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2408 *Current Topics in Medicinal Chemistry,* 2014, *14,* 2408-2423

Phosphonate Analogues of 1α, 25 Dihydroxyvitamin D_3 are Promising Candidates for Antitumoural Therapies

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Abstract: The active metabolite of vitamin D, 1α, 25 dihydroxyvitamin D_3 (calcitriol) is classically known to regulate calcium and phosphate homeostasis and bone mineralization. In addition, calcitriol has also been documented to act as a potent anticancer agent in multiple cell culture and animal models of cancer. However, major side effects, such as hypercalcemia, hinder broad-spectrum therapeutic uses of calcitriol in cancer chemotherapy. Synthesis of calcitriol analogues with the same or increased antiproliferative and pro-differentiating activities, and with reduced undesired effects on calcium and bone metabolism, is getting significant attention towards rational therapeutics to treat cancer. In this regard, phosphonate analogues have been shown to display a certain degree of dissociation between the vitamin D activity *in vitro* and undesired hypercalcemia *in vivo*. However, few phosphonates have been described in the literature and fewer of them tested for antitumoral effects. Our group has synthesized a novel vitamin D analogue (EM1) bearing an alkynylphosphonate moiety that combines the low calcemic properties of phosphonates with the decreased metabolic inactivation due to the presence of a triple bond between C-23 and C-24. Biological assays demonstrated that this analogue has potent antiproliferative effects in a wide panel of tumour cell lines, even in those resistant to calcitriol treatment. Importantly, EM1 does not show toxic effects in animals, even administered at high doses and for extended periods of time. In the current review we discuss the effects and the potential application in cancer of vitamin D and its derivatives, with an emphasis on phosphonate analogues.

Keywords: Analogues, Calcitriol, Cancer, Hypercalcemia, Phosphonate, Therapy.

VITAMIN D IS A PRO-HORMONE

Vitamin D_3 (also known as cholecalciferol) is the precursor to the potent steroid hormone calcitriol (1α,25 dihydroxyvitamin D_3) that regulates the expression of many genes and has widespread actions throughout the body [1]. Vitamin D_3 is not really a vitamin as, in addition to being obtained from the diet, it can be synthesized in adequate amounts in the skin by ultraviolet radiation of sunlight [2]. Vitamin D_3 is converted in the liver into 25 hydroxyvitamin D_3 (25(OH)D_3 hereafter) which is the circulating form of vitamin D [3]. This is subsequently hydroxylated by the enzyme CYP27B1 to form calcitriol in the kidneys. In addition, calcitriol is also synthesized locally by the CYP27B1 present in most extrarenal tissues, including many cancer cells, where it acts in a paracrine manner. This local synthesis depends on the circulating levels of 25(OH) D_3. Levels of calcitriol are additionally regulated by the enzyme CYP24A1, which begins the inactivation of calcitriol through 24-hydroxylation [4].

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The biological actions of calcitriol are mediated by the vitamin D receptor (VDR), mostly through genomic actions [5-6] through which calcitriol binds to VDR, causing it to dimerize with the retinoid X receptor (RXR); this complex, in turn, binds to vitamin D response elements (VDREs) in regulatory regions of target genes and recruits co-modulators. In addition, calcitriol can also exert its effects through rapid, non-genomic pathways [7]. The fact that VDR is present in most cells in the body [8] and that calcitriol regulates, directly or indirectly, as much as 3-5% of the human genome indicates that vitamin D activity is widespread.

Vitamin D has been traditionally known to regulate calcium and phosphate metabolism and to be essential for bone mineralization [2]. However, over the past three decades it has become clear that vitamin D has many extra skeletal actions, such as those associated with a reduction in cancer risk and progression. The following sections discuss the evidence linking vitamin D status with cancer prevention and survival and the evidence of the activity of vitamin D compounds on cancer therapeutics.

VITAMIN D AND CANCER PREVENTION AND SURVIVAL

In humans the first evidence of the existence of a relation between vitamin D and cancer prevention originated in geo-

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graphical and ecological studies which suggested that a good exposure of people to sunlight (UV), or their living in lower latitudes were related to a lower incidence of colon cancer [9]. Subsequently, other studies showed similar correlations in prostate cancer [10-11]. These ecological studies prompted research on cancer incidence and mortality at the individual level. From 2000 on, numerous studies have investigated the association between circulating 25(OH) D_3 levels in individuals and incidence or mortality/survival for different cancers [12]. Currently, analysis of serum levels of 25(OH) D_3 is the widely accepted measure of the nutritional status of vitamin D. Although there is no general consensus as to which are the optimal levels of circulating 25(OH) D_3, the majority of the experts define them as those higher than 30 ng/ml [2].

Several studies have reported an inverse association between the risk of getting cancer and circulating levels of 25(OH) D_3 [13]. With respect to colorectal cancer, most studies support an inverse relationship between 25(OH) D_3 levels and risk [14-17] whereas, for prostate and breast cancer, the relationship is less clear [15].

This association between the status of vitamin D and cancer risk does not necessarily imply a cause-effect relationship. Information from randomized clinical trials could establish with greater precision the existence or not of this causal relationship, but it is scarce. One of the most important clinical trials carried out was not able to demonstrate significant differences in the incidence of cancer among patients who ingested vitamin D and those to whom a placebo was administered [18]; however, it was later pointed out that there had been significant limitations in the development of the trial.

Evidence of the beneficial effects of the status of vitamin D on cancer progression and survival of the patients surpasses that collected on prevention. There is abundant and consistent evidence that indicates that high levels of 25(OH)D_3 correlate with better survival of the patients with colorectal cancer [19-22], breast cancer [20, 23-24], prostate cancer [25-26], lung cancer [27] and hematologic cancers [28-29]. Importantly, these various correlations observed in the literature are likely to be causal when viewed in the light of most criteria for assessing causality [30].

ANTITUMOR ACTIVITY OF VITAMIN D COMPOUNDS

Vitamin D, besides its known classical actions on calcium homeostasis, has been shown to modulate the majority of the capabilities that cancer cells acquire in their progression to a malignant state and metastatic dissemination, capabilities that were named the "hallmarks of cancer" [31]. Eight capabilities and two enabling characteristics which have been described [32] are: sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, activating invasion and metastasis, altering genome stability, inducing inflammation, reprogramming energy metabolism and evading immune destruction. Therefore, compounds that have the potential to modulate these capabilities are of interest in cancer therapeutics. The accumulating evidence linking calcitriol with these capabilities (Fig. **1**) will be described in the following sections.

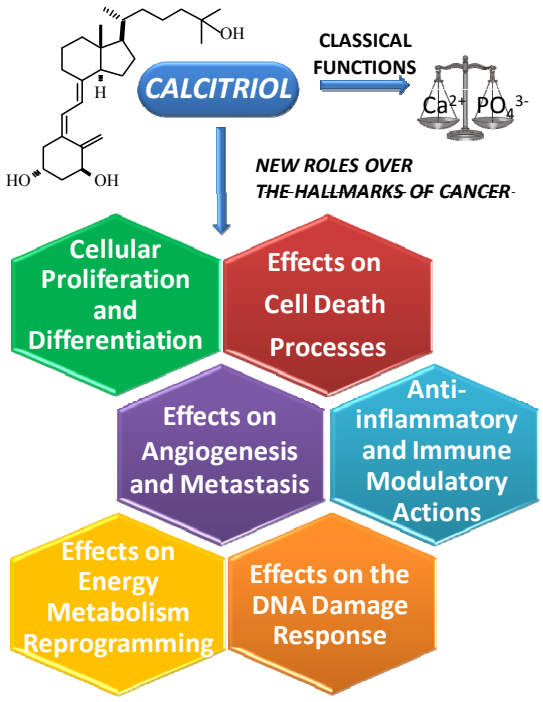


Fig. (1). Calcitriol modulate the majority of the capabilities that cancer cells acquire in their progression to a malignant state and metastatic dissemination.

Effects on Cellular Proliferation and Differentiation

The effect of calcitriol on cell proliferation was initially described for melanoma cells [33]; after that report many other groups have described the anti-proliferative effects of the hormone, not only in tumour cells but also in non-malignant ones [34-40].

In terms of cellular differentiation, the first investigators who demonstrated a relationship between calcitriol and the induction of differentiation were Abe and collaborators in myeloid leukemia cells [41]. Following this initial report it was demonstrated that calcitriol induces differentiation in several other cell types [42-45] and that it exerts antineoplastic activity in several *in vitro* and *in vivo* models [1, 46]. An important feature of the effects of calcitriol on differentiation and cellular survival is that it possesses specificity in relation to the cell type and its context [45]. However, in most cells there seems to be a common molecular mechanism which consists in the arrest of the cell cycle in G_0/G_1 [35-36, 39, 47]. The more in-depth analysis of the mechanisms that underlie cell-cycle arrest led to demonstrate that the hormone produces inhibition of cyclins and cyclin-dependent kinases [47], either by directly modulating their expression or through the induction of the inhibitors $p21^{waf1}$ and $p27^{kip1}$ [36, 47-49]. In relation to the activation of p27, it has been demonstrated that calcitriol acts, in several cancer cell lines, mostly by inducing an increase in the half-life of this protein instead of modulating its transcription. In relation to the modulation of p21, it has been demonstrated that the hor-

mone binds to the VDRE in the gene promoter of this inhibitor and thus increases the levels of both the mRNA and the protein [48]. In addition, calcitriol stimulates the expression of E-cadherin [50], inhibits the transcriptional activity of B-catenin [51], modulates the activation of the TGF-β, IGFBP3 and EGFR pathways and reduces the levels of c-myc and c-jun [36, 52].

Effects on Cell Death Processes

In addition to the effects on cell proliferation and differentiation, calcitriol may induce apoptosis in cells of different tumour types such as those of breast carcinoma, squamous cell carcinoma, colorectal carcinoma, prostate cancer, myeloma, retinoblastoma and lymphocytic leukemias of B cell lineage [53-58]. It has been observed that the hormone suppresses the expression of apoptotic proteins Bcl-2 and Bcl-XL and induces the expression of the pro-apoptotic Bax, Bak and Bad [40, 59-60]. In addition, it has been observed that it can directly activate the effector molecules of caspases [61]. More specifically, it has been shown that the antiapoptotic protein Bcl-2 decreases in response to treatment with the hormone in prostate cancer [56], chronic leukemias B [58], cells of retinoblastoma [57] and in the MCF-7 breast adenocarcinoma cell line [62], and thus leading to an increase in apoptosis. In cell lines of invasive breast cancer, the reduction of Bcl-2 is accompanied by an increase in Bax and cytochrome c release followed by the cleavage of PARP [63]. Other suggested mechanisms which mediate the effect of calcitriol on cell death are: a reduction in the expression of IFGR [64], the induction of MEKK1 [65], the activation of the signalling pathway sphingomyelin/ ceramide/ ganglioside GD3 [66], the reduction in the expression of Akt [67-68] and the increase in TNFα [69].

In addition, calcitriol increases the sensitivity of cancer cells to treatment with various cytotoxic drugs and radiation. For example, breast cancer cells are more sensitive to apoptosis induced by reactive oxygen species (ROS) when they are treated with calcitriol [70].

An alternative type of programmed cell death independent of caspases action, and which is sometimes deregulated in tumours, has recently been described under the name autophagy. Autophagy is characterized by the increase in the number of autophagosomes, the vacuoles which are surrounded by a double membrane and which sequester cytoplasm and organelles to be digested when merged with the lysosomes. It has been shown that some agents that mobilize Ca^{2+}, such as calcitriol, induce a massive increase in the number of autophagosomes in breast cancer cells, thereby inducing autophagy [71-72].

Effects on Angiogenesis and Metastasis

Angiogenesis is the process of formation of new blood vessels from existing vasculature, and is a crucial event for the continuous growth, progression and metastasis of tumours [73]. Tumour cells acquire the ability to induce the formation of new vessels in order to ensure an adequate supply of oxygen, and for this end, they often induce the synthesis of vascular endothelial growth factor (VEGF) which, in turn, stimulates the endothelial cells of the adjacent vessels. In this regard, it has been described in *in vitro* assays that 1α, 25 dihydroxyvitamin D_3 inhibits the proliferation of endothelial cells derived from tumours [68, 74] and also the elongation and branching observed in endothelial cells after exposure to the VEGF [75], thereby reducing the angiogenesis. Furthermore, it increases the mRNA levels of the potent antiangiogenic factor thrombospondin in colorectal cancer [76], interrupts the signalling of IL-8-dependent nuclear factor NF in prostate cancer [77] and reduces the expression of VEGF through the transcriptional repression of HIF-1 in cancer cells of the pancreas [78].

There is also evidence that indicates that calcitriol is a potent inhibitor of angiogenesis *in vivo*, capable of reducing the vascularization of tumours in animal models of breast [75] and colon cancer [79]. The formation of new vessels in the peritumoural area is important not only for the oxygen supplies to the tumour cells but also to make the cancer cells invade the circulatory system and migrate to distant places. Therefore, the effect of calcitriol on angiogenesis ultimately results in a decrease in the metastatic process. However, this hormone also affects directly the metastatic cascade through the reduction of the migratory and invasive capacity of tumour cells. Specifically it was found that it produces inhibition of serine proteases and metallo-proteinases of the family of the plasminogen system [80-81], proteases necessary for the degradation of the extracellular matrix that allow tumour cells to migrate to distant sites of the primary tumour. More specifically, it is known that calcitriol decreases the expression and activity of MMP-9 at the same time that it increases the activity of its counterpart, the inhibitor of metalloproteinase 1 (TIMP-1) [77]. On the other hand, it causes a decrease in the expression of integrins [82] and increases that of E-cadherin [46, 83], proteins that participate in the process of epithelial-mesenchymal transition involved in the metastatic tumour process.

ANTI-INFLAMMATORY AND IMMUNE-MODULATORY ACTIONS

Tissue inflammation contributes to the development and progression of many types of cancer [84-86]. Furthermore, as mentioned above, it has been described as one of the enabling characteristics that tumour cells acquire during tumour progression [32]. Inflammatory mediators such as cytokines, chemokines, the prostaglandins (PGs) and reactive oxygen and nitrogen species favour tumorigenesis through the activation of multiple signalling pathways that lead to sustained proliferation, inhibition of cell death, promotion of angiogenesis and metastasis and the acquisition of additional mutations in the DNA of tumour cells [84-86].

Studies in cells of prostate and breast cancer revealed that calcitriol exerts important regulatory effects in some of the major molecular pathways involved in inflammation; for example, it has been shown to inhibit the synthesis and the activity of prostaglandins (PGs) [87-88] and to decrease the levels of the angiogenic cytokine and pro-inflammatory IL-8 [77].

On the other hand it is known that calcitriol blocks the activation of NF-κB, an important regulator of the innate immune response and inflammation [89], thus increasing the expression of IκB [90-91].

As regards its immunomodulatory action, it has been shown that calcitriol exerts immunosuppressive effects by inhibiting the activation of the effector T lymphocytes and consequently the production of their cytokines and by stimulating regulatory T lymphocytes [92]. The hormone also inhibits the antibody-producing B cells. All of these actions show the inhibitory action of 1α, 25 dihydroxyvitamin D_3 on the adaptive immune response. In addition, it has also been demonstrated that calcitriol modulates the innate immune response [93].

In contrast with the immune response associated with the inflammation and infection, the tumoural immune response is affected in various ways by calcitriol or vitamin D analogues. Although some studies have shown that these compounds stimulate immune reactivity in cancer patients, other studies have shown they have immune-inhibitory effects. In tumour squamous cell carcinomas the hormone has been shown both to activate the immune system by promoting the differentiation of immature CD34+ cells into antigen-presenting cells [94] and to reverse the immunosuppressive mechanisms in patients [95]. In non-muscle invasive bladder cancer, calcitriol was shown to enhance the response of the tumour to treatment with the bacillus Calmette-Guerin [96].

Effects on Energy Metabolism Reprogramming

As observed originally by Otto Warburg, cancer cells can reprogram their glucose metabolism, and thus their energy production, by limiting their energy metabolism largely to glycolysis, leading to a state that has been termed "aerobic glycolysis". They do so, in part, by up-regulating glucose transporters, notably Glut-1 which substantially increases glucose import into the cytoplasm [97-98]. Calcitriol has been shown to modulate Glut-1 in various human cancer cells [78], play a pivotal role in maintaining the glucose transport in the brainstem of diabetic rats [99] and enhance insulin action in muscle tissue [100].

Effects on the DNA Damage Response

It has been demonstrated that calcitriol increases the expression of several genes that aid in the repair of DNA, such as Rad50 and ATM [101]. Keratinocytes in the epidermis of mice lacking VDR are deficient in the DNA damage response (DDR) as demonstrated by a reduced rate of clearing of the DNA adducts DNA cyclobutane pyrimidine dimers (CPD) and pyrimidine (6–4) pyrimidone photoproducts (6–4PP), following UVB irradiation [102]. Moreover, calcitriol increases CPD clearance and also p53 levels in VDR-intact mice [103-104]. It also induces upregulation of two genes important for DDR: XPC (xeroderma pigmentosum complementation group C) and DDB2 (damage-specific DNA binding protein 2, also known as XPE) [102, 105]. In normal proliferating lymphocytes as well as in A549 lung adenocarcinoma and lymphoblastic TK6 tumour cells the level of constitutive DNA damage likely induced by products of oxidative metabolism was attenuated by calcitriol [106].

THERAPEUTIC APPLICATIONS OF VITAMIN D COMPOUNDS IN CANCER

As previously described, both the epidemiologic studies and the numerous pre-clinical testing led to the conclusion that the vitamin D compounds have important effects on cancer progression. On the basis of this overwhelming evidence of the antitumoral action of vitamin D compounds, some clinical trials were carried out evaluating their effects, either alone or in combination regimens with cytotoxic agents, in the treatment of different types of cancer [107].

The first clinical studies were conducted in patients with myelodysplastic syndrome (MDS) and acute leukemia (AML) [108-111]. A daily regimen of cytarabine and calcitriol achieved a prolonged remission in elderly patients with both diseases although a severe hypercalcemia was also observed in 10-30 % of the patients. Subsequent clinical trials showed no effect [112] while others had a limited success [113-114]. Some of the ongoing or completed clinical trials evaluating the effects of vitamin D compounds in cancer can be seen in the site of the World Health Organization International Clinical Trials Registry Platform. Some of these clinical studies have been criticized either for having used very low doses of the compound, or for having employed continuous schemes rather than intermittent ones or for not having been properly designed [115]. The formulations of calcitriol commercially available do not have a good bioavailability and also present a great variability among patients in terms of the serum levels that can be obtained [116]. At present, trials evaluating a combination of calcitriol and non-cytotoxic compounds, such as glucocorticoids, or the use of intermittent doses, have shown better results by reducing the hypercalcemia [117]. An alternative approach to the problem of the hypercalcemia lies in designing analogues of vitamin D that retain or even increase the antitumoral capacities but lack the toxic side effects. This alternative is described below.

Analogues of Vitamin D

There have been tremendous efforts in both industry and academia to develop new vitamin D_3 analogues able to retain the beneficial effects but minimal or no undesired hypercalcemic side effects for a variety of potential indications such as psoriasis, cancer and inflammation. At present, several thousand vitamin D analogues have been synthesized and, in preclinical assays, many have demonstrated to exert anticancer effects with reduced hypercalcemic activity. Some of these were more potent than calcitriol as antitumoral agents but only a few of these were evaluated in clinical trials. For a list of the most promising vitamin D analogues and their possible target diseases see [4]. However, there are still no government-approved vitamin D_3 analogues to be used in cancer therapeutics [118-119].

Most analogues have largely appeared not by design but, instead, from obvious chemical modifications and subsequent screening for specific activities, i.e., antiproliferative effects, induction of hypercalcemia, induction of differentiation. The differential response and activity of each new analogue synthesized might be explained by the differential properties of the compound over the following parameters: affinity for VDR, affinity for vitamin D binding protein (DBP), ability to recruit RXR and coactivators, rate of target cell metabolism and rate of hepatic or non-specific clearance [120-121] (Fig. **2**).

Since it is not possible to predict which of these properties will be affected by a given chemical modification, it is always necessary to evaluate the activity of the new synthesized compound by performing biological assays.

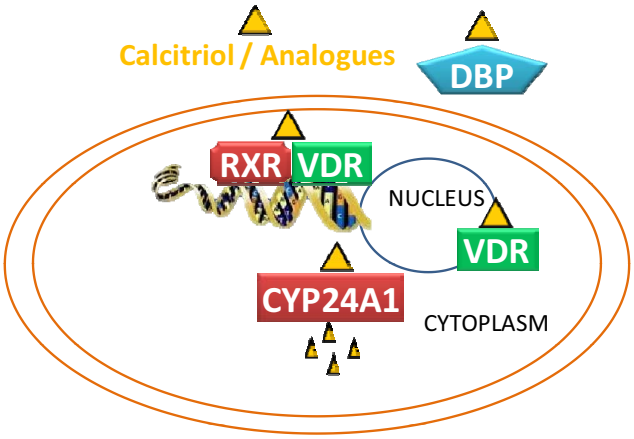


Fig. (2). Differential response and activity of each new analogue synthesized might be explained by the differential properties of the compound over the following parameters: affinity for VDR, affinity for vitamin D binding protein (DBP), ability to recruit RXR and coactivators, rate of target cell metabolism and rate of hepatic or non-specific clearance.

Numerous routes have been developed with the aim of obtaining a versatile and rapid way to prepare vitamin D analogues [122-125]. Fig. (**3**) summarizes the main methodologies currently in use. Among them, some strategies involve the use of the whole steroid precursor, thus offering the advantage of most of the vitamin D skeleton, including its absolute stereochemistry. Other approaches are based on the construction of the trienic system by coupling small synthons in the last step of the biosynthetic pathway. Such convergent synthesis work better to modify different remote regions, allowing ample versatility. Some approaches are more suited than others, depending on the part of the structure to modify, and the well-known Wittig-Horner coupling is probably the most used.

Vitamin D Analogues and Clinical Trials

Some analogues of vitamin D have been evaluated in clinical trials for some types of cancer either individually or in combination with other cytotoxic agents. However, the same analogue can have varied effects on different types of cancer and it is necessary to carry out the studies in each case in order to define its therapeutic utility. For example, although a phase II trial suggested that the analogue EB1089 (seocalcitol) was beneficial in patients with hepatocellular carcinoma [112], subsequent clinical trials showed negative results in pancreatic cancer [126], breast adenocarcinoma and colorectal cancer [127]. A phase I/II study of the analogue 19-nor-1α-25-dihydroxyvitamin D_2 (paricalcitol) in patients with advanced prostate cancer showed that, despite the fact that the drug was well tolerated, there was no significant response [128]. This same analogue administered orally in patients with metastatic breast carcinoma proved to be well tolerated [129].

The 1α-hydroxy vitamin D_3 has been tested in phase I and II clinical trials in patients with prostate cancer either alone or in combination with docetaxel, having demonstrated only a limited antitumoral response [130-132]. This analogue has also been tested in phase II trials in glioma, and a response (involving blockade of the tumoral extension, decrease in the gadolinium enhanced area and slow shrinking of the lesion) was observed in 20% of the patients [133].

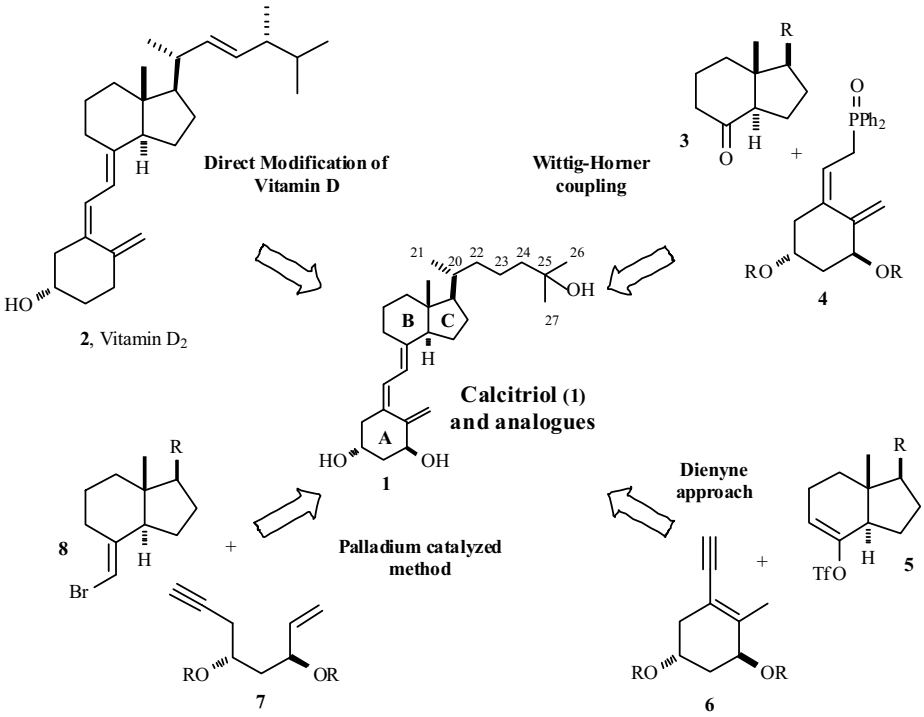


Fig. (3). Main methodologies developed to obtain a versatile and rapid way to prepare vitamin D analogues.

Inecalcitol (19 nor-, 14 epi-, 23-yne, 1α,25-dihydroxyvitamin D_3) is a recent analogue that has been developed by Hybrigenics Corporation. In patients with advanced prostate cancer this analogue, in combination with docetaxel, showed a reduced propensity to generate hypercalcemia while maintaining its antitumoral activity [134].

In summary, in some of the clinical trials conducted to date the daily administration of the analogues generated hypercalcemia; in other trials there were no toxic effects but neither were there any beneficial ones. This lack of response may be, in part, due to the fact that the effective doses of the vitamin D analogue needed to retard cell growth (approximately 1 nM or higher) cannot be usually attained *in vivo* owing to the possible low bioavailability of the compound [135]. Another explanation may be that cancer cells usually express high levels of the catabolic enzyme CYP24A1, and this may limit the effective drug concentration reached in the tumour. All these data suggest the need for synthesizing new analogues with the aim of finding one that overcome the problems described above.

Synthesis of Phosphonate Analogues

In the context of analogue development, structural modifications have been introduced in each part of the vitamin D molecule: in the side chain, in the central CD-ring and in the A ring. However, most of the synthetic studies involve side-chain modification, and in a lesser extent in the A-ring. The modifications usually follow two main strategies, which are either to increase the affinity of the compounds for binding to the VDR or to modulate the metabolic stability of the compounds [136]. As previously mentioned, the metabolic stability of vitamin D analogues in target cells contributes to their abundance, and thus increases their biologic activities. Taking into account that the depletion of calcitriol is mostly due to the metabolic cascade initiated mainly in the side chain by C-24 hydroxylation, the direct chemical interference and prevention of the inactivating characteristic pathways is a logical choice for the design of new analogues [137].

The synthesis of the first phosphorus-containing analogue of vitamin D was reported by W. Dauben [138] (Fig. **4**). In such analogue, the heteroatom was introduced in the side chain by replacing C-25 as phosphine oxide moiety (**9**). Subsequently, this author reported the synthesis and the biological activity data of the corresponding 1α-hydroxy derivative **10**, and the methyl phosphonates analogues **21** and **22** [139]. The phosphorus functionalities were introduced in that position in order to test the structural parameters involved in 25-hydroxylation and the binding to the 25-hydroxyvitamin D receptor sites. It was also expected that the introduction of the polar phosphorus functionalities into vitamin D skeleton would alter the solubility and transport properties of the new analogues, as well as their toxic effects, such as hypercalcemia.

The synthesis of these compounds involves the convergent Wittig-Horner coupling between ketone **17**, which contains the phosphonate side chain fragment, and the A-ring phosphine oxide **18** [140] and **4** [141-142]. For the preparation of ketone **17** they took advantage of the synthetic strategy planned in the synthesis of **9**.

The CD-ring portion **11** was obtained by degradation of vitamin D_2 using the procedure of Okamura [140] (Scheme **1**). After protection of the hydroxyl group as the silyl ether, the side chain was ozonolytically cleaved affording the aldehyde **12**.

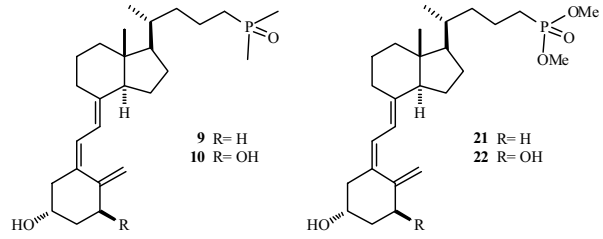


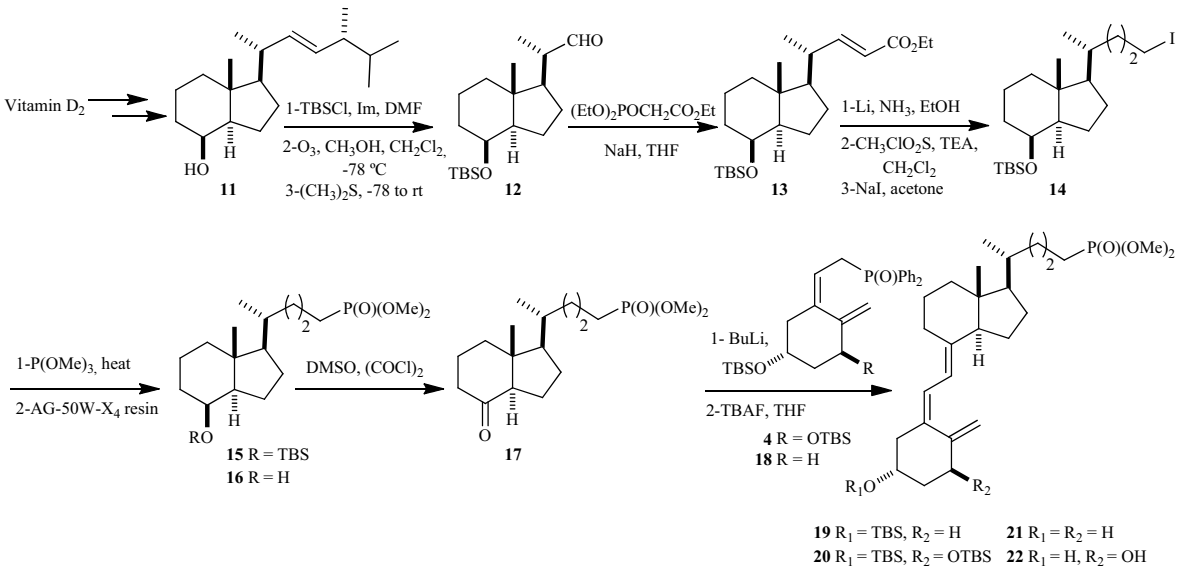
Fig. (4). First phosphorus-containing analogues of vitamin D reported by W. Dauben.

A two carbon homologation of **12** by Horner-Wadsworth-Emmons conditions afforded αβ-unsaturated ester **13,** which was reduced with lithium-ammonia in ethanol to alcohol; which was transformed to mesylate and subsequently to iodide **14**. Arbusov reaction of **14** with trimethylphosphite gave the phosphonate **15** in 26% yield. Further desilylation of C8 hydroxyl with activated AG-50W-X4 cationic exchange resin in methanol afforded alcohol **16**, which under Swern oxidation gave the CD-ring ketone **17** in 84% yield.

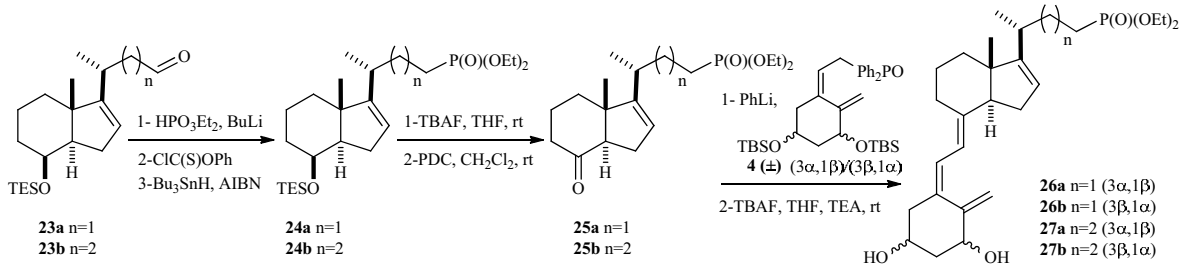
The Wittig-Horner reaction of ketone **17** with the anion derived from the phosphine oxide **18** and **4** yielded protected vitamin derivates **19** (74%) and **20** (83%) respectively. Finally, the silyl ether groups were removed by treatment with TBAF to give the phosphonate analogues **21** (67%) and **22** (83%) in good yields.

Some years later, Han G. [143] reported the synthesis and biological evaluation of analogues containing a phosphonate moiety in the side chain and 16, 17-double bond in the CD ring, which have either natural or unnatural A-ring [144]. They designed these analogues containing phosphonyl group (P=O) instead of C-24 carbonyl group (C=O) (**26a,b**), taking into consideration that the main metabolite of side chain degradation, lα,25-dihydroxy-24-oxo-vitamin D_3, shows equal antiproliferative potency to calcitriol but 10-fold less calcemic effect. They also prepared the corresponding homoanalogues (**27a, b**), to evaluate side chain length effect.

The reported synthesis of 16-ene-phosphonates analogues **26a,b** and **27a,b**, outlined in Scheme **2**, was carried out in a convergent manner by employing Wittig-Horner coupling. The side chain construction starting from 16-ene aldehydes **23a** and **23b** [145], was followed by reaction with lithium diethyl phosphate to afford diasteromeric hydroxyphosphonates, which after further deoxygenation under Barton reaction gave phosphonates **24a** and **b** (43-56%). Following the classical sequence of deprotection of the C8 hydroxyl group with TBAF, further oxidation of the resulting hydroxyl group with PDC, Wittig–Horner reaction of compounds **25a,b** with the anion derived from racemic phosphine oxide **4**, and final deprotection by treatment with TBAF yields diastereomeric 16-ene-phosphonate calcitriol analogues **26a,b** and **27a,b** in 22 and 41% yield respectively. The diastereomers analogues (**a,b**) were separated by reverse phase HPLC.



Scheme 1.



Scheme 2.

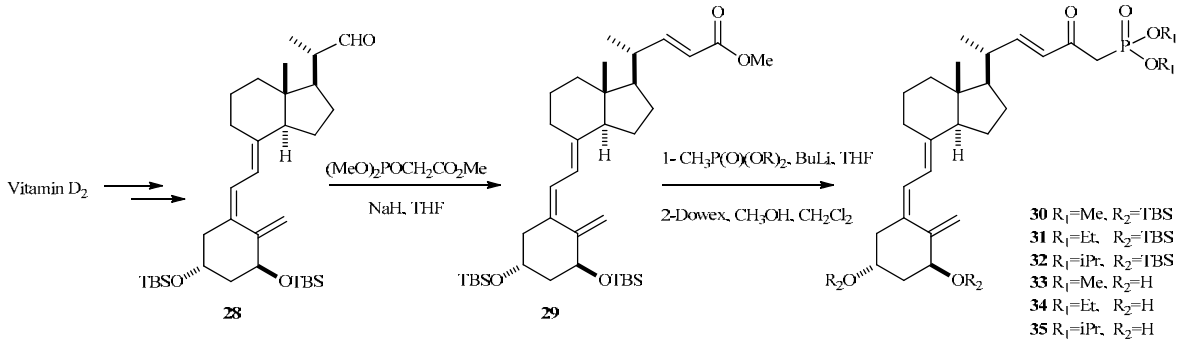
In the search for new vitamin D derivatives which have advantageous or improved spectrum of action and better suited for systemic administration owing to their higher metabolic stability, Steinmeyer *et al.* by Schering [146-147] reported a new study of vitamin D phosphonate hybrids which involved phosphonate and bisphosphonate substructures in the side chain, and their biological evaluation. They took into consideration that the C-24 hydroxylated 1α-hydroxyvitamin D_3 derivatives have lower toxicity than the corresponding non-hydroxylated 1α-hydroxyvitamin D_3; and also that 25-carboxylic acid derivatives of Calcitriol that were hydroxylated at C-24 exhibit a more advantageous spectrum of action than Calcitriol. Generally, however, the introduction of the 24-hydroxyl group results in metabolic destabilization of the derivatives.

The study describes the synthesis of series 25-phosphonates vitamin D analogues like β-ketophosphonates (**33**-**35**), β-hydroxyphosphonates (**39**-**41**), enol phosphate derivatives (**43**) and bisphosphonates (**47** and **49**). The synthetic approach implies the direct modification of vitamin D_2, *via* protection of the triene system, selective cleavage of the isolated side chain double bond, 1α-hydroxilation and subsequent introduction of the modified side chain.

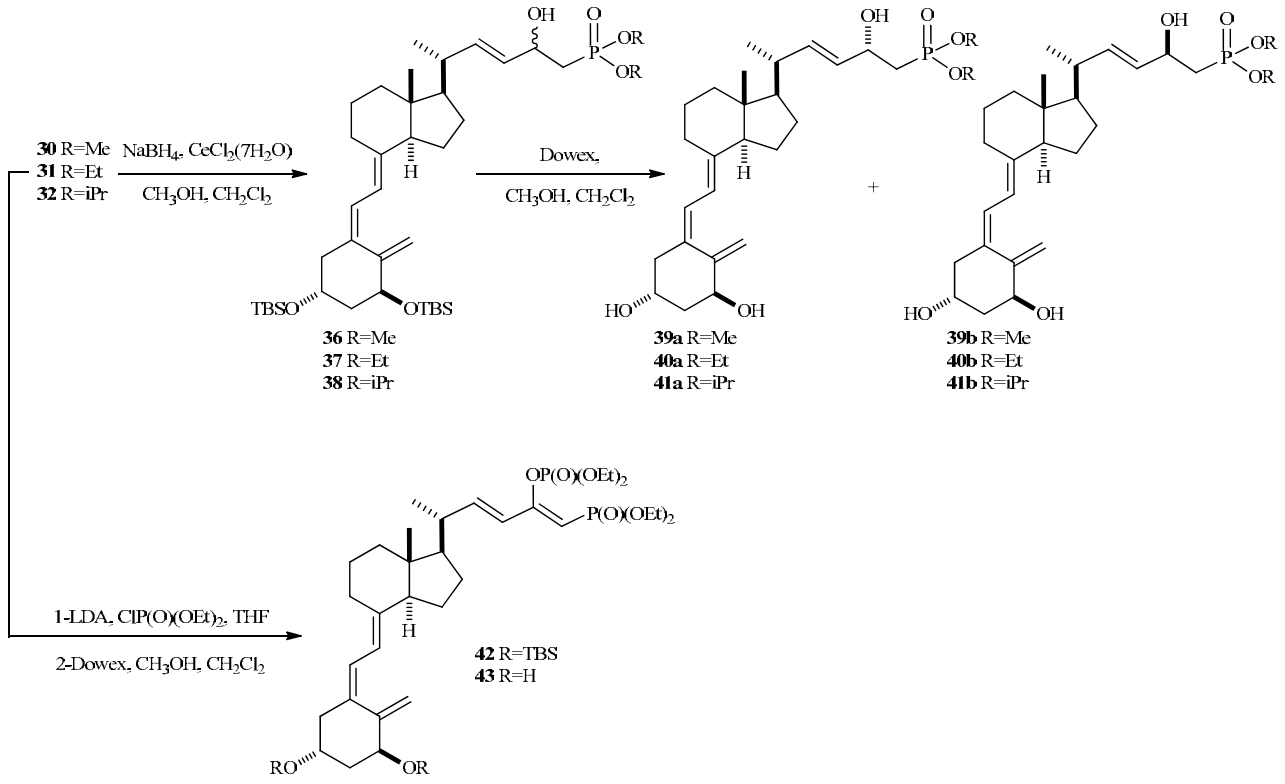
The synthesis of series 25-phosphonates started from aldehyde **28**, which was obtained by using the Barton-Hesse procedure from vitamin D_2, [148-149] and followed by photoisomerization of the triene system using antracene (Scheme **3**).

The synthesis of β-ketophosphonates (**33**-**35**) analogues was achieved by Wadsworth-Emmons reaction of **28** using methyl (dimethoxyphospinyl) acetate to afford α,β-unsaturated ester **29** (71% yield), which was reacted with different deprotonated dialkyl methylphosphonates to give access to β-ketophosphonates **30**, **31** and **32** (74-77%). Successive deprotection of silyl groups using acidic Dowex ion exchange resin furnished the desired vitamin D analogues **33**, **34** and **35** in 67%, 85% and 69% yield respectively. In addition, Luche reduction of keto group in compounds **30**-**32**, and subsequently desilylation afforded the diastereomeric β-hydroxyphosphonates **39**, **40** and **41**, which were separated by HPLC (series **a** and **b**) with yields of 14-19% (Scheme **4**).

The synthesis of structures with two phosphorus units in the side chain took advantage of the intermediate compounds generated previously in the described synthetic sequence. The treatment of β-ketophosphonate **31** with LDA and subsequent trapping of the anion with diethyl chlorophosphate yielded the enol phosphate **42** (81%) as a single isomer. Further deprotection with Dowex resin afforded the dihydroxy enol phosphate analogue **43** in 69% yield. The synthesis of



Scheme 3.



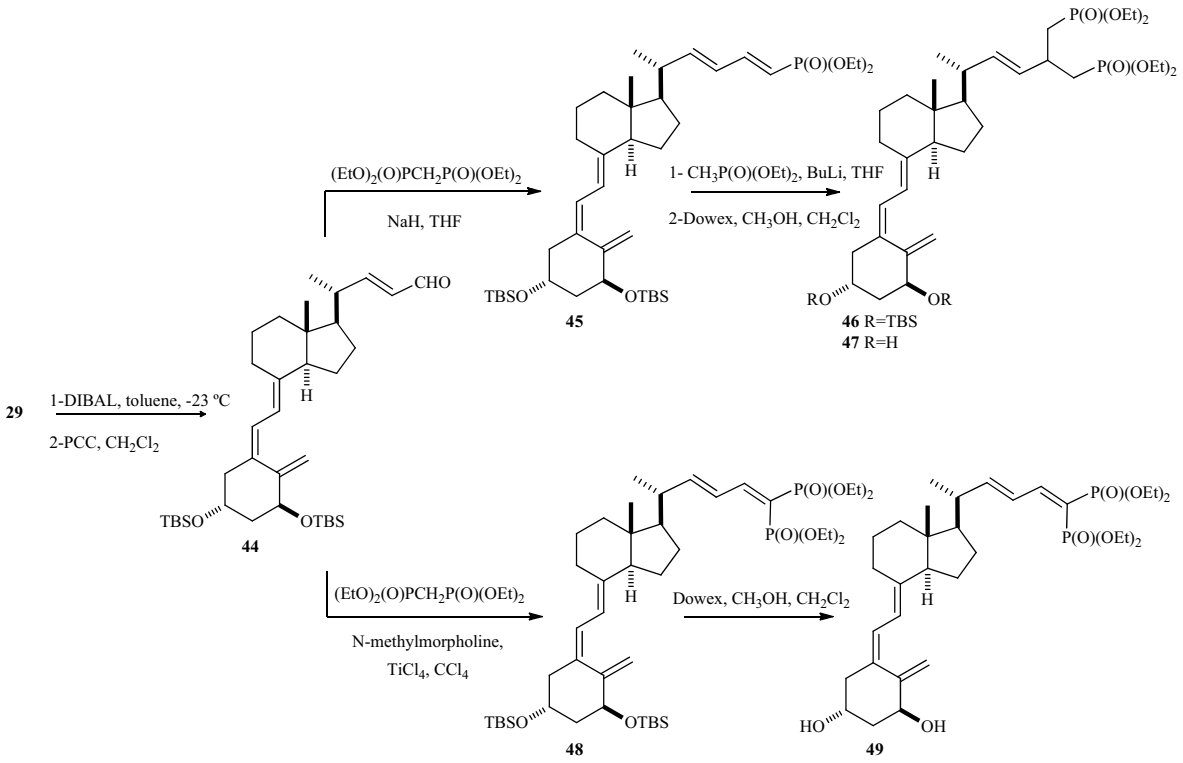
Scheme 4.

analogues with two phosphonate units started with reduction of ester **29** using DIBAL-H in toluene and subsequent oxidation of the resulting allylic alcohol with PDC to obtain the aldehyde **44** (74%) (Scheme **5**). The synthesis of derivative **47** was followed by Wadsworth-Emmons reaction using tetraethyl methylenebisphosphonate to afford the diene phosphonate **45** (58%), whose Michael addition with diethyl methylphosphonate gave the bisphosphonate **46** (62%). Finally, removing of the silyl ethers groups produced the bisphosphonate analogue **47** in 43% yield. Furthermore, the treatment of aldehyde **29** with tetraethyl methylenebisphosphonate under Knoevenagel conditions gave the bisphosphonate **48** (56 %) which after deprotection furnished the bisphosphonate calcitriol analogue **49** in 68% yield.

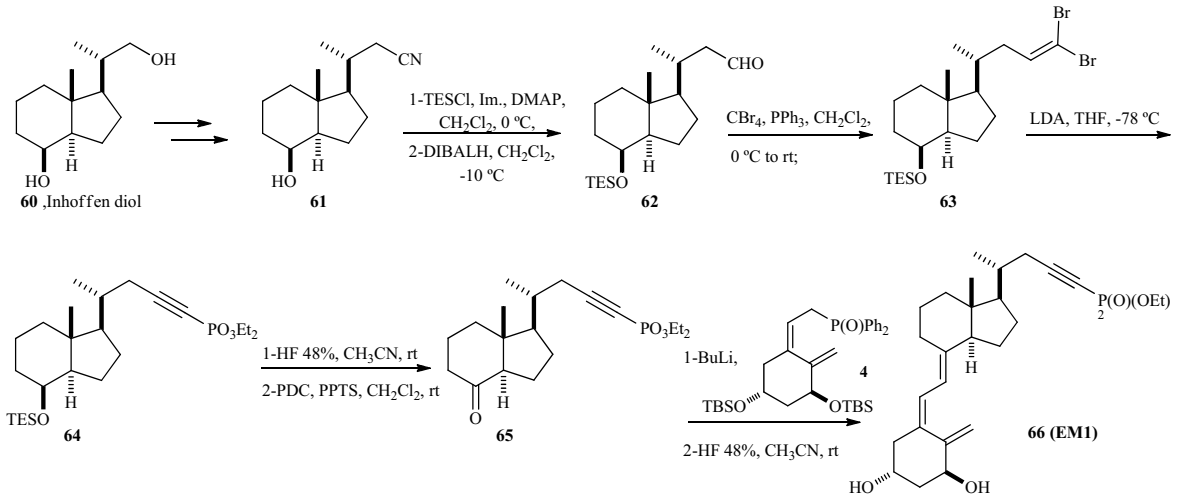
More recently, we designed a convergent route for the synthesis of alkynylphosphonate calcitriol analogue (**66**) [150]. This analogue combines the low calcemic properties of phosphonates [146] with decreased metabolic inactivation due to the presence of a triple bond in C-24 [137].

The synthetic approach to rebuild the vitamin D triene system involves the convergent Wittig-Horner coupling between the ketone **65** and the phosphine oxide **4**. The synthesis of the alkynylphosphonate precursor **65** takes advantage of the readily available Inhoffen diol (**60**) to afford the nitrile **61** (Scheme **6**) [140, 151].

Protection of the C-8 hydroxyl group of **61** as a triethylsilyl ether, followed by treatment with DIBAL-H in dichloromethane yielded the aldehyde **62**, which was submitted under Corey-Fuchs conditions to give a dibromoalkene **63**. Treatment of **63** with an excess of LDA led to the corresponding acetylenic anion, which was trapped with diethyl chlorophosphate to afford the alkynylphophonate **64**.



Scheme 5.



Scheme 6.

Further cleavage of the silyl group of **64** using HF 48% and oxidation of the resulting C-8 hydroxyl group with PDC afforded ketone **65**.

Finally, coupling reaction of **65** with the anion derived from the phosphine oxide **4** yielded the protected analogue. Subsequent removal of the silyl protecting groups led to desired analogue **66** (EM1).

BIOLOGICAL STUDIES OF PHOSPHONATE ANALOGUES OF CALCITRIOL

The biological activities of vitamin D derivatives possessing phosphorus atoms in the side chain were first reported by Dauben and col. [139]. These analogues (shown in Fig. **4**) had either a phosphine (**10**) or a phosphonate moiety (**22**) at position 25. As mentioned, the authors anticipated that the introduction of a heteroatom at the 25-position would inhibit 25-hydroxylation and that the polar phosphorous should alter the solubility and transport properties of the analogue. For the biological assays, the authors assessed analogue binding to chick intestinal receptor and observed that the phosphonates were more active than the phosphine oxides and the lα-hydroxylated compounds were more active than the non-lα-hydroxylated analogues. The results were expressed as Relative Competitive Index (RCI), where RCI is defined as 100 for calcitriol. Compound **22** showed a RCI

of 7% in binding, under *in vitro* conditions, to chick intestinal receptor. In addition, this analogue showed low intestinal calcium absorption (2%) and low bone calcium mobilization (1%) and consequently these analogues presented low hypercalcemic effects. Subsequently, Zhou and col. [152] used these compounds to perform additional biological assays. These analogues again demonstrated reduced hypercalcemic effects when compared to calcitriol (around 0.2% of intestinal calcium absorption and 0.14% bone calcium mobilization) but had only weak activity over clonal proliferation of HL-60 promielocytic leukemic cell line, and displayed almost no activity in differentiation assays.

Han [143] prepared a new series of 16-ene-phosphonate side chain analogues of calcitriol by increasing side chain length (Scheme **2**) with the aim to increase desirable antiproliferative activity over those described above. They analysed analogue antiproliferative activity using murine keratinocytes. Overall, the four analogues synthesized demonstrated a potent antiproliferative activity that was greater than that observed with compounds **10** and **22** and was similar at the μM level to that exerted by calcitriol.

Subsequently, Steinmeyer and col. [146] demonstrated that the introduction of phosphonate and bisphosphonate units into the vitamin D side chain (Schemes **3-4**) resulted in different activity profiles. These authors evaluated the activity of the analogues with various biological assays such as binding to the vitamin D receptor, study of the induction of differentiation of the HL-60 promielocytic leukemic cell line, synthesis of osteocalcin in the rat osteosarcoma cell line ROS 17/2.8, *in vivo* assessment of bone anabolic activity and the induction of hypercalcemia in rats. Analogues which have 24β-hydroxy groups in the side chain showed considerable vitamin D activities *in vitro*, and hypercalcemic effects were less pronounced than for calcitriol. The 24α-hydroxy derivatives, on the other hand, were considerably less active. The corresponding 24-keto analogue stimulated bone matrix formation, while hypercalcemic effects were remarkably reduced in comparison with calcitriol, thus indicating the existence of a therapeutic window for treatment of bone formation. Finally, vitamin D bisphosphonate hybrids turned out to be rather inactive in terms of vitamin D actions *in vitro* but induced osteoid formation *in vivo*. In summary, these analogues demonstrated potential as agents against osteoporosis. No further studies were reported in the literature describing antitumoral effects of these compounds.

Recently, our group has synthesized and evaluated the antitumoral effects of a new phosphonate analogue of calcitriol, Diethyl [(5Z,7E)-(1S,3R)-1,3-dihidroxy-9,10-secocola-5,7,10(19)-trien-23-in-24-yl] phosphonate (EM1). The antitumoral effects were assayed by using a panel of human and murine tumour cell lines derived from different types of cancer [150]. We observed a significant decrease in cell counting after treatment with EM1 in human squamous cell carcinoma HN12, human glioma T98G, human T47D and murine LM05 hormone-sensitive breast adenocarcinoma and Kaposi sarcoma SVEC vGPCR cell lines. Furthermore, in HN12 and in T98G cell lines, the antiproliferative effects exerted by EM1 were greater than those elicited by calcitriol and, importantly, no antiproliferative effects were observed in primary human astrocyte cultures. Similarly, although calcitriol was more potent than EM1 in inhibiting the growth of Kaposi sarcoma cells, EM1 had no effects on the non-malignant parental cell line SVEC. On the other hand, no effects were observed in the colorectal carcinoma HCT116 and in the murine non-hormone responsive breast carcinoma LM3 cell lines. It is known that cancer cell lines display a range of sensitivities to the antiproliferative effects of calcitriol and its derivatives, although the reason for this is largely unknown and could result from defects in any of the components in the VDR signaling pathway including VDR and 24-hidroxylase (CYP24A1). Calcitriol action is limited by its catabolism, occurring mainly by the CYP24A1 resulting in 1α,24,25-$(OH)_3$-D_3, a metabolite with substantially lower affinity for the VDR. Although this enzyme is located primarily in liver, many studies have demonstrated that it can also be expressed by many tissues [61]. An abnormally high basal expression level of CYP24A1 occurs in several cancer cells, thereby making them resistant to calcitriol action [87, 153-154]. In this regard, EM1 presents limitations in its metabolization through 24-hydroxylation due to the presence of a triple bond between the carbons 23 and 24, so its metabolic transformation might be reduced [137]. This is relevant in cells showing important activity of CYP24A1 such as astrocytes [155], prostate cells [156], colon, ovary, lung tumours [157], breast cancer [158] and glioma [159]. In human glioma cell lines, the natural hormone either does not exert antiproliferative activity or it increases proliferation [159-160] whereas EM1 potently inhibits cellular proliferation. The potential differences in the metabolic degradation between calcitriol and EM1 might account for the differences observed in the antiproliferative response. The analyses of the mechanisms possibly involved in EM1 action showed that VDR is not totally necessary for the antiproliferative effects observed in the glioma cell line, thus suggesting the involvement, at least in part, of non-genomic effects elicited by this analogue [150]. Our results demonstrating EM1 antiproliferative effects on breast cancer cell lines that are hormone-responsive are also supported by previous observations showing that the sensitivity to calcitriol is higher in those mammary cancer cell lines that express estrogen receptors [34]. The induction of hypercalcemia was also evaluated *in vivo* for EM1 in mice and, similarly to previously reported phosphonate analogues, EM1 showed no calcemic activity, even administered at high doses (5 and 20 μg/kg weight). These concentrations have been shown to exert antitumoral effects in mice, at least for calcitriol [161]. As expected, calcitriol was effective at causing an increase in plasma calcium and caused the death of the animals within 3 days. On the contrary, mice treated with EM1 remained alive and healthy during the entire examination period (20 days), and observation of the internal organs of the animals such as liver, duodenum, lungs and kidneys showed no macroscopic morphological alterations. Thus, this compound appears to be well tolerated even at high doses. This analogue as also effective in reducing the tumour burden in a glioma animal model and the number of pulmonary metastasis in a breast carcinoma animal model (unpublished data). Fig. (**5**) integrates the preclinical studies performed so far with this analogue. Further characterization of the potential application of EM1 in cancer is warranted.

In the following Table **1** we summarize the main biological studies performed so far with the above mentioned phosphonate analogues of calcitriol.

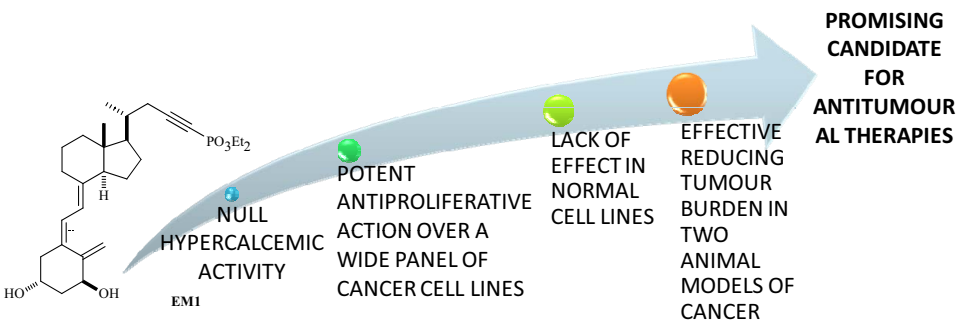


Fig. (5). Preclinical studies performed so far with EM1.

Table 1. Summary of the biological studies performed so far with phosphonate analogs.

Analog	Cancer Cell Lines Which Showed Inhibition of Proliferation	Binding Affinities to VDR	Calcemic Activity in *In vivo* Experiments (Doses, Animal)	Others
21[a] **22**	Leukemia HL-60	Chick and HL-60 VDR	Low hypercalcemic effects (dose not reported, vitamin D deficient chickens)	Instestinal Ca absorption (ICA) Bone Ca mobilization (BCM)
26[b] **27**	Keratinocyte cells	Not determined	Not determined	-
33-35[c] **39b-41b** **43** **47** **49**	Leukemia HL-60	Porcine VDR	Low hypercalcemic effects (2 µg/kg, Wistar rats)	Bone anabolic activity (ROS17/2.8)
66[d]	HN12 (head and neck squamous cell carcinoma), T98G (glioma), T47D (breast carcinoma), HCT116 (colorectal carcinoma); SVEC vGPCR (Kaposi sarcoma), LM05 and LM3 (breast carcinoma).	Not determined	No hypercalcemic effects (5 and 20 µg/kg, CF1 mice)	Reduced tumour burden in a glioma animal model and decreased number of pulmonary metastasis in a breast carcinoma animal model[e]

[a]see ref. 139,152; [b]see ref. 143; [c]see ref. 146, 147; [d]see ref. 150; [e]unpublished data.

CONCLUDING REMARKS

As a disease, cancer still remains to be intimidating and lethal, and the search for agents that may effectively be used to combat this deadly disease is in the focus of cancer research. Preclinical studies in cells and animal models, some observational studies and smaller interventional trials support an anticancer role for vitamin D compounds [1]. Unfortunately, administration of the dose required to obtain a positive response is usually accompanied by hypercalcemic effects. In some clinical studies, partial responses were observed following administration of calcitriol. However, the lack of properly designed regimens has generated doubts about its clinical usefulness. Some calcitriol analogues might be more effective in inhibiting tumour growth than calcitriol at equivalent doses, while having reduced calcemic effects. Many calcitriol analogues have been synthesized and evaluated at the preclinical setting, showing promising results although few of them reached clinical trials. Therefore the search for analogues that behave with a certain level of dissociation between their hypercalcemic and their antitumoural effects and that could display a good bioavailability continues. We have described herein the potential utility of phosphonate analogues for cancer treatment. Few analogues containing a phosphonate moiety have been described in the literature and still fewer were assayed for their antitumoral effects. One of them, the alkynylphosphonate EM1, has been shown to affect the growth properties of various cancer cell lines (even those that were resistant to calcitriol treatment) without affecting the growth of non-malignant counterparts. In addition, EM1 showed no calcemic effects when administered at high doses to mice. These data highlight the need for additional studies that evaluate the full potential of these vitamin D derivatives as anticancer agents.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by grants from CONICET, Agencia Nacional de Promoción Científica y Tecnológica

(ANPCyT) and Secretaría de Ciencia y Técnica de la Universidad Nacional del Sur. D.G. Salomón, M.J Ferronato and S.M. Grioli are recipients of fellowship from CONICET. We apologize to those colleagues whose papers and work we could not cite owing to space limitations.

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Received: May 12, 2014 **Revised: September 01, 2014** **Accepted: October 12, 2014**