Biological Activity Profiles of $1\alpha,25$ -Dihydroxyvitamin D_2 , D_3 , D_4 , D_7 , and 24-Epi- $1\alpha,25$ -dihydroxyvitamin D_2

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We have synthesized several $1\alpha,25$ -dihydroxyvitamin D [$1\alpha,25(OH)_2D$] derivatives and evaluated their biological activity in terms of their binding affinity for the vitamin D receptor (VDR) and vitamin D-binding protein (DBP), antiproliferative or differentiation-inducing effects on human promyelocytic leukemic HL-60 cells, and transcriptional activity on a rat 25-hydroxyvitamin D_3 -24-hydroxylase gene promoter, including two vitamin D-responsive elements (VDREs), and human osteocalcin gene promoter, including a VDRE in transfected human osteosarcoma MG-63 cells. Furthermore, human VDR- or retinoic acid X receptor α (RXR α)-mediated luciferase activities of the derivatives were also measured by a one-hybrid system in human epitheloid carcinoma, cervix HeLa cells and African green monkey kidney CV-1 cells. Binding affinity for VDR, bone-resorbing activity, antiproliferative and cell-differentiating effects, transactivation potencies on target genes and VDR- or RXR α -mediated gene regulations of $1\alpha,25(OH)_2D_2$ and $1\alpha,25(OH)_2D_4$ were almost comparable to the effects of $1\alpha,25(OH)_2D_3$, while 24-epi- $1\alpha,25(OH)_2D_2$ and $1\alpha,25(OH)_2D_7$ were much less active than $1\alpha,25(OH)_2D_3$ in these respects. This is the first report concerning biological assessment of $1\alpha,25(OH)_2D_2$, $1\alpha,25(OH)_2D_3$, $1\alpha,25(OH)_2D_4$, 24-epi- $1\alpha,25(OH)_2D_2$ and $1\alpha,25(OH)_2D_7$ at the molecular level, especially with regards to the structural differences at the 24R- or 24S-methyl group and a double bond between carbons 22 and 23 in the side chain of $1\alpha,25(OH)_2D$ derivatives.

Key words $1\alpha,25$ -dihydroxyvitamin D derivative; receptor binding; cell proliferation; cell differentiation; transactivation; bone resorption

The physiologically active metabolite of vitamin D_3 , $1\alpha,25$ -dihydroxyvitamin D_3 [$1\alpha,25(OH)_2D_3$],¹⁾ regulates calcium metabolism in intestine, kidney and bone, as well as maturation and differentiation of normal and malignant cells and immune responsiveness.^{2–4)} Biological activities of $1\alpha,25(OH)_2D_3$ are predominantly expressed through transcriptional regulation resulting from the interaction of $1\alpha,25(OH)_2D_3$ with the vitamin D receptor (VDR),⁵⁾ which is a member of the superfamily of nuclear receptors,^{6,7)} and is similar to the receptors for thyroid hormone, retinoic acid, estrogen, and glucocorticoids.^{8,9)} The $1\alpha,25(OH)_2D_3$ -liganded VDR heterodimerizes with the retinoid X receptor (RXR)¹⁰⁾ and this complex binds to specific DNA sequences, the vitamin D-responsive element (VDRE), to induce gene transcription.¹¹⁾ The finding that $1\alpha,25(OH)_2D_3$ serves as a potent multifunctional biological response modifier in many tissues and cells¹²⁾ implies promising therapeutic applications of this metabolite for the treatment of cancers,¹³⁾ psoriasis^{14,15)} and immune disorders.¹⁶⁾ The major limitation of $1\alpha,25(OH)_2D_3$ therapy is, however, its accompanying calcemic activity.¹⁷⁾ For this reason, $1\alpha,25(OH)_2D_3$ analogues having side-chain modifications with low calcemic activity have been extensively studied.¹⁸⁾ Among these analogues, $1\alpha,24R$ -dihydroxyvitamin D_3 ($1\alpha,24R(OH)_2D_3$), which has a (R)-hydroxy group at carbon 24 of the side chain skeleton of $1\alpha,25(OH)_2D_3$, is a potent growth inhibitor of human psoriatic keratinocytes with reduced hypercalcemic activity.^{19,20)} 1α -Hydroxyvitamin D_2 (a prodrug of $1\alpha,25(OH)_2D_2$) is a synthetic analogue with a (S)-methyl group of carbon 24 and a double bond between carbons 22 and 23 in its side chain

skeleton. This has been shown to be readily metabolized in the liver to $1\alpha,25(OH)_2D_2$ and reduces the number of osteoclasts in bone, resulting in a significant increase in bone mineral contents in normal rats as compared to $1\alpha,25(OH)_2D_3$, which increases the osteoclast number and slightly reduces bone mineral contents.^{21,22)} The mortality rate was five to fifteen times higher in rats intoxicated with 1α -hydroxyvitamin D_3 than for rats intoxicated with 1α -hydroxyvitamin D_2 .^{23,24)} Furthermore, it has been demonstrated that the relative potencies of the antirachitic activity of vitamin D_2 ,²⁵⁾ vitamin D_3 , vitamin D_4 (a 24S-methyl derivative of vitamin D_3),²⁶⁾ vitamin D_5 (a 24R-ethyl derivative of vitamin D_3),²⁷⁾ vitamin D_6 (a derivative with a 24S-ethyl and a double bond between carbons 22, 23 of vitamin D_3),²⁸⁾ and vitamin D_7 (a 24R-methyl derivative of vitamin D_3)²⁹⁾ on rachitic rats bone healing are 100, 100, 75, 2, 1 or 10, respectively. The above observations indicate that substitution of a (R)-methyl or a (S)-methyl group at carbon 24, dehydrogenation at carbons 22, 23 or a combination of both may induce remarkable changes in the biological activity of vitamin D_3 and its active metabolite, $1\alpha,25(OH)_2D_3$.

Our present study indicates that $1\alpha,25(OH)_2D_2$ and $1\alpha,25(OH)_2D_4$, both of which have a 24S-methyl group in the side chain, have comparable calcemic and non-calcemic activity to $1\alpha,25(OH)_2D_3$, but 24-epi- $1\alpha,25(OH)_2D_2$ and $1\alpha,25(OH)_2D_7$ are slightly or much less potent than $1\alpha,25(OH)_2D_3$ in the regulation of cell proliferation and differentiation and transactivation of target genes. This is the first report concerning the biological assessments of $1\alpha,25(OH)_2D_2$, $1\alpha,25(OH)_2D_3$, $1\alpha,25(OH)_2D_4$, 24-epi- $1\alpha,25$ -

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Fig. 1. Chemical Structures of $1\alpha,25(\text{OH})_2\text{D}$ Derivatives Tested in the Present Study

$(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_7$ at the molecular level, especially with respect to the structural differences in the configuration of the 24-methyl group and the role of a double bond between carbons 22 and 23 in the side chain of $1\alpha,25(\text{OH})_2\text{D}$ derivatives.

MATERIALS AND METHODS

Chemicals $1\alpha,25(\text{OH})_2\text{D}_3$ was obtained from Solvay-Duphar Co., Weesp, The Netherlands. $[23,24(n)\text{-}^3\text{H}]\text{-}25(\text{OH})\text{D}_3$ (specific activity; 3.3 TBq/mmol) and $[26,27\text{-methyl-}^3\text{H}]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ (specific activity; 6.7 TBq/mmol) were obtained from Amersham Co., Buckinghamshire, UK. $1\alpha,25(\text{OH})_2\text{D}_2$, $1\alpha,25(\text{OH})_2\text{D}_4$, 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_7$ were synthesized at Nisshin Flour Milling Co., Ltd., Saitama, Japan. The chemical structures of the $1\alpha,25(\text{OH})_2\text{D}$ derivatives tested are shown in Fig. 1. All derivatives were dissolved in aldehyde-free absolute ethanol as stock solutions at 10^{-4} M and stored at -35°C protected from light. All other reagents were of the highest analytical grade commercially available.

Binding Assay for Rat Serum Vitamin D-Binding Protein (DBP) Competitive displacement of $[23,24(n)\text{-}^3\text{H}]\text{-}25(\text{OH})\text{D}_3$ from vitamin D-deficient rat serum DBP by the $1\alpha,25(\text{OH})_2\text{D}$ derivatives was determined under equilibrium ligand-binding conditions.³⁰⁾ 82 fmol $[^3\text{H}]\text{-}25(\text{OH})\text{D}_3$ in 50 μl ethanol was mixed with increasing amounts of the derivatives (0.24–1000 ng) in 100 μl ethanol. Next, 1 ml of vitamin D-deficient rat serum diluted 1:70000 with freshly prepared barbital acetate buffer (3.5 mM acetic acid, 3.5 mM sodium barbiturate, 0.13 M sodium chloride, pH 8.6) was added, and the samples were incubated at 0°C for 1 h. To separate bound and free $[^3\text{H}]\text{-}25(\text{OH})\text{D}_3$, 500 μl of dextran charcoal (0.025% dextran T-150, 0.25% Norit A Charcoal Decolorizing Neutral in freshly prepared barbital acetate buffer, treated by ultrasonication and stirred well before addition) was used. The assay tubes were vortexed and centrifuged at 3000 rpm for 15 min at 4°C . Following centrifugation, 1 ml of each supernatant was collected and transferred into a scintillation vial to measure radioactivity.

Binding Assay for Calf-Thymus VDR Displacement of $[^3\text{H}]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ from calf-thymus cytosol receptor (Yamasa Shoyu Co. Ltd., Chiba, Japan) by $1\alpha,25(\text{OH})_2\text{D}$ derivatives was determined as described elsewhere.³¹⁾ Solutions containing 500 μl of the calf-thymus cytosol receptor prepared with phosphate buffer (0.3 M KCl, 0.05 M K_2HPO_4 ,

0.05 M KH_2PO_4 , pH 7.4) were mixed with increasing amounts of the derivatives (0.25–256 pg) in 20 μl ethanol and the samples were incubated at 20°C for 1 h. Next, 34 fmol $[^3\text{H}]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ in 25 μl ethanol was added and the samples were incubated at 20°C for 1 h. The addition of 200 μl of dextran charcoal (0.05% dextran T-150, 0.5% Norit A Charcoal Decolorizing Neutral) in freshly prepared phosphate buffer (0.05 M Na_2HPO_4 , 0.05 M NaH_2PO_4 , pH 7.5 buffer, treated by ultrasonication and stirred well before addition) was used to separate bound and free $[^3\text{H}]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$. The assay tubes were incubated for 10 min on ice and centrifuged at 3000 rpm for 10 min at 4°C . 500 μl of each supernatant was collected and transferred into a scintillation vial to measure radioactivity.

Assessment of Cell Proliferation and Differentiation Human promyelocytic leukemic HL-60 cells were cultured in RPMI-1640 medium supplemented with L-glutamine (0.29 mg/ml), kanamycin (0.06 mg/ml) and 10% heat-inactivated fetal bovine serum at 37°C in a humidified atmosphere of 5% CO_2 in air. To examine the effects of the $1\alpha,25(\text{OH})_2\text{D}$ derivatives on cell proliferation, HL-60 cells (10^5 cells/ml) were placed in twenty four-well tissue culture plates and cultured for 3 d with or without different concentrations (10^{-10} – 10^{-7} M) of the derivatives. At the end of incubation, cells were harvested and viable cell numbers were counted by the trypan-blue extrusion method. For cell differentiation studies, the number of cells exhibiting monocyte/macrophage-like morphological changes associated with differentiation were counted under a light microscope, and expressed as a percentage of the total number of the cells. This measurement was used for indexing the differentiation-inducing activity of each $1\alpha,25(\text{OH})_2\text{D}$ derivative. All experiments were performed twice and each data point represents the average of six determinations.

Transfection and Luciferase Activity Assay Human osteosarcoma MG-63 cells, which were positive for VDR and RXR gene expression, were maintained in Dulbecco's modification eagle medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) supplemented with 1% penicillin, 1% streptomycin and 10% dextran-coated charcoal-treated fetal calf serum (Gibco BRL, Grand Island, NY, U.S.A.). 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., Tokyo, Japan) inserted with a rat 25-hydroxyvitamin D_3 -24-hydroxylase gene promoter (–291/+9) including the two VDREs or a human osteocalcin gene pro-

moter ($-848/+10$) including the VDRE and $0.25\text{ }\mu\text{g}$ of the pRL-CMV vector (Toyo Ink Co., Ltd.) as an internal control using the Tfx-50 reagent (Promega Corp., Tokyo, Japan). Human epitheloid carcinoma, cervix HeLa cells were maintained in Eagle-modified essential medium (Nissui Seiyaku Co., Ltd.) supplemented with 1% L-glutamine and 10% dextran-coated charcoal-treated fetal calf serum (Gibco BRL). Cells (2×10^5) were suspended in 2 ml of the medium and transfected with $0.5\text{ }\mu\text{g}$ of an expression plasmid (pM vector, Promega Corp.) inserted with a human VDR-ligand binding domain cDNA connected with a yeast GAL4 DNA-binding domain cDNA, $0.5\text{ }\mu\text{g}$ of luciferase reporter plasmid (pGVB2 vector) inserted with GAL4 binding site (GAL2) DNA and a pRL-CMV vector as an internal control. African green monkey kidney CV-1 cells were maintained in Eagle-minimum essential medium supplemented with 1% L-glutamine and 10% dextran-coated charcoal-treated fetal calf serum. Cells (2×10^5) were suspended in 2 ml of the medium and transfected with $0.5\text{ }\mu\text{g}$ of a one-hybrid plasmid^(32,33) (pM vector) inserted with a human RXR α -ligand binding domain cDNA connected with a yeast GAL4 DNA-binding domain cDNA, $0.5\text{ }\mu\text{g}$ of human VDR expression plasmid (pSG5-hVDR), $0.5\text{ }\mu\text{g}$ of luciferase reporter plasmid (pGVB2 vector) inserted with GAL4 binding site (GAL2) DNA and a pRL-CMV vector as an internal control. The cells were incubated with the $1\alpha,25(\text{OH})_2\text{D}$ derivatives at a concentration of 10^{-8} M for 2 d. 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 (Toyo Ink Co. Ltd.)⁽³⁴⁾ as a control. Each set of experiments was repeated at least three times, and the results are presented in terms of fold-induction as means $\pm$ standard errors.

Measurements of *in Vitro* Bone-Resorbing Activity

Bone-resorbing activity was measured using a modification of the method of Raisz.⁽³⁵⁾ $^{45}\text{CaCl}_2$ (740 kBq) was injected subcutaneously into a mouse (ICR strain) on day 16 of gestation. On the following day, the injected mouse was sacrificed, the embryos removed, and the calvaria were separated, cut into two pieces, and precultured for 1 d at 37°C in BGJb medium (Gibco BRL) containing 0.1% bovine serum albumin (BSA) for efflux of readily exchangeable bone ^{45}Ca into the medium. The bones were cultured in fresh BGJb medium containing 0.1% BSA with or without the $1\alpha,25(\text{OH})_2\text{D}$ derivatives for 5 d. The medium was changed after 2 d. The ^{45}Ca content of the medium at 2 and 5 d and of the 5% trichloroacetic acid extract of the bone was measured by liquid scintillation counting and used to calculate the total ^{45}Ca release and the cumulative percentage of released ^{45}Ca .

Statistical Analysis Data were analyzed with the unpaired Student's *t*-test.

RESULTS

Binding Studies As shown in Fig. 2, the binding affinity of $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ for the calf-thymus cytosol VDR was almost the same as for $1\alpha,25(\text{OH})_2\text{D}_3$, while 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_7$ had about 80% and 60% less binding affinity for the VDR compared with

Fig. 2. Displacement Curves of $[^3\text{H}]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ from the VDR with $1\alpha,25(\text{OH})_2\text{D}$ Derivatives

The derivatives at different concentrations were incubated with calf-thymus VDR and then subsequently incubated with $[^3\text{H}]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$. Unbound compounds were removed by dextran charcoal stripping and radioactivity was counted as described in Materials and Methods. The values in parentheses represent the relative binding affinity in comparison with $1\alpha,25(\text{OH})_2\text{D}_3$ (expressed as 1.0), calculated from the concentration of each derivative needed to achieve 50% displacement of $[^3\text{H}]\text{-}1\alpha,25(\text{OH})_2\text{D}_3$ from thymus VDR.

Fig. 3. Competitive Displacement of $[^3\text{H}]\text{-}25(\text{OH})\text{D}_3$ from DBP with the $1\alpha,25(\text{OH})_2\text{D}$ Derivatives

Mixtures containing $[^3\text{H}]\text{-}25(\text{OH})\text{D}_3$ and the derivatives at different concentrations were incubated with vitamin D-deficient rat serum DBP. Unbound compounds were removed by dextran charcoal stripping and the radioactivity was counted as described in Materials and Methods. The values in parentheses represent the relative binding affinity in comparison with $1\alpha,25(\text{OH})_2\text{D}_3$ (expressed as 1.0), calculated from the concentration of each derivative needed to achieve 50% displacement of $[^3\text{H}]\text{-}25(\text{OH})\text{D}_3$ from rat DBP.

that of $1\alpha,25(\text{OH})_2\text{D}_3$, respectively. This implies that the introduction of a 24S-methyl group or the combination of a 24S-methyl group and unsaturation at C-22/C-23 in the side chain of $1\alpha,25(\text{OH})_2\text{D}_3$ does not affect VDR binding affinity. On the other hand, the introduction of a 24R-methyl group or a combination of a 24R-methyl group and unsaturation at C-22/C-23 reduces VDR binding affinity significantly. Accordingly, a 24S-methyl group, not a 24R-methyl group, may be preferred for VDR binding and therefore, biological activity. As shown in Fig. 3, except for 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$, the binding affinity of the $1\alpha,25(\text{OH})_2\text{D}$ derivatives for rat plasma DBP was much higher than that of $1\alpha,25(\text{OH})_2\text{D}_3$. 24-Epi- $1\alpha,25(\text{OH})_2\text{D}_2$ exhibited 50% less binding affinity for DBP compared with $1\alpha,25(\text{OH})_2\text{D}_3$. This implies that the introduction of a 24S-methyl group or a 24R-methyl group into the side chain of $1\alpha,25(\text{OH})_2\text{D}_3$ increases DBP binding affinity significantly, while the introduction of a 24S-methyl group or a 24R-methyl group in the presence of a C-22/C-23 double bond in the side chain, increases or decreases DBP binding affinity, respectively. Accordingly, it appears that a C-22/C-23 double bond may serve to reduce DBP binding.

Fig. 4. Antiproliferative Effect of the $1\alpha,25(\text{OH})_2\text{D}$ Derivatives on Human Promyelocytic Leukemic HL-60 Cells

HL-60 cells (10^5 cells/ml) were treated with different concentrations of the derivatives. After 3 d, cell proliferation was determined by counting the cells exhibiting trypan-blue extrusion, as described in Materials and Methods. The values in parentheses represent the relative antiproliferative activities of the derivatives in comparison with $1\alpha,25(\text{OH})_2\text{D}_3$ (expressed as 1.0), calculated from the concentration of each derivative needed to achieve 50% inhibition of cell proliferation.

Fig. 5. Differentiation-Inducing Effect of the $1\alpha,25(\text{OH})_2\text{D}$ Derivatives on Human Promyelocytic Leukemic HL-60 Cells

HL-60 cells (10^5 cells/ml) were treated with different concentrations of the derivatives. After 3 d, cell differentiation was determined by counting the number of cells exhibiting morphological changes indicative of monocytes/macrophages by light microscopy. The values in parentheses represent the relative differentiation-inducing activities of the derivatives in comparison with $1\alpha,25(\text{OH})_2\text{D}_3$ (expressed as 1.0), calculated from the concentration of each derivative needed to achieve 50% induction of cell differentiation.

Cell Proliferation and Differentiation As shown in Figs. 4 and 5, the four $1\alpha,25(\text{OH})_2\text{D}$ derivatives exhibited 60–110% of the antiproliferative effect and 40–100% of the differentiation-inducing effect of $1\alpha,25(\text{OH})_2\text{D}_3$. It has been shown that the ability of $1\alpha,25(\text{OH})_2\text{D}_3$ metabolites and their analogues to inhibit proliferation of HL-60 cells is almost proportional to their ability to induce differentiation of the cells.^{36,37} We also observed the same tendency for the derivatives tested. There have been conflicting reports concerning a positive or negative correlation between VDR binding affinity and biological activities of $1\alpha,25(\text{OH})_2\text{D}$ metabolites and their analogues. In the present study, the antiproliferative and differentiation-inducing effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_4$ were nearly proportional to their binding

Fig. 6. Transcriptional Activity of the $1\alpha,25(\text{OH})_2\text{D}$ Derivatives on Human Osteosarcoma MG-63 Cells Cotransfected with a Luciferase Reporter Plasmid (pGVB2 Vector) Inserted with a Rat 25-Hydroxyvitamin D_3 -24-hydroxylase Gene Promoter (−291/+9) Including two VDREs and a pRL-CMV Vector as an Internal Control

After 2 d culture with the derivatives, cell lysates were prepared, and light production from luciferase was measured, as described in Materials and Methods. Luciferase activities induced by the $1\alpha,25(\text{OH})_2\text{D}$ derivatives in MG-63 cells was quantified and represented as fold-induction as compared with luciferase activity observed in the absence of the $1\alpha,25(\text{OH})_2\text{D}$ derivatives. Results represent the mean of three experiments (values in column) and standard errors (vertical bars) at 10^{-8} M. Values between the letters a and a', b and b', c and c', and d and d' are significantly different ($p < 0.01$).

affinity for VDR. In contrast, despite the comparable ability to bind to VDR, the antiproliferative and differentiation-inducing effects of $1\alpha,25(\text{OH})_2\text{D}_2$ were slightly lower than that of $1\alpha,25(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_4$. Furthermore, despite the lower binding affinity for VDR, 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ had a comparable ability to suppress cell proliferation and 70% of the ability of that of $1\alpha,25(\text{OH})_2\text{D}_3$ in inducing cell differentiation of HL-60 cells. $1\alpha,25(\text{OH})_2\text{D}_7$ was the least potent derivative tested, nevertheless it exhibited a higher binding affinity for VDR than 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$.

Transcriptional Activities At 10^{-8} M, the transcriptional activity of $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ on a rat 25-hydroxyvitamin D_3 -24-hydroxylase gene promoter containing two VDREs in transfected MG-63 cells were significantly higher than that observed with $1\alpha,25(\text{OH})_2\text{D}_3$, whereas 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_7$ had about 70% and 20% of the effect of $1\alpha,25(\text{OH})_2\text{D}_3$, as shown in Fig. 6. The derivatives had similar effects on a human osteocalcin gene promoter containing a VDRE in transfected MG-63 cells (Fig. 7). In a one-hybrid system using an expression plasmid for a human VDR-ligand binding domain fused with a yeast GAL4 DNA-binding domain and a luciferase reporter plasmid for a yeast GAL4 binding site (GAL2) DNA in cotransfected HeLa cells, the luciferase activity of $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ were almost comparable to that of $1\alpha,25(\text{OH})_2\text{D}_3$, whereas the luciferase activity of 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_7$ were significantly lower than that of $1\alpha,25(\text{OH})_2\text{D}_3$ (Fig. 8). The results correlate well with their binding affinity for VDR, as shown in Fig. 2. To further examine the efficacy for dimerization of VDR with RXR α , we employed a different one-hybrid system using an expression plasmid for a human RXR α -ligand binding domain fused with a yeast GAL4 DNA-binding domain and a luciferase reporter plasmid for a yeast GAL4 binding site (GAL2) DNA in cotransfected CV-1 cells. As shown in Fig. 9, it is clear that, except for $1\alpha,25(\text{OH})_2\text{D}_7$, all other derivatives strongly induce VDR/RXR α heterodimerization.

Bone-Resorbing Activity The ^{45}Ca releasing activity of $1\alpha,25(\text{OH})_2\text{D}_2$, $1\alpha,25(\text{OH})_2\text{D}_3$ and $1\alpha,25(\text{OH})_2\text{D}_4$ from fetal mouse calvaria previously labeled with ^{45}Ca over a 5 d cul-

Fig. 7. Transcriptional Activity of the 1α,25(OH)₂D Derivatives on Human Osteosarcoma MG-63 Cells Cotransfected 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

After 2 d culture with the derivatives, cell lysates were prepared, and light production from luciferase was measured, as described in Materials and Methods. Luciferase activity induced by the 1α,25(OH)₂D derivatives in MG-63 cells was quantified and represented as fold-induction as compared with luciferase activity observed in the absence of the 1α,25(OH)₂D derivatives. Results represent the mean of three experiments (values in column) and standard errors (vertical bars) at 10^{−8} M. Values between the letters a and a', b and b', c and c', and d and d' are significantly different ($p < 0.01$).

Fig. 8. Transcriptional Activity of the 1α,25(OH)₂D Derivatives on Human Epitheloid Carcinoma, Cervix HeLa Cells

The cells were cotransfected with a plasmid (pM vector) inserted with a human VDR-ligand binding domain cDNA, connected with yeast GAL4 DNA-binding domain cDNA, a luciferase reporter plasmid, inserted with yeast GAL4 binding site (GAL2) DNA, and a pRL-CMV vector as an internal control. After 2 d culture with the derivatives, cell lysates were prepared, and light production from luciferase was measured, as described in Materials and Methods. Luciferase activity induced by the 1α,25(OH)₂D derivatives in HeLa cells was quantified and represented as fold-induction as compared with luciferase activity observed in the absence of the 1α,25(OH)₂D derivatives. Results represent the mean of three experiments (values in column) and standard errors (vertical bars) 10^{−8} M. This one-hybrid system enabled us to assess direct VDR-mediated transcriptional activity of the derivatives in the cells. In this system, neither RXRα nor VDRE takes part in the transcriptional activity of the derivatives. Values between the letters a and a', b and b', and c and c' are significantly different ($p < 0.01$).

ture period is shown in Fig. 10. The bone-resorbing activities of 1α,25(OH)₂D₂ and 1α,25(OH)₂D₄ were almost the same as for 1α,25(OH)₂D₃. Unfortunately, we were unable to perform the bone resorbing assay of 24-epi-1α,25(OH)₂D₂ and 1α,25(OH)₂D₇ due to shortage of Material. Sato *et al.*³⁸⁾ previously reported that the bone calcium mobilization activity of 24-epi-1α,25(OH)₂D₂ and 1α,25(OH)₂D₇ in vitamin D-deficient rats were about 50% of 1α,25(OH)₂D₃ by assessing serum Ca changes after intravenous administration.

DISCUSSION

In the present study, we examined the *in vitro* biological activity of the five 1α,25(OH)₂D derivatives. The results show that the calcemic effects of 1α,25(OH)₂D₂ and 1α,25(OH)₂D₄, which are 24S-methyl and/or C-22/C-23 double bond derivatives of 1α,25(OH)₂D₃, are comparable to that of 1α,25(OH)₂D₃, as expected from their comparable

Fig. 9. Transcriptional Activity of the 1α,25(OH)₂D Derivatives on African Green Monkey Kidney CV-1 Cells

The cells were cotransfected with a plasmid (pM vector), inserted with human retinoid X receptor (RXRα) ligand binding domain cDNA, connected with yeast GAL4 DNA-binding domain cDNA, a luciferase reporter plasmid inserted with a yeast GAL4 binding site (GAL2) DNA, and a pRL-CMV vector as an internal control. After 2 d culture with the derivatives, cell lysates were prepared, and light production from luciferase was measured, as described in Materials and Methods. Luciferase activity induced by the 1α,25(OH)₂D derivatives in HeLa cells was quantified and represented as fold-induction as compared with luciferase activity observed in the absence of 1α,25(OH)₂D derivatives. Results represent the mean of three experiments (values in column) and standard errors (vertical bars) 10^{−8} M. This one-hybrid system enabled us to assess the VDR/RXRα heterodimer-mediated transcriptional activity of the derivatives in the cells. In this system, a VDR/RXRα heterodimer bound to the derivative directly induces an increase in luciferase activity without interactions with VDRE. Values between the letters a and a' are significantly different ($p < 0.01$).

Fig. 10. *In Vitro* Bone-Resorbing Activity of 1α,25(OH)₂D₂, 1α,25(OH)₂D₃ and 1α,25(OH)₂D₄

Bone-resorbing activity was measured by ⁴⁵Ca release from fetal mouse calvariae previously labeled with ⁴⁵Ca during 5 d culture with the derivatives. Data are shown as the mean ± S.E.M. of seven to nine bones at each data point. The values in parentheses represent the relative bone-resorbing activity of the derivatives in comparison with 1α,25(OH)₂D₃ (expressed as 1.0), calculated from the concentration of each derivative needed to reach 50% of maximum ⁴⁵Ca release with 1α,25(OH)₂D₃.

binding affinity for calf-thymus VDR. It has been shown that vitamin D₂ and vitamin D₄ are 100% and 75% as potent as vitamin D₃ in healing rickets in rachitic mammals, respectively.^{25,26)} Vitamin D₄, similar to vitamin D₂ and vitamin D₃, appears to be metabolized to 25-hydroxyvitamin D₄ in the liver and subsequently to 1α,25(OH)₂D₄ in the kidney. This suggests that the antirachitic activity of vitamin D₄ may correlate directly to the activity of 1α,25(OH)₂D₄. It has also been shown that the bone-resorbing activity of 1α,25(OH)₂D₃ metabolites and analogues is positively related to binding affinity for VDR.^{39,40)} In the present study, the relative binding affinity of 1α,25(OH)₂D₂, 1α,25(OH)₂D₃ and 1α,25(OH)₂D₄ for VDR were identical. Therefore, the difference between the antirachitic activity of vitamin D₂ and vitamin D₄ reported previously may not be due to the direct effects of their active metabolites on bone, but

probably due to other influencing factors, such as different metabolism (activation and inactivation), pharmacokinetic properties or metabolic clearance rate. Among these factors, DBP is particularly important. It has been shown that biological properties and extracellular pharmacokinetics of $1\alpha,25(\text{OH})_2\text{D}$ derivatives can be largely predicted by their affinity for DBP.^{41,42)} In general, derivatives with high affinity for DBP are more stable in circulation than those with low affinity, however, an increased affinity for DBP induces decreased availability for target cells because of decreased uptake into the cells.^{43,44)} The fact that $1\alpha,25(\text{OH})_2\text{D}_4$ has a five times greater binding potency for DBP and a comparable binding potency for VDR than $1\alpha,25(\text{OH})_2\text{D}_3$ explains in part the difference between the antirachitic activity of vitamin D_2 and vitamin D_4 . The assay for bone-resorbing activity of 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_7$, which is the 24 epimer of $1\alpha,25(\text{OH})_2\text{D}_2$ and the 24 epimer of $1\alpha,25(\text{OH})_2\text{D}_4$, respectively, could not be performed because of the limited amounts of compounds available for testing. DeLuca *et al.* reported that 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ is inactive in the *in vivo* mobilization of bone calcium while on the other hand, it was only five times less active than $1\alpha,25(\text{OH})_2\text{D}_2$ in inducing bone resorption in fetal rat bone in culture.⁴⁵⁾ As a possible explanation of the difference between the results of the *in vivo* and *in vitro* experiments, they suggested that 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ is more rapidly degraded *in vivo* than $1\alpha,25(\text{OH})_2\text{D}_2$ and, as a result, it shows preferential activity on intestine, whose response to a single dose of $1\alpha,25(\text{OH})_2\text{D}_2$ remains for several days, whereas short-lived *in vitro* bone systems do not remain stimulated for as long. Our finding that 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ has 20% and 40% of the binding affinity of $1\alpha,25(\text{OH})_2\text{D}_3$ for VDR and DBP respectively, supports, at least in part, the above hypothesis. Sato *et al.* reported that $1\alpha,25(\text{OH})_2\text{D}_7$ stimulates intestinal calcium absorption and calcium mobilization from bone in rats, however, the effect is about 50% of that of $1\alpha,25(\text{OH})_2\text{D}_3$.³⁸⁾ In the present study, it was found that the binding affinity of $1\alpha,25(\text{OH})_2\text{D}_7$ for VDR and DBP was 30% and 1280% of that of $1\alpha,25(\text{OH})_2\text{D}_3$. This also supports the reduced bone-resorbing activity demonstrated for $1\alpha,25(\text{OH})_2\text{D}_7$. Since $1\alpha,25(\text{OH})_2\text{D}_7$ is the 22, 23 dihydro derivative of 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$, it is obvious that the presence of a carbon 22, 23 double bond in the 24R-methyl derivatives induces a significant decrease in binding affinity for DBP without changing binding affinity for VDR.

As shown in Fig. 4, the antiproliferative activity of $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ was much weaker at the lower concentrations (10^{-10} – 10^{-9} M) but almost comparable at the high concentrations (10^{-8} – 10^{-7} M) as compared to that of $1\alpha,25(\text{OH})_2\text{D}_3$ and 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$. Since $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ have relatively high binding affinity for DBP, the inhibitory effect of DBP on the antiproliferative activity of the derivatives occurs more at the low concentrations than at the high concentrations. In comparing the antiproliferative and cell differentiating effects of the derivatives at 10^{-8} M, we conclude that all of the derivatives tested, except for $1\alpha,25(\text{OH})_2\text{D}_7$, are at least as potent as $1\alpha,25(\text{OH})_2\text{D}_3$ in regulation of cell proliferation and differentiation. The transactivation potencies of $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ on both the 25-hydroxyvitamin D_3 -24-hydroxylase and osteocalcin genes are comparable to, or exceed

the potency of $1\alpha,25(\text{OH})_2\text{D}_3$, but have comparable potencies for binding to VDR. As shown in Figs. 2 and 8, there is little doubt that both $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ can bind to VDR as efficiently as $1\alpha,25(\text{OH})_2\text{D}_3$ in either *in vitro* binding studies or the one-hybrid assay system. We do not have a clear explanation for the large transactivation potency of $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$, but it is likely that $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$, and probably 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ as well, more efficiently form a VDR/RXR α heterodimer than $1\alpha,25(\text{OH})_2\text{D}_3$, as reported previously.⁴⁶⁾

In conclusion, we found that binding affinity for VDR, bone-resorbing activity, antiproliferative and cell-differentiating effects, transactivation potencies on target genes and VDR- or RXR α -mediated gene regulation by $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_4$ are almost comparable to the effects of $1\alpha,25(\text{OH})_2\text{D}_3$, while 24-epi- $1\alpha,25(\text{OH})_2\text{D}_2$ and $1\alpha,25(\text{OH})_2\text{D}_7$ are much less active than $1\alpha,25(\text{OH})_2\text{D}_3$ in these respects.

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