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VITAMIN D AND CANCER: AN UPDATE OF *IN VITRO* AND *IN VIVO* DATA

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1. ABSTRACT

1alpha,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, Calcitriol) is a pleiotropic hormone with anti-proliferative, pro-apoptotic and pro-differentiation effects on numerous cell types, which suggest anti-cancer activity in addition to its classical regulatory action on calcium and phosphate metabolism. $1,25(OH)_2D_3$ exerts its actions mainly *via* its high affinity receptor VDR through a complex network of genomic (transcriptional and post-transcriptional) and also non-genomic mechanisms, which are partially coincident in the different cells and tissues studied. Epidemiological and experimental *in vitro* and *in vivo* data support a cancer preventive role of $1,25(OH)_2D_3$. The anti-cancer activity of $1,25(OH)_2D_3$ and multiple analogs with reduced calcemic properties, which are thus less toxic, is under investigation in a long list of cultured cell types and in several *in vivo* models of wild-type and genetically-modified animals. Some vitamin D compounds have reached clinical trials, but results are still scarce.

2. INTRODUCTION

2.1. Vitamin D and cancer: an overview

The relation between vitamin D and cancer is the focus of very intense research. The active form of vitamin D, the seco-steroid hormone 1alpha,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$, Calcitriol) has pleiotropic effects in the organism, regulating calcium and phosphate metabolism and bone biology (classical actions) and also the proliferation, differentiation, apoptosis, invasiveness and/or angiogenic or metastatic potential of many cell types (non-classical actions). Because of these non-classical actions and also as a result of epidemiological studies indicating chemopreventive activity against colon, prostate cancer and other neoplasias (1-6), $1,25(OH)_2D_3$ has received great interest as a candidate anti-cancer compound. Anti-tumor activity in experimental animals largely increased these expectations. However, toxicity resulting from the hypercalcemic effect of the doses required for *in vivo* activity has hampered the clinical anti-tumoral use of

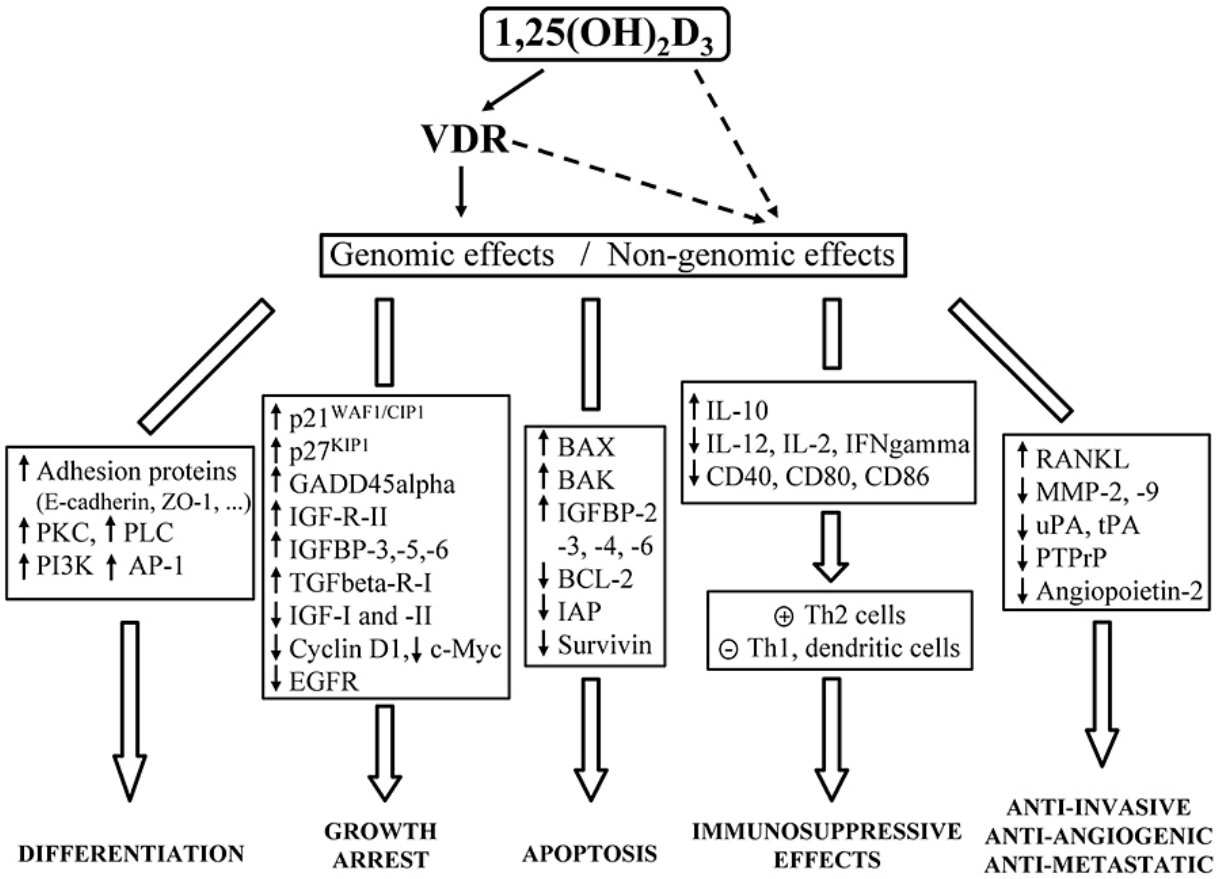

Figure 1. Summary of the cancer-related biological actions and target genes of $1,25(OH)_2D_3$. Discontinous lanes refer to non-confirmed processes.

$1,25(OH)_2D_3$. In parallel to investigations aimed to define acceptable administration schedules for $1,25(OH)_2D_3$, a few thousand analogs generically termed deltanoids have been synthesized in the search for clinically useful compounds that retain the anti-cancer activity but are less toxic (7).

A number of excellent reviews deal with vitamin D biology, its gene regulatory effects and the mechanism of vitamin D receptor (VDR) action (8-12) or the pre-clinical and clinical development of $1,25(OH)_2D_3$ and analogs against cancer (13-15). Here, we review the data on the mechanism of action of $1,25(OH)_2D_3$ in human cancer cells and the situation and perspectives of its analogs in the clinic, with special emphasis on the use of animal models to investigate $1,25(OH)_2D_3$ anti-tumoral action.

Vitamin D_3 is produced in the skin by the action of sunlight on 7-dehydrocholesterol or, to a much lesser extent, ingested in the diet. Vitamin D_3 is then hydroxylated at carbon 25 in the liver by the 25-hydroxylase product of the cytochrome *CYP27A1* gene to form 25-hydroxyvitamin D_3 ($25(OH)D_3$). This is again hydroxylated to render $1,25(OH)_2D_3$ in the kidney and several cell types, by the action of 25-hydroxyvitamin D_3 1alpha-hydroxylase (product of the *CYP27B1* gene). Over 30 cell types express VDR and are thus targets of $1,25(OH)_2D_3$. The same pattern of expression is shared by the 24-hydroxylase enzyme encoded by the cytochrome *CYP24* gene, which converts its substrates $1,25(OH)_2D_3$ and $25(OH)D_3$ respectively to $1,24,25(OH)_3D_3$ and $24,25(OH)_2D_3$. These compounds have much lower activity and are progressively oxidized to less active compounds, and finally to calcitroic acid, which is secreted (9, review). $1,25(OH)_2D_3$ regulates its own synthesis and degradation through the induction of *CYP24* and the repression of *CYP27B1* genes. Parathyroid hormone is a sensor of low calcium and acts as a major inducer of *CYP27B1* expression. The finding that 25-hydroxyvitamin D_3 1alpha-hydroxylase and VDR are both expressed at early stages of colon tumor progression at higher levels than those found in normal mucosa or in undifferentiated tumors indicates that colon carcinoma cells can synthesize and respond to $1,25(OH)_2D_3$ (16). Thus, $1,25(OH)_2D_3$ behaves as a hormone-cytokine, acting in endocrine, paracrine and probably autocrine fashion.

2.2. Anti-cancer effects and target genes of $1,25(OH)_2D_3$

The anti-cancer activity of $1,25(OH)_2D_3$ is mostly based on the inhibition of proliferation and the induction of differentiation or apoptosis of cancer cells (Figure 1). The predominant effect depends on the type of cancer cells. $1,25(OH)_2D_3$ also has several immunomodulatory actions that, unfortunately, are opposite to those expected of an anti-cancer agent. It inhibits differentiation and maturation,

and promotes apoptosis of antigen-presenting dendritic cells (17, review). Through these actions on dendritic cells, and also *via* direct inhibition of Th1 cells and potentiation of Th2 and regulatory T cells, $1,25(OH)_2D_3$ attenuates T-cell response. In line with these immunosuppressive effects, $1,25(OH)_2D_3$ analogs prevent autoimmune disorders and prolong allograft survival in experimental animals (17 and refs therein). Future work may clarify the putative clinical use of $1,25(OH)_2D_3$ analogs against autoimmune diseases or allograft rejection and also if analogs with reduced immunosuppressive effects have improved anti-tumor activity.

Gene regulatory actions of $1,25(OH)_2D_3$ and its analogs are mediated by the VDR, a member of the superfamily of nuclear receptors. VDR is a ligand-regulated transcription factor that binds to specific sequences termed vitamin D response elements (VDRE) in its target genes and modulates their expression (9-12). VDR interacts with DNA forming heterodimers with the retinoid X receptor (RXR) or, sometimes, other proteins. Genes that are induced *via* positive VDRE by $1,25(OH)_2D_3$ remain silenced by unliganded VDR through recruitment of transcriptional co-repressors such as SMRT, N-CoR, or Alien/TRIP15. Hormone binding induces a conformational change in VDR that causes co-repressor release. Subsequently, VDR binds co-activators (SRC-1, ACTR, GRIP-1 and others) and interacts with the vitamin D-receptor interacting protein (DRIP) complex and chromatin remodeller complexes, which leads to gene activation (9-12). The mechanism by which $1,25(OH)_2D_3$ represses the expression of target genes harboring negative VDRE is much less characterized. In some cases such as the granulocyte-macrophage colony-stimulating factor (*GM-CSF*) gene, VDR competes with the binding of other transcription factors (NFAT1) to their DNA binding sites, or alters their binding dynamics and/or transactivation activity (AP-1) *via* protein-protein interaction (18).

Several phosphorylated residues have been identified in human VDR located at the ligand- and DNA-binding domains, and the level of phosphorylation is increased by hormone binding (9). However, how specific phosphorylations affect VDR transcriptional activity or whether VDR is subjected to other post-translational modifications remains unclear.

In addition, $1,25(OH)_2D_3$ induces rapid, gene-expression-independent (non-genomic) effects such as changes in the activity of ion (calcium, chloride) channels or some enzymes (kinases, phospholipases), which might be mediated by membrane or cytosolic VDR or by other unknown receptors (19-22). $1,25(OH)_2D_3$ is a flexible molecule capable of rotation about its 6,7 single carbon bond, which can generate a potential series of ligand shapes ranging from 6-s-*cis* (6C) to 6-s-*trans* (6T). Strikingly, the 6-s-*cis* configuration favors activation of the non-genomic pathway, whereas the 6-s-*trans* preferentially mediates genomic responses (23). Supporting the dependence of genomic and non-genomic actions on VDR, both were abrogated in osteoblasts expressing a mutated VDR lacking the DNA binding domain (24), and recent data show that VDR is present in caveolae-enriched plasma membranes (25). The relation of non-genomic effects of $1,25(OH)_2D_3$ to its anti-cancer activities is however poorly understood.

The anti-proliferative effect of $1,25(OH)_2D_3$ is usually based on the induction of cell cycle arrest at the G1 phase through the increase in the expression of the cyclin-dependent kinase (CDK) inhibitors $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ and the repression of cyclin D1 (*CCND1*) and c-*MYC* genes (Figure 1). The mechanisms of these regulatory effects are different. While $p21^{WAF1/CIP1}$ gene has been reported to have a VDRE and is induced in many cell types but inhibited in others (5, 26, 27), the induction of $p27^{KIP1}$ gene lacking VDRE seems to be mediated by NF-Y and Sp1 transcription factors, and additional effects occur at the level of protein stability (28-31). These different data probably reflect the combination of differences among cell types and techniques (time of exposure, assay) used in the studies. Moreover, the repression of c-*MYC* in leukemic HL60 cells is due to the induction of HOXB4 and other proteins that bind to intron 1 sequences and cause transcription elongation block (32), whereas in SW480-ADH colon carcinoma cells $1,25(OH)_2D_3$ blocks c-*MYC* activation by the beta-catenin-TCF4 complex (33). Other target genes involved in proliferation arrest of colon and head and neck squamous carcinoma cells such as growth-arrest and DNA damage 45 alpha (*GADD45alpha*) have arisen from transcriptional profiling studies using $1,25(OH)_2D_3$ or analogs (27, 34, 35). In mouse osteoblasts, one fifth of the genes down-regulated 12 h after treatment are genes associated with DNA replication such as those coding for subunits of several DNA polymerases (36).

Differentiation is often linked to proliferation arrest, but $1,25(OH)_2D_3$ has specific pro-differentiation effects in several cell types through the induction of differentiation genes (Figure 1). For instance, in human head and neck, colon, prostate carcinoma and leukaemia cells $1,25(OH)_2D_3$ and some analogs induce the expression of a vast array of candidate target genes responsible for the differentiated phenotype (34, 35, 37-43). In contrast, $1,25(OH)_2D_3$ inhibits differentiation and activation of dendritic and Th1 cells respectively through the repression of interleukin (IL)-12 and co-stimulatory molecules CD40, CD80 and CD86 (and enhancement of IL-10 production) (44, 45), and of IL-2 and interferon (IFN)-gamma. Liganded VDR blocks human *IL-2* gene by impairing the binding of NFAT to the promoter, whereas in the case of *IFN-gamma* it binds to a VDRE in the promoter region (46-48). Expectedly, transcriptional profiling studies using dendritic cells have also provided a number of candidate target genes of 1alpha,$25(OH)_2$-19-ene-23-yne-26,27-hexafluoro-19-nor-D_3 involved in immunoregulation including immunoreceptors, chemokines, cytokines, and receptors of both, some of which have been confirmed (*MIP-1alpha* and *RANTES*) (49, 50). The regulation of keratinocyte differentiation by $1,25(OH)_2D_3$ seems to involve several signaling pathways including protein kinase C (PKC) and phospholipase C (PLC) activation (51), whereas the induction of myeloid differentiation of THP-1 cells requires the formation of complexes between VDR and the phosphatidylinositol 3-kinase (PI3K) and the

activation of this enzyme (52). In human CaCo-2 colon cancer cells, $1,25(OH)_2D_3$ increases differentiation through PKC-alpha- and JNK-dependent AP-1 activation (53).

Apoptosis induction by $1,25(OH)_2D_3$ relies on the regulation of genes controlling cell death pathways. $1,25(OH)_2D_3$ causes in a cell type-dependent fashion the activation of *BAX* and *BAK* and the inhibition of *BCL-2* and *IAP*, which individually and coordinately potentiate the effect of apoptosis-inducing signals (5, and refs therein) (Figure 1). Additionally, Apaf-1 is regulated in prostate cells (42), and novel targets involved in apoptosis regulation such as Grim19 and Stat3 have recently been found in breast cancer cells (J. Welsh, unpublished data). Putatively, at least some of the effects on apoptosis, as well as on proliferation and differentiation in some cell types, might be linked to the regulation of genes coding for several insulin-like growth factor-binding proteins (IGFBP)-2, -3, -4 and -6 (35, 40, 54, 55). However, some controversy exists on the regulatory effect of $1,25(OH)_2D_3$ on apoptosis. Thus, $1,25(OH)_2D_3$ protects thyrocytes against the induction of apoptosis by either staurosporine or ultraviolet irradiation through the increase of *BCL-2* expression (56). In addition, at pharmacological doses, $1,25(OH)_2D_3$ and the 6-s-*cis*-locked $1,25(OH)_2$lumisterol$_3$ analog protect murine osteoblasts and osteocytes and human HeLa cells from apoptosis by mechanisms mediated at least in part by Src, JNK and PI3K (57). At the same high (10^{-6} M) dose, $1,25(OH)_2D_3$ also inhibits ultraviolet B-induced apoptosis of human primary keratinocytes reducing JNK activation and IL-6 production (58). Although the pro-apoptotic action is more general, these controversial reports indicate that the effects of $1,25(OH)_2D_3$ on apoptosis are cell- and context-dependent.

The anti-invasive action of $1,25(OH)_2D_3$ reported in several systems is probably based, at least in part, on the inhibition of genes encoding metalloproteinases such as MMP-2 and MMP-9, and urokinase and tissue-type plasminogen activators (59) (Figure 1). In contrast, the anti-angiogenic effect of $1,25(OH)_2D_3$ in mice (60-62) is far from being understood, as in some cultured cells such as osteoblasts, head and neck squamous carcinoma cells and mouse embryo fibroblasts it induces the expression of the potent pro-angiogenic agent vascular endothelial growth factor (VEGF) (34, 63, 64). Supposedly, the anti-angiogenic effect is due to the regulation of other genes such as angiopoietin-2 in tumoral cells (65), or to anti-proliferative and/or pro-apoptotic effects on endothelial cells (62, 65, 66). In one study, $1,25(OH)_2D_3$ and three analogs (Ro-25-6760, EB1089, ILX23-7553) were potent inhibitors of the proliferation of tumor-derived endothelial cells, and interestingly $1,25(OH)_2D_3$ showed less potency against normal endothelial cells (65). $1,25(OH)_2D_3$ analogs also inhibit the formation of spontaneous metastasis in chemically-induced colon carcinogenesis in mice (67), which may result from a combination of effects at different stages of tumor progression. However, specific effects on the formation of bone metastasis may also be related to the regulation of other genes such as parathyroid hormone-related protein (*PTPrP*) in prostate cancer cells *via* a negative VDRE (68) or receptor activator of NF-kappaB ligand (*RANKL*) in osteoblasts *via* a positive VDRE (69).

Since a large number of genes respond to $1,25(OH)_2D_3$, it is reasonable to assume that only a subset are direct transcription targets regulated *via* VDRE, while many others are secondary targets controlled in cascade at either the transcriptional or post-transcriptional level by the primarily-regulated genes, or by non-genomic mechanisms.

3. EFFECTS OF $1,25(OH)_2D_3$ AND ANALOGS ON CULTURED HUMAN CANCER CELLS

3.1. Colon cancer cells

Colon cancer is the most frequent neoplasia in the United States and Europe for which no satisfactory therapy exists when surgery is not curative (70, 71). One of the earliest observations suggesting that vitamin D may play a role in colon cancer prevention and treatment was that people who lived at higher latitudes and so, received less solar radiation, had an increased risk of dying of colon cancer (72). It was also shown that low $25(OH)D_3$ serum levels correlated with a higher risk of developing colon cancer (2, 73). These data have led to an increasing interest in the effects of $1,25(OH)_2D_3$ on colon cancer cells. Numerous studies have shown that, as in other cancer cell types, $1,25(OH)_2D_3$ and its non-hypercalcemic analogs inhibit cell growth and induce epithelial differentiation and apoptosis in cells of the large intestine (5, 13, 74).

In the human colon, normal epithelial cells express VDR and 25-hydroxyvitamin D_3 1alpha-hydroxylase. Therefore, they can respond to $1,25(OH)_2D_3$ and synthesize it from serum $25(OH)D_3$ (16, 75, 76). Furthermore, VDR and 25-hydroxyvitamin D_3 1alpha-hydroxylase are up-regulated in early stages of colon cancer progression, suggesting that the local production of this hormone could be a tumor response that affects its own progression (16, 75, 76). Up-regulation of VDR and 25-hydroxyvitamin D_3 1alpha-hydroxylase can thus be considered as intrinsic tumor-suppressive functions. Unfortunately, this capacity is lost in late carcinomas, in which VDR and 25-hydroxyvitamin D_3 1alpha-hydroxylase are down-regulated (16, 75-77). Accordingly, high VDR expression is considered a good prognosis marker in colon cancer (78, 79).

The first demonstration of the inhibitory role of $1,25(OH)_2D_3$ on the growth of colon cancer cells was performed in LoVo cells (80). Similar effects were later observed in other human colon adenocarcinoma cell lines, such as CaCo-2, HT29 and SW480 (33, 81-86). Furthermore, the growth-inhibiting action of $1,25(OH)_2D_3$ has also been confirmed in cultured primary human colon adenoma- and carcinoma-derived cells (87, 88). $1,25(OH)_2D_3$ and some analogs reduce the high mitogenic rate of adenoma cells to that of normal colonocytes and significantly suppress the growth of carcinoma cells (87). In addition, $1,25(OH)_2D_3$ inhibits the tumor cell capacity for anchorage-independent growth (81).

Whereas $1,25(OH)_2D_3$ concentrations from 10^{-8} to 10^{-7} M induce G0/G1 arrest and $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ expression in other cancer cell types (89, 90), they inhibit proliferation without arresting cell cycle in colon cancer cells (91, 92), and higher doses (10^{-6} M) are required for G0/G1 arrest and $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ modulation in these cells (86, 91-93). This may be explained by apoptotic effects or by a uniform retardation of the cell cycle (92). In contrast, concentrations from 10^{-10} to 10^{-7} M of some potent $1,25(OH)_2D_3$ analogs can arrest colon cancer cells in G0/G1 and induce $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ (86, 91, 92). $1,25(OH)_2D_3$ inhibits c-*MYC* expression in SW480-ADH cells (33) but a number of studies have shown that in CaCo-2 cells its anti-proliferative effect is independent of c-*MYC* down-regulation (83, 94, 95).

Growth inhibition of colon cancer cells by $1,25(OH)_2D_3$ involves additional mechanisms, that include regulation of growth factor synthesis and signaling. One example is the effect of $1,25(OH)_2D_3$ on the transforming growth factor (TGF)-beta pathway, which inhibits epithelial cell growth (74). Progression from colonic adenoma to carcinoma is accompanied by increasing resistance to TGF-beta action (96). Whereas the growth of normal colonic epithelial cells is inhibited by TGF-beta, most human colon cancer cells such as SW480 and CaCo-2, are resistant to TGF-beta action (97). Activation of the TGF-beta signaling pathway contributes to the anti-proliferative action of $1,25(OH)_2D_3$ in colon cancer cells. First, $1,25(OH)_2D_3$ sensitizes cells to the growth inhibitory action of TGF-beta by inducing type I TGF-beta receptor expression (35, 97). Second, $1,25(OH)_2D_3$ increases the expression of type II insulin-like growth factor receptor (IGF-R), which facilitates the proteolytic activation of the inactive TGF-beta precursor (97). Additionally, SMAD3, a TGF-beta signaling downstream protein, binds to SRC-1 and acts as a co-activator of VDR, and therefore positively regulates $1,25(OH)_2D_3$ target genes (5, 74, 98). This effect is abrogated by SMAD7, an inhibitory transducer of TGF-beta signaling (99).

IGF-I and IGF-II are among the most abundant growth factors in the body, acting as mitogens and survival factors (13). IGF-II has been described to be over-expressed in colorectal adenomas (100). Accordingly, HT29 adenocarcinoma colon cells secrete IGF-II, which stimulates their growth (101). It has been shown that $1,25(OH)_2D_3$ and certain analogs inhibit IGF-II secretion in this cell line (55). Furthermore, they also increase the production of IGFBP-6, which negatively modulates the bioavailability of IGF-II by blocking its binding to type I IGF-R (35, 55). Moreover, the induction of type II IGF-R by $1,25(OH)_2D_3$ also contributes to the blockade of the IGF-II pathway because binding of IGF-II to this receptor results in an accelerated degradation of IGF-II (97). It is therefore likely that interference with the IGF-II signaling pathway contributes to the anti-cancer action of $1,25(OH)_2D_3$.

Epidermal growth factor (EGF) induces DNA synthesis and proliferation of colon epithelial cells. The stimulatory effect of EGF on cell division is effectively counteracted by $1,25(OH)_2D_3$ in primary cultures of human colon adenocarcinoma as well as in CaCo-2 cells (88). $1,25(OH)_2D_3$ reduces EGF receptor (*EGFR*) mRNA and protein expression and decreases the number of membrane EGFRs promoting their ligand-induced internalization (88). Thus, inhibition of EGF signaling is part of the anti-mitotic action of $1,25(OH)_2D_3$. However, EGF down-regulates VDR expression in CaCo-2 cells (84, 94). Therefore, activation of the EGF pathway could allow colon carcinoma cells to escape from the anti-mitogenic action of $1,25(OH)_2D_3$ (84, 94).

The pro-differentiation effects of $1,25(OH)_2D_3$ in human colon cancer cells have been widely studied. CaCo-2 and HT29 cells undergo enterocyte differentiation upon $1,25(OH)_2D_3$ treatment, developing a prominent brush-border membrane with high activity of alkaline phosphatase and other brush-border-associated enzymes (82, 83, 85, 86). In addition, the differentiated phenotype induced by $1,25(OH)_2D_3$ involves an increase in the amount of intermediate filaments and desmosomes as well as the number of microvilli (83, 102). Alkaline phosphatase activity is also induced by $1,25(OH)_2D_3$ in primary cultured colon carcinoma cells and in other colon cancer cell lines (87, 91). Results from our group have revealed that $1,25(OH)_2D_3$ and several analogs induce epithelial differentiation in a subpopulation of SW480 cells that express VDR (SW480-ADH cells) (33) (Figure 2). This differentiation is linked to the induction of E-cadherin, the main component of the adherent junctions and important factor responsible for the maintenance of the epithelial phenotype, and other adhesion proteins such us vinculin, occludin, ZO-1 and ZO-2 (Figure 2). The Wnt/beta-catenin signaling pathway is deregulated in almost all colon cancers due to mutation of *APC* (*Adenomatous Poliposis Coli*) gene or, less frequently, the *CTNNB1* gene coding for beta-catenin. These alterations lead to the nuclear accumulation of beta-catenin, which then forms complexes with the TCF4 transcription factor and regulates numerous genes involved in proliferation and invasiveness (103). $1,25(OH)_2D_3$ represses beta-catenin transcriptional activity through at least two mechanisms: first, by direct interaction between ligand-bound VDR and beta-catenin in the nucleus, which prevents the formation of beta-catenin-TCF4 complexes; and, later, by inducing nuclear export of beta-catenin as a consequence of E-cadherin accumulation at the plasma membrane (33) (Figure 2). These effects of $1,25(OH)_2D_3$ on E-cadherin expression and beta-catenin signaling have also been observed in other colon cancer cell lines such as CaCo-2, HT29 and SW1417 (33).

Apoptosis induced by $1,25(OH)_2D_3$ in colon adenoma and carcinoma cells seems to be subsequent to the induction of differentiation, since the apoptotic cells express enterocyte differentiation markers like alkaline phosphatase (91). In this system, $1,25(OH)_2D_3$ and its analogs induce p53-independent apoptosis (13, 91). This is particularly relevant because a high percentage of colon tumors presents alterations in the *TP53* tumor suppressor gene. In human colon adenoma and carcinoma cell lines the apoptotic action of $1,25(OH)_2D_3$ is associated with an increase in BAK protein levels (91). However, the effect of

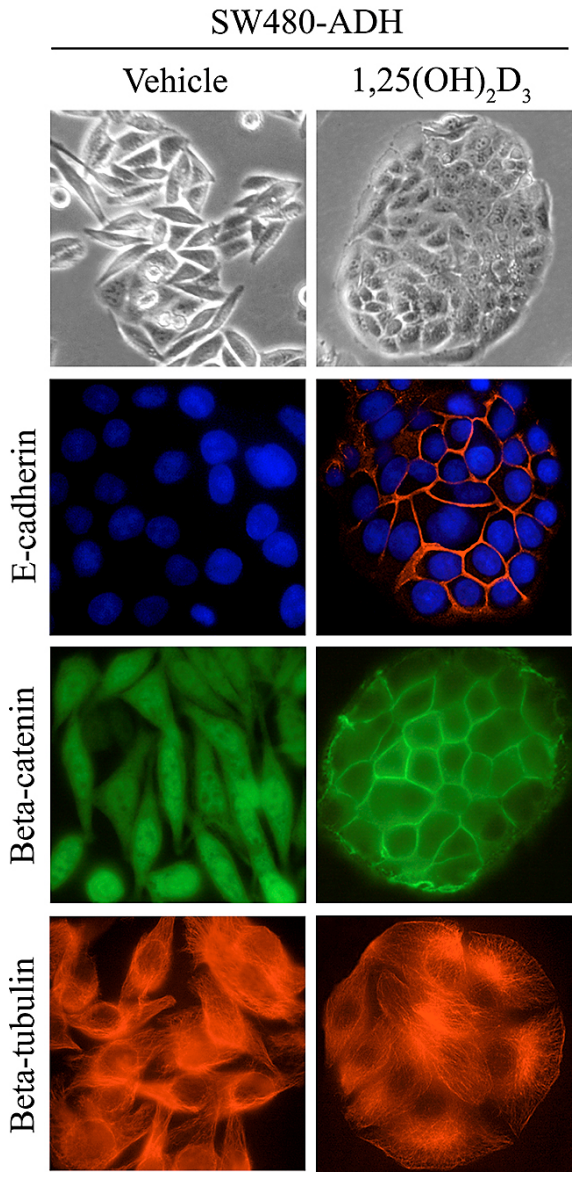

Figure 2. Effects of $1,25(OH)_2D_3$ on the phenotype and expression of E-cadherin and beta-catenin in human SW480-ADH colon cancer cells. Phase contrast (upper panels) and confocal (lower panels) microscopy images of cells treated with vehicle or 100 nM $1,25(OH)_2D_3$ for 48 h. Immunofluorescence analysis using antibodies against E-cadherin, beta-catenin or beta-tubulin was performed before confocal microscopy to examine the expression and localization of these proteins. Nuclei were counterstained with DAPI (in blue).

$1,25(OH)_2D_3$ on the expression of other pro-apoptotic (BAX) or anti-apoptotic (BCL-2, BCL-X_L) proteins, which are regulated by $1,25(OH)_2D_3$ in other systems (13), is not clear in colon cancer, and appears to be cell line specific (35, 91).

The gene expression profile associated with $1,25(OH)_2D_3$ treatment of SW480-ADH cells is compatible with its anti-proliferative and pro-differentiation effects (35). The long list of target genes includes some involved in DNA synthesis and cell cycle, apoptosis, cytoskeleton and adhesion, transcription and translation, intracellular signaling and redox metabolism. This list partially overlaps with that obtained by transcriptional profiling in head and neck squamous carcinoma cells treated with $1,25(OH)_2D_3$ or its analog EB1089 (24a,26a,27a,-trihomo-22,24-diene-1alpha,$25(OH)_2D_3$; Seocalcitol) (34, 37). The differences could be due to the distinct set of genes expressed, the pathways activated or other alterations present in each cell type.

In summary, the anti-proliferative, pro-differentiation and pro-apoptotic effects of $1,25(OH)_2D_3$ in colon cancer cells are well established and support a beneficial effect of its analogs in colon cancer prevention and treatment. However, down-regulation of *VDR* expression during colon tumor progression causes loss of responsiveness to $1,25(OH)_2D_3$ and its analogs. *VDR* gene is subjected to a complex transcriptional regulation: a number of transcription factors such as Wilm´s tumor suppressor, Zeb-1, Cdx-2 and Sp1 induce human or rodent *VDR* gene expression (104-107). Recently, we have reported that the transcription factor SNAIL binds to the human *VDR* gene promoter and represses its expression leading to $1,25(OH)_2D_3$ unresponsiveness in SW480-ADH cells (77). Furthermore, *SNAIL* gene up-regulation in human colon tumors correlates with *VDR* down-regulation (77), whose clinical implications will be discussed in section 5.2. Clearly, further studies are needed for a better understanding of *VDR* gene regulation in colon cancer cells.

3.2. Breast cancer cells

The mammary gland is responsive to $1,25(OH)_2D_3$. Normal breast cells express VDR (108), and this expression is dynamically regulated during pregnancy and lactation (109, 110). Studies in animal models suggest that $1,25(OH)_2D_3$ may play a role in differentiation and milk production (109, 110). Multiple data indicate that $1,25(OH)_2D_3$ and its analogs may act as cancer-preventing factors in normal mammary epithelial cells, and epidemiological studies have suggested an association between vitamin D deficiency and breast cancer risk (111).

Colston and others have shown that VDR is also expressed in breast cancer cells and that $1,25(OH)_2D_3$ has profound effects in this cell type, modulating cell growth, differentiation, and invasion (112-114). There appears to be an association between VDR levels and prognosis, as tumor receptor status may be positively related to disease-free survival (115-116). Moreover, a link between polymorphisms of the *VDR* gene and breast cancer risk has been reported (117-120). In contrast to its action in colon cells, EGF (also IGF-I and estradiol) induces *VDR* expression in MCF-7 breast cells (121). Also the phytoestrogens resveratrol and genistein, and the 12-O-tetradecanoylphorbol-13-acetate (TPA) phorbol ester have this same effect, as does activation of the JNK and p38 MAPK pathways (121-123). In contrast, treatment of MCF-7 cells with tamoxifen or a combination of estrogen and

tamoxifen decreases VDR levels (121). These results might explain the increased sensitivity to vitamin D compounds of estrogen receptor (ER)-positive breast cancer cells compared to ER-negative cells (114). In fact, ERalpha and VDR seem to regulate each other's transcription in breast cancer cells (121, 124). The sensitivity to selective estrogen-receptor modulators (SERMs) and vitamin D analogs has been proposed to be inverse, indicating that the sensitivity to vitamin D analogs might increase after development of anti-estrogen resistance, and *vice versa* (125). Cooperative effects of sequential or combined treatment with vitamin D analogs and SERMs have been demonstrated in both MCF-7 and ZR75-1 cells (126, 127). Recently, Swami and colleagues have studied the gene expression patterns in ERalpha positive MCF-7 and ERalpha negative MDA-MB-231 human breast cancer cells following $1,25(OH)_2D_3$ treatment using transcriptional profiling. The gene expression profiles of the two cell lines were different, with a few overlapping genes suggesting that different cellular pathways might be regulated by $1,25(OH)_2D_3$ to trigger its actions in ERalpha positive and ERalpha negative cells (39).

$1,25(OH)_2D_3$ and certain of its analogs induce both cell cycle arrest and morphological and biochemical features of apoptosis in breast cancer cells (128-133). Arrest in G0/G1 has been associated with up-regulation of the cell cycle inhibitors $p21^{WAF1/CIP1}$ (130, 133) and $p27^{KIP1}$ (134, 135), down-regulation of cyclins A and D1, decreased Cdk2 and Cdk2-associated histone H1 kinase activity (134) and de-phosphorylation of the retinoblastoma protein (136). The effects of $1,25(OH)_2D_3$ on the cell cycle have been linked to down-regulation of growth promoting signals, such as IGF-I or EGF, as well as up-regulation of negative growth regulators, such as TGF-beta (127, 135, 137-142). $1,25(OH)_2D_3$ and analogs have been reported to increase IGFBP-3 in MCF-7 and Hs578t cells (141), while EB1089 enhanced the expression of IGFBP-5 mRNA and protein in MCF-7 cells (139, 140). In addition, $1,25(OH)_2D_3$ and its analogs reduced the expression of type I IGF-R in these cells, although this is a late event and might not be relevant to the effects on the IGF-I signaling pathway (143, 144). In fact, the precise mechanism responsible for the attenuation by vitamin D compounds of IGF-I effects in breast cancer cells remains to be determined. $1,25(OH)_2D_3$ also inhibits serum-induced activation of ERK-1 and ERK-2 kinases in MCF-7 cells through a non-genomic mechanism that involves Src tyrosine kinase phosphorylation and binding to VDR (145). Interestingly, the anti-proliferative effect of $1,25(OH)_2D_3$ in MCF-7 (and also in some prostate) cancer cells has been associated with the induction of *BRCA1* mRNA and protein *via* transcriptional activation (146).

While the molecular mechanisms by which vitamin D derivatives induce apoptosis in breast cancer cells are not fully understood, there is growing evidence of an involvement of the BCL-2 family of proteins. A decrease in the relative expression of anti-apoptotic BCL-2 and $BCL\text{-}X_L$ to pro-apoptotic BAX and BAK proteins has been reported in a number of systems in response to vitamin D compounds (130, 147). Overexpression of *BCL-2* renders MCF-7 cells resistant to $1,25(OH)_2D_3$ mediated apoptosis (148). Up-regulation of other pro-apoptotic related proteins, such as clusterin, cathepsin B and TGF-beta, has also been reported in response to $1,25(OH)_2D_3$ (130, 131, 147). Additionally, $1,25(OH)_2D_3$ down-regulates the anti-apoptotic protein survivin in MCF-7 cells through a mechanism involving activation of the TGF-beta and p38 MAPK pathways. In turn, forced expression of survivin blocks $1,25(OH)_2D_3$-mediated G1 arrest and increases S and G2/M populations (149).

Although caspase inhibitors block the late stages of $1,25(OH)_2D_3$-induced MCF-7 cell apoptosis, the commitment to death occurs even if caspase activation is prevented (148). Induction of apoptosis by vitamin D compounds appears to be independent of *TP53* gene as it occurs in T47D breast cancer cells which have a mutated *TP53* (148). Cell death in MCF-7 cells treated with $1,25(OH)_2D_3$ involves dissipation of mitochondrial membrane potential, a hallmark of apoptosis, and is associated with a release of cytochrome C and an increase in the generation of reactive oxygen species (ROS) (150). The increase in ROS after $1,25(OH)_2D_3$ treatment is concomitant with the down-regulation of thioredoxin, a small redox protein that neutralizes ROS and can prevent oxidative stress-induced apoptosis (121). Interestingly, mitochondrial disruption in MCF-7 cells treated with $1,25(OH)_2D_3$ persists even in the presence of caspase inhibitors, which in contrast can inhibit this process when it is induced by tumor necrosis factor (TNF)-alpha, a classical pro-apoptotic factor (150). Emphasizing the involvement of mitochondrial signaling in $1,25(OH)_2D_3$-mediated apoptosis, the pro-apoptotic BAX protein translocated from the cytosol to the mitochondria in MCF-7 cells treated with the hormone (150).

Cellular calcium has been implicated in the induction of apoptosis. Treatment of MCF-7 breast cancer cells with $1,25(OH)_2D_3$ and its analogs induces a sustained increase in intracellular calcium leading to the activation of the calcium-dependent pro-apoptotic proteases micro-calpain and caspase-12 (151). The selective inhibition of calcium binding sites of micro-calpain or the ectopic expression of the calcium-binding protein calbindin-D decreased apoptotic indices in $1,25(OH)_2D_3$-treated cells (151, 152). Furthermore, $1,25(OH)_2D_3$ treatment has been shown to enhance cellular sensitivity to other triggers of apoptosis such as anti-estrogens, TNF-alpha, interleukins, radiation and chemotherapeutic drugs, such as adriamycin and taxol (153-160). These findings suggest a novel apoptotic pathway in breast cancer cells treated with vitamin D compounds, and that cross-talk between distinct apoptosis pathways may occur.

$1,25(OH)_2D_3$ affects the invasive capacity of human breast cancer cells. ER-negative MDA-MB-231 cells respond poorly to vitamin D and its analogs when grown in monolayer culture. However, Hansen and colleagues demonstrated that $1,25(OH)_2D_3$ and two of its analogs inhibit invasion of these cells in the Boyden chamber assay (161). Reduced invasiveness of MDA-MB-231 cells in response to $1,25(OH)_2D_3$ is 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 MMP inhibitor 1 (59).

3.3. Prostate cancer cells

Several studies support an association between $1,25(OH)_2D_3$ and/or $25(OH)D_3$ and prostate cancer, while others have yielded conflicting results. $1,25(OH)_2D_3$ has anti-proliferative and pro-differentiating effects in prostate cancer cell lines, and levels of circulating $25(OH)D_3$ may be important as prostate cancer cells possess 25-hydroxyvitamin D_3 1-alpha-hydroxylase activity (162).

Campbell and co-workers (163) observed an inhibition of proliferation of LNCaP, PC3 and DU145 cells by a 19-nor-hexafluoride vitamin D_3 analog, and a synergistic effect of this compound with a retinoid (SR11238) having anti-AP-1 activity. This effect involved the induction of $p21^{WAF1/CIP1}$, $p27^{KIP1}$ and E-Cadherin. EB1089 extensively inhibited the growth of LNCaP cells, causing accumulation in G0/G1 (164). $1,25(OH)_2D_3$ analogs have also been used in combination with other compounds in order to check the effects on the proliferation of malignant prostate cancer cells. Thus, combination therapy with ketoconazole (a general inhibitor of P450 enzymes, some of which are necessary for androgen biosynthesis and the metabolism of vitamin D compounds) and calcitriol or EB1089 may enhance the anti-tumor activities of these compounds in prostate cancer and alleviate side effects of $1,25(OH)_2D_3$ deficiency that may be associated with ketoconazole therapy (165).

Yang and co-workers (166) showed that $1,25(OH)_2D_3$ inhibited G1-to-S progression in LNCaP prostate cancer cells through $p27^{KIP1}$ stabilization and Cdk2 mislocalization to the cytoplasm. Using the same cell line, Rao *et al* (167) showed that $1,25(OH)_2D_3$ and genistein cooperate to increase the stability of the VDR protein and to up-regulate the levels of $p21^{WAF1/CIP1}$. Bao *et al* (168) have shown that androgen signaling is required for $1,25(OH)_2D_3$-mediated growth inhibition in human prostate cancer cells. They demonstrated that the androgen receptor (AR)-positive LNCaP and CWR22R cell lines are more sensitive than the AR-negative PC3 or DU145 lines. $1,25(OH)_2D_3$ treatment inhibited Cdk2 activity and induced G0/G1 arrest. Supporting these data, $1,25(OH)_2D_3$ inhibits fatty acid synthase (FAS) expression by stimulating the expression of long-chain fatty acid CoA ligase 3 (FACL3/ACS3) in LNCaP cells (169). This takes place through an androgen/AR-mediated pathway (170), which might be one of the mechanisms of the anti-proliferative effect of $1,25(OH)_2D_3$ in LNCaP cells.

Another study has shown that combined treatment with $1,25(OH)_2D_3$ and 9-*cis*-retinoic acid (9cRA) inhibits the transcription of the telomerase reverse transcriptase (*TERT*) gene in LNCaP and PC3 cells. *TERT* codes for the catalytic subunit of telomerase, and contains in its promoter a VDRE bound by the VDR-RXR heterodimer (171). In ALVA-31 cells, complete G0/G1 arrest and approximately 50% inhibition of tumor stromal cell growth has been observed (42). By transcriptional profiling, a number of genes related to potential mechanisms of prostate growth regulation have been found up- and down-regulated by $1,25(OH)_2D_3$. These include those coding for ER, heat shock proteins 70 and 90, Apaf-1, HER-2/NEU, and paxillin (42).

The finding that normal prostate epithelial cells are acutely sensitive to the anti-proliferative action of $1,25(OH)_2D_3$, while prostate cancer cell lines and primary cultures display a range of sensitivities suggests that cancer cells have a number of mechanisms (genetic, epigenetic, post-translational modifications) that inhibit the expression of VDR and its target genes. $1,25(OH)_2D_3$ sensitivity (estimated as *GADD45alpha* induction) can be restored by co-treatment with low doses of histone deacetylation inhibitors such as trichostatin A (TSA), which is concordant with the finding that insensitive prostate cancer cell lines showed epigenetic repression of *VDR* transcription (172). A possible explanation for this finding is that PC3 and DU145 cells express respectively 1.8- and 2-fold higher level of the VDR co-repressor *SMRT* mRNA relative to normal prostate cells, which impedes *GADD45alpha* induction by $1,25(OH)_2D_3$ (173). Additionally, prostate cancer cells have greatly decreased activity of 25-hydroxyvitamin D_3 1alpha-hydroxylase and are therefore very inefficient in the conversion of circulating $25(OH)D_3$ into $1,25(OH)_2D_3$ (174).

An interesting study has shown that $1,25(OH)_2D_3$ enhances ionizing radiation (IR)-induced apoptosis of LNCaP cells, and that nanomolar doses of $1,25(OH)_2D_3$ and 19-nor-1alpha,$25(OH)_2D_2$ showed synergistic inhibition of growth of LNCaP cells at radiobiologically relevant doses of IR (175). At higher doses of IR, the combination of $1,25(OH)_2D_3$ and IR or 19-nor-1alpha,$25(OH)_2D_2$ and IR resulted in moderate antagonism.

3.4. Cancer cells of diverse origin

3.4.1. Head and neck squamous carcinoma

Early stage head and neck squamous cell carcinoma (HNSCC) is often treated with surgery and radiotherapy. However, a high percentage of patients develop second primary carcinomas. For this reason, there is an intense search for chemotherapeutic drugs for HNSCC treatment (176). $1,25(OH)_2D_3$ and EB1089 have anti-proliferative, genoprotective and pro-differentiation effects in HNSCC cell lines (37, 177). Both inhibit proliferation by arresting HNSCC cells in G0/G1 phase rather than by inducing apoptosis (27, 34, 178, 179). However, the mechanism of this anti-proliferative action is not entirely clear. While $p27^{KIP1}$ expression does not change after $1,25(OH)_2D_3$ treatment in human head and neck SCC25 cells (34), $1,25(OH)_2D_3$ increases $p27^{KIP1}$ protein levels in mouse AT-84 and SCCVII/SF cell lines (27, 30, 178). It has been reported that EB1089 raises $p27^{KIP1}$ levels by increasing $p27^{KIP1}$ protein stability through the repression of cyclin kinase subunit 1 and F-box protein $p45^{SKP2}$, which are components of the SCF^{SKP2} ubiquitin ligase complex implicated in $p27^{KIP1}$ proteasomal degradation (30). $p45^{SKP2}$ repression is relevant because its over-expression is

associated with a poor prognosis in HNSCC (180). On the other hand, $1,25(OH)_2D_3$ does not change or even inhibits $p21^{WAF1/CIP1}$ expression in HNSCC cell lines (27, 34, 178).

Transcriptional profiling has helped to identify novel target genes mediating the anti-proliferative effects of $1,25(OH)_2D_3$ in HNSCC (34, 37, 177). One example is amphiregulin, a member of the EGF family that inhibits growth of SCC25 cells (34, 179). Amphiregulin induction by $1,25(OH)_2D_3$ has also been observed in human breast and colon carcinoma cells (43, 179), and therefore may contribute in an autocrine or paracrine manner to the growth inhibitory effects of $1,25(OH)_2D_3$ in different cell systems. In addition, transcriptome analyses have revealed that $1,25(OH)_2D_3$ down-regulates the expression of several mitogenic factors in HNSCC cells including VEGF-related protein, midkine and Cyr61, a growth factor implicated in angiogenesis and tumorigenesis (37, 177).

As in colon cancer and insulinoma cells (35, 181), $1,25(OH)_2D_3$ induces the expression of *GADD45alpha* gene in HNSCC cells (27, 34). *GADD45alpha* promotes cell cycle arrest and DNA repair in response to p53 induction due to DNA damage, and is required for the maintenance of genomic stability. However, $1,25(OH)_2D_3$ and its analogs are not DNA damaging agents and their induction of *GADD45alpha* is p53-independent (27, 34). As happens in colon SW480 cells (35), $1,25(OH)_2D_3$ also induces the expression of enzymes that are implicated in the control of redox balance, implicating $1,25(OH)_2D_3$ as an anti-oxidant agent (37, 177). The identification of several target genes has provided evidence that $1,25(OH)_2D_3$ and EB1089 drive SCC25 cells towards a more differentiated state and revert their malignant phenotype (37, 177). EB1089 represses the expression of several markers associated with cancer progression, such as squamous cell carcinoma antigen (SCCA), tenascin C and N-cadherin. Repression of N-cadherin by EB1089 is associated with restoration of an epithelial phenotype (37, 177). In addition, EB1089 induces several genes associated with epithelial cell differentiation: cystatin M, protease M, type XIII collagen, desmoglein-3 and calmodulin-like protein. In view of these results, $1,25(OH)_2D_3$ analogs could be considered as candidates for HNSCC treatment, alone or in combination with radiotherapy or other chemotherapeutic drugs.

3.4.2. Pancreatic cancer

There is an increasing interest in the study of possible effects of $1,25(OH)_2D_3$ and analogs against this highly aggressive and incurable cancer. The effects of 9cRA and the vitamin D analogs EB1089 and CB1093 on three pancreatic adenocarcinoma cell lines (AsPc-1, Bx-PC-3, T3M-4) were investigated (182). All these compounds caused growth inhibition, but the vitamin D analogs were generally more potent. They were also more effective on their own than in combination with 9cRA. Growth arrest correlated with an increased proportion of cells in the G0/G1 phase. Apoptosis was induced in all three cell lines by 9cRA, whereas neither EB1089 nor CB1093 had this effect. Furthermore, addition of EB1089 or CB1093 together with 9cRA significantly reduced apoptosis.

Colston *et al* (183) observed that EB1089 inhibits the growth of the GER cell line expressing significant amounts of VDR, although the mechanism of action was not studied. More biochemical data were obtained by Kawa and co-workers (184). These authors analyzed the growth-inhibitory effect of $1,25(OH)_2D_3$ and 22-oxa-$1,25(OH)_2D_3$. In responsive cells (Bx-PC-3, Hs 700T and SUP-1), there was marked up-regulation of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ after 24 h treatment and marked down-regulation of cyclins, Cdks and Cdk inhibitors seven days after treatment. In non-responsive cells (Hs 766T and Capan-1), no such changes were observed.

Remarkably, VDR has been found expressed in pancreatic tumors and in all cell lines established from primary cultures of them. VDR expression in cancer cells was higher (3-fold) than in normal pancreas, and treatment of the cancer cell lines with high doses (10^{-5} M or higher) of EB1089 decreased cell number (185). Recently, Ohlsson *et al* (186) reported that $1,25(OH)_2D_3$ decreases the proliferation of several pancreatic cancer cell lines at low concentrations (10^{-10} M) and Schwartz *et al* (187) showed that the pro-hormone $25(OH)D_3$ inhibited the growth of three of the four lines tested in a manner that correlated with the level of induction of $p21^{WAF1/CIP1}$ and $p27^{KIP1}$ and with the induction of cell cycle arrest at the G1/S checkpoint. Furthermore, the anti-proliferative effect of $25(OH)D_3$ is independent of the presence of mutated *K-Ras* gene.

3.4.3. Blood cancers

There is increasing evidence that $1,25(OH)_2D_3$ and its analogs could be used in the prevention and treatment of several forms of blood-related cancer, although some of the results are slightly contradictory. Twenty years ago $1,25(OH)_2D_3$ was found to induce the maturation of the promyelocytic leukemia HL60 cell line to macrophage-like cells (188). High doses (10^{-6} M) also induced differentiation *in vitro* of leukemia blasts from patients, though no enduring therapeutic effect could be seen (189). Remarkably, while cells from myeloid leukemic lines that contained relatively mature cells (HL60, U937, THP, HEL, M1) were induced to differentiate and were inhibited in their clonal growth, the myeloblast KG-1 line and normal human granulocyte-monocyte stem cells which depend on colony-stimulating factor were stimulated in their clonal proliferation by the hormone in the presence of suboptimal concentrations of this factor (190). Exposure of myeloid leukemia cells to $1,25(OH)_2D_3$ analogs results in monocytic-like maturation. It has been reported that brief treatment of HL60 cells with differentiation-inducing concentrations of $1,25(OH)_2D_3$ makes these cells resistant to cell death by apoptosis, although Northern and Western blot analysis showed that the expression of *BCL-2* proto-oncogene was rapidly reduced by $1,25(OH)_2D_3$ (191). This anti-apoptotic effect of $1,25(OH)_2D_3$ seems to be related with increased mitochondrial MCL-1 and RAF-1 proteins and reduced release of cytochrome C (192). Treatment of HL60 cells with various combinations of retinoids and $1,25(OH)_2D_3$ resulted in cell differentiation to neutrophils or monocytes, or in a failure to differentiate and apoptosis (193). Combination of either 9cRA or all-*trans* RA and

$1,25(OH)_2D_3$ resulted in a different cell fate, which has important implications for the use of combinations of these agents in differentiation therapy. The combination of the 20-epi-vitamin D_3 analog KH1060 with 9cRA irreversibly inhibits clonal growth, decreases BCL-2 expression, and induces apoptosis in HL60 cells (194). CB1093 has proved to be the most efficient $1,25(OH)_2D_3$ analog tested so far in inducing differentiation and inhibiting proliferation of HL60 cells (195).

Jung *et al* (196) showed EB1089-induced inhibition of HL60 cell proliferation, with up-regulation of the expression of type I and II TGF-beta receptors and TGF-beta1. VDR expression was increased by TGF-beta1, suggesting synergistic action of TGF-beta1 and EB1089. Combined treatment of EB1089 and TGF-beta1 increased expression of $p27^{KIP1}$ protein compared to either ligand alone. Moreover, EB1089 induces apoptosis via a p53-independent mechanism involving p38 MAPK activation and suppression of ERK activity in B-cell chronic lymphocytic leukemia cells *in vitro* (197). Additionally, $1,25(OH)_2D_3$ and bryostatin-1 synergistically induce monocytic differentiation of NB4 acute promyelocytic leukemia cells by modulating cell cycle progression (198). The combined treatment decreases expression of CDK2, CDK1, CDK4, Cyclins E and D3, and RB binding protein (RBBP) (199).

NB4 and HL60 cell growth is also inhibited by the analog 21-(3-methyl-3-hydroxy-butyl)-19-norD_3 (Gemini-19-nor). This compound induces differentiation and apoptosis, strongly inducing the expression of CD11b and CD14 on HL60 cells, and also of $p27^{KIP1}$ and PTEN (200). The combination of Paricalcitol (19-nor-1alpha,$25(OH)_2D_2$) with arsenic trioxide (As_2O_3) shows marked enhanced anti-proliferative effect and induces monocytic differentiation of NB4 and HL60 cells, probably because As_2O_3 increases the transcriptional activity of Paricalcitol by increasing its intracellular levels *via* the reduction of the function of the 24-hydroxylase (201).

The effects of $1,25(OH)_2D_3$ analogs have also been explored in myeloma cells. Puthier and co-workers (202) showed that $1,25(OH)_2D_3$ and EB1089, alone and in synergy with dexamethasone, leukemia inhibitory factor and Oncostatin M, induced cell growth arrest and apoptosis in LP1, NCI-H929, RPMI 8226 and SBN1 cells. EB1089 down-regulated the expression and production of soluble interleukin-6 receptor alpha chain (gp80), and inhibited its deleterious up-regulation by dexamethasone. In FO mouse myeloma cells, $1,25(OH)_2D_3$ decreased cell count and increased apoptosis (203).

3.4.4. Melanoma

In 1981 Colston K., Colston M. J. and Feldman D. described the presence of specific, high-affinity receptors for $1,25(OH)_2D_3$ in malignant melanoma and that the growth of human melanoma cells *in vitro* was inhibited by the hormone. This was the first demonstration of an action of $1,25(OH)_2D_3$ on tumor cells (204). Melanoma cells show cell-type specific responses to $1,25(OH)_2D_3$. MeWo and WM1341 cells express similar amounts of *VDR* mRNA and show functional gene regulatory effects of $1,25(OH)_2D_3$ but, nevertheless, only WM1341 cells undergo apoptosis (205). Further investigations indicated that physiological concentrations of $1,25(OH)_2D_3$ did not induce apoptosis in primary melanocytes despite a cell growth inhibitory effect (206). $1,25(OH)_2D_3$ treatment renders melanocytes resistant to several apoptosis inducers including TNF-alpha and ultraviolet radiation. This anti-apoptotic effect was completely abolished by the addition of N,N-dimethylsphingosine, which blocks the formation of the sphingolipid degradation product sphingosine 1-phosphate (S1P), suggesting a crucial role for this sphingolipid in $1,25(OH)_2D_3$-mediated cytoprotection (206). This anti-apoptotic effect of S1P contrasts with its reported mitogenic action in fibroblasts and other cells types. In contrast, again, to what happens in epithelial cells, neither $1,25(OH)_2D_3$ nor S1P altered the BCL-2/BAX ratio in melanocytes.

High RNA expression of *VDR*, *CYP27A1*, *CYP27B1* and *CYP24* has been found in MeWo, SKMEL28 and BU47HOM cells (207). In these cell lines, $1,25(OH)_2D_3$ and several analogs suppressed proliferation, but did not induce apoptosis, by mechanisms that include induction of VDR and 24-hydroxylase expression as well as histone deacetylation and calpain activity.

3.4.5. Miscellaneous

Studies on $1,25(OH)_2D_3$ action in human liver cancer cells are scarce. Liver has long been considered negative for VDR expression. However, recent studies have shown expression of functional VDR in some liver cell types and human hepatocarcinomas (208). Accordingly, Morris´ group (209-211) has reported that EB1089 and CB1093 profoundly inhibit the proliferation of HepG2 cells *in vitro*.

Data on $1,25(OH)_2D_3$ effects on bladder cancer are still rather preliminary. Nevertheless, it has been reported to inhibit proliferation and induce apoptosis in at least two human bladder cancer cell lines, 253j and T-24, by unknown mechanisms (212).

4. ANTI-TUMOR ACTION OF $1,25(OH)_2D_3$ AND ANALOGS IN EXPERIMENTAL ANIMALS

Studies with experimental animals play a pivotal role in late pre-clinical agent optimization, and guide the selection of candidates for phase I clinical trials. The anti-cancer activity of $1,25(OH)_2D_3$ and its analogs has been demonstrated *in vitro* and *in vivo* by evaluating their activity and toxicity in different animal models. Four models have been used: mice/rats fed with high-fat diets or subjected to carcinogenic treatment (Table 1), immunodeficient mice implanted with human tumor xenografts (Table 2), and mice with germline mutations in genes involved in the process of carcinogenesis or in vitamin D biology (*VDR*, *CYP27B1*).

4.1. Results in diet- or carcinogen-treated animal models

Epidemiological and laboratory studies have suggested that a high-fat diet, with low levels of vitamin D,

Table 1. Effects of vitamin D compounds on animal models of chemical carcinogenesis

Organ	Carcinogen	Treatment	Comments	Ref.
Breast	DMBA	Vitamin D deficient diet	Increase in the incidence of mammary lesions	217
	MNU	1alpha(OH)D_5	Reduction of tumor incidence and multiplicity	219
		1alpha(OH)D_5	Tumor inhibition if treatment during the promotion phase	220
		EB1089	Tumor regression, partially due to apoptosis	221, 222
		9cRA + 1,25$(OH)_2D_3$	Decrease of mammary cancer incidence	223
		Ro24-5531	Reduction of tumor incidence	224
		Ro24-5531 + tamoxifen	Reduction of total tumor load and prevention of carcinogenesis	224
Colon	MNU	1alpha$(OH)D_3$	Reduction of colonic tumorigenesis	225
	DMH	Vitamin D deficient diet	Abolition of the protective effects of calcium supplementation	228
		1,25$(OH)_2D_3$	Tumor incidence reduced if treatment prior to the carcinogen	212
		24R,25$(OH)_2D_3$	Decrease of ACF incidence if treatment in the post-initiation phase	229
		Ro25-5317 or Ro25-9022	Reduction of tumor incidence and of spontaneous metastases	64
	AOM	Ro24-5531	Reduction of cyclin D1 increase and E-cadherin decrease in ACF	231
		1,25$(OH)_2D_3$ or 1alpha$(OH)D_3$	Decrease of proliferation and angiogenesis	58
Skin	DMBA/TPA	1,25$(OH)_2D_3$	Reduction of tumor incidence	233
		1beta-hydroxymethyl hybrid	Reduction of tumor incidence and multiplicity of papilloma formation	234

DMBA: 7,12-dimethylbenzanthracene; MNU: N-methyl-N-nitrosourea; DMH: 1,2-dimethylhydrazine; AOM: azoxymethane; TPA: 12-*O*-tetradecanoylphorbol-13-acetate; EB1089: 24a,26a,27a-trihomo-22,24-diene-1,25$(OH)_2D_3$; 9cRA: 9-*cis*-retinoic acid; Ro24-5531: 1,25$(OH)_2$-16-ene-23-yne-26,27-hexafluoro-D_3; Ro25-5317: 1,25$(OH)_2$-16,23Z-diene-26,27-hexafluoro-D_3; Ro25-9022: 1,25$(OH)_2$-16,23E-diene-26,27-hexafluoro-19-nor-D_3; ACF: aberrant crypt foci

Table 2. Xenograft models showing anti-tumor activity of vitamin D compounds

Tumor cell type	Cell line injected	Treatment	Comments	Ref.
Breast	MDA-MB-231	EB1089	Prevention of metastasic bone lesions	243
	MCF-7	EB1089/EB1089 + IR	Induction of apoptosis	153, 244
	BT-474	1alpha$(OH)D_5$	Analog crosslinked to HER-2 antibody	258
	UISO-BCA-4	1alpha$(OH)D_5$	Absence of hypercalcemia	257
	MX-1/MCF-7	OCT	Injected ER-negative and ER-positive breast cells	123, 250
	MCF-7	OCT + tamoxifen	Injected ER-positive breast cells	123
	MCF-7/MDA-435S	1,25$(OH)_2D_3$	MCF-7 overexpressing VEGF/tumors less vascularized	59
Colon	SW480-ADH	EB1089	Ineffective for SW480-R cells (VDR-negative)	74
	LoVo	EB1089	Hypercalcemia	242
	HT29	Ro25-6760	Ineffective for SW620 expressing very low levels of VDR	248
	HT29	Paricalcitol	Reduction of tumor growth	247
Prostate	LNCaP	EB1089	Reduction of tumor growth	161
	Mat-LyLu	EB1089	Reduction of lung metastases	260
Pancreas	GER	EB1089	Absence of hypercalcemia	180
	Bx-PC-3	OCT	Absence of hypercalcemia	251
	FA-6, PAN-7	OCT	Useful for treatment of cancer-associated hypercalcemia	252
	FA-6	OCT + AHPrBP	Synergistic effect	252
Retinoblastoma	Y-79	16,23-D_3	Effective in large tumor study, but not in long-term study	249
	Y-79	1alpha$(OH)D_2$	Effective in large tumor study and in long-term study	249
Squamous	KCC-1, LC-6, PHA-1	OCT	Useful for treatment of cancer-associated hypercalcemia	252
	HPK1A*ras*	EB1089	Reversal of hypercalcemia	245
Liver	SKHEP-1	1,25$(OH)_2D_3$	Absence of hypercalcemia	254
Thyroid	WRO	1,25$(OH)_2D_3$	Restores differentiation and prevents metastasic growth	255
Melanoma	COLO 239F	1,25$(OH)_2D_3$	Ineffective for RPMI 7932 cells (VDR-negative)	253
Kaposi sarcoma	KS Y-1	1,25$(OH)_2D_3$	Tumor growth retardation	256

EB1089: 24a,26a,27a-trihomo-22,24-diene-1,25$(OH)_2D_3$; IR: ionizing radiation; OCT: 22-oxa-1,25$(OH)_2D_3$; Ro25-6760: 1alpha,25$(OH)_2$-16-ene-23-yne-26,27-hexafluoro-19-nor-D_3; Paricalcitol: 19-nor-1alpha$(OH)D_2$; AHPrBP: disodium 3-amino-1-hydroxypropylidene-1,1-bisphosphonate pentahydrate; 16,23 D_3: 1,25-$(OH)_2$-16-ene-23-yne-D_3; ER: estrogen receptor; VEGF: vascular endothelial growth factor; VDR: vitamin D receptor

increases the risk of cancer development in the pancreas, prostate, colon, and breast (213). In long-term studies, it has been reported that wild-type mice fed with a Western-style diet (high fat and phosphate and low vitamin D and calcium content) showed hyperproliferation in epithelial cells of these tissues in the absence of carcinogen exposure (213-216). Short periods (12 weeks) on these diets were sufficient to induce colon-crypt hyperplasia (217). Furthermore, these effects were markedly suppressed when Western-style diets were supplemented with calcium and vitamin D, suggesting that hyperproliferation could be prevented by increasing dietary calcium and vitamin D (213). The effects of a Western-style diet were also tested in mutant Apc^{min} mice, a model of intestinal carcinogenesis that develops multiple neoplasias throughout the intestinal tract within a few weeks of birth. Mice used in this study carried a truncated *Apc* allele with a nonsense mutation in exon 15 (Apc1638). The numerous polyps that developed in normal conditions (standard AIN-76A diet) were significantly increased when the mice were fed the

Western-style diet, and their survival diminished (218). Apc^{min} mice were also treated with $1,25(OH)_2D_3$ and the synthetic analog Ro26-9114 (1alpha,25$(OH)_2$-16-ene-19-nor-24-oxo-D_3). After 10 weeks of treatment tumor number was not significantly affected, although there was a significant decrease in total tumor load (sum of all polyp areas) over the entire gastrointestinal tract. In the group treated with the vitamin D analog there was a 36% decrease in total tumor load, with no severe toxic side effects, while the reduction was 46% in the $1,25(OH)_2D_3$-treated group, which showed significantly increased calcemia (219). Another study in different tissues (skin, liver, colon, stomach) of normal mice, demonstrated that $1,25(OH)_2D_3$ inhibited the induction by tumor promoters of ornithine decarboxylase (ODC) (220). Importantly, ODC is the rate-limiting enzyme in the polyamine biosynthetic pathway, and its activity increased in normal proliferating cells and also in intestinal cells of carcinogen-treated rats (221) and Apc^{min} mice (222).

Prevention of colorectal, breast, and prostate cancer may depend on optimal synthesis of $1,25(OH)_2D_3$. Soy feeding or, more effectively, genistein feeding for 4 months increased *CYP27B1* and reduced *CYP24* expression in the mouse colon, which resulted in high $1,25(OH)_2D_3$ levels (223). These results are consistent with the extremely low incidence of prostate, breast, and possibly colon cancer in countries such as China and Japan, which have a traditional Asian diet, rich in Soybean products containing high levels of genistein and other phytoestrogens (224).

As common laboratory rodents do not develop cancer spontaneously, they are usually treated with an initiator to induce the neoplasia so that any influence of dietary factors can be observed. The effects of vitamin D compounds on experimental carcinogenesis have mainly been studied in the mammary gland, colon and skin (Table 1). The carcinogens most frequently used to induce mammary adenocarcinoma in rats are 7,12-dimethylbenzanthracene (DMBA) and N-methyl-N-nitrosourea (MNU), with nearly 100% incidence (225). Initial studies showed that DMBA-treated female Sprague-Dawley rats fed with a high-fat diet, low in calcium and vitamin D, showed an increase in the incidence of mammary lesions from 31% to 55%, and a significant increase in average lesion burden/rat (226).

Synthetic analogs have also been tested in chemical carcinogenesis protocols, showing that they are potent inhibitors of the development of neoplasic lesions and less toxic than $1,25(OH)_2D_3$. 1alpha$(OH)D_5$ inhibited the development of DMBA-induced pre-neoplasic lesions in mammary glands in organ culture (227). Similar results showing significant reduction of tumor incidence and multiplicity were obtained in MNU-induced rat mammary carcinogenesis (228). In a recent study using MNU, diet supplementation with 1alpha$(OH)D_5$ provided no protection during the initiation phase, but it did provide protection during the promotion phase, leading to 37% inhibition of mammary cancer incidence. These results show for the first time that the effects of this analog may be mediated selectively during the promotion or progression phases of carcinogenesis (229). EB1089 causes regression of MNU-induced rat mammary tumors due at least in part to the induction of apoptosis, as tumor sections showed a marked loss of cellularity, only few mitotic cells and a considerable nuclear DNA fragmentation (230, 231). In the same model, the combination of 9cRA and $1,25(OH)_2D_3$ caused a statistically significant decrease of 44% in mammary cancer incidence in comparison with a 23% decrease obtained with 9cRA alone (232). Ro24-5531 (1alpha,25$(OH)_2$-16-ene-23-yne-26,27-hexafluoro-D_3) extended tumor latency and reduced tumor incidence in MNU-treated rats without raising serum calcium levels. Its combination with tamoxifen enhanced the reduction of total tumor load and prevention mammary carcinogenesis, suggesting that these two compounds act through independent mechanisms (233).

Similar beneficial results were obtained using the carcinogens MNU, 1,2 dimethylhydrazine (DMH) and azoxymethane (AOM) in models of colon carcinogenesis. Colonic tumorigenesis induced by the chronic treatment with MNU in rats was reduced by oral supplementation of 1alpha$(OH)D_3$ (234). Around one-third of the colonic carcinomas induced by DMH had mutated K-*ras* oncogene (235); however, no mutations were detected in the tumors developed by rats fed with calcium-supplemented diet. In this model, concomitant vitamin D deficiency abolished the anti-mutagenic effect of dietary calcium supplementation, abolishing the protective effects of calcium on tumor formation (236, 237). Further studies have shown that $1,25(OH)_2D_3$ administered prior to (but not simultaneously to or after) DMH significantly reduced (50%) the incidence of colon tumors (221). 24R,25$(OH)_2D_3$ was used in this same experimental model, causing a dose-dependent decrease in the number of aberrant crypt foci (ACF) only when administered in the post-initiation phase (238). In DMH-treated rats, Ro25-5317 (1alpha,25$(OH)_2$-16,23Z-diene-26,27-hexafluoro-D_3) and more potently Ro25-9022 (1alpha,25$(OH)_2$-16,23E-diene-26,27-hexafluoro-9-nor-D_3) inhibited tumor incidence and the rate of spontaneous metastases (67). In another study, the analog Ro24-5531 demonstrated chemopreventive effect in AOM-treated rats, reducing the incidence of tumors, most of which were benign adenocarcinomas, by 70% (239). AOM-induced hyperproliferation was accompanied by a 5-fold increase in cyclin D1 and >50% decrease in E-cadherin protein in ACF. These alterations were significantly inhibited by Ro24-5531 in ACF and tumors. Likewise, the up-regulation of cyclooxygenase-2 and inducible nitric oxide synthase in AOM-induced tumors were also blocked by this analog (240). Moreover, this compound blocked the changes in PKC-zeta expression induced by AOM in adenomas and carcinomas and induced PKC-epsilon, which may underlie its ability to prevent malignancy (241). In addition, $1,25(OH)_2D_3$ decreased proliferation and angiogenesis in AOM-treated rats, as reflected by a decrease in microvessel counts and immunohistochemical VEGF staining (61).

Topically administered $1,25(OH)_2D_3$ during the promotion phase halved the formation of chemically-induced (DMBA as initiator and 12-*O*-tetradecanoylphorbol-13-acetate (TPA) as promoter)

tumors in the mice skin (242). The analogs 1beta-hydroxymethyl hybrid and 24- or 25-t-butyl sulfones, lacking the 25-hydroxyl group, efficiently inhibited the incidence and multiplicity of papilloma formation in a similar protocol. The greatest efficacy was shown by the hybrid analog, with reduction in tumor incidence (28%) and multiplicity (63%) (243).

Taken together, these data confirm that vitamin D compounds are preventive and therapeutic agents in animal tumor models, but very few mechanistic studies have been carried out *in vivo*. In one of them, VDR has been defined as a potential bile acid sensor in the enteric tract, where elevated concentrations of the secondary bile acid lithocholic acid (LCA), which promotes colon carcinogenesis, may activate VDR (244). High-fat diet leads to LCA accumulation in the colon, which induces DNA damage and inhibits DNA repair enzymes. Accordingly, LCA promotes colon cancer in animals and high levels of LCA have been found in colorectal cancer patients (245). To confirm the results *in vitro*, Makishima *et al* treated mice with agonists for VDR ($1,25(OH)_2D_3$ and EB1089), pregnane X receptor (PXR) (pregnenolone-16alpha-carbonitrile) and farnesoid X receptor (LCA as a pan-agonist for all three receptors) (244). *CYP3A11*, a cytochrome P450 enzyme that detoxifies LCA in colon cells, increased its mRNA expression in response to both VDR- and PXR-specific ligands, as well as to LCA. In contrast, the VDR-specific target gene *calbindin 9K* was activated by LCA and also by the VDR-selective agonists, indicating that LCA can function as a VDR agonist *in vivo*. Activation of VDR by LCA or $1,25(OH)_2D_3$ induced *CYP3A11* transcription in a feed-back mechanism that results in colon LCA degradation (244, 246, 247). Therefore, this mechanism of LCA detoxification could partly explain the protective action of $1,25(OH)_2D_3$ against colon cancer. This protection may be overridden when the detoxification is saturated, for example by increased levels of LCA provided by Western-style diet (5, 244).

In rats, EB1089 caused a marked prostate regression accompanied by an increase in the expression of *IGFBP-2,-3, -4*, and *-5* mRNA and *IGF-I* gene expression. Furthermore, a significant increase in the number of apoptotic cells was observed (248). These findings suggest an interfering effect of ligand-activated VDR with the IGF-I signaling pathway that correlate with results obtained in cultured cells (35, 54, 55, 139-141).

4.2. Action of $1,25(OH)_2D_3$ and analogs against tumor xenografts

Human tumor xenografts implanted subcutaneously into immunosuppressed mice play a significant role in pre-clinical anti-cancer drug development. Studies using human cancer cells in athymic (nude) mice were first performed with $1,25(OH)_2D_3$ and $24R,25(OH)_2D_3$, and rendered optimal results of tumor growth inhibition, induction of differentiation and activation of apoptosis, but unfortunately with a simultaneous hypercalcemic effect (249, 250).

Studies from our laboratory have shown that EB1089 inhibits (30%) the growth of tumors generated by VDR-positive SW480-ADH cells, but not those generated by VDR-negative SW480-R human colon cancer cells implanted in severely immunosuppressed SCID mice (77, and unpublished results) (Table 2). EB1089 also shows activity against other human colon (LoVo) (251), breast (MDA-MB-231, MCF-7) (156, 252, 253), pancreas (GER) (183), squamous (HPK1A*ras*) (254), and prostate (LNCaP) (164) xenografts. It causes apoptotic regression of MCF-7 cells (156, 253) and prevents skeletal metastasis originated by MDA-MB-231 cells in nude mice (252). Furthermore, EB1089 acts in synergy with the effects of ionizing radiation against xenografts of breast MCF-7 cells (156). In addition, long-term administration of EB1089 significantly decreases the incidence of spontaneous hepatocellular carcinoma in C3H/Sy mice, which indicates chemopreventive activity (255). Other non-toxic analogs, Paricalcitol and Ro25-6760 ($1alpha,25(OH)_2$-16-ene-23-yne-26,27-hexafluoro-19-nor-D_3), tested in colon HT29 xenografts, have similar anti-tumoral effects (256, 257). In retinoblastoma xenografts (Y-79 cells), $16,23\text{-}D_3$ ($1alpha,25(OH)_2$-16-ene-23-yne-D_3) and $1alpha(OH)D_2$ were successfully used in a large-tumor study, but effective doses of both compounds caused toxic effects and an increase in mortality (258). OCT (22-oxa-$1alpha,25(OH)_2D_3$) has an anti-tumor effect against xenografts generated by ER-negative and ER-positive breast cells and pancreatic carcinoma cells (126, 259, 260). This analog has been used alone and in combination with AHPrBP, a biphosphonate that inhibits osteoclastic bone resorption, against cancer-induced hypercalcemia in xenografts of pancreatic (FA-6, PAN-7) and squamous cell carcinoma of the lung (KCC-1, LC-6) and pharynx (PHA-1) cells (261). OCT has synergistic action with tamoxifen against ER-positive breast cancer xenografts (126). Studies using malignant melanoma, liver, thyroid and Kaposi sarcoma cells have demonstrated anti-tumoral action of $1,25(OH)_2D_3$ in xenotransplanted nude mice (262-265). In the case of thyroid carcinoma, the reduction in tumor burden and prevention of metastasic growth after $1,25(OH)_2D_3$ administration was associated with a marked accumulation of $p27^{KIP1}$ in the cell nucleus (264).

$1alpha(OH)D_5$, which is non-toxic in short-term studies but calcemic in long-term treatments, inhibited the growth of breast UISO-BCA-4 cancer cells in athymic mice (266). Mehta and colleagues have investigated the therapeutic potential of targeted delivery of this analog to HER-2 overexpressing BT-474 breast cancer cells. The growth of BT-474 cells transplanted into athymic mice was significantly inhibited by $1alpha(OH)D_5$ covalently linked to an anti-HER-2 antibody (267).

Xenograft tumors have several limitations. They cannot be used to study anti-metastasic and anti-angiogenic strategies, since many of the components involved are of murine, rather than human, origin (268). One approach to a suitable model is the use of the androgen-insensitive metastasic rat Mat-LyLu prostate cells. $1,25(OH)_2D_3$ and EB1089 caused not only inhibition of tumor growth, but also a significant reduction in the number and size of lung

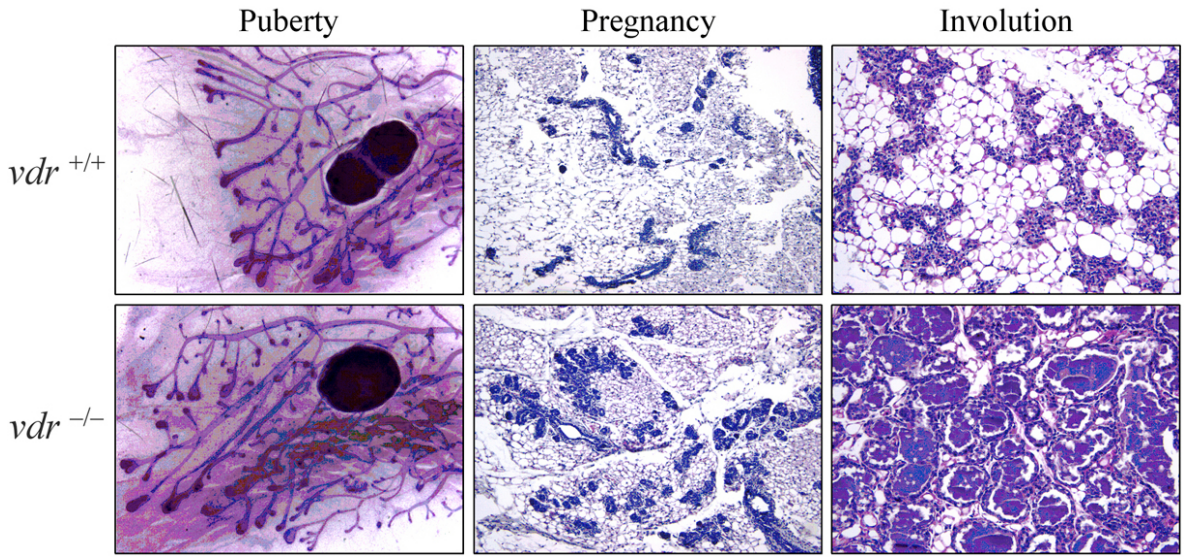

Figure 3. *vdr* ablation accelerates mammary gland development and delays post-lactational involution. Mammary glands from age-matched wild-type ($vdr^{+/+}$) and *vdr* knock-out ($vdr^{-/-}$) mice were compared during puberty (six weeks of age), pregnancy (day 9 after timed mating) and involution (day 4 after pup withdrawal). Left panels show whole mounted glands to visualize ductal development. Middle and right panels show glands that were formalin fixed, sectioned, and stained with hematoxylin and eosin. Adapted from refs. 276 and 307.

metastases generated by cells injected in Copenhagen rats. The effect of $1,25(OH)_2D_3$ was accompanied by hypercalcemia and loss of body weight, while EB1089 was less toxic and did not induce severe weight loss (269). Anti-angiogenic activity *in vivo* has been investigated in MCF-7 cells overexpressing VEGF, which were xenografted subcutaneously together with MDA-435S cells into nude mice. The administration of $1,25(OH)_2D_3$ for eight weeks produced tumors that were less vascularized than those forming in mice treated with vehicle alone, although tumor volume was not significantly altered probably due to the low dose of $1,25(OH)_2D_3$ used (62). These results highlight the potential use of $1,25(OH)_2D_3$ in both the prevention and regression of conditions characterized by pathological angiogenesis.

4.3. Results in genetically-modified mice

Transgenic mouse models have been used successfully to elucidate the molecular and cellular processes that lead to cancer initiation, progression and metastasis, and are often appropriate for therapeutic and chemopreventive trials (270).

To examine the functional role of VDR and the effects of disruption of the vitamin D signaling pathway, four independent strains of *vdr*-deficient mice were generated by different targeting strategies: Demay´s group generated animals lacking exons 3-5 of the *vdr* gene encoding the second zinc finger of the DNA-binding domain (271), while Kato´s group ablated exon 2 of the gene, which encodes the first zinc finger of the same domain (272). Later, Carmeliet´s group (273) generated mice lacking exons 1 and 2 encompassing the ligand-independent transactivation domain and the first zinc-finger. Also, Balling *et al* (24) have generated mice expressing a VDR protein lacking the first zinc-finger of the DNA-binding domain, which abrogates genomic and non-genomic hormonal actions. None of four types of homozygous mutant mice shows defects in development or growth before weaning, irrespective of the reduced expression of vitamin D target genes. After weaning, however, mutants fail to thrive, and developed alopecia, hypocalcemia, secondary hyperparathyroidism, osteomalacia, rickets and elevated serum $1,25(OH)_2D_3$. In addition, bone formation was severely impaired, a typical feature of vitamin D-dependent rickets type II (VDDR II) (271, 272). Studies in these mice confirmed a marked impairment in duodenal calcium absorption (273). However, differences have been observed between the different *vdr*-deficient mice. Homozygous null mice of Demay´s group were fertile, and survived for six months without complications (271). Also, mice generated by Balling´s group showed normal uterine, testicular, and seminal vesicle weight (24). In contrast, mice generated by Kato´s group had a shorter lifespan, and female animals showed uterine hypoplasia and impaired ovarian folliculogenesis, and male animals had also reproductive dysfunction (272). Altogether, these findings establish a critical role for VDR in promotion of intestinal calcium absorption, growth and bone formation (271, 272). In addition, mice lacking VDR display cardiac hypertrophy (274) enhanced thrombogenicity (275) and, as reviewed below, altered sensitivity to tumorigenesis.

Mammary glands from *vdr* knock-out mice are heavier and exhibit enhanced growth, as evidenced by the higher number of terminal end buds, greater ductal outgrowth, and enhanced secondary branch points compared with mammary glands from age- and weight-matched wild-type mice (276). Moreover, in the absence of VDR it has been observed accelerated glandular development during pregnancy and a delayed post-lactational involution (277) (Figure 3). In addition, their

proliferative response to exogenous estrogen and progesterone, both *in vivo* and in organ culture, is stronger (276). Disruption of VDR signaling is associated with accelerated mammary tumor development, supporting the idea that $1,25(OH)_2D_3$ and VDR participate in pathways that inhibit proliferation and induce differentiation in the mammary gland (277). Experiments in which *vdr*-deficient mice were crossed with mouse mammary tumor virus (MMTV)-*neu* transgenic mice showed that the loss of either one or both copies of *vdr* was associated with increased incidence of pre-neoplasic lesions and abnormal ductal morphology (278). Another study analyzed the sensitivity of *vdr* knock-out and wild-type mice to DMBA. Although the protocol was optimized for the induction of mammary tumors, 85% of *vdr* knock-out mice developed persistent skin tumors within 60 days of carcinogen exposure. Most of these skin tumors were classified histologically as sebaceous, squamous, or follicular papillomas. In contrast, no papillomas or any other skin lesions were observed in wild-type mice, suggesting that VDR may act as a tumor suppressor gene in the epidermis (279). In addition, there was higher percentage of DMBA-induced pre-neoplasic mammary lesions in glands from *vdr* knock-out than in wild-type mice. Furthermore, the histopathologic features of mammary tumors developed by *vdr* knock-out mice (primarily pilar tumors) were different from those of wild-type mice (primarily myoepithelial tumors) (277).

vdr-deficient mice have confirmed the protective function of VDR against colon tumor progression. These mice display enhanced proliferation in colon *descendens*, a tissue whose malignant transformation is particularly sensitive to diet. The inverse relationship between VDR levels and proliferation was determined by measuring the levels of proliferating cell nuclear antigen (PCNA) and cyclin D1. In parallel to enhanced proliferation, a highly significant increase of oxidative stress was determined with the marker of oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine, which was significantly augmented in *vdr* null mice (76, 280). In the prostate, these animals showed fat necrosis and apoptosis in the periprostatic adipose tissue, but there were no gross differences between the experimental and control group (281). Taken together, these results confirm that disruption of VDR signaling predisposes to neoplasia, enhancing sensitivity to tumorigenesis, and that *vdr* knock-out mice are an excellent model in which to examine induction and prevention of pre-malignant changes.

Several studies have used *vdr* knock-out mice to investigate the anti-metastasic effect of $1,25(OH)_2D_3$. The metastasic capacity of Lewis lung carcinoma cells expressing the green fluorescent protein (LLC-GFP) was strongly reduced in *vdr* null mice fed a normal or high calcium diet as compared to wild-type mice (282, 283). The reasons are unclear: the authors propose that the inhibition of metastasis must be due to a factor(s) that is enhanced or suppressed by *vdr* deficiency, but the possibility that the very high levels of circulating $1,25(OH)_2D_3$ in these animals due to the overexpression of the 1alpha-hydroxylase gene may act through other uncharacterized receptors cannot be ruled out. As expected, wild-type mice displayed an inverse relationship between the circulating levels of serum $1,25(OH)_2D_3$ and tumorigenesis by LLC-GFP cells.

Another interesting animal model was generated by targeted inactivation of the 25-hydroxyvitamin D_3 1alpha-hydroxylase (*CYP27B1*) gene (284). Homozygous *CYP27B1* null mice are phenotypically normal at birth, but progressively develop the symptoms of pseudovitamin D-deficiency rickets (PDDR): hypocalcemia, hypophosphatemia, hyperparathyroidism and, in the young adults, marked osteomalacia (284). $1,25(OH)_2D_3$ is not detected in these animals, which show a reduction in the expression of skin epidermal differentiation markers (285). However, no studies on tumor susceptibility have been reported to date.

The results obtained with vitamin D compounds in different animal models demonstrate their ability to inhibit the formation of chemically-induced tumors, cause regression of tumors, prevent the development of metastases and inhibit angiogenesis. Further work to substantiate their value as chemopreventive and chemotherapy agents for clinical application should be encouraged.

5. $1,25(OH)_2D_3$ AND ANALOGS AS ANTI-NEOPLASIC AGENTS IN THE CLINIC

5.1. Clinical data

Based on their effects *in vitro* and in experimental animals, $1,25(OH)_2D_3$ and certain analogs have been used in a number of clinical studies. Originally, Calcitriol was mainly used in leukemia and myelodisplasia and although there was some evidence of response, the results were largely disappointing (286, 287). The therapeutic application of $1,25(OH)_2D_3$ has been hampered by predictable hypercalcemia when it is given daily, but recently several groups have demonstrated that high-dose intermittent therapy with Calcitriol is safe (288-290). Beer and colleagues developed a phase I trial where they reported that weekly dosing of oral $1,25(OH)_2D_3$ permitted substantial dose escalation with minimal toxicity. Weekly high-dose Calcitriol (0.5 microg/kg) was then combined with weekly docetaxel in a phase II clinical trial carried out in 37 men with metastasic androgen-independent prostate cancer (AIPC). Treatment resulted in prostate specific antigen (PSA) response (defined as a confirmed 50% reduction) in 81% of the patients. This level of activity, as well as the median time to progression of 11.4 months and median survival of 19.5 months, compared favorably to results with docetaxel alone and has led to the development of a randomized trial of docetaxel with Calcitriol or placebo in the same patient population (291-293). Calcitriol has also been used in combination with paclitaxel and carboplatin in phase I studies (294, 295). In a phase II trial, 17 patients with AIPC were treated weekly with Calcitriol (0.5 microg/kg) and carboplatin. However, only one patient achieved a confirmed PSA response and four had PSA reductions ranging from 24% to 38%, which is similar to that obtained with carboplatin alone (296). In another phase

II study, Trump and colleagues combined three doses per week of Calcitriol with a weekly dose of dexamethasone in 43 patients with AIPC. 80% of the men showed a lower rate of PSA rise and 34% had stable disease or decrease in PSA (50% reduction) (297).

Much effort has been directed to identifying new analogs with potent cell regulatory effects but with a weaker calcemic effect than $1,25(OH)_2D_3$. One of the most widely used up to now is EB1089 (298). A phase I trial was designed to evaluate the calcemic effect of EB1089 in 36 patients with advanced breast and colorectal cancer. On the basis of this study, the estimated maximum tolerated dose was found to be seven microg/m^2/day for prolonged use. Six patients on treatment for more than 90 days showed stabilization of disease (299). In a phase II study, 56 patients with advanced hepatocellular carcinoma were included in an uncontrolled study of oral EB1089 treatment for one year. EB1089 was shown to reduce tumor dimensions and induce complete remission in two out of 33 evaluable patients, while 12 had stable disease and 19 had progressive disease (300). This is important because most patients with this malignant tumor are inoperable and most chemotherapy agents have minimal activity on this disease. Likewise, EB1089 has been used against inoperable cancer of the exocrine pancreas, which responds poorly to most conventional anti-cancer agents. However, the data obtained from a phase II study with 36 patients with advanced pancreatic cancer who received once-daily oral treatment with dose escalation every two weeks showed that EB1089 is well tolerated but has no objective anti-tumor activity and is ineffective in advanced pancreatic cancer (301).

Phase I (302) and phase II (303) studies using 1alpha-hydroxyvitamin D_2 in patients with AIPC have been reported. The compound was well tolerated, with no unexpected toxicity. 30% of the evaluable patients (six out of 20) experienced stable disease for more than six months suggesting possible cytostatic activity.

$25(OH)D_3$ was used in a phase IB study in patients with head and neck squamous cell carcinoma (HNSCC). Two groups of six patients were orally administered 20 or 40 microg/day $25(OH)D_3$ for six weeks. Although no clinical responses were observed, results of these pilot studies showed that $25(OH)D_3$ reduced the presence of immune suppressive $CD34^+$ cells and improved immune competence of HNSCC patients (304).

Moreover, Wieder and colleagues have reported a phase I clinical trial with another $1,25(OH)_2D_3$ analog, ILX23-7553 ($1,25(OH)_2$-16-ene-23-yne-D_3) (305). This compound has pre-clinically demonstrated anti-tumor and differentiating effects and diminished hypercalcemic effects. The drug is safe and has potential benefits at serum concentrations assessed in patients with a variety of advanced malignancies. Further studies are needed with a reformulated higher unit dose compound to determine the safety and efficacy of higher serum concentrations.

Clearly, the available clinical data on vitamin D compounds as anti-cancer agents are unsatisfactory. However, studies have been limited to a few compounds and it is evident that this therapy is still in its infancy. Ongoing and future studies will define whether any vitamin D analog can be useful in the treatment of human neoplasias.

5.2. Mechanisms of resistance to vitamin D compounds

Resistance to vitamin D compounds can appear *de novo* or be acquired by a panel of mechanisms that among others may include *VDR* repression or mutation, deficient $1,25(OH)_2D_3$ synthesis by attenuated 25-hydroxyvitamin D_3 1alpha-hydroxylase expression or activity, accelerated $1,25(OH)_2D_3$ elimination by 24-hydroxylase over-expression, and abnormal pattern of expression of co-repressors or co-activators.

The clinical response to $1,25(OH)_2D_3$ and analogs requires the expression of VDR in tumoral cells, and so the diminished levels found at late stages of colon cancer progression may be responsible for the limited number of responses found in this and perhaps other types of neoplasias. Importantly, the up-regulation of the transcription factor SNAIL has been associated with diminished *VDR* RNA expression in a series of 32 colon cancer patients (77). This result has been confirmed in a larger series of 114 individuals (F. Bonilla, unpublished data). Since *SNAIL* is up-regulated in advanced colon cancers, these data suggest that $1,25(OH)_2D_3$ analogs could be used, if toxicity is acceptable, as chemopreventive and chemotherapeutic agents to be administered during the early stages of carcinogenesis (306). In addition, the possibility that the up-regulation of *SNAIL* reported in cancers other than colon (melanoma, breast, gastric, hepatocellular) can be linked to *VDR* down-regulation, and so to unresponsiveness to vitamin D compounds, deserves to be studied.

In cultured cancer cells, other agents have been found to regulate *VDR*, *CYP24* or *CYP27B1* expression. Among them, EGF, IGF-I, estradiol, tamoxifen and phytoestrogens (84, 94, 121-123, 223, 224). Whether they modulate VDR levels, and thus vitamin D sensitivity in tumor cells *in vivo* is not well established.

6. CONCLUSIONS AND PERSPECTIVES

In recent years, there have been an increasing number of studies *in vitro*, in cultured cells, and *in vivo*, in animal models, aimed to elucidate the anti-cancer action and therapeutic potential of $1,25(OH)_2D_3$ and its analogs with reduced calcemic properties. Results indicate that these compounds are strong regulators of many processes involved in cancer initiation and progression, and thus candidates for clinical use. The use of genetically-modified mice deficient for VDR or enzymes involved in $1,25(OH)_2D_3$ metabolism are and will be in the near future, important to our understanding of the array of vitamin D activities. Undoubtedly, clinical studies will finally establish their definitive position among the anti-cancer armamentarium. In this regard, the development of analogs with additional reduced immunosuppressive effects

remains mostly unexplored. It is also obvious that a better knowledge of the molecular mechanism of action of these compounds will allow the rational design of combined treatments with other drugs and of compounds with more potent and useful biological activities.

VDR is critical for the action of $1,25(OH)_2D_3$ and its analogs. Therefore, the elucidation of the agents and conditions controlling its expression in normal and tumoral cells is crucial for the selection of patients suitable to receive vitamin D compounds as chemopreventive and chemotherapeutics agents.

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