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Review

Vitamin D analogues: from molecule to clinical application

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During the last 70 years our understanding of the significance of vitamin D as an important regulator of calcium and phosphate homeostasis has greatly increased. It is now well established that vitamin D is metabolized first in the liver to 25-hydroxyvitamin D_3 and then in the kidney to the active metabolite 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$). The seco-steroid hormone 1,25-$(OH)_2D_3$ exerts its principal biological activities through specific intracellular receptors, which are nuclear transcription factors belonging to the steroid receptor superfamily (Haussler, 1986; Reichel *et al.*, 1989; Pike, 1991).

A series of new discoveries, however, has made it apparent that the vitamin D endocrine system plays a much wider role in biology than was previously thought. The list of target tissues of 1,25-$(OH)_2D_3$ not only includes well known tissues such as bone, intestine, kidney and parathyroids, but also a broad range of cells and organs involved in physiological functions not directly related to systemic calcium homeostasis (Walters, 1992; Bikle 1992). Vitamin D receptors (VDR) have been found in various elements of the haematopoietic and immune system, endocrine glands and skin, while receptors have also been identified in a number of malignant cell types (Braidman & Anderson, 1985; Walters, 1992).

Based on current knowledge the new actions of 1,25-$(OH)_2D_3$ can roughly be divided into two sorts: (1) modulation of hormone and cytokine production, and (2) regulation of cellular differentiation and proliferation. Modulation of hormone production and secretion has been described for parathyroid hormone (PTH) from parathyroid glands, prolactin from the pituitary and insulin from the pancreas. In-vitro studies indicate that 1,25-$(OH)_2D_3$ can also regulate the production and secretion of several cytokines, such as interleukin-2 from lymphocytes and tumour necrosis factor from monocytes. 1,25-$(OH)_2D_3$ decreases proliferation and increases differentiation of lymphocytes, monocytes, fibroblasts, keratinocytes, melanocytes, and bone cells (osteoblasts and osteoclasts). Similar effects on cell proliferation and/or differentiation have been observed in malignant cell lines, e.g. myeloid leukaemia and breast carcinoma cells (for review see Reichel *et al.*, 1989; Pols *et al.*, 1990; Bikle, 1992; Walters, 1992). Moreover, the antiproliferative effect of 1,25-$(OH)_2D_3$ has been confirmed *in vivo*. The sterol considerably prolongs survival of nude mice inoculated with murine myeloid leukaemia cells (M1-cells) (Homna *et al.*, 1983), while it also inhibits growth of human malignant melanoma, colonic cancer cell and breast cancer cell xenografts in immune suppressed mice (Eisman *et al.*, 1987; Colston *et al.*, 1989). Administration of 1α-hydroxyvitamin D_3 also reduces the number of lung metastases after implantation of Lewis lung carcinoma cells into mice (Sato *et al.*, 1982).

The exciting new properties of 1,25-$(OH)_2D_3$ in a number of its non-classic target tissues underlie current efforts to explore new clinical indications for the use of 1,25-$(OH)_2D_3$ (Bikle, 1992). Clinical studies in patients with psoriasis have already shown favourable effects of especially topically administered 1,25-$(OH)_2D_3$ (Holick, 1989; Bikle & Pillai, 1993). Of course there is also significant interest in the therapeutic potential of 1,25-$(OH)_2D_3$ for treatment of malignancies. However, for the antiproliferative (antitumour) effect supraphysiological doses of systemically administered 1,25-$(OH)_2D_3$ are needed. Thus doses optimal in terms of preventing tumour growth, will inevitably result in complications, like hypercalcaemia and hypercalciuria. These severe complications have prompted the development of vitamin D analogues in an attempt to separate the calcaemic activity from the antiproliferative and cell differentiation activity.

Comparison of the mechanisms of action of vitamin D and its analogues

Most of the analogues currently available are based on extensive modifications of the side-chain of vitamin D (Calverley & Jones, 1992). The selection of analogues for future clinical research has in particular been focused on their ability to decrease proliferation and enhance differentiation of malignant or normal cells *in vitro* without having apparent calcaemic effects. Nevertheless, attempts have also been undertaken to develop analogues for the more classic indications. For instance, to treat osteoporosis we need analogues which stimulate bone formation, but do not affect bone resorption (Nishii *et al.*, 1993).

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For the development and understanding of the action of vitamin D analogues it is important to define the mechanisms involved in the biological action of vitamin D, because the interplay between these mechanisms will determine the ultimate biological responses. Therefore, this review will be focused on the relevance of (1) metabolism and vitamin D binding proteins, (2) receptor binding and (3) aspects which have come forward more recently, namely non-classic steroid hormone messenger systems such as calcium and protein kinase C. It is not our purpose to discuss in detail individual characteristics of the various analogues.

Metabolism and vitamin D binding proteins

Tissue and plasma levels of 1,25-$(OH)_2D_3$ are dependent upon both synthesis and catabolism. In the kidney 1,25-$(OH)_2D_3$ is a negative regulator of its own synthesis. Therefore, a possible site of action for vitamin D analogues is inhibition of the endogenous synthesis of 1,25-$(OH)_2D_3$. For the analogue 22-oxacalcitriol (OCT) such an inhibition of 1,25-$(OH)_2D_3$ formation *in vivo* has been observed (Dusso *et al.*, 1992). However, it remains to be established whether such a mechanism is related to the low calcaemic activity of OCT or other vitamin D analogues.

In kidney and intestine, and in most other target tissues, 1,25-$(OH)_2D_3$ induces the enzyme 24-hydroxylase. This enzyme initiates a catabolic cascade for the side-chain oxidation, cleavage and ultimate metabolic elimination of 1,25-$(OH)_2D_3$ and its precursor 25-$(OH)D_3$ (Kumar, 1986; Haussler, 1986). This so-called C24-oxidation pathway is regarded as a mechanism whereby 1,25-$(OH)_2D_3$ can regulate/limit its own biological action in cells. It is conceivable that vitamin D analogues can interact with this local regulatory mechanism in several ways. The ability of analogues to induce the enzyme 24-hydroxylase could be different or even absent (Pols *et al.*, 1991), and modifications in the side-chain could reduce susceptibility to side-chain oxidation.

C24-oxidation contributes only 35–40% to the catabolism of 1,25-$(OH)_2D_3$; the remainder consists of other side-chain oxidations and biliary excretion of polar metabolites. In contrast to the C24-oxidation pathway these processes are regulated neither by 1,25-$(OH)_2D_3$ nor by calcium or phosphate (Kumar, 1986). Nevertheless these metabolic pathways may also interact with the regulation of the eventual response of vitamin D analogues. It is even possible that in contrast to the metabolism of 1,25-$(OH)_2D_3$, the metabolism of analogues results in the accumulation of active metabolites in target cells, as has been reported for 26,27-hexafluoro-1,25-$(OH)_2D_3$ (Nagata *et al.*, 1988). Whether this is applicable only to fluorinated analogues, which have an altered side-chain metabolism, remains to be elucidated.

An aspect directly related to 1,25-$(OH)_2D_3$ catabolism, and thereby its action, is binding to transport proteins in blood. Vitamin D binding protein (DBP) is characterized by a high affinity and high capacity for vitamin D_3 and its metabolites. Besides binding to DBP vitamin D_3 metabolites can also bind to albumin and low-density lipoproteins (both low affinity, high capacity) (Haddad, 1987). Pharmacological studies showed that for several analogues a low affinity to DBP seems to be related to a shorter half-life (Kissmeyer & Binderup, 1991; Bouillon *et al.*, 1991). In addition low binding to DBP may decrease the immediate availability of the analogues to target tissues (Bouillon *et al.*, 1991; Norman *et al.*, 1991). These observations are supported by the observation that reduced binding to DBP is mostly correlated with diminished in-vivo activity (Binderup & Bramm, 1988; Bouillon *et al.*, 1991). Also, data obtained with analogues with increased binding to DBP demonstrate a decreased in-vivo activity in rachitic chick (Bouillon *et al.*, 1992). However, in this latter situation not only reduced bioavailability, but also reduced affinity for the VDR has to be taken into account.

The fact that the analogue OCT still has profound effects *in vivo*, despite its low affinity to DBP and its rapid clearance from the circulation, may be related to its higher affinity for lipoproteins compared to various vitamin D_3 metabolites (Kobayashi *et al.*, 1991; Abe *et al.*, 1989; 1991). It is thought that OCT is transported to target tissues bound to lipoproteins and then incorporated into cells via lipoprotein-membrane receptors. In general, this mechanism indicates that, dependent on the presence on target cells of membrane receptors for transporters of vitamin D_3 and analogues, the compounds may be delivered in a selective manner.

Receptor binding

In the classical model (see also below) 1,25-$(OH)_2D_3$ binds to a specific cytosolic/nuclear receptor which subsequently binds to DNA and initiates transcription of various genes resulting in an altered cellular activity. The VDR is a member of the steroid hormone receptor family which have a characteristic structure with distinct ligand and DNA binding domains separated by a hinge-region. Characteristic of the DNA binding domain are the zinc-fingers in which the so-called P-box and D-box are responsible for recognition of DNA sequences and receptor dimerization (O'Malley, 1990; Carson-Jurica *et al.*, 1990). In contrast to the closely related thyroid hormone and retinoic acid hormone receptors only one type of VDR has been identified. Therefore, one can argue that it is unlikely that vitamin D analogues exert tissue

specific actions via tissue specific receptors. However, after receptor binding several processes take place which can be subject to different activation by analogues. Firstly, in response to 1,25-$(OH)_2D_3$ binding, phosphorylation of the VDR on specific sites appears to be crucial for activation of gene transcription (Pike & Sleator, 1985; Brown & Deluca, 1990; Hsieh *et al.*, 1991; Jones *et al.*, 1991; Orti *et al.*, 1992; Jurutka *et al.*, 1993; Darwish *et al.*, 1993). Secondly, it has been shown that interaction with a nuclear factor (heterodimerization), recently identified as the retinoic X receptor (RXR), is necessary for the binding of the 1,25-$(OH)_2D_3$-VDR complex to DNA (Liao *et al.*, 1990; Ozono *et al.*, 1991; Ross *et al.*, 1992). Although it remains to be established whether altered VDR phosphorylation and heterodimerization play a role in the action of vitamin D_3 analogues, results with the analogue 26,27-hexafluoro-1,25-$(OH)_2D_3$, showing a higher binding affinity of the VDR-ligand complex for DNA, at least do not exclude the involvement of these mechanisms (Inabe *et al.*, 1989).

Non-classic steroid hormone mechanisms

Over the last five years responses to 1,25-$(OH)_2D_3$ have been observed which were previously exclusively associated with the action of peptide hormones and not with the action of steroid hormones. Both 1,25-$(OH)_2D_3$-induced, but actinomycin D-insensitive, rapid changes in intestinal calcium transport (termed transcaltachia) (Nemere & Norman, 1987) and rapid changes in intracellular calcium levels in osteoblasts, muscle cells, hepatocytes, keratinocytes, and parathyroid cells (Baran & Milne, 1986; De Boland & Boland, 1987; Lieberherr, 1987; MacLaughlin *et al.*, 1987; Oshima *et al.*, 1987; Sugimoto *et al.*, 1988; Caffrey & Farach-Carson, 1989) in response to the seco-steroid indicate a genomic-independent action. Also, rapid effects of 1,25-$(OH)_2D_3$ on cellular cyclic GMP levels (Barsony & Marx, 1988) and membrane lipid metabolism in a variety of cells have been reported (Matsumoto *et al.*, 1981; Baran *et al.*, 1990; Baran & Kelly, 1988; Okazaki *et al.*, 1989; Lieberherr *et al.*, 1989; Civitelli *et al.*, 1990). This latter observation might be related to changes in activity of the intracellular signal system protein kinase C (Martell *et al.*, 1987; Obeid *et al.*, 1990; Simboli-Campbell *et al.*, 1992). Whether these apparently non-genomic effects of 1,25-$(OH)_2D_3$ are exerted via the classic VDR or via a new membrane-associated receptor remains to be established. However, recent findings in osteoblast-like cells with different vitamin D analogues demonstrate dissociations between VDR binding and stimulation of calcium influx (Farach-Carson *et al.*, 1991). These observations together with data showing a rapid 1,25-$(OH)_2D_3$-induced increase in intracellular calcium in rat osteosarcoma cells lacking the classical VDR (Baran *et al.*, 1991) support the existence of a distinct plasma-membrane receptor. Studies to isolate and characterize a possible vitamin D membrane receptor are in progress. Nevertheless the functional involvement of calcium and protein kinase C in various biological responses to 1,25-$(OH)_2D_3$ has already been demonstrated in HL-60 cells and bone (Simpson *et al.*, 1989; Van Leeuwen *et al.*, 1990; 1992) and support the importance of both genomic and non-genomic mechanisms. Therefore, the difference in the ability of the various analogues to activate both genomic and non-genomic mechanisms may contribute to their selective actions.

Clinical applications

The characteristics of the relatively few analogues tested *in vivo* give some indications of their potential clinical applications. In Table 1, in-vivo studies with several vitamin D analogues are summarized, with the exception of studies which examined only effects on serum and urinary calcium.

The ability of 1,25-$(OH)_2D_3$ to inhibit PTH synthesis and/or secretion has led to its use in patients with secondary hyperparathyroidism due to chronic renal failure. However, in these patients oral 1,25-$(OH)_2D_3$ frequently induces hypercalcaemia. Ten years ago Slatopolsky *et al.* (1984) observed that the intravenous administration of 1,25-$(OH)_2D_3$ reduced PTH levels more effectively and with considerably less increment of serum calcium than did oral therapy. The recently developed analogue OCT has similar effects to 1,25-$(OH)_2D_3$ on PTH synthesis and release, but is without a calcaemic effect. Therefore, OCT may provide a useful treatment of secondary hyperparathyroidism (reviewed by Slatopolsky *et al.*, 1992). Consequently, it is tempting to speculate that noncalcaemic analogues can be used in the treatment of primary hyperparathyroidism. At present the use of analogues for modulation of the secretion of other hormones like insulin seems to be more distant (Bikle, 1992).

The synthesis of vitamin D analogues that, at much lower concentrations than 1,25-$(OH)_2D_3$, are able to inhibit T-lymphocyte activation, may give rise to a new class of immunosuppressive compounds (reviewed by Binderup, 1992). In this respect the analogue, KH 1060, has been shown to be several orders of magnitude more potent than cyclosporin A (Lillevang *et al.*, 1992). In addition, preliminary in-vivo experiments indicate that i.p. administered low doses of KH 1060 significantly prolong mouse skin allograft survival (Chiocchia *et al.*, 1991). These findings, together with in-vivo studies showing that 1,25-$(OH)_2D_3$ can modulate the course of autoimmune disorders such as thyroiditis, encephalomyelitis, rheumatoid arthritis and systemic lupus

Table 1 In-vivo effects of vitamin D analogues*

Analogue	Effect	Dose	Administration†	Species	Ref.
OCT	Increased primary immune response	0·2–200 ng/kg BW	Oral	Mouse	Abe *et al.* (1989)
OCT	Inhibition of PTH synthesis	130 ng/kg BW	i.p. Injection	Rat	Brown *et al.* (1989)
OCT	Beneficial effect on autoimmune disorder	2 or 100 ng/kg BW	Oral	Mouse	Abe *et al.* (1990)
OCT	Inhibition of embryonic angiogenesis	1–3 ng/egg	Pellet implantation	Chicken	Oikawa *et al.* (1990)
OCT	Inhibition of breast cancer growth	1–10000 ng/kg BW	Oral and intratumour	Mouse	Abe *et al.* (1991)
OCT	Enhancement of vasoconstrictor response	200 ng/kg BW	Osmotic minipump	Rat	Shimisawa *et al.* (1993)
KH1060	Immunosuppressive in autoimmune disease	3–300 ng/kg BW	i.p. Injection	Rat	Lillevang *et al.* (1992)
EB1089	Inhibition of breast cancer growth	0·5–2·5 μg/kg BW	Oral	Rat	Colston *et al.* (1991; 1992b)
EB1089	Inhibition of PTH-related peptide production and prevention of related hypercalcaemia	100–850 ng/kg/24 h	Permanent i.p. infusion	Rat	Haq *et al.* (1993)
MC903	Treatment of psoriasis	25–100 μg/g vehicle	Topical application	Human	Kragballe *et al.* (1991)
MC903	Treatment of cutaneous metastatic breast cancer	100 μg/g vehicle	Topical application	Human	Bower *et al.* (1991)
MC903	Inhibition of breast cancer growth	50 μg/kg BW	i.p. Injections	Rat	Colston *et al.* (1992b)
MC903	Reduction of HIV-related psoriasis	30 g/day	Topical application	Human	Gray *et al.* (1992)
CB966	Inhibition of breast cancer growth	1 μg/kg BW	Oral	Rat	Colston *et al.* (1991)
RO 24-2637	Immunosuppressive in transplantation of cardiac allografts	150–200 μg/animal	i.p. Injections	Mouse	Lemire *et al.* (1992)
RO 23-7553	Increased survival of mice with myeloid leukaemia	0·8–1·6 μg animal	i.p. Injections	Mouse	Zhou *et al.* (1990)
26,27-HF-1,25	Treatment of uraemic patients on haemodialysis	0·05–0·3 μg/day	Oral	Human	Nishizawa *et al.* (1991)
26,27-HF-1,25	Treatment of hypoparathyroidism	0·35 μg/kg BW 0·05–1·0 μg/day	Oral Oral	Rat Human	Nakatsuka *et al.* (1992)
24-F-1,25	Reduction of incidence of parturient paresis	250 μg	Pellet implantation	Cow	Goff & Horst (1989)

OCT (22-oxacalcitriol) Chugai Pharmaceuticals Co., Ltd., Japan; MC903 (Calcipotriol), KH1060, EB1089, and CB966 Leo Pharmaceuticals Products Ltd A/S, Denmark; Ro 24-2637 (1,25-dihydroxy-16-ene-vitamin D_3), RO 23-7553 (1,25-dihydroxy-16-ene-23-yne-vitamin D_3), and 24-F-1,25 (24-fluoro-1,25-$(OH)_2D_3$) Hoffmann-La Roche, Inc., USA; 26,27-HF-1,25 (26,27-hexafluoro-1,25-$(OH)_2D_3$) Sumitomo Pharmaceutical Co Ltd, Japan.

* No studies are mentioned which merely examined effects on serum calcium and urinary calcium.

† Duration of administration, diet, and effects on serum calcium varies between the various studies.

erythematosus in murine models, suggest that immunology is a potential field to further explore clinical application of vitamin D analogues (Manolagas *et al.*, 1991; Binderup, 1992).

The use of vitamin D analogues as antiproliferative compounds is also surrounded with an air of optimism. After the observation of Morimoto and Kumahara (1985), who showed that treatment of an osteoporotic woman with 1α-(OH)D_3 resulted in clearing of her psoriasis, other clinical studies indicate that topical treatment with 1,25-$(OH)_2D_3$ improves psoriasis (Holick, 1989). Topical application of the analogue Calcipotriol (MC903) in patients with psoriasis resulted in clearing of their skin lesions in over 80% of the patients (Kragballe *et al.*, 1991). No changes in serum calcium levels were observed. Also, topical application of Calcipotriol on cutaneous deposits did prove effective in some patients with advanced breast cancer (Bower *et al.* 1991). The low calcaemic response to Calcipotriol after systemic administration has been related to its poor affinity to DBP and (consequently) its rapid clearance from the circulation (Bouillon *et al.*, 1991; Kissmeyer & Binderup, 1991). Based on these observations one could argue that the usefulness of Calcipotriol hinges simply on its mode of delivery, enabling it to reach its target tissues, but without exerting calcaemic effects. However, despite its low binding to DBP, experiments with the analogue 1,25-$(OH)_2$-16-ene-

23-yne-D_3 clearly indicate systemic effects. This compound increased survival time of mice inoculated with fulminant, moderate or slowly progressive leukaemia significantly, while no hypercalcaemia was observed (Zhou *et al.*, 1990). In contrast, the control group treated with a 16-fold lower dose of 1,25-$(OH)_2D_3$ developed mild hypercalcaemia. The reason for enhanced antitumour activity of 1,25-$(OH)_2$-16-ene-23-yne-D_3 over calcaemic action is unknown, although one can speculate that the 23-yne conformation decreases its susceptibility for C24-oxidation of the side-chain.

An enhanced in-vivo antitumour activity relative to 1,25-$(OH)_2D_3$, without causing marked hypercalcaemia, has also been described for other analogues (Table 1) (Colston *et al.*, 1991). In this respect it is interesting that the serum half-life of the potent antitumour analogue EB 1089 was comparable to 1,25-$(OH)_2D_3$ (2·8 *vs* 2·4 hours), while EB 1089 has nevertheless a threefold lower calcaemic activity *in vivo* (Binderup *et al.*, 1991). Taken together these in-vivo observation suggest that the calcaemic action of vitamin D analogues can not be explained simply by their serum half-lifes.

Another promising aspect of the clinical use of vitamin D analogues might be its combination with other antitumour agents. A realistic combination in this respect could be addition of a vitamin D analogue to the treatment with tamoxifen of breast cancer. Recent in-vitro findings in our laboratory indicate the potential of this combination (Vink-van Wijngaarden *et al.*, 1994). A similar approach, for instance combination with cyclosporin A, could be advantgeous as immunosuppressive treatment (Manolagas *et al.*, 1991; Binderup, 1992).

Conclusions

The picture that emerges from the recent research with vitamin D analogues clearly shows the possibility of developing analogues with properties especially capable of modulating non-classic actions of 1,25-$(OH)_2D_3$. As discussed, several mechanisms at the cellular level may be candidates to explain the properties of the new analogues. However, more detailed structure and function studies of the vitamin D molecule, together with studies on the molecular mechanisms of gene activation and possible tissue-specific effects of vitamin D, will be needed to assess whether it is indeed possible to develop analogues devoid of calcaemic actions. Finally, it has to be emphasized that in-vitro screening of new analogues provides insufficient data for selecting potential candidates for future clinical applications. It is clear that intestinal absorption, metabolism (both target-specific and general), and transport to target cells (binding proteins) are major determinants of the ultimate effect of an analogue *in vivo*.

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