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Novel Ring A Stereoisomers of 2-Methyl-1α,25-dihydroxyvitamin D_3 and 2-Methyl-20-epi-1α,25-dihydroxyvitamin D_3: Transactivation of Target Genes and Modulation of Differentiation in Human Promyelocytic Leukemia (HL-60) Cells

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ABSTRACT. We evaluated the biological activity of two sets of ring A stereoisomers of 2-methyl-1α,25-dihydroxyvitamin D_3 (2-methyl-1α,25$(OH)_2D_3$) and 2-methyl-20-epi-1α,25-dihydroxyvitamin D_3 (2-methyl-20-epi-1α,25$(OH)_2D_3$) in terms of the following: transactivation of a rat 25-hydroxyvitamin D_3-24-hydroxylase gene promoter including two vitamin D response elements (VDREs) and a human osteocalcin gene promoter including a VDRE in transfected human osteosarcoma (MG-63) cells; a vitamin D receptor (VDR)-mediated response using a VDR–GAL4 one-hybrid luciferase reporter system and a retinoid X receptor α (RXRα)-mediated response using an expressed VDR/RXRα–GAL4 modified two-hybrid luciferase reporter system in transfected human epitheloid carcinoma, cervix (HeLa) cells; and modulation of cell surface CD11b antigen expression in human leukemia (HL-60) cells. All the diastereomers of both analogues exhibited unique biological activity profiles depending upon the configurations of the C-1 and C-3 hydroxyl groups, the C-2 methyl group in ring A, and the C-20 methyl group in the side chain. Of the eight possible diastereomers of the 2-methyl analogues, 2α-methyl-1α,25$(OH)_2D_3$ was the most potent and exhibited comparable or even greater biological potency than 1α,25$(OH)_2D_3$. Of the eight possible diastereomers of the 2-methyl-20-epi analogues, 2α-methyl-20-epi-1α,25$(OH)_2D_3$ was the most potent and exhibited 100- to 200-fold higher transcriptional potencies than 1α,25$(OH)_2D_3$ and exceptionally high cell regulatory activities. 2β-Methyl-20-epi-1α,25$(OH)_2D_3$ was nearly as potent as its 2-epimer, 2α-methyl-20-epi-1α,25$(OH)_2D_3$, whereas its 20-epimer, 2β-methyl-1α,25$(OH)_2D_3$, was almost completely biologically inactive. In these respects, it can be postulated that the double modification of 2-methyl substitution and 20-epimerization to 1α,25$(OH)_2D_3$ induces remarkable changes in a VDR/RXRα/VDRE-mediated signaling response and greatly enhances biological activity. The other striking finding was that 2β-methyl-20-epi-3-epi-1β,25$(OH)_2D_3$ is transcriptionally more active than 1α,25$(OH)_2D_3$ despite lacking the 1α-hydroxyl group, which was believed to be essential for expressing VDR-mediated gene transcription. Since the C-20 natural counterpart, 2β-methyl-3-epi-1β,25$(OH)_2D_3$, was almost completely biologically inactive, 20-epimerization is probably responsible for activation of gene expression. Although earlier extensive structure–activity studies of vitamin D analogues showed stereochemistry at the C-1, C-3, and C-20 of 1α,25$(OH)_2D_3$ to be the key structural motif for vitamin D action, our results clearly demonstrated that stereochemistry at the C-2 is also an important structural motif for vitamin D action and imply that 2-methyl substitution possibly induces conformational changes in ring A depending upon the combinations of configurations of the C-1 and C-3 hydroxyl groups with C-20 stereochemistry. Consequently, several of these analogues exhibit exceptionally high or unexpected biological activities at the molecular and cellular levels. These results suggest that 2-methyl substitution together with alterations of stereochemistry in both ring A and the side chain of 1α,25$(OH)_2D_3$ will provide useful analogues for structure–activity studies and development of therapeutic agents with unique biological activity profiles. BIOCHEM PHARMACOL **59**;6:691–702, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. 1α,25-dihydroxyvitamin D_3; 2-methyl or 2-methyl-20-epi analogues; diastereoisomers; transactivation; cell surface CD11b antigen expression

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The physiologically active form of vitamin D_3, $1\alpha,25(OH)_2D_3$* [1], plays a key role in the regulation of calcium homeostasis in mammals. $1\alpha,25(OH)_2D_3$ also exerts cell regulatory effects in target cells [2–4]. The exact mechanism of action of $1\alpha,25(OH)_2D_3$ in target cells has not been clarified, but it is well documented that $1\alpha,25(OH)_2D_3$ first binds to the nuclear VDR [5], a member of the superfamily of steroid receptors [6, 7]. The $1\alpha,25(OH)_2D_3$-liganded VDR then heterodimerizes with the RXR [8], and this complex subsequently binds to the VDREs in the promoter regions of the primary responding genes, leading to either activation or suppression of gene transcription [9]. The broad distribution of VDR in many tissues and the fact that $1\alpha,25(OH)_2D_3$ modulates proliferation and differentiation of normal and malignant cells make this hormone a potentially useful agent for the treatment of diseases such as cancer [10], psoriasis [11, 12], and immune disorders [13]. However, the major limitation to its clinical use is that it causes hypercalcemia [14]. Therefore, vitamin D analogues with potent cell regulatory effects but with weaker calcemic effects than $1\alpha,25(OH)_2D_3$ are needed [15]. It has been shown that highly potent vitamin D analogues can be generated by epimerization of carbon 20 in the side chain. Among them, KH-1060 has high cell antiproliferation and differentiation-inducing activities with relatively low calcemic effects [16]. Modifications in ring A also produced analogues with a unique biological profile. Thus, hybrid analogues with both 20-epimerization and ring A modification may generate a unique analogue with potent cell regulatory effects and low calcemic activity as well. Recently, Konno *et al.* [17] and Fujishima *et al.* [18] designed and synthesized all possible ring A diastereomers of 2-methyl-$1\alpha,25(OH)_2D_3$ and 2-methyl-20-epi-$1\alpha,25(OH)_2D_3$ and evaluated their biological properties in terms of binding affinity for VDR and serum DBP, morphological changes in HL-60 cells, and calcium mobilization from bone in normal rats. The results showed that double modification of 2α-methyl substitution and 20-epimerization generates analogues with exceptionally high biological activity. To gain more insight into the biological action of the analogues at the molecular level, we examined their transactivation of gene expression potencies on specific target genes in transfected cells and cell surface CD11b antigen expression in human leukemia (HL-60) cells [19]. We report here that both 2-methyl and 2-methyl-20-epi analogues of $1\alpha,25(OH)_2D_3$ exhibited unique transcriptional potencies and cell regulatory activities that depended upon the configuration of ring A and/or the side chain. We found that modification with 2α-methyl substitution and/or 20-epimerization to $1\alpha,25(OH)_2D_3$ resulted in large increases in transcriptional potencies and cell regulatory activities. We also demonstrated for the first time that 2β-methyl-20-epi-3-epi-$1\beta,25(OH)_2D_3$ was 2- to 4-fold as potent as $1\alpha,25(OH)_2D_3$ despite lacking the 1α-hydroxyl group, which was believed to be essential for expressing VDR-mediated gene transcription.

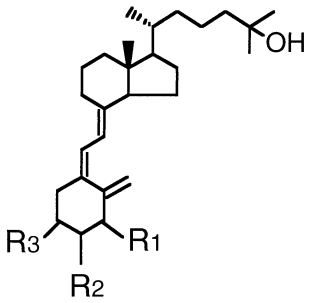
* *Abbreviations:* 2-methyl-$1\alpha,25(OH)_2D_3$, 2-methyl-1α,25-dihydroxyvitamin D_3; 2-methyl-20-epi-$1\alpha,25(OH)_2D_3$, 2-methyl-20-epi-1α,25-dihydroxyvitamin D_3; $1\alpha,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; D_3, vitamin D_3; OH or $(OH)_2$, hydroxy or dihydroxy; KH-1060, 20-epi-22-oxa-24a,26a,27a-trihomo-$1\alpha,25(OH)_2D_3$; DBP, vitamin D-binding protein; VDR, vitamin D receptor; RXR, retinoid X receptor; VDRE, vitamin D response element; CMV, cytomegalovirus; DBD, DNA-binding domain; BS, binding site; and FITC, fluorescein isothiocyanate.

MATERIALS AND METHODS

Chemicals, Antibody, and Cell Culture

$1\alpha,25(OH)_2D_3$ was obtained from Solvay-Duphar Co. and HL-60 cells from Dr. Y. Seino of the Okayama University School of Medicine. The cells were maintained in continuous culture in RPMI-1640 medium (Nissui Seiyaku Co. Ltd.) supplemented with 10% dextran-coated charcoal-treated fetal bovine serum (GIBCO BRL), 0.06 mg/mL kanamycin (Sigma). The doubling time of HL-60 cells was approximately 24 hr. The human fluorescein isothiocyanate (FITC) conjugated antibody CD11b was obtained from Sigma. All possible ring A diastereomers of 2-methyl-$1\alpha,25(OH)_2D_3$ and 2-methyl-20-epi-$1\alpha,25(OH)_2D_3$ were synthesized according to Konno *et al.* [17] and Fujishima *et al.* [18]. On the basis of the configuration of a methyl group at C-2, the 2-methyl ring A diastereomers were classified into two groups, namely 2β-methyl or 2α-methyl ring A diastereomers. The 2β-methyl ring A diastereomers included 2β-methyl-$1\alpha,25(OH)_2D_3$, as exemplified by the 2β-(1α,3β) isomer (the Greek letters denote the configurations at the C-1 and C-3 hydroxyl groups and the C-2 methyl group in the vitamin D numbering system), and the 2β-methyl-3-epi-$1\alpha,25(OH)_2D_3$ [2β-(1α,3α)], 2β-methyl-$1\beta,25(OH)_2D_3$ [2β-(1β,3β)], and 2β-methyl-3-epi-$1\beta,25(OH)_2D_3$ [2β-(1β,3α)] isomers. Similarly, the 2α-methyl ring A diastereomers included 2α-methyl-$1\alpha,25(OH)_2D_3$ [2α-(1α,3β)], 2α-methyl-3-epi-$1\alpha,25(OH)_2D_3$ [2α-(1α,3α)], 2α-methyl-$1\beta,25(OH)_2D_3$ [2α-(1β,3β)], and 2α-methyl-3-epi-$1\beta,25(OH)_2D_3$ [2α-(1β,3α)] isomers. The 2-methyl-20-epi ring A diastereomers were also classified into two groups, namely 2β-methyl-20-epi or 2α-methyl-20-epi ring A diastereomers. The 2β-methyl-20-epi ring A diastereomers included 2β-methyl-20-epi-$1\alpha,25(OH)_2D_3$ [2β-20-epi-(1α,3β)], 2β-methyl-20-epi-3-epi-$1\alpha,25(OH)_2D_3$ [2β-20-epi-(1α,3α)], 2β-methyl-20-epi-$1\beta,25(OH)_2D_3$[2β-20-epi-(1β,3β)], and 2β-methyl-20-epi-3-epi-$1\beta,25(OH)_2D_3$[2β-20-epi-(1β,3α)] isomers. The 2α-methyl-20-epi ring A diastereomers included 2α-methyl-20-epi-$1\alpha,25(OH)_2D_3$ [2α-20-epi-(1α,3β)], 2α-methyl-20-epi-3-epi-$1\alpha,25(OH)_2D_3$ [2α-20-epi-(1α,3α)], 2α-methyl-20-epi-$1\beta,25(OH)_2D_3$ [2α-20-epi-(1β,3β)], and 2α-methyl-20-epi-3-epi-$1\beta,25(OH)_2D_3$ [2α-20-epi-(1β,3α)] isomers. All sixteen analogues tested are shown in Figs. 1 and 2. The analogues were dissolved in aldehyde-free absolute ethanol as stock solutions at 10^{-4} M and stored at −35° protected from light. All other reagents were of the highest analytical grade commercially available.

Ring-A Diastereoisomers of 2-Methyl-1,25(OH)$_2$D$_3$



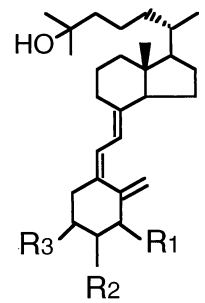
code name	R1	R2	R3
2β - (1α,3β)	1α-OH	2β-CH3	3β-OH
2β - (1α, 3α)	1α-OH	2β-CH3	3α-OH
2β - (1β, 3β)	1β-OH	2β-CH3	3β-OH
2β - (1β, 3α)	1β-OH	2β-CH3	3α-OH
2α - (1α, 3β)	1α-OH	2α-CH3	3β-OH
2α - (1α, 3α)	1α-OH	2α-CH3	3α-OH
2α - (1β, 3β)	1β-OH	2α-CH3	3β-OH
2α - (1β, 3α)	1β-OH	2α-CH3	3α-OH

FIG. 1. Chemical structure and code name assigned to the 2-methyl analogues of 1α,25(OH)$_2$D$_3$.

Transfection and Luciferase Activity Assay

MG-63 cells, which are positive for VDR and RXR gene expression, were maintained in Dulbecco's modified Eagle's medium (GIBCO BRL) supplemented with 1% penicillin, 1% streptomycin, and 10% dextran-coated charcoal-treated fetal bovine serum (GIBCO BRL). Cells (2×10^5) were suspended in 2 mL of the medium and transfected with 0.5 μg luciferase reporter plasmid (pGVB2 vector, Toyo Ink Co., Ltd.) inserted with a rat 25(OH)D_3-24-hydroxylase gene promoter (−291/+9) including the two VDREs [20] or a human osteocalcin gene promoter (−848/+10) including the VDRE [21] and 0.25 μg of the pRL-CMV vector (Toyo Ink Co., Ltd.) as an internal control using the Tfx-50 reagent (Promega Corp.). HeLa cells were maintained in Eagle's modified essential medium (Nissui Seiyaku Co., Ltd.) supplemented with 1% L-glutamine and 10% dextran-coated charcoal-treated fetal bovine serum (GIBCO BRL). Cells (2×10^5) were suspended in 2 mL of medium and transfected with 0.5 μg of a one-hybrid plasmid (pM vector, Promega Corp.) containing a human VDR cDNA connected with a yeast GAL4–DBD cDNA, 0.5 μg of luciferase reporter plasmid (pGVP2 vector, Toyo Ink Co., Ltd.) containing GAL-BS, and a pRL-CMV vector as an internal control using the LipofectAMINE reagent (GIBCO BRL). HeLa cells (2×10^5) were suspended in 2 mL of the medium and transfected with 0.5 μg of a pM vector containing a human RXRα cDNA connected to GAL–DBD, 0.5 μg of human VDR expression plasmid (pSG5-hVDR) [22], 0.5 μg of pGVP2 vector con-

Ring-A Diastereoisomers of 2-Methyl-20-epi-1,25(OH)$_2$D$_3$



code name	R1	R2	R3
2β-20-epi- (1α,3β)	1α-OH	2β-CH3	3β-OH
2β-20-epi- (1α, 3α)	1α-OH	2β-CH3	3α-OH
2β-20-epi- (1β, 3β)	1β-OH	2β-CH3	3β-OH
2β-20-epi- (1β, 3α)	1β-OH	2β-CH3	3α-OH
2α-20-epi- (1α, 3β)	1α-OH	2α-CH3	3β-OH
2α-20-epi- (1α, 3α)	1α-OH	2α-CH3	3α-OH
2α-20-epi- (1β, 3β)	1β-OH	2α-CH3	3β-OH
2α-20-epi- (1β, 3α)	1β-OH	2α-CH3	3α-OH

FIG. 2. Chemical structure and code name assigned to the 2-methyl-20-epi analogues of 1α,25(OH)$_2$D$_3$.

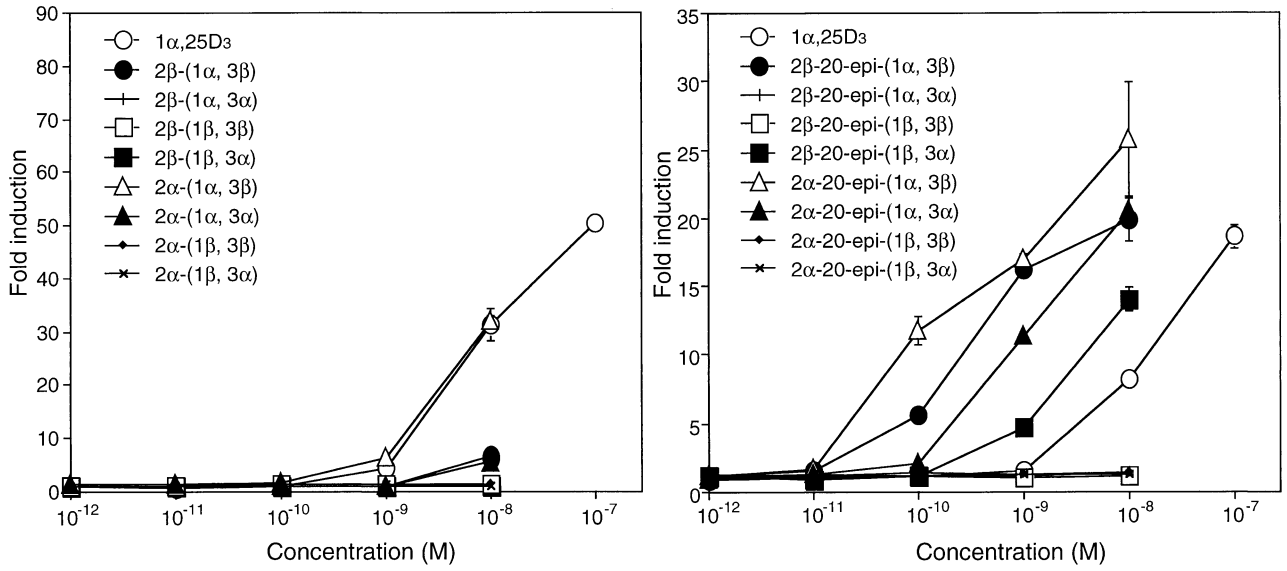


FIG. 3. Dose–response curves for $1\alpha,25(OH)_2D_3$ and its analogues induced rat $25(OH)D_3$-24-hydroxylase gene luciferase activity in MG-63 cells. The cells were co-transfected with a luciferase reporter plasmid (pGVB2 vector) containing a rat $25(OH)D_3$-24-hydroxylase gene promoter (−291/+9) including two VDREs and a pRL-CMV vector as an internal control. After 2 days of culture with the compounds, cell lysates were prepared and light production from luciferase was measured as described in Materials and Methods. Luciferase activities induced by $1\alpha,25(OH)_2D_3$ and its analogues in MG-63 cells were quantified and represented as fold induction as compared with luciferase activity observed in the vehicle-treated cells. Results represent the means of three experiments and standard errors at 10^{-12}–10^{-8} M.

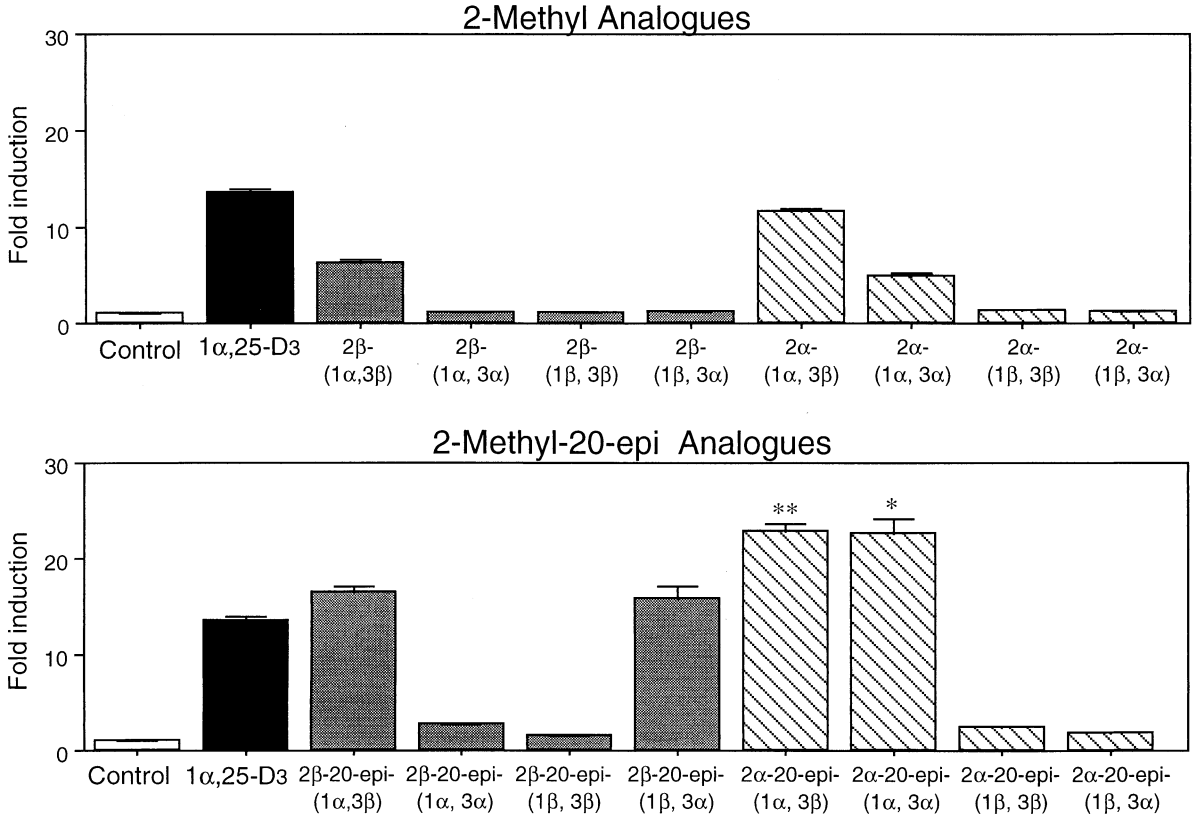


FIG. 4. Transcriptional potency of $1\alpha,25(OH)_2D_3$ and its analogues on a human osteocalcin gene in MG-63 cells. The cells were co-transfected with a luciferase reporter plasmid (pGVB2 vector) containing a human osteocalcin gene promoter (−848/+10) including a VDRE and a pRL-CMV vector as an internal control. After 2 days of culture with the compounds, cell lysates were prepared and light production from luciferase was measured as described in Materials and Methods. Luciferase activities induced by $1\alpha,25(OH)_2D_3$ and its analogues in MG-63 cells were quantified and represented as fold induction as compared with luciferase activity observed in the control cells. Results represent the means of three experiments (values in column) and standard errors (vertical bars) at 10^{-8} M. *$P < 0.05$; **$P < 0.01$.

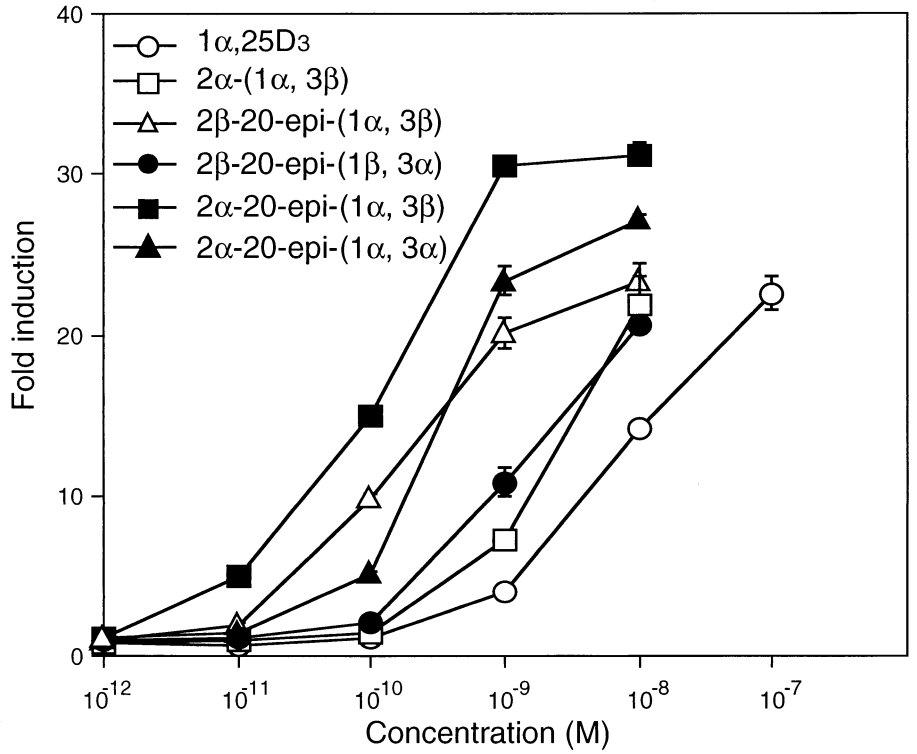


FIG. 5. Dose–response curves for 1α,25(OH)$_2$D$_3$, 2α-(1α,3β), 2β-20-epi-(1α,3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3β), and 2α-20-epi-(1α,3α) induced human osteocalcin gene luciferase activity in MG-63 cells. The cells were co-transfected with a luciferase reporter plasmid (pGVB2 vector) inserted with a human osteocalcin gene promoter (−848/+10) including a VDRE and a pRL-CMV vector as an internal control. Results represent the means ± standard errors of three separate experiments.

taining GAL-BS, and a pRL-CMV vector as an internal control using the LipofectAMINE reagent. The cells were incubated with various concentrations of 1α,25(OH)$_2$D$_3$ or an analogue for 2 days [23, 24]. The luciferase activities of the cell lysates were measured with a luciferase assay system (Toyo Ink Co., Ltd.), according to the manufacturer's instructions. Transactivation measured by luciferase activity was standardized with the luciferase activity of the same cells determined by the Sea Pansy luciferase assay system as a control (Toyo Ink Co., Ltd.) [25]. Each set of experiments was repeated at least three times, and the results are presented in terms of fold induction as means ± standard errors. Both luciferase reporter systems are based on VDR-induced transactivation in HeLa cells. In a VDR–GAL4 one-hybrid system, a GAL4-fused VDR is induced by an expression vector in transiently transfected HeLa cells and binds to a GAL-BS of co-transfected luciferase reporter plasmid which, in turn, stimulates luciferase activity. Thus, the stimulations of luciferase activity by 1α,25(OH)$_2$D$_3$ and analogues are mediated only by the chimeric VDR independent of the VDR–RXR heterodimer formation and its binding to VDRE. This system allows us to estimate *in situ* the VDR binding affinity of 1α,25(OH)$_2$D$_3$ and analogues. In an expressed VDR/RXRα–GAL4 modified two-hybrid system, a GAL4-fused RXRα is induced by an expression vector in transiently transfected HeLa cells. A VDR expressed by pSG5-hVDR forms a heterodimer with a GAL4-fused RXRα, and this complex binds to a GAL-BS of co-transfected luciferase reporter plasmid which, in turn, stimulates luciferase activity. Thus, stimulations of luciferase activity by 1α,25(OH)$_2$D$_3$ and analogues are mediated by the VDR–RXRα heterodimer independent of its binding to VDRE.

Cell Surface Antigen Expression Analysis

HL-60 cells were seeded at 10^5 cells/well in 24-well plates and incubated for 72 hrs with between 10^{-10} M and 10^{-7} M of 1α,25(OH)$_2$D$_3$ or an analogue at 37° in a humidified atmosphere of 5% carbon dioxide in air. The cells were then washed with PBS and adjusted to 2×10^6 cells/100 μL of diluent solution (without calcium and magnesium) containing 1% BSA and 1% NaN_3. Aliquots of cell suspension (100 μL) were incubated with 10 μL of the human monoclonal FITC-conjugated CD11b antibody for 30 min at room temperature without light. The cells were washed once with diluent solution and then fixed in 500 μL of PBS containing 2% paraformaldehyde. Fluorescence was read on a Beckton Dickinson FACScan™ at excitation wavelength of 490 nm and emission wavelength of 520 nm. Results for this measurement were recorded as the mean fluorescence index, which is the product of the % fluorescence and the mean fluorescence intensity, with 10^4 cells being counted per treatment.

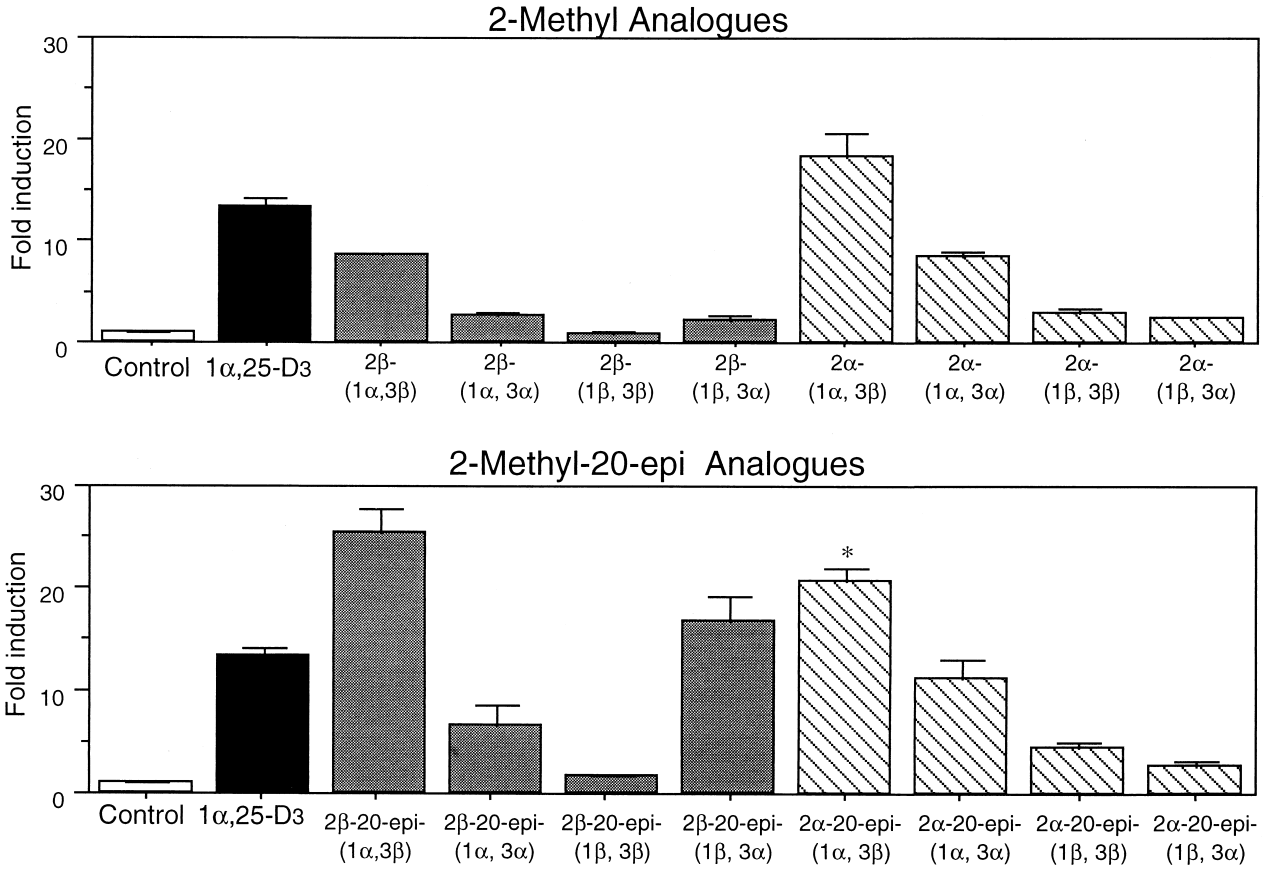


FIG. 6. Transcriptional potencies of $1\alpha,25(OH)_2D_3$ and its analogues on a human VDR–GAL4 expression gene in transfected HeLa cells. The cells were co-transfected with an expression plasmid (pM vector) inserted with a human VDR cDNA connected with GAL–DBD, a luciferase reporter plasmid (pGVP2 vector) containing GAL-BS, and a pRL-CMV vector as an internal control. After 2 days of culture with the compounds, cell lysates were prepared and light production from luciferase was measured as described in Materials and Methods. Luciferase activities induced by $1\alpha,25(OH)_2D_3$ and its analogues in HeLa cells were quantified and are represented as fold induction as compared with luciferase activity observed in the control cells. Results represent the means of three experiments (values in column) and standard errors (vertical bars) at 10^{-8} M. This system enabled us to assess direct VDR-mediated transcriptional activity of the compounds in the cells. In this system, neither RXRα nor VDRE takes part in transcriptional activity of $1\alpha,25(OH)_2D_3$ and its analogues. *$P < 0.05$.

Statistical Analysis

Statistical significances were determined using Student's *t*-test and expressed as means ± SEM.

RESULTS

Transactivations on Target Genes

Transcriptional potencies of $1\alpha,25(OH)_2D_3$ and the analogues at different concentrations on a rat $25(OH)D_3$-24-hydroxylase gene promoter containing two VDREs in transfected MG-63 cells are shown in Fig. 3. Of the 2-methyl analogues, the 2α-(1α,3β) isomer exhibited comparable potency to $1\alpha,25(OH)_2D_3$, while the rest of the isomers had weak or virtually no potency. 2β-Methyl analogues and 2α-methyl analogues with a 1β-hydroxyl group also had no potency. On the other hand, of the 2-methyl-20-epi analogues, the 1α-isomers [2β-20-epi-(1α,3β), 2α-20-epi-(1α,3β), and 2α-20-epi-(1α,3α)], except for the 2β-20-epi-(1α,3α) isomer, exhibited remarkably high potencies in a concentration-dependent manner. The 1β-isomers [2β-20-epi-(1β,3β), 2α-20-epi-(1β,3β), and 2α-20-epi-(1β,3α)], except for the 2β-20-epi-(1β,3α) isomer, had virtually no potency. Interestingly, the 2β-20-epi-(1β,3α) isomer, which has different configurations of the C-1 and C-3 hydroxyl groups and of the C-20 methyl group from the natural configurations of $1\alpha,25(OH)_2D_3$, exhibited a greater potency than $1\alpha,25(OH)_2D_3$ at concentrations of 10^{-9} and 10^{-8} M. While the 2β-20-epi-(1α,3β) and 2β-20-epi-(1β,3α) isomers exhibited significantly higher potencies than $1\alpha,25(OH)_2D_3$, the corresponding 2β-methyl counterparts [(2β-(1α,3β) and 2β-(1β,3α)] had almost no potency. The transcriptional potencies of $1\alpha,25(OH)_2D_3$ and the analogues on a human osteocalcin gene promoter including a VDRE in transfected MG-63 cells are shown in Figs. 4 and 5. The human osteocalcin gene transactivation results for the analogues were very consistent with those for rat $25(OH)D_3$-24-hydroxylase gene transactivation. To de-

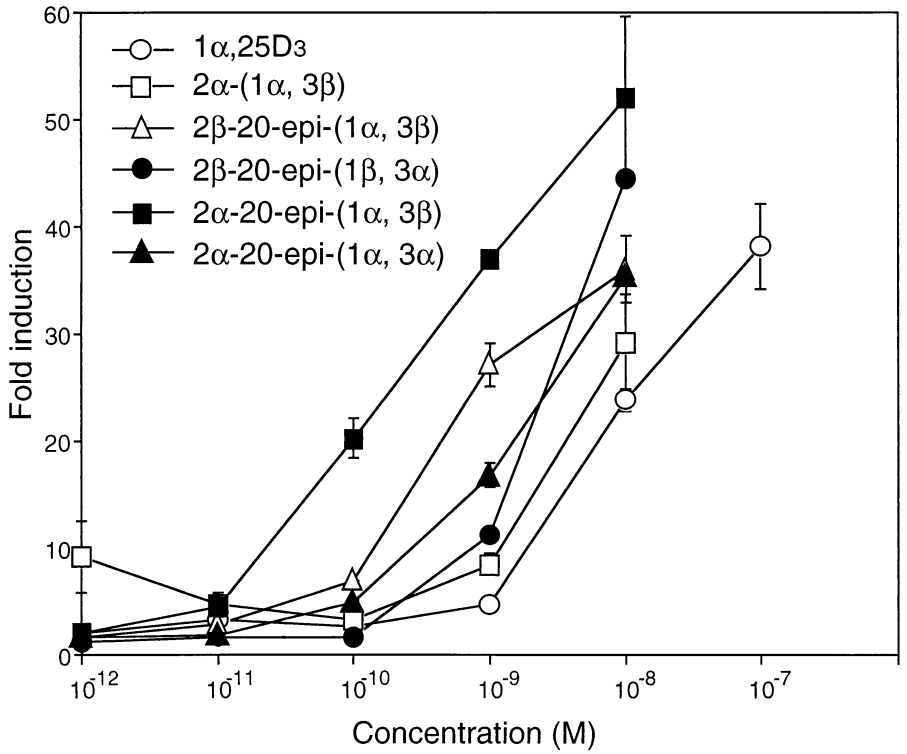


FIG. 7. Dose–response curves for 1α,25(OH)$_2$D$_3$, 2α-(1α,3β), 2β-20-epi-(1α,3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3β), and 2α-20-epi-(1α,3α)-induced human VDR–GAL4 expression gene luciferase activity in transfected HeLa cells. The cells were co-transfected with an expression plasmid (pM vector) containing a human VDR cDNA connected with GAL–DBD, a luciferase reporter plasmid (pGVP2 vector) containing GAL-BS, and a pRL-CMV vector as an internal control. Results represent the means ± standard errors of three separate experiments.

termine whether transcriptional potency is associated with binding potency to VDR, we co-transfected a one-hybrid plasmid inserted with a human VDR cDNA connected with GAL–DBD and a luciferase reporter plasmid containing GAL-BS into HeLa cells and treated the cells with various concentrations of 1α,25(OH)$_2$D$_3$ and the analogues. We obtained similar results to those observed in the above experiments (Figs. 6 and 7). To further investigate whether transcriptional potency is associated with binding potency to RXRα via binding to VDR, we co-transfected a one-hybrid plasmid inserted with a human RXRα cDNA connected with GAL–DBD, a human VDR expression plasmid, and a luciferase reporter plasmid containing GAL-BS and treated the cells with 10^{-8} M of 1α,25(OH)$_2$D$_3$ and the analogues. We obtained similar results to those observed in Fig. 8.

Effect on Cell Surface CD11b Antigen Expression

To confirm the phenotypic maturation of HL-60 cells by 1α,25(OH)$_2$D$_3$ and the analogues, cell surface CD11b antigen expression was measured using FACS analysis. Figures 9 and 10 depict the effects of 1α,25(OH)$_2$D$_3$ and the analogues at various concentrations for 72 hrs on cell surface CD11b antigen expression in HL-60 cells. A significant increase in the CD11b antigen positive cells was observed in the 1α,25(OH)$_2$D$_3$, 2α-(1α,3β), 2β-20-epi-(1α,3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3α), and 2α-20-epi-(1α,3β) isomer-treated cells as compared to the vehicle-treated cells. The transcriptional potencies of the 2-methyl or 2-methyl-20-epi analogues in the present study and the previous results of biological activities as reported by Konno *et al.* [17] and Fujishima *et al.* [18] are summarized in Table 1. The rank orders of the transcriptional potencies of the analogues were almost parallel to those of the VDR-binding affinity and HL-60 cell differentiation, except for the 2β-20-epi-(1β,3α) and 2α-20-epi-(1α,3α) isomers, whose VDR binding affinities were only 7% and 17%, respectively of that of 1α,25(OH)$_2$D$_3$, but whose cell differentiation potencies were 135% and 705%, respectively of that of 1α,25(OH)$_2$D$_3$.

DISCUSSION

We evaluated a large number of analogues of 1α,25(OH)$_2$D$_3$ to investigate the structure–activity relationships and to develop potential therapeutic agents. A majority of analogues had an altered side chain. 20-epi-1α,25(OH)$_2$D$_3$ belongs to a group of 1α,25(OH)$_2$D$_3$ analogues characterized by an inverted stereochemistry at C-20 of the side chain. A previous study suggested that 20-epi-1α,25(OH)$_2$D$_3$ is a highly potent growth inhibitor and an inducer of differentiation of many

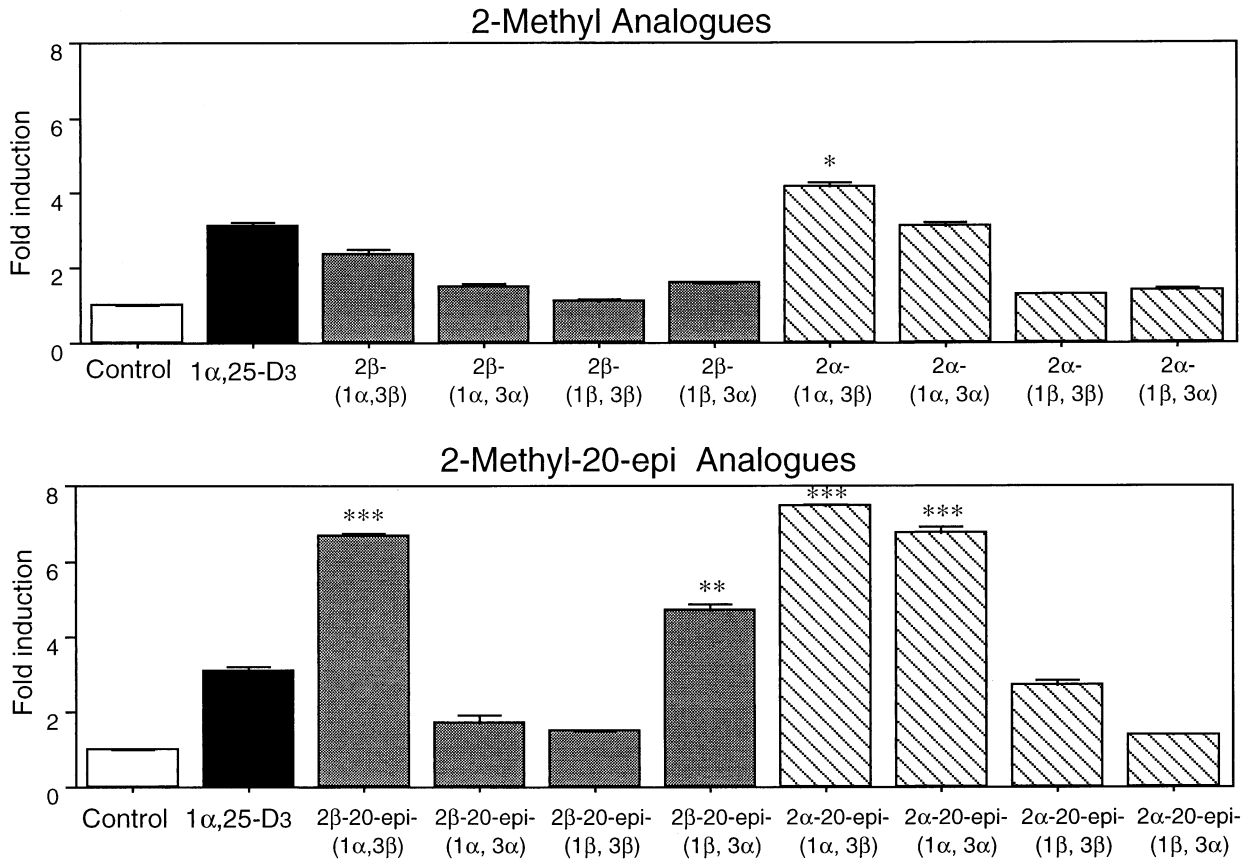


FIG. 8. Transcriptional potencies of $1\alpha,25(OH)_2D_3$ and its analogues on a human RXRα–GAL4 expression gene in transfected HeLa cells. The cells were co-transfected with an expression plasmid (pM vector) containing a human RXRα cDNA connected with GAL–DBD, human VDR expression plasmid (pSG5-hVDR), a luciferase reporter plasmid containing GAL-BS, and a pRL-CMV vector as an internal control. After 2 days of culture with the compounds, cell lysates were prepared and light production from luciferase was measured as described in Materials and Methods. Luciferase activities induced by $1\alpha,25(OH)_2D_3$ and its analogues in HeLa cells were quantified and are represented as fold induction as compared with luciferase activity observed in the control cells. Results represent the means of three experiments (values in column) and standard errors (vertical bars) at 10^{-8} M. This system enabled us to assess direct VDR/RXRα-mediated transcriptional activity of the compounds in the cells. In this system, a VDR/RXRα heterodimer bound to the derivative directly induced an increase in luciferase activity without interactions with VDRE. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

malignant cells. It also exerts calcemic actions comparable to those of $1\alpha,25(OH)_2D_3$ when tested in rats. The alteration of stereochemistry at C-20 on the side chain is the only difference between $1\alpha,25(OH)_2D_3$ and 20-epi-$1\alpha,25(OH)_2D_3$. The reason for this is not yet known, but several possibilities have been proposed. Since the side chain of $1\alpha,25(OH)_2D_3$ is very flexible, it can be expected that numerous low-energy conformations are accessible. It is not yet known which side-chain conformation(s) represents that of the ligand bound to the VDR. Inversion of the stereochemistry at C-20 allows the side chain to attain a different population of side-chain conformations, of which a small proportion is common to those attained by the natural orientation [26]. Elstner *et al.* [27] suggested that the exceptional potency of 20-epi-$1\alpha,25(OH)_2D_3$ may be attributed to the greater ease with which the side chain of 20-epi-$1\alpha,25(OH)_2D_3$ can access the appropriate side-chain hydroxyl topology and be more optimally reorganized into the biologically competent orientation. It has been shown that 20-epimerization is an element enhancing biological activity in many vitamin D analogues. Konno *et al.* [17] and Fujishima *et al.* [18] previously reported that 2α-methyl-20-epi-$1\alpha,25(OH)_2D_3$ exhibited 12-fold higher binding affinity for VDR, 600-fold higher potency in inducing HL-60 cell differentiation, and 6.55-fold higher bone calcium mobilization activity in normal rats as compared to $1\alpha,25(OH)_2D_3$. These results indicate that 2α-methyl-20-epi-$1\alpha,25(OH)_2D_3$ is a potent analogue having comparable activity to KH-1060, the most potent analogue reported to date. Further, 2α-methyl-20-epi-$1\alpha,25(OH)_2D_3$ is generated with a 2α-methyl substitution to ring A as opposed to KH-1060, which is generated by a combination of 22-oxa and elongation of C-26 and C-27 in the side chain of 20-epi-$1\alpha,25(OH)_2D_3$.

In the present study, we demonstrated, using transient transfection luciferase assay systems, that 2α-methyl-20-epi-$1\alpha,25(OH)_2D_3$ also exhibits exceptionally high potency at the transcriptional level. Our study revealed that it binds to the VDR 68.75 times stronger than $1\alpha,25(OH)_2D_3$ and its liganded VDR binds to RXRα 2.4 times stronger

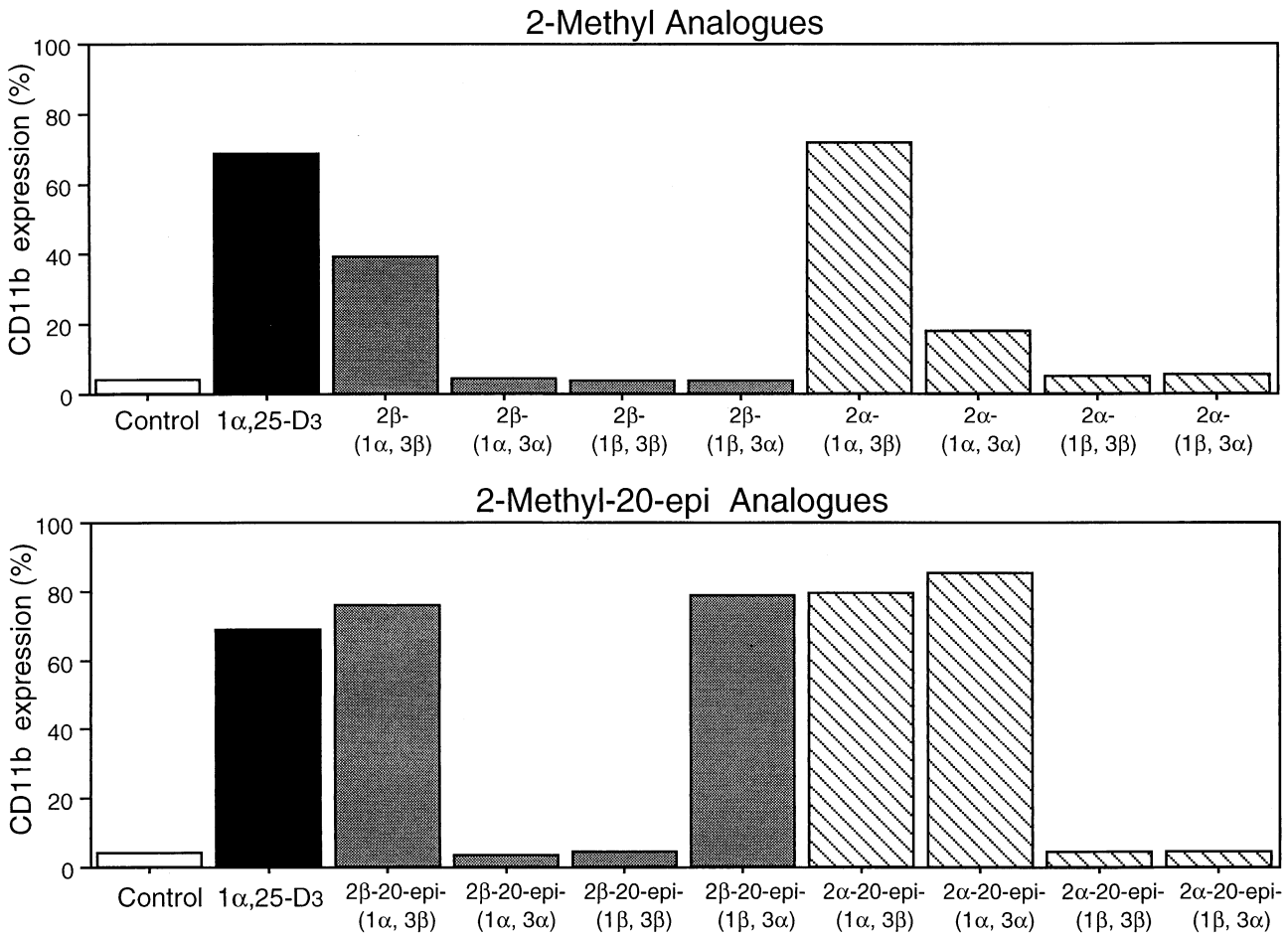


FIG. 9. Expression of cell surface CD11b antigen in HL-60 cells treated with 1α,25(OH)$_2$D$_3$ or its analogues. HL-60 cells were incubated for 72 hrs with 10^{-8} M of 1α,25(OH)$_2$D$_3$ or the analogues at 37° in a humidified atmosphere of 5% carbon dioxide in air. The cells were then incubated with 10 μL of the human monoclonal FITC-conjugated CD11b antibody for 30 min at room temperature without light. Fluorescence was read on a Beckton Dickinson FACScan™ at an excitation wavelength of 490 nm and an emission wavelength of 520 nm as described in Materials and Methods. Results were recorded as the mean fluorescence index ± SEM, which is the product of the % fluorescence and the mean fluorescence intensity, with 10^4 cells being counted per treatment. Values are representative of three independent experiments.

than 1α,25(OH)$_2$D$_3$, resulting in 236.36- and 119.05-fold higher potencies than 1α,25(OH)$_2$D$_3$ in transactivating the rat 25(OH)D$_3$-24-hydroxylase gene and human osteocalcin gene in transfected MG-63 cells. It has recently been shown that the increased activity induced by vitamin D analogues was associated with a more efficient heterodimerization with the RXR and a more stable form of the heterodimer due to conformational changes of the VDR. It is thus conceivable that 2α-methyl-20-epi-1α,25(OH)$_2$D$_3$ might form a liganded VDR/RXR heterodimer more efficiently or that the heterodimer might be more stable than the 1α,25(OH)$_2$D$_3$-liganded VDR/RXR heterodimer. Since our modified two-hybrid system using the RXRα–GAL4 luciferase reporter assay (Fig. 8) can only detect VDR/RXRα heterodimerization efficacy, these possibilities should be tested by gel mobility shift assays for VDR/RXR/VDRE complexes. It was found that there was a poor correlation between HL-60 cell differentiation activity and VDR-binding affinity for both 2β-20-epi-(1β,3α) and 2α-20-epi-(1α,3α) analogues (Table 1). We also found that the transcriptional potencies of the two analogues on the rat 25(OH)D$_3$-24-hydroxylase gene and the human osteocalcin gene in transfected MG-63 cells were almost parallel to their VDR–GAL4 luciferase activity in transfected HeLa cells. The reason for the remarkable differences between the *in vitro* VDR-binding potencies (7% and 17% compared to 1α,25(OH)$_2$D$_3$) and the *in situ* VDR-binding potencies (324% and 423% compared to 1α,25(OH)$_2$D$_3$) is unknown. It is generally accepted that *in vitro* displacement assays using 1α,25(OH)$_2$D$_3$ as a radioactive ligand can predict the apparent binding affinity of vitamin D analogues for VDR, but stability for liganded VDR cannot be predicted by this method. In contrast, the *in situ* VDR-binding assay can indicate the magnitude of direct interaction of vitamin D analogues with the VDR. It is thus conceivable that both the 2β-20-epi-(1β,3α) and 2α-20-epi-(1α,3α) analogues are able to mediate

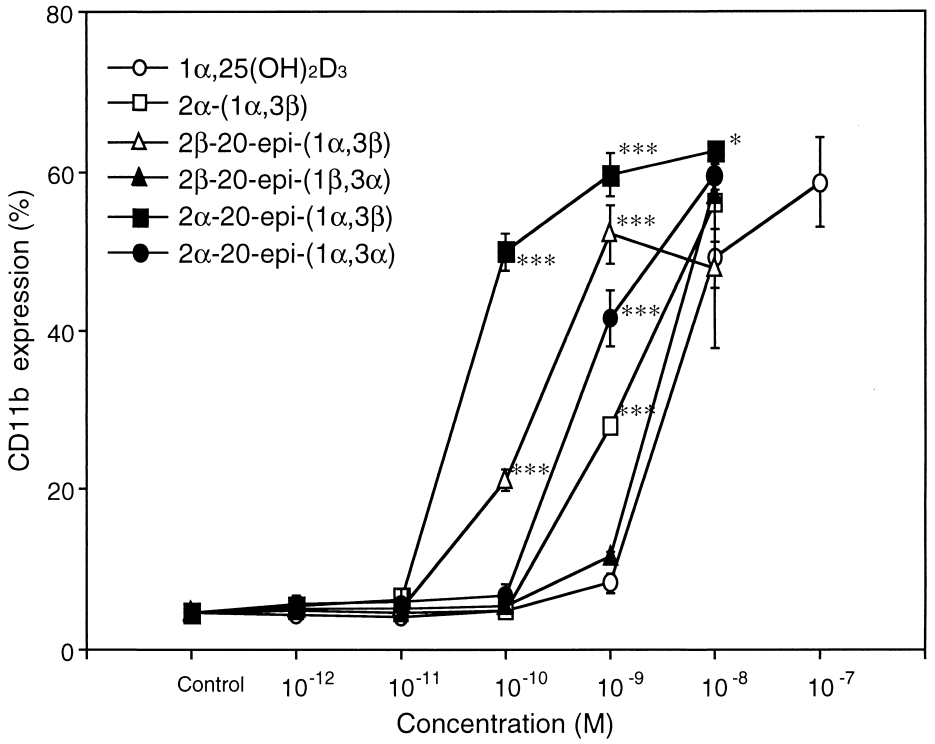


FIG. 10. Dose–response curves for $1\alpha,25(OH)_2D_3$, 2α-(1α,3β), 2β-20-epi-(1α,3β), 2β-20-epi-(1β,3α), 2α-20-epi-(1α,3β), and 2α-20-epi-(1α,3α)-induced expression of cell surface CD11b antigen in HL-60 cells. Results were recorded as the mean fluorescence index ± SEM, which is the product of the % fluorescence and the mean fluorescence intensity, with 10^4 cells being counted per treatment. Values are representative of three independent experiments. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

TABLE 1. The relative potency of the 2-methyl-1α,25-dihydroxyvitamin D_3 and 2-methyl-20-epi-1α,25-dihydroxyvitamin D_3 analogues

	Binding affinity [17, 18]		Transcriptional potency					
	Bovine serum DBP	Bovine thymus VDR	Rat 24-OHase	Human osteocalcin	Human VDR–GAL4	Human RXRα–GAL4	HL-60 cell differentiation	Ca regulation [17, 18]
$1\alpha,25(OH)_2D_3$	100	100	100	100	100	100	100	100
2-methyl analogues								
2β-(1α, 3β)	76	13	24†	46†	64†	76†	54†	2
2β-(1α, 3α)	21	0.3	17†	8†	20†	48†	0.5†	NT
2β-(1β, 3β)	1000	<0.1	13†	8†	7†	36†	0†	NT
2β-(1β, 3α)	1300	0.8	15†	9†	16†	51†	0†	NT
2α-(1α, 3β)	66	400	111*	278*	172*	134†	258*	400
2α-(1α, 3α)	44	4	29†	36†	64†	100†	22†	NT
2α-(1β, 3β)	200	<0.1	12†	10†	22†	41†	2†	NT
2α-(1β, 3α)	1200	<0.1	13†	9†	18†	45†	2†	NT
2-methyl-20-epi analogues								
2β-20-epi-(1α, 3β)	<0.3	160	5909*	3571*	1447*	215†	1722*	115
2β-20-epi-(1α, 3α)	<0.3	<0.1	27†	20†	49†	55†	0†	NT
2β-20-epi-(1β, 3β)	<0.3	<0.1	12†	11†	13†	49†	0.5†	NT
2β-20-epi-(1β, 3α)	<0.3	7	433*	417*	324*	143†	135*	19
2α-20-epi-(1α, 3β)	<0.3	1200	23636*	11905*	6875*	239†	9688*	655
2α-20-epi-(1α, 3α)	<0.3	17	2281*	2273*	423*	217†	705*	NT
2α-20-epi-(1β, 3β)	<0.3	<0.1	14†	18†	34†	70†	0.6†	NT
2α-20-epi-(1β, 3α)	<0.3	<0.1	14†	14†	20†	46†	0.5†	NT

*All results are expressed as percentage activity (at 50% of the dose–response) in comparison with $1\alpha,25(OH)_2D_3$ (= 100% activity).
†All results are expressed as percentage activity at 10^{-8} M in comparison with $1\alpha,25(OH)_2D_3$ (= 100% activity). NT, not tested.

their effects through a VDR-mediated signaling mechanism much more efficiently than 1α,25$(OH)_2D_3$, which is consistent with the results of cell differentiation (Figs. 9 and 10).

Another possible explanation for the difference in the *in vitro* and *in situ* VDR binding potencies might be the higher transmembrane permeability of the analogues, since the two analogues had almost no binding affinity for DBP, which inhibits cellular uptake of vitamin D analogues from the medium. It is surprising that the 2β-(1β,3α) analogue is virtually biologically inactive, but its 20-epimer 2β-20-epi-(1β,3α) analogue is much more active than 1α,25$(OH)_2D_3$ despite its only 7% VDR affinity relative to 1α,25$(OH)_2D_3$. A mechanistic explanation for the high potency of the 2β-20-epi-(1β,3α) analogue cannot be given at present. From the results of the VDR–GAL4 and RXRα–GAL4 luciferase assays, it is clear that the 2β-20-epi-(1β,3α) analogue, having inverted stereochemistry at the C-1 and C-3 hydroxyl groups and the C-20 side chain, can bind to VDR, form a heterodimer with RXRα, and transactivate specific target genes more efficiently than 1α,25$(OH)_2D_3$. To our knowledge, there are no similar findings from the hybrid analogues with ring A and side-chain modifications. Future studies are required to elucidate this unique phenomenon.

In summary, we have evaluated transcriptional activity and cell regulatory effects of all possible ring A diastereomers of the 2-methyl and 2-methyl-20-epi analogues of 1α,25$(OH)_2D_3$. These analogues exhibited unique biological activity profiles depending upon ring A and/or side chain configurations. Of the sixteen analogues tested, 2α-methyl-20-epi-1α,25$(OH)_2D_3$ was the most potent stimulator of specific target gene expressions and the most potent modulator of HL-60 cell differentiation, presumably having comparable potency to KH-1060, the most potent analogue reported to date. In addition, 2β-20-epi-3-epi-1β,25$(OH)_2D_3$ was the most unique analogue, exhibiting both inverted stereochemistry and high biological potency. These analogues may be useful for the development of analogues of 1α,25$(OH)_2D_3$ for biomedical applications.

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