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# Natural Metabolites of 1α,25-Dihydroxyvitamin $D_3$ Retain Biologic Activity Mediated Through the Vitamin D Receptor

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**Abstract** 1α,25-dihydroxyvitamin $D_3$ (1α,25$(OH)_2D_3$), the active metabolite of vitamin D, mediates many of its effects through the intranuclear vitamin D receptor (VDR, NR1I1), that belongs to the large superfamily of nuclear receptors. Vitamin D receptor can directly regulate gene expression by binding to vitamin D response elements (VDREs) located in promoter or enhancer regions of various genes. Although numerous synthetic analogs of 1α,25$(OH)_2D_3$ have been analysed for VDR binding and transactivation of VDRE-driven gene expression, the biologic activity of many naturally occuring metabolites has not yet been analyzed in detail. We therefore studied the transactivation properties of 1α,24R,25-trihydroxyvitamin $D_3$ (1α,24R,25$(OH)_3D_3$), 1α,25-dihydroxy-3-epi-vitamin $D_3$ (1α,25$(OH)_2$-3-epi-$D_3$), 1α,23S,25-trihydroxyvitamin $D_3$ (1α,23S,25$(OH)_3D_3$), and 1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin $D_3$ (1α(OH)-24,25,26,27-tetranor-23-COOH-$D_3$; calcitroic acid) using the human G-361 melanoma cell line. Cells were cotransfected with a VDR expression plasmid and luciferase reporter gene constructs driven by two copies of the VDRE of either the mouse osteopontin promoter or the 1α,25$(OH)_2D_3$ 24-hydroxylase (CYP24) promoter. Treatment with 1α,25$(OH)_2D_3$ or the metabolites 1α,24R,25$(OH)_3D_3$, 1α,25$(OH)_2$-3-epi-$D_3$, and 1α,23S,25$(OH)_3D_3$ resulted in transactivation of both constructs in a time- and dose-dependent manner, and a postitive regulatory effect was observed even for calcitroic acid in the presence of overexpressed VDR. The metabolites that were active in the reporter gene assay also induced expression of CYP24 mRNA in the human keratinocyte cell line HaCaT, although with less potency than the parent hormone. A ligand-binding assay based on nuclear extracts from COS-1 cells overexpressing human VDR demonstrated that the metabolites, although active in the reporter gene assay, were much less effective in displacing [$^3$H]-labeled 1α,25$(OH)_2D_3$ from VDR than the parent hormone. Thus, we report that several natural metabolites of 1α,25$(OH)_2D_3$ retain significant biologic activity mediated through VDR despite their apparent low affinity for VDR. J. Cell. Biochem. 78:112–120, 2000. 

**Key words:** 1α,25$(OH)_2D_3$; metabolites; reporter gene assay; VDR, CYP24

1α,25-dihydroxyvitamin $D_3$ (1α,25$(OH)_2D_3$), the active metabolite of vitamin $D_3$, exerts pleiotropic effects including regulation of bone mineralization and calcium homeostasis, regulation of proliferation and differentiation, and has immunomodulatory activity [reviewed by Bouillon et al., 1995; Christakos, et al., 1996; Issa et al., 1998]. 1α,25$(OH)_2D_3$ exerts most of its effects via the vitamin D receptor (VDR; NR1I1), which belongs to the large superfamily of nuclear receptors [Mangelsdorf et al., 1995]. Vitamin D receptor has been shown to heterodimerize with retinoid X receptor and binds to vitamin D response elements (VDREs), which are located in promoter or enhancer regions of certain genes, such as those encoding mouse osteopontin [Noda et al., 1990], rat and human osteocalcin [Demay et al., 1990; Ozono et al., 1990], and rat and human 1α,25$(OH)_2D_3$ 24-hydroxylase (CYP24) [Zierold et al., 1994; Chen and DeLuca, 1995]. 1α,25$(OH)_2D_3$ can also suppress the transcription of some genes, including parathyroid hormone (PTH) [Demay et al., 1992], and several cytokines, including interleukin-2 (IL-2) [Alroy et al., 1995], interferon-γ [Cippitelli and Santoni,

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1998], IL-12 [D'Ambrosio et al., 1998], and IL-8 [Harant et al., 1997]. $1\alpha,25(OH)_2D_3$ is converted in the body via two major pathways into catabolic end products. One pathway starts with hydroxylation at C-24 catalyzed by CYP24, leading to the intermediary metabolites 1α,24R,25-trihydroxyvitamin $D_3$ ($1\alpha,24R,25(OH)_3D_3$), 1α,25-dihydroxy-24-oxo-vitamin $D_3$ ($1\alpha,25(OH)_2$-24-oxo-$D_3$), 1α,23S,25-trihydroxy-24-oxo-vitamin $D_3$ ($1\alpha,23,25(OH)_3$-24-oxo-$D_3$), 1α,23-dihydroxy-24,25,26,27-tetranorvitamin $D_3$ ($1\alpha,23(OH)_2$-24,25,26,27-tetranor $D_3$), and finally 1α-hydroxy-23-carboxy-24,25,26,27-tetranorvitamin $D_3$ (1α(OH)24,25,26,27-tetranor 23-COOH-$D_3$; calcitroic acid) [Ishizuka et al., 1984; Reddy et al., 1987; Makin et al., 1989; Reddy and Tserng, 1989]. The second pathway involves hydroxylation of C-23, leading to 1α,23S,25-trihydroxyvitamin $D_3$ ($1\alpha,23S,25(OH)_3D_3$) [Horst et al., 1984], 1α,23S,25R,26-tetrahydroxyvitamin $D_3$ ($1\alpha,23S,25R,26(OH)_4D_3$), 1α,23S,25R-dihydroxyvitamin $D_3$-26,23S-lactol (1α,23S,25R$(OH)_2$-26,23S-lactol-$D_3$), and finally to 1α,23S,25R-dihydroxyvitamin $D_3$-26,23S-lactone ($1\alpha,23S,25R(OH)_2$-26,23S-lactone-$D_3$) [Ishizuka and Norman, 1987; Siu-Caldera et al., 1995].

It is generally thought that $1\alpha,25(OH)_2D_3$ is the major biologically active hormone, whereas metabolic products downstream of $1\alpha,25(OH)_2D_3$ have lost their capacity to activate VDR, due to lower binding affinities [Bouillon et al., 1995]. However, recent reports demonstrate that some of the metabolites derived from $1\alpha,25(OH)_2D_3$, such as $1\alpha,23S,25(OH)_3$-24-oxo-$D_3$ [Lee et al., 1997] or $1\alpha,25(OH)_2$-3-epi-$D_3$ [Brown et al., 1999] have significant biologic activity and can regulate VDR-responsive genes. In a previous report we tested metabolites derived from 25-OH-$D_3$ and found that several of the 1α-hydroxylated metabolites were active in a VDRE transactivation assay [Harant et al., 1997].

In the present study we show that selected metabolites of $1\alpha,25(OH)_2D_3$, such as $1\alpha,24R,25(OH)_3D_3$, $1\alpha,23S,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and under certain conditions even calcitroic acid, have the capacity to transactivate a VDRE via VDR, although the binding of these metabolites to VDR is attenuated compared to that of the parent hormone. Most of these metabolites are also able to upregulate CYP24 mRNA levels in the human keratinocyte cell line HaCaT, although less markedly than $1\alpha,25(OH)_2D_3$. These data demonstrate that, along the catabolic pathway, biologic activity of some natural metabolites is retained despite an apparently lower affinity for VDR.

## MATERIALS AND METHODS

### Vitamin D Metabolites

$1\alpha,25(OH)_2D_3$ and 25-OH-$D_3$ were obtained from Calbiochem. The vitamin D metabolites $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, $1\alpha,23S,25(OH)_3D_3$, and 1α,(OH)-24,25,26,27-tetranor-23-COOH-$D_3$ (calcitroic acid) were generated by a kidney perfusion system and high performance liquid chromatography purification [Reddy et al., 1987]. All metabolites were dissolved in ethanol and stored at −20°C.

### Cell Culture

The human melanoma cell line G-361 was purchased from ATCC and cultivated in minimum essential medium (MEM) supplemented with 5% fetal calf serum (FCS). The human keratinocyte cell line HaCaT was provided by Dr. N. Fusenig (DKFZ, Heidelberg, Germany) and cultivated in Dulbecco's modified Eagle's medium supplemented with 10% FCS. COS-1 cells were obtained from ATCC and cultivated in RPMI 1640 supplemented with 10% FCS. All cell lines were incubated at 37°C in a 5% $CO_2$ atmosphere and passaged twice a week.

### Plasmids

The plasmid 2×VDRE(MOP)tkLUC contains two copies of the VDRE of the mouse osteopontin promoter (gatcTAACAAGGTTCACGAGGTTCA CGTCTTg) linked to a 155-bp portion of the herpes simplex virus (HSV) thymidine kinase promoter (−105 to +50 relative to the transcription start) cloned upstream of the luciferase coding region into the *Bgl*II–*Hind*III sites of the pGL2-basic vector (Promega). The plasmid 2×VDRE(CYP24)tkLUC contains two copies of VDRE1 (gatcTCCGGACGCCCTCGCTCACCTCGCTg) of the human 1α, $25(OH)_2D_3$ 24-hydroxylase (CYP24) promoter linked to the 155-bp portion of the HSV promoter, cloned into the *Kpn*I–*Hind*III sites of the pGL2 basic vector. The coding region of the human vitamin D receptor was cloned into the *EcoR*I–*BamH*I sites of the pSG5 mammalian expression vector (Stratagene).

### Transient Transfection

G-361 cells were transfected using lipofectamine (Gibco-BRL). Cells ($1.4 \times 10^6$) were seeded into a 100-mm petri dish and trans-

fected with 7 μg of reporter plasmid and 3 μg of either VDR expression vector or pSG5 vector. Twenty-four hours post-transfection, cells were scraped off, seeded into 96-well plates (Packard), and treated with the individual vitamin D metabolites for 6 and 24 h in FCS-free MEM. Cells were then directly lysed with 10 μl lysis buffer (Promega), and luciferase activity was recorded using luciferase assay reagent (Promega) in a microplate scintillation counter (Packard).

### Northern Analysis

HaCaT cells were seeded at a density of $1 \times 10^6$ cells per well into a six-well plate and treated with the individual vitamin D metabolites at a concentration of $2 \times 10^{-8}$ M for 6 and 24 h before preparation of total RNA using Tri reagent (Molecular Research Center, Cincinnati, OH). Approximately 20 μg of total RNA was loaded on to a formaldehyde-agarose gel. After capillary transfer to a nylon membrane (Schleicher & Schuell) and fixing of the RNA by baking, hybridization was carried out at 65°C using Rapid hyb buffer (Amersham) and a [$^{32}$P]-labeled 400-bp *Pst*I fragment of the human CYP24 cDNA (kindly provided by K.-S. Chen, Wisconsin) [Chen et al., 1993]. The blot was then stripped and rehybridized with a glyceraldehyde-3-phosphate dehydrogenase cDNA.

### Ligand Binding Assay

COS-1 cells ($1.4 \times 10^6$) were transfected with 5 μg VDR expression plasmid and lipofectamine as described above. Twenty-eight hours post-transfection, nuclear extracts were prepared from cells (100 μl nuclear extract/$1.4 \times 10^6$ cells) as described previously [Harant et al., 1996]. Nuclear extract (8 μl) was diluted with phosphate-buffered saline (PBS) to 98 μl, and 1 μl of cold ligand at different concentrations was added and incubated for 1 h at room temperature before addition of 1 μl of 1α,25-dihydroxy [26,27-*methyl*-$^3$H] cholecalciferol (Amersham; specific activity, 180 Ci/mmol) to give a final concentration of $5.5 \times 10^{-10}$ M. After a further 1-h incubation at 22°C, 40 μl of charcoal/dextran (Sigma; 0.5% in PBS, freshly prepared) were added and the samples were vortexed and centrifuged for 10 min at 3,000 rpm at 4°C. Fifty microliters of the supernatants were incubated with 200 μl Microscint 40 (Packard) and measured in a microplate scintillation counter (Packard).

## RESULTS

### Natural Metabolites Derived from 1α,25(OH)$_2$D$_3$ Transactivate a VDRE-Driven Promoter Through VDR

Four metabolites of 1α,25(OH)$_2$D$_3$ were analyzed for their transcriptional activation of a vitamin D response element in the human melanoma cell line G-361. Cells were transfected with a luciferase reporter plasmid driven by two copies of the VDRE of the mouse osteopontin promoter linked to a portion of the HSV thymidine kinase promoter in the presence or absence of overexpressed VDR. G-361 cells have been previously used by us to investigate the biologic activity of various vitamin D metabolites [Harant et al., 1997]. In the present study we found that, in comparison to this earlier study, G-361 cells responded less to 1α,25(OH)$_2$D$_3$ through endogenous receptors, an effect that may be caused by changes in the experimental format or passage-dependent variable levels of endogenous VDR. However, although absolute activation of luciferase gene expression was reduced, there was no difference in the relative activation of VDRE-driven reporter gene expression by 1α,25(OH)$_2$D$_3$ and the metabolites. Moreover, overexpression of VDR resulted in a markedly enhanced transactivation response by 1α,25(OH)$_2$D$_3$ and the metabolites, suggesting that the effects on the VDRE were mediated through VDR.

Transfected cells were treated with 1α,25(OH)$_2$D$_3$ or the metabolites 1α,24R,25(OH)$_3$D$_3$, 1α,25(OH)$_2$-3-epi-D$_3$, 1α,23S,25(OH)$_3$D$_3$, and calcitroic acid, each at a concentration of $2 \times 10^{-8}$ M in FCS-free medium, and luciferase activity was recorded after 6 and 24 h. No significant transactivation via endogenous receptors was seen after 6 h treatment with 1α,25(OH)$_2$D$_3$ or the metabolites (Fig. 1a), while a 24-h incubation resulted in a twofold to threefold transactivation by 1α,25(OH)$_2$D$_3$ and the metabolites 1α,24R,25(OH)$_3$D$_3$, 1α,25(OH)$_2$-3-epi-D$_3$, and 1α,23S,25(OH)$_3$D$_3$ (Fig. 1b). No transactivation was induced by calcitroic acid or 25-hydroxyvitamin D$_3$ (25-OH-D$_3$). Overexpression of VDR resulted in a significant enhancement of transactivation by all the metabolites tested. Treatment with 1α,25(OH)$_2$D$_3$ and the metabolites 1α,24R,25(OH)$_3$D$_3$, 1α,25(OH)$_2$-3-epi-D$_3$, and 1α,23S,25(OH)$_3$D$_3$ for 6 h induced a fivefold to eightfold transactivation of the VDRE-driven promoter (Fig. 1c). Incubation for 24 h in the presence of overexpressed VDR resulted in a

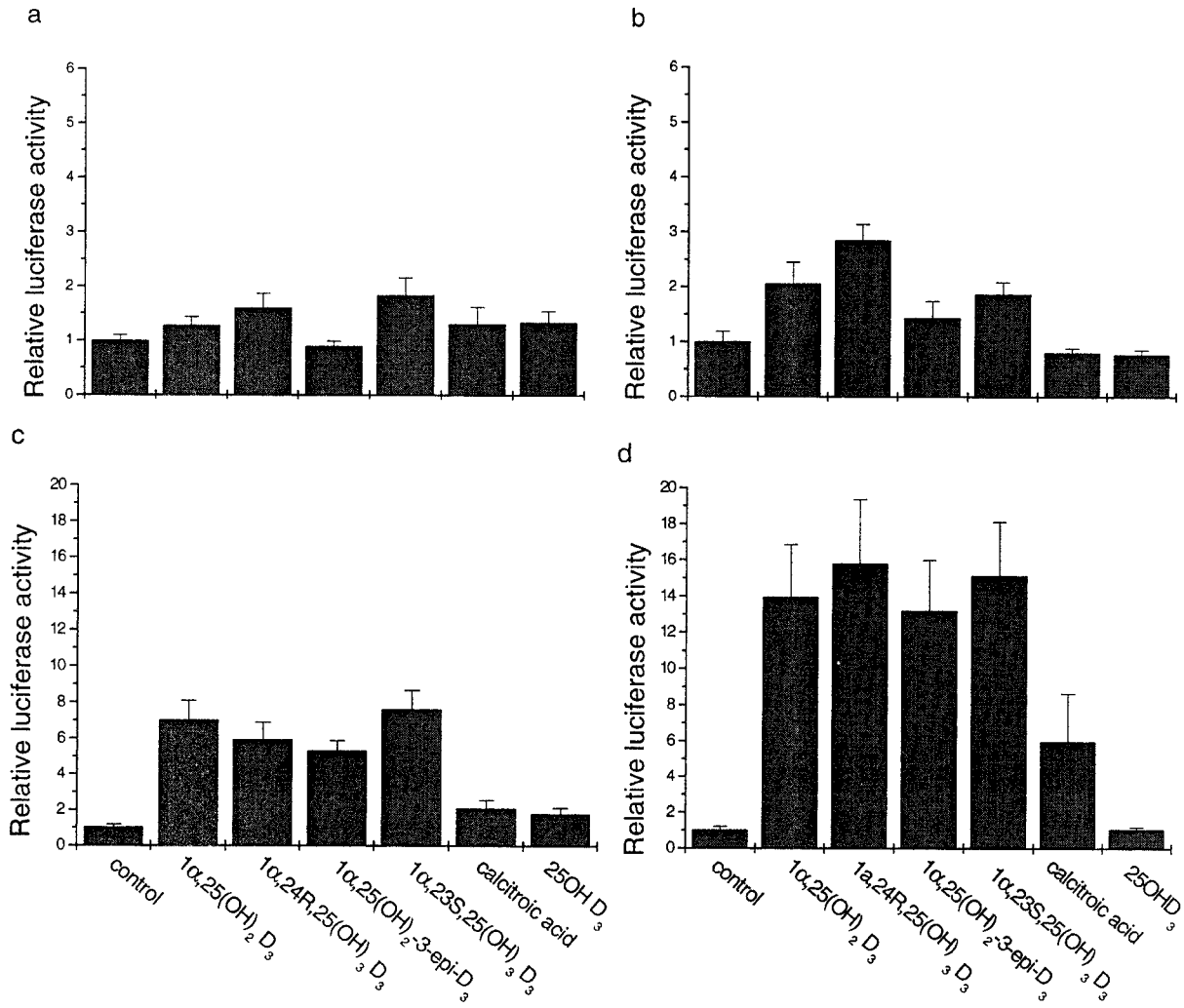


**Fig. 1.** Natural metabolites derived from $1\alpha,25(OH)_2D_3$ transactivate a vitamin D response element (VDRE)-driven promoter through vitamin D receptor (VDR). G-361 human melanoma cells were transfected with a reporter gene construct containing two copies of the VDRE of the mouse osteopontin promoter linked to the herpes simplex virus (HSV) thymidine kinase promoter and the luciferase coding region in pGL2 basic vector. Cotransfections were performed with either pSG5 vector or pSG5-VDR expression plasmid. Cells were then treated with ethanol (0.1%; control), $1\alpha,25(OH)_2D_3$, $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, $1\alpha,23S,25(OH)_3D_3$, calcitroic acid, or 25-OH-$D_3$, each at a concentration of $2 \times 10^{-8}$ M, and luciferase activity was measured as described in Materials and Methods. **a:** Endogenous VDR, 6 h treatment. **b:** Endogenous VDR, 24 h. **c:** Overexpressed VDR, 6 h. **d:** Overexpressed VDR, 24 h. Data points are means ± SEM from triplicate samples from three individual experiments.

14- to 16-fold transactivation of the VDRE by $1\alpha,25(OH)_2D_3$, $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and $1\alpha,23S,25(OH)_3D_3$ at $2 \times 10^{-8}$ M, whereas a sixfold transactivation was induced by calcitroic acid (Fig. 1d). As negative control, a promoter construct containing only the HSV thymidine kinase promoter together with the VDR expression plasmid was used. Treatment of transfected G-361 cells with $1\alpha,25(OH)_2D_3$ or the metabolites did not result in induction of luciferase (data not shown).

To determine whether the vitamin D metabolites also transactivate another VDRE, the same transfection experiments were performed with a construct containing two copies of VDRE1 of the human CYP24 gene [Chen and DeLuca, 1995], linked to the same minimal promoter as for the mouse osteopontin VDRE. The metabolites that caused transactivation of the mouse osteopontin VDRE significantly enhanced reporter gene expression driven by the CYP24 VDRE after 6 and 24 h treatment (Fig. 2a,b).

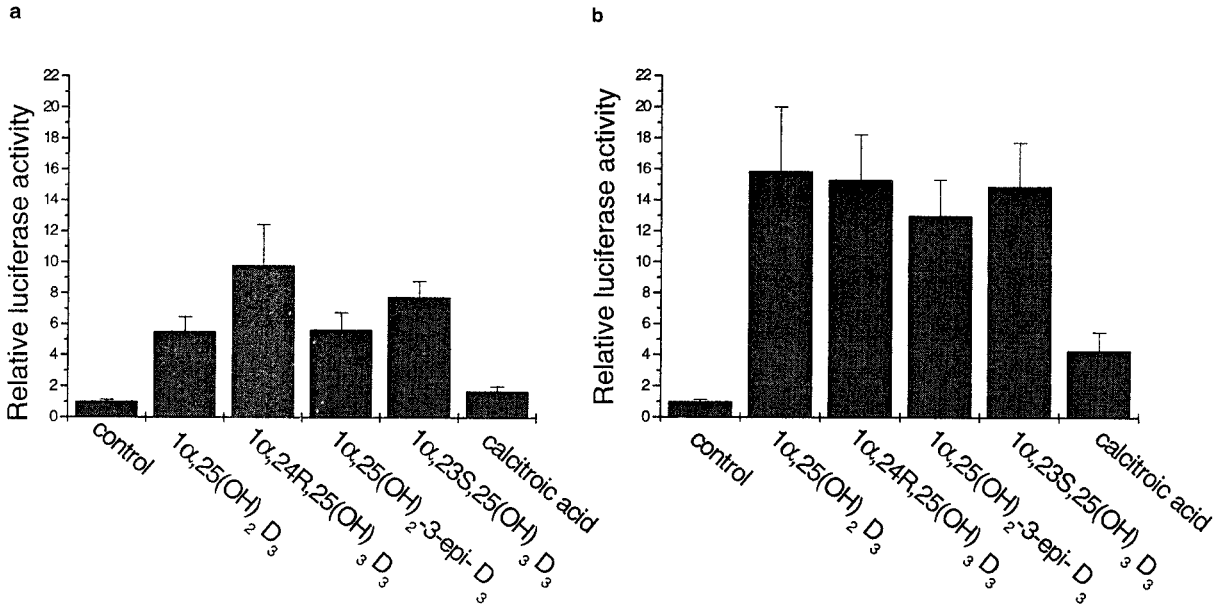


**Fig. 2.** Metabolites derived from $1\alpha,25(OH)_2D_3$ transactivate the vitamin D response element 1 (VDRE1) of the human $1\alpha,25(OH)_2D_3$ 24-hydroxylase (CYP24) promoter. G-361 cells were transfected with a construct containing two copies of the VDRE1 of the human CYP24 promoter fused to the herpes simplex virus (HSV) thymidine kinase promoter and the luciferase coding region in pGL2 basic vector together with the vitamin D receptor (VDR)-expression plasmid. Transfected cells were treated with ethanol (0.1%; control), $1\alpha,25(OH)_2D_3$, $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, $1\alpha,23S,25(OH)_3D_3$, and calcitroic acid, each at a concentration of $2 \times 10^{-8}$ M and luciferase activity measured as described in Materials and Methods. **a:** 6 h treatment. **b:** 24 h treatment. Data points are means ± SEM from triplicate samples from three individual experiments.

A dose-response study for VDR transactivation by the individual metabolites was then performed. G-361 cells were transfected with the VDR expression plasmid and the reporter construct containing the VDRE of the mouse osteopontin promoter and treated with $1\alpha,25(OH)_2D_3$ or metabolites for 6 h. Serial dilutions of $1\alpha,25(OH)_2D_3$ and the metabolites $1\alpha,24R,25(OH)_3D_3$ and $1\alpha,23S,25(OH)_3D_3$ showed that these still were active at $2 \times 10^{-10}$ M, while we could not detect any transactivation by $1\alpha,25(OH)_2$-3-epi-$D_3$ at this concentration (Fig. 3). Using this approach, we conclude that the potency of the individual metabolites for VDRE transactivation is $1\alpha,25(OH)_2D_3 > 1\alpha,24R,25(OH)_3D_3 > 1\alpha,23S,25(OH)_3D_3 > 1\alpha,25(OH)_2$-3-epi-$D_3$ > calcitroic acid.

## Metabolites of $1\alpha,25(OH)_2D_3$ Induce Expression of CYP24 mRNA in HaCaT Cells

Because $1\alpha,25(OH)_2D_3$ can transcriptionally activate the CYP24 gene and has been reported to exert various biologic effects in keratinocytes [Holick et al., 1987; Bikle, 1997; Kragballe, 1997], we investigated by Northern blot analysis whether metabolites can induce CYP24 mRNA expression in the human keratinocyte cell line HaCaT. Cells were exposed to $1\alpha,25(OH)_2D_3$, $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, $1\alpha,23S,25(OH)_3D_3$, calcitroic acid, or 25-OH-$D_3$ at a concentration of $2 \times 10^{-8}$ M for 6 and 24 h before preparation of total RNA. By 6 h, $1\alpha,25(OH)_2D_3$ had upregulated CYP24 mRNA levels, which remained elevated at 24 h. $1\alpha,24R,25(OH)_3D_3$ also increased CYP24 mRNA levels at 6 and 24 h, although less than $1\alpha,25(OH)_2D_3$. The metabolites $1\alpha,25(OH)_2$-3-epi-$D_3$ and $1\alpha,23S,25(OH)_3D_3$, which were active in the reporter gene assay using G-361 cells, also enhanced CYP24 mRNA levels, but much less than $1\alpha,25(OH)_2D_3$. Calcitroic acid, however, was not able to elevate CYP24 mRNA levels in HaCaT cells. Although apparently having less activity in HaCaT cells than in the more sensitive G-361 cell reporter gene assays, the metabolites $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and $1\alpha,23S,25(OH)_3D_3$ were nonetheless able to induce expression of CYP24 mRNA (Fig. 4).

## The Metabolites Derived From $1\alpha,25(OH)_2D_3$ Bind Human VDR With Lower Affinity Than $1\alpha,25(OH)_2D_3$

A ligand-binding assay based on human VDR was developed using nuclear extract from COS-1 cells overexpressing human VDR. Bind-

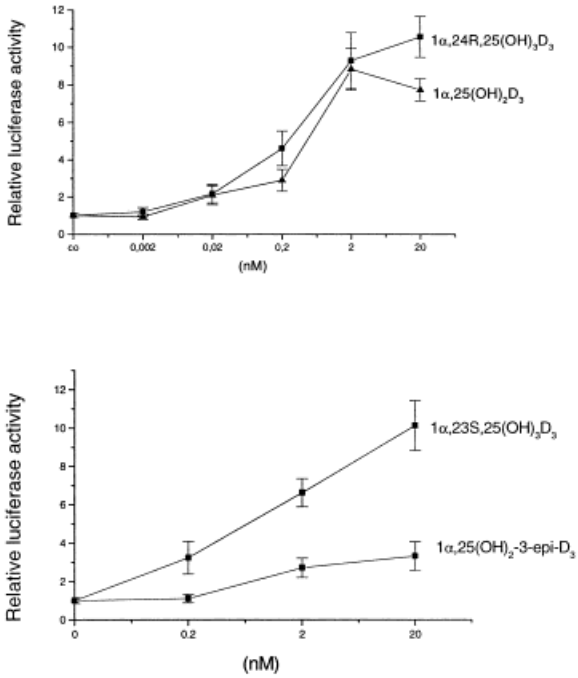


**Fig. 3.** Dose-dependent transactivation of the mouse osteopontin vitamin D response element (VDRE) by $1\alpha,25(OH)_2D_3$ and the metabolites $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and $1\alpha,23S,25(OH)_3D_3$. Cells were transfected with the mouse osteopontin VDRE promoter construct and the vitamin D receptor (VDR) expression plasmid and treated with different concentrations of $1\alpha,25(OH)_2D_3$ or the metabolites $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and $1\alpha,23S,25(OH)_3D_3$ for 6 h. Data shown are means ± SEM from triplicate samples from three individual experiments.

ing of [$^3$H]-labeled $1\alpha,25(OH)_2D_3$ to this extract was assayed in the presence of increasing concentrations of cold competitors. After removal of the free ligand using charcoal/dextran, the remaining VDR-bound ligand was quantified by measuring radioactivity. When COS-1 extracts containing hVDR were incubated with increasing concentrations of $1\alpha,25(OH)_2D_3$ or the metabolites, differences in competition for binding of the radioligand were observed. $1\alpha,25(OH)_2D_3$ efficiently competed the radioligand for binding to hVDR, whereas $1\alpha,24R,25(OH)_3D_3$ was less efficient, although it significantly competed the [$^3$H]-labeled $1\alpha,25(OH)_2D_3$ at higher concentrations. $1\alpha,25(OH)_2$-3-epi-$D_3$ was also capable of competing for binding of the radioligand, but much less than $1\alpha,25(OH)_2D_3$ or $1\alpha,24R,25(OH)_3D_3$. However, $1\alpha,23S,25(OH)_3D_3$, although active in the reporter gene assay and in induction of CYP24 mRNA expression, exhibited extremely low activity in displacing the radiolabeled $1\alpha,25(OH)_2D_3$ (Fig. 5).

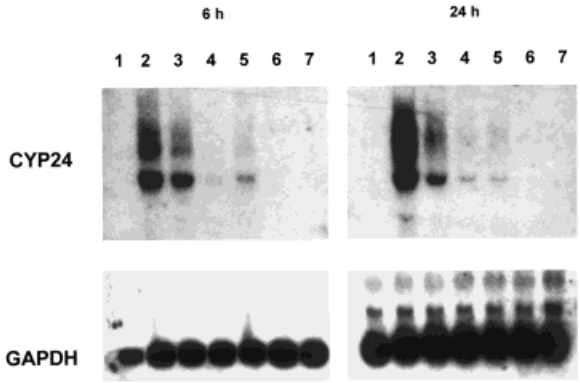


**Fig. 4.** $1\alpha,25(OH)_2D_3$ and the metabolites $1\alpha,24R,25(OH)_3$-$D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and $1\alpha,23S,25(OH)_3D_3$ induce expression of $1\alpha,25(OH)_2D_3$ 24-hydroxylase (CYP24) mRNA in the human keratinocyte cell line HaCaT. **C**ells ($1 \times 10^6$) were treated with 0.1% ethanol (1), $1\alpha,25(OH)_2D_3$ (2), the metabolites $1\alpha,24R,25(OH)_3D_3$ (3), $1\alpha,25(OH)_2$-3-epi-$D_3$ (4), $1\alpha,23S,25(OH)_3D_3$ (5), calcitroic acid (6), and 25-OH-$D_3$ (7) at a concentration of $2 \times 10^{-8}$ M for 6 and 24 h before preparation of total RNA. Expression of CYP24 mRNA was assessed by Northern blot analysis. Blots were hybridized with a [$^{32}$P]-labeled fragment of the CYP24 cDNA and rehybridized with a GAPDH cDNA.

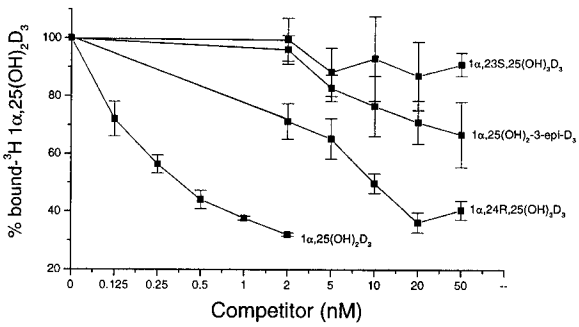


**Fig. 5.** Metabolites derived from $1\alpha,25(OH)_2D_3$ bind human vitamin D receptor (VDR) with lower affinity than $1\alpha,25(OH)_2D_3$. A ligand-binding assay based on human VDR, expressed in COS-1 cells was used to analyse VDR binding of the individual metabolites. COS-1 nuclear extracts were incubated with increasing concentrations of competitor and then with [$^3$H]-labeled $1\alpha,25(OH)_2D_3$. Free ligand was removed by charcoal/dextran treatment and the remaining radioactivity measured as described in Materials and Methods. Data shown are results from three ($1\alpha,25(OH)_2D_3$) or four ($1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, $1\alpha,23S,25(OH)_3D_3$) independent experiments.

## DISCUSSION

$1\alpha,25(OH)_2D_3$ is converted into $1\alpha,24R,25(OH)_3D_3$ by CYP24, an enzyme that tightly regulates the levels of $1\alpha,25(OH)_2D_3$. It is then converted further via $1\alpha,25(OH)_2$-24-oxo-$D_3$, $1\alpha,23,25(OH)_3$-24-oxo-$D_3$, $1\alpha,23(OH)_2$-24,25,26,27-tetranor $D_3$, and finally into 1α(OH)-24,25,26,27-tetranor 23-COOH-$D_3$ (calcitroic acid)

[Reddy et al., 1987; Reddy and Tserng, 1989; Makin et al., 1989]. CYP24 is a multicatalytic enzyme that catalyzes most, if not all, of these reactions [Beckman et al., 1996; Sakaki et al., 1999]. Another pathway of inactivation of $1\alpha,25(OH)_2D_3$ is oxidation at C-23, generating $1\alpha,23S,25(OH)_3D_3$, which can be converted by different steps into $1\alpha,25R(OH)_2$-26,23S-lactone-$D_3$ [Horst et al., 1984; Ishizuka et al., 1984; Ishizuka and Norman, 1987; Siu-Caldera et al., 1995]. Outside these major pathways of $1\alpha,25(OH)_2D_3$ conversion, other metabolites of $1\alpha,25(OH)_2D_3$ have been isolated. These include $1\alpha,25(OH)_2$-3-epi-$D_3$, which was shown to be generated in bovine-cultured parathyroid cells [Brown et al., 1999], rat osteosarcoma cells (Siu-Caldera et al., 1999), Caco-2 colon carcinoma cells [Bischof et al., 1998], and human primary keratinocytes [Astecker et al., 1997]. Although it is generally accepted that the metabolites derived from $1\alpha,25(OH)_2D_3$ exert attenuated biologic activity due to reduced binding to VDR, recent investigations now suggest that some of the metabolites still retain biologic activity similar to that of the parent hormone. For instance, $1\alpha,23S,25(OH)_2$-24-oxo-$D_3$ has been reported to be an inhibitor of PTH secretion [Lee et al., 1997]. $1\alpha,25(OH)_2$-3-epi-$D_3$ also has the ability to downregulate PTH secretion with a potency comparable to that of $1\alpha,25(OH)_2D_3$, although it binds VDR with an affinity 30 times lower than $1\alpha,25(OH)_2D_3$ [Brown et al., 1999].

In a previous report on the transactivation of the VDRE and repression of a nuclear factor-κB-driven reporter gene by vitamin D metabolites, we observed activation of the VDRE by 1α-hydroxylated metabolites with variously hydroxylated, but intact side-chains, namely $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-24-oxo-$D_3$, $1\alpha,23S,25(OH)_3D_3$, and $1\alpha,25R,26(OH)_3D_3$. However, only minor transactivation was seen with calcitroic acid and no transactivation with $1\alpha,25R(OH)_2$-26,23S-lactone-$D_3$, the end product of the C-23 oxidation pathway [Harant, et al. 1997]. In the present study we investigated the biologic activity of some selected metabolites of $1\alpha,25(OH)_2D_3$ in more detail. The metabolites used were $1\alpha,24R,25(OH)_3D_3$, a product of the C-24 oxidation pathway; $1\alpha,23S,25(OH)_3D_3$, a metabolite derived from the C-23 oxidation pathway; the naturally occurring A-ring stereoisomer $1\alpha,25(OH)_2$-3-epi-$D_3$; and calcitroic acid, the end product of the metabolic vitamin D cascade. Using G-361 human melanoma cells transfected with a reporter gene driven by the VDRE of the mouse osteopontin promoter or a VDRE of the human CYP24 gene, we observed transactivation by $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and $1\alpha,23S,25(OH)_3D_3$ in a concentration range between $2 \times 10^{-8}$ and $2 \times 10^{-11}$ M, depending on the metabolite used. The highest activation was observed for $1\alpha,24R,25(OH)_3D_3$, whereas $1\alpha,25(OH)_2$-3-epi-$D_3$ and $1\alpha,23S,25(OH)_3D_3$ were less potent. These activities were clearly VDR-dependent, as in all cases the response to the metabolites was greatly enhanced by overexpression of VDR. These three metabolites, $1\alpha,24R,25(OH)_3D_3$, $1\alpha,25(OH)_2$-3-epi-$D_3$, and $1\alpha,23S,25(OH)_3D_3$, were also able to enhance expression of CYP24 mRNA levels in HaCaT cells, again with $1\alpha,24R,25(OH)_3D_3$ being the most active metabolite. The G-361 cell-based reporter gene assay represents an artificial system that is optimized via VDR overexpression to identify the transactivation potential of the metabolites and does not fully correlate with the in vitro VDR-binding affinities of the metabolites. However, analysis of CYP24 mRNA levels in HaCaT cells shows regulation of a VDR-responsive gene through the endogenous receptor, which agrees with the in vitro binding affinities and thus better reflects the natural situation. Although calcitroic acid, the end product of the C-24 oxidation pathway, was weakly active in the reporter gene assays, it was unable to upregulate CYP24 mRNA levels in HaCaT cells. This suggests that calcitroic acid has the potential to transactivate, but does not exert significant activity on regulation of VDR-responsive genes under natural conditions.

Extensive studies on the structure-activity relationship of vitamin D derivatives showed that for binding to VDR, the 1α-hydroxyl group is essential together with the 25 (or 24)-hydroxyl group in the side-chain. The importance of the 3β-hydroxyl group was underlined by the fact that epimerization of this moiety causes a significant reduction in VDR binding [Bouillon et al., 1995]. Most of the naturally occurring vitamin D metabolites have been analyzed for their binding to VDR, mainly using chick VDR [Bouillon et al., 1995]. In the present study we analyzed the binding of these metabolites using an assay based on human VDR. In this assay, $1\alpha,25(OH)_2D_3$ efficiently displaced [$^3$H]-labeled $1\alpha,25(OH)_2D_3$, whereas $1\alpha,24R,25(OH)_3D_3$ was able to compete the radioligand at higher concentration. Both $1\alpha,23S,25(OH)_3D_3$ and $1\alpha,25(OH)_2$-3-epi-$D_3$

exhibited weaker affinity for human VDR. In our study, the competition by $1\alpha,25(OH)_2$-3-epi-$D_3$ on hVDR is comparable to the ligand binding assay results obtained in cultured bovine parathyroid cells [Brown et al., 1999]. $1\alpha,23S,25(OH)_3D_3$ showed extremely low affinity for VDR, in agreement with a previously determined chick VDR binding affinity [Horst et al., 1984; Bouillon et al., 1995]. However, this metabolite could transactivate the VDRE and cause induction of CYP24 mRNA in HaCaT cells. It cannot be excluded that $1\alpha,23S,25(OH)_3D_3$ is converted into some other unknown metabolite in the cells, which could account for its activity in the reporter gene assay. Although $1\alpha,23S,25(OH)_3D_3$ shows weak affinity for VDR, $1\alpha,23S,25(OH)_3$-24-oxo-$D_3$, a product of the C24-oxidation pathway, has higher affinity for VDR and is biologically active, as shown by downregulation of PTH secretion [Lee et al., 1997]. It is unlikely that C-24 oxidation of $1\alpha,23S,25(OH)_3D_3$ would be an explanation for the discrepancy between transcriptional activation of VDR and binding to VDR, since we could not detect CYP24 mRNA in G-361 cells by Northern blot analysis (data not shown), and the accepted metabolic fate of $1\alpha,23S,25(OH)_3D_3$ is the formation of the respective lactone without C-24 oxidation [Horst et al., 1984; Ishizuka and Norman, 1987]. Analysis of synthetic derivatives of $1\alpha,25(OH)_2D_3$ have shown that weak affinity for VDR may correlate with weak transcriptional activity, although this appears not to be a general rule. Derivatives of 16-ene with high or moderate affinity for VDR caused transcriptional activation of a VDRE-driven reporter gene at much lower concentrations than the parent compound $1\alpha,25(OH)_2D_3$ [Ferrara et al., 1994], whereas other analogs, such as the A-ring analogs of 19-nor-$1\alpha,25(OH)_2D_3$ and 19-nor-22-oxa-$1\alpha,25(OH)_2D_3$ have been shown to be potent transcriptional activators, although they bind VDR with very low affinity [Okano et al., 1998]. The observation on synthetic derivatives of $1\alpha,25(OH)_2D_3$ raises the possibility that, in addition to binding affinity for VDR, other factors influence the biologic activity of a given $1\alpha,25(OH)_2D_3$ derivative. One possible explanation is a difference in bioavailability caused by differential binding to serum components or the metabolic fate, which could give rise to a more potent metabolite. Alternatively, it is also possible that a conformational change of the VDR ligand binding domain induced by derivatives would favor the recruitment of coactivators, which then enhance the transcriptional response. This could be true for the metabolites which transactivated strongly but bound VDR with lower affinity than $1\alpha,25(OH)_2D_3$ .

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