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COMMENTARY

EB 1089, a Novel Vitamin D Analog with Strong Antiproliferative and Differentiation-Inducing Effects on Target Cells

Christina Mørk Hansen and Pekka H. Mäenpää†‡*

*Biological Research and Development, Leo Pharmaceutical Products, Ballerup, Denmark; and
†Department of Biochemistry and Biotechnology, University of Kuopio, Kuopio, Finland

ABSTRACT. The physiologically active form of vitamin D, 1α,25-dihydroxyvitamin D_3, plays an important role not only in the establishment and maintenance of calcium metabolism, but also in regulating cell growth and differentiation. As the clinical usefulness of 1α,25-dihydroxyvitamin D_3 is limited by its tendency to cause hypercalcemia, new analogs with a better therapeutic profile have been synthesized. One of these new synthetic vitamin D analogs is EB 1089, which is characterized by an altered side chain structure featuring 26,27-dimethyl groups and two double bonds. This analog has been shown to be more potent than 1,25-dihydroxyvitamin D_3 in inhibiting proliferation, stimulating differentiation, and inducing apoptosis in a number of different cell types, including cancer cells. Despite being more potent than 1α,25-dihydroxyvitamin D_3 with respect to its cell regulatory effects, EB 1089 displays weaker calcemic side-effects. These characteristics make EB 1089 a potentially useful compound for the treatment of a diversity of clinical disorders, including cancer and metabolic bone diseases. A promising phase I study with EB 1089 in patients with advanced breast and colon cancer has already been carried out, and more clinical trials evaluating the clinical effectiveness of EB 1089 in other types of cancer are in progress. BIOCHEM PHARMACOL 54;11:1173–1179, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. vitamin D; calcitriol; analog; cancer; bone; differentiation

The field of vitamin D research has gained much attention due to the fact that this hormone is involved in essential cell regulatory processes such as proliferation, differentiation, and apoptosis in a number of different cell types, including cancer cells. Vitamin D has long been known to play a key role in the establishment and maintenance of calcium homeostasis. Besides this classical effect on calcium metabolism, 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$, calcitriol) has also been found to exert cell regulatory effects that are mediated via binding to specific receptors in the target cells. During the last 15 years, numerous reports have emerged that show the presence of VDR§ in almost every cell type in the human body. Epithelial tissues such as liver, kidney, thyroid, adrenal, gastrointestinal tract, and breast cells have all been shown to possess VDR. Moreover, pituitary, parathyroid, pancreas, bone, muscle, and skin cells, as well as a number of different cancer cell types and some activated cells of the immune system, also express high affinity receptors for 1α,25-dihydroxyvitamin D_3 [1–3]. VDR abundance appears to be regulated by growth factors and activators of the major signalling systems in association with changes in cell proliferation and differentiation. Among others, estrogens, glucocorticoids, retinoic acid, parathyroid hormone, and 1α,25-dihydroxyvitamin D_3 itself have been found to be capable of regulating the levels of VDR or mRNA of VDR [4–7]. At present, it is well documented that the biological function of 1α,25-dihydroxyvitamin D_3, in cells that are not directly associated with calcium metabolism, is to regulate cell growth and differentiation by its ability to inhibit proliferation, stimulate differentiation, and induce apoptosis [1, 2].

The exact mechanism of action of 1α,25-dihydroxyvitamin D_3 is not fully elucidated, but it is well accepted that the main effects of the compound are mediated by its binding to specific high affinity intracellular receptors that belong to the superfamily of steroid receptors. The receptor-ligand complex functions as a transcription factor by interacting with specific DNA sequences (response elements) in the promoter regions of the primary responding genes, leading to either activation or suppression of gene transcription [1, 8–10]. To date, several natural VDREs have been identified, in which the core binding motifs of six nucleotides appear to be arranged into either direct repeats or inverted palindromes with a number of spacing

‡ Corresponding author: Pekka Mäenpää, M.D., Professor of Biochemistry, Department of Biochemistry and Biotechnology, University of Kuopio, P.O.B. 1627, FIN-70211 Kuopio, Finland. Tel. 358-17-163040; FAX 358-17-2811510; E-mail: pekka.maenpaa@uku.fi

§ *Abbreviations:* AUC∞, area under serum level/time curve; DBP, vitamin D binding protein; EB 1089, 1α,25-dihydroxy-22,24-diene-24,26,27-trihomovitamin D_3; EGF, epidermal growth factor; IGF-1, insulin-like growth factor-1; RAR, retinoic acid receptor; RXR, retinoid X receptor; TGF-β, transforming growth factor-β; TR, thyroid hormone receptor; VDR, vitamin D receptor(s); and VDRE, vitamin D response element.

nucleotides. Recent evidence suggests that the VDR binds to these response elements either as a homodimer or as a heterodimer with other nuclear receptors, preferentially with the RXR, but also with the RAR, and the TR [8]. In this way, multiple combinations and a number of different signalling pathways are possible, which may explain the great diversity of vitamin D actions.

Apart from this genomic pathway, a number of investigations have indicated the presence of another, non-genomic, pathway, in which 1α,25-dihydroxyvitamin D_3 binds to a membrane-associated receptor that has ligand binding properties different from those of the classical vitamin D receptor described above. This receptor-ligand complex is thought to act via regulation of voltage-sensitive calcium channels, which then initiate various biological responses [3].

The broad distribution of VDR in the human body and the fact that 1α,25-dihydroxyvitamin D_3 is able to affect cell growth and differentiation make this hormone a potentially useful agent in the treatment of diseases such as cancer, psoriasis, and immunological disorders [2]. However, the major drawback of using 1α,25-dihydroxyvitamin D_3 as a therapeutic agent is that it causes hypercalcemia. Therefore, much effort has been put into the search for new vitamin D analogs with potent cell regulatory effects, but with weaker calcemic side-effects than 1α,25-dihydroxyvitamin D_3.

BASIC ASPECTS OF EB 1089

One of the new promising analogs is EB 1089, which has been shown to be approximately 50–200 times more potent than 1α,25-dihydroxyvitamin D_3 with respect to regulation of cell growth and differentiation in almost every cell type studied [11–16]. Compared with 1α,25-dihydroxyvitamin D_3, EB 1089 has an altered side chain structure that is characterized by an extra carbon atom (24a) and two double bonds at positions C22 and C24, respectively, and 26, 27 dimethyl groups (Fig. 1). It has been suggested that the introduction of double bonds in the side chain inhibits the hydroxylation of C23 or C24a, leading to a metabolically more stable compound [13, 17]. This hypothesis has been further supported by recent pharmacokinetic investigations that have demonstrated that EB 1089, in contrast to

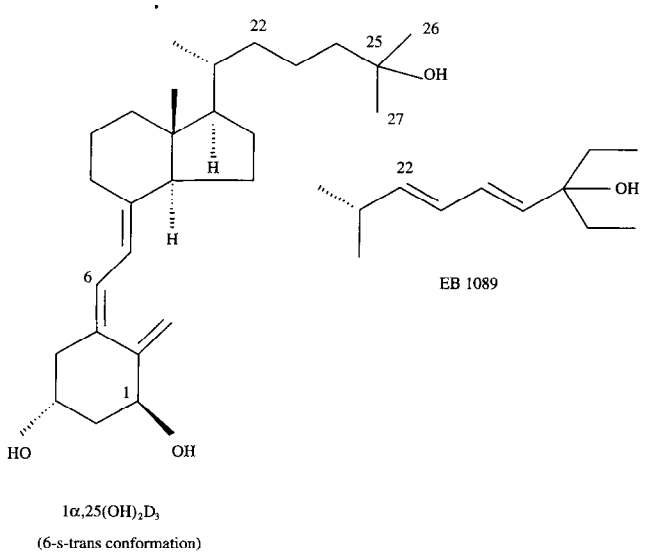

FIG. 1. Structure of 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$D$_3$) and the side-chain structure of EB 1089.

most other synthetic vitamin D analogs, is metabolically more stable, having a serum half-life comparable to that of 1α,25-dihydroxyvitamin D_3 (Table 1). On the other hand, the AUC∞, which indicates the concentration and persistence of a compound in the serum, has been found to be considerably higher for 1α,25-dihydroxyvitamin D_3 than for EB 1089 (Table 1). This is apparently due to the lower affinity of EB 1089 for the DBP compared with 1α,25-dihydroxyvitamin D_3, as the DBP binding affinity of vitamin D analogs correlates with their serum levels or the volume of distribution, which then influences the clearance of the compounds [13, 18]. Moreover, the effect of EB 1089 on calcium metabolism *in vivo* in rats has been shown to be approximately 50% lower than that of 1α,25-dihydroxyvitamin D_3 [13, 19]. This may also be due, in part, to its relatively low binding affinity for DBP and the increased clearance, but other mechanisms such as tissue-specific differences in the binding affinities of 1α,25-dihydroxyvitamin D_3 and EB 1089 for the VDR, and different signalling pathways have also been suggested to contribute to the reduced calcemic activity of EB 1089 [11, 17, 20, 21].

The majority of synthetic vitamin D analogs appear to bind with a lower affinity to VDR than 1α,25-dihydroxyvi-

TABLE 1. Pharmacological data on 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2$D$_3$) and EB 1089

Compound	Binding to DBP 50% displacement* (M)	Serum half-life *in vivo* ($T_{1/2}$)* (hr)	AUC∞* (ng/mL · hr)	Calcemic activity *in vivo*† (%)
EB 1089	7.9×10^{-6}	2.1	255	50
1α,25(OH)$_2$D$_3$	$1.5–6.0 \times 10^{-7}$	2.2	7355	100

The binding affinity for DBP was assessed by displacing [^{3}H]-1,25(OH)$_2$D$_3$ from a DBP-Affi-Gel. $T_{1/2}$ and AUC∞ were measured following a single i.v. dose of 200 μg/kg to rats, and the calcemic activity was determined after p.o. administration for 7 days in rats.

* Data were obtained from Ref. 18.

† Data were obtained from Ref. 13.

TABLE 2. Antiproliferative effects of 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2D_3$) and EB 1089 on three different human cell lines *in vitro*

Compound	U937 lymphoma cells* (IC_{50}) (M)	MCF-7 breast cancer cells† (IC_{50}) (M)	HaCaT keratinocytes* (IC_{50}) (M)
EB 1089	5.8×10^{-10}	2.3×10^{-10}	7.9×10^{-10}
1α25(OH)$_2D_3$	4.6×10^{-8}	1.0×10^{-8}	7.1×10^{-8}

* Data obtained from Ref. 13.
† Data obtained from Ref. 19.

tamin D_3, as demonstrated by displacement studies using isolated VDR from different cell sources [11, 16, 22, 23]. Despite being more potent than 1α,25-dihydroxyvitamin D_3 in the regulation of cell growth and differentiation, EB 1089 was found to have a reduced binding affinity for VDR compared with 1α,25-dihydroxyvitamin D_3 in most of the cell systems studied [11, 13, 16, 19, 24]. Recent evidence suggests, however, that the biological activity of vitamin D compounds is not determined by the receptor binding affinity alone. Other important factors such as the mode of interaction of the compounds with the VDR, their ability to form receptor–dimer complex, and their preference for specific VDRE types all contribute to the activation of VDR-mediated transcription of the effector genes, and thus determine the biological activity [8, 11, 14, 20, 25]. Interestingly, it has been demonstrated that EB 1089 is clearly more efficient than 1α,25-dihydroxyvitamin D_3 in stimulating gene activity, presumably due to its ability to enhance the stability of the occupied receptor, to stimulate the formation of the dimerization of VDR with RXR, and/or to cause a stronger, longer-lasting binding of the receptor complex to VDRE [8, 11, 14, 20, 25]. Moreover, recent investigations have suggested that EB 1089, in contrast to 1α,25-dihydroxyvitamin D_3, preferentially activates a VDRE (IP9-type), which seems to be more closely related to the regulation of cell growth than to the classical actions of vitamin D, i.e. the effects on calcium metabolism [15, 26].

EFFECTS OF EB 1089 ON EARLY GENOMIC CHANGES

Today more than 50 genes have been reported to be sensitive to vitamin D, including several genes that are known to be involved in the regulation of cell proliferation and differentiation [1, 8]. Very recently it has been shown that one of the key regulators in the cell cycle, the cyclin-dependent kinase-inhibitor $p21^{WAF1,CIP1}$, contains a VDRE and is transcriptionally induced by 1α,25-dihydroxyvitamin D_3 [27, 28]. Further studies at the protein level have demonstrated that EB 1089 is more potent than 1α,25-dihydroxyvitamin D_3 in up-regulating the p21 protein, suggesting that EB 1089 acts as a more effective blocker of cell transition from G_1 to S-phase in the cell cycle than 1α,25-dihydroxyvitamin D_3 [29]. Another newly identified primary vitamin D responding gene of particular importance for cell cycle progression is the gene encoding for c-*fos* [30]. Previous investigations have shown that this gene is transcriptionally regulated by vitamin D, and that EB 1089 is approximately 50 times more potent in terms of up-regulating the expression of c-fos mRNA in MCF-7 breast cancer cells, compared with 1α,25-dihydroxyvitamin D_3 [19]. Moreover, EB 1089 has been found to exert stronger effects than 1α,25-dihydroxyvitamin D_3 on several early responding genes. These include the proto-oncogene c-*myc*, the growth regulator gene TGF-β, and the apoptosis related gene *TRPM-2* (clustrin), which all show altered mRNA expression in breast cancer cells treated with EB 1089 [19, 29, 31].

EFFECTS OF EB 1089 ON NEOPLASTIC CELLS

It is well documented that 1α,25-dihydroxyvitamin D_3 inhibits cell growth and promotes cell differentiation in several different cancer cell types *in vitro*. These include cells derived from tumors of the breast, prostate, pancreas, colon, bladder, thyroid, pituitary, and skin as well as melanoma, glioma, neuroblastoma, leukemia, and lymphoma cells [1, 22, 23, 32–34]. In addition to its antiproliferative and differentiation-inducing effects, 1α,25-dihydroxyvitamin D_3 has been found to induce apoptosis and to decrease the invasiveness of a number of different cancer cells *in vitro* [29, 32, 33, 35, 36*]. *In vivo*, inhibition of the development of metastases, regression of tumors, inhibition of angiogenesis, prevention of malignancy-associated hypercalcemia, and prolongation of survival time have been observed in tumor-bearing animals treated with the hormone [29, 36–39].

In Vitro *Effects*

In most cancer cell systems *in vitro*, EB 1089 has been found to be approximately 50–200 times more potent than 1α,25-dihydroxyvitamin D_3 with respect to inhibition of cell proliferation [11, 13–16, 19, 33, 40] (Table 2). This may be due, in part, to its more pronounced effects on cell cycle regulators like c-*myc*, c-*fos*, p21, and p53, but also

* Hoelting T, Duh QY, Clark OH and Herfarth C, Vitamin D and its analog EB-1089 inhibit growth, invasion and protease activity in follicular thyroid cancer cells *in vitro*. 10th International Congress on Endocrinology, San Francisco, CA, 12–15 June, 1996, Abstr. 160.

modulation of the levels of estrogen receptors and of TGF-β type II receptors seems to contribute to the antiproliferative effect of the compound. In MCF-7 breast cancer cells, EB 1089 has been shown to cause a more potent reduction of the estrogen receptor concentration than 1α,25-dihydroxyvitamin D_3, leading to a more effective block of the mitogenic effect of estradiol [41–43]. In addition, besides being able to increase the level of TGF-β [31], a recent study has indicated that TGF-β type II receptors are up-regulated in MCF-7 cells in response to treatment of the cells with EB 1089, resulting in an increase in TGF-β autocrine negative growth activity [44]. Also, the mitogenic activity of insulin, IGF-1, and EGF in human breast cancer cells has been shown to be blocked by vitamin D analogs, with EB 1089 being more potent than 1α,25-dihydroxyvitamin D_3 [40, 45].

Another important factor for the regulation of cell growth is apoptosis, and a number of recent studies have indicated that EB 1089 is more efficient than 1α,25-dihydroxyvitamin D_3 in inducing this process. In MCF-7 human breast cancer cells, EB 1089 has proven to be approximately 50–100 times more potent than 1α,25-dihydroxyvitamin D_3 in stimulating the expression of TRPM-2 and cathepsin B and in down-regulating the expression of bcl-2, which all serve as early markers of apoptosis [29, 43, 46]. Moreover, an increased fragmentation of genomic DNA was seen when the cells were treated with EB 1089, suggesting that active cell death is occurring in the cells [29, 46]. Interestingly, the apoptosis-inducing effect of EB 1089 could be further augmented by cotreatment of the cells with EB 1089 and 9-*cis*-retinoic acid [46]. Co-operative effects of the combination of EB 1089 with retinoids have also been observed in other cell types, including human pancreatic cells, human breast cancer cells, and in certain colon carcinoma cell lines, where the antiproliferative effect of EB 1089 was shown to be enhanced in the presence of 9-*cis*- or all-*trans*-retinoic acid [46–48]. In leukemia cells, the growth-reducing effects of EB 1089 were accompanied by a marked increase in the expression of differentiation markers like CD11b, CD14, and CD18 [49]. This differentiation-inducing effect and the antiproliferative effect of EB 1089 on these cells were increased further by simultaneous treatment of the cells with EB 1089 and 9-*cis*- or all-*trans*-retinoic acid [49].

Differentiation-inducing effects in response to treatment of the cells with EB 1089 have also been documented in other cancer cell types. These include transformed human keratinocytes, human prostate cancer cells, and human neuroblastoma cells, in which up-regulation of, respectively, fillagrin and cytokeratin K10, prostate specific antigen, and acetylcholinesterase activity have been observed [33, 34, 50]. In human neuroblastoma cells, the increased activity of acetylcholinesterase was found to be accompanied by a decreased expression of N-*myc*, which is known to precede neuroblastoma differentiation. Moreover, the N-*myc* expression was also shown to be correlated directly with a reduced ability of the cells to invade an artificial basement membrane [33]. The anti-invasive effect of EB 1089 was further confirmed by other studies using human breast cancer cells and human follicular thyroid cancer cells [33–35]. Interestingly, these studies have proven that the anti-invasive effect of vitamin D analogs is not associated directly with their growth-inhibiting potential. Other mechanisms, such as decreased expression of collagenases and a reduction of the laminin receptor expression, may also be involved [11, 34, 35, 51].

In Vivo *Effects*

The studies described above have demonstrated that EB 1089 has potent cell regulatory effects *in vitro*, which could be of great interest for the treatment of neoplastic diseases. As its calcemic activity was found to be approximately 50% lower than that of 1α,25-dihydroxyvitamin D_3 [13, 19], EB 1089 has been selected for further investigations *in vivo* and has been tested in a series of assays. In rats with mammary tumors induced by nitrosomethylurea, oral treatment of the animals with EB 1089 at 0.5 μg/kg/day for 4 weeks was shown to result in a significant inhibition of tumor growth and a significant regression of the tumor size without changes in serum calcium levels [11, 29]. In addition, in rats bearing the Leydig tumor H-500, EB 1089 was found to prolong the survival time and to prevent the induction of malignancy-associated hypercalcemia when administered at non-calcemic doses [11, 37].

EB 1089 is currently undergoing clinical evaluation in patients suffering from a variety of cancer types. A phase I study in patients with advanced breast and colon cancer has indicated that EB 1089 can be administered at relatively high doses without causing hypercalcemia or other toxicities [52].

EFFECTS OF EB 1089 ON BONE CELLS

Vitamin D has long been known to affect bone growth and mineralization and serve as an important agent for the treatment of metabolic bone diseases. *In vitro*, 1α,25-dihydroxyvitamin D_3 has proven to be a potent regulator of bone cell functions, and it is capable of inducing differentiation of the two major bone cells, osteoblasts and osteoclasts [53]. VDRs have been demonstrated in the osteoblasts, while osteoclasts, which are derived from immature bone marrow cells, do not contain VDR [9, 53]. The differentiation-inducing effect of 1α,25-dihydroxyvitamin D_3 in osteoclasts is therefore indirect and is apparently mediated by a factor that is released from the osteoblastic cells in response to treatment of the cells with 1α,25-dihydroxyvitamin D_3 [9, 53]. Differentiation of the osteoclast precursors results in an increased bone resorbing activity of the cells by affecting the matrix attachment phase of the resorption process [54]. In the osteoblasts, 1α,25-dihydroxyvitamin D_3 has been shown to regulate the expression of several maturation associated proteins such as alkaline phosphatase, collagen, osteocalcin, osteopontin,

matrix Gla-protein, and the third component of complement (C3) [53]. Some of these effects are mediated through changes in the local production of cytokines such as TGF-β, interleukin-4, and EGF, and their receptors [55–57].

EB 1089 and 1α,25-dihydroxyvitamin D_3 were found to be equipotent stimulators of osteoclast recruitment in murine marrow cells *in vitro* [24]. However, in *in vitro* resorption experiments using bone cells from fetal mice, EB 1089 appeared to be approximately 10 times more potent than 1α,25-dihydroxyvitamin D_3 in stimulating the bone resorption activity of the cells. This stimulatory effect could be reduced considerably by addition of the antiestrogen, tamoxifen, suggesting a role for such a combination therapy in reducing the risk of skeletal metastases in cancer patients treated with vitamin D analogs [58].

Other *in vitro* studies using MG-63 osteosarcoma cells have shown that EB 1089 inhibits cell proliferation more potently than 1α,25-dihydroxyvitamin D_3 and that it causes a marked increase in the synthesis of procollagen type I and osteocalcin [14, 16]. Moreover, EB 1089 appears to be more potent than 1α,25-dihydroxyvitamin D_3 with respect to the stimulation of alkaline phosphatase activity and to increasing the level of VDR [14, 16]. One explanation for the stronger effects on bone cells of EB 1089 than 1α,25-dihydroxyvitamin D_3 may be the fact that the binding of VDR to the composite AP-1 plus VDRE promoter region of the human osteocalcin gene after EB 1089 is stronger and longer lasting than after 1α,25-dihydroxyvitamin D_3 treatment [14, 16].

SUMMARY AND DIRECTIONS FOR FUTURE RESEARCH

On the basis of both *in vitro* and *in vivo* studies, EB 1089 emerges as one of the promising new vitamin D analogs. It has a conjugated double-bond side chain structure, which probably makes it metabolically more stable than the parent compound, 1α,25-dihydroxyvitamin D_3. EB 1089 belongs to the group of vitamin D analogs characterized by having a low affinity for the DBP, a relatively long serum half-life, and weak calcemic side-effects compared with those of 1α,25-dihydroxyvitamin D_3. It is more potent than 1α,25-dihydroxyvitamin D_3 with respect to regulation of cell growth and differentiation, probably due to the fact that its effects on target cell DNA, mediated by the vitamin D receptor, are stronger and longer lasting than those of 1α,25-dihydroxyvitamin D_3. Moreover, EB 1089 has also been found to exert potent effects on bone metabolism, affecting both osteoblastic and osteoclastic cells.

In basic research, it is anticipated that the X-ray structures of VDR and DBP will be determined in the near future. Knowledge of these structures may help clarify their structure-function relationships and thus greatly assist in the development and search for new vitamin D analogs with specific pharmacological profiles. Thus, in the future, the use of vitamin D analogs may hopefully extend beyond its classical role in bone diseases, to encompass new areas in the field of neoplastic diseases either as a monotherapy or in combination with other anticancer agents.

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