4 Metabolism and Catabolism of Vitamin D, Its Metabolites and Clinically Relevant Analogs

Glenville Jones

Abstract This chapter discusses the current state of knowledge of vitamin D metabolism and the specific enzymes involved. Vitamin D_3 undergoes a two-step metabolic activation involving sequential hydroxylations at 25- and 1α-carbons by cytochrome P450-based hydroxylases (CYP2R1 and CYP27B1) to give first the main circulating form 25-hydroxyvitamin D_3 and then a hormonally active form 1α,25-dihydroxyvitamin D_3. The plant-derived vitamin D_2 undergoes the same activation steps. This review highlights the recent finding of extra-renal sites of CYP27B1 expression and the physiological implications of this discovery. 1α,25-Dihydroxyvitamin D_3 is inactivated by another cytochrome P450 enzyme (CYP24A1) which produces a series of metabolic products culminating in either a side chain-truncated biliary excretory form, calcitroic acid, or a 26,23-lactone derivative. This chapter also discusses the current knowledge of the metabolism of the clinically relevant analogs of vitamin D, ranging from prodrug forms (e.g. 1(OH)D) that require a step or more of activation to produce a biologically active form to the calcitriol analogs, which are active as administered. Differences between metabolism-sensitive and metabolism-resistant vitamin D analogs are discussed in the context of evaluating the relative importance of analog metabolism in their mechanism of action. The review ends by attempting to predict future directions in the field, focussing on determination of CYP structure, the knowledge gained from mouse CYP knockouts and future vitamin D drug design.

Key Words: Vitamin D metabolism; vitamin D analogs; 25-hydroxyvitamin D; 1α,25-dihydroxyvitamin D; 25-hydroxylase; 1α-hydroxylase; 24-hydroxylase; cytochrome P450

1. METABOLISM OF VITAMIN D_3 AND 25(OH)D_3

The elucidation of the metabolism of vitamin D_3 is arguably one of the most important developments in nutritional sciences over the latter half of the twentieth century. An appreciation that vitamin D_3 represents a precursor to the functionally active form and that two steps of activation are necessary to produce the hormone 1α,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) constitute historical landmarks in modern vitamin research *(1)*. These developments not only spawned detailed studies of the biological properties of vitamin D metabolites produced and the regulation of cytochrome

From: *Nutrition and Health: Vitamin D*
Edited by: M.F. Holick, DOI 10.1007/978-1-60327-303-9_4,

P450-containing enzymes involved in their production but also provided the stimulus for the chemical synthesis of a plethora of vitamin D analogs (~thousands at last count). Furthermore, it appears that susceptibility to vitamin D catabolic pathways together with other important parameters such as binding to the vitamin D receptor (VDR) functional complex and binding to the vitamin D-binding protein (DBP) are probably key elements in dictating the differences in the actions of so-called calcaemic and non-calcaemic vitamin D analogs. Therefore, from the perspective of its historical significance and relevance, it seems entirely logical to consider the metabolism of vitamin D and its analogs together at this stage of a general text on vitamin D. It should be stated that since the publication of the first edition of this book, there has been significant progress made on the nature of the cytochromes P450 involved in vitamin D metabolism including the release of the first X-crystal structure, namely of CYP2R1.

1.1. 25- and 1α-Hydroxylation

Vitamin D_3 can be synthesized in the skin (see Chapter 1) or be derived from dietary sources. As such, this precursor does not circulate for long in the bloodstream, but, instead, is immediately taken up by adipose tissue or liver for storage. In humans, tissue storage of vitamin D can last for months or even years. Ultimately, the vitamin D_3 undergoes its first step of activation, namely 25-hydroxylation in the liver (Fig. 1). Over

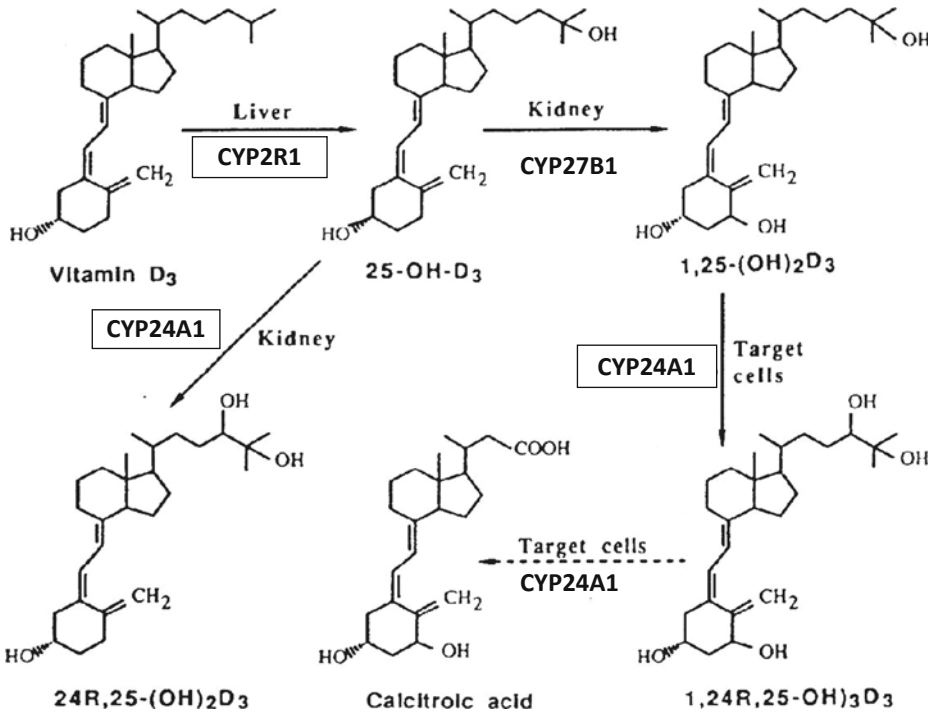


Fig. 1. Metabolism of vitamin D_3.

the years, there has been much controversy over whether 25-hydroxylation is carried out by one enzyme or two and whether this cytochrome P450-based enzyme is found in the mitochondrial or microsomal fractions of liver *(2)*. In the past few years, this has been clarified substantially by work which suggests that the mitochondrial enzyme is CYP27A1 and the microsomal enzyme is CYP2R1. The mitochondrial form CYP27A1 has been purified to homogeneity, subsequently cloned from several species *(3–5)*, and appears to be a bifunctional cytochrome P450 which in addition to synthesis of 25-hydroxylating vitamin D_3 also carries out the side chain hydroxylation of intermediates involved in bile acid biosynthesis. Even though 25-hydroxylation of vitamin D_3 has been clearly demonstrated in cells transfected with CYP27A1, there was continuing scepticism in the vitamin D field that a single cytochrome P450 could explain all the metabolic findings observed over the past two decades of research. These unexplained observations include

(A) Using the perfused rat liver, Fukushima et al. *(6)* demonstrated *two* 25-hydroxylase enzyme activities: a high-affinity, low-capacity form (presumably microsomal) and a low-affinity, high-capacity form (presumably mitochondrial; CYP27A1).
(B) Regulation, albeit weak, of the liver 25-hydroxylase in animals given normal dietary intakes of vitamin D after a period of vitamin D deficiency *(7)* is not explained by a transcriptional mechanism since the gene promoter of CYP27A1 lacks a VDRE.
(C) No obvious 25(OH)D_3 or 1,25$(OH)_2D_3$ deficiency in patients suffering from the genetically inherited disease, cerebrotendinous xanthomatosis, where CYP27A1 is mutated. Although a subset of these patients can suffer from osteoporosis, this is more likely due to biliary defects leading to altered enterohepatic circulation of 25(OH)D *(8)*.
(D) CYP27A1 does not appear to 25-hydroxylate vitamin D_2.

Despite the persistent doubts, one thing that the existence of CYP27A1 does explain is the occasional reports of extra-hepatic 25-hydroxylation of vitamin D_3 *(9)*. CYP27A1 mRNA has been detected in a number of extra-hepatic tissues including kidney and bone (osteoblast) *(10, 11)*.

Over the past 5 years, the "missing" liver *microsomal* 25-hydroxylase has been identified as CYP2R1 and cloned from several species *(12)*. Work has shown that it fits the activity profile predicted above in the points A–D. First, CYP2R1 has a high substrate affinity for vitamin D_2, vitamin D_3 and its 1α-hydroxylated counterparts, 1(OH)D_2 and 1(OH)D_3 *(12–15)*, making it a strong candidate to be called the physiologically relevant 25-hydroxylase. Second, Chen et al. *(13)* demonstrated that there is a human mutation of CYP2R1 L99P which results in null enzyme activity and in vitamin D-dependent rickets (VDDR type 1a). Lastly, CYP2R1 shows a mainly liver tissue distribution *(12)*. A slightly modified, fully active version of human CYP2R1 was recently crystallized with vitamin D_3 docked in its active site and its X-ray crystal structure determined to 2.7 Å *(15)*. The product of the 25-hydroxylation step, 25-hydroxyvitamin D_3 [25(OH)D_3], is the major circulating form of vitamin D_3 and in humans is present in plasma at concentrations in the range 10–40 ng/ml (25–125 nM). The main reason for the stability of this

metabolite is its strong affinity for the vitamin D-binding (globulin) protein of blood (DBP) which is around 5×10^{-8} M.

The second step of activation, 1α-hydroxylation, occurs in the kidney *(12)* and the synthesis of the *circulating* hormone in the normal mammal appears to be the *exclusive* domain of that organ. The main evidence for this comes from clinical medicine, where patients with chronic kidney disease gradually deplete their circulating levels of $1,25(OH)_2D_3$ over the five-stage history of their disease and if left untreated go on to develop frank renal osteodystrophy caused by a deficiency of $1,25(OH)_2D_3$ caused by lack of 1α-hydroxylase and secondary hyperparathyroidism. This condition is prevented by $1,25(OH)_2D_3$ administration. In 1997, as the result of a tremendous amount of attention over 25 years of research, the cytochrome P450, CYP27B1, representing the 1α-hydroxylase enzyme was finally cloned from a rat renal cDNA library by St Arnaud's group in Montreal *(16)*. This was rapidly followed by cloning of cDNAs representing mouse and human CYP27B1 *(17–19)* as well as the human and mouse genes *(16, 18–20)*. It had been known for some time that the mitochondrially located 1α-hydroxylase enzyme comprises three proteins: a cytochrome P450, ferredoxin and ferredoxin reductase for activity and is strongly down-regulated by $1,25(OH)_2D_3$ & FGF23 and upregulated by PTH *(21–23)*. It is now evident that the promoter for the CYP27B1 gene contains the transcriptional regulatory elements necessary to explain the observed physiological up-regulation by PTH *(24)* and down-regulation by $1,25(OH)_2D_3$ *(25)*. The human CYP27B1 gene co-localizes to the chromosome 12q14 where vitamin D dependency rickets type 1 (VDDR-Ib), a human disease state, was first proposed to be caused by a mutation of the 1α-hydroxylase 35 years ago *(17, 21, 26, 113)*.

Over the past 20 years, suggestions of extra-renal 1α-hydroxylase expression have gone from incidental findings to a new concept that vitamin D works through an autocrine/paracrine mechanism in addition to the long-standing endocrine mechanism involving circulating calcitriol *(27, 28)*. Extra-renal expression of the 1α-hydroxylase enzyme activity in various tissues (e.g. placenta, bone and skin *(29)*) has been placed on a firmer footing by the development of highly specific antibodies to CYP27B1 protein and PCR-based detection techniques for CYP27B1 mRNA after the cloning of the CYP27B1 gene. Also, Adams and Gacad *(30)* had demonstrated the pathological significance of extra-renal 1α-hydroxylase expression by showing

(a) the existence of a 25-OH-D_3-1α-hydroxylase in sarcoid tissue in sarcoidosis patients;
(b) that this poorly regulated enzyme results in elevated plasma $1,25(OH)_2D_3$ levels; and
(c) that this causes hypercalciuria and hypercalcaemia in such sarcoidosis patients.

The availability of new specific CYP27B1 tools has confirmed the molecular basis of the disease as an over-expression of CYP27B1 *(31)*. The regulation of this 1α-hydroxylase in macrophages by cytokines and other growth factors has been demonstrated to involve interferon-gamma *(32)*. But the real value of the probes for CYP27B1 has been emergence of the widespread distribution of extra-renal 1α-hydroxylase in normal tissues including osteoblast, keratinocyte, monocyte and colonic, prostatic and breast epithelial cells *(27, 28, 31, 33–35)*. The existence of CYP27B1 in various extra-renal sites around the body implies that the enzyme boosts local $1,25(OH)_2D_3$ production allowing for higher intracellular concentrations of $1,25(OH)_2D_3$ in order to trigger

transcriptional regulation of genes unaffected by the plasma concentrations of the hormone *(27, 28, 31)*. However, it should be noted that this hypothesis is currently unproven at the level of measurement of intracellular $1,25(OH)_2D_3$ concentrations.

1.2. 24-Hydroxylation

24-Hydroxylation of both $25(OH)D_3$ and $1,25(OH)_2D_3$ has been shown to occur in vivo *(36, 37)*. The importance of this step has been immersed in controversy since it has been claimed that 24-hydroxylated metabolites might play a role in (a) bone mineralization *(38, 39)* and (b) egg hatchability *(40)*. Experimental evidence favours a different function for 24-hydroxylation, namely *inactivation* of the vitamin D molecule.

This concept comes from four main lines of evidence:

(i) The levels of $24,25(OH)_2D_3$ do not appear to be regulated, reaching >100 ng/ml in hypervitaminotic animals *(41)*.
(ii) There is no apparent $24,25(OH)_2D_3$ receptor similar to VDR within the orphan class of the steroid receptor superfamily.
(iii) Synthesis of vitamin D analogs blocked with fluorine atoms at the various carbons of the side chain (e.g. $24F_2$-$1,25(OH)_2D_3$) results in molecules with the *full* biological activity of vitamin D in vivo *(42)*.
(iv) 24-Hydroxylation appears to be the first step in a degradatory pathway demonstrable in vitro *(43, 44)* (Fig. 2), which culminates in a biliary excretory form, calcitroic acid, observed in vivo *(45)*.

The 25-OH-D_3-24-hydroxylase was originally characterized as a P450-based enzyme over 35 years ago *(46)* and more recently the cytochrome P450 species purified and

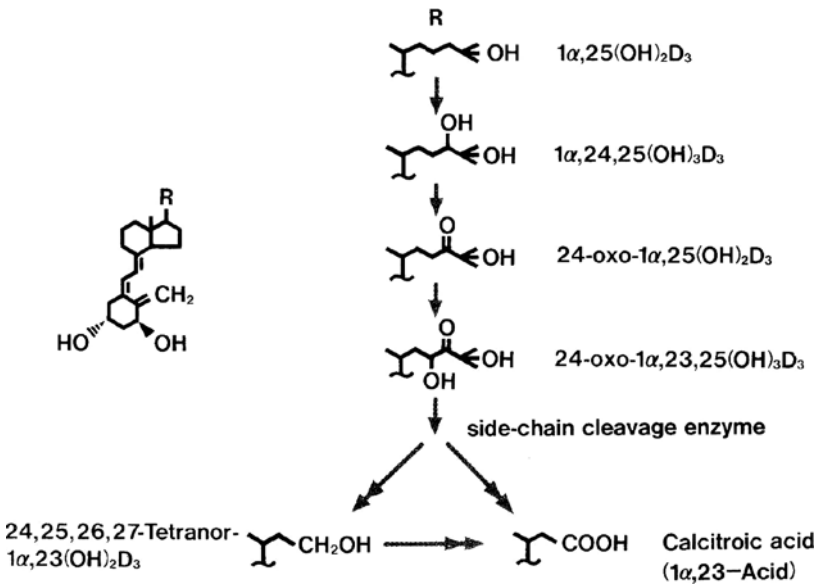


Fig. 2. C24-oxidation pathway. (Reproduced from *(43)* with permission).

cloned by Okuda's group *(47)*. The enzyme appears to 24-hydroxylate both $25(OH)D_3$ and $1,25(OH)_2D_3$, the latter with a 10-fold higher efficiency *(48, 49)*. However, since the circulating level of $25(OH)D_3$ is ~1,000 times higher than $1,25(OH)_2D$, the role of the enzyme in vivo is not clear. The enzyme, particularly the renal form which appears to be expressed at high constitutive levels in the normal animal, may be involved in the inactivation and clearance of excess $25(OH)D_3$ in the circulation *(43)*. On the other hand, the 24-hydroxylase may be involved in target cell destruction of $1,25(OH)_2D_3$. This topic will be discussed further under Section 2.1.

1.3. 26-Hydroxylation and 26,23-Lactone Formation

$25,26\text{-}(OH)_2D_3$ was the first dihydroxylated metabolite to be identified back in the late 1960s *(50)* and yet it is still the most poorly understood. The metabolite is readily detectable in the plasma of animals given large doses of vitamin D_3 and it retains strong affinity for DBP *(51)*. However, its biological activity is inferior to other endogenous vitamin D compounds and it is presumed to be a minor catabolite. The knowledge that CYP27A1 is involved in vitamin D_3 activation and that 26(27)(OH)D and $1,27(OH)_2D_3$ are formed from vitamin D_3 and 1(OH)D, respectively, in CYP27A1 transfection systems *(5)* when taken together suggests that 26(27)-hydroxylation may be a consequence of errant side chain hydroxylation.

The most abundant 26-hydroxylated analog appearing in vivo is the 26,23-lactone derivative of $25(OH)D_3$. $25\text{-}OH\text{-}D_3$-26,23-Lactone accumulates in hypervitaminotic animals in vivo because of its extremely strong affinity for DBP *(51)*. The route of synthesis of this metabolite is depicted in Fig. 3 and research indicates that 26-hydroxylation follows 23-hydroxylation in this process *(52)* and a wealth of evidence *(53, 54)* now suggests that CYP24A1 is responsible for 23- as well as 24-hydroxylation and is also involved in 26,23-lactone formation. Indeed, mutational analysis of human CYP24A1 *(54)* has shown that a A326G modification changes the enzyme from a 24-hydroxylating enzyme synthesizing predominantly calcitroic acid to a 23-hydroxylating enzyme making 26,23-lactone (Fig. 4). The role of $25\text{-}OH\text{-}D_3$-26,23-lactone or its $1,25(OH)_2D_3$ counterpart, which has also been reported *(54, 55)*, is currently unknown but members of the synthetic 26,23-lactone family are strong VDR antagonists *(56)* with potential therapeutic value in Paget's disease *(57)*. Since some species (e.g. opossum, guinea pig) make predominantly 26,23-lactone over calcitroic acid, it is theorized that 26,23-lactone formation represents a backup degradatory pathway for $25(OH)D_3$ and/or $1,25(OH)_2D_3$ or helps to reinforce the inactivation of the vitamin D endocrine system by antagonizing the further action of remaining $1,25(OH)_2D_3$ at the VDR *(54)*.

2. CATABOLISM OF $1,25(OH)_2D_3$

2.1. C-24 Oxidation Pathway to Calcitroic Acid

As described in Section 1.2 above, $1,25(OH)_2D_3$ is a very good substrate for the 24-hydroxylase. Using a variety of cell lines representing specific vitamin D target organs (intestine: CaCo2 cells; osteosarcoma: UMR-106 cells; kidney: LLC-PK1

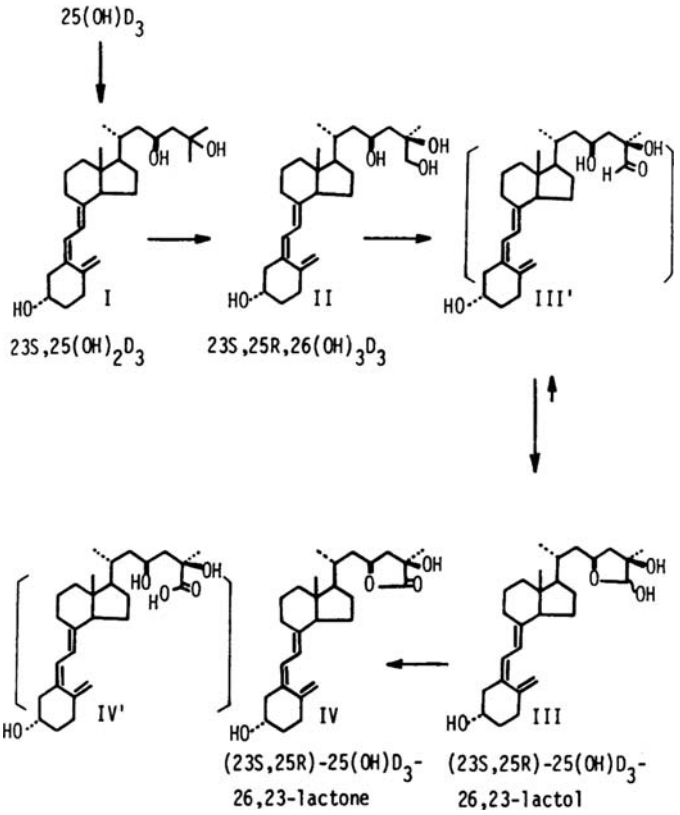
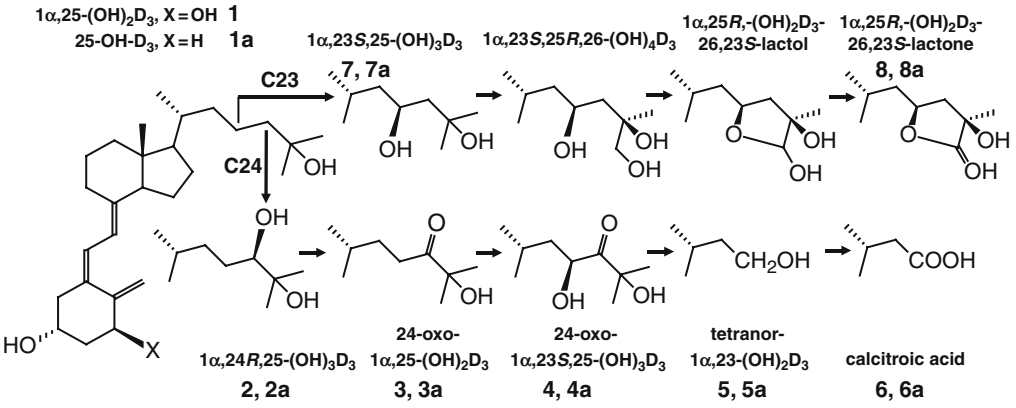


Fig. 3. 26,23-Lactone pathway. The pathway shown is for 25-OH-D_3. An analogous pathway exists for 1α,25-$(OH)_2D_3$. (Reproduced from *(52)* with permission).

cells; keratinocyte: HPK1A and HPK1A-ras) a number of researchers have shown that 24-hydroxylation is the first step in the C-24 oxidation pathway, a 5-step, vitamin D-inducible, ketoconazole-sensitive pathway which changes the vitamin D molecule to water-soluble truncated products (Figs. 2 and 4) *(43, 44)*. In most biological assays, the intermediates and truncated products of this pathway possess lower or negligible activity. Furthermore, many of these compounds have little or no affinity for DBP making their survival in plasma tenuous at best. The cloning of the cytochrome P450 component (CYP24) of the 24-hydroxylase enzyme has led to detection of CYP24mRNA in a wide range of tissues *(58)*, corroborating the earlier studies reporting widespread 24-hydroxylase enzyme activity in most, if not all, vitamin D target cells *(59)*. Additional studies have shown that mRNA transcripts for CYP24 are virtually undetectable in naive target cells not exposed to $1,25(OH)_2D_3$ but increase dramatically by a VDR-mediated mechanism within hours of exposure to $1,25(OH)_2D_3$ *(58)*. It is therefore attractive to propose that 24-hydroxylation is not only an important step in inactivation of excess $25(OH)D_3$ in the circulation but also involved in the inactivation of $1,25(OH)_2D_3$ inside target cells. As such one can hypothesize that C-24 oxidation is a target cell attenuation or desensitization process which constitutes a molecular switch



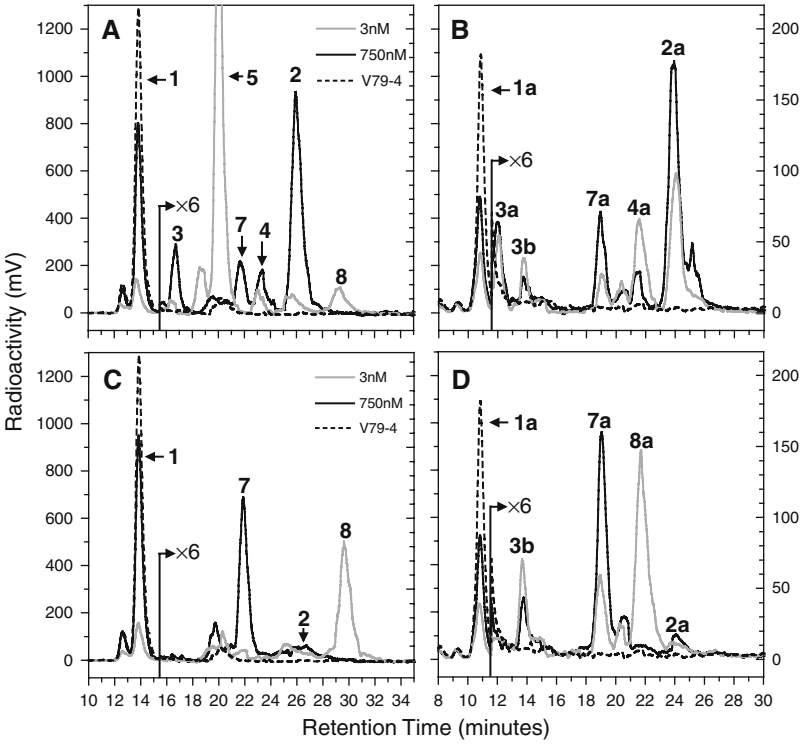


Fig. 4. Effect of A326G mutation on the hydroxylation properties of human CYP24A1. Chromatograms of the lipid extracts of V79 cells expressing either human CYP24A1 (**a** and **b**) or A326G human CYP24A1 (**c** and **d**) incubated with either 25-OH-D_3 (**b** and **d**) or 1α,25-$(OH)_2D_3$ (**a** and **c**). Note that human CYP24A1 makes mainly 24-hydroxylated metabolites and calcitroic acid whereas A326G human CYP24A1 makes mainly 23-hydroxylated metabolites and 26,23-lactones. The numbered metabolites are shown on the pathways above the chromatograms. (Reproduced from *(54)* with permission).

to turn off vitamin D responses inside target cells *(59)*. The recent development of a CYP24A1 knockout mouse *(60)* resulting in hypercalcaemia, hypercalciuria, nephrocalcinosis and premature death in 50% of null animals seems to support this hypothesis. On the other hand, surviving animals have changes in bone morphology which could suggest an alternative role for 24-hydroxylase in bone mineralization although these cyp24–/– animals are rescued by crossing them with vdr–/– animals, suggesting that abnormal bone is more likely due to $1,25(OH)_2D_3$ toxicity *(60, 61)*. Surviving CYP24 null animals exhibit altered pharmacokinetics of exogenously administered $1,25(OH)_2D_3$ and appear to have much reduced ability to get rid of the hormone, suggesting that the mammal has little in the way of backup systems to catabolize $1,25(OH)_2D_3$ when CYP24A1 is absent *(53)*. Nonetheless, recent work by Thummel's group has shown that $1,25(OH)_2D_3$ is subject to glucuronidation by UGT1A4 *(62)*.

Calcitroic acid, the final product of $1,25(OH)_2D_3$ catabolism, is probably not synthesized in the liver because C-24 oxidation does not occur in hepatoma cells and therefore must presumably be transferred from target cells to liver via some plasma carrier. Though calcitroic acid has been found in various tissues in vivo *(63)*, the nature of any transfer mechanism has not been elucidated.

3. METABOLISM AND CATABOLISM OF THE ANALOGS OF VITAMIN D

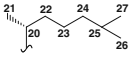
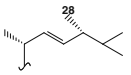
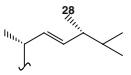
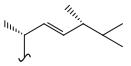
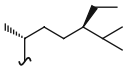
3.1. Activation of Prodrugs

Prodrugs are synthetic analogs of vitamin D_3 requiring *one or more step(s)* of activation by endogenous enzyme systems before they are biologically active (e.g. *one step*: $1(OH)D_2$, $1(OH)D_3$ or $25(OH)D_3$; *multiple steps*: vitamin D_2 and dihydrotachysterol).

3.2. Vitamin D_2

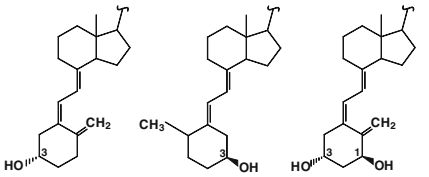
Though vitamin D_2 (for structure see Table 1) can be synthesized naturally by irradiation of ergosterol, little finds its way into the human diet unless it is provided as a dietary supplement. Indeed, vitamin D_2 has been used as a dietary supplement in lieu of vitamin D_3 since the 1930s. Since vitamin D_2 is an artificial form of the vitamin and it is dependent on the same activation steps as vitamin D_3 to become biologically active, one could make a strong case for considering it as a prodrug. In many ways the metabolism of vitamin D_2 is analogous to that of vitamin D_3. For instance, it has been established that vitamin D_2 gives rise to a similar series of metabolites in the form of $25(OH)D_2$ *(64)*, $1,25(OH)_2D_2$ *(65)* and $24,25(OH)_2D_2$ *(66)*. The formation of these metabolites suggests that the enzymes involved in side chain metabolism namely 25-, 1α- and 24-hydroxylases do not discriminate against compounds bearing the vitamin D_2 side chain. However, metabolic studies have also revealed the formation of several additional metabolites including $24(OH)D_2$ *(67)*, $1,24S(OH)_2D_2$ *(68)*, $24,26(OH)_2D_2$ *(69)* and $1,25,28(OH)_3D_2$ *(70)*. Pharmaceutical companies have exploited these subtle differences in the metabolism of vitamin D_2 by synthesizing molecules incorporating the features of the vitamin D_2 side chain, namely the C22=C23 double bond or the C-24 methyl group (see Table 1), into the structure of other analogs (e.g. calcipotriol).

Table 1
Vitamin D Prodrugs

Vitamin D prodrug [ring structure][a]	Side chain structure	Company	Status	Possible target diseases	Mode of delivery	References
1α-OH-D_3 [3]		Leo	In use Europe	Osteoporosis	Systemic	Barton *et al. (85)*
1α-OH-D_2 [3]		Genzyme	In use USA	Secondary hyperparathyroidism	Systemic	Paaren *et al. (89)*
Dihydrotachysterol [2]		Duphar	Withdrawn	Renal failure	Systemic	Jones *et al. (33)*
Vitamin D_2 [1]		Various	In use USA	Rickets Osteomalacia	Systemic Systemic	Fraser *et al. (151)*
1α-OH-D_5 [3]		NCI	Clinical trials	Cancer	Systemic Systemic	Mehta *et al. (160)*

[a] Structure of the vitamin D nucleus (secosterol ring structure).

Vitamin D nucleus



[1] [2] [3]

Biologically active vitamin D_2 compounds, such as $1,25(OH)_2D_2$ and $1,24S(OH)_2D_2$, are also subject to further metabolism although it differs from that of $1,25(OH)_2D_3$, essentially because the modifications in the vitamin D_2 side chain prevent the C23C24 cleavage observed during calcitroic acid production. Instead, the principal products are more polar tri- and tetra-hydroxylated metabolites such as $1,24,25(OH)_3D_2$, $1,25,28(OH)_3D_2$ and $1,25,26(OH)_3D_2$ from $1,25(OH)_2D_2$ *(70–72)* and $1,24,26(OH)_3D_2$ from $1,24S(OH)_2D_2$ *(73)*, all likely produced by the action of CYP24A1. In this latter case, the rate of $1,24S(OH)_2D_2$ metabolism appears slower than that of $1,25(OH)_2D_3$ *(73)*. Some catabolites retain considerable biological activity and at least one, $1,25,28(OH)_3D_2$, is patented for use as a drug.

3.2.1. Dihydrotachysterol

This example of a vitamin D prodrug represents the oldest vitamin D analog and was developed in the 1930s as a method of stabilizing the triene structure of one of the photoisomers of vitamin D. The structure of dihydrotachysterol$_2$ shown in Table 1 contains an A-ring rotated through 180°, a reduced C10=C19 double bond and the side chain structure of ergosterol/vitamin D_2. The side chain depicted is that of vitamin D_2 because the clinically approved drug version of dihydrotachysterol is dihydrotachysterol$_2$.

However, it should be noted that dihydrotachysterol$_3$ (DHT_3) can also be chemically synthesized with the side chain of vitamin D_3. The metabolism of both dihydrotachysterol$_2$ (DHT_2) and dihydrotachysterol$_3$ (DHT_3) has been extensively studied over the past four decades *(74–77)*. Initial studies performed in the early 1970s showed that DHT is efficiently converted to its 25-hydroxylated metabolite *(78)*.

The effectiveness of DHT to relieve hypocalcaemia of chronic renal failure in the absence of functional renal 1α-hydroxylase led to the hypothesis *(79)* that

> 25-OH-DHT might represent the biologically-active form of DHT, by virtue of its 3ß-hydroxy group being rotated 180° into a "pseudo 1α-hydroxyl position".

It was thus believed that 1α-hydroxylation of 25(OH)DHT was unnecessary. This viewpoint prevailed for at least a decade but debate was renewed when Bosch et al. *(75)* were able to provide evidence for the existence of a mixture of 1α- and 1β-hydroxylated products of 25(OH)DHT_2 in the blood of rats dosed with DHT_2. Studies involving the perfusion of kidneys from vitamin D-deficient rats with an incubation medium containing 25(OH)DHT_3 and using diode array spectrophotometry to analyse the extracts showed this molecule to be subject to extensive metabolism by renal enzymes but failed to give the expected 1-hydroxylated metabolites (Fig. 5), opening up the possibility that the 1α- and 1β-hydroxylated metabolites observed by Bosch et al. *(75)* might be formed by a putative extra-renal 1-hydroxylase activity *(80)*. Following the synthesis of appropriate authentic standards, subsequent research *(77)* has confirmed the in vivo formation and identity of 1α,25$(OH)_2$DHT and 1β,25$(OH)_2$DHT in both rat and human. The ability of these 1α- and 1β-hydroxylated forms of both DHT_2 and DHT_3 to stimulate a VDRE-inducible growth hormone reporter system exceeded that of 25(OH)DHT and in the process established 1α,25$(OH)_2$DHT and 1β,25$(OH)_2$DHT as the most potent derivatives of DHT identified to date. The importance of the "pseudo 1α-hydroxyl group" hypothesis now stands in question though current findings do not rule out that the biological activity of DHT might be due to the collective action of a group of metabolites including 25(OH)DHT, 1α,25$(OH)_2$DHT and 1β,25$(OH)_2$DHT. The enzymatic origin of these dihydroxylated metabolites is still undetermined despite the availability of recombinant CYP27A1, CYP27B1 and CYP24A1 although work suggests that extra-renal hydroxylases of bone marrow origin might be involved *(80, 81)*.

Though the enzymes involved in the activation of DHT, especially the 1-hydroxylation step, have an altered specificity towards this molecule, the enzymes involved in the catabolism of DHT_3 appear to treat the molecule as they would 25$(OH)D_3$ or 1,25$(OH)_2D_3$. Side chain hydroxylated derivatives of both 25(OH)DHT_3 and 1,25$(OH)_2DHT_3$ have been identified and appear to be analogous to intermediates of the C-24 oxidation and 26,23-lactone pathways of vitamin D_3 metabolism *(82, 83)*.

3.2.2. 1(OH)D_2 AND 1(OH)D_3

The prodrug, 1(OH)D_3, was developed in the early 1970s *(84, 85)* following the discovery of the hormone, 1α,25-$(OH)_2D_3$, and the realization that the kidney was the main site of its synthesis *(21, 22, 86)*. The rationale behind its use was to circumvent the 1α-hydroxylation step involved in vitamin D activation thereby providing a molecule

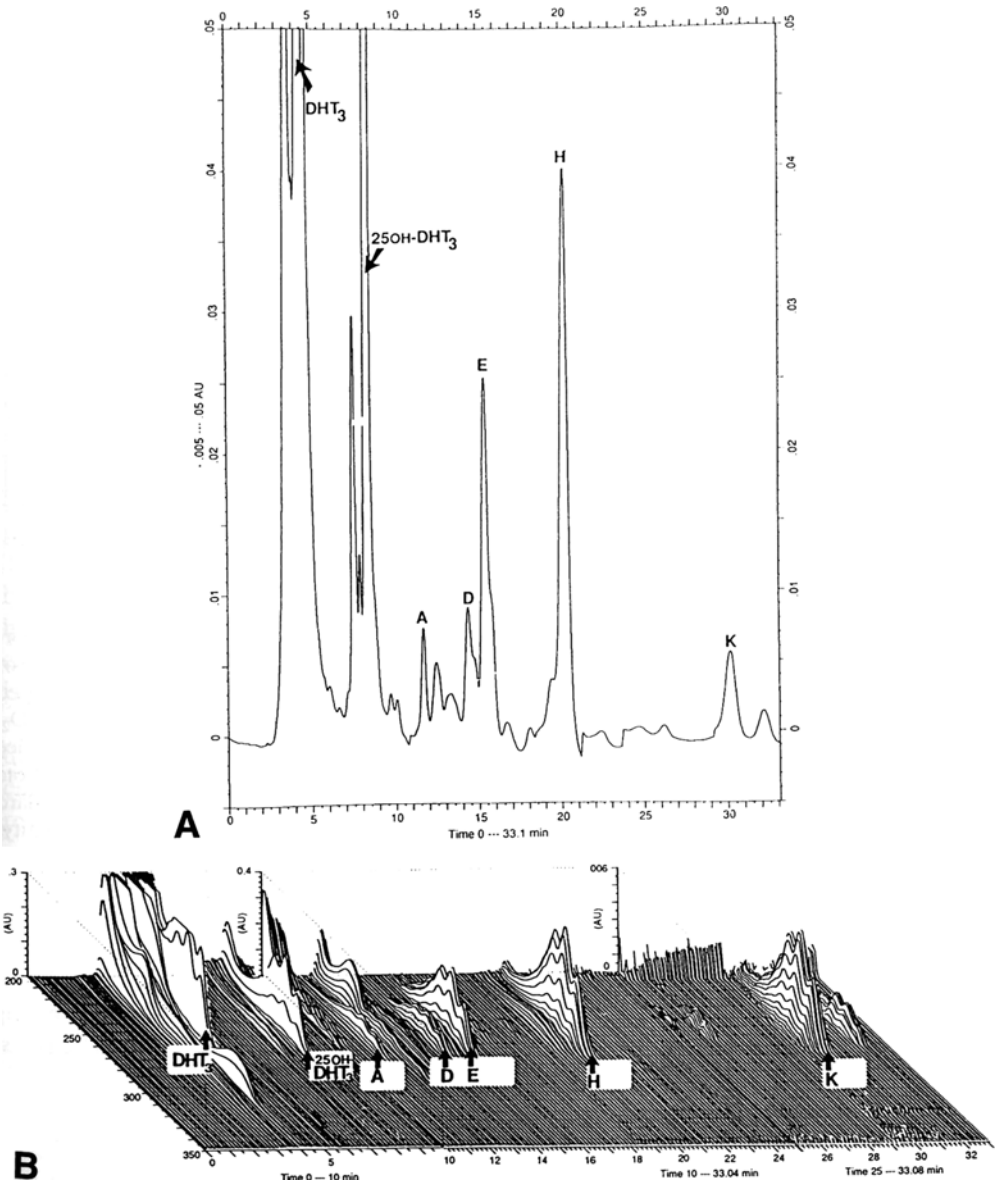


Fig. 5. In vivo metabolism of dihydrotachysterol$_3$ in the rat. Diode array HPLC of the plasma extract of a rat administered 1 mg DHT_3 18 h prior to sacrifice. Metabolites are labelled 25-OH-DHT_3 and peaks A–L. All possess the distinctive tricuspid UV spectrum (λ_{max} 242.5, 251 and 260.5 nm). Metabolites A–L were subsequently identified as side chain-modified compounds analogous to vitamin D metabolites of the C-24 oxidation and 26,23-lactone pathways depicted in Figs. 1 and 2. (Reproduced from *(76)* with permission).

which could still be activated even in the absence of a functional kidney. It soon became an alternative drug therapy to synthetic $1,25(OH)_2D_3$ in renal osteodystrophy and other hypocalcaemic conditions. Aside from the advantage of a reduced cost of synthesis, $1(OH)D_3$ offers the potential biological edge of requiring a step of

activation in the form of 25-hydroxylation to produce an active molecule. It was believed that the requirement for an activation step might alter the pharmacokinetics of the drug compared to $1,25(OH)_2D_3$, delaying slightly its initial effects and extending its duration of action thereby making the drug less likely to cause acute hypercalcaemia. The 25-hydroxylation of $1(OH)D_3$ was first investigated using the isolated perfused rat liver *(6)* and confirmed that the liver represents the main site of activation. More recent work has confirmed that both CYP27A1 and CYP2R1 are able to 25-hydroxylate $1(OH)D_3$ efficiently *(6, 15)*, and there are no data to support promoter-mediated regulation of these enzymes by $1,25(OH)_2D_3$. It is widely assumed that either 25-hydroxylase enzyme is only loosely regulated and therefore constitutes an insignificant barrier to drug activation. The theoretical advantages of $1(OH)D_3$ over $1,25(OH)_2D_3$ have not materialized in clinical practice *(87)*.

A prodrug based on vitamin D_2 has also been synthesized in the form of $1(OH)D_2$ *(88, 89)*. Although developed as a potential anti-osteoporosis drug and currently approved for the treatment of secondary hyperparathyroidism and renal osteodystrophy as a result of chronic kidney disease *(90, 91)*, this molecule has proved to be a valuable tool in studying hydroxylation reactions in the liver. At low substrate concentrations, $1(OH)D_2$, like $1(OH)D_3$, is 25-hydroxylated by liver hepatomas, Hep3B and HepG2, producing the well-established, biologically active compound $1,25(OH)_2D_2$ *(92)*. However, when the substrate concentration is increased to micromolar values the principal site of hydroxylation of $1(OH)D_2$ becomes the C-24 position, the product being $1,24S(OH)_2D_2$ (Fig. 6), another compound with significant biological activity in several calcaemia and cell proliferation assay systems *(68, 73, 92)*. This metabolite has been previously reported in cows receiving massive doses of vitamin D_2 *(68)*. Transfection studies using the liver cytochrome P450, CYP27A1, expressed in COS-1 cells suggest that $1,24S(OH)_2D_2$ is a product of this cytochrome *(6)*. Whether the formation of this unique metabolic product of $1(OH)D_2$ is the reason for the relative lower toxicity of $1(OH)D_2$ as compared to $1(OH)D_3$ *(93)* has not been established definitively. Recent data *(14, 15)* suggest that CYP2R1 is responsible for the 25-hydroxylation of $1(OH)D_2$ at physiologically relevant concentrations of substrate (Fig. 6).

3.3. Metabolism-Sensitive Analogs

These synthetic analogs of $1,25(OH)_2D_3$ require *no* activation in vivo but are susceptible to attack by catabolic enzyme systems, in most cases rendering them biologically inactive (e.g. calcipotriol, OCT, KH1060).

3.3.1. Cyclopropane Ring Containing Analogs of Vitamin D

These analogs are modified in their side chains such that C-26 is joined to C-27 to give a cyclopropane ring consisting of C-25, C-26 and C-27. The best-known member of this group of compounds is MC 903 or calcipotriol *(94)*, the structure of which is shown in Table 2. In addition to the cyclopropane ring, calcipotriol features a C22=C23 double bond and a 24*S*-hydroxyl group which has been proposed to act as a surrogate C-25 hydroxyl in interactions of the molecule with the VDR. Calcipotriol was the first

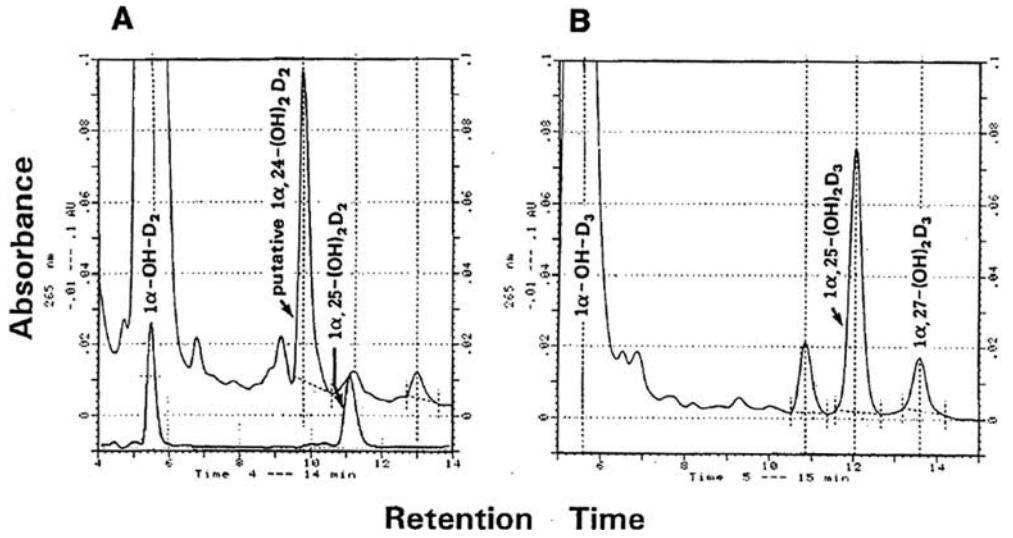
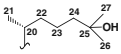
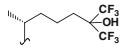
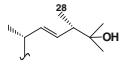
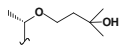
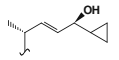
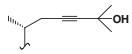
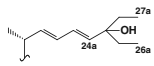
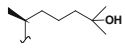
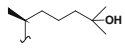
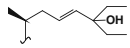
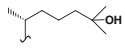
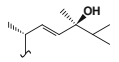
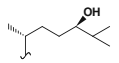


Fig. 6. In vitro metabolism of 1α-OH-D_2 and 1α-OH-D_3 by the hepatoma Hep3B. (**a**) HPLC trace using diode array detector at 265 nm of an extract of Hep3B cells incubated with 10 μM 1α-OH-D_2. The peak at 9.79 min was later conclusively identified by GC-MS and co-migration with authentic standard as 1α,24*S*-$(OH)_2D_2$. Note that inset is a trace of standards: 1α-OH-D_2, 5.6 min; 1α,25-$(OH)_2D_2$, 11.1 min. (**b**) HPLC trace using diode array detector at 265 nm of an extract of Hep3B cells incubated with 10 μM 1α-OH-D_3. Note that the peak at 12.04 min comigrated with authentic 1α,25-$(OH)_2D_3$. Subsequent work with standard 1α,26(27)-$(OH)_2D_3$ (synthesized by Martin Calverley, Leo Pharmaceuticals) has confirmed its identity. (From *(92)* with permission).

vitamin D analog to be approved for topical use in psoriasis and is currently used worldwide for the successful control of this skin lesion *(95, 96)*.

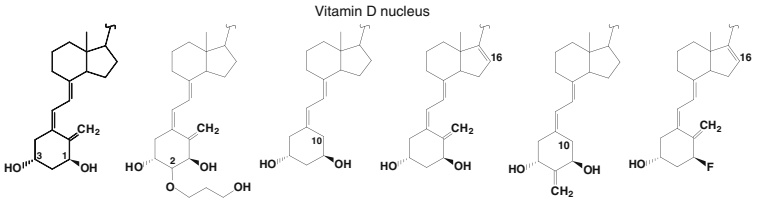
Pharmacokinetic data acquired for calcipotriol showed that it had a very short $t_{1/2}$, in the order of minutes, results that are consistent with the lack of a hypercalciuric/hypercalcaemic effect when administered in vivo *(97)*. The first metabolic studies *(98)* revealed that calcipotriol was rapidly metabolized by a variety of different liver preparations from rat, minipig and human to two novel products. These workers *(99)* were able to isolate and identify the two principal products as a C22=C23 unsaturated, 24-ketone (MC1046) and a C22–C23 reduced, 24-ketone (MC1080). These results were confirmed and extended by others *(99)* who showed that calcipotriol metabolism was not confined to liver tissue, but could be carried out by a variety of cells including those cells exposed to topically administered calcipotriol in vivo, namely keratinocytes. Furthermore, these workers *(99)* proposed further metabolism of the 24-ketone in these vitamin D target cells to side chain cleaved molecules including calcitroic acid (Fig. 7). The main implications of this work are that calcipotriol is subject to rapid metabolism initially by non-vitamin D-related enzymes, then by vitamin D-related pathways probably including CYP24A1 *(14)* to a side chain cleaved molecule *(99)*. Catabolites are produced in a variety of tissues and appear to have lower biological activity than the parent molecule. Since calcipotriol is administered topically the work suggests that it acts and is broken down locally and may never reach detectable levels in the

Table 2
Analogs of 1α,25-$(OH)_2D_3$

Vitamin D analog [ring structure][a]	Side chain structure	Company	Status	Possible target diseases	Mode of delivery	Reference
Calcitriol, 1α,25-$(OH)_2D_3$ [3]		Roche, Duphar	In use worldwide	Hypocalcemia Psoriasis	Systemic Topical	Baggiolini *et al. (152)*
26,27-F_6-1α,25-$(OH)_2D_3$ [3]		Sumitomo-Taisho	In use Japan	Osteoporosis Hypoparathyroidism	Systemic Systemic	Kobayashi *et al. (112)*
19-Nor-1α,25-$(OH)_2D_2$ [5]		Abbott	In use USA	Secondary hyperparathyroidism	Systemic	Perlman *et al. (153)*
22-Oxacalcitriol (OCT) [3]		Chugai	In use Japan	Secondary hyperparathyroidism Psoriasis	Systemic Topical	Murayama *et al. (100)*
Calcipotriol (MC903) [3]		Leo	In use worldwide	Psoriasis Cancer	Topical Topical	Calverley *(94)*
1α,25-$(OH)_2$-16-ene-23-yne-D_3 (Ro 23–7553) [6]		Roche	Pre-clinical	Leukemia	Systemic	Baggiolini *et al. (154)*
EB1089 [3]		Leo	Clinical trials	Cancer	Systemic	Binderup *et al. (121)*
20-epi-1α,25-$(OH)_2D_3$ [3]		Leo	Pre-clinical	Immune diseases	Systemic	Calverley *et al. (155)*
2-methylene-19-nor-20-epi-1α,25-$(OH)_2D_3$ (2MD) [7]		Deltanoids	Pre-clinical	Osteoporosis	Systemic	Shevde *et al. (161)*
BXL-628 (formerly Ro-269228) [8]		Bioxell	Clinical trials	Prostate Cancer	Systemic	Marchiani *et al. (159)*
ED71 [4]		Chugai	Clinical trials	Osteoporosis	Systemic	Nishii *et al. (156)*
1α,24(*S*)-$(OH)_2D_2$ [3]		Genzyme	Pre-clinical	Psoriasis	Topical	Strugnell *et al. (92)*
1α,24(*R*)-$(OH)_2D_3$(TV-02) [3]		Teijin	In use Japan	Psoriasis	Topical	Morisaki *et al. (157)*

[a]Structure of the vitamin D nucleus (secosterol ring structure).

Vitamin D nucleus



[3] [4] [5] [6] [7] [8]

bloodstream. Should calcipotriol enter the circulation, the ability of liver and target cells to breakdown calcipotriol provides a backup system to prevent hypercalcaemia.

The reduction of the C22=C23 double bond during the earliest phase of calcipotriol catabolism was an unexpected event given that the C22=C23 double bond in vitamin D_2

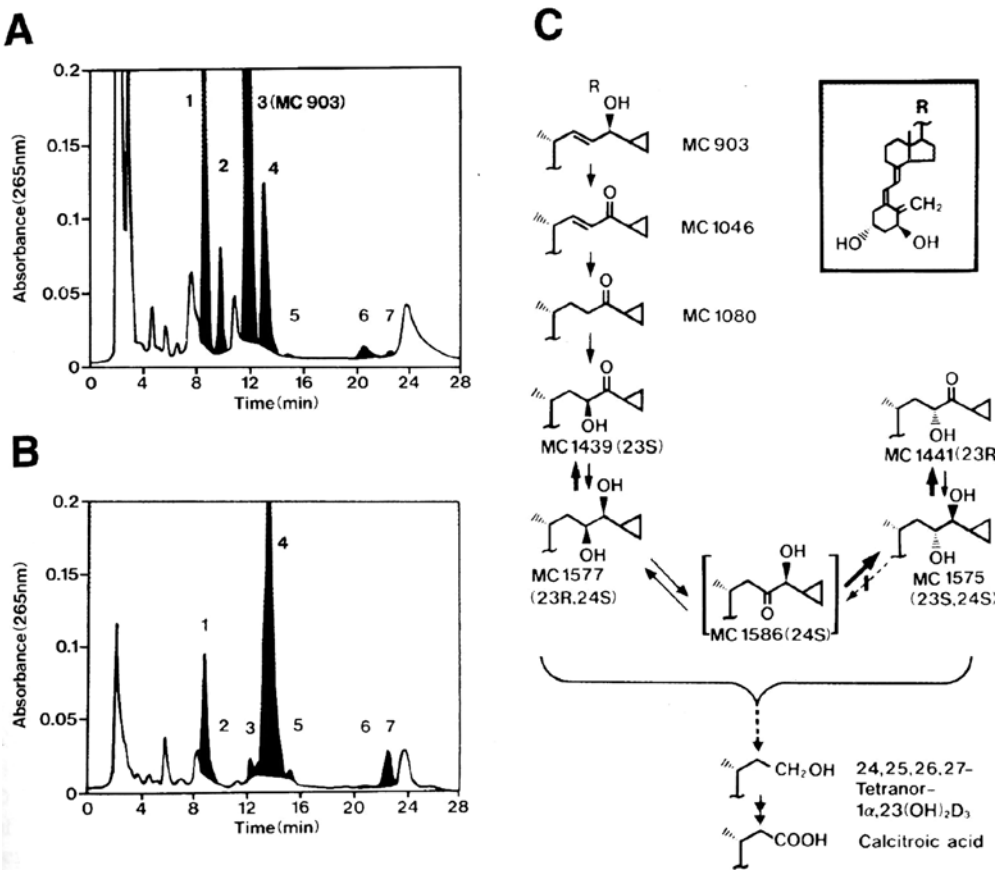


Fig. 7. In vitro metabolism of calcipotriol (MC903) by HPK1A-ras cells. HPLC of lipid extracts following incubation of MC903 with (**a**) HPK1A human keratinocytes and (**b**) HPK1A-ras human keratinocytes. Peak 1 = MC1080; Peak 2 = MC1046; Peak 3 = MC903 (calcipotriol); Peak 4 = mixture of MC1439 and MC1441; Peak 5 = Tetranor-1α,23(OH)$_2$D$_3$; Peak 6 = MC1577; Peak 7 = MC1575. 7C: Proposed Pathway of calcipotriol metabolism in cultured keratinocytes. (Reproduced from *(99)* with permission).

compounds is extraordinarily stable to metabolism. It thus appears that metabolism of calcipotriol provides evidence that the C-24 methyl group in the vitamin D_2 side chain must play a stabilizing role, preventing the formation of the 24-ketone which facilitates the reduction of the C22=C23 double bond. However, it is still unknown which enzyme is responsible for this reduction in the side chain of calcipotriol, although it is widely suspected that the extremely versatile CYP24A1 may once again be involved.

3.3.2. Oxa-Group-Containing Analogs

These compounds involve the replacement of a carbon atom (usually in the side chain) with an oxygen atom. The best known of these are the 22-oxa-analogs including 22-oxa-calcitriol (OCT) and KH1060 *(100, 101)*. Both of these molecules are metabolically fascinating to study because *the oxa-atom makes the molecule inherently unstable*

should it be hydroxylated at the adjacent carbon atom. The hydroxylation at an adjacent carbon generates an unstable hemi-acetal which spontaneously breaks down to eliminate the carbons distal to the oxa-group. In the case of the 22-oxa-compounds the expected product(s) would be C-20 alcohol/ketone.

The metabolism of OCT has been extensively studied in a number of different biological systems including primary parathyroid *(102)*, primary keratinocyte cells *(103)* as well as cultured osteosarcoma, hepatoma and keratinocyte cell lines *(104)*. In all these systems, OCT is rapidly broken down. Judicious use of two different radioactive labels in the form of [26-^{3}H]OCT and [2β-^{3}H]OCT enabled Brown et al. *(102)* to suggest that the side chain was truncated, though definitive proof of the identity of the products was not immediately forthcoming. It was not until later work *(104)* that the principal metabolites were unequivocably identified by GC-MS as 24(OH)OCT, 26(OH)OCT and hexanor-1α,20-dihydroxyvitamin D_3 (Fig. 8). In the case of the keratinocyte-derived cell line, HPK1A-ras, an additional product, hexanor-20-oxo-1α-hydroxyvitamin D_3, is also formed. These latter two truncated products are suggestive of hydroxylation of OCT at

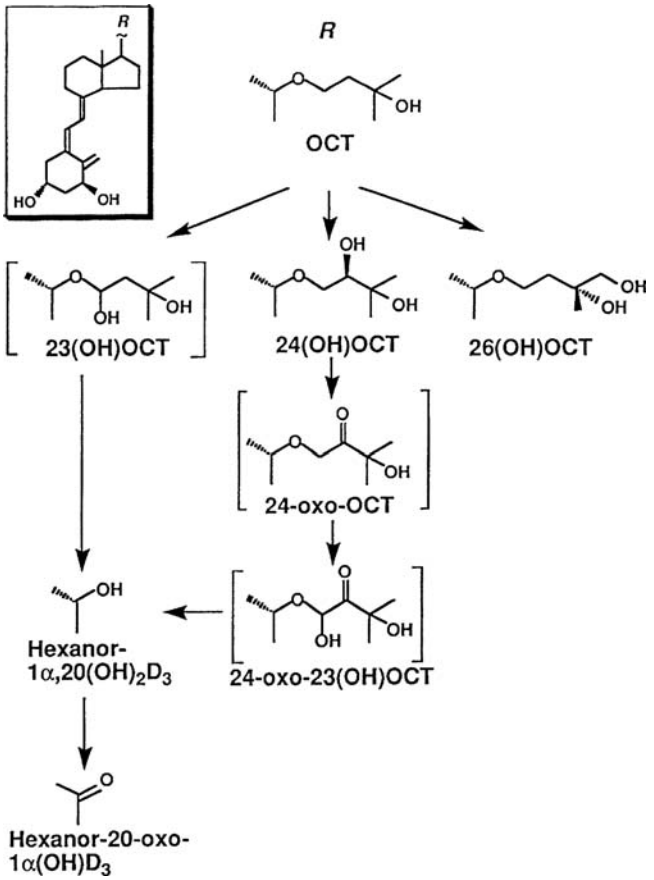


Fig. 8. Proposed pathways of OCT metabolism in cultured vitamin D target cells in vitro. Metabolic pathways worked out using cultured cell lines representing hepatoma, osteosarcoma and keratinocyte. (Taken from *(104)* with permission).

the C-23 position to give the theoretical unstable intermediate. Though all of these products were isolated from in vitro systems, there is evidence that the processes also occur in vivo because Kobayashi et al. *(105)* have generated data which suggest that the biliary excretory form of OCT in the rat is a glucuronide ester of the truncated 20-alcohol.

The above example of a simple oxa-analog provides useful knowledge which can help in predicting the metabolic fate of a complex oxa-analog such as KH1060. This highly potent compound which possesses in vitro cell-differentiating activity exceeding that of any other analog synthesized to date has four different modifications to the side chain of $1,25(OH)_2D_3$, namely (1) 22-oxa-group, (2) the 20-epi side chain stereochemistry, (3) 24a-homologation and (4) 26- and 27-dimethyl homologation (see Table 2 for structure).

Since all of these changes are known to separately affect biological activity in vitro and in vivo as well as side chain metabolism *(106, 107)*, it comes as no surprise that the metabolism of KH1060 is extremely complex. KH1060 has a very short $t_{1/2}$ in pharmacokinetic studies in vivo *(108)* giving a metabolic profile with at least 16 unknown metabolites *(109)*. Dilworth et al. *(109)* reported the first in vitro study using micromolar concentrations of KH1060 incubated with the keratinocyte-derived cell line HPK1A-ras in which these workers were able to discern 22 different metabolites after multiple HPLC steps and assigned structures to 12 of these metabolites (see Fig. 9). As would be expected from consideration of the studies of other oxa-compounds, two of these were truncated products and were identical to the molecules formed from another 22-oxa compound, OCT. As would be expected from consideration of the studies of other homologated compounds, other products are hydroxylated at specific carbons of the side chain including C-26 and C-26a. As with the metabolism-resistant analog, EB1089 (see following section 3.3.2) and 26,27-dimethyl-$1,25(OH)_2D_3$ (Leo code: MC1548), the presence of dimethyl groups in the terminus of the side chain appears to attract hydroxylation to these sites in KH1060. One novel metabolite found only for KH1060 is 24a-OH-KH1060, observed both in broken cell and intact cell models *(109, 110)*.

An important facet of this complex metabolic profile is that rather than simplifying our understanding of the mechanism of action of KH1060, this data complicates it. This is because biological assays performed on each of the metabolic products of KH1060 have shown that several of the principal and long-lived metabolites retain significant vitamin D-dependent gene inducing activity in reporter gene expression systems *(111)*. While current published assays have demonstrated a high biological activity for KH1060, these assays are performed in whole cell assay systems (cell culture, organ culture, transfected cell systems) over extended time periods (usually 24–72 h) where metabolism is known to occur. Yet analysts do not employ inhibitors of metabolism and often assume that the biological effects observed are due to the parent compound, not to its metabolic products. In the case of KH1060 where metabolism is rapid, it would seem to be prudent to assess the rate of metabolism in the bioassay model or else attempt to block metabolism by the use of appropriate inhibitors (e.g. ketoconazole).

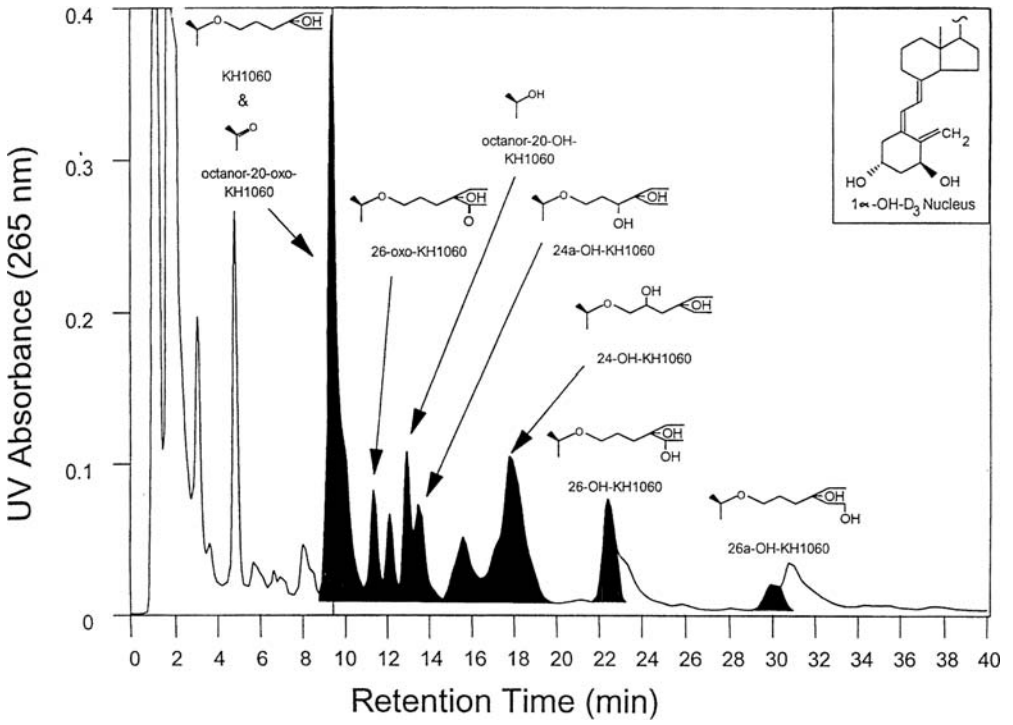


Fig. 9. In vitro metabolism of KH1060 by HPK1A-ras cells. HPLC of lipid extracts following incubation of KH1060 (10 μM) with the human keratinocyte, HPK1A-ras for 72 h. Nine peaks (*darkened*) possessing the characteristic UV chromophore of vitamin D (λ_{max} 265 nm, λ_{min} 228 nm) are visible in the HPLC profile reproduced here. Rechromatography of these peaks on a second HPLC system resulted in the further resolution of these 9 peaks into 22 separate metabolites. Many of these metabolites were identified *(81)* by comparison to synthetic standards on HPLC and GC-MS. Some examples of the types of structures corresponding to each peak are provided. These include Peak at 13.39 min = 24a-OH-KH1060; Peak at 22.14 min= 26-OH-KH1060. (Reproduced with permission from *(109)*).

3.4. Metabolism-Resistant Analogs

These synthetic analogs of $1,25(OH)_2D_3$ require *no* activation in vivo and are resistant to attack by catabolic enzyme systems because of blocking groups in metabolically sensitive regions (e.g. 26,27-F_6-$1,25(OH)_2D_3$; $1,25(OH)_2D_3$-16-ene-23-yne; EB1089).

3.4.1. F_6-1,25-$(OH)_2$D

This analog was first synthesized in the early 1980s *(112)*, along with a number of other side chain fluorinated analogs, to test the importance of certain key hydroxylation sites (e.g. C-23, C-24, C-25, C-26(27), C-1) to biological activity. It was noted immediately that 26,27-F_6-$1,25(OH)_2D_3$ was extremely potent (10-fold higher than $1,25(OH)_2D_3$) in assays to measure calcaemic activity both in vitro and in vivo *(114–116)*. Lohnes and Jones *(60)* presented evidence using a bone cell line, UMR106, that 26,27-F_6-$1,25(OH)_2D_3$ had a longer $t_{1/2}$ inside target cells due to the apparent lack of 24-hydroxylation of 26,27-F_6-$1,25(OH)_2D_3$. At around the same time, Morii's group

noted the appearance of a metabolite of 26,27-F_6-1,25$(OH)_2D_3$ which they have identified as 26,27-F_6-1,23,25$(OH)_3D_3$ *(117)*. This compound possesses excellent calcaemic activity in its own right *(117)* but whether this derivative is in part responsible for the biological activity of 26,27-F_6-1,25$(OH)_2D_3$ is not conclusively proven. Nonetheless, 26,27-F_6-1,25$(OH)_2D_3$ has undergone clinical trials for hypocalcaemia associated with hypoparathyroidism and uraemia *(118, 119)*.

3.4.2. Unsaturated Analogs

The idea of introducing double bond(s) into the side chain of vitamin D analogs arose from experience with vitamin D_2. Vitamin D_2 metabolites have similar biological activity to those of vitamin D_3, so that the introduction of the double bond is not deleterious. As mentioned earlier the metabolism of the side chain is significantly altered by this relatively minor change.

The modification has not been confined to the introduction of a C22=C23 double bond. Roche has developed molecules with two novel modifications:

(a) Introduction of a C16=C17 double bond and (b) introduction of a C23≡C24 triple bond that when combined produce the well-studied 16-ene, 23-yne analog of 1,25$(OH)_2D_3$ *(120)* (see Table 2 for structure). Leo Pharmaceuticals has introduced the unsaturated analog EB1089 which contains a conjugated double bond system at C22=C23 and C24=C24a, in addition to both main side chain and terminal dimethyl types of homologation *(121)* (see Table 2 for structure). These two series of Roche and Leo compounds have shown strong anti-proliferative activity both in vitro and in vivo *(120, 122, 123)*.

The metabolism of the 16-ene compound by the perfused rat kidney has been studied *(124)*. These workers *(124)* found that the introduction of the C16=C17 double bond reduces 23-hydroxylation of the molecule and the implication is that the D-ring modification must alter the conformation of the side chain sufficiently to subtly change the site of hydroxylation by CYP24, the cytochrome P450 thought to be responsible for 23- and 24-hydroxylation. Both Dilworth et al. *(106)* studying the metabolism of 20-epi-1,25$(OH)_2D_3$ and Shankar et al. studying the metabolism of 20-methyl-1,25$(OH)_2D_3$ have noted the absence or much reduced 23-hydroxylation reinforcing the view that modifications around the C17-C20 bond profoundly influence the rate of 23-hydroxylation.

The metabolism of the 16-ene,23-yne analog of 1,25$(OH)_2D_3$ by WEHI-3 myeloid leukaemic cells has recently been reported *(126)*. Because this molecule is blocked in the C-23 and C-24 positions one might predict that this molecule might be stable to C-24 oxidation pathway enzyme(s); however, it was found experimentally that the 16-ene,23-yne analog has the same $t_{1/2}$ as 1,25$(OH)_2D_3$ when incubated with this cell line ($t_{1/2}$=6.8 h). The main product of [25-^{14}C]1,25$(OH)_2$-16-ene,23-yne-D_3 was not identified by these workers but appeared to be more polar than the starting material. Similar work *(127)* used the perfused rat kidney and GC-MS to identify the main metabolite as 1,25,26$(OH)_3$-16-ene,23-yne-D_3. As with 24-F_2-1,25$(OH)_2D_3$, it appears that the C-26 becomes vulnerable to attack when the C-23 and C-24 positions are metabolically blocked. As in the case of calcipotriol, not all vitamin D analogs containing

unsaturation are resistant to metabolism suggesting that the type of unsaturation, exact position and context (neighbouring groups) must also be considered.

Another unsaturated analog which one might predict would be relatively metabolically stable is EB1089 with its conjugated double bond system. However, as pointed out earlier EB1089 contains three structural modifications: the conjugated double bond system is accompanied by two types of side chain homologation. Nevertheless, as expected the conjugated double bond system dominates the metabolic fate of EB1089, there being no C-24 oxidation activity due to the blocking action of the conjugated diene system. When metabolism is studied with either in vitro liver cell systems or the cultured keratinocyte cell line, HPK1A-ras, disappearance of EB1089 is much slower than that of $1,25(OH)_2D_3$ *(128, 129)*. Such data are consistent with the fairly long $t_{1/2}$ observed in pharmacokinetic studies in vivo *(108)*. Since the conjugated system of EB1089 blocks C-24 oxidation reactions, it is not surprising that a different site in the molecule becomes

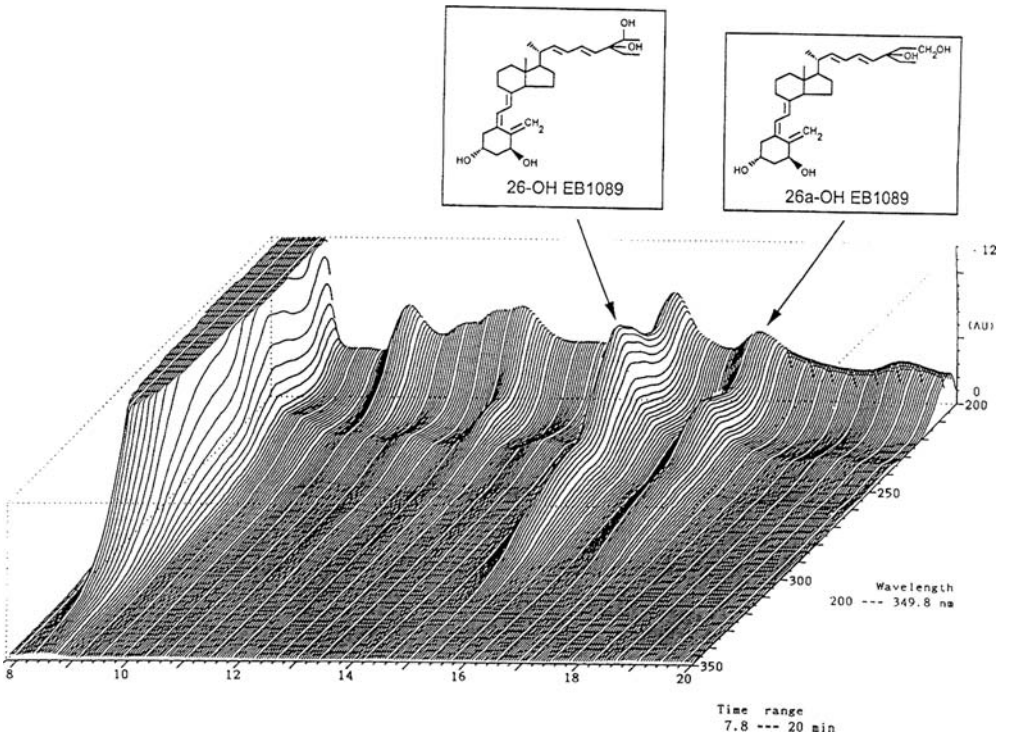


Fig. 10. In vitro metabolism of EB1089 by HPK1A-ras cells. Diode array HPLC of a lipid extract following incubation of EB1089 (10 μM) with the human keratinocyte, HPK1A-ras, for 72 h. In addition to the substrate at 8.5 min, two metabolites showing the distinctive UV chromophore of EB1089 (λ_{max} 235 nm, shoulder 265 nm) are visible in the part of the HPLC profile reproduced here (8–20 min). Metabolite peaks at 15.03 min and 16.55 min were isolated by extensive HPLC and identified *(128, 129)* by comparison to synthetic standards on HPLC, GC-MS and NMR. The identifications are Peak A at 15.03 min= 26-OH-EB1089; Peak B at 16.55 min= 26a-OH-EB1089. The structures of each of the metabolite peaks are depicted in the insets. (Reproduced from *(128)* with permission).

the target for hydroxylation, albeit at a much reduced rate. Diode array spectrophotometry has allowed for the identification of the principal metabolic products of EB1089 as 26- and 26a-hydroxylated metabolites *(128, 129)* (Fig. 10). These metabolites of EB1089 have been chemically synthesized and, as in the case of KH1060, have been shown to retain significant biological activity in cell differentiation and anti-proliferative assays *(129)*.

Again, it is interesting to note that with EB1089 and other molecules blocked in the C-23 and C-24 positions such as 1,24*S*$(OH)_2D_2$ *(73, 92)*, the terminal carbons C-26 and C-26a become the sites of further hydroxylation. However, it should also be considered that even in molecules not blocked in the C-23 and C-24 positions but containing the terminal 26- and 27-dimethyl homologation such as 26,27-dimethyl-1,25$(OH)_2D_3$ (MC1548) *(107)*, there seems to be significant terminal 26a-hydroxylation occurring. Thus, the hydroxylation of EB1089 at C-26 and C-26a may be in part a consequence of the introduction of the conjugated double bond system and only in part a consequence of the introduction of the terminal homologation.

When the C22=C23 double bond is present in the side chain in the absence of a C-24 methyl group, as in calcipotriol, the double bond appears vulnerable to reduction. As pointed out earlier, the *principal metabolites of calcipotriol are reduced in the C22–C23 bond* except for one, the C22=C23 unsaturated, 24-ketone (MC1046) *(98, 99)*. This suggests a C-24 ketone must be present to allow for reduction of the double bond to occur. Work using the Roche compound, Δ^{22}-1,25$(OH)_2D_3$, an analog which contains the C22=C23 double bond but lacks a C-24 substituent, tends to indirectly support this theory *(130)*. When incubated with the chronic myelogenous leukaemic cell line, RWLeu-4, this molecule, like 1,25$(OH)_2D_3$, is converted, presumably via metabolites analogous to intermediates in the C-24 oxidation pathway, to the side chain-truncated product 24,25,26,27-tetranor-1,23$(OH)_2D_3$, a molecule which lacks the C22=C23 double bond.

4. IMPORTANT IMPLICATIONS DERIVED FROM METABOLISM STUDIES

4.1. Relative Importance of Metabolism in the Mechanism of Action of Vitamin D Analogs

There is currently tremendous interest in explaining the mechanism of action of vitamin D analogs at the molecular level, in particular clarification of the difference between "calcaemic" and "non-calcaemic" analogs.

The susceptibility of a specific vitamin D analog to metabolism and excretion undoubtedly plays a significant role in determining the biological activity of the analog in vivo. In practice, the rate of metabolism of an analog can be studied in cultured cells in vitro, and several cell lines from liver or target cell sources are available to act as valuable tools to reflect this process occurring in vivo *(131)*. In the 10 years since the publication of the first edition of this book, all the individual vitamin D-related CYPs (CYP2R1, CYP27A1, CYP27B1, CYP24A1) have been introduced into appropriate expression vectors (for *E. coli*, yeast, insect and mammalian cell expression) to

enable the study of the metabolism of any vitamin D analog reproducibly and outside of the influence of hormonal or ionic regulatory factors *(132)*. In addition, the emergence of CYP24 inhibitors as well as general CYP inhibitors such as ketoconazole *(133, 134)* has opened up the possibility of studying the action of any analog without metabolic complications. Perhaps the ultimate degree of sophistication in current metabolic studies is the use of mice genetically engineered to exhibit over-expression or ablation of any of the vitamin D-related CYPs *(53, 135, 136)*.

Use of the above metabolic approaches allows for the assessment of the relative importance of metabolism within the framework of the full list of biological parameters:

(1) Susceptibility to metabolism.
(2) *Affinity of the analog for plasma DBP* which dictates transport, cell entry and plasma clearance *(137)*.
(3) *Affinity of the analog for target cell VDR–RXR heterodimeric complex* and the resultant affinity of this complex for the VDRE found in the promoter of target genes *(138, 139)*.
(4) Possible *tissue-specific recruitment of downstream coactivators (140)* to the VDR–transactivation complex which might allow for action in cell proliferative or differentiation roles without actions at classical calcium and phosphate homeostatic tissues.

Data for each one of these parameters can be collected in various different ways. Binding assays for DBP and VDR *(137)* and VDRE-mediated transactivation assays have been available for some time now *(138, 139)*. Patterns of coactivator (DRIP) recruitment are now being added in recent analog VDR transcriptional analyses *(140–143, 158)*.

The more detailed studies of the post-VDR steps of the transactivation process are now starting to reveal differences in the affinity of different coactivators for the liganded VDR–RXR heterodimeric complex *(140–145)*. Furthermore, the data are suggestive that analogs which form stable complexes are more active than $1,25(OH)_2D_3$ in vitro. However, taking the reports together no consistent pattern of coactivator recruitment has emerged that might explain analog selectivity on a general scale *(140–145)*.

Rather this author concludes that the application of these assays has shown that none of the parameters, VDR binding, DBP binding *(146, 147)*, recruitment of specific coactivators, transactivation activity *or* rate of metabolism, *when considered separately,* is able to fully explain why some analogs have superior biological activity as compared with $1,25(OH)_2D_3$. However, when these metabolic VDR and DBP parameters *are considered together* for a given analog such as 20-epi-$1,25(OH)_2D_3$ *(106)* as in Fig. 11 then one begins to understand the complexity and the fact that many components might contribute to explain the apparent overall superiority of 20-epi-$1,25(OH)_2D_3$ over $1,25(OH)_2D_3$ observed in gene transactivation models in vitro (Fig. 10d). One might anticipate this complexity to be even greater in vivo when additional pharmacokinetic parameters (e.g. target cell or hepatic clearance) are added to the in vitro picture.

In the whole animal in vivo, pharmacokinetic data can be acquired for each analog which probably reflects more than one of these parameters to different degrees. Pharmacokinetic data reflect the following important parameters including

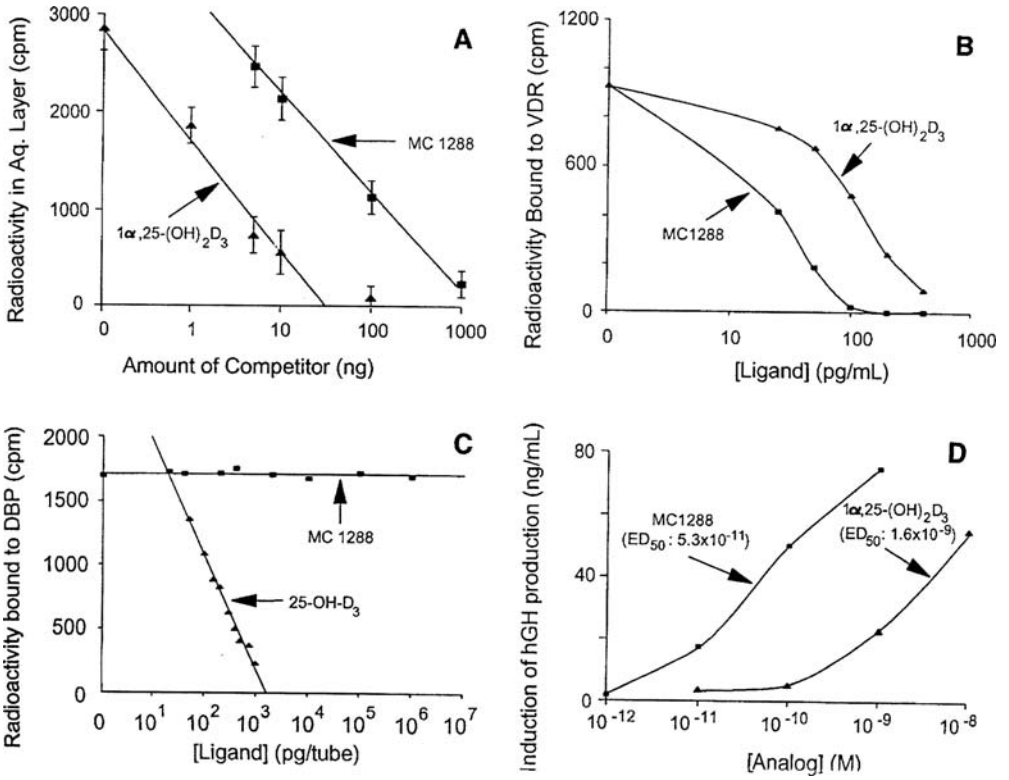


Fig. 11. Biological parameters for 20-epi-1,25$(OH)_2D_3$ (MC1288) **a**: Ability of 20-epi-1, 25$(OH)_2D_3$ to compete for C24-oxidation pathway enzymes. **b**: VDR-binding affinity of 20-epi-1, 25$(OH)_2D_3$ compared to 1,25$(OH)_2D_3$. **c**: DBP-binding affinity of 20-epi-1,25$(OH)_2D_3$ compared to 1,25$(OH)_2D_3$. **d**: Gene transactivation (VDRE placed upstream of GH reporter gene) by 20-epi-1,25$(OH)_2D_3$ as compared to 1,25$(OH)_2D_3$ in the COS-1 cell line. (Reproduced from *(106)* with permission).

(a) The affinity of the vitamin D analog for DBP in the bloodstream.
(b) The rate of target cell uptake and metabolism by target cell enzymes.
(c) The rate of liver cell uptake, hepatic metabolism and biliary clearance.
(d) The rate of storage depot uptake and release.

In the case of some of the analogs shown in Tables 1 and 2, pharmacokinetic data *(81, 109, 110, 128, 129)* are available and can be compared to the data provided by in vitro metabolic studies. It is apparent from perusal of pharmacokinetic and metabolic data that the analogs which we have defined as *metabolically resistant are usually "calcaemic"* analogs and those termed *metabolically sensitive are "non-calcaemic"*. In fact, this classification can be refined along the lines suggested by Kissmeyer et al. *(81)*, all of their compounds segregate into at least two groups (perhaps more) on the basis of their pharmacokinetic parameters:

Calcaemic Analogs (Strong or Weak): Those analogs with a long $t_{1/2}$ which is either a function of strong DBP binding *or* a reduced rate of metabolism (or both). There appear

to be a group of analogs in which a long $t_{1/2}$ is correlated with a slower rate of metabolism (e.g. 26,27-F_6-1,25$(OH)_2D_3$, EB1089, 2-MD and ED-71). With the exception of ED-71, which has a strong affinity for DBP, most of these active analogs bind DBP poorly.

Non-calcaemic Analogs: Those analogs with a short $t_{1/2}$ which is either a function of poor DBP binding *or* a rapid rate of metabolism (or both) (e.g. calcipotriol, KH1060 and OCT).

It should be noted that certain classifications used in the vitamin D literature are somewhat artificial since analogs that are *purely* "non-calcaemic" have not been created. All "non-calcaemic" analogs will stimulate in vitro "calcaemic gene" expression and will eventually cause hypercalcaemia in vivo if their concentration is raised sufficiently. The crucial question to the development of anti-cancer or immune-suppressive analogs is whether systemically administered, "weakly calcaemic" or "non-calcaemic" analogs can (e.g. 1$(OH)D_5$, 19-nor-1,25$(OH)_2D_2$ and BXL-628) produce their anti-cell proliferation/pro-cell differentiation effects in vivo at concentrations lower than that required to produce calcaemia. Various in vivo clinical trials currently underway will be the acid test for this question.

When considering molecular mechanisms of action at the target cell level, metabolism is often disregarded or given too little emphasis. Furthermore, certain invalid metabolic assumptions made during biological activity testing include (i) the analog is biologically active as administered and (ii) the analog is stable in the in vitro target cell model used, whether organ culture, cultured cell or transfected cell. The validity of this approach is made more tenuous *when data acquired with different in vitro models where metabolic considerations may or may not apply are compared to data acquired in vivo where metabolic considerations definitely apply*. Invalid comparisons of in vivo and in vitro data have led to frequent false expectations when taking promising analogs from in vitro assays to pre-clinical animal testing.

In summary, from studies performed thus far, metabolism appears to be one of a number of key parameters which dictates the survival and hence biological activity of the analog when administered topically or systemically in vivo. At this point in time, a pattern is emerging that suggests "non-calcaemic" or "calcaemic" analogs are metabolically sensitive and metabolically resistant, respectively. However, it seems unlikely that permutations of these biochemical parameters will translate into pure "non-calcaemic" and "calcaemic" analogs, but rather therapeutic agents with improved cell-differentiating or calcaemic activities suitable for different applications.

4.2. Future Directions

4.2.1. Structural Aspects of Vitamin D-Related Cytochrome P450s

One of the highlights of the past 10 years has been the emergence of X-ray crystal structures for the ligand-binding domain of VDR, DBP and certain CYPs. The recent over-expression, crystallization and X-ray crystallographic determination of the structure of CYP2R1 will spur the study of the mitochondrial vitamin D-related CYPs 24A1, 27A1 and 27B1. As alluded to earlier, mutagenesis studies of human CYP24A1

have already revealed the important information that explains why some species synthesize calcitroic acid via a 24-hydroxylation pathway and others 26,23-lactones via a 23-hydroxylation pathway *(54)*. This study has also revealed that computer homology modelling of the mitochondrial CYPs has also reached a new level of sophistication that has allowed the prediction of active site lining residues *(54, 148)*. One anticipates that emerging polymorphisms of any of the human vitamin D-related CYPs will be tested through such in vitro expression systems such as the Chinese hamster lung fibroblast cell line, V-79 and rationalized using these new CYP structures and computer models.

The complex regulation of the 1α-hydroxylase (CYP27B1) in kidney and extra-renal sites by PTH, FGF23, $1,25(OH)_2D_3$, IFN-γ and potentially other factors is of central interest to physiologists trying to understand the role of CYP27B1 around the body *(27, 28)*. Over the coming years, the relative importance of the endocrine $1,25(OH)_2D_3$ produced by the kidney CYP27B1 and the paracrine/autocrine $1,25(OH)_2D_3$ produced by the extra-renal CYP27B1 should emerge. Perhaps equally important will be to clarify the roll of the cell membrane receptors megalin/cubilin in the uptake of 25-OH-D_3 bound to its DBP carrier into vitamin D target cells. The fact that the megalin knockout mouse *(149)* suffers from vitamin D deficiency supports the view that a special mechanism exists in the kidney and there is evidence that extra-renal tissues also need to express this receptor protein in order to concentrate and metabolize $25(OH)D_3$ *(150)*.

4.2.2. Hydroxylase Gene Knockouts

The advent of the genetically engineered mouse has opened up new possibilities of studying the influence of metabolism on the biological activity of an analog within the in vivo context. In particular, the CYP24A1 knockout mouse *(53, 60, 61)* offers the advantage of comparing biological activity or alternate catabolism of a particular analog in the presence and absence of CYP24A1. Despite the availability of this CYP24A1-null model, few analogs except $1,25(OH)_2D_3$ have been studied to date in this way, though we might expect more in the years to come.

Since the first edition of this book, the emergence of CYP2R1 as the best candidate for physiologically relevant 25-hydroxylase *(14)* also opens up the possibility of engineering a CYP2R1 null mouse and testing the veracity of this hypothesis in a whole animal context in the near future. There has been no shortage of other 25-hydroxylases, i.e. CYP27A1, CYP3A4, CYP2J1/2, so that the deletion of CYP2R1 will allow us to observe the degree of redundancy among these liver-related CYPs. One might predict from various kinetic data for these alternative liver CYPs that none is able to operate effectively at the nanomolar concentrations of substrate found in vivo. The study of the molecular structure, properties and regulation of vitamin D-related cytochrome P450s will continue over the coming decade.

4.2.3. Future Vitamin D Analog Design and Development

The success of vitamin D analogs in a variety of clinical applications, particularly chronic kidney disease, hypocalcaemic conditions and psoriasis, will continue to fuel interest in the development of more effective vitamin D analogs for use in these

conditions as well as in osteoporosis, cancer and immunosuppression. Over the immediate future we can anticipate (1) a continuing search for novel synthetic modifications to the vitamin D molecule (e.g. 2-MD); (2) combination of "useful" modifications in order to fine-tune the best analogs (e.g. BXL-628); (3) synthesis of coactivator-specific molecules based on emerging transactivation and structure–activity information gained from earlier generations of molecules; (4) clinical testing of a new class of vitamin D analogs, the CYP24-inhibitors which have already been used with promising results in Phase 11B psoriasis and are also going into Phase 11B for testing in the treatment of secondary hyperparathyroidism associated with chronic kidney disease.

In particular, one can envision that VDR ligand-binding pocket studies and cytochrome P450 substrate-binding pocket studies just beginning to emerge will provide valuable information for the design of further generations of non-steroidal vitamin D analogs. With the application of molecular modelling techniques to the study of vitamin D-related proteins for the first time, we will be in the position to view the fit of vitamin D analog to active site topology and fine-tune this fit. The future of vitamin D metabolism remains bright while the pursuit of novel vitamin D analogs continues to be attractive to the pharmaceutical industry.

ACKNOWLEDGEMENTS

The author wishes to acknowledge the help of F. Jeffrey Dilworth and David Prosser in assembly of the tables and figures contained within this document. Some of the work cited here is supported through grants to the author from the Medical Research Council of Canada/Canadian Institutes of Health Research. Over the past 20 years, the research described here has involved some excellent research trainees: David Lohnes, Fuad Qaw, Stephen Strugnell, Sonoko Masuda, Jeffrey Dilworth, VN Shankar, David Prosser and Martin Kaufmann as well as the interdisciplinary collaborations from talented scientists from around the world from Hugh LJ Makin, Martin Calverley, Joyce Knutson, Charles Bishop, Noboru Kubodera, Anne-Marie Kissmeyer, Richard Kremer, Rene St-Arnaud, Mark R. Haussler and Hector F.DeLuca. I sincerely thank them all.

REFERENCES

1. Holick MF (1995) Vitamin D: Photobiology, Metabolism and Clinical Applications. In: Degroot L (ed) Endocrinology, vol 2, 3rd edn. Saunders, Philadelphia, 990–1014
2. Bhattacharyya MH, DeLuca HF (1973) The regulation of the rat liver calciferol-25-hydroxylase. J Biol Chem 248:2969–2973
3. Andersson S, Davis DL, Dahlback H, Jornvall H, Russell DW (1989) Cloning, structure and expression of the mitochondrial cytochrome P450 sterol 26-hydroxylase, a bile acid biosynthetic enzyme. J Biol Chem 246:8222–8229
4. Okuda KI, Usui E, Ohyama Y (1995) Recent progress in enzymology and molecular biology of enzymes involved in vitamin D metabolism. J Lipid Res 36:1641–1652
5. Guo Y-D, Strugnell S, Back DW, Jones G (1993) Transfected human liver cytochrome P-450 hydroxylates vitamin D analogs at different side-chain positions. Proc Natl Acad Sci USA 90: 8668–8672
6. Fukushima M, Suzuki Y, Tohira Y, Nishii Y, Suzuki M, Sasaki S, Suda T (1976) 25-Hydroxylation of 1α-hydroxyvitamin D_3 in vivo and in the perfused rat liver. FEBS Lett 65:211–214

7. Baran DT, Milne ML (1986) 1,25-Dihydroxyvitamin D increases hepatocyte cytosolic calcium levels: a potential regulator of vitamin D-25-hydroxylase. J Clin Invest 77:1622–1626
8. Berginer VM, Shany S, Alkalay D, Berginer J, Dekel S, Salen G, Tint GS, Gazit D (1993) Osteoporosis and increased bone fractures in cerebrotendinous xanthomatosis. Metabolism 42:69–74
9. Tucker G, Gagnon RE, Haussler MR (1973) Vitamin D_3-25-hydroxylase: tissue occurrence and lack of regulation. Arch Biochem Biophys 155:47–57
10. Axen E, Postlind H, Wikvall K (1995) Effects of CYP27 mRNA expression in rat kidney and liver by 1α,25-dihydroxyvitamin D_3, a suppressor of renal 25-hydroxyvitamin D_3-1α-hydroxylase activity. Biochem Biophys Res Commun 215:136–141
11. Ichikawa F, Sato K, Nanjo M, Nishii Y, Shinki T, Takahashi N, Suda T (1995) Mouse primary osteoblasts express vitamin D_3 25-hydroxylase mRNA and convert 1α-hydroxyvitamin D_3 into 1α,25-dihydroxyvitamin D_3. Bone 16:129–135
12. Cheng JB, Motola DL, Mangelsdorf DJ, Russell DW (2003) Deorphanization of cytochrome P450 2R1: a microsomal vitamin D 25-hydroxylase. J Biol Chem 278:38084–38093
13. Cheng JB, Levine MA, Bell NH, Mangelsdorf DJ, Russell DW (2004) Genetic evidence that the human CYP2R1 enzyme is a key vitamin D 25-hydroxylase. Proc Natl Acad Sci U S A 101: 7711–7715
14. Jones G, Byford V, West S, Masuda S, Ibrahim G, Kaufmann M, Knutson J, Strugnell S, Mehta R (2006) Hepatic activation & inactivation of clinically-relevant vitamin D analogs and prodrugs. Anticancer Res 26:2589–2596
15. Strushkevich N, Usanov SA, Plotnikov AN, Jones G, Park H-W (2008) Structural analysis of CYP2R1 in complex with vitamin D_3. J Mol Biol 380:95–106
16. St-Arnaud R, Messerlian S, Moir JM, Omdahl JL, Glorieux FH (1997) The 25-hydroxyvitamin D 1α-hydroxylase gene maps to the pseudovitamin D-deficiency rickets (PDDR) disease locus. J Bone Miner Res 12:1552–1559
17. Takeyama K-I, Kitanaka S, Sato T, Kobori M, Yanagisawa J, Kato. S (1997) 25-Hydroxyvitamin D_3 1α-hydroxylase & vitamin D synthesis. Science 277:1827–1830
18. Monkawa T, Yoshida T, Wakino S, Shinki T, Anazawa H, DeLuca HF, Suda T, Hayashi M, Saruta T (1997) Molecular cloning of cDNA and genomic DNA for human 25-hydroxyvitamin D_3 1α-hydroxylase. Biochem Biophys Res Commun 239:527–533
19. Fu GK, Lin D, Zhang MY, Bikle DD, Shackleton CH, Miller WL, Portale AA (1997) Cloning of human 25-hydroxyvitamin D-1α-hydroxylase and mutations causing vitamin D dependent rickets type 1. Mol Endocrinol 11:1961–1970
20. Fu GK, Portale AA, Miller WL (1997) Complete structure of the human gene for the vitamin D 1α-hydroxylase, P450c1. DNA Cell Biol 16:1499–1507
21. Gray RW, Omdahl JL, Ghazarian JG, DeLuca HF (1972) 25-Hydroxycholecalciferol-1-Hydroxylase: subcellular location and properties. J Biol Chem 247:7528–7532
22. Henry HL (1979) Regulation of the hydroxylation of 25-hydroxyvitamin D_3 in vivo and in primary cultures of chick kidney cells. J Biol Chem 254:2722–2729
23. Stubbs J, Liu S, Quarles LD (2007) Role of fibroblast growth factor 23 in phosphate homeostasis and pathogenesis of disordered mineral metabolism in chronic kidney disease. Semin Dial 20:302–308
24. Brenza HL, Kimmel-Jehan C, Jehan F, Shinki T, Wakino S, Anazawa H, Suda T, DeLuca HF (1998) Parathyroid hormone activation of the 25-hydroxyvitamin D_3-1α-hydroxylase gene promoter. Proc Natl Acad Sci USA 95:1387–1391
25. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Hosoya T, Kato S (1998) The promoter of the human 25-hydroxyvitamin D_3 1α-hydroxylase gene confers positive and negative responsiveness to PTH, calcitonin, and 1α,25$(OH)_2D_3$. Biochem Biophys Res Commun 249:11–16
26. Kitanaka S, Takeyama K, Murayama A, Sato T, Okumura K, Nogami M, Hasegawa Y, Niimi H, Yanigisawa J, Tanaka T, Sato K (1998) Inactivating mutations in the 25-hydroxyvitamin D_3-1α-hydroxylase gene in patients with pseudovitamin D deficiency rickets. N Engl J Med 338:653–661
27. Jones G (2007) Expanding role for vitamin D in chronic kidney disease: Importance of blood 25-OH-D levels & extra-renal 1α-hydroxylase in the classical and non-classical actions of 1α,25-dihydroxyvitamin D_3. Semin Dial 20:316–324

28. Holick MF (2007) Vitamin D deficiency. N Engl J Med 357:266–281
29. Lester GE, Gray TK, Williams ME (1981) In vitro 1α-hydroxylation of ^{3}H-25-hydroxyvitamin D_3 by isolated cells from rat kidneys and placentae. In: Cohn DV, Talmage RV, Matthews JL (eds) Hormonal control of calcium metabolism. Excerpta Medica, Amsterdam, 376
30. Adams JS, Gacad MA (1985) Characterization of 1α-hydroxylation of vitamin D_3 sterols by cultured alveolar macrophages from patients with sarcoidosis. J Exp Med 161:755–765
31. Hewison M, Burke F, Evans KN, Lammas DA, Sansom DM, Liu P, Modlin RL, Adams JS (2007) Extra-renal 25-hydroxyvitamin D_3-1α-hydroxylase in human health and disease. J Steroid Biochem Mol Biol 103:316–321
32. Stoffels K, Overbergh L, Giulietti A, Verlinden L, Bouillon R, Mathieu C (2006) Immune regulation of 25-hydroxyvitamin-D_3-1α-hydroxylase in human monocytes. J Bone Miner Res 21: 37–47
33. Jones G, Ramshaw H, Zhang A, Cook R, Byford V, White J, Petkovich M (1998) Expression and activity of vitamin D-metabolizing cytochrome P450 s (CYP1α & CYP24) in human non-small cell lung carcinomas. Endocrinology 140:3303–3310
34. Bises G, Kállay E, Weiland T, Wrba F, Wenzl E, Bonner E, Kriwanek S, Obrist P, Cross HS (2004) 25-hydroxyvitamin D_3-1α-hydroxylase expression in normal and malignant human colon. J Histochem Cytochem 52:985–989
35. Chen TC, Wang L, Whitlatch LW, Flanagan JN, Holick MF (2003) Prostatic 25-hydroxyvitamin D-1α-hydroxylase and its implication in prostate cancer. J Cell Biochem 88:315–322
36. Holick MF, Schnoes HK, DeLuca HF, Gray RW, Boyle IT, Suda T (1972) Isolation and identification of 24,25-dihydroxycholecalciferol: A metabolite of vitamin D_3 made in the kidney. Biochemistry 11:4251–4255
37. Holick MF, Kleiner-Bossaller A, Schnoes HK, Kasten PM, Boyle IT, DeLuca HF (1973) 1,24,25-Trihydroxyvitamin D_3. A metabolite of vitamin D_3 effective on intestine. J Biol Chem 248: 6691–6696
38. Ornoy A, Goodwin D, Noff D, Edelstein S (1978) 24,25-Dihydroxyvitamin D is a metabolite of vitamin D essential for bone formation. Nature 276:517–519
39. Rasmussen H, Bordier P (1978) Vitamin D & bone. Metab Bone Dis Rel Res 1:7–13
40. Henry HL, Norman AW (1978) Vitamin D: two dihydroxylated metabolites are required for normal chicken egg hatchability. Science 201:835–837
41. Jones G, Vriezen D, Lohnes D, Palda V, Edwards NS (1987) Side chain hydroxylation of vitamin D_3 and its physiological implications. Steroids 49:29–55
42. Brommage R, Jarnagin K, DeLuca HF, Yamada S, Takayama H (1983) 1- but not 24-hydroxylation of vitamin D is required for skeletal mineralization in rats. Am J Physiol 244:E298–E304
43. Makin G, Lohnes D, Byford V, Ray R, Jones G (1989) Target cell metabolism of 1,25-dihydroxyvitamin D_3 to calcitroic acid. Evidence for a pathway in kidney and bone involving 24-oxidation. Biochem J 262:173–180
44. Reddy GS, Tserng K-Y (1989) Calcitroic acid, end product of renal metabolism of 1,25-dihydroxyvitamin D_3 through C-24 oxidation pathway. Biochemistry 28:1763–1769
45. Esvelt RP, Schnoes HK, DeLuca HF (1979) Isolation and characterization of 1α-hydroxy-23-carboxytetranorvitamin D: a major metabolite of 1,25-dihydroxyvitamin D_3. Biochemistry 18: 3977–3983
46. Knutson JC, DeLuca HF (1974) 25-Hydroxyvitamin D_3-24-hydroxylase. Subcellular location and properties. Biochemistry 13:1543–1548
47. Ohyama Y, Noshiro M, Okuda K (1991) Cloning and expression of cDNA encoding 25-hydroxyvitamin D_3 24-hydroxylase. FEBS Lett 278:195–198
48. Ohyama Y, Okuda K (1991) Isolation and characterization of a cytochrome P450 from rat kidney mitochondria that catalyzes the 24-hydroxylation of 25-hydroxyvitamin D3. J Biol Chem 266: 8690–8695
49. Tomon M, Tenenhouse HS, Jones G (1990) Expression of 25-hydroxyvitamin D_3-24-hydroxylase activity in CaCo-2 cells. An In Vitro model of intestinal vitamin D catabolism. Endocrinology 126:2868–2875

50. Suda T, DeLuca HF, Schnoes HK, Tanaka Y, Holick MF (1970) 25,26-dihydroxyvitamin D_3, a metabolite of vitamin D_3 with intestinal transport activity. Biochemistry 9:4776–4780
51. Horst RL (1979) 25-OH-D_3-26,23-Lactone: a metabolite of vitamin D_3 that is 5 times more potent than 25-OH-D_3 in the rat plasma competitive protein binding radioassay. Biochem Biophys Res Commun 89:286–293
52. Yamada S, Nakayama K, Takayama H, Shinki T, Takasaki Y, Suda T (1984) Isolation, identification and metabolism of (23S,25R)-25-hydroxyvitamin D_3-26,23-lactol: a biosynthetic precursor of (23S,25R)-25-hydroxyvitamin D_3-26,23-lactone. J Biol Chem 259:884–889
53. Masuda S, Byford V, Arabian A, Sakai Y, Demay MB, St-Arnaud R, Jones G (2005) Altered Pharmacokinetics of 1α,25-dihydroxyvitamin D_3 and 25-hydroxyvitamin D_3 in the blood and tissues of the 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) null mouse. Endocrinology 146: 825–834
54. Prosser D, Kaufmann M, O'Leary B, Byford V, Jones G (2007) Single A326G mutation converts hCYP24A1 from a 25-OH-D_3-24-hydroxylase into -23-hydroxylase generating 1α,25-$(OH)_2D_3$-26,23-lactone. Proc Natl Acad Sci USA 104:12673–12678
55. Ishizuka S, Ishimoto S, Norman AW (1984) Isolation and identification of 1α,25-dihydroxy-24-oxovitamin D_3, 1α,25-dihydroxyvitamin D_3-26,23-lactone, 1α,24(S),25-trihydroxy-vitamin D_3: in vivo metabolites of 1α,25-dihydroxyvitamin D_3. Biochemistry 23:1473–1478
56. Toell A, Gonzalez MM, Ruf D, Steinmeyer A, Ishizuka S, Carlberg C (2001) Different molecular mechanisms of vitamin D_3 receptor antagonists. Mol Pharmacol 59:1478–1485
57. Ishizuka S, Kurihara N, Reddy SV, Cornish J, Cundy T, Roodman GD (2005) (23S)-25-Dehydro-1α-hydroxyvitamin D_3-26,23-lactone, a vitamin D receptor antagonist that inhibits osteoclast formation and bone resorption in bone marrow cultures from patients with Paget's disease. Endocrinology 146:2023–2030
58. Shinki T, Jin CH, Nishimura A, Nagai Y, Ohyama Y, Noshiro M, Okuda K, Suda T (1992) Parathyroid hormone inhibits 25-hydroxyvitamin D_3-24-hydroxylase mRNA expression stimulated by 1α,25-dihydroxyvitamin D_3 in rat kidney but not in intestine. J Biol Chem 267:13757–13762
59. Lohnes D, Jones G (1992) Further metabolism of 1α,25-dihydroxyvitamin D_3 in target cells. J Nutr Sci Vitaminol Special Issue:75–78
60. St-Arnaud R, Arabian A, Travers R, Glorieux FH (1997) Abnormal intramembranous ossification in mice deficient for the vitamin D 24-hydroxylase gene. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D. Chemistry, biology and clinical applications of the steroid hormone. Vitamin D Workshop Inc., Riverside, CA, pp 635–639
61. St-Arnaud R, Arabian A, Travers R, Glorieux FH (1997) Partial rescue of abnormal bone formation in 24-hydroxylase knock-out mice supports a role for 24,25-$(OH)_2D_3$ in intramembranous ossification. J Bone Miner Res 12:33 (abstract S111)
62. Hashizume T, Xu Y, Mohutsky MA, Alberts J, Hadden C, Kalhorn TF, Isoherranen N, Shuhart MC, Thummel KE (2008) Identification of human UDP-glucuronosyltransferases catalyzing hepatic 1α,25-dihydroxyvitamin D_3 conjugation. Biochem Pharmacol 75:1240–1250
63. Esvelt RP, DeLuca HF (1980) Calcitroic acid: biological activity and tissue distribution studies. Arch Biochem Biophys 206:404–413
64. Suda T, DeLuca HF, Schnoes HK, Blunt JW (1969) Isolation and identification of 25-hydroxyergocalciferol. Biochemistry 8:3515–3520
65. Jones G, Schnoes HK, DeLuca HF (1975) Isolation and identification of 1,25-dihydroxyvitamin D_2. Biochemistry 14:1250–1256
66. Jones G, Rosenthal A, Segev D, Mazur Y, Frolow F, Halfon Y, Rabinovich D, Shakked Z (1979) Isolation and identification of 24,25-dihydroxyvitamin D_2 using the perfused rat kidney. Biochemistry 18:1094–1101
67. Jones G, Schnoes HK, Levan L, DeLuca HF (1980) Isolation and identification of 24-hydroxyvitamin D_2 and 24,25-dihydroxyvitamin D_2. Arch Biochem Biophys 202:450–457
68. Horst RL, Koszewski NJ, Reinhardt TA (1990) 1α-Hydroxylation of 24-hydroxyvitamin D_2 represents a minor physiological pathway for the activation of vitamin D_2 in mammals. Biochemistry 29:578–582

69. Koszewski NJ, Reinhardt TA, Napoli JL, Beitz DC, Horst RL (1988) 24,26-Dihydroxyvitamin D_2: a unique physiological metabolite of vitamin D_2. Biochemistry 27:5785–5790
70. Reddy GS, Tserng K-Y (1986) Isolation and identification of 1,24,25-trihydroxyvitamin D_2, 1,24,25,28-tetrahydroxyvitamin D_2, 1,24,25,26-tetrahydroxyvitamin D_2: new metabolites of 1,25-dihydroxyvitamin D_2 produced in the rat kidney. Biochemistry 25:5328–5336
71. Clark JW, Reddy GS, Santos-Moore A, Wankadiya KF, Reddy GP, Lasky S, Tserng K-Y, Uskokovic MR (1993) Metabolism and biological activity of 1,25-dihydroxyvitamin D_2 and its metabolites in a chronic myelogenous leukemia cell line, RWLEU-4. Bioorg Med Chem Lett 3:1873–1878
72. Masuda S, Strugnell S, Knutson JC, St-Arnaud R, Jones G (2006) Evidence for the activation of 1α-hydroxyvitamin D_2 by 25-hydroxyvitamin D-24-hydroxylase: delineation of pathways involving 1α,24-dihydroxyvitamin D_2 & 1α,25-dihydroxyvitamin D_2. Biochim Biophys Acta (Mol Cell Biol Lipids) 1761:221–234
73. Jones G, Byford V, Kremer R, Makin HLJ, Rice RH, deGraffenreid LA, Knutson JC, Bishop CA (1996) Anti-proliferative activity and target cell catabolism of the vitamin D analog, 1α,24(S)-dihydroxyvitamin D_2 in normal and immortalized human epidermal cells. Biochem Pharmacol 52:133–140
74. Suda T, Hallick RB, DeLuca HF, Schnoes HK (1970) 25-hydroxydihydrotachysterol$_3$ Synthesis and biological activity. Biochemistry 9:1651–1657
75. Bosch R, Versluis C, Terlouw JK, Thijssen JHH, Duursma SA (1985) Isolation and identification of 25-hydroxydihydrotachysterol$_2$, 1α,25-dihydroxydihydrotachysterol$_2$ and 1β,25-dihydroxydihydrotachysterol$_2$. J Steroid Biochem 23:223–229
76. Jones G, Edwards N, Vriezen D, Porteous C, Trafford DJH, Cunningham J, Makin HLJ (1988) Isolation and identification of seven metabolites of 25-hydroxy-dihydrotachysterol$_3$ formed in the isolated perfused rat kidney: A model for the study of side-chain metabolism of vitamin D. Biochemistry 27:7070–7079
77. Qaw F, Calverley MJ, Schroeder NJ, Trafford DJH, Makin HLJ, Jones G (1993) *In vivo* metabolism of the vitamin D analog, dihydrotachysterol. Evidence for formation of 1α,25- and 1β,25-dihydroxydihydrotachysterol metabolites and studies of their biological activity. J Biol Chem 268:282–292
78. Bhattacharyya MH, DeLuca HF (1973) Comparative studies on the 25-hydroxylation of vitamin D_3 and dihydrotachysterol$_3$. J Biol Chem 248:2974–2977
79. Wing RM, Okamura WH, Pirio MP, Sine SM, Norman AW (1974) Vitamin D in solution: conformations of vitamin D_3, 1,25-dihydroxyvitamin D_3 and dihydrotachysterol$_3$. Science 186: 939–941
80. Shany S, Ren S-Y, Arbelle JE, Clemens TL, Adams JS (1993) Subcellular localization and partial purification of the 25-hydroxyvitamin D-1-hydroxylation reaction in the avian myelomonocytic cell line HD-11. J Bone Miner 8:269–276
81. Qaw F, Schroeder NJ, Calverley MJ, Maestro M, Mourino A, Trafford DJH, Makin HLJ, Jones G (1992). In vitro synthesis of 1,25-dihydroxydihydrotachysterol in the myelomonocytic cell line, HD-11. J Bone Miner Res 7:S161 (Abstract 274)
82. Qaw FS, Makin HLJ, Jones G (1992) Metabolism of 25-hydroxy-dihydrotachysterol$_3$ in bone cells *in vitro*. Steroids 57:236–243
83. Schroeder NJ, Qaw F, Calverley MJ, Trafford DJH, Jones G, Makin HLJ (1992) Polar metabolites of dihydrotachysterol$_3$ in the rat: Comparison with *in vitro* metabolites of 1α,25-dihydroxy dihydrotachysterol$_3$. Biochem Pharm 43:1893–1905
84. Holick MF, Semmler E, Schnoes HK, DeLuca HF (1973) 1α-Hydroxy derivative of vitamin D_3: a highly potent analog of 1α,25-dihydroxyvitamin D_3. Science 180:190–191
85. Barton DH, Hesse RH, Pechet MM, Rizzardo E (1973) A convenient synthesis of 1α-hydroxy-vitamin D_3. J Am Chem Soc 95:2748–2749
86. Fraser DR, Kodicek E (1970) Unique biosynthesis by kidney of a biologically active vitamin metabolite. Nature 228:764–766
87. Gallagher JC, Goldgar D (1990) Treatment of postmenopausal osteoporosis with high doses of synthetic calcitriol. A randomized control study. Ann Intern Med 113:649–655

88. Lam HY, Schnoes HK, DeLuca HF (1974) 1α-Hydroxyvitamin D_2: a potent synthetic analog of vitamin D_2 . Science 186:1038–1040
89. Paaren HE, Hamer DE, Schnoes HK, DeLuca HF (1978) Direct C-1 hydroxylation of vitamin D compounds: convenient preparation of 1α-hydroxyvitamin D_3, 1α,25-dihydroxyvitamin D_3 and 1α-hydroxyvitamin D_2. Proc Natl Acad Sci USA 75:2080–2081
90. Gallagher JC, Bishop CW, Knutson JC, Mazess RB, DeLuca HF (1994) Effects of increasing doses of 1α-hydroxyvitamin D_2 on calcium homeostasis in post-menopausal osteopenic women. J Bone Miner Res 9:607–614
91. Tan AU Jr, Levine BS, Mazess RB, Kyllo DM, Bishop CW, Knutson JC, Kleinman KS, Coburn JW (1997) Effective suppression of parathyroid hormone by 1α-hydroxy-vitamin D_2 in hemodialysis patients with moderate to severe secondary hyperparathyroidism. Kidney Int 51:317–323
92. Strugnell S, Byford V, Makin HLJ, Moriarty RM, Gilardi R, LeVan LW, Knutson JC, Bishop CW, Jones G (1995) 1α,24(*S*)-dihydroxyvitamin D_2: A biologically active product of 1α-hydroxyvitamin D_2 made in the human hepatoma, Hep3B. Biochem J 310:233–241
93. Sjoden G, Smith C, Lindgren V, DeLuca HF (1985) 1α-hydroxyvitamin D_2 is less toxic than 1α-hydroxyvitamin D_3 in the rat. Proc Soc Exp Biol Med 178:432–436
94. Calverley MJ (1987) Synthesis of MC-903, a biologically active vitamin D metabolite analog. Tetrahedron 43:4609–4619
95. Kragballe K, Gjertsen BT, De Hoop D, Karlsmark T, van de Kerkhof PC, Larko O, Nieboer C, Roed-Petersen J, Strand A, Tikjob G (1991) Double-blind, right/left comparison of calcipotriol and betamethasone valerate in treatment of psoriasis vulgaris. Lancet 337:193–196
96. Jones G, Calverley MJ (1993) A dialogue on analogues: newer vitamin-D drugs for use in bone disease, psoriasis, and cancer. Trends Endocrinol Metab 4:297–303
97. Binderup L (1988) MC903 - A novel vitamin D analogue with potent effects on cell proliferation and cell differentiation. In: Norman AW, Schaefer K, Grigoleit H-G, von Herrath D (eds) Vitamin D. Molecular, cellular and clinical endocrinology. De Gruyter, Berlin, pp 300–309
98. Sorensen H, Binderup L, Calverley MJ, Hoffmeyer L, Rastrup Anderson N (1990) *In vitro* metabolism of calcipotriol (MC 903), a vitamin D analogue. Biochem Pharmacol 39:391–393
99. Masuda S, Strugnell S, Calverley MJ, Makin HLJ, Kremer R, Jones G (1994) In vitro metabolism of the anti-psoriatic vitamin D analog, calcipotriol, in two cultured human keratinocyte models. J Biol Chem 269:4794–4803
100. Murayama E, Miyamoto K, Kubodera N, Mori T, Matsunaga I (1986) Synthetic studies of vitamin D analogues. VIII. Synthesis of 22-oxavitamin D_3 analogues. Chem Pharm Bull (Tokyo) 34:4410–4413
101. Hansen K, Calverley MJ, Binderup L (1991) Synthesis and biological activity of 22-oxa vitamin D analogues. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D: gene regulation, structure-function analysis and clinical application. De Gruyter, Berlin, pp 161–162
102. Brown AJ, Berkoben M, Ritter C, Kubodera N, Nishii Y, Slatopolsky E (1992) Metabolism of 22-oxacalcitriol by a vitamin D-inducible pathway in cultured parathyroid cells. Biochem Biophys Res Commun 189:759–764
103. Bikle DD, Abe-Hashimoto J, Su MJ, Felt S, Gibson DFC, Pillai S (1995) 22-Oxa-calcitriol is a less potent regulator of keratinocyte proliferation and differentiation due to decreased cellular uptake and enhanced catabolism. J Invest Dermatol 105:693–698
104. Masuda S, Byford V, Kremer R, Makin HLJ, Kubodera N, Nishii Y, Okazaki A, Okano T, Kobayashi T, Jones G (1996) *In vitro* metabolism of the vitamin D analog, 22-oxacalcitriol, using cultured osteosarcoma, hepatoma and keratinocyte cell lines. J Biol Chem 271:8700–8708
105. Kobayashi T, Tsugawa N, Okano T, Masuda S, Takeuchi A, Kubodera N, Nishii Y (1994) The binding properties with blood proteins and tissue distribution of 22-oxa-1α,25-dihydroxyvitamin D_3, a noncalcemic analogue of 1α,25-dihydroxyvitamin D_3 in rats. J Biochem 115:373–380
106. Dilworth FJ, Calverley MJ, Makin HLJ, Jones G (1994) Increased biological activity of 20-epi-1,25-dihydroxyvitamin D_3 is due to reduced catabolism and altered protein binding. Biochem Pharmacol 47:987–993
107. Dilworth FJ, Scott I, Green A, Strugnell S, Guo Y-D, Roberts EA, Kremer R, Calverley MJ, Makin HLJ, Jones G (1995) Different mechanisms of hydroxylation site selection by liver and kidney

cytochrome P450 species (CYP27 and CYP24) involved in vitamin D metabolism. J Biol Chem 270:16766–16774
108. Kissmeyer A-M, Mathiasen IS, Latini S, Binderup L (1995) Pharmacokinetic studies of vitamin D analogues: relationship to vitamin D binding protein (DBP). Endocrine 3:263–266
109. Dilworth FJ, Williams GR, Kissmeyer A-M, Løgsted-Nielsen J, Binderup E, Calverley MJ, Makin HLJ, Jones G (1997) The vitamin D analog, KH1060 is rapidly degraded both in vivo and in vitro via several pathways: principal metabolites generated retain significant biological activity. Endocrinology 138:5485–5496
110. Rastrup-Anderson N, Buchwald FA, Grue-Sorensen G (1992) Identification and synthesis of a metabolite of KH1060, a new potent 1α,25-dihydroxyvitamin D_3 analogue. Bioorg Med Chem Lett 2:1713–1716
111. Van Den Bemd GJ-CM, Dilworth FJ, Makin HLJ, Prahl JM, DeLuca HF, Jones G, Pols HAP, Van Leeuwen JPTM (2000) Contribution of several metabolites of the vitamin D analog 20-epi-22-oxa-24a,26a,27a-trihomo-1,25-$(OH)_2$vitamin D_3 (KH1060) to the overall biological activity of KH1060 by a shared mechanism of action. Biochem Pharmacol 59:621–627
112. Kobayashi Y, Taguchi T, Mitsuhashi S, Eguchi T, Ohshima E, Ikekawa N (1982) Studies on organic fluorine compounds. XXXIX. Studies on steroids. LXXIX. Synthesis of 1α,25-dihydroxy-26,26,26,27,27,27-hexaflurovitamin D_3. Chem Pharm Bull (Tokyo) 30:4297–4303
113. Koeffler HP, Armatruda T, Ikekawa N, Kobayashi Y, DeLuca HF (1984) Induction of macrophage differentiation of human normal and leukemic myeloid stem cells by 1α,25-dihydroxyvitamin D_3 and its fluorinated analogs. Cancer Res 44:6524–6528
114. Inaba M, Okuno S, Nishizawa Y, Yukioka K, Otani S, Matsui-Yuasa I, Morisawa S, DeLuca HF, Morii H (1987) Biological activity of fluorinated vitamin D analogs at C-26 and C-27 on human promyelocytic leukemia cells, HL-60. Arch Biochem Biophys 258:421–425
115. Kistler A, Galli B, Horst R, Truitt GA, Uskokovic MR (1989) Effects of vitamin D derivatives on soft tissue calcification in neonatal and calcium mobilization in adult rats. Arch Toxicol 63: 394–400
116. Inaba M, Okuno S, Nishizawa Y, Imanishi Y, Katsumata T, Sugata I, Morii H (1993) Effect of substituting fluorine for hydrogen at C-26 and C-27 on the side chain of 1α,25-dihydroxyvitamin D_3. Biochem Pharmacol 45:2331–2336
117. Sasaki H, Harada H, Hanada Y, Morino H, Suzawa M, Shimpo E, Katsumata T, Masuhiro Y, Matsuda K, Ebihara K, Ono T, Matsushige S, Kato S (1995) Transcriptional activity of a fluorinated vitamin D analog on VDR-RXR-Mediated gene suppression. Biochemistry 34:370–377
118. Nakatsuka K, Imanishi Y, Morishima Y, Sekiya K, Sasao K, Miki T, Nishizawa Y, Katsumata T, Nagata A, Murakawa S (1992) Biological potency of a fluorinated vitamin D analogue in hypoparathyroidism. Bone Miner 16:73–81
119. Nishizawa Y, Morii H, Ogura Y, DeLuca HF (1991) Clinical trial of 26,26,26,27,27,27-hexafluro-1α,25-dihydroxyvitamin D_3 in uremic patients on hemodialysis: preliminary report. Contrib Nephrol 90:196–203
120. Zhou J-Y, Norman AW, Chen D-L, Sun G, Uskokovic M, Koeffler HP (1990) 1,25-Dihydroxy-16-ene-23-yne-vitamin D_3 prolongs survival time of leukemic mice. Proc Natl Acad Sci USA 87: 3929–3932
121. Binderup E, Calverley MJ, Binderup L (1991) Synthesis and biological activity of 1α-hydroxylated vitamin D analogues with poly-unsaturated side chains. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D: gene regulation, structure-function analysis and clinical application. de Gruyter, Berlin, pp 192–193
122. Colston KW, Mackay AG, James SY, Binderup L, Chandler S, Coombes RC (1992) EB1089: A new vitamin D analogue that inhibits the growth of breast cancer cells *in vivo* and *in vitro*. Biochem Pharmacol 44:2273–2280
123. James SY, Mackay AG, Binderup L, Colston KW (1994) Effects of a new synthetic analogue, EB1089, on the oestrogen-responsive growth of human breast cancer cells. J Endocr 141:555–563
124. Reddy GS, Clark JW, Tserng K-Y, Uskokovic MR, McLane JA (1993) Metabolism of 1,25-$(OH)_2$-16-ene D_3 in kidney: influence of structural modification of D-ring on side chain metabolism. Bioorg Med Chem Lett 3:1879–1884

125. Shankar VN, Byford V, Prosser DE, Schroeder NJ, Makin HLJ, Wiesinger H, Neef G, Steinmeyer A, Jones G (2001) Metabolism of a 20-methyl substituted series of vitamin D analogs by cultured human cells: Apparent reduction of 23-hydroxylation of the side chain by 20-methyl group. Biochem Pharmacol 61:893–902
126. Satchell DP, Norman AW (1996) Metabolism of the cell differentiating agent 1,25-$(OH)_2$-16-ene-23-yne vitamin D_3 by leukemic cells. J Steroid Biochem Molec Biol 57:117–124
127. Dantuluri PK, Haning C, Uskokovic MR, Tserng K-Y, Reddy GS (1994) Isolation and identification of 1,25,26-$(OH)_3$-16-ene-23-yne D_3, a metabolite of 1,25-$(OH)_2$-16-ene-23-yne D_3 produced in the kidney. In: Ninth workshop on vitamin D abstract book, Orlando, May 28–June 2 1994, abstract #43, p 32.
128. Shankar VN, Dilworth FJ, Makin HLJ, Schroeder NJ, Trafford DAJ, Kissmeyer A-M, Calverley MJ, Binderup E, Jones G (1997) Metabolism of the vitamin D analog EB1089 by cultured human cells: redirection of hydroxylation site to distal carbons of the side chain. Biochem Pharmacol 53:783–793
129. Kissmeyer A-M, Binderup E, Binderup L, Hansen CM, Andersen NR, Schroeder NJ, Makin HLJ, Shankar VN, Jones G (1997) The metabolism of the vitamin D analog EB 1089: Identification of in vivo and in vitro metabolites and their biological activities. Biochem Pharmacol 53:1087–1097
130. Wandkadiya KF, Uskokovic MR, Clark J, Tserng K-Y, Reddy GS (1992) Novel evidence for the reduction of the double bond in Δ^{22}-1,25-dihydroxyvitamin D_3 . J Bone Miner Res 7:S171 (Abstract 315)
131. Jones G, Lohnes D, Strugnell S, Guo Y-D, Masuda S, Byford V, Makin HLJ, Calverley MJ (1994) Target cell metabolism of vitamin D and its analogs. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D. A pluripotent steroid hormone: structural studies, molecular endocrinology and clinical applications. deGruyter, Berlin, pp 161–169
132. Masuda S, Kaufmann M, Byford V, Gao M, St-Arnaud R, Arabian A, Makin HLJ, Knutson JC, Strugnell S, Jones G (2004) Insights into vitamin D metabolism using CYP24 over-expression and knockout systems in conjunction with liquid chromatography/mass spectrometry (LC/MS). J Steroid Biochem & Mol Biol 89–90:149–153
133. Schuster I, Egger H, Astecker N, Herzig G, Schüssler M, Vorisek G (2001) Selective inhibitors of CYP24: mechanistic tools to explore vitamin D metabolism in human keratinocytes. Steroids 66: 451–462
134. Posner GH, Crawford KR, Yang HW, Kahraman M, Jeon HB, Li H, Lee JK, Suh BC, Hatcher MA, Labonte T, Usera A, Dolan PM, Kensler TW, Peleg S, Jones G, Zhang A, Korczak B, Saha U, Chuang SS (2004) Potent low-calcemic selective inhibitors of CYP24 hydroxylase: 24-sulphone analogs of the hormone 1α,25-dihydroxyvitamin D_3. J Steroid Biochem & Mol Biol 89–90: 5–12
135. Dardenne O, Prud'homme J, Arabian A, Glorieux FH, St-Arnaud R (2001) Targeted inactivation of the 25-hydroxyvitamin D_3-1α-hydroxylase gene (CYP27B1) creates an animal model of pseudovitamin D-deficiency rickets. Endocrinology 142:3135–3141
136. Panda DK, Miao D, Tremblay ML, Sirois J, Farookhi R, Hendy GN, Goltzman D (2001) Targeted ablation of the 25-hydroxyvitamin D 1α-hydroxylase enzyme: evidence for skeletal, reproductive, and immune dysfunction. Proc Natl Acad Sci USA 98:7498–7503
137. Bouillon R, Okamura WH, Norman AW (1995) Structure-function relationships in the vitamin D endocrine system. Endocr Rev 16:200–257
138. Terpening CM, Haussler CA, Jurutka PW, Galligan MA, Komm BS, Haussler MR (1991) The vitamin D-responsive element in the rat bone Gla protein gene is an imperfect direct repeat that cooperates with other cis-elements in 1,25-dihydroxyvitamin D_3- mediated transcriptional activation. Mol Endocrinol 5:373–385
139. Cheskis B, Lemon BD, Uskokovic MR, Lomedico PT, Freedman LP (1995) Vitamin D_3-retinoid X receptor dimerization, DNA binding, and transactivation are differentially affected by analogs of 1,25-dihydroxyvitamin D_3. Mol Endocrinol 9:1814–1824
140. Freedman LP, Reszka AA (2005) Vitamin D receptor cofactors: Function, regulation and selectivity. In: Feldman D, Pike JW, Glorieux FH (eds) Vitamin D, 2nd edn. Elsevier-Academic Press, New York, pp 263–279

141. Issa LL, Leong GM, Sutherland RL, Eisman JA (2002) Vitamin D analogue-specific recruitment of vitamin D receptor coactivators. J Bone Miner Res 17:879–890
142. Takeyama K, Masuhiro Y, Fuse H, Endoh H, Murayama A, Kitanaka S, Suzawa M, Yanagisawa J, Kato S (1999) Selective interaction of vitamin D receptor with transcriptional coactivators by a vitamin D analog. Mol Cell Biol 19:1049–1055
143. Peleg S, Ismail A, Uskokovic MR, Avnur Z (2003) Evidence for tissue- and cell-type selective activation of the vitamin D receptor by Ro-26-9228, a noncalcemic analog of vitamin D_3 . J Cell Biochem 88:267–273
144. Peleg S, Sastry M, Collins ED, Bishop JE, Norman AW (1995) Distinct conformational changes induced by 20-epi analogues of 1α,25-dihydroxyvitamin D_3 are associated with enhanced activation of the vitamin D receptor. J Biol Chem 270:10551–10558
145. Nayeri S, Danielsson C, Kahlen J, Schräder M, Mathiasen IS, Binderup L, Carlberg C (1995) The anti-proliferative effect of vitamin D_3 analogues is not mediated by inhibition of the AP-1 pathway, but may be related to promoter selectivity. Oncogene 11:1853–1858
146. Bouillon R, Allewaert K, Xiang DZ, Tan BK, Van Baelen H (1991) Vitamin D analogs with low affinity for the vitamin D binding protein: Enhanced *in vitro* and decreased *in vivo* activity. J Bone Miner Res 6:1051–1057
147. Dusso AS, Negrea L, Gunawardhana S, Lopez-Hilker S, Finch J, Mori T, Nishii Y, Slatopolsky E, Brown AJ (1991) On the mechanisms for the selective action of vitamin D analogs. Endocrinology 128:1687–1692
148. Prosser DE, Guo Y-D, Geh KR, Jia Z, Jones G (2006) Molecular modelling of CYP27A1 and site-directed mutational analyses affecting vitamin D hydroxylation. Biophys J 90:1–21
149. Willnow TE, Nykjaer A (2005) Chapter 10: Endocytic Pathways for 25-hydroxyvitamin D_3. In: Feldman D, Pike JW, Glorieux FH (eds) Vitamin D, 2nd edn. Elsevier Academic Press, New York, pp 153–163
150. Rowling MJ, Kemmis CM, Taffany DA, Welsh J (2006) Megalin-mediated endocytosis of vitamin D binding protein correlates with 25-hydroxycholecalciferol actions in human mammary cells. J Nutr 136:2754–2759
151. Fraser D, Kooh SW, Kind P, Holick MF, Tanaka Y, DeLuca HF (1973) Pathogenesis of hereditary vitamin D dependency rickets. N Engl J Med 289:817–822
152. Baggiolini EG, Wovkulich PM, Iacobelli JA, Hennessy BM, Uskokovic MR (1982) Preparation of 1-alpha hydroxylated vitamin D metabolites by total synthesis. In: Norman AW, Schaefer K, von Herrath D, Grigoleit H-G (eds) Vitamin D: chemical, biochemical and clinical endocrinology of calcium metabolism. DeGruyter, Berlin, pp 1089–1100
1523. Perlman KL, Sicinski RR, Schnoes HK, DeLuca HF (1990) 1α,25-Dihydroxy-19-nor-vitamin D_3, a novel vitamin D-related compound with potential therapeutic activity. Tetrahedron Lett 31: 1823–1824
154. Baggiolini EG, Partridge JJ, Shiuey S-J, Truitt GA, Uskokovic MR (1989) Cholecalciferol 23-yne derivatives, their pharmaceutical compositions, their use in the treatment of calcium-related diseases, and their antitumor activity, US 4,804,502 [Abstract]. Chem Abstr 111:58160d
155. Calverley MJ, Binderup E, Binderup L (1991) The 20-epi modification in the vitamin D series: Selective enhancement of "non-classical" receptor-mediated effects. In: Norman AW, Bouillon R, Thomasset M (eds) Vitamin D: gene regulation, structure-function analysis and clinical application. de Gruyter, Berlin, pp 163–164
156. Nishii Y, Sato K, Kobayashi T (1993) The development of vitamin D analogues for the treatment of osteoporosis. Osteoporosis Int 1:S190–S193 (Suppl)
157. Morisaki M, Koizumi N, Ikekawa N, Takeshita T, Ishimoto S (1975) Synthesis of active forms of vitamin D. Part IX. Synthesis of 1α,24-dihydroxycholecalciferol. J Chem Soc Perkin Trans 1(1): 1421–1424
158. Whitfield GK, Jurutka PW, Haussler C et al. (2005) Chapter 13: Nuclear Receptor: Structure-Function, Molecular Control of gene Transcription and Novel Bioactions. In: Feldman D, Pike JW, Glorieux FH (eds) Vitamin D, 2nd edn. Elsevier Academic Press, New York, pp. 219–262

159. Marchiani S, Bonaccorsi L, Ferruzzi P, Crescioli C, Muratori M, Adorini L, Forti G, Maggi M, Baldi E (2006) The vitamin D analogue BXL-628 inhibits growth factor-stimulated proliferation and invasion of DU145 prostate cancer cells. J Cancer Res Clin Oncol 132:408–416
160. Mehta R, Hawthorne M, Uselding L, Albinescu D, Moriarty R, Christov K, Mehta R (2000) Prevention of N-methyl-N-nitrosourea-induced mammary carcinogenesis in rats by 1α-hydroxyvitamin D_5. J Natl Cancer Inst 92:1836–1840
161. Shevde NK, Plum LA, Clagett-Dame M, Yamamoto H, Pike JW, DeLuca HF (2002) A potent analog of 1α,25-dihydroxyvitamin D3 selectively induces bone formation. Proc Natl Acad Sci USA 99:13487–13491