M, Koeffler HP.

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We have studied the in vitro biological activities and mechanism of action of 1,25-dihydroxyvitamin D3 (1,25D3) and four potent 1,25D3 analogues [20-epi-22oxa-24a,26a,27a-tri-homo-1,25(OH)2D3 (KH 1060); 20-epi-1,25(OH)2D3; 1,25(OH)2-16ene-D3; and 1,25(OH)2-16ene-23yne-D3] on proliferation and differentiation of estrogen receptor-negative (MDA-MB-436, BT-20, SK-BR-3, and MDA-MB-231), estrogen receptor-weakly positive (BT474), and estrogen receptor-positive (MCF-7) breast cancer cell lines. Dose-response studies showed that KH 1060 was the most potent analogue, because it was able to induce differentiation in all seven breast cancer cell lines (measured by lipid staining) and to suppress more than 50% clonal proliferation (ED50) at 10<sup>-10</sup> M in all cell lines, except MDA-MB-436 and BT-20. To explore how these compounds mediated antiproliferative actions, their effects on the cell cycle, on expression of bcl-2 and p53, and on apoptosis were assessed. Five of six

cell lines have a mutant p53 gene, whereas MCF-7 has wild-type p53. Immunohistochemical staining showed that the p53 protein was predominantly localized in the nucleus in each of the breast cancer cell lines except for MCF-7, which expressed the protein predominantly in the cytoplasm. After incubation with KH 1060 (3 days;  $10^{-7}$  M), expression of bcl-2 protein as determined by immunohistochemical localization was markedly decreased in BT-474, MCF-7, and MDA-MB-231; these same cells were profoundly inhibited in their clonal proliferation and arrested in the G0/G1 phase of the cell cycle when cultured with KH 1060. In contrast, BT-20 and MDA-MB-436 cells that were refractory to the antiproliferative effect of KH 1060 ( $ED_{50} < 10^{-6}$  M) had no down-regulation of their bcl-2 expression and no cell cycle changes after exposure to KH 1060. MCF-7 showed morphological changes and DNA fragmentation, indicative of apoptosis after 48 h incubation with KH 1060 ( $10^{-6}$  M), during which time p53 protein accumulated in the nucleus and decreased in the cytoplasm. In contrast, no apoptosis was detected in three other breast lines (MDA-MB-231, SK-BR-3, and BT-474) that had a mutated p53. In conclusion, the data indicate that KH 1060 is an extremely potent 1,25D3 analogue inducing differentiation of all six breast cancer lines and potently inhibiting clonal growth of four of them with concomitant decreased bcl-2 and cell cycle arrest at G0/G1. (ABSTRACT TRUNCATED AT 400 WORDS)

Bone. 1996 Dec;19(6): 615-20.

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## Influence of vitamin D and retinoids on the gammacarboxylation of osteocalcin in human osteosarcoma MG63 cells.

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Osteocalcin (OC) is a bone matrix protein, synthesized by osteoblasts, which contains three residues of gammacarboxyglutamic acid (GLA). A fraction of circulating OC, which is not fully carboxylated and does not bind to hydroxyapatite, is called undercarboxylated OC (ucOC). In elderly institutionalized women, we have shown an increase of circulating ucOC level which may result not only from vitamin K deficiency but also from vitamin D deficiency (Szulc et al., *J Clin Invest* 91: 1769; 1993). This intriguing finding prompted us to study the effect of vitamin D on the secretion of ucOC by osteoblastic cells in vitro in the presence of warfarin, an inhibitor of gammacarboxylation of GLA-containing proteins. The potential influence of retinoic acid (RA) was also studied, because its mechanism of action involves pathways that are similar to vitamin D. In the presence of warfarin (0.05 microg/mL),  $1\alpha,25(OH)_2D$  ( $10^{-8}$ - $10^{-6}$  mol/L) decreased dose dependently ucOC secretion by human osteosarcoma MG63 cells (from  $3.87 \pm 0.96$  to  $2.12 \pm 0.13$  ng/10(6) cells). When expressed as a fraction of total OC, secretion ucOC decreased from  $47.4 \pm 1.4\%$  to  $24.8 \pm 3.2\%$  in the MG63 cells. The secretion of total OC was stimulated by RA and by Ro 13-7410, which is a specific ligand of retinoic acid receptor (RAR), but not by 9-cis retinoic acid (9-cisRA), which is a physiologic ligand of retinoid X receptor (RXR). RA and Ro 13-7410 decreased ucOC secretion and ucOC% in warfarin-treated MG63 cells (RA: from  $50.4 \pm 13.3\%$  to  $13.5 \pm 2.8\%$ ; Ro 13-7410: from  $28.4 \pm 8.2\%$  to  $11.3 \pm 8.4\%$ ). 9-cisRA had no effect on OC gammacarboxylation. These results show that vitamin D, RA, and Ro 13-7410, but not 9-cisRA, may modify the gammacarboxylation of OC in human MG63 cells.

Cancer Res. 1999 Feb 15;59(4):862-7.

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## 1,25-Dihydroxyvitamin D3 enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage.

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1,25-Dihydroxyvitamin D3 (1,25(OH)2D3), the hormonal form of vitamin D, has anticancer activity *in vivo* and *in vitro*. Doxorubicin exerts its cytotoxic effect on tumor cells mainly by two mechanisms: (a) generation of reactive oxygen species (ROS); and (b) inhibition of topoisomerase II. We studied the combined cytotoxic action of 1,25(OH)2D3 and doxorubicin on MCF-7 breast cancer cells. Pretreatment with 1,25(OH)2D3 resulted in enhanced cytotoxicity of doxorubicin. The average enhancing effect after a 72-h pretreatment with 1,25(OH)2D3 (10 nM) followed by a 24-h treatment with 1 microg/ml doxorubicin was 74+/-9% (mean +/- SE). Under these experimental conditions, 1,25(OH)2D3 on its own did not affect cell number or viability. 1,25(OH)2D3 also enhanced the cytotoxic activity of another ROS generating quinone, menadione, but did not affect cytotoxicity induced by the topoisomerase inhibitor etoposide. The antioxidant N-acetylcysteine slightly reduced the cytotoxic activity of doxorubicin but had a marked protective effect against the combined action of 1,25(OH)2D3 and doxorubicin. These results indicate that ROS are involved in the interaction between 1,25(OH)2D3 and doxorubicin. 1,25(OH)2D3 also increased doxorubicin cytotoxicity in primary cultures of rat cardiomyocytes. Treatment of MCF-7 cells with 1,25(OH)2D3 alone markedly reduced the activity, protein, and mRNA levels of the cytoplasmic antioxidant enzyme Cu/Zn superoxide dismutase, which indicated that the hormone inhibits its biosynthesis. This reduction in the antioxidant capacity of the cells could account for the synergistic interaction between 1,25(OH)2D3 and doxorubicin and may also suggest increased efficacy of 1,25(OH)2D3 or its analogues in combination with other ROS-generating anticancer therapeutic modalities.

Anticancer Res. 2003 Jan-Feb; 23(1A):283-9.

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## Ability of potent vitamin D3 analogs to inhibit growth of prostate cancer cells *in vivo*.

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**BACKGROUND:** Studies have identified analogs of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3], which *in vitro* are 10- to 3,000-fold more active than 1,25(OH)2D3. We compared *in vivo* the anti-cancer activity of three potent vitamin D3 analogs and

1,25(OH)2D3 at near to each of their maximal tolerated dose (MTD). MATERIALS AND METHODS: Human LNCaP prostate cancer xenografts were grown in nude mice and the animals were treated with intraperitoneal injections of either diluant; 1,25(OH)2D3; 1,25-Dihydroxy-20 $\beta$ -22-oxa-24,26,27-trisho-mocholecalciferol (KH 1060); 1,25-Dihydroxy-22E,24E-diene-24,26,27-trishomocholecalciferol (EB 1039); and 1,25-Dihydroxy-16-ene-24-oxo-19-norcholecalciferol (RO 26-9114). Tumor sizes were measured weekly and tumor weights were measured at autopsy on the 12th week. RESULTS: Each of the analogs equally and markedly inhibited growth of the prostate cancer xenografts. The 1,25(OH)2D3 initially inhibited growth but, by the time of sacrifice, the tumors were nearly the same size as diluant controls. The histological examination of the tumors showed that the analogs produced tumor necrosis and microcalcification. None of the mice developed hypercalcemia, which is the major toxicity of vitamin D3 compounds. CONCLUSION: The MTD of the analogs varied by 400-fold but each had similar efficacy suggesting that, when choosing a vitamin D analog for clinical study, overall efficacy without toxicity is more important than the total amount of the compound that can be administered. In summary, we have identified three vitamin D analogs that show marked potency *in vivo* to inhibit growth of human prostate cancer xenografts; each had no detectable toxicity.

Endocrinology. 2000 Jul; 141(7): 2567-73.

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## Hepatocyte growth factor and vitamin D cooperatively inhibit androgen-unresponsive prostate cancer cell lines.

Qadan LR, Perez-Stable CM, Schwall RH, Burnstein KL, Ostenson RC, Howard GA, Roos BA.

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Expression of MET, the receptor for hepatocyte growth factor (HGF), has been associated with androgen-insensitive prostate cancer. In this study we evaluated MET activation by HGF and HGF action in prostate cancer cell lines. HGF causes phosphorylation (activation) of the MET receptor in three androgen-unresponsive cell lines (DU 145, PC-3, and ALVA-31) together with morphological change. Although HGF is known to stimulate the growth of normal epithelial cells, including those from prostate, we found that HGF inhibited ALVA-31 and DU 145 (hormone-refractory) cell lines. Moreover, HGF and vitamin D additively inhibited growth in each androgen-unresponsive cell line, with the greatest growth inhibition in ALVA-31 cells. Further studies in ALVA-31 cells revealed distinct cooperative actions of HGF and vitamin D. In contrast to the accumulation of cells in G1 seen during vitamin D inhibition of androgen-responsive cells (LNCaP), growth inhibition of the androgen-unresponsive ALVA-31 cell line with the HGF and vitamin D combination decreased, rather than increased, the fraction of cells in G1, with a corresponding increase in the later cell cycle phases. This cell cycle redistribution suggests that in androgen-unresponsive prostate cancer cells, HGF and vitamin D act together to slow cell cycle progression via control at sites beyond the G1/S checkpoint, the major regulatory locus of growth control in androgen-sensitive prostate cells.

Mol Cell Endocrinol. 2002 Jan 15; 186(1): 69-79.

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## Vitamin D-mediated growth inhibition of an androgen-ablated LNCaP cell line model of human prostate cancer.

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1,25-(OH)<sub>2</sub> vitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub> D), the active metabolite of vitamin D, exerts antiproliferative effects on a variety of tumor cells including prostate. This inhibition requires vitamin D receptors (VDRs) as well as downstream effects on the G1 to S phase checkpoint of the cell cycle. Recent data raise the possibility that androgen plays a role in the antiproliferative effects of 1,25-(OH)<sub>2</sub> D in prostate cancer cells; however, this hypothesis has been difficult to test rigorously as the majority of prostate cancer cell lines (unlike human prostate tumors) lack androgen receptors (ARs). We utilized two different models of androgen-independent prostate cancer that express functional ARs and VDRs to evaluate a possible role of androgen in 1,25-(OH)<sub>2</sub> D mediated growth inhibition. We stably introduced the AR cDNA into the human prostate cancer cell line ALVA 31, which expresses functional VDR but is relatively resistant to growth inhibition by 1,25-(OH)<sub>2</sub> D. Neither ALVA-AR nor the control cells, ALVA-NEO, exhibited substantial growth inhibition by 1,25-(OH)<sub>2</sub> D in the presence or absence of androgen. This observation suggests that the basis for the resistance of ALVA 31 to 1,25-(OH)<sub>2</sub> D-mediated growth inhibition is not the lack of AR. The second model was LNCaP-104R1, an AR-expressing androgen independent prostate cancer cell line derived from androgen dependent LNCaP. 1,25-(OH)<sub>2</sub> D inhibited the growth of LNCaP-104R1 cells in the absence of androgen and this effect was not blocked by the antiandrogen Casodex. As was observed in the parental LNCaP cells, this effect was correlated with G1 phase cell cycle accumulation and upregulation of the cyclin dependent kinase inhibitor (CKI) p27, as well as increased association of p27 with cyclin dependent kinase 2. These findings suggest that the antiproliferative effects of 1,25-(OH)<sub>2</sub> D do not require androgen-activated AR but do involve 1,25-(OH)<sub>2</sub> D induction of CKIs required for G1 cell cycle checkpoint control.

Endocrinology. 1997 Apr; 138(4): 1491-7.

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## 1,25-dihydroxyvitamin D<sub>3</sub> and 9-cis-retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G1.

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Recent studies have suggested that the active metabolite of vitamin D<sub>3</sub>, 1,25-dihydroxyvitamin D<sub>3</sub>, can inhibit the growth and/or induce the differentiation of a variety of cell types and that these characteristics might be useful in the treatment of some cancers. Retinoids also promote the differentiation and inhibit the growth of some cells. That the vitamin D receptor acts as a heterodimer with the retinoid X receptor

(RXR) suggests that there may be functional interactions between 1,25-dihydroxyvitamin D3 and retinoids. In this study, we show that the combination of 1,25-dihydroxyvitamin D3 and 9-cis retinoic acid synergistically inhibits the growth of LNCaP prostate cancer cells. That this effect is mediated by RXR rather than retinoic acid receptors was shown using RXR- and retinoic acid receptor-specific ligands. The vitamin D3 analog, EB1089, inhibited growth more effectively than 1,25-dihydroxyvitamin D3 and also acted synergistically with 9-cis-retinoic acid. These treatments caused cells to accumulate in the G1 phase of the cell cycle, suggesting that 1,25-dihydroxyvitamin D3 can regulate one or more factors critical for the G1/S transition.

J Biol Chem. 2003 Nov 21; 278(47): 46862-8. Epub 2003 Sep 03.

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### Vitamin D inhibits G1 to S progression in LNCaP prostate cancer cells through p27Kip1 stabilization and Cdk2 mislocalization to the cytoplasm.

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1,25-(OH)<sub>2</sub> vitamin D3 (1,25-(OH)<sub>2</sub>D<sub>3</sub>) exerts antiproliferative effects via cell cycle regulation in a variety of tumor cells, including prostate. We have previously shown that in the human prostate cancer cell line LN-CaP, 1,25-(OH)<sub>2</sub>D<sub>3</sub> mediates an increase in cyclin-dependent kinase inhibitor p27Kip1 levels, inhibition of cyclin-dependent kinase 2 (Cdk2) activity, hypophosphorylation of retinoblastoma protein, and accumulation of cells in G1. In this study, we investigated the mechanism whereby 1,25-(OH)<sub>2</sub>D<sub>3</sub> increases p27 levels. 1,25-(OH)<sub>2</sub>D<sub>3</sub> had no effect on p27 mRNA levels or on the regulation of a 3.5-kb fragment of the p27 promoter. The rate of p27 protein synthesis was not affected by 1,25-(OH)<sub>2</sub>D<sub>3</sub> as measured by luciferase activity driven by the 5'- and 3'-untranslated regions of p27 that regulate p27 protein synthesis. Pulse-chase analysis of 35S-labeled p27 revealed an increased p27 protein half-life with 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment. Because Cdk2-mediated phosphorylation of p27 at Thr187 targets p27 for Skp2-mediated degradation, we examined the phosphorylation status of p27 in 1,25-(OH)<sub>2</sub>D<sub>3</sub>-treated cells. 1,25-(OH)<sub>2</sub>D<sub>3</sub> decreased levels of Thr187 phosphorylated p27, consistent with inhibition of Thr187 phosphorylation-dependent p27 degradation. In addition, 1,25-(OH)<sub>2</sub>D<sub>3</sub> reduced Skp2 protein levels in LNCaP cells. Cdk2 is activated in the nucleus by Cdk-activating kinase through Thr160 phosphorylation and by cdc25A phosphatase via Thr14 and Tyr15 dephosphorylation. Interestingly, 1,25-(OH)<sub>2</sub>D<sub>3</sub> decreased nuclear Cdk2 levels as assessed by subcellular fractionation and confocal microscopy. Inhibition of Cdk2 by 1,25-(OH)<sub>2</sub>D<sub>3</sub> may thus involve two mechanisms: 1) reduced nuclear Cdk2 available for cyclin binding and activation and 2) impairment of cyclin E-Cdk2-dependent p27 degradation through cytoplasmic mislocalization of Cdk2. These data suggest that Cdk2 mislocalization is central to the antiproliferative effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub>.