Mechanisms implicated in the growth regulatory effects of vitamin D in breast cancer

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Abstract

It is now well established that, in addition to its central role in the maintenance of extracellular calcium levels and bone mineralization, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$), the active form of vitamin D, also acts as a modulator of cell growth and differentiation in a number of cell types, including breast cancer cells. The anti-proliferative effects of 1,25$(OH)_2D_3$ have been linked to suppression of growth stimulatory signals and potentiation of growth inhibitory signals, which lead to changes in cell cycle regulators such as p21$^{WAF-1/CIP1}$ and p27^{kip1}, cyclins and retinoblastoma protein as well as induction of apoptosis. Such studies have led to interest in the potential use of 1,25$(OH)_2D_3$ in the treatment or prevention of certain cancers. Since this approach is limited by the tendency of 1,25$(OH)_2D_3$ to cause hypercalcaemia, synthetic vitamin D analogues have been developed which display separation of the growth regulating effects from calcium mobilizing actions. This review examines mechanisms by which 1,25$(OH)_2D_3$ and its active analogues exert both anti-proliferative and pro-apoptotic effects and describes some of the synthetic analogues that have been shown to be of particular interest in relation to breast cancer.

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Introduction

$1\alpha,25$-Dihydroxyvitamin D_3 is the biologically active form of vitamin D_3. Vitamin D_3 was identified in the 1920s as a new lipid soluble substance with anti-rachitic properties. It is now clear that vitamin D_3 is normally synthesized in adequate amounts in the skin by the action of ultra violet light on the precursor molecule 7-dehydrocholesterol. Vitamin D_3 is activated in the body by two metabolic steps. The first is in the liver where hydroxylation in the C-25 position produces 25-hydroxyvitamin D_3, the major circulating metabolite. The second metabolic step takes place in the kidney where the active hormonal form, $1\alpha,25$-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) is synthesised. This reaction is catalysed by a mitochondrial P450 enzyme, 1α-hydroxylase. The initial step in the catabolism of 1,25$(OH)_2D_3$ is via 24-hydroxylation leading to formation of the biologically inactive calcitroic acid. It is now known that 1,25$(OH)_2D_3$, acting via its receptor, transcriptionally regulates the expression of the 24-hydroxylase (CYP24) gene thus promoting this catabolic pathway (Norman *et al*. 1982, Zierold *et al*. 1994).

Adequate synthesis of vitamin D_3 and intake of dietary calcium are essential for skeletal health. 1,25$(OH)_2D_3$ mediates its calciotrophic actions primarily by stimulating intestinal absorption of calcium and phosphate to provide mineral for bone. The hormone has also been demonstrated to have effects on bone cells and promotes differentiation of both osteoblasts and osteoclasts (Tanaka & Seino 1997). It is generally accepted that the main actions of 1,25$(OH)_2D_3$ are receptor mediated and involve modulation of the transcription of target genes. The cloning of the vitamin D receptor (VDR) was reported in 1987 and subsequent studies have indicated that this receptor protein is a member of the nuclear receptor super-family, which share a common functional domain structure (Carlberg 1995). VDRs form dimer complexes with other nuclear receptors, preferentially retinoid X receptors (RXR), and bind to specific DNA sequences (vitamin D response elements, VDREs) which are located in the promotor region of primary responding genes. In addition to this pathway, the presence of another non-genomic pathway has been demonstrated in a number of tissues leading to rapid biological responses mediated by a putative membrane receptor (Nemere *et al*. 1998).

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VDR expression in mammary tissue and breast tumours

VDRs are present in normal breast and many other epithelial tissues (Berger *et al*. 1988). Studies in experimental animals have demonstrated that the VDR is dynamically regulated during pregnancy and lactation, but little is known about its specific functions. The VDR is expressed at low levels in mammary gland in virgin rats and is upregulated in response to the differentiation inducing hormones cortisol, prolactin and insulin (Mezzetti *et al*. 1987). Highest levels of VDR in mammary gland are seen during lactation, being maximal at 3 days post partum when the concentration of calcium in milk is highest (Colston *et al*. 1988). Addition of $1,25(OH)_2D_3$ to mammary gland explants increased VDR expression and enhanced calcium uptake (Mezzetti *et al*. 1988). Circulating concentrations of $1,25(OH)_2D_3$ are increased during pregnancy and lactation and recent studies have demonstrated the presence of 1α-hydroxylase in normal breast tissue (Friedrich *et al*. 2000). These studies suggest that $1,25(OH)_2D_3$ may play a role in differentiation and milk production by the mammary gland.

Further studies have indicated that vitamin D can protect against transformation of mammary cells. Animal studies have demonstrated that dietary vitamin D can abrogate the tumorigenic effects of a high fat diet on mammary tissue (Jacobson *et al*. 1989) and treatment with a vitamin D analogue can prevent the development of carcinogen-induced mammary tumours (Anzano *et al*. 1994). Furthermore, treatment with $1,25(OH)_2D_3$ prevented the development of pre-neoplastic lesions in mammary gland explants following treatment with the carcinogen 7,12-dimethylbenz(a)-anthracene (DMBA; Mehta *et al*. 1997). Finally, in the female VDR-knockout mouse, abnormalities in mammary gland terminal end bud development have been identified which could lead to an increased susceptibility to chemical carcinogens (Narvaez *et al*. 2001). Taken together, these findings suggest that $1,25(OH)_2D_3$ and its analogues may suppress tumorigenesis of normal mammary epithelial cells and that disruption of VDR regulated pathways may predispose to transformation.

Several studies have demonstrated that a high proportion of breast cancer biopsy specimens contains vitamin D receptors (Freake *et al*. 1984, Eisman *et al*. 1986, Berger *et al*. 1987). Furthermore, there appears to be an association between VDR levels and prognosis, as tumour receptor status may be positively related to disease-free survival (Colston *et al*. 1989, Berger *et al*. 1991). In addition, epidemiological studies have suggested an association between vitamin D deficiency and breast cancer risk (Janowsky *et al*. 1999) and disease activity (Mawer *et al*. 1997). More recently, a link between polymorphisms in the gene encoding the VDR and breast cancer risk has also been reported (Curran *et al*. 1999, Lundin *et al*. 1999, Ingles *et al*. 2000, Bretherton Watt *et al*. 2001).

$1,25(OH)_2D_3$ and its synthetic analogues inhibit growth of breast cancer cells

The anti-proliferative effect of $1,25(OH)_2D_3$ on cultured human cancer cells was first demonstrated in 1981 (Colston *et al*. 1981). These first experiments demonstrated that $1,25(OH)_2D_3$ at nanomolar concentrations inhibited the growth of human amelanotic melanoma cells in culture. At the same time it was shown that $1,25(OH)_2D_3$ could promote the differentiation of mouse cultured myeloid leukaemia cells (Abe *et al*. 1981). Over the past two decades many reports have confirmed that $1,25(OH)_2D_3$ can affect growth and differentiation of a wide variety of cancer cell types *in vitro*, including breast cancer cells (Feldman *et al*. 1997). Such findings have prompted considerable interest in the development of synthetic analogues with reduced calcaemic activity and which may have therapeutic potential in malignancy. A wide variety of analogues have been developed (Fig. 1), many of which display modification in the C-17 side chain of the molecule (Binderup *et al*. 1997, Mørk Hansen *et al*. 2001*a*). The most promising of these compounds display an improved activity profile, with enhanced cell regulatory effects relative to the native hormone but with weaker effects on calcium metabolism. Thus, these new analogues provide both a new class of agents with potential in the treatment and prevention of certain cancers as well as additional experimental tools with which to elucidate the mechanisms underlying the anti-cancer effects of $1,25(OH)_2D_3$. Many of the studies published to date have utilized established breast cancer cell lines as well as animal models of breast cancer to gain a clearer understanding of the signalling pathways involved.

Mechanisms associated with inhibitory effects of vitamin D compounds in breast cancer cells

Effects on cell cycle regulation compounds

A number of investigations have indicated that $1,25(OH)_2D_3$ and its analogues may induce inhibition of breast cancer cell growth by regulating cell cycle progression. Treatment of oestrogen receptor-positive MCF-7 breast cancer cells with $1,25(OH)_2D_3$ induces cell cycle arrest in G0/G1 (Simboli-Campbell *et al*. 1997, Wu *et al*. 1997). These effects are accompanied by alterations in the expression of important cell cycle regulators such as increases in cyclin-dependent kinase (cdk) inhibitors and dephosphorylation of the retinoblastoma protein (Fan & Yu 1995, Mørk Hansen *et al*. 2001*b*). Increases in p21[WAF-1/CIP1]
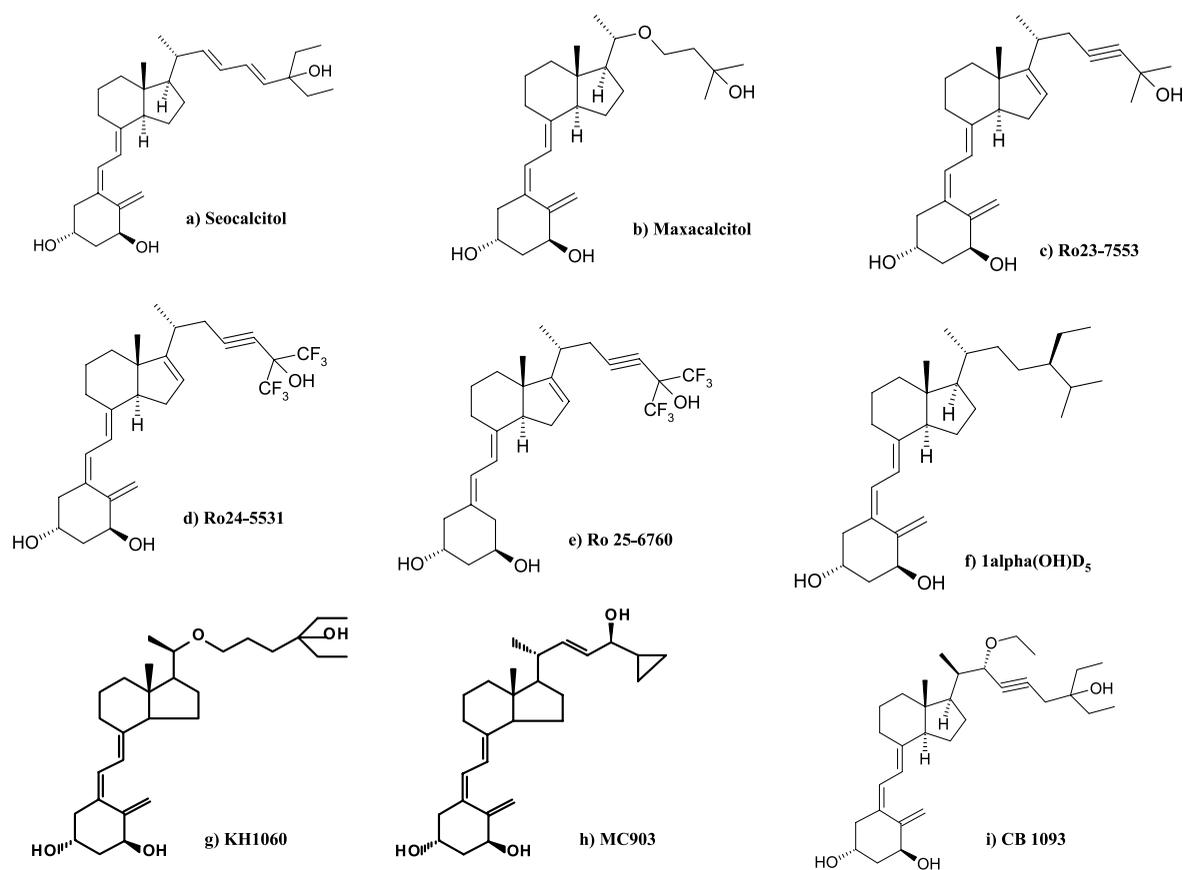
Figure 1 Structures of synthetic analogues of vitamin D with anti-tumour activity.

expression at the mRNA and protein level have been documented in association with G1 arrest mediated by $1,25(OH)_2D_3$ or certain of its synthetic analogues (James *et al.* 1996, Mørk Hansen *et al.* 2001*b*). A study in MCF-7 breast cancer cells treated with the vitamin D analogue EB1089 demonstrated a correlation between increased p21[WAF-1/CIP1] protein levels, inhibition of Cdk2-associated histone H1 kinase activity and G1 arrest (Wu *et al.* 1997). Effects of $1,25(OH)_2D_3$ on p27[kip1] appear to vary with cell type. Some studies have reported unchanged levels of p27[kip1] in human MCF-7 cells treated with $1,25(OH)_2D_3$ or its analogue EB1089 (Wu *et al.* 1997, Jensen *et al.* 2001, Mørk Hansen *et al.* 2001*b*) but another reported an increase in this cyclin-dependent kinase inhibitor (Verlinden *et al.* 1998). Increased p27[kip1] expression was also identified in response to treatment with the analogue EB1089 in BT20 and ZR-75-1 breast cancer cells (Wu *et al.* 1997). The effect of EB1089 on the regulation of growth of MCF-7 cells has been further studied at the level of expression of the c-*myc* and c-*fos* proto-oncogenes. Treatment of cells with this vitamin D analogue decreased the level of c-*myc* mRNA and transiently increased c-*fos* expression, being approximately 50 times more potent

than $1,25(OH)_2D_3$ (Mathiasen *et al.* 1993). The observation that $1,25(OH)_2D_3$ is able to regulate c-*myc* at the mRNA level is in accordance with the finding of a putative VDRE which has been reported in the human c-*myc* gene (Okano *et al.* 1999).

Many studies addressing the effects of vitamin D compounds on breast cancer cells have been undertaken in MCF-7 cells, which are oestrogen receptor (ER)-positive. However, $1,25(OH)_2D_3$ and its analogues also exert inhibitory effects on certain oestrogen-independent cell lines (Abe *et al.* 1991, Colston *et al.* 1998, Xie *et al.* 1999). Sensitivity to $1,25(OH)_2D_3$ is generally reported as being higher in breast cancer cells which express the oestrogen receptor than in those that do not (Narvaez *et al.* 2001). Effects of vitamin D compounds on oestrogen response pathways have been assessed by a number of laboratories. The analogue EB1089 has been shown to down-regulate the expression of ER in MCF-7 cells and to limit responsiveness to both the mitogenic effects of 17β-oestradiol and the induction by this steroid of the progesterone receptor protein and pS2 mRNA (James *et al.* 1994, Colston *et al.* 1995) (Fig. 2). $1,25(OH)_2D_3$ has been shown similarly to down-regulate ER levels and
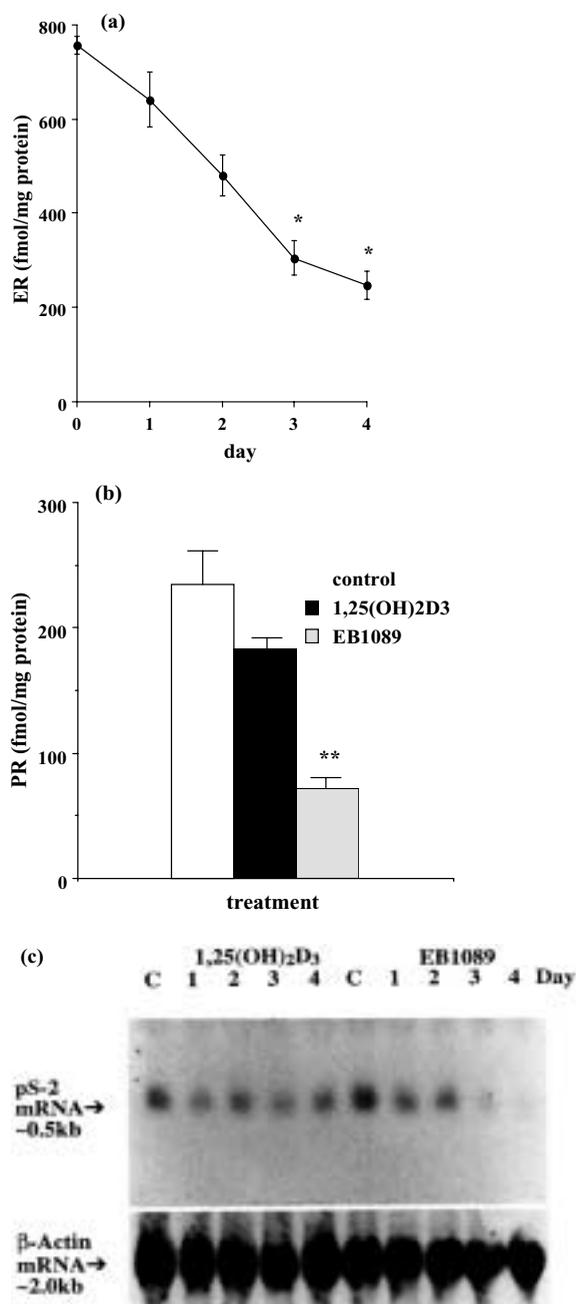
Figure 2 Vitamin D derivatives down-regulate oestrogen and progesterone receptor abundance and pS2 transcript levels in MCF-7 human breast cancer cells. (a) Regulation of oestrogen receptor (ER) expression by the vitamin D analogue EB1089 in MCF-7 cells. Cells were grown in phenol-red-free DMEM medium supplemented with 5% charcoal-stripped fetal calf serum and treated for 1–4 days with 10^{-8} M EB1089 or ethanol vehicle as control. Cell cytosols were prepared and ER content was quantitated by the Abbott ER-enzyme immunoassay (EIA) method (Abbott Laboratories, Chicago, IL, USA). Results are expressed as mean ER concentration (fmol/mg cytosol protein) ± S.E.M. *$P<0.05$, significantly different from control. (b) Effects of EB1089 and $1,25(OH)_2D_3$ on progesterone receptor protein (PR) expression. MCF-7 cells were treated for 4 days with ethanol vehicle or the vitamin D derivatives (both at 10^{-8} M). Cell cytosols were prepared and PR content was quantitated by the Abbott PR-EIA method. Results are expressed as mean PR concentration (fmol/mg cytosol protein) ± S.E.M. **$P<0.01$, significantly different from control. (c) Effects of EB1089 and $1,25(OH)_2D_3$ on pS2 transcript levels. MCF-7 cells were cultured in phenol-red-free DMEM with 5% charcoal-stripped serum in the presence of 10^{-8} EB1089, 10^{-8} M $1,25(OH)_2D_3$ or ethanol vehicle (C) for 1–4 days prior to extraction of total RNA and northern blot analysis. (Adapted from Colston *et al.* 1995.)

suppress oestrogen action in the MCF-7 cell line (Swami *et al*. 2000). Another study has indicated that 1,25(OH)$_2$D$_3$ inhibits oestrogen-induced transcription of the pS2 gene in the absence of a change in ER abundance (Demirpence *et al*. 1994). Thus, it has been suggested that vitamin D compounds may act at several points on the oestrogen response pathway, including having effects on both the abundance of ER protein and the ability of this receptor to act as a transcriptional activator. Recent sequence analysis of the ERα gene has demonstrated a potential VDRE within the promotor, which may point to a direct regulatory effect of 1,25(OH)$_2$D$_3$ on ER gene transcription (Stoica *et al*. 1999).

Interactions of the vitamin D analogue EB1089 and the pure anti-oestrogen ICI 182,780 (Wakeling *et al*. 1991) on the oestradiol-stimulated growth of MCF-7 cells have been investigated. Treatment of cell cultures with EB1089 in combination with ICI 182,780 and in the presence of 17β-oestradiol produced an augmented inhibition of proliferation compared with the actions of either compound alone (James *et al*. 1994). Cooperative effects of combined treatment with vitamin D analogues and tamoxifen have also been demonstrated both in MCF-7 and ZR-75-1 cells (Abe-Hashimoto *et al*. 1993, Vink-van Wijngaarden *et al*. 1994). However, breast cancer cells selected for anti-oestrogen resistance and those negative for ER retain sensitivity to 1,25(OH)$_2$D$_3$-mediated cell cycle arrest (Love-Schimenti *et al*. 1996, Welsh *et al*. 1998, Flanagan *et al*. 1999).

Induction of apoptosis by vitamin D derivatives

Cell growth requires both proliferation signals and survival signals. Tumour cells gain a growth advantage by abnormal proliferation and a defect in the regulation of cell death. The failure of cancer cells to undergo 'programmed' cell death (apoptosis) is a major determining factor in the development of many types of tumour. In addition to inhibitory effects on cell growth, 1,25(OH)$_2$D$_3$ and certain of its analogues have been shown to induce morphological and biochemical features of apoptosis in breast cancer cells (Welsh 1994, James *et al*. 1995, 1996, Simboli-Campbell *et al*. 1996, Narvaez & Welsh 1997, Mørk Hansen *et al*. 2001b). MCF-7 cells treated with 1,25(OH)$_2$D$_3$ exhibit cytoplasmic condensation and hyperchromatic, pycnotic nuclei resulting from chromatin condensation typical of apoptotic cells. Morphological assessment of MCF-7 cells treated with 1,25(OH)$_2$D$_3$ and tamoxifen in combination show enhanced induction of apoptosis (Welsh 1995). While the molecular mechanisms by which vitamin D derivatives may induce apoptosis in breast cancer cells are not fully understood, there is growing evidence for an involvement of the bcl-2 family of proteins. A decrease in the relative expression of anti-apoptotic (bcl-2/bcl-XL) to pro-apoptotic family members (bax, bak) has been reported in a number of systems in response to vitamin

D compounds (James *et al*. 1996, Danielsson *et al*. 1997, Simboli-Campbell *et al*. 1997, James *et al*. 1998). It has been reported that treatment of MCF-7 cells with 1,25(OH)$_2$D$_3$ leads to a redistribution of bax from the cytosol to the mitochondria (Narvaez & Welsh 2001) and forced expression of bcl-2 renders MCF-7 cells resistant to 1,25(OH)$_2$D$_3$ and its analogues (Mathiasen *et al*. 1999). These studies suggest that sensitivity to vitamin D-mediated apoptosis may be determined by the relative expression or subcellular distribution of pro- and anti-apoptotic members of the bcl-2 family. Cytochrome c release with a concomitant decrease in mitochondrial membrane potential has recently been shown to take place in response to vitamin D-mediated apoptosis, but the relationship between this observation and changes in the bcl-2 family of proteins is poorly understood (Narvaez & Welsh 2001). While cytochrome c release from mitochondria to cytosol is associated with caspase activation in a number of systems, recent *in vitro* studies have indicated that, in MCF-7 cells, vitamin D-mediated apoptosis is not dependent on the activation of any known caspase (Mathiasen *et al*. 1999, Narvaez & Welsh 2001, Pirianov & Colston 2001a). In addition, induction of apoptosis by vitamin D derivatives appears to be independent of the mutational status of the p53 tumour suppressor gene. 1,25(OH)$_2$D$_3$ and its analogues are capable of inducing apoptosis in T47-D breast cancer cells (Mathiasen *et al*. 1999) which possess a mutated p53 gene (Bartek *et al*. 1990). Upregulation of apoptotic related proteins such as clusterin, cathepsin B and transforming growth factor β (TGFβ) has been reported in MCF-7 cells undergoing apoptosis in response to 1,25(OH)$_2$D$_3$ and its analogues (Colston *et al*. 1995, James *et al*. 1996, Simboli-Campbell *et al*. 1996, 1997). In addition, 1,25(OH)$_2$D$_3$ treatment has been shown to enhance sensitivity of breast cancer cells to a number of anti-cancer drugs and a number of other agents, indicating that vitamin D compounds may be useful in combination with conventional chemotherapy (Table 1). Thus, vitamin D compounds have been demonstrated to potentiate apoptosis induced by adriamycin and taxol as well as by radiation (Ravid *et al*. 1999, Sundaram *et al*. 2000, Wang *et al*. 2000). These findings suggest that cross talk between distinct apoptosis pathways might exist. This point has been addressed by identification of potential cross talk between the tumour necrosis factor α (TNFα) and vitamin D systems. TNFα induces apoptosis in MCF-7 breast cancer cells by a well-defined pathway that is triggered by activation of TNF-R1, a cell surface 'death receptor' whose signalling is linked to activation of caspases. Pretreatment of MCF-7 cells with 1,25(OH)$_2$D$_3$ or active vitamin D analogues potentiates the effects of TNFα on induction of apoptosis (Rocker *et al*. 1994, Pirianov *et al*. 1999, Mathiasen *et al*. 2001). Pirianov and associates provided evidence that this potentiation could be attributable to enhanced accumulation of ceramide and cytosolic phospholipase A2 (cPLA2) activation but was independent of changes in TNF-R1 or TNFα

Table 1 Summary of studies showing enhancement by vitamin D compounds of effects of anticancer agents in breast cancer cells.

Anticancer agent	Vitamin D analogue	Cell line	Reference
Antioestrogen			
Tamoxifen	EB1089, KH1060	MCF-7, ZR-75-1	Vink-van Wijngaarden *et al.* (1994)
	OCT	MCF-7, ZR-75-1	Abe-Hashimoto *et al.* (1993)
	1,25-D	MCF-7	Demirpence *et al.* (1994)
IC182,780	EB1089	MCF-7	James *et al.* (1994)
	1,25-D	MCF-7	Nolan *et al.* (1998)
IC164,384	1,25-D, EB1089	BT-474, MCF-7, MDA-MB-453	Love-Schimenti *et al.* (1996)
Retinoids	EB1089	MCF-7	James *et al.* (1995)
	1,25-D	T47-D	Koga & Sutherland (1991)
Doxorubicin	1,25-D	MCF-7	Ravid *et al.* (1999)
(Adriamycin)	EB1089	MCF-7	Sundaram *et al.* (2000)
	ILX-23-7553	MCF-7	Chaudhry *et al.* (2001)
	EB1089 sulphone	MCF-7	Posner *et al.* (2001)
Taxol	1,25-D	MCF-7, MDA-MB-231, T47-D	Wang *et al.* (2000)
Carbo/cisplatin	1,25-D	MCF-7	Cho *et al.* (1991)
Cytokines			
IL-6	1,25-D	MCF-7	Koren *et al.* (2000)
TNFα	CB1093	MCF-7	Pirianov *et al.* (1999)
	CB1093	MCF-7, Hs578t, T47-D	Pirianov & Colston (2001*a*)
	1,25-D	MCF-7	Rocker *et al.* (1994)
	1,25-D	MCF-7, T47-D	Mathiasen *et al.* (2001)
Ceramide	CB1093	MCF-7	Pirianov *et al.* (1999)
	CB1093	MCF-7, Hs578t, T47-D	Pirianov & Colston (2001*a*)
Radiation	EB1089	MCF-7	Sundaram & Gewirtz (1999)
	ILX-23-7553	MCF-7	Chaudry *et al.* (2001)

OCT, maxacalcitol; 1,25-D, 1,25(OH)$_2$D$_3$; IL-6, interleukin 6.

expression (Pirianov *et al.* 1999, Pirianov & Colston 2001*a*). Mathiasen and associates (2001) similarly demonstrated enhanced induction of apoptosis in MCF-7 cells in response to 1,25(OH)$_2$D$_3$ and TNFα combinations, but found that this treatment led to increased surface expression of TNF-R1, enhanced TNFα-induced nuclear factor-κB (NF-κB) activation and increased release of lysosomal cathepsin B. Both laboratories have reported that T47-D human breast cancer cells are resistant to TNFα-mediated apoptosis. Mathiasen *et al.* (2001) demonstrated that 1,25(OH)$_2$D$_3$ enhanced TNFα-induced NF-κB activation in T47-D cells, suggesting a potentiation of this aspect of the TNFα pathway. Another study has provided evidence that resistance of T47-D cells to TNFα-mediated apoptosis is associated with impaired activation of cPLA2 (Pirianov & Colston 2001*a*). However, these cells retain their capacity to undergo apoptosis and cPLA2 activation in response to exogenous ceramide, suggesting a block in TNFα signalling at the level of the sphingomyelin pathway and ceramide generation. Interestingly, pre-treatment of T47-D cells with the vitamin D analogue CB1093 potentiated DNA fragmentation and cPLA2 activation in response to exogenous ceramide. Taken together, these various studies indicate cross talk between the vitamin D and TNFα pathways in breast cancer cells.

Modulation of growth factor signalling

An additional mechanism by which vitamin D derivatives may influence breast cancer cell growth and viability is through modulation of growth factor signalling. It is well documented that breast cancer cells both elaborate and respond to a variety of paracrine/autocrine growth factors. The vitamin D analogue EB1089 can reverse the growth stimulatory effects of epidermal growth factor (EGF; Saez *et al*. 1994) and regulation of EGF receptor levels by 1,25(OH)$_2$D$_3$ has been demonstrated (Koga *et al*. 1988, Desprez *et al*. 1991). In addition, it has recently been reported that the gene encoding amphiregulin, a heparin-binding EGF-related growth factor, is transcriptionally regulated by 1,25(OH)$_2$D$_3$ (Akutsu *et al*. 2001). In most epithelial cells, including breast cancer cells, TGFβ has been shown to be a negative growth regulator and thus increased TGFβ activity is expected to decrease breast cancer cell growth. It is thus of interest that 1,25(OH)$_2$D$_3$ has been shown to enhance the expression of TGFβ1 and its latent form binding protein in cultured breast cancer cells (Koli & Keski-Oja *et al*. 1994). Further, *in vitro* studies using both human BT20 or MCF-7 breast cancer cells have demonstrated a dose-dependent increase in TGFβ1 mRNA and TGFβ protein secreted into the medium in

response to treatment of cells with $1,25(OH)_2D_3$ or EB1089, with the analogue being more potent (Mercier *et al.* 1996). These effects were abrogated by addition of neutralizing antibodies to TGFβ, suggesting that the anti-proliferative effect of vitamin D compounds could, in part, be mediated by increased expression of this growth inhibitory peptide (Mercier *et al.* 1996, Verlinen *et al.* 1998, Yang *et al.* 2001).

Insulin-like growth factor I (IGF-I) is a potent mitogen and survival factor for many cell types, including normal breast epithelium and breast cancer cells (Pollak 1998). IGF-I is known to contribute to the loss of growth regulation by inhibiting cell death in tumour cells. Furthermore, high plasma IGF-I levels are associated with increased risk of breast cancer in premenopausal women (Hankinson *et al.* 1998) and the IGF-I receptor is overexpressed in many breast cancer cell lines (Papa *et al.* 1993). The influence of IGF-I on cell survival *in vivo* is determined both by the extracellular concentration of the growth factor and the levels of several IGF binding proteins (IGFBPs) which modulate the availability of the free cytokine for interaction with its membrane receptor (IGF-IR). Evidence suggests that the wild-type IGF-IR and/or its ligands have a widespread anti-apoptotic effect against many signals including serum and growth factor deprivation (Dews *et al.* 1997). In addition, IGF-I has been shown to be an effective inhibitor of apoptosis induced by diverse chemotherapeutic agents and tamoxifen (Dunn *et al.* 1997).

Interestingly, studies with a number of breast cancer cell lines have indicated that vitamin D compounds are able to block the mitogenic effects of IGF-I. This effect is accompanied by a decrease in proliferation and an increase in apoptosis (Vink-van Wijngaarden 1996, Xie *et al.* 1997, 1999, Pirianov & Colston 2001*b*). It remains to be determined whether the attenuation by vitamin D compounds of IGF-I effects in breast cancer cells are mediated by decreased expression of the IGF-IR, an accumulation of inhibitory IGFBPs or modulation of down stream effectors of IGF-I signalling. Studies with MCF-7 cells maintained under serum-free conditions and treated with $1,25(OH)_2D_3$ or EB1089 showed no direct effect on IGF-I binding (Vink-van Wijngaarden *et al.* 1996). In contrast, our own studies demonstrated a decrease in IGF-IR expression in response to vitamin D treatment by the techniques of both ligand binding assay and immunoblotting (Xie *et al.* 1997) (Fig. 3, left panel). However, we have observed that decreased expression of IGF-IR in response to vitamin D analogues is a late event and is not detected at 4 days of treatment, at which time we have observed inhibition of IGF-I effects in cultures co-treated with vitamin D analogues (Xie *et al.* 1999). The contribution of changes in expression of IGFBPs to the inhibitory effects of vitamin D compounds on IGF-I actions is still unclear. Rozen and colleagues have suggested a role for IGFBP-5 in the modulation of IGF-I signalling by the

analogue EB1089 in MCF-7 cells, as they observed enhanced expression of IGFBP-5 mRNA as well as increased accumulation of IGFBP-5 in culture (Rozen *et al.* 1997, Rozen & Pollak 1999). This group also reported that the vitamin D compounds were unable to inhibit the mitogenic activity of long R3 IGF-I, an IGF-I analogue with greatly reduced affinity for IGFBPs but similar affinity for IGF-I receptors (Nickerson *et al.* 1997). Our own findings have indicated that vitamin D compounds increased expression of IGFBP-3 in MCF-7 cells and also in Hs578t breast cancer cells, which are not growth stimulated by IGF-I (Colston *et al.* 1998). We addressed the ability of vitamin D analogues to abrogate the anti-apoptotic effects of IGF-I by co-incubation of MCF-7 cells maintained in serum-free medium with IGF-I. We observed that the analogue CB1093 prevented the anti-apoptotic effects of IGF-I as assessed by cell viability and DNA fragmentation assays (Pirianov & Colston 2001*b*). In contrast to the report from Rozen and Pollak (1999), we found that the stimulatory effect of long R3 IGF-I was also attenuated by co-treatment with vitamin D analogues (Fig. 3, right panel). This suggests that the ability of these compounds to prevent the anti-apoptotic effects of IGF-I is not predominantly mediated via increased expression of inhibitory IGF binding proteins. Further studies are needed to determine if vitamin D compounds can directly modulate down stream effectors of IGF-I signalling.

Invasion and metastasis

Metastatic spread constitutes the major clinical problem of breast cancer patients. Invasion and metastasis involve complex processes by which tumour cells detach, degrade extracellular matrix and disseminate to form secondary deposits at distant sites. The highly metastatic ER-negative human breast cancer cell line, MDA-MB-231, has previously been used to evaluate these aspects of cancer cell biology. These cells have been shown to be poorly responsive to the growth inhibitory actions of $1,25(OH)_2D_3$ and its analogues when grown in monolayer culture (Koike *et al.* 1997, Koli & Keski-Oja 2000). However, using a well established assay of tumour cell invasion, Mørk Hansen and colleagues (1994) demonstrated that $1,25(OH)_2D_3$ and two of its analogues were capable of inhibiting this aspect of cell activity. Furthermore, reduced invasiveness of these cells in response to vitamin D has been shown to be associated with diminished activity of the metalloproteinase MMP-9 and the two serine proteases urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). These effects were associated with an increase in PA inhibitor 1 and the MMP inhibitor 1 (Koli & Keski-Oja 2000).

In addition, the anti-angiogenic activity of $1,25(OH)_2D_3$ may contribute to its anti-invasive and anti-metastatic actions. In the chick embryo chorioallantoic membrane assay,
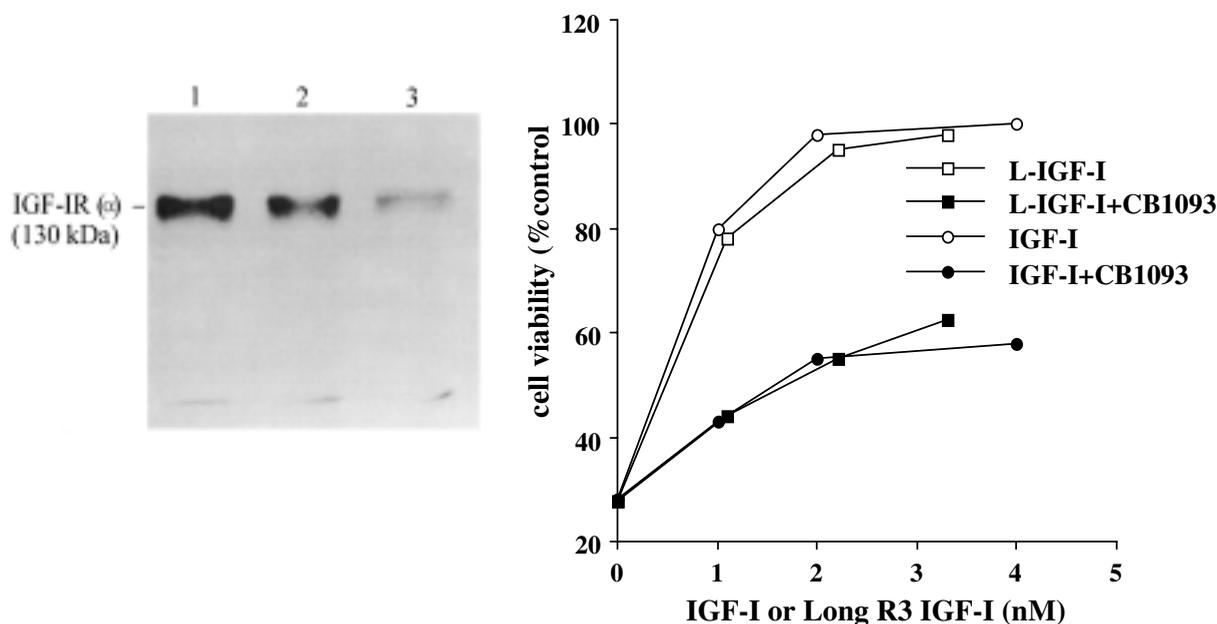
Figure 3 Anti-IGF-I effects of vitamin D analogues. (Left panel) Dose-dependent inhibition of IGF-IR expression by the vitamin D analogue EB1089 in MCF-7 cells. Cells were treated for 6 days with ethanol vehicle (lane 1) or EB1089 (10^{-9} and 10^{-8} M, lanes 2 and 3). Lysates were prepared from these cultures and fractionated by SDS-PAGE. Immunoblotting was performed using a rabbit polyclonal antibody against the α subunit of IGF-IR. (Right panel) Effects of recombinant human (rh) IGF-I and long R3 IGF-I (L-IGF-I) on MCF-7 cell growth in serum-free medium. MCF-7 cells (2×10^4/well) were seeded into 24-well plates in growth medium and allowed to adhere overnight. Cell layers were extensively washed and cultures incubated in RPMI 1640 medium supplemented with 1% FCS or in serum-free medium. Cells were treated for 4 days with increasing concentrations of rhIGF-I or long R3 IGF-I in the presence or absence of 10^{-7} M CB1093. Cell viability was assessed by neutral red assay (Rocker *et al.* 1996). Results are expressed as a percentage of untreated control cultures maintained in medium supplemented with 1% FCS.

$1,25(OH)_2D_3$ concentrations in the picomolar range have been shown to inhibit angiogenesis (Oikawa *et al*. 1990). These studies are now supported by several *in vivo* studies (Majewski *et al*. 1993, 1996, Iseki *et al*. 1999).

Development of synthetic analogues: preclinical and clinical trials

The potentially toxic effect of conventional vitamin D metabolites with regard to calcium handling prompted the development of synthetic vitamin D analogues. Thus, while beneficial effects of $1,25(OH)_2D_3$ on cancer cells have been supported by results obtained with 1α-hydroxy D_3, which is converted to $1,25(OH)_2D_3$ *in vivo*, the therapeutic window is extremely narrow (reviewed in Mørk Hansen *et al*. 2000). A number of pharmaceutical companies have addressed this problem by investigating whether structural modification of the parent hormone can produce compounds which display separation of the growth regulating effects from calcium mobilizing actions. Several hundred new vitamin D analogues have been designed and tested and many display modification of the parent molecule at the C-17 side chain structure whilst

keeping the 1α-hydroxylated A ring and *cis* triene the same as in the $1,25(OH)_2D_3$ molecule (Jones & Calverly 1993). Initially, compounds with modifications at carbons 22, 23 and 24 were designed, as binding to the vitamin D receptor is not substantially altered by this change, while metabolic degradation as well as binding to the vitamin D serum transport protein, vitamin D binding protein (DBP), are both reduced. Thus introduction of double or triple bonds, fluoro groups and/or aromatic rings into the side chain have produced compounds with improved activity profile. In addition, epimerization at C-20 in the side chain or introduction of a double bond at position C-16 in the D ring are approaches that have been shown to improve differentiation of the two classes of activity (Bouillon *et al*. 1995, Binderup *et al*. 1997, Yamada *et al*. 2000). Initially, the effectiveness of these various analogues has been evaluated using *in vitro* methods. The human leukaemic cell lines HL-60 and U937 have been extensively utilized to assess the effects of conventional vitamin D metabolites and synthetic analogues on cell growth and differentiation (DeLuca & Ostrem 1988). A large number of these compounds have also been assessed for their effects on breast cancer cell growth and apoptosis.

Calcipotriol (MC903)

The compound MC903 (calcipotriol; Leo Pharmaceutical Products, Ballerup, Denmark) contains a cyclopropyl substitution in the side chain (Binderup & Bramm 1988). This analogue appears to be equipotent with $1,25(OH)_2D_3$ in inhibiting the growth of MCF-7 breast cancer cells *in vitro* (Colston *et al*. 1992*a*), but displays calcaemic activity 100–200 times less than the natural hormone. This profile of activity was subsequently found to be largely due to rapid inactivation of the analogue *in vivo* and subsequently this compound has been developed and marketed for topical treatment of psoriasis (Binderup & Kragballe 1992, Fogh & Kragballe 2000). With regard to breast cancer, the efficacy of topical treatment of cutaneous nodules with calcipotriol has been investigated (Bower *et al*. 1991). In this study, 19 women with locally advanced or cutaneous metastatic disease were treated topically with calcipotriol ointment. Of 14 patients who completed treatment, 3 showed partial response and 1 a minimal response.

Maxacalcitol (OCT)

22-Oxa-$1\alpha,25(OH)_2D_3$ (Chugai Pharmaceutical Co. Ltd, Tokyo, Japan), in which an oxygen atom is substituted for the methyl group at C-22, displays reduced calcaemic activity *in vivo* and has been extensively studied with regard to its effects on cancer cell growth *in vitro* and in animal models (Kubodera *et al*. 1997). Abe and associates (1991) have documented potent anti-proliferative effects of OCT in ER-positive and -negative cell lines. In animal models, OCT has been shown to retard the growth of breast tumour xenografts developed in athymic mice from ER-positive MCF-7 cells and the ER-negative tumour MX-1 (Abe *et al*. 1991). Using the xenograft model, these workers also observed a synergistic anti-tumour effect of submaximal doses of OCT and tamoxifen (Abe-Hashimoto *et al*. 1993). Furthermore, OCT has also been demonstrated to exert a growth inhibitory effect in DMBA-induced rat mammary tumours, alone and in combination with the aromatase inhibitor CGS 16949A (Andoh & Iino 1996).

Seocalcitol (EB1089)

Seocalcitol (EB1089), a second generation analogue from Leo Pharmaceutical Products, contains a conjugated double bond system and is approximately 50 times more potent than $1,25(OH)_2D_3$ *in vitro*, while the actions of EB1089 on calcium metabolism *in vivo* are markedly reduced (Colston *et al*. 1992*b*, Mathiasen *et al*. 1993). Studies in our laboratory have demonstrated that oral administration of this compound to rats bearing nitrosomethyl urea (NMU)-induced mammary tumours leads to a dose-dependent inhibition of tumour progression. No significant increase in serum calcium concentration was seen at the lower doses tested (Colston *et al*.

1992*b*, Mackay *et al*. 1996). These findings have also been supported by subsequent studies with both DMBA-induced mammary tumours and MCF-7 xenograft models (Saez *et al*. 1994, VanWeelden *et al*. 1998). In both the NMU-induced tumour model and MCF-7 cell xenografts, treatment of animals with EB1089 led to tumour regression and evidence in tumour tissue of DNA fragmentation indicative of apoptotic cell death (James *et al*. 1998, VanWeelden *et al*. 1998). Furthermore, the beneficial effects of EB1089 on tumour progression in the MCF-7 xenograft model can be enhanced by combination of the analogue with paclitaxel (Koshizuka *et al*. 1999*a*) or retinoic acid (Koshizuka *et al*. 1999*b*).

Breast cancer metastasizes frequently to bone, and a well established animal model that has been utilized to test potential therapeutic agents is the development of bone metastases following intracardiac inoculation of athymic mice with MDA-MB-231 cells. Using this model, it has recently been shown that administration of EB1089 results in a marked increase in survival and an inhibition of development of bone metastases without development of hypercalcaemia (El Abdaimi *et al*. 2000).

The safety of EB1089 has been evaluated by a dose finding study in 13 healthy volunteers who received the analogue orally for 4 consecutive days. This study indicated that doses in the range of 5 to 20 µg/day could be considered for future use in clinical trials. A phase I trial of oral EB1089 in patients with advanced breast and colorectal cancer has been completed. This trial was an open, non-controlled single-centre study with sequentially assigned dose levels (Gulliford *et al*. 1998). Twenty-five females had breast cancer and four females and seven males had colorectal carcinoma. Patients received the analogue twice daily for 5 days with a 3-week post-dosing follow-up. Twenty patients received compassionate treatment after this post-dosing interval for between 10 and 234 days (mean 90±62 days). On the basis of this study, the estimated maximum tolerated dose (MTD) was determined to be 7 µg/m² per day for prolonged use. Ten patients developed hypercalcaemia, which resolved within 7 days of stopping treatment and no other serious adverse reactions were observed. Although no clear anti-tumour effects were seen in this study, 6 patients (2 colorectal, 4 breast cancer) showed disease stabilization for at least 3 months.

Additional clinical trials are currently under way to evaluate the efficacy of oral administration of EB1089 in various malignant conditions. A phase I/II trial involving patients with non-resectable pancreatic carcinoma has been undertaken as well as evaluation of the efficacy and safety of EB1089 in patients with advanced hepatocellular carcinoma. These studies indicate that EB1089 is well tolerated within a dose range of 5 to 25 µg/day, with dose limiting hypercalcaemia as the only consistently reported adverse

effect. This was found to be reversible with cessation of treatment. Furthermore, preliminary data suggest that a small number of patients with hepatocellular carcinoma have shown reduction in tumour size (Evans *et al.* 2000).

16-ene analogues

The 16-ene vitamin D analogues are characterized by the introduction of a double bond at the C-16 position in the D ring of the molecule (Uskokovic *et al.* 1997). Ro-23-7553 (ILX-23-7553, Hoffmann-LaRoche Ltd, Nutley, NJ, USA) is 1α,25-dihydroxy-16-ene-23-yne-cholecalciferol and this compound shows increased potency compared with $1,25(OH)_2D_3$ in regulating cell growth and has been tested in animal models of prostatic carcinoma with encouraging results (Schwartz *et al.* 1995). This analogue is currently in phase I trials in patients with advanced metastatic cancer. A related analogue, 1α,25-dihydroxy-16-ene-23-yne-26-27-hexafluorocholecalciferol (Ro-24-5531) has been evaluated for its efficacy in preventing the development of NMU-induced rat mammary tumours (Anzano *et al.* 1994).

Other vitamin D analogues with antitumour effects in breast cancer models

The Hoffmann-LaRoche compound Ro-25-6760 is a 19-nor-hexafluoride analogue that has been demonstrated to suppress growth of human breast cancer cells *in vitro* (Koike *et al.* 1997) and to inhibit tumour progression in nude mice bearing MCF-7 xenografts. Enhanced effects on tumour progression were reported in combination with paclitaxel (Koshizuka *et al.* 1999*a*). Other 19-nor analogues have recently been described that also demonstrate anti-cancer effects in human breast cancer cells *in vitro* and *in vivo* (Verlinden *et al.* 2000). In addition, 1α-hydroxyvitamin D_5 was reported to prevent the development of mammary tumours in rats treated with NMU (Mehta *et al.* 2000). Elstner and colleagues (1995) have demonstrated inhibitory effects on growth of cultured breast cancer cells of a series of novel 20-epi vitamin D analogues. We have evaluated the efficacy of CB1093 (20-epi(S)-ethoxy-23-yne-24a,26a,27a-trihomo 1α, 25-dihydroxyvitamin D_3) on the progression of established NMU-induced rat mammary tumours and demonstrated that oral administration of this compound leads to significant

Table 2 *In vivo* effects of vitamin D analogues in animal models of breast cancer: summary of studies.

Vitamin D analogue	Animal model of mammary cancer	Reference
Single agent		
1αOHD3	rat/NMU-induced	Colston *et al.* (1992*a*)
1αOHD3	rat/DMBA-induced	Iino *et al.* (1994)
OCT	mouse/MCF-7 + MX-1 xenograft	Abe-Hashimoto *et al.* (1993)
OCT	rat/DMBA-induced	Andoh & Iino (1996)
OCT	rat/DMBA-induced	Oikawa *et al.* (1991)
EB1089	rat/NMU-induced	Colston *et al.* (1992*b*)
EB1089	mouse/Sum159T cells	Flanagan *et al.* (1997)
EB1089	mouse/MCF-7 xenografts	VanWeelden *et al.* (1998)
EB1089	rat/DMBA-induced	Saez *et al.* (1994)
CB1093	rat/NMU-induced	Danielsson *et al.* (1997)
EB1089	mouse/MDA/MB-231 bone mets	El Abdaimi *et al.* (2000)
1αOHD5	rat/NMU-induced	Mehta *et al.* (1997)
Ro-25-6760	mouse/MCF-7 xenograft	Koshizuka *et al.* (1999*a*)
Ro-24-5331	rat/NMU-induced	Anzano *et al.* (1994)
TX 522	mouse/MCF-7 xenograft	Verlinden *et al.* (2000)
TX 527	mouse/MCF-7 xenograft	Verlinden *et al.* (2000)
Combination treatment		
CB1093 + taxol	mouse/MCF-7 xenografts	Koshizuka *et al.* (1998)
CB1093 + cisplatin	mouse/MCF-7 xenografts	Koshizuka *et al.* (1998)
EB1089 + tamoxifen	rat/NMU-induced	Mackay *et al.* (1996)
EB1089 + ICI 182,780	mouse/MCF-7 xenograft	Packman *et al.* (2000)
OCT + tamoxifen	mouse/MCF-7 xenograft	Abe-Hashimoto *et al.* (1993)
OCT + aromatase inhibitor	rat/DMBA-induced	Andoh & Iino (1996)
EB1089 + ATRA	mouse/MCF-7 xenograft	Koshizuka *et al.* (1999*b*)
Ro-25-6760 + ATRA	mouse/MCF-7 xenograft	Koshizuka *et al.* (1999*b*)
Ro-25-6760 + taxol	mouse/MCF-7 xenograft	Koshizuka *et al.* (1999*a*)
EB1089 + taxol	mouse/MCF-7 xenograft	Koshizuka *et al.* (1999*a*)
Ro-24-5331	rat/NMU-induced	Anzano *et al.* (1994)

OCT, maxacalcitol; mets, metastases; 1αOHD5, 1α-hydroxyvitamin D_5; ATRA, all-*trans* retinoic acid.

tumour regression (Danielsson *et al.* 1997). In addition, CB1093 has been shown to inhibit growth of MCF-7 xenografts when combined with paclitaxel and cisplatin (Koshizuka *et al.* 1998). Table 2 summarizes studies to date on effects of vitamin D analogues in animal models of breast cancer.

Future prospects

The expanding body of research with new synthetic analogues of vitamin D has demonstrated the possibility of developing compounds with differentiation of calcaemic from cell regulatory effects. This research also indicates a wider role than formerly recognized for the vitamin D endocrine system in the control of mammary epithelial cell proliferation, differentiation and apoptosis. The discovery that the VDR is detectable in cancer cells of both epithelial and haemopoietic origin and that $1,25(OH)_2D_3$ and its analogues display the ability to affect a number of processes known to be involved in tumorigenesis establishes these compounds as potential agents in cancer treatment and prevention. Of major importance in recognizing this goal is the development of analogues with selective biological profiles, and to this end an improved understanding of the mechanisms implicated in the growth modulating actions of $1,25(OH)_2D_3$ and its analogues is needed. An increasing number of studies has indicated that an important aspect of the anti-tumour effects of vitamin D analogues in breast cancer cells is activation of the cell death pathway. Further characterization of the apoptosis-related genes that are directly or indirectly regulated by vitamin D derivatives may provide a basis for the design of new compounds that can target these pathways in breast cancer cells. A greater understanding of how the apoptotic pathway mediated by vitamin D may modulate or overlap with more established pathways leading to cell death is likely to provide clinically useful information. Studies addressing this question will suggest new ways to optimize the apoptotic response of breast cancer cells by combinations of vitamin D analogues with conventional cytotoxic agents.

References

Abe E, Miyaura C, Sakagami H, Takeda M, Konno K, Yamazaki T, Yoshiki S & Suda T 1981 Differentiation of mouse myeloid leukaemia cells induced by 1,25-dihydroxyvitamin D_3. *PNAS* **78** 4990–4995.

Abe J, Nakano T, Nishii Y, Matsumoto T, Ogata E & Ikeda K 1991 A novel vitamin D_3 analog, 22-oxa-1,25-dihydroxyvitamin D_3, inhibits the growth of human breast cancer *in vitro* and *in vivo* without causing hypercalcemia. *Endocrinology* **129** 832–837.

Abe-Hashimoto J, Kikuchi T, Matsumoto T, Nishii Y, Ogata E & Ikeda K 1993 Anti-tumor effect of 22-oxa-calcitriol, a non-calcaemic analog of calcitriol in athymic mice implanted

with human breast carcinoma and its synergism with tamoxifen. *Cancer Research* **53** 2534–2537.

Akutsu N, Bastien Y, Lin R, Mader S & White JH 2001 Amphiregulin is a vitamin D_3 target gene in squamous cell and breast carcinoma. *Biochemical and Biophysical Research Communications* **281** 1051–1056.

Andoh T & Iino Y 1996 Usefulness of 22-oxa-1,25-dihydroxyvitamin D_3 (OCT) as a single agent or combined therapy with aromatase inhibitor (CGS 16949A) on 7,12-dimethylbenz[a]anthracene-induced rat mammary tumors. *International Journal of Oncology* **9** 79–82.

Anzano MA, Smith JM, Uskokovic MR, Peer CW, Mullen LT, Letterio JJ, Welsh MC, Shrader MW, Logsdon DL, Driver CL, Brown CC, Roberts AB & Sporn MB 1994 1α-Dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol (Ro24-5531), a new deltanoid (vitamin D analogue) for prevention of breast cancer in the rat. *Cancer Research* **54** 1653–1656.

Bartek J, Iggo R, Gannon J & Lane DP 1990 Genetic and immunochemical analysis of mutant p53 in human breast cancer cell lines. *Oncogene* **5** 893–899.

Berger U, Wilson P, McClelland R, Colston K, Haussler MR, Pike JW & Coombes RC 1987 Immunocytochemical detection of 1,25-dihydroxyvitamin D_3 receptor in primary breast cancer. *Cancer Research* **47** 6793–6795.

Berger U, Wilson P, McClelland RA, Colston K, Haussler MR, Pike JW & Coombes RC 1988 Immunocytochemical detection of 1,25-dihydroxyvitamin D_3 receptor in normal human tissues. *Journal of Clinical Endocrinology and Metabolism* **67** 607–613.

Berger U, McClelland RA, Wilson P, Greene GL, Haussler MR, Pike JW, Colston K, Easton D & Coombes RC 1991 Immunocytochemical detection of oestrogen receptor, progesterone receptor and 1,25-dihydroxyvitamin D_3 receptor in breast cancer and relation to prognosis. *Cancer Research* **51** 239–244.

Binderup L & Bramm E 1988 Effects of a novel vitamin D analogue MC903 on cell proliferation and differentiation *in vitro* and on calcium metabolism *in vivo*. *Biochemical Pharmacology* **37** 887–895.

Binderup L & Kragballe K 1992 Origin of the use of calcipotriol in psoriasis treatment. *Reviews in Contemporary Pharmacotherapeutics* **3** 357–365.

Binderup L, Binderup E & Godtfredsen WO 1997 Development of new vitamin D analogs. In *Vitamin D*, pp 1027–1043. Eds D Feldman, FH Glorieux & JW Pike. New York: Academic Press.

Bouillon R, Okamura WH & Norman AW 1995 Structure–function relationships in the vitamin D endocrine system. *Endocrine Reviews* **16** 200–257.

Bower M, Colston KW, Stein RC, Hedley A, Gazet J-C, Ford HT & Coombes RC 1991 Topical calcipotriol treatment in advanced breast cancer. *Lancet* **337** 701–702.

Bretherton-Watt D, Given-Wilson R, Mansi JL, Thomas V, Carter N & Colston KW 2001 Vitamin D receptor gene polymorphisms are associated with breast cancer risk in a UK Caucasian population. *British Journal of Cancer* **85** 171–176.

Carlberg C 1995 Mechanisms of nuclear signalling by vitamin D_3. Interplay with retinoid and thyroid hormone signalling. *European Journal of Biochemistry* **231** 517–527.

Chaudhry M, Sundaram S, Gennings C, Carter H & Gewirtz DA 2001 The vitamin D analog ILX-23-7553 enhances the response to adriamycin and irradiation in MCF-7 breast tumor cells. *Cancer Chemotherapy and Pharmacology* **47** 429–436.

Cho YL, Christensen C, Saunders DE, Lawrence WD, Deppe G, Malviya VK & Malone JM 1991 Combined effects of 1,25-dihydroxyvitamin D_3 and platinum drugs on the growth of MCF-7 cells. *Cancer Research* **51** 2848–2853.

Colston KW, Colston MJ & Feldman D 1981 1,25-dihydroxyvitamin D_3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. *Endocrinology* **108** 1083–1086.

Colston K, Berger U, Wilson P, Hadcocks L, Naeem I, Earl H & Coombes R 1988 Mammary gland 1,25-dihydroxyvitamin D_3 receptor content during pregnancy and lactation. *Molecular and Cellular Endocrinology* **60** 15–22.

Colston KW, Berger U & Coombes RC 1989 Possible role for vitamin D in controlling breast cancer cell proliferation. *Lancet* **1** 185–191.

Colston KW, Chander SK, Mackay AG & Coombes RC 1992*a* Effects of synthetic vitamin D analogues in breast cancer cell proliferation *in vivo* and *in vitro*. *Biochemical Pharmacology* **44** 1153–1155.

Colston KW, Mackay AG, James SY, Binderup L, Chander S & Coombes RC 1992*b* EB1089: a new vitamin D analogue that inhibits the growth of breast cancer cells *in vivo* and *in vitro*. *Biochemical Pharmacology* **44** 2273–2280.

Colston KW, Mackay AG & James SY 1995 Vitamin D_3 derivatives and breast cancer. In Ernst Schering Research Foundation Workshop 14 *Apoptosis in Hormone-Dependent Cancers*, vol 4, p 201–224. Eds M Tenniswood & H Michna. Heidelberg: Springer Verlag.

Colston KW, Perks CM, Xie SP & Holly JMP 1998 Growth inhibition of both MCF-7 and Hs578T human breast cancer cell lines by vitamin D analogues is associated with increased expression of insulin-like binding protein-3. *Journal of Molecular Endocrinology* **20** 157–162.

Curran JE, Vaughn T, Lea RA, Weinstein SR, Morrison NA & Griffiths LR 1999 Association of a vitamin D receptor polymorphism with sporadic breast cancer development. *International Journal of Cancer* **83** 723–726.

Danielsson C, Mathiasen IS, James SY, Nayeri S, Bretting S, Mørk Hansen C, Colston KW & Carlberg C 1997 Sensitive induction of apoptosis in breast cancer cells by a novel 1,25-dihydroxyvitamin D_3 analogue shows relation to promoter selectivity. *Journal of Cellular Biochemistry* **66** 552–562.

DeLuca HF & Ostrem VK 1988 Analogs of the hormonal form of vitamin D and their possible use in leukaemia. *Progress in Clinical and Biological Research* **259** 41–55.

Demirpence E, Balaguer P, Trousse F, Nicolas J-C, Pons M & Gagne D 1994 Antiestrogenic effects of all-trans retinoic acid and 1,25-dihydroxyvitamin D_3 in breast cancer cells occur at the estrogen response element level but through different mechanisms. *Cancer Research* **54** 1458–1464.

Desprez P-Y, Poujol D, Falette N, Lefebvre M-F & Saez 1991 1,25-Dihydroxyvitamin D_3 increases epidermal growth factor receptor gene expression in BT-20 breast carcinoma cells. *Biochemical and Biophysical Research Communications* **176** 1–6.

Dews M, Nishimoto I & Basgra R 1997 IGF-I receptor protection from apoptosis in cells lacking the IRS proteins. *Receptor Signal Transduction* **7** 231–240.

Dunn SE, Hardman R, Kari F & Barrett JC 1997 Insulin-like growth factor I (IGF-I) alters drug sensitivity of HBL100 human breast cancer cells by inhibition of apoptosis induced by diverse anticancer drugs. *Cancer Research* **57** 2687–2693.

Eisman JA, Suva LJ & Martin TJ 1986 Significance of 1,25-dihydroxyvitamin D_3 receptor in primary breast cancers. *Cancer Research* **46** 5406–5408.

El Abdaimi K, Dion N, Papavasiliou V, Cardinal P-E, Binderup L, Goltzman D, Ste-Marie L-G & Kremer R 2000 The vitamin D analogue EB1089 prevents skeletal metastasis and prolongs survival time in nude mice transplanted with human breast cancer cells. *Cancer Research* **60** 4412–4418.

Elstner E, Linker-Israli M, Said J, Umiel T, deVos S, Shintaku IP, Heber D, Binderup L Uskokovic M & Koeffler HP 1995 20-Epi-vitamin D analogues: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. *Cancer Research* **55** 2822–2830.

Evans TRJ, Hamberg KJ, Skov T, Haahr HLO, Menday P, Bay C & Binderup L 2000 Seocalcitol (EB1089) – clinical experience to date. In *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects*, pp 485–488. Eds AW Norman, R Bouillon & M Thomasset. Riverside: University of California.

Fan FS & Yu W 1995 1,25 Dihydroxyvitamin D_3 suppresses cell growth, DNA synthesis and phosphorylaton of retinoblastoma protein in a breast cancer cell line. *Cancer Investigation* **13** 280–286.

Feldman D, Glorieux FH & Pike JW (Eds) 1997 *Vitamin D*. San Diego: Academic Press.

Flanagan L, Ethier S & Welsh JE 1997 Vitamin D induced apoptosis in estrogen independent breast cancer cells and tumors. In *Vitamin D, a Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications*, pp 459–460. Eds AW Norman, R Bouillon & M Thomasset. Berlin: De Gruyter.

Flanagan L, VanWeelden K, Ammerman C, Ethier S & Welsh JE 1999 SUM-159PT cells, a novel estrogen independent human breast cancer model system. *Breast Cancer Research Treatment* **58** 193–204.

Fogh K & Kragballe K 2000 Recent developments in vitamin D analogs. *Current Pharmaceutical Design* **6** 961–972.

Freake HC, Abeasekekera G, Iwasaki J, Marocci C, MacIntyre I, McClelland RA, Skilton RA, Easton DF & Coombes RC 1984 Measurement of 1,25-dihydroxyvitamin D_3 receptors in breast cancer and relationship to biochemical and clinical indices. *Cancer Research* **44** 1677–1681.

Friedrich M, Reichrath J, Chen TC, Tanpricha V, Gherson I, Tilgen W, Schmidt W & Holick MF 2000 Expression of 25-hydroxyvitamin D_3-1α-hydroxylase in breast tissue. In *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects*, pp 189–192. Eds AW Norman, R Bouillon & M Thomasset. Riverside: University of California.

Gulliford T, English J, Colston KW, Menday P, Moller S & Coombes RC 1998 A phase I study of the vitamin D analogue EB1089 in patients with advanced breast and colorectal cancer. *British Journal of Cancer* **78** 6–13.

Hankinson SE, Willett WC, Colditz GA, Hunter DJ, Michaud DS, Deroo B, Rosner B, Speizer FR & Pollak M 1998 Circulating concentrations of insulin-like growth factor-I and breast cancer risk. *Lancet* **351** 1393–1396.

Iino Y, Yoshida M, Sugamata N, Maemura M, Ohwada S, Yokoe T, Ishikita R, Horiuchi R & Morishita Y 1994 1α-Hydroxyvitamin D_3, hyperclcaemia, and growth suppression of 7,12-dimethylbenz(a)anthracene-induced rat mammary tumors. *Breast Cancer Research Treatment* **22** 133–140.

Ingles SA, Garcia DG, Wang W, Nieters A, Henderson BE & Coetzee GA 2000 Vitamin D receptor genotype and breast cancer in Latinas (United States). *Cancer Causes and Control* **11** 25–30.

Iseki K, Tatsuta M, Uehara H, Yano H, Sakai N & Ishiguro S 1999 Inhibition of angiogenesis as a mechanism for inhibition by 1alpha-hydroxyvitamin D_3 and 1,25-dihydroxyvitamin D_3 of colon carcinogenesis induced by azoxymethane in Wistar rats. *International Journal of Cancer* **81** 730–733.

Jacobson E, James K, Newmark H & Carroll K 1989 Effects of dietary fat, calcium, and vitamin D on growth and mammary tumorigenesis induced by 7,12-dimethylbenz(a)anthracene in female Sprague-Dawley rats. *Cancer Research* **49** 6300–6303.

James SY, Mackay AG, Binderup L & Colston KW 1994 Effects of a new synthetic vitamin D analogue, EB1089, on the oestrogen-responsive growth of human breast cancer cells. *Journal of Endocrinology* **141** 555–546.

James SY, Mackay AG & Colston KW 1995 Vitamin D derivatives in combination with 9-*cis* retinoic acid promote active cell death in breast cancer cells. *Journal of Molecular Endocrinology* **14** 391–394.

James SY, Mackay AG & Colston KW 1996 Effects on 1,25 dihydroxyvitamin D_3 and its analogues on induction of apoptosis in breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **58** 395–401.

James SY, Mercer E, Brady M, Binderup L & Colston KW 1998 EB1089, a synthetic analogue of vitamin D_3, induces apoptosis in breast cancer cells *in vivo* and *in vitro*. *British Journal of Pharmacology* **125** 953–962.

Janowsky EC, Lester GE, Weinberg CR, Millikan RC, Schildkraut JM, Garrett PA & Hulka BS 1999 Association between low levels of 1,25–dihydroxyvitamin D and breast cancer risk. *Public Health Nutrition* **2** 283–291.

Jensen S, Madsen MW, Lukas J, Binderup L & Bartek J 2001 Inhibitory effects of 1α,25-dihydroxyvitamin D_3 on the G1–S phase controlling machinery. *Molecular Endocrinology* **15** 1370–1380.

Jones G & Calverly MJ 1993 A dialogue on analogues, newer vitamin D drugs for use in bone disease, psoriasis and cancer. *Trends in Endocrinology and Metabolism* **4** 292–297.

Koga M & Sutherland RL 1991 Retinoic acid acts synergistically with 1,25-dihydroxyvitamin D_3 or antiestrogen to inhibit T47-D human breast cancer cell growth. *Journal of Steroid Biochemistry and Molecular Biology* **39** 455–466.

Koga M, Eisman JA & Sutherland RL 1988 Regulation of epidermal growth factor receptor levels by 1,25-dihydroxyvitamin D_3 in human breast cancer cells. *Cancer Research* **48** 2734–2739.

Koike M, Elstner E, Campbell MJ, Asou H, Uskokovic M, Tsuruoka N & Koeffler HP 1997 19-Nor-hexafluoride analogue of vitamin D_3: a novel class of potent inhibitors of proliferation of human breast cell lines. *Cancer Research* **57** 4545–4550.

Koli K & Keski-Oja J 1994 1,25-Dihydroxyvitamin D_3 has been shown to enhance the expression of transforming growth factor B1 and its latent form binding protein in breast carcinoma cells. *Cancer Research* **55** 1540–1547.

Koli K & Keski-Oja J 2000 1α,25-Dihydroxyvitamin D_3 and its analogues down-regulate cell invasion-associated proteases in cultured malignant cells. *Cell Growth and Differentiation* **11** 221–229.

Koren R, Rocker D, Kotestiano O, Liberman UA & Ravid A 2000 Synergistic anticancer activity of 1,25-dihydroxyvitamin D_3 and immune cytokines: the involvement of reactive oxygen species. *Journal of Steroid Biochemistry and Molecular Biology* **73** 105–112.

Koshizuka K, Koike M, Said J, Biderup L & Koeffler HP 1998 Novel vitamin D_3 analog (CB1093) when combined with paclitaxel and cisplatin inhibit growth of MCF-7 human breast cancer cells *in vivo*. *International Journal of Oncology* **13** 421–428.

Koshizuka K, Koike M, Asou H, Cho SK, Stephen T, Rude RK, Binderup L, Uskokovic M & Koeffler HP 1999*a* Combined effect of vitamin D_3 analogs and paclitaxel on the growth of MCF-7 breast cancer cells *in vivo*. *Breast Cancer Research Treatment* **53** 113–120.

Koshizuka K, Kubota T, Said J, Koike M, Binderup L, Uskokovic M & Koeffler HP 1999*b* Combination therapy of a vitamin D analog and all trans retinoic acid. Effect on human breast cancer in nude mice. *Anticancer Research* **19** 519–524.

Kubodera N, Sato K & Nishi Y 1997 Characteristics of 22-oxacalcitriol (OCT) and 2β-(3-hydroxypropoxy)-calcitriol (ED-71). In *Vitamin D*, pp 1071–1086. Eds D Feldman, FH Glorieux & JW Pike. San Diego: Academic Press.

Love-Schimenti C, Gibson D, Ratman A & Bikle D 1996 Antiestrogen potentiation of antiproliferative effects of vitamin D_3 analogues in breast cancer cells. *Cancer Research* **56** 2789–2794.

Lundin AC, Soderkvist P, Eriksson B, Bergmann-Jungestrom M & Wingren S 1999 Association of breast cancer progression with a vitamin D receptor polymorphism. *Cancer Research* **59** 2332–2334.

Mackay AG, Ofori-Kuragu EA, Lansdown A, Coombes RA, Binderup L & Colston KW 1996 Effects of the synthetic vitamin D analogue EB1089 and tamoxifen on the growth of experimental rat mammary tumours. *Endocrine-Related Cancer* **3** 327–335.

Majewski S, Skopinska M, Szmurio & Bollag W 1993 Inhibition of tumor cell-induced angiogenesis by retinoids, 1,25-dihydroxyvitamin D_3 and their combination. *Cancer Letters* **75** 35–39.

Majewski S, Szmurio A, Marczak M, Jablonska S, Bollag W & Jablonska J 1996 Vitamin D_3 is a potent inhibitor of tumor cell-induced angiogenesis. *Journal of Investigative Dermatology Symposium Proceedings* **1** 97–101.

Mathiasen IS, Colston KW & Binderup 1993 EB1089, a novel vitamin D analogue, has strong antiproliferative and differentiation inducing effects on cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **46** 365–371.

Mathiasen IS, Lademann U & Jäättelä M 1999 Apoptosis induced by vitamin D compounds in breast cancer cells is inhibited by Bcl-2 but does not involve known caspases or p53. *Cancer Research* **59** 4848–4856.

Mathiasen I S, Mørk Hansen C, Foghsgaard L & Jäättelä M 2001 Sensitisation to TNF-induced apoptosis by 1,25-dihydroxyvitamin D_3 involves up-regulation of the TNF receptor 1 and cathepsin B. *International Journal of Cancer* **93** 224–231.

Mawer EB, Walls J, Howell A, Davies M, Ratcliffe W & Bundred NJ 1997 Serum 1,25-Dihydroxyvitamin D may be related inversely to disease activity in breast cancer patients with bone metastases. *Journal of Clinical Endocrinology and Metabolism* **82** 118–122.

Mehta R, Moriarty R, Mehta R, Penmasta R, Lazzaro G, Constantinou A & Guo L 1997 Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1α hydroxyvitamin D$_5$. *Journal of the National Cancer Institute* **89** 212–218.

Mehta R, Hawthorne M, Uselding L, Albinescu D, Moriarty R, Christov K & Mehta R 2000 Prevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats by 1α-hydroxyvitamin D$_5$. *Journal of the National Cancer Institute* **92** 1836–1840.

Mercier T, Chaumontet C, Gaillard-Sanchez I, Martel P & Heberden C 1996 Calcitriol and lexicalcitol (KH1060) inhibit the growth of human breast adenocarcinoma cells by enhancing transforming growth factor-β production. *Biochemical Pharmacology* **52** 505–510.

Mezzetti G, Barbiroli B & Oka T 1987 1,25-Dihydroxy-cholecalciferol receptor regulation in hormonally induced differentiation of mouse mammary gland in culture. *Endocrinology* **120** 2488–2493.

Mezzetti G, Monti M, Casolo L, Piccinini G & Moruzzi M 1988 1,25-Dihydroxycholecalciferol-dependent calcium uptake by mouse mammary gland in culture. *Endocrinology* **122** 389–394.

Mørk Hansen C, Frandsen TL, Brunner N & Binderup L 1994 1α,25-Dihydroxyvitamin D$_3$ inhibits the invasive potential of human breast cancer cells *in vitro*. *Clinical and Experimental Metastasis* **12** 195–202.

Mørk Hansen C, Hamberg KJ, Binderup E & Binderup L 2000 Seocalcitol (EB1089): a vitamin D analogue of anti-cancer potential. Background, design, synthesis, pre-clinical and clinical evaluation. *Current Pharmaceutical Design* **6** 803–828.

Mørk Hansen C, Binderup L, Hamberg K & Carlberg C 2001*a* Vitamin D and cancer: effects of 1,25(OH)$_2$D$_3$ and its analogues on growth control and tumorigenesis. *Frontiers in Bioscience* **6** 820–848.

Mørk Hansen C, Rhode L, Madsen MW, Hansen D, Colston KW, Pirianov G, Holm PK & Binderup 2001*b* MCF-7/VDR: a new vitamin D resistant cell line. *Journal of Cellular Biochemistry* **82** 422–436.

Narvaez CJ & Welsh J 1997 Differential effects of 1,25-dihydroxyvitamin D$_3$ and tetradecanoylphorbol acetate on cell cycle and apoptosis of MCF-7 cells and a vitamin D$_3$ resistant variant. *Endocrinology* **138** 4690–4698.

Narvaez CJ & Welsh J 2001 Role of mitochondria and caspases in vitamin D mediated apoptosis of MCF-7 breast cancer cells. *Journal of Biological Chemistry* **276** 9101–9107.

Narvaez CJ, Zinser G & Welsh JE 2001 Functions of 1α,25-dihydroxyvitamin D$_3$ in mammary gland: from normal development to breast cancer. *Steroids* **66** 301–308.

Nemere I, Schwartz Z, Pedrozo H, Sylvia VL, Dean DD & Boyan BD 1998 Identification of a membrane receptor for 1,25-dihydroxyvitamin D$_3$ which mediates rapid activation of protein kinase C. *Journal of Bone and Mineral Research* **13** 1353–1359.

Nickerson T, Huynh H & Pollak 1997 Insulin-like growth factor binding protein-3 induces apoptosis in MCF-7 breast cancer cells. *Biochemical and Biophysical Research Communications* **237** 690–693.

Nolan E, Donepudi M, VanWeelden K, Flanagan L & Welsh JE 1998 Dissociation of vitamin D$_3$ and anti-estrogen mediated growth regulation in MCF-7 breast cancer. *Molecular and Cellular Biochemistry* **188** 13–20.

Norman AW, Roth J & Orci L 1982 The vitamin D endocrine system: steroid metabolism, hormone receptors and biological response (calcium binding proteins). *Endocrine Reviews* **3** 331–366.

Oikawa T, Hirotani K, Ogasawara H, Katayama T, Nakamura O, Iwaguchi T & Hiragun A 1990 Inhibition of angiogenesis by vitamin D$_3$ analogues. *European Journal of Pharmacology* **178** 247–250.

Oikawa T, Yoshida Y, Shimamura M, Ashino-Fuse H, Iwaguchi T & Tominaga T 1991 Antitumor effect of 22-oxa-1α, 25-dihydroxyvitamin D$_3$, a potent angiogenesis inhibitor, on rat mammary tumors induced by 7,12–dimethylbenz(a)anthracene. *Anti-Cancer Drugs* **2** 475–480.

Okano K, Usa T, Ohtsuru A, Tsukazaki T, Miyazaki Y, Yonekura A, Namba H, Shindoh H & Yamashita S 1999 Effect of 22-oxa-1,25-dihydroxyvitamin D$_3$ on human thyroid cancer cell growth. *Endocrine Journal* **46** 243–252.

Packman K, Flanagan L, Zinser G, Mitsch R, Tenniswood M & Welsh J 2000 Combination treatment of MCF-7 xenografts with the vitamin D$_3$ analog EB1089 and antiestrogens. In *Vitamin D Endocrine System: Structural, Biological, Genetic and Clinical Aspects*, pp 511–514. Eds AW Norman, R Bouillon & M Thomasset. Riverside: University of California.

Papa V, Gliozzo B, Clark GM, McGuire WL, Moore D, Fujita-Yamaguchi Y, Vigneri R, Goldfine ID & Pezzino V 1993 Insulin-like growth factors are over expressed and predict a low risk in human breast cancer. *Cancer Research* **53** 3736–3740.

Pirianov G & Colston KW 2001*a* Interactions of vitamin D analogue CB1093, TNFα and ceramide on breast cancer cell apoptosis. *Molecular and Cellular Endocrinology* **172** 69–78.

Pirianov G & Colston KW 2001*b* Interaction of vitamin D analogs with signalling pathways leading to apoptosis in breast cancer cells. *Steroids* **66** 309–318.

Pirianov G, Danielsson C, Carlberg C, James SY & Colston KW 1999 Potentiation by vitamin D analogues of TNFα and ceramide-induced apoptosis is associated with activation of cytosolic phospholipase A2. *Cell Death and Differentiation* **6** 890–901.

Pollak MN 1998 Endocrine effects of IGF-I on normal and transformed breast epithelial cells: potential relevance to strategies for breast cancer treatment and prevention. *Breast Cancer Research Treatment* **47** 209–217.

Posner GH, Crawford KRC, Peleg S, Welsh J, Romu S, Gewirtz DA, Gupta MS, Dolan P & Kensler TW 2001 A non-calemic sulfone version of the vitamin D$_3$ analogue seocalcitol (EB1089): chemical synthesis, biological evaluation and potency enhancement of the anticancer drug adriamycin. *Bioorganic and Medical Chemistry* **9** 2365–2371.

Ravid A, Rocker D, Machlenkin A, Rotem C, Hochman A, Kessler-Icekson G, Liberman UA & Koren R 1999 1,25-Dihydroxyvitamin D$_3$ enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage. *Cancer Research* **59** 862–867.

Rocker D, Ravid A, Liberman UA, Gurach-Jehoshua O & Koren R 1994 1,25-Dihydroxyvitamin D$_3$ potentiates the cytotoxic effect of TNF on human breast cancer cells. *Molecular and Cellular Endocrinology* **106** 157–162.

Rozen F & Pollak M 1999 Inhibition of insulin-like growth factor receptor signaling by the vitamin D analogue EB1089 in MCF-7 breast cancer cells: a role for insulin-like growth factor binding proteins. *International Journal of Oncology* **15** 589–594.

Rozen F, Yang X-F, Huynh H & Pollak M 1997 Antiproliferative action of vitamin D-related compounds and insulin-like growth factor binding protein 5 accumulation. *Journal of the National Cancer Institute* **89** 652–656.

Saez S, Meggough F, Lefebvre M-F, Descotes F, Pampile R, Adam L & Crepin M 1994 Potential direct and indirect influence of 1,25(OH)$_2$D$_3$ on the growth of human colonic and breast carcinoma. In *Vitamin D, a Pluripotent Steroid Hormone: Structural Studies, Molecular Endocrinology and Clinical Applications,* pp 469–476. Eds AW Norman, R Bouillon & M Thomasset. Berlin: De Gruyter.

Schwartz GG, Hill CC, Oeler TA, Becich MJ & Bahnson RR 1995 1,25-Dihydroxy-16-ene-23-yne-vitamin D$_3$ and prostate cancer cell proliferation *in vivo*. *Urology* **46** 365–369.

Simboli-Campbell M, Narvaez CJ, Tenniswood M & Welsh JE 1996 1α,25(OH)$_2$D$_3$ induces morphological and biochemical indices of apoptosis in MCF-7 breast cancer cells. *Journal of Steroid Biochemistry and Molecular Biology* **58** 367–376.

Simboli-Campbell M, Narvaez CJ, VanWeelden K, Tenniswood M & Welsh JE 1997 Comparative effects of 1α,25(OH)$_2$D$_3$ and EB1089 on cell cycle kinetics and apoptosis in MCF-7 cells. *Breast Cancer Research Treatment* **42** 31–41.

Stoica A, Saceda M, Fakhro A, Solomon HB, Fenester BD & Martin MB 1999 Regulation of estrogen receptor-α gene expression by 1,25-dihydroxyvitamin D in MCF-7 cells. *Journal of Cellular Biochemistry* **75** 640–651.

Sundaram S & Gewirtz DA 1999 The vitamin D analog EB1089 enhances the response of human breast tumor cells to radiation. *Radiation Research* **152** 479–486.

Sundaram S, Chaudhry M, Reardon D, Gupta M & Gewirtz DA 2000 The vitamin D$_3$ analog EB1089 enhances the antiproliferative and apoptotic effects of adriamycin in MCF-7 breast tumour cells. *Breast Cancer Research Treatment* **63** 1–10.

Swami S, Krishnan AV & Feldman D 2000 1α,25-Dihydroxy-vitamin D$_3$ down-regulates estrogen receptor abundance and suppresses estrogen action in MCF-7 human breast cancer cells. *Clinical Cancer Research* **6** 3371–3379.

Tanaka H & Seino Y 1997 Vitamin D metabolites and bone. In *Vitamin D*, pp 305–311. Eds D Feldman, FH Glorieux & JW Pike. San Diego: Academic Press.

Uskokovic MR, Studzinski GP, Gardner JP, Reddy SG, Campbell MJ & Koeffler HP 1997 The 16-ene vitamin D analogs. *Current Pharmaceutical Design* **3** 99–123.

VanWeelden K, Flanagan L, Binderup L, Tenniswood M & Welsh JE 1998 Apoptotic regression of MCF-7 xenografts in nude mice treated with the vitamin D analog EB1089. *Endocrinology* **139** 2102–2110.

Verlinden L, Verstuyf A, Convents R, Marcelis S, van Camp M & Bouillon R 1998 Action of 1,25(OH)$_2$D$_3$ on the cell cycle genes, cyclin D1, p21 and p27 in MCF-7 cells. *Molecular and Cellular Endocrinology* **142** 57–65.

Verlinden L, Verstuyf A, van Camp M, Marcelis S, Sabbe K, Zhao X-Y, de Clercq P, Vandewalle M & Bouillon R 2000 Two novel 14-epi analogues of 1,25-dihydroxyvitamin D$_3$ inhibit the growth of human breast cancer cells *in vitro* and *in vivo*. *Cancer Research* **60** 2673–2679.

Vink-van Wijngaarden T, Pols HAP, Buurman CJ, van der Bemd GJCM, Dorssers CJ, Birkenhager JC & van Leeuwen JPTM 1994 Inhibition of breast cancer cell growth by combined treatment with vitamin D analogues and tamoxifen. *Cancer Research* **54** 5711–5717.

Vink-van Wijngaarden T, Pols HAP, Buurman CJ, Birkenhager JC, & van Leeuwen JPTM 1996 Inhibition of insulin- and insulin-like growth factor stimulated growth of human breast cancer cells by 1,25-dihydroxyvitamin D$_3$ and the vitamin D analogue EB1089. *European Journal of Cancer* **32A** 842–848.

Wakeling AE, Dukes M & Bowler J 1991 A potent specific pure antiestrogen with clinical potential. *Cancer Research* **51** 3867–3387.

Wang Q, Yang W, Uytingco MS, Christakos S & Weider R 2000 1,25-Dihydroxyvitamin D$_3$ and all *trans* retinoic acid sensitize breast cancer cells to chemotherapy-induced cell death. *Cancer Research* **60** 2040–2048.

Welsh JE 1994 Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens. *Biochemistry and Cell Biology* **72** 537–545.

Welsh JE 1995 Induction of apoptosis in breast cancer cells in response to vitamin D and antiestrogens. *Biochemistry and Cell Biology* **72** 537–545.

Welsh J, VanWeelden K, Flanagan L, Byrne I, Nolan E & Narvaez CJ 1998 The role of vitamin D$_3$ and anti-estrogens in modulating apoptosis of breast cancer cells and tumors. *Subcellular Biochemistry* **30** 245–270.

Wu G, Fan RS, Li W, Ko T & Brattain MG 1997 Modulation of cell cycle by vitamin D$_3$ and its analogue EB1089 in human breast cancer cells. *Oncogene* **15** 1555–1563.

Xie S, James SY & Colston KW 1997 Vitamin D derivatives inhibit the mitogenic effects of IGF-I on MCF-7 human breast cancer cells. *Journal of Endocrinology* **154** 495–504.

Xie SP, Pirianov G & Colston KW 1999 Vitamin D analogues suppress IGF-I signalling and promote apoptosis in breast cancer cells. *European Journal of Cancer* **35** 1717–1723.

Yamada S, Yamamoto K & Masuno H 2000 Structure–function analysis of vitamin D and VDR model. *Current Pharmaceutical Design* **6** 733–748.

Yang L, Yang J, Venkateswarlu S, Ko T & Brattain MG 2001 Autocrine TGF beta signalling mediates vitamin D$_3$ analog-induced growth inhibition in breast cells. *Journal of Cellular Physiology* **188** 383–393.

Zierold C, Darwish HM & DeLuca HF 1994 Identification of a vitamin D responsive element in the rat calcidiol (25-hydroxyvitamin D$_3$) 24-hydroxylase gene. *PNAS* **91** 900–902.