

VITAMIN D AND CANCER: EFFECTS OF 1,25(OH)₂D₃ AND ITS ANALOGS ON GROWTH CONTROL AND TUMORIGENESIS

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1. ABSTRACT

Today, it is well established that besides playing a crucial role in the establishment and maintenance of the calcium homeostasis in the body, the active form of vitamin D, 1,25(OH)₂D₃, also acts as an effective regulator of cell growth and differentiation in a number of different cell types, including cancer cells. This has led to an increased interest in using 1,25(OH)₂D₃ in the treatment or prevention of cancer patients and to a substantial number of studies investigating the effect of 1,25(OH)₂D₃ on cancer cells. The results are encouraging, but clearly demonstrate that the therapeutic window of 1,25(OH)₂D₃ is extremely narrow due to the calcemic adverse effects of this compound. Much effort has consequently been directed into identifying vitamin D analogs with potent cell regulatory effects but with weaker effects on the calcium metabolism than those of 1,25(OH)₂D₃. In an attempt to clarify the mechanisms implicated in the cell regulatory effects of 1,25(OH)₂D₃ and eventually facilitate the process

of developing new specific vitamin D analogs, numerous investigations have been carried out with 1,25(OH)₂D₃ and its analogs. The present review will focus on the results obtained in these studies and describe some of the synthetic analogs, which have shown to be of particular interest in relation to cancer.

2. INTRODUCTION

The term "vitamin D" was first used in 1922 when McCollum and colleagues reported the identification of a new lipid-soluble substance, which they named vitamin D (1). This finding appeared to be of great importance as the new vitamin showed to be capable of preventing rickets, a bone disease, which at that time had reached epidemic proportions. It was assumed that dietary sources like cod liver oil and corn contained the nutrient, but other factors such as sunlight were also suggested to be

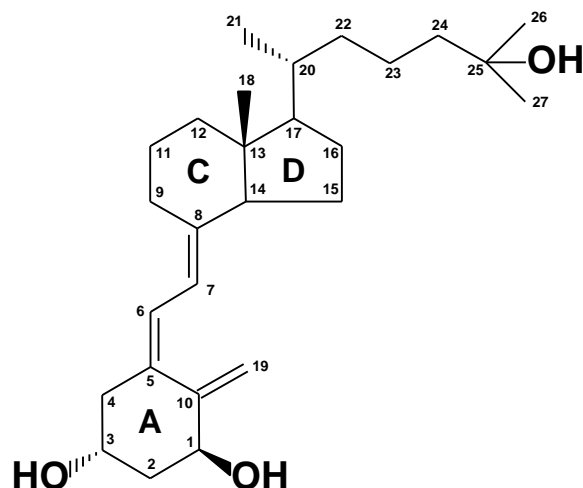


Figure 1. Chemical structure of the active metabolite of vitamin D₃, 1,25(OH)₂D₃. The numbers refer to the carbon atoms in the molecule.

involved in the positive effect on the bones (2, 3). Obviously, this led to an intensive search for the active substrate and in 1931 Askew and colleagues succeeded in isolating vitamin D₂ from irradiated plant sterols (4). Later in 1936, vitamin D₃ was identified by Windaus and colleagues (5), but it was not until the 1960's that it was demonstrated that vitamin D₃ required conversion to more biologically active metabolites in order to mediate its biological effects (6, 7). 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the metabolically active form of vitamin D₃ was identified by Holick and colleagues in 1971 and then chemically synthesised by DeLuca and coworkers in 1969-72 (8, 9).

1,25(OH)₂D₃ is structurally related to the classic steroid hormones which include the androgens, estrogens, progestins, glucocorticoids and mineralocorticoids. However, in contrast to the classic steroid hormones, the B-ring of the ring structure has undergone fission by breakage of the 9,10-carbon bond and 1,25(OH)₂D₃ is consequently designated as a seco-steroid. In addition, 1,25(OH)₂D₃ contains a side chain and is conformationally more flexible than the classic steroid hormones (figure 1) (10).

3. SYNTHESIS AND REGULATION OF 1,25(OH)₂D₃ IN THE HUMAN BODY

Today, it is well established that under normal conditions the main source of vitamin D₃ occurs via skin exposure to ultraviolet (UV) light from the sun rather than by food intake. By definition, a vitamin is an essential nutritional factor, which means that the term "vitamin D" is somewhat misleading. Vitamin D, therefore, should technically be referred to as a prohormone. Pre-vitamin D₃ is formed from 7-dehydrocholesterol in the skin in a reaction, which is catalyzed by UV light (figure 2). The thermodynamically unstable *cis*-isomer of vitamin D₃ is rapidly transformed to the more stable vitamin D₃, which is then bound to and transported by the vitamin D binding protein (DBP) from the skin into the circulation. Vitamin

D₃-bound DBP is transported to the liver where it is hydroxylated by the mitochondrial cytochrome P450 enzyme, 25-hydroxylase (CYP27A1), to 25-hydroxyvitamin D₃ (25(OH)D₃) which is the major circulating form of vitamin D. The production of 25(OH)D₃ is virtually uncontrolled and seems to correlate with substrate availability rather than with physiological needs. Therefore, measuring circulating 25(OH)D₃ levels provides a useful indicator of the actual vitamin D status in the body and is widely applied in clinical practice (reviewed in (7, 11)).

The 25(OH)D₃ present in the circulating blood pool is subsequently converted into the proximal tubule of the kidneys by another mitochondrial cytochrome P450 enzyme, 1 α -hydroxylase (CYP27B1), to the active metabolite of vitamin D, 1,25(OH)₂D₃ (12). Although, other tissues and cell types have been shown to contain CYP27B1 and to produce 1,25(OH)₂D₃ from 25(OH)D₃, the kidney is supposed to be the major site of production of circulating 1,25(OH)₂D₃ (13). In contrast to the hepatic production of 25(OH)D₃, renal synthesis of 1,25(OH)₂D₃ is a strictly regulated process that responds to the physiological needs for calcium and phosphate. Several agents have been suggested to be involved in this regulation, including protein kinase C (PKC), estrogens, prolactin, calcitonin and glucocorticoids, but the two most important physiological regulators are 1,25(OH)₂D₃ itself and parathyroid hormone (PTH) (13-15).

1,25(OH)₂D₃ acts via a negative feedback mechanism which has been suggested to take place at the transcriptional level and which eventually leads to suppression of the CYP27B1-activity (16). In addition, 1,25(OH)₂D₃ is able to stimulate 24-hydroxylase (CYP24) gene expression and thus increase the level of renal CYP24, which catalyzes the conversion of 1,25(OH)₂D₃ to the biologically inactive calcitric acid along the major catabolic pathway of 1,25(OH)₂D₃ (17-19). PTH, on the other hand, stimulates the activity of the CYP27B1 and down-regulates the CYP24 activity by interfering with both the protein kinase A (PKA) and the cAMP signaling pathways (15, 20, 21). Moreover, the level of PTH is further regulated by a direct action of 1,25(OH)₂D₃ on the PTH gene in a negative manner (22-24). Thus, together 1,25(OH)₂D₃ and PTH form a complex regulatory mechanism which serves to maintain the level of calcium and phosphate within a narrow interval.

4. BIOLOGICAL ACTIONS OF 1,25(OH)₂D₃

The classic actions of vitamin D involve establishment and maintenance of the calcium homeostasis in the body. 1,25(OH)₂D₃ mediates these effects primarily by regulating calcium absorption from the intestine, calcium excretion from the kidneys, and calcium resorption and mobilization from the skeleton. Substantial evidence has now demonstrated that one mechanism by which 1,25(OH)₂D₃ exerts its action in the intestine and in the kidneys is by interfering with specific high affinity calcium binding proteins, the calbindins, which in turn act to facilitate and regulate transcellular calcium transport. Two

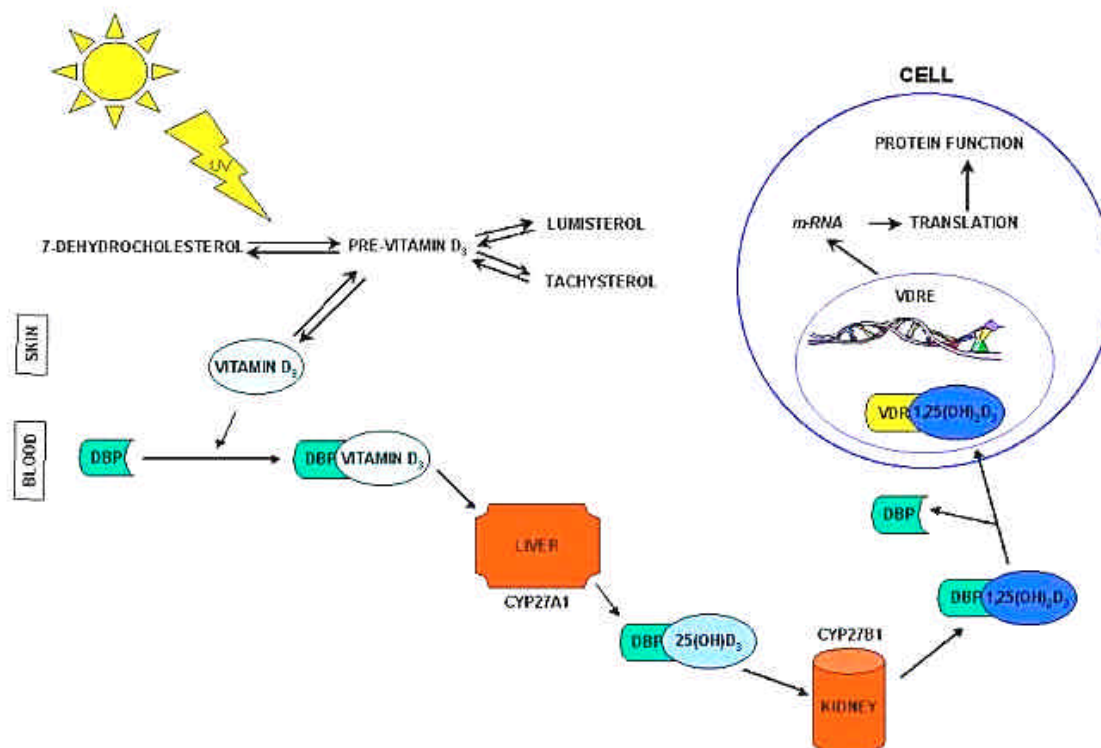


Figure 2. Synthesis of 1,25(OH)₂D₃ and generation of the vitamin D receptor mediated biological response. This figure has been modified from Fig. 1 in Ref. 47.

major classes of calbindin have been identified, the calbindin-D_{9K} of approximately 9 kDa and the calbindin-D_{28K} of approximately 28 kDa and both forms have been shown to be transcriptionally regulated by 1,25(OH)₂D₃ (25-28). Moreover, the uptake of calcium from the intestinal lumen to the blood is facilitated by a calcium pump, which has been shown to be induced at the mRNA level by 1,25(OH)₂D₃. However, whether this induction also occurs in the kidney is uncertain (29-31). In the skeleton, 1,25(OH)₂D₃ regulates both formation and resorption of bone by interacting with the osteoblasts and the osteoclasts, respectively. Proliferation and differentiation of the bone forming osteoblasts are directly stimulated by 1,25(OH)₂D₃, while the effect of 1,25(OH)₂D₃ on formation and activity of the bone-resorbing osteoclast is believed to be mediated indirectly via the osteoblasts (13, 29). The importance of the calbindins in the transportation of calcium in the bone is not clear. However, several studies have indicated that calbindins play an essential role in the process of calcification of the chondrocyte and during mineralization, processes which are both influenced by 1,25(OH)₂D₃ (32, 33).

Besides these classic actions, 1,25(OH)₂D₃ has also been shown to exert potent regulatory effects on growth and differentiation in a variety of cells which are not directly involved in the calcium metabolism. This has led to an increased clinical interest for vitamin D compounds as potential drugs in the treatment of diseases that are characterized by a dysregulation of cell growth and

differentiation, such as cancer and psoriasis. Numerous reports describing the growth regulatory effects of vitamin D compounds have emerged during the last 20 years and much effort has been directed into clarifying the mechanisms underlying the actions of vitamin D. However, a complete understanding of these mechanisms has still not been achieved.

4.1. Genomic effects of 1,25(OH)₂D₃

It is generally accepted that the main actions of 1,25(OH)₂D₃ are mediated via the so-called genomic pathway which involves binding of the hormone to a specific high affinity intracellular vitamin D receptor (VDR) with a molecular weight of 48-50 Kda (13). The cloning of the VDR in 1987 and subsequent functional analysis studies have demonstrated that the VDR belongs to the super family of steroid receptors, which share a common functional domain structure (34, 35). This structure is characterized by a variable amino (N)-terminal, a central DNA-binding domain (DBD) composed of two highly conserved zinc fingers and a less conserved ligand-binding domain (LBD) located in the carboxy (C)-terminal and linked to the DBD via a hinge region (13, 36, 37). The DBD mediates binding of the VDR to the DNA and is involved in protein-protein interactions, while the LBD binds the ligand and is critical for the formation of receptor dimerization and contact with cofactors via a helix region known as the activation function (AF-2) domain. In addition, the LBD contains a number of serine residues that serve as substrates for phosphorylation, which is regarded as an essential factor in regulation of the transcriptional activity of VDR (13, 29, 36, 38-40).

Although VDR is a nuclear receptor, the presence of the receptor has also been demonstrated in the cytoplasm. Such studies have shown that in some cell types, exposure of the cells to vitamin D compounds results in translocation of the unliganded VDR from the cytoplasm into the nucleus (41, 42). However, further investigation is needed to fully clarify this aspect and at present, the general assumption is that the VDRs are primarily located in the nucleus where they form dimer complexes with other nuclear receptors, preferentially with the retinoid X receptor (RXR). As $1,25(\text{OH})_2\text{D}_3$ is a relatively small (416.6 Da) lipophilic molecule it can easily penetrate the cell membrane and is thus taken up by the cell by simple diffusion. In the cell, $1,25(\text{OH})_2\text{D}_3$ binds with high affinity to the VDR ($K_d = 0.1 \text{ nM}$) which then leads to stabilization of the agonistic conformation of the LBD. In this agonistic conformation, the VDR is capable of interacting with co-activator proteins, which mediate the opening of the chromatin via recruiting histone acetylases. As a consequence, a second type of co-activators form contact with the basal transcriptional machinery via so-called mediator proteins, which eventually results in stimulation of the target genes. In contrast, in the absence of ligand, the VDR interacts with co-repressor proteins, which in turn recruit histone deacetylases that close the chromatin and thus lead to repression of gene transcription (43, 44). The VDR binds to specific DNA sequences, the so-called vitamin D response elements (VDREs), which are located in the promoter regions of the primary responding genes. Numerous genes have been described to be sensitive to vitamin D (table 1), including 26 in which a natural vitamin D response element (VDRE) has been identified (45-47). These VDREs are characterized by the presence of a core binding motif of 6 nucleotides which are arranged into either direct repeats (DR) or inverted palindromes (IP) with a number of spacing nucleotides (38, 45, 46, 48).

4.2. Non-genomic effects of $1,25(\text{OH})_2\text{D}_3$

In addition to the genomic pathway, the presence of another non-genomic pathway has been demonstrated in a number of different tissues. The non-genomic pathway involves regulation of voltage-gated calcium channels, opening of chloride channels, modulation of PKC activity, and activation of mitogen-activated protein kinases (MAP kinases), which eventually lead to the onset of rapid biological responses (seconds to 1-2 minutes), including inhibition of cell proliferation and stimulation of cell differentiation (13, 49-53). Recent studies have indicated that the generation of these rapid responses is mediated via a putative membrane receptor with ligand binding properties different from those of the nuclear VDR (54-56). However, the existence of such a receptor is still controversial. At present, studies have only been performed in a limited number of cell types and the results still remain to be supported by *in vivo* investigations. In addition, as an alternative to a specific membrane receptor for $1,25(\text{OH})_2\text{D}_3$, it has been suggested that membrane-associated annexin II might serve as a receptor for $1,25(\text{OH})_2\text{D}_3$ -mediated rapid responses or that extracellular binding sites for the VDR-ligand complex may exist (57, 58).

5. THE RATIONALE FOR CONSIDERING VITAMIN D IN THE TREATMENT OF CANCER

The nuclear VDR has been demonstrated in almost every cell type in the body. Epithelial tissues such as kidney, liver, adrenal, thyroid, bladder, cells of the gastrointestinal tract, prostate and breast cells have all been shown to possess VDR. Moreover, pituitary, parathyroid, pancreatic, bone, muscle and skin cells as well as some activated cells of the immune system also express high affinity receptors for $1,25(\text{OH})_2\text{D}_3$ (40, 45, 50, 60). Interestingly, many cancer cells derived from these tissues also possess VDR and have thus retained the ability to respond to the growth regulating effects of $1,25(\text{OH})_2\text{D}_3$ (47, 61, 62). In fact, it has been shown that a high expression of VDR is associated with a high degree of tumor cell differentiation and a favorable prognosis in colon cancer patients (63-65). Also, in breast cancer patients a significantly longer disease free survival has been observed in patients with VDR positive tumors as opposed to patients with VDR negative tumors (66-68).

The molecular mass and the ligand binding property of VDR isolated from cancer cells appear to be identical to those of the wild-type VDRs found in normal cells, suggesting that the receptors are identical (63, 64, 69-72). However, a series of polymorphisms in the VDR gene has recently been reported to be associated with malignant melanoma, prostate and breast cancer. These include a *FokI* restriction fragment length polymorphism (RFLP) in exon 2, *BmsI* and *ApaI* polymorphisms in intron 8 and an adjacent *TaqI* RFLP in exon 9 (73-76).

The correlation between vitamin D and cancer is further supported by epidemiological investigations showing an inverse relationship between vitamin D deficiency and cancer. As mentioned above, the vitamin D status in the body is mainly dependent on the exposure to UV light, but also dietary intake of vitamin D provides a small amount of the daily requirement. Accordingly, several studies have demonstrated an increased risk of colorectal and breast cancer and an increased mortality of prostate cancer with low serum concentrations of $25(\text{OH})\text{D}_3$ resulting from inadequate exposure to sunlight (77-80). Moreover, an inverse relationship between dietary intake of vitamin D and cancer has been observed in colorectal and breast cancer (81, 82), and a high intake of vitamin D from fish oils in the diet among Japanese men has been shown to be associated with a lower incidence of prostate cancer (83).

Taken together, these observations strongly indicate that vitamin D is a potentially useful agent in the treatment or prevention of various cancer types.

5.1. Direct effects of $1,25(\text{OH})_2\text{D}_3$ on cancer cells

The first evidence for a direct growth regulating effect of $1,25(\text{OH})_2\text{D}_3$ on cancer cells emerged in 1981 when Colston and colleagues demonstrated that growth of malignant melanoma cells *in vitro* was significantly inhibited by incubating the cells with nanomolar

Table 1. Vitamin D-regulated genes in cancer cells

Gene	Cancer type	Species	Direction of regulation	Reference
Apolipoprotein D	breast cancer	human	↑	(180)
Amphiregulin	breast cancer	human	↑	(162)
	Squamous cell carcinoma	human	↑	(162)
Androgen receptor	prostate cancer	human	↑	(72)
Aromatase P450	breast cancer	human	↑	(269)
c-fms	leukemia	human	↑	(270)
c-fos	breast cancer	human	↑	(116)
c-jun	colon cancer	human	↑	(49)
c-myc	breast cancer	human	↓	(107, 116)
	leukemia	human	↓	(110-114, 271)
	thyroid	human	↓	(115)
	glioma	rat	↑	(272)
Cathepsin B	breast cancer	human	↑	(273)
CD14	leukaemia	human	↑	(101)
Com1	breast cancer	human	↑	(108)
Cyclin A	breast cancer	human	↓	(107)
Cyclin E	breast cancer	human	↓	(107)
CYP24	breast cancer	human	↑	(102, 135)
	colon cancer	human	↑	(274)
	prostate cancer	human	↑	(209)
Cytidine deaminase	leukemia	human	↑	(275)
Estrogen receptor	breast cancer	human	↓	(167, 168)
FGF-7/KGF	breast cancer	human	↑	(163)
Gadd45	glioma	rat	↑	(272)
IGF-II	prostate cancer	human	↑	(152)
IGFBP-3	prostate cancer	human	↑	(152)
IGFBP-5	breast cancer	human	↑	(151)
IL-1beta	leukemia	human	↓	(164)
IL-6	glioma	rat	↑	(272)
Laminin	fibrosarcoma	human	↓	(184)
Laminin receptor (α6)	melanoma	human	↓	(191)
NM23.H1	leukemia	human	↓	(112)
NM23.H1	leukemia	human	↓	(112)
p21	breast cancer	human	↑	(108, 109)
	leukemia	human	↑	(101, 103, 175,
	squamous cell carcinoma	mouse	↓	276) (119)
p27	breast cancer	human	↑	(109)
	leukemia	human	↑	(101, 276)
	squamous cell carcinoma	mouse	↑	(119)
p53	glioma	rat	↑	(272)
PA inhibitor 2	leukemia	human	↓	(187)
pRb	leukemia	human	↑	(175)
PSA	prostate cancer	human	↑	(72, 277)
PTHrP	breast cancer	rat	↓	(278)
	lung squamous carcinoma	human	↓	(279)
	oral squamous carcinoma	human	↓	(280)
	melanoma	human	↓	(228)
Telomerase	leukemia	human	↓	(174, 281)
TGF-beta1	breast cancer	human	↑	(155, 156)
	leukemia	human	↑	(157)
	prostate cancer	mouse	↑	(282)
Tissue transglutaminase	leukemia	human	↑	(283)
TRPM-2	breast cancer	human	↑	(273)
	prostate cancer	mouse	↑	(282)
uPA	breast cancer	human	↓	(186)
	leukemia	human	↑	(187)
VEGF	glioma	rat	↑	(272)
VDR	breast cancer	human	↑	(260, 262)
	leukemia	human	↓	(284)
	prostate cancer	human	↑	(285)
	glioma	rat	↑	(272)
VLA-4	leukemia	human	↓	(192)

concentrations of $1,25(\text{OH})_2\text{D}_3$ for 2-3 weeks (84). At the same time, it was shown that $1,25(\text{OH})_2\text{D}_3$ was also able to stimulate differentiation of immature myeloid leukemia cells of mouse origin. Abe and colleagues found that a 2-3 days incubation of the cells with $1,25(\text{OH})_2\text{D}_3$ at physiological plasma concentrations (0.1nM) resulted in an induction of differentiation characteristics, such as morphological changes towards mature macrophages, adhesion of cells to the substrate, phagocytotic activity and the appearance of Fc and C3 receptors on the cell surface (85). Numerous *in vitro* investigations have been carried out since the early studies by Colston and Abe, and it is now well known that $1,25(\text{OH})_2\text{D}_3$ is able to affect growth and differentiation in most cancer cell types possessing VDRs (13, 29, 47, 61).

Based on the many encouraging results obtained with $1,25(\text{OH})_2\text{D}_3$ *in vitro*, several *in vivo* investigations have subsequently been performed. These studies have clearly demonstrated that $1,25(\text{OH})_2\text{D}_3$, besides being a potent regulator of cancer cell growth *in vitro*, also possesses the ability to suppress the formation of chemically induced tumors, cause regression of tumors, prevent the development of metastases, inhibit angiogenesis and prolong survival time in tumor-bearing animals (Reviewed in (61)). Obviously these observations have led to an increased interest not only in using $1,25(\text{OH})_2\text{D}_3$ in the clinical treatment or prevention of cancer in the clinic, but also in elucidating the mechanisms responsible for the anti-cancer effects of $1,25(\text{OH})_2\text{D}_3$.

6. MECHANISMS INVOLVED IN THE ANTI-CANCER EFFECTS OF VITAMIN D

Despite an intense activity within this area of the vitamin D field, the exact mechanisms responsible for the anti-cancer effects of $1,25(\text{OH})_2\text{D}_3$ are still to be identified. However, based on an immense number of studies it is now well accepted that several different signaling pathways may be involved, some of which include directly vitamin D-regulated target molecules.

6.1. Cell cycle regulators

Recent *in vitro* investigations have shown that one mechanism by which vitamin D compounds exert their growth inhibiting effects is by regulating cell cycle progression. Treatment of most cell types with vitamin D compounds has been found to cause an arrest of cell cycle progression in G₁-phase resulting in a decreased number of cells in the S-phase complemented by an accumulation of cells in the G₀-G₁ phase (86-93). The number of cells in the G₂-M compartment is relatively unaffected by treatment with vitamin D. However, in some cell types such as in the human leukemia cell line HL-60, block of cells in the G₂-phase has been observed in response to treatment with vitamin D (94, 95). Subsequent studies have indicated that this is due to an effect of vitamin D on the p34^{cdc2} protein kinase that controls this phase of the cell cycle (96).

Based on results obtained in a number of different cancer cell types it can be concluded that blocking of the G₁-phase by vitamin D is associated with alterations

of the expression of important cell cycle regulators. Increased expression of the cyclin-dependent kinase inhibitors (CKIs) results in a decreased activity of cyclin-dependent kinases (Cdks), which are strongly implicated in the phosphorylation of the retinoblastoma protein (pRb). Under normal conditions, pRb is maintained in a hypophosphorylated state through most of G₁, but undergoes hyper-phosphorylation in late G₁, subsequently resulting in release of transcription factors such as E2F, and entry of the cells into the S-phase (Reviewed in (97-100)). The two CKIs p21^{WAF1/CIP1} and p27^{kip1} appear to be the main mediators of the vitamin D-induced G₁-phase block. In 1996, Liu and colleagues identified a VDRE in the gene encoding for p21^{WAF1/CIP1} and their results suggested that $1,25(\text{OH})_2\text{D}_3$ was able to directly stimulate the transcriptional activity of this CKI (101). Additional studies have later demonstrated an up-regulation of p21^{WAF1/CIP1} at both mRNA and protein levels in most cancer cell types in response to treatment with vitamin D (Reviewed in (47, 61)). Interestingly, the effect of the vitamin D compounds on the p21^{WAF1/CIP1} expression has been shown to be independent of the p53 tumor suppressor status which further supports the assumption that vitamin D directly regulates the p21^{WAF1/CIP1} gene (101-103).

No VDRE has yet been identified for p27^{kip1}, but an up-regulation of both p27^{kip1} mRNA and protein levels has been observed in a variety of different cancer cell types after treatment with vitamin D (86, 92, 93, 101, 104-106). However, the effect of vitamin D on p27^{kip1} appears to be dependent on the cell type, as several independent studies have reported unchanged levels of p27^{kip1} in the human MCF-7 breast cancer cell line in relation to vitamin D-induced G₁-phase blockage (102, 107, 108).

As expected, the induction of p21^{WAF1/CIP1} and p27^{kip1} in response to treatment of vitamin D is accompanied by an up-regulation of the hypophosphorylated form of pRb and a down-regulation of the hyper-phosphorylated form of pRb (89, 107). In addition, the levels of a number of Cdks and cyclins as well as their complex formation have been shown to be modulated by vitamin D. These include Cdk2, Cdk4, Cdk6, cyclin D1, cyclin A and cyclin E, which are all crucial in regulating progression through the different phases of the cell cycle (86, 93, 94, 98, 100, 105, 107, 109).

Other important cell growth regulators, such as *c-fos* and *c-myc* proto-oncogenes have also been suggested to be involved in the growth inhibitory effect induced by vitamin D in cancer cells. Induction of differentiation and inhibition of DNA synthesis have been found to be closely associated with decreased levels of *c-myc* in a number of different cancer cell lines (107, 110-115). Moreover, in MCF-7 cell cultures, a decreased expression of *c-myc* mRNA was shown to be accompanied by a transient increase of *c-fos* mRNA expression (116). The gene encoding for *c-fos*, has been shown to contain a VDRE, which demonstrates that vitamin D compounds may regulate directly the transcriptional activity of *c-fos* (117). Substantial evidence has suggested that the effect of vitamin D compounds on *c-myc* expression also occurs at

the transcriptional level (110, 114, 118), and recently a putative response element responding to the vitamin D analog 22-oxa-1,25(OH)₂D₃ (OCT)-VDR complex was identified in the human *c-myc* gene. Although this element differs from most known VDREs, it contains sequences similar to those of the PTH gene. The results therefore suggest that regulation of the *c-myc* gene by vitamin D is mediated directly through this VDRE (115).

Among the cell cycle regulators, only p21^{WAF1/CIP1} has been shown to be affected by vitamin D *in vivo*. In mice carrying squamous cell carcinoma derived tumors, daily administration of 1,25(OH)₂D₃ for 3 days was found to cause a significant reduction of tumor size which was associated with a statistically significant decrease in p21^{WAF1/CIP1} within the tumors. In contrast, no difference in the p27^{kip1} expression was observed between the animals treated with 1,25(OH)₂D₃ and the control animals (119).

6.2. Apoptosis

Cell death by apoptosis occurs as a part of a natural regulatory process in the body, serving to establish and maintain a proper control of the cellular turnover. Apoptosis is closely linked to the cell cycle and is thus controlled in part by the same cell cycle regulatory machinery (110, 120, 121). Cancer is often associated with cells that fail to undergo apoptosis. This leads to the survival of aberrant cells, which would normally die, and, in turn, to malignant outgrowth. The fact that vitamin D is able to induce apoptosis in a number of different cancer cell types is therefore of immense interest and suggests that induction of apoptosis is likely to be one mechanism by which vitamin D exerts its anti-cancer effects. Induction of apoptotic features in response to vitamin D has been demonstrated in breast, colon, and prostate cancer cells as well as in melanoma, myeloma, and glioblastoma cells *in vitro*. However, the effect may vary between the different subtypes of these cells. Moreover, human leukemia cell lines such as HL-60 and U937 appear to be resistant to induction of apoptosis by vitamin D (Reviewed in (47, 61, 122, 123)).

Recent *in vitro* studies have indicated that, at least in breast cancer cells, vitamin D-induced apoptosis is not dependent on the activation of any known caspase from the cellular proteolytic machinery (124-126). In addition, induction of apoptosis by vitamin D in these cells appeared to be independent of the p53 tumor suppressor status, as both cells containing wild-type p53 and cells carrying a mutated form of p53 have been found to undergo apoptosis in response to treatment with vitamin D (124, 125). In contrast, accumulating evidence points to the bcl-2 family of apoptosis regulatory proteins as playing a critical role in vitamin D-induced apoptosis. In myeloma cells, breast, colon, and prostate cancer cell lines, a down-regulation of the two anti-apoptotic members of this family, bcl-2 and bcl-X_L, and an up-regulation of the pro-apoptotic bax and bak proteins have been demonstrated after treatment with vitamin D (89, 104, 127-132). Especially, down-regulation of bcl-2 in conjunction with translocation of bax to the mitochondria has recently been shown to be of particular importance for the induction of vitamin D-mediated

apoptosis in the MCF-7 cells (126). Moreover, over-expression of bcl-2 has been shown to block vitamin D-induced apoptosis in both MCF-7 breast cancer cells and in human LNCaP prostate cancer cells, further supporting the key role of the bcl-2 family of proteins in vitamin D-mediated apoptosis (125, 131). This latter effect may be due to bcl-2 preventing the efflux of cytochrome c from the mitochondria, which is assumed to be a necessary step in the initiation of the apoptotic process (133). 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 (126), but the exact link to the bcl-2 family of proteins is still poorly characterized.

However, it should be mentioned that in the human NCI-H929 myeloma cell line, down-regulation of bcl-2 in response to treatment with vitamin D compounds, has been reported to be associated with an increased activity of caspase 3. No regulation of the bax protein was seen in this study. In contrast, a down-regulation of the p44 extracellular related kinase (ERK) activity and an up-regulation of the p38 kinase activity was observed, indicating that these two MAP kinases are involved in the induction of apoptosis in myeloma cells (127).

Other signaling pathways like the sphingomyelin pathway, which mediates tumor necrosis factor- α (TNF) induced apoptosis, have also been suggested to be involved in apoptosis induced by vitamin D. Activation of sphingomyelinases hydrolyses sphingomyelin to generate ceramide, which acts as a second messenger to activate apoptosis. *In vitro* studies in breast cancer cells have shown that vitamin D is able to potentiate TNF-induced apoptosis and that an enhanced accumulation of intracellular ceramide is likely to be involved in this effect (134, 135). This correlates well with previous results obtained in human glioblastoma cells and in the HL-60 cell line demonstrating that 1,25(OH)₂D₃ induces the hydrolysis of sphingomyelin with the consequent generation of ceramide and phosphorylcholine (136). However, in the HL-60 cells the ceramide was found to act as a second messenger for induction of differentiation and not apoptosis (137). Also, an up-regulation of the TNF receptor 1 and a modulation of cathepsin B, a supposed key mediator of TNF-induced apoptosis, have been shown to be involved in the stimulating effect of vitamin D on TNF-induced apoptosis (138).

Besides being able to enhance TNF-induced apoptosis, vitamin D has also been demonstrated to promote apoptosis induced by other anti-cancer agents, such as adriamycin, paclitaxel, and radiation, in breast cancer cells *in vitro* (139-142). The results clearly suggest that a cross talk between the distinct apoptosis pathways may exist. Moreover, they imply that vitamin D may be useful in combination with conventional chemotherapy and/or radiotherapy in the treatment of cancer.

The theory that induction of apoptosis contributes to the anti-cancer effect of vitamin D has recently been supported by *in vivo* studies demonstrating large areas of

apoptotic cells in mammary tumors from rats and mice which have been treated with vitamin D. Using the NMU-model in which mammary tumors are induced in rats by injection of nitrosomethylurea (NMU), it was shown that tumor sections from animals treated with the vitamin D analog EB 1089 exhibited a marked loss of cellularity, only few mitotic cells and a considerable nuclear DNA fragmentation (130). Similar results were obtained with the same analog in nude mice carrying established MCF-7 breast cancer xenografts (143), strongly indicating that regression of tumors in response to vitamin D treatment is, at least in part, due to induction of apoptosis.

6.3. Growth factors and hormones

Growth factors and hormones are important factors in regulating growth and differentiation in normal cells. Often the expression and activity of these proteins are disturbed in cancer cells, further promoting the malignant process. The insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are among the most abundant growth factors in the body. Besides being general mitogens, IGF-I and IGF-II also act as potent survival factors for both normal cells and cancer cells (144). The IGF-I receptor (IGF-IR) has been described to be over-expressed in breast cancer tissues, and clinical investigations have shown that the total plasma IGF-I level is increased in women with breast cancer compared to healthy women (144-146). Interestingly, studies in breast cancer cell lines have demonstrated that vitamin D compounds are able to block the mitogenic activity of IGF-I and that this effect is accompanied by a decrease of proliferation and an increase of apoptosis (124, 147). Moreover, the block of IGF-I activity by vitamin D has been linked to a suppression of IGF-RI expression (148, 149). It is therefore likely, that interfering with the IGF-I/IGF-II signaling pathway contribute to the anti-cancer effect of vitamin D. This hypothesis is further supported by results showing that vitamin D treatment leads to modulation of the expression and activity of insulin-like growth factor binding proteins (IGFBPs). The IGFBPs control the bioavailability of the IGF-I and IGF-II growth factors, but in addition, they also act as independent mediators of growth regulation. Vitamin D has been described to cause an up-regulation of IGFBP-3 and IGFBP-5 in breast cancer cells where these binding proteins are known to serve as inducers of apoptosis (150, 151). Moreover, it has recently been reported that vitamin D-mediated growth inhibition of prostate cancer cells *in vitro* is associated with an increased IGFBP-3 mRNA abundance, IGFBP-3 mRNA stability, IGFBP-3 protein accumulation, and a decreased IGF-II gene expression (152).

Consistent with the *in vitro* results that support a role for the IGF-I/IGF-II signaling pathway in contributing to the anti-cancer effect of vitamin D are results from a recent *in vivo* study on prostate regression in normal rats. Treatment of the rats with the vitamin D analog EB 1089 was found to cause a marked ventral regression accompanied by an increase in the expression of IGFBP-2, -3, -4 and -5 mRNA and IGF-I gene expression. In addition, a significant increase in the number of apoptotic

cells was observed in tissue sections from the vitamin D-treated animals compared to sections from control animals. The results suggest that vitamin D exerts its growth inhibiting effects on prostate cells by interfering with the IGF-I signaling pathway, which eventually leads to induction of apoptosis (153).

Other growth factors of interest with reference to vitamin D and cancer are the transforming growth factor-beta (TGF-beta) and the epidermal growth factor (EGF). In most epithelial cells, including leukemia and breast cancer cells, TGF-beta acts as a negative growth regulator and an up-regulation of TGF-beta activity is thus expected to decrease the growth potential of these cells (154). *In vitro* studies have demonstrated that growth inhibition by vitamin D in leukemia and breast cancer cells is correlated with an increase in TGF-beta1 mRNA and protein secretion into the culture medium. In breast cancer cells, this effect can be abrogated by addition of neutralizing TGF-beta antibodies, which strongly indicates that the growth inhibitory effect of vitamin D on these cells is due, at least in part, to an increased activity of TGF-beta (109, 155, 156). This observation is consistent with results obtained in leukemia cells, which demonstrated an enhanced expression of TGF-beta receptors in response to treatment of the cells with vitamin D (157). TGF-beta has previously been shown to affect some of the same intracellular targets as vitamin D, including c-myc, pRb, p21 and p27, suggesting that TGF-beta and vitamin D share parts of the same signaling pathways (109, 158).

In the case of EGF, studies using breast cancer cells in which the EGF receptor (EGFR) gene is known to be amplified, have suggested that vitamin D exerts its anti-proliferative effects on such cells, partly by altering the expression of the cellular sensitivity to autocrine factors that act via the EGFR (159-161). Moreover, results obtained in two recent studies indicate that amphiregulin (a member of the EGFR-family) and the keratinocyte growth factor, FGF-7/KGF (a member of another family of growth factors with important cell regulatory properties) may be involved in the growth inhibitory effect of vitamin D in certain cell types (162, 163). Similarly, based on results obtained in acute myelogenous leukemia cells, it has been suggested that interruption of interleukin 10 (IL-10) mediated growth may also contribute to the growth inhibitory effect of vitamin D (164).

The development and progression of breast cancer is also under the influence of the hormonal milieu in the body. In breast cancer cells, which have retained their ability to respond to estrogen, the hormone has been shown to stimulate proliferation by a receptor-mediated pathway (165). Treatment of the estrogen receptor positive (ER⁺) MCF-7 cell line with vitamin D compounds has been found to cause a time- and dose-dependent decrease in estrogen receptor (ER) at both protein and mRNA levels. The mechanism by which vitamin D inhibits growth of ER⁺ breast cancer cells may therefore partially be mediated via a down-regulation of the ER which eventually leads to a diminished responsiveness of the cells to the growth-stimulatory effect of estrogen (166-168). Other results showing an inhibition of estrogen-dependent gene

transcription in response to treatment of MCF-7 cells with $1,25(\text{OH})_2\text{D}_3$ have indicated that the effect occurs at the estrogen response element level (169). This is in agreement with recent sequence analysis studies on the estrogen receptor- α gene suggesting a putative VDRE within the ER promoter (167). Finally, a close interaction between the vitamin D and the estrogen signaling pathways has also been observed in studies revealing co-operative effects of vitamin D compounds and anti-estrogens on breast cancer cell growth (Reviewed in (170, 171)).

6.4. Telomerase

Telomerase is a ribonucleoprotein enzyme that regulates the length of eukaryotic chromosomes, known as telomeres. In most human somatic cells, the telomerase activity is repressed, and telomeres shorten progressively with each cell division. In contrast, 80-90% of human cancers have been found to possess telomerase activity, resulting in stabilization of telomere length and continued proliferation. Clinical studies have shown a correlation of high levels of telomerase activity and poor prognosis in neuroblastoma, gastric and breast tumors, indicating that telomerase plays a critical role in tumor progression (172, 173).

In the human HL-60 leukemia cell line, which expresses high levels of telomerase activity, inhibition of growth, up-regulation of p21WAF1/CIP1 and induction of differentiation by vitamin D have been shown to correlate with a down-regulation of telomerase activity (174, 175). However, the results indicated that the decrease in telomerase activity was independent of the growth pathway, but rather occurred as an early event in the differentiation process in these cells (175). This observation correlates with results from another study showing repression of telomerase activity during differentiation of a number of different tumor cell lines (176). It is therefore likely that the differentiation-inducing effect of vitamin D compounds in the HL-60 cells is closely associated with the effects of the compounds on both p21WAF1/CIP1 and telomerase activity.

6.5. Differentiation

Whether induction of cell differentiation by vitamin D compounds is a consequence of blockage of cell cycle progression or vice versa is still a matter of considerable debate. Moreover, it has been questioned whether it is possible to dissociate between these two processes. A few studies on vitamin D effects in the HL-60 line have indicated that the anti-proliferative and the differentiation-inducing effects of the compounds are uncoupled (87, 95, 176, 177). However, the consensus view emerging from studies in most other cell systems is that inhibition of cell growth is accompanied by a stimulation of cell differentiation

Induction of differentiation is difficult to measure in many cell types *in vitro*, but in others an altered expression of specific molecular, biochemical or morphological markers indicates that the cells have reached a more mature phenotype. In human leukemia cells, the onset of differentiation towards mature monocytes is

accompanied by an increased expression of the surface antigens CD11b, CD14 and CD18. In addition, the phagocytotic activity of the cells, induction of super oxide production, expression of glycoprotein IIb/IIa, and the expression of enzymes like non-specific esterase (NSE) and tissue transglutaminase are enhanced when the cells are induced to differentiate. These markers have all been shown to be significantly up-regulated in response to treatment of leukemia cells by vitamin D compounds *in vitro* (Reviewed in (47, 61, 178)).

Secretion of prostate specific antigen (PSA) is a characteristic of mature prostate cancer cells and PSA is therefore often used as a differentiation marker. A number of studies have demonstrated that in the LNCaP cell line, vitamin D stimulates the secretion of PSA and increases the steady state level of its mRNA. In contrast, prostate cancer in the clinic is associated with leak of PSA into the serum, and an increase in the level of serum PSA is consequently used as a prognostic factor. In accordance with this, $1,25(\text{OH})_2\text{D}_3$ has been shown to delay the rate of increase of serum PSA in a pilot trial involving men with early recurrent prostate cancer after primary therapy with radiation or surgery (179). In breast cancer cells *in vitro*, vitamin D has been demonstrated to cause an up-regulation of the apolipoprotein D, which has been suggested to be associated with the level of differentiation and clinical outcome in women with breast cancer (180). In addition, other differentiation markers such as alkaline phosphatase activity in human colon cancer cells, acetylcholinesterase activity in human neuroblastoma cells, nerve growth factor (NGF) synthesis in transformed murine fibroblasts, and fillagrin expression in human squamous carcinoma cells have also been found to be augmented after treatment of the cells with vitamin D (Reviewed in (47, 61, 178)).

6.6. Invasion and metastasis

Invasion and metastasis constitute the main clinical problems of cancer and are the major causes of death in most cancer patients. Both are highly complex processes, which involve multiple factors, including attachment of the tumor cells to extracellular matrix components, degradation of the extracellular matrix, locomotion of the tumor cells through the degraded area, induction of angiogenesis and an overall coordination of the secretion of proteolytic enzymes and their inhibitors (181, 182). Moreover, the ability of tumor cells to invade and to metastasize depends on the degree of dedifferentiation within the tumor. Poorly differentiated cells seem to be more invasive than well-differentiated tumor cells (182).

This correlates well with observations in human neuroblastoma cells *in vitro* where induction of differentiation after treatment with vitamin D has been demonstrated to be accompanied by a significantly reduced invasiveness of these cells (183). The anti-invasive effect of vitamin D has further been substantiated by other studies showing that these compounds are able to inhibit cancer cell progression by interfering with specific steps in the metastatic cascade, including the proteolytic enzymes. In the mouse B16 melanoma and the human HT1080

fibrosarcoma cell lines, inhibition of invasion by vitamin D has been shown to be associated with a decreased activity of the type IV collagenases (184, 185). Moreover, in the human MDA-MB-231 breast cancer cell line, treatment of the cells with vitamin D was found to result in a diminished activity of the matrix metalloproteinase MMP-9 and the two serine proteases urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) concomitant with a reduced invasiveness of the cells. Furthermore, these effects were accompanied by an increase of the PA inhibitor 1 and the MMP inhibitor 1. Interestingly, the regulation of uPA by vitamin D appeared to be at the transcriptional level, suggesting that uPA is a primary target for vitamin D (186). The ability of vitamin D to modulate the plasminogen activator system in cancer cells has previously been reported for human U937 leukemia cells. However, in these cells, the expression of uPA is linked to induction of differentiation and is consequently up-regulated in response to treatment with vitamin D (187, 188).

Also, the granulocyte-macrophage colony-stimulating factor (GM-CSF) has been suggested to be involved in the anti-invasive effect of vitamin D. This is based on *in vitro* studies in mouse lung carcinoma cells where inhibition of invasion and migration by $1,25(\text{OH})_2\text{D}_3$ was found to be accompanied by a decreased production of GM-CSF, a reduced activity of PKA and increased levels of polymerized actin. This observation is in accordance with the hypothesis that GM-CSF stimulates tumor cell motility through a PKA signal transduction pathway and that reduction in PKA serves as an intermediate signal through which $1,25(\text{OH})_2\text{D}_3$ affects the cytoskeleton and diminishes tumor cell invasiveness (189, 190). However, studies using the MDA-MB-231 cell line have demonstrated that inhibition of cell proliferation and cell migration is not sufficient to account for the decreased invasive potential after treatment of the cells with vitamin D (70). Other mechanisms, such as regulation of the proteolytic enzymes, as mentioned above, modulation of extracellular matrix proteins and adhesion molecules, are therefore likely to be involved. The latter hypothesis is in accordance with the observation that $1,25(\text{OH})_2\text{D}_3$ reduces the expression of laminin in human fibrosarcoma cells and the number of laminin-binding integrins and the expression of very late antigen-4 (VLA-4) in human melanoma cells (184, 191, 192).

Finally, the anti-angiogenic activity of vitamin D may contribute to its overall anti-cancer effects. In the chick embryo chorioallantoic membrane assay, concentrations of vitamin D compounds in the picomolar range have been found to attenuate angiogenesis (181). Likewise, vascular endothelial growth factor (VEGF)-induced endothelial cell sprouting and elongation *in vitro* have recently been shown to be significantly inhibited by $1,25(\text{OH})_2\text{D}_3$. In the latter study, the anti-angiogenic effect of $1,25(\text{OH})_2\text{D}_3$ was further supported by experiments showing a decreased formation of networks of elongated endothelial cells within 3D collagen gels and a regression of sprouting endothelial cells in established cultures after treatment with $1,25(\text{OH})_2\text{D}_3$. Interestingly,

the results indicated that induction of apoptosis accounts for the regression in already established cultures of sprouting endothelial cells (193).

Using different animal models it has now been confirmed that also *in vivo* vitamin D suppresses invasion and metastasis and exerts an anti-angiogenic activity. In melanoma, lung, prostate, colon and breast cancer models a reduction in the number and size of metastases has been observed in animals which have been treated with $1,25(\text{OH})_2\text{D}_3$ or one of its analogues as compared to non-treated animals (185, 189, 194-197). Moreover, administration of $1,25(\text{OH})_2\text{D}_3$ to mice inoculated with cancer cells of different origin and rats bearing chemically induced colon tumors has been shown to result in a significant decrease in tumor-induced angiogenesis (193, 198-201). Consistent with the *in vitro* findings, results obtained *in vivo* indicate that regulation of VEGF is involved in the anti-angiogenic activity of vitamin D. This is based on immuno-histochemical analysis of colon tumors from vitamin D treated rats, which demonstrated a significant decrease in VEGF expression and microvessel counts compared to tumors from untreated animals (199).

Taken together, it can be concluded that vitamin D affects a number of molecules and signaling pathways known to play a key role in the progression of cancer cell growth. Some targets appear to be under direct transcriptional control by vitamin D, while others are regulated indirectly and regarded as secondary effects. Moreover, some kind of target cell specificity seems to exist as diverse responses are observed in different cell types. Obviously, the mechanisms underlying the anti-cancer effects of vitamin D are complex and further investigation within this area is required in order to obtain a better understanding.

7. VITAMIN D RESISTANCE

Development of drug resistance is a major clinical problem with most anti-cancer agents. Only limited data on the long-term use of vitamin D compounds in cancer patients are yet available. On the other hand, clinical studies in psoriasis patients, where vitamin D is successfully used as a long-term treatment, have demonstrated a sustained efficacy and safety of the drug (202). Despite the fact that no signs of the development of a pharmacological tolerance to the drug have been observed in the clinic, it is well known that the responsiveness to the drug varies in different psoriasis patients. The reason for this phenomenon is unclear, but studies on the VDR expression in psoriasis lesions have indicated that the presence and quantity of the receptors might not be the only explanation (203). Also, vitamin D resistance is known from the rare recessive disorder hereditary hypocalcemic rickets, but in this case the resistance is caused by congenital mutations in the gene encoding VDR (204).

It is well accepted that the presence of functional VDRs is a prerequisite for most vitamin D-mediated effects to occur. Also, a direct relationship between the anti-proliferative effect of vitamin D and the level of receptors

has been observed in some cell types *in vitro*, while in others there seems to be no correlation (Reviewed in (61)). However, despite being unable to respond to the growth inhibiting effect of vitamin D, these cells may still be capable of responding to other effects of vitamin D, such as induction of differentiation and inhibition of invasion (70, 129, 184).

Partial blockage of the vitamin D-mediated actions has also been observed in two recently established and stable vitamin D resistant cell lines derived from the human MCF-7 breast cancer cell line (102, 205-207). No inhibition of cell growth, modulation of cell cycle regulators or induction of apoptosis was seen in response to treatment of the cells with different vitamin D compounds. Despite these characteristics, both cell lines were found to contain fully functional VDRs, although in a slightly lower number than seen in the parental cell line. Also, the regulation of CYP24 appeared to be intact and no significant differences were observed with regard to growth rate and morphological appearance between the parental cells and the vitamin D resistant cells. However, one of the cell lines, the MCF-7/VD^R cell line, appeared to be by far more sensitive to the anti-estrogen ICI 182,780 than the parental cell line, supporting the results from other studies demonstrating a close interaction between the vitamin D and the estrogen signaling pathways. Moreover, this observation argues against a complete cell cycle control in the vitamin D resistant cells (102). In the other cell line, the MCF-7D^{res} cell line, the phorbol ester TPA was found to be able to sensitize the cells to a growth inhibiting effect of 1,25(OH)₂D₃, suggesting that even the vitamin D-mediated growth control is only partially blocked (207). This, together with the fact that functional VDRs are present in both the MCF-7/VD^R and the MCF-7D^{res} cell line, suggests involvement of a defect downstream of VDR complex formation as the main reason for the development of vitamin D resistance. This hypothesis is further supported by results obtained with vitamin D resistant HL-60 leukemia cell variants, which showed that these cells possessed high levels of VDRs and that the initial steps of the vitamin D signaling pathway were intact (177).

Other vitamin D resistant cell lines have also been described, including the human JMRD₃ leukemia cell line, in which alterations of the DNA-binding properties of activator protein-1 (AP-1) transcription factors, particular those complexes containing JunD proteins, have been suggested to be involved in the development of vitamin D resistance (208). Moreover, recent results obtained in prostate cancer cells with a low sensitivity to vitamin D, indicate that these cells dysregulate the normal growth regulating signals of vitamin D during cancer progression by a mechanism involving histone deacetylation (209). Another factor which has been suggested to be involved in the sensitivity of prostate cancer cells to vitamin D is the level and rate of induction of CYP24, as this enzyme metabolizes and eventually inactivates 1,25(OH)₂D₃ in the cells (106, 210, 211).

Obviously, more studies are required in order to clarify the mechanisms responsible for the development of

vitamin D resistance. However, together the different vitamin D resistant cell lines provide a useful tool for examining these aspects in more detail and serve as interesting models for studying the mechanisms of actions of vitamin D.

8. THE DEVELOPMENT OF NEW VITAMIN D ANALOGS

The beneficial effects of 1,25(OH)₂D₃ on cancer cells have been supported by results obtained with 1α-hydroxyvitamin D₃ (1α(OH)D₃), which *in vivo* is converted into 1,25(OH)₂D₃ in the liver (212-215). Also, a number of clinical trials have been performed with 1,25(OH)₂D₃. However, despite a few encouraging results, these trials have confirmed that the therapeutic window of 1,25(OH)₂D₃ is extremely narrow, i.e. effective doses cannot be administered without inducing hypercalcemia (Reviewed in (47)). Therefore much effort has been directed to identify new analogs with potent cell regulatory effects but with decreased risk of inducing calcemic adverse effects.

Several hundred new vitamin D analogs, most of which feature chemical modifications in the C17 side chain structure, have been synthesized with the aim of separating the growth regulating effects from the calcemic adverse effects. One of the early analogues that appeared to meet these requirements was calcipotriol (MC 903). MC 903 exerts growth regulating effects similar to those of 1,25(OH)₂D₃, but displays a calcemic activity which is 100-200 times less than that of 1,25(OH)₂D₃ (216). The latter was found to be due to a very rapid metabolism of the analog to inactive metabolites (217), a feature that makes MC 903 ideal for topical use. Today MC 903 is successfully used in the treatment of psoriasis and sold under the trade names Dovonex[®] and Daivonex[®] (202, 218). However, as systemical administration is a prerequisite for an ideal treatment of cancer, attempts to develop analogs with a longer biological half-life have been made. Introduction of conjugated double or triple bonds, heteroatoms, fluor atoms, and/or aromatic rings in the side chain represent chemical modifications that have resulted in analogues with promising biological profiles. In addition, epimerization at C20 in the side chain or introduction of a double bond at position C16 in the D-ring have shown to improve the therapeutic window of the analogues. A number of excellent and very detailed structure-activity relationship studies have been carried out suggesting that at least theoretically, it should be possible to design analogs with selective biological profiles (219-221). However, a better understanding of the mechanisms implicated in the growth regulating effects of the vitamin D analogs would greatly facilitate this process. Moreover, identification of genes, which are directly or indirectly regulated by vitamin D as well as more knowledge about the VDR-DNA interactions, may allow for the identification of more selective analogs.

9. PROMISING VITAMIN D ANALOGS: RESULTS FROM *IN VIVO* STUDIES AND CLINICAL DATA

As shown in figure 3, several synthetic vitamin D analogs have been reported to exert promising anti-cancer effects. These analogs have been extensively studied

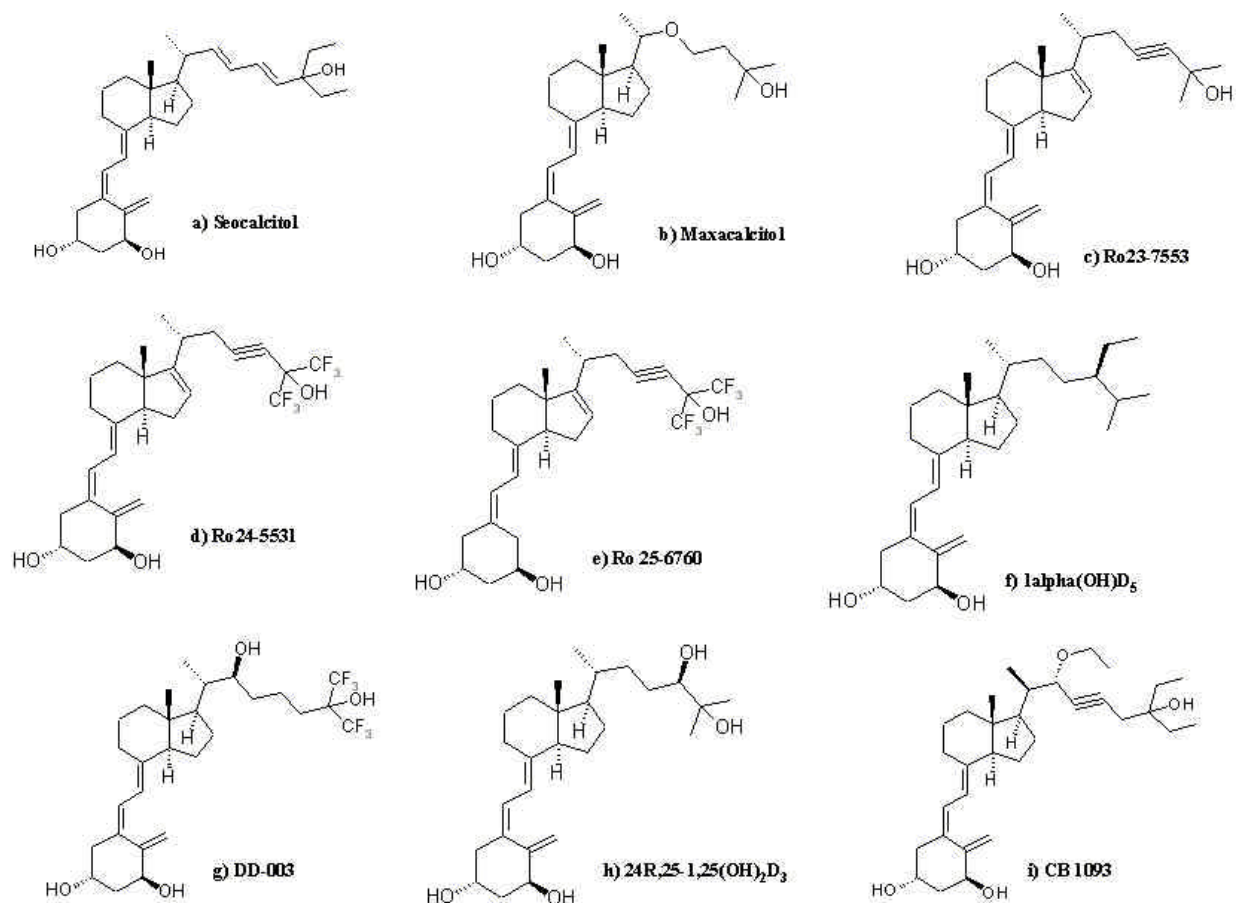


Figure 3. Examples of synthetic vitamin D analogs, which have shown promising anti-cancer effects both *in vitro* and *in vivo*. **a)** Seocalcitol (EB 1089), **b)** Maxacalcitol (OCT), **c)** Ro23-7553 (ILX-23-7553), **d)** Ro24-5531, **e)** Ro25-6760, **f)** 1 α (OH) D_5 , **g)** DD-003, **h)** 24R,25-1,25(OH) $_2D_3$, and **i)** CB 1093.

both *in vitro* and *in vivo* and in a variety of different cancer cell types.

9.1 Seocalcitol (EB 1089)

At the moment, one of the most promising synthetic analogs is Seocalcitol (EB 1089, Leo Pharmaceutical Products), which is characterized by an altered side chain structure featuring 26,27-dimethyl groups and two double bonds. A considerable number of *in vitro* studies have been carried out with EB 1089 and the results clearly show that EB 1089 is more potent than 1,25(OH) $_2D_3$ with respect to regulation of cancer cell growth and differentiation. However, the effect of EB 1089 on the calcium metabolism *in vivo* is approximately 50% weaker than that of 1,25(OH) $_2D_3$, while the biological half-life is similar to that of 1,25(OH) $_2D_3$ (47, 89, 116, 222-225). Together, these characteristics make EB 1089 a better candidate than 1,25(OH) $_2D_3$ for *in vivo* use and point to EB 1089 as a potential useful analog for systemic use in the clinical treatment of cancer.

Colston and colleagues were the first to show that the growth inhibitory effects obtained with EB 1089 *in vitro* could be transferred to the *in vivo* situation. Oral

treatment of rats carrying NMU-induced mammary tumors with EB 1089 at 0.5 μ g/kg/day for 28 days was shown to result in a significant inhibition of tumor progression without changes in the serum calcium levels. In contrast, treatment with 1,25(OH) $_2D_3$ at 0.5 μ g/kg/day was demonstrated to have no significant effect on tumor growth, but induced hypercalcaemia. EB 1089 administered at a higher dose (2.5 μ g/kg/day), appeared to cause a marked regression of the tumors, but the effect was accompanied by induction of hypercalcaemia (226). These findings have now been reproduced in several subsequent studies using the same *in vivo* model (128, 130, 227). In addition, they have been supported by results obtained in a rat breast cancer model, using 7,12-dimethylbenz[a]anthracene (DMBA) as the chemical breast tumor inducer, in immunodeficient mice inoculated into the mammary fat pad with melanoma cells and in nude mice carrying established MCF-7 breast cancer xenografts (47, 143, 228). Also more recently, it has also been shown that administration of EB 1089 to nude mice transplanted with human breast cancer cells results in a marked increase in survival time and an inhibition of the development of bone metastases without causing hypercalcemia (197).

The beneficial effects of EB 1089 on breast cancer in tumor-bearing animals can further be enhanced by combination of the analog with other anti-cancer agents such as paclitaxel and retinoic acid, suggesting that EB 1089 may also have potential as an adjuvant therapy of breast cancer (229, 230). Of particular interest in relation to breast cancer is also the observation that tumors derived from anti-estrogen resistant breast cancer cell lines have been shown to be fully capable of responding to the growth inhibiting effect of EB 1089 (205). In fact, such anti-estrogen resistant cells seem to be even more sensitive to EB 1089 than their parental cells when grown *in vitro* (231). This, together with the fact that EB 1089 inhibits growth of both ER⁺ and ER⁻ breast cancer tumors, indicates that EB 1089 is effective in the treatment of ER⁺ breast cancer patients who have relapsed on anti-estrogen treatment or as well as in ER⁻ breast cancer patients.

Other *in vivo* investigations supporting the beneficial effects of EB 1089 cancer cells include studies on prostate cancer, colon cancer and pancreatic cancer. In the Dunning MAT LyLu prostate rat cancer model EB 1089 has been shown to cause a reduced incidence of lung metastases (194), while administration of EB 1089 to nude mice carrying LNCaP prostate cancer xenografts was demonstrated to result in inhibition of tumor growth. In addition, in the latter study, tumors from EB 1089 treated animals were found to be significantly less vascularized than tumors from untreated animals, strongly suggesting that the process of angiogenesis was repressed (232). The colonic and the pancreatic *in vivo* models involve immunodeficient mice bearing colon cancer xenografts and pancreatic xenografts, respectively, and in both models EB 1089 was shown to inhibit tumor growth without causing hypercalcemia (233, 234).

Interestingly, a number of studies have demonstrated that EB 1089 is also capable of inhibiting the development of humoral malignancy-associated hypercalcemia which may be caused either by local osteolytic activity or by secretion of parathyroid hormone-related peptide (PTHrP) by the tumor. In rats implanted with Leydig cell tumors, administration of EB 1089 was found to result in an increased survival time of the animals accompanied by a decrease in the level of PTHrP mRNA and in the concentration of PTHrP in the plasma (235). Moreover, in a mouse model of squamous cancer, EB 1089 appeared to efficiently block the PTHrP production and was shown to be able to reverse established hypercalcemia (236). Thus, based on these observations it is assumed that EB 1089 inhibits the development of malignant hypercalcemia by interfering with the production of PTHrP from the tumor cells. This theory is further supported by studies showing that vitamin D directly represses PTHrP transcription by interacting with a VDRE within the gene encoding for PTHrP (237-239).

Of particular interest are the clinical data, which are now available on EB 1089. Unlike most anti-cancer agents, the toxicity profile of EB 1089 permits its evaluation in healthy volunteers. Accordingly, a dose-finding study in which EB 1089 was orally administered

for 4 consecutive days was performed in 13 healthy volunteers (47). The results clearly demonstrated that EB 1089 was well tolerated for the 4 days treatment period and that doses in the range of 5-20 µg/day should be considered for use in future clinical trials. Moreover, it was found that EB 1089 had predictable vitamin D-like effects on the calcium metabolism with only minimal reversible adverse effects of which none led to withdrawal from the study.

The safety of EB 1089 has been further evaluated in a study including 36 patients with advanced cancer of either breast or colorectal origin (240). On the basis of this study, the estimated maximum tolerated dose (MTD) was determined to be 7 µg/m²/day for prolonged use. 10 patients developed hypercalcemia, but this effect resolved within 7 days and no other serious adverse reactions were observed. Although no clear-cut anti-tumor effects were seen in this study, 6 patients (2 colorectal, 4 breast cancer) were found to have disease stabilization for ≥ 3 months, which indicate that EB 1089 is able to sustain or prolong the disease-free survival.

Several phase II and III studies using oral administration of EB 1089 are currently ongoing in various malignant indications and at present, only data in abstract form are available from these studies. These include data from a trial involving previously treated patients with blastic myelodysplastic syndromes or acute myeloid leukemia in remission, a trial involving patients with non-resectable colorectal cancer, a trial involving patients with non-resectable pancreatic cancer and data from the first 22 enrolled patients in a trial investigating the efficacy and safety of EB 1089 in patients with advanced hepatocellular carcinoma (47). Collectively, the data demonstrate that EB 1089 is well tolerated within a dose-range of 5-25 µg/day with dose limiting hypercalcemia as the only consistently reported adverse effect. However, the hypercalcemia is usually mild and reversible within one week after discontinued treatment with the drug. Moreover, an indication of a potential anti-cancer effect of EB 1089 has been observed in hepatocellular carcinoma and in colorectal cancer, in which reductions in tumor size have been demonstrated.

Taken together, the data from the clinical trials with EB 1089 confirm that the low calcemic activity observed with EB 1089 in animal studies can be reproduced in the clinic. Moreover, the preliminary data indicate that EB 1089 is effective in certain types of cancer. However, further studies including trials involving patients with less advanced cancer, controlled trials with survival as the primary endpoint and combination treatment with other conventional cancer therapies are required to draw a final conclusion on the fate of EB 1089.

9. 2. Maxacalcitol (OCT)

22-oxy-1α,25(OH)₂D₃ (OCT, Maxacalcitol, Chugai Pharmaceutical Co. Ltd.), which is characterized by the introduction of an oxygen atom at position 22 in the side chain, is another relatively low-calcemic vitamin D analog that has been extensively studied with regard to its effects on cancer cells *in vitro* and *in vivo* (241, 242). *In*

vitro, OCT appears to display more profound cell regulatory effects than those of $1,25(\text{OH})_2\text{D}_3$ (181, 243).

OCT has been demonstrated to reduce tumor size and tumor weight significantly without increasing the serum calcium concentration in a number of different breast cancer *in vivo* models including nude mice implanted with human breast cancer cells and rats carrying DMBA-induced breast tumors (242, 243). Moreover, it has been shown that the beneficial effects of OCT on breast cancer can be further augmented by combination of the compound with the aromatase inhibitor, CGS 16949A or with the anti-estrogen, tamoxifen (244, 245). Other promising results obtained with OCT *in vivo* include reduction of pancreatic tumor growth and inhibition of tumor development in the intestines of mice, further supporting the anti-cancer effect of this analog (91, 246).

Similarly to EB 1089, OCT has been shown to inhibit the development of malignancy-associated hypercalcemia in tumor-bearing animals. In nude mice carrying human pancreatic tumors, administration of OCT was found to result in a significant reduction of the steady-state levels of PTHrP mRNA accompanied by a marked prolongation of the survival time of the animals. In addition, OCT appeared to exert a therapeutic effect on malignancy-associated hypercalcemia in animals with already elevated calcium concentrations in the blood (247).

However, despite the promising effects of OCT in animal models of cancer, no clinical cancer trials are currently ongoing. Instead OCT has been approved and launched (September 2000, Chugai Pharmaceutical Co. Ltd.) for the treatment of secondary hyperparathyroidism (248).

9.3. 16-ene analogs

A group of 16-ene vitamin analogs have also produced encouraging results in a number of *in vivo* cancer models. These analogs are all characterized by the introduction of a double bond at the C16 position in the D-ring (249). Ro23-7553 (ILX-23-7553, Hoffmann LaRoche Inc.) and Ro24-5531 (Hoffmann LaRoche Inc.), which is a $1\alpha,25$ -dihydroxy-16-ene-23-yne-cholecalciferol and a $1\alpha,25$ -dihydroxy-16-ene-23-yne-26,26,26,27,27-hexafluorocholecalciferol analog, respectively, have been shown to be of particular interest when considering new potential anti-cancer agents. Several studies have demonstrated that these two analogs are approximately 50-100 times more potent than $1,25(\text{OH})_2\text{D}_3$ with respect to their cell growth regulatory effects *in vitro*, while they exert less effects on the calcium metabolism *in vivo* compared to $1,25(\text{OH})_2\text{D}_3$ (249).

In mice inoculated with human prostate cancer cells and in transgenic mice with retinoblastoma, Ro23-7553 has been shown to be capable of inhibiting tumor growth without affecting serum calcium levels (250-252). This was further supported by results obtained in a murine squamous cell carcinoma model system, where a significant anti-tumor activity was observed with the treatment of established tumors and in the prevention of tumor initiation or induction with both $1,25(\text{OH})_2\text{D}_3$ and Ro23-7553. However, in contrast to $1,25(\text{OH})_2\text{D}_3$, Ro23-7553 did not

induce hypercalcemia (253). Using the same model system, subsequent studies have demonstrated that Ro23-7553 is a potent enhancer of the cytotoxic effect of the conventional chemotherapeutic cisplatin (254).

The $1\alpha,25$ -dihydroxy-16-ene-23-yne-26,27-hexafluorocholecalciferol analog, Ro24-5531, has been shown to possess promising anti-cancer effects when fed to tumor-bearing animals as a supplement to the diet. In rats with NMU-induced breast tumors, azoxymethane-induced colonic tumors or androgen promoted carcinoma of the seminal vesicle or prostate gland, long-term feeding (5-7 months) of the animals with Ro24-5531 was shown to reduce the number of tumors and the occurrence of invasive carcinomas (255-257).

Ro23-7553 and Ro24-5531 were originally developed by Hoffmann LaRoche Inc. However, ILEX Oncology has recently licensed Ro23-7553 and initiated phase I clinical trials with the compound under the name ILX-23-7553. The current trials include cancer patients with advanced metastatic cancer and are designed to identify the maximum tolerated dose and to demonstrate the safety of ILX-23-7553, both as a single agent and in combination with dexamethasone. In contrast, no clinical trials are currently reported with Ro24-5531 (248).

9.4. 19-nor analogs

Another Hoffmann LaRoche Inc. analog which has been shown to possess promising anti-cancer effects *in vitro* as well as *in vivo* is the 19-nor-hexafluoride analog Ro25-6760 (106, 258, 259). Using the Dunning MAT LyLu prostate rat cancer model it was demonstrated that administration of Ro25-6760 to the animals resulted in inhibition of tumor volume and a reduction in the number and size of lung metastases (196). In addition, studies in nude mice carrying MCF-7 breast cancer tumors have shown that Ro25-6760 is able to suppress tumor growth without inducing hypercalcemia, an effect that could be further enhanced by combination with Taxol. However, the effect of Ro25-6760, either alone or in combination with Taxol, was still found to be less pronounced than the corresponding effects obtained with EB 1089 in the same study (229). Despite the initial encouraging results obtained with Ro25-6760, subsequent studies have indicated that the analog has a tendency to cause hypercalcemia and clinical trials have therefore not been initiated with Ro25-6760 (248). A few other 19-nor analogs have already been mentioned as follow up compounds for Ro25-6760, including the two new 19-nor-14-epi analog TX 522 and TX 527. These two analogs have very recently been described to exert promising anti-cancer effects in human breast cancer cells *in vitro* as well as *in vivo* (260).

9.5. 1α -hydroxyvitamin D₅

Also recently, Metha and colleagues have reported on a new vitamin D analog, 1α -hydroxyvitamin D₅ ($1\alpha(\text{OH})\text{D}_5$), which appears to be approximately 4 times less calcemic than $1,25(\text{OH})_2\text{D}_3$ when tested in rats (261). *In vitro* investigations in breast cancer cells have shown that this analog exerts similar or slightly weaker cell regulating effects than those of

1,25(OH)₂D₃ (262). However, *in vivo* 1α(OH)D₅ was found to be able to prevent the development of chemically induced breast tumors in rats without inducing hypercalcemia or any other side-effects. Thus, the results suggest that despite its relatively low potency *in vitro*, 1α(OH)D₅ may have potential as a chemopreventive agent, as it can be administered at relatively high doses (up to 50 μg/kg) without inducing undesirable toxicity (263). Obviously, more information about the pharmacological profile of 1α(OH)D₅ is required before considering clinical trials, but the possibilities of the analog warrant further investigation.

9.6. Other analogs of interest

Based on promising pre-clinical results, a few other vitamin D analogs have been mentioned as possible agents for treatment of cancer patients, including the 22(s)-24-homo-26,26,26,27,27-hexafluoro-1α,22,25-trihydroxyvitamin D₃ (DD-003, Daikin Industries Ltd.), the 24R,25-dihydroxyvitamin D₃ and the 20-epi(S)-ethoxy-23-yne-24a,26a,27a-trihomocalcitriol (CB 1093, Leo Pharmaceutical Products). Besides, being more potent than 1,25(OH)₂D₃ in inhibiting proliferation of human colon carcinoma cells *in vitro*, DD-003 has also been shown to be able to inhibit growth and invasion of colon tumors in mice *in vivo* (195).

In the case of the 24R,25-dihydroxyvitamin D₃ analog, studies using rats with chemically induced colonic aberrant crypt foci (early signs of colon cancer) or glandular stomach cancer have suggested that this analog exerts chemopreventive effects (264, 265). No rise in serum calcium was seen in former study, but urinary calcium levels were found to be markedly increased in the treated animals, indicating that 24R,25-dihydroxyvitamin D₃ have a narrow therapeutic window.

The 20-epi(S)-ethoxy-23-yne-24a,26a,27a-trihomocanalogue, CB 1093, has been shown to be even more potent than EB 1089 with regard to inhibition of proliferation, induction of apoptosis and inhibition of invasion in leukemia and breast cancer cells *in vitro* (128, 266, 267). In addition, studies in mice and rats carrying breast tumors have demonstrated that CB 1093 efficiently reduces tumor growth and size *in vivo*. In the mice, this effect could be further enhanced by combination of CB 1093 with other chemotherapeutic agents such as cisplatin and paclitaxel (128, 268). However, due to a relatively low bioavailability of CB 1093, EB 1089 was preferred for clinical development and consequently no clinical trials with CB 1093 in cancer patients have been initiated.

10. PERSPECTIVES

Worldwide cancer claims millions of lives every year and new effective agents in the prevention and treatment of cancer are therefore constantly being sought. Experimental, clinical, and epidemiological studies have revealed that hormones such as vitamin D provide promising effects in certain cancer types. The fact that receptors for vitamin D are widely distributed in cancer cells of both epithelial and myelopoietic origin and that 1,25(OH)₂D₃ and its analogs are able to affect a number of

processes known to be involved in the progression of tumorigenesis, point to vitamin D as a potential useful class of compounds in cancer treatment and prevention. A major focus in the research field of vitamin D has been to develop analogs with potent cell regulatory effects but with reduced risk of inducing calcemic adverse effects. A number of such synthetic analogs are now available and a few of them are currently undergoing clinical evaluation in cancer patients. So far, the development of vitamin D analogs as anti-cancer agents holds promise, but a final answer to the role of these compounds in the prevention and treatment of cancer awaits further results. Moreover, many potential perspectives are still to be explored, such as optimal treatment schedule and combination of vitamin D analogs with other anti-cancer agents.

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Abbreviations:

1 α OH-D₃ = 1 α -hydroxyvitamin D₃
 1 α OH-D₅ = 1 α -hydroxyvitamin D₅
 1,25(OH)₂D₃ = 1 α ,25 dihydroxyvitamin D₃
 25(OH)D₃ = 25-hydroxyvitamin D₃
 AF-2 = activation function domain
 AP-1 = activator protein-1
 B16 cells = murine melanoma cell line
 cAMP = cyclic adenosine monophosphate
 CB 1093 = 20-epi(S)-ethoxy-23-yne-24a,26a,27a-trihomo-1,25(OH)₂D₃
 CYP24 = mitochondrial cytochrome P450 enzyme 24-hydroxylase
 CYP27A1 = mitochondrial cytochrome P450 enzyme 25-hydroxylase
 CYP27B1 = mitochondrial cytochrome P450 enzyme 1 α -hydroxylase
 Cdk = cyclin-dependent kinase
 CKI = cyclin-dependent kinase inhibitor
 Da = dalton
 DBD = DNA binding domain
 DBP = vitamin D binding protein
 DD-003 = 22(s)-24-homo-26,26,26,27,27,27-hexafluoro-1 α ,22,25-trihydroxyvitamin D₃
 DMBA = 7,12-dimethylbenzanthracene
 DR = direct repeats
 EB 1089 = seocalcitol, 24a,26a,27a-trihomo-1 α ,25(OH)₂D₃
 EGF = epidermal growth factor
 EGFR = epidermal growth factor receptor
 ER⁺ = estrogen receptor positive
 ER⁻ = estrogen receptor negative
 ERK = extracellular signal-related kinase
 FGF-7/KGF = keratinocyte growth factor
 GM-CSF = granulocyte-macrophage colony-stimulating factor
 HL-60 cells = human promyelocytic leukemia cell line
 HT1080 cells = human fibrosarcoma cell line
 IGFBP = insulin-like growth factor binding protein
 IGF-I = insulin-like growth factor-I
 IGF-II = insulin-like growth factor-II
 IGF-IR = insulin-like growth factor-I receptor
 IL-10 = interleukin 10
 IP = inverted palindromes
 JMRD₃ = vitamin D resistant human chronic myelogenous leukemia cell line
 kDa = kilodalton
 LBD = ligand binding domain
 LNCaP cells = human prostate cancer cell line
 MAP kinase = Mitogen-Activated Protein Kinase
 MC 903 = calcipotriol (Daivonex[®], Dovonex[®])
 MCF-7 cells = human breast adenocarcinoma cell line
 MCF-7/VD^R = vitamin D resistant cell line derived from the MCF-7 cell line (ref. (102))
 MCF-7D^{res} = vitamin D resistant cell line derived from the MCF-7 cell line (ref. 207))
 MDA-MB-231 cells = human breast adenocarcinoma cell line

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MTD = maximum tolerated dose
NCI-H929 cells = human myeloma cell line
NGF = nerve growth factor
NMU = nitrosomethylurea
NSE = non-specific esterase
OCT = 22-oxa-1 α ,25(OH)₂D₃ (Maxacalcitol)
PKA = protein kinase A
PKC = protein kinase C
pRb = retinoblastoma protein
PSA = prostate specific antigen
PTH = parathyroid hormone
PTHrP = parathyroid hormone-related peptide
Ro23-7553 = ILX-23-7553, 1 α ,25-dihydroxy-16-ene-23-yne-cholecalciferol
Ro24-5531 = 1 α ,25-dihydroxy-16-ene-23-yne-26,26,26,27,27,27-hexafluorocholecalciferol
Ro25-6760 = 1 α ,25-dihydroxy-16-ene-23-yne-26,26,26,27,27,27-hexafluoro-19-nor-D₃
RFLP = restriction fragment length polymorphism
RXR = retinoid X receptor
TGF-beta = transforming growth factor-beta
TNF = tumor necrosis factor-alpha
tPA = tissue-type plasminogen activator
TRPM-2 = testosterone repressed prostatic message 2
TX 522 = 19-nor-14-epi-23-yne-1,25(OH)₂D₃
TX 527 = 19-nor-14,20-bisepi-23-yne-1,25(OH)₂D₃
U937 cells = human histiocytic leukemia cell line
uPA = urokinase-type plasminogen activator
UV = ultraviolet
VDR = vitamin D receptor
VDRE = vitamin D response element
VEGF = vascular endothelial factor
Vitamin D = vitamin D compounds in general
Vitamin D₃ = pro-vitamin D₃, inactive form
VLA-4 = very late antigen-4

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