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

1α,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, 1α,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 preneoplastic 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 $p21^{WAF-1/CIP1}$

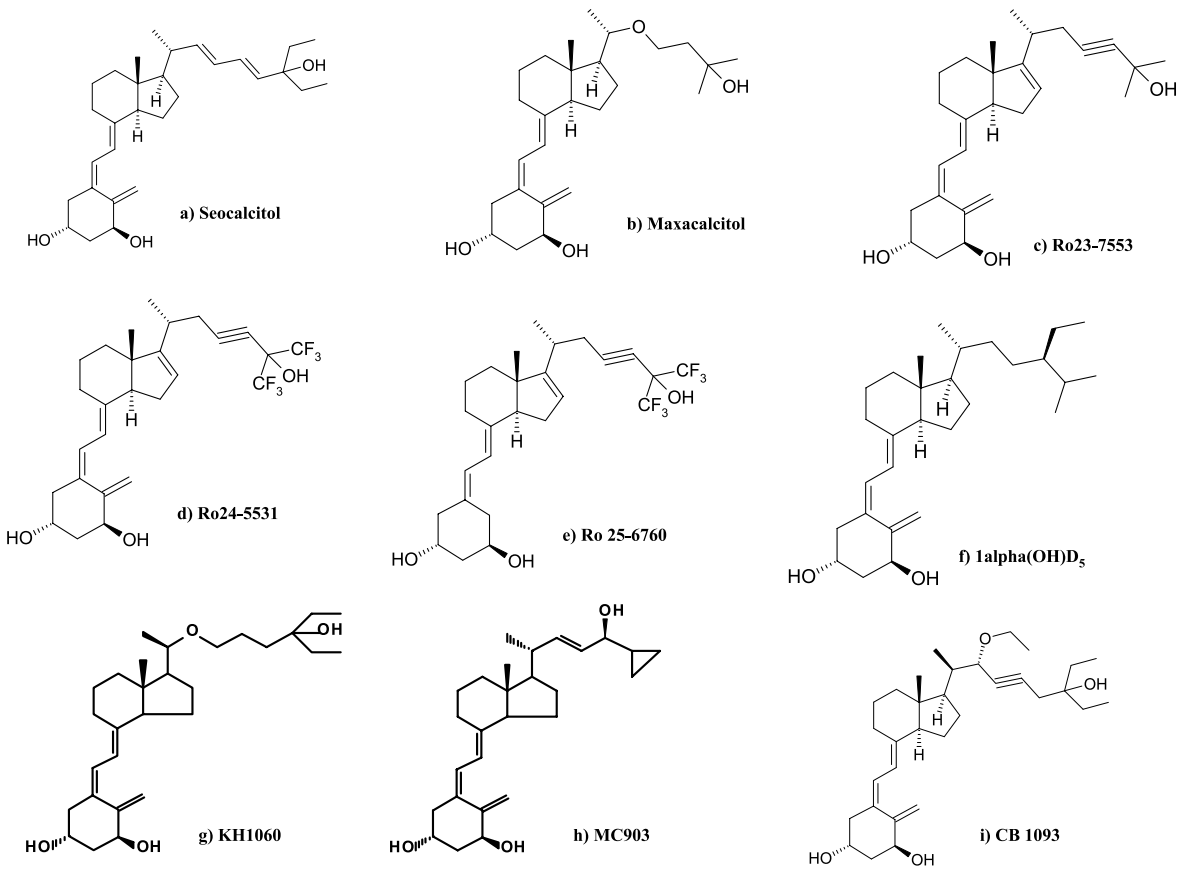

Figure 1 Structures of synthetic analogues of vitamin D with anti-tumour activity.

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 $p21^{WAF\text{-}1/CIP1}$ 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

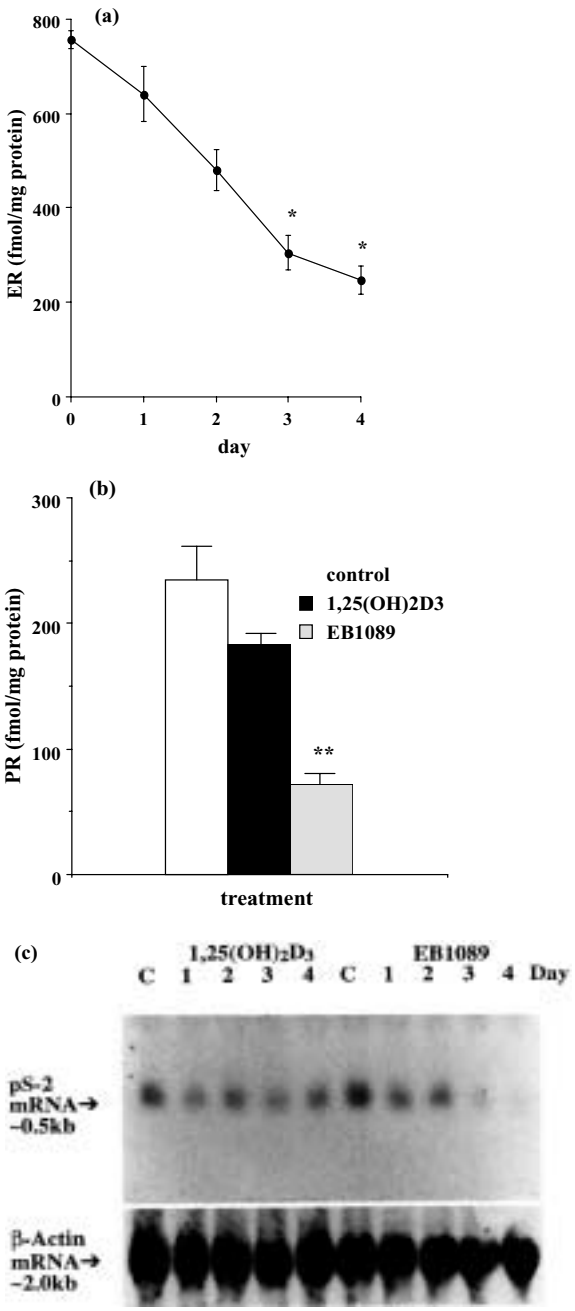

Figure 2 Vitamin D derivatives down-regulate oestrogen and progesterone receptor abundance and pS2 transcript levels in MCF-7 human breast cancer cells. (a) Regulation of oestrogen receptor (ER) expression by the vitamin D analogue EB1089 in MCF-7 cells. Cells were grown in phenol-red-free DMEM medium 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$-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, $1,25(OH)_2D_3$ 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*. 2001*b*). MCF-7 cells treated with $1,25(OH)_2D_3$ exhibit cytoplasmic condensation and hyperchromatic, pycnotic nuclei resulting from chromatin condensation typical of apoptotic cells. Morphological assessment of MCF-7 cells treated with $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 2001*a*). In addition, induction of apoptosis by vitamin D derivatives appears to be independent of the mutational status of the p53 tumour suppressor gene. $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ and its analogues (Colston *et al*. 1995, James *et al*. 1996, Simboli-Campbell *et al*. 1996, 1997). In addition, $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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, $1,25(OH)_2D_3$; 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ 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 $1,25(OH)_2D_3$ (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 $1,25(OH)_2D_3$ 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,

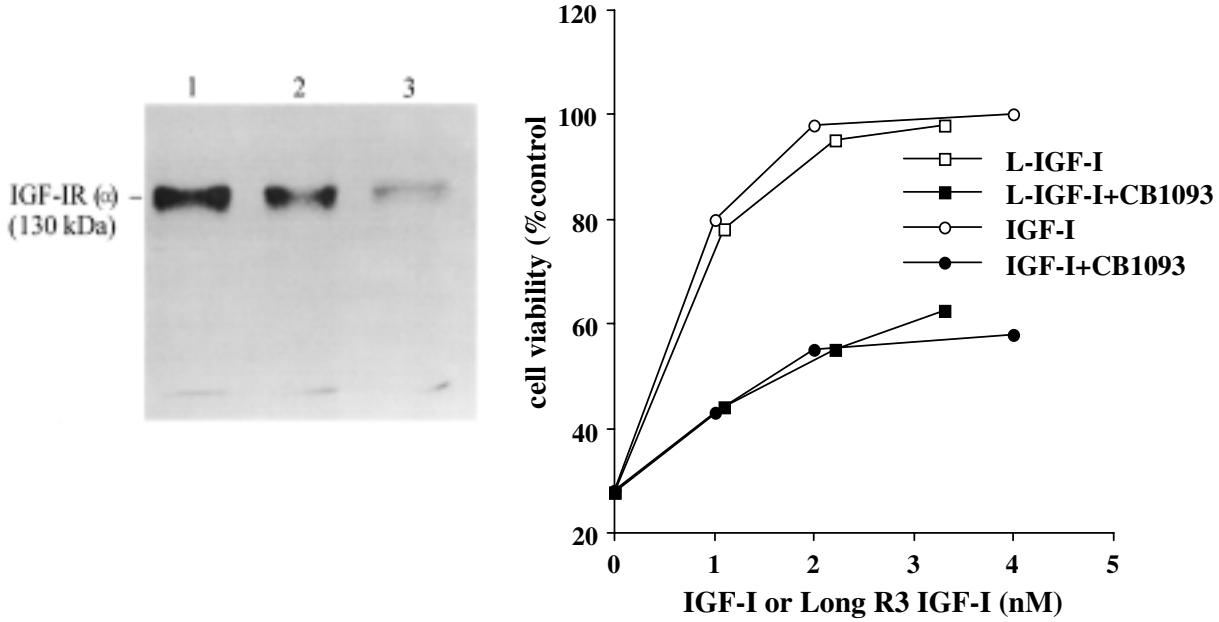

Figure 3 Anti-IGF-I effects of vitamin D analogues. (Left panel) Dose-dependent inhibition of IGF-IR expression by the vitamin D analogue EB1089 in MCF-7 cells. Cells were treated for 6 days with ethanol vehicle (lane 1) or EB1089 (10^{-9} and 10^{-8} M, lanes 2 and 3). Lysates were prepared from these 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-1α,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 $\mu g/m^2$ 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.

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