

Relative Potencies of 1,25-(OH)₂D₃ and 19-Nor-1,25-(OH)₂D₂ on Inducing Differentiation and Markers of Bone Formation in MG-63 Cells

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Abstract. 19-Nor-1,25-(OH)₂D₂, an analog of 1,25-(OH)₂D₃, is used to treat secondary hyperparathyroidism because it suppresses parathyroid hormone synthesis and secretion with lower calcemic and phosphatemic activities. 19-Nor-1,25-(OH)₂D₂ is approximately 10 times less active than 1,25-(OH)₂D₃ in promoting bone resorption, which accounts in part for the low potency of this analog in increasing serum calcium and phosphorus. Concern that 19-nor-1,25-(OH)₂D₂ also could be less potent than 1,25-(OH)₂D₃ on bone formation led to a comparison of the potency of both compounds on osteoblasts. In the human osteoblast-like cell line MG-63, 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ had a similar potency in upregulating vitamin D receptor content and suppressing proliferation. Both sterols caused a similar reduction in DNA content and proliferating cell nuclear antigen protein expression. Time-

course and dose-response studies on 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ induction of the marker of bone formation, osteocalcin, showed overlapping curves. The effects on alkaline phosphatase (ALP) activity also were studied in MG-63 cells that had been co-treated with either sterol and transforming growth factor-β, an enhancer of 1,25-(OH)₂D₃-induced ALP activity in this cell line. Transforming growth factor-β alone had no effect, whereas 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ increased ALP activity similarly. These studies demonstrate that 19-nor-1,25-(OH)₂D₂ has the same potency as 1,25-(OH)₂D₃ not only in inducing vitamin D receptor content, osteocalcin levels, and ALP activity but also in controlling osteoblastic growth. Therefore, it is unlikely that 19-nor-1,25-(OH)₂D₂ would have deleterious effects on bone remodeling.

19-Nor-1,25-(OH)₂D₂, an analog of 1,25-(OH)₂D₃, is being used for the treatment of secondary hyperparathyroidism because of its ability to suppress parathyroid hormone (PTH) synthesis and secretion while at the same time having low calcemic and phosphatemic activities (1–3). Previous studies from our laboratory demonstrated that the decreased calcemic and phosphatemic activities of 19-nor-1,25-(OH)₂D₂ are due in part to decreased bone resorption (4). Because 1,25-(OH)₂D₃ is not only a potent stimulator of bone resorption but also a promoter of bone formation, these studies examined whether 19-nor-1,25-(OH)₂D₂ also is less active than 1,25-(OH)₂D₃ in bone-forming activity.

1,25-(OH)₂D₃ promotes bone formation by inhibiting proliferation of osteoblast precursors and promoting their differentiation into mature osteoblasts (5,6). Most biologic responses to 1,25-(OH)₂D₃ in vitamin D-target tissues are mediated through the vitamin D receptor (VDR). Because 1,25-(OH)₂D₃

is known to upregulate its own receptor (7–9), we compared the ability of 19-nor-1,25-(OH)₂D₂ to mimic 1,25-(OH)₂D₃ in the regulation of VDR content in osteoblasts before assessing the relative potencies of these two sterols in suppressing osteoblast proliferation and inducing the expression of markers of bone-forming activity in osteoblasts. Studies were conducted with the use of the well-characterized human osteoblastic cell line MG-63, which was derived from a human osteosarcoma and is capable of expressing the phenotype of the most differentiated osteoblast (10,11). The antiproliferative properties of 1,25-(OH)₂D₃ on MG-63 cells (12) led us to compare the effects of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ on MG-63 proliferation by measuring DNA content and proliferating cell nuclear antigen (PCNA). PCNA, a processivity factor for DNA polymerase-δ and an important regulator of cell cycle progression (13,14), is increased in the nuclei of cells that are undergoing mitosis.

To assess the ability of both sterols to induce the expression of markers of osteoblast bone formation, we compared the potencies of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ in increasing osteocalcin (OC) levels and alkaline phosphatase (ALP) activity. In MG-63 cells, 1,25-(OH)₂D₃ treatment increases OC secretion and ALP activity (15–17), the latter only when cells also are exposed to transforming growth factor-β (TGF-β) (18). By itself, TGF-β has little effect on ALP activity in these cells. Although the exact mechanism by which TGF-β

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enhances 1,25-(OH)₂D₃-mediated ALP expression is unclear, it is thought that TGF- β induces these cells to an intermediate stage of differentiation in which they are held (11). In the present studies, the comparison of the potencies of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ in inducing ALP activity were performed in MG-63 cells that had been co-treated with TGF- β .

The results of these *in vitro* studies demonstrate that 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ have a similar potency in controlling the proliferation of osteoblasts and their differentiation into mature bone-forming cells.

Materials and Methods

Cell Culture

MG-63 cells were grown to confluence in 75-cm² tissue culture flasks in Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/ml), and streptomycin (100 μ g/ml) in a humidified atmosphere containing 5% CO₂ in air at 37°C. Cells were subcultured weekly with trypsin-ethylenediaminetetraacetate. For experimental protocols, cells were plated in Eagle's minimum essential medium with 10% FBS at a concentration of 100,000 cells/10-cm² well. Twenty-four h later, the medium was removed and the cells were washed three times with phosphate-buffered saline (PBS). The medium was replaced with fresh medium containing 2% FBS and vehicle, 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂. ALP measurements were performed both under basal conditions and after co-treatment with either sterol and TGF- β (10 ng/ml). Dose-response experiments were carried out for 72 h with concentrations of 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ ranging from 10⁻¹¹ to 10⁻⁶ M. Time-course experiments were performed from 0 to 72 h with the use of 10⁻⁷ M 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂.

Western Blot Analysis for VDR and PCNA

For Western blot analysis, cells were maintained in culture as described above and plated at a density of 3 \times 10⁶ cells/10-cm-diameter culture dish. After 24 h, cells were washed three times with PBS and fresh media containing vehicle or 10⁻⁷ M 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ was added. Cell lysates were prepared with the use of a modified RIPA buffer containing 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris (pH 8), and 1 \times protease inhibitor cocktail (Roche Analytics, Newark, NJ). Lysates were analyzed for total protein with the use of the Bradford Method (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (40 μ g) were resolved by polyacrylamide gel electrophoresis in 4 to 20% gradient gels (Fisher Scientific, St. Louis, MO) and wet-transferred to 0.45- μ m nitrocellulose membranes in 20% methanol in Tris-glycine at 100 volts for 1 h. Gel loading and transfer efficiencies were examined by staining total protein in the nitrocellulose membranes with Ponceau red. After blocking, membranes were exposed to either a monoclonal antibody against PCNA (PC10 mouse anti-PCNA; Zymed Laboratories Inc., San Francisco, CA) or VDR (a kind gift from Dr. Paul N. McDonald, Case Western Reserve University, Cleveland, OH) at 4°C overnight. Blots then were incubated with horseradish peroxidase-conjugated second antibody (Pierce, Rockford, IL) for 1 h at room temperature. Specific bands were detected with the use of the Supersignal Substrate Working Solution (Pierce) and enhanced autoradiographic film (Amersham, Arlington Heights, IL). Gels were scanned, and the densitometric

analysis was performed with the use of the Gel-Pro Analyzer (Media Cybernetics, Silver Spring, MD).

OC Measurement

At the end of each experiment, medium was frozen at -20°C for later OC determinations and cells again were washed three times with PBS. A buffer containing TRIZMA-HCl 100 mM and NaCl 100 mM (pH 7.5; 500 ml) was added, and the cells were sonicated on ice for 45 s. Sonicates were frozen at -20°C for later DNA analysis. For OC levels, media samples were assayed in duplicate by specific competitive RIA with the use of a goat anti-human-OC antibody, human OC as the standard and ¹²⁵I-labeled OC (19). Antibody/OC complexes were separated from free iodinated tracer with an anti-goat IgG. The assay buffer consisted of 132 mM NaCl, 10 mM NaH₂PO₄, 25 mM Na-ethylenediaminetetraacetate, 0.1% Tween 20 (wt/vol), and 0.1% bovine serum albumin (pH 7.4).

ALP Measurement

At the end of each experiment, cells were washed three times with PBS. Triton X-100 (0.05%) was added, and the cells were sonicated on ice for 45 s. ALP was analyzed immediately by autoanalyzer (Miraplus; COBAS, Indianapolis, IN) with the use of p-nitrophenyl phosphate as substrate. The remainder of the sonicate was frozen at -20°C for later DNA analysis.

DNA Assay

Cell sonicates were prepared from MG-63 cells that had been subjected to the experimental conditions described previously. DNA was measured with the use of the fluorometric quantitation of ethidium bromide complexes according to the method of LePecq and Paoletti (20).

Materials

TGF- β was purchased from R & D Systems (Minneapolis, MN). 1,25-(OH)₂D₃ was a kind gift of Dr. Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ). 19-Nor-1,25-(OH)₂D₂ was supplied by Dr. Hector DeLuca (University of Wisconsin, Madison, WI). Unless otherwise stated, all other reagents were purchased from Sigma Chemical Company (St. Louis, MO).

Statistical Analyses

Results are expressed as mean \pm SEM. ANOVA was used to examine statistical significance in dose-response or time-course protocols for either 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ treatment. The Bonferroni correction identified the dose or time points responsible for the statistical significance revealed by ANOVA tests. Comparisons between 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ at a given time point or dose were performed with the use of the unpaired *t* test. *P* < 0.05 was considered statistically significant.

Results

These studies compared the relative potencies of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ in suppressing proliferation and inducing differentiation in human osteoblasts. Because most biologic responses to 1,25-(OH)₂D₃ are mediated by the VDR, we first compared the abilities of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ to upregulate VDR content in MG-63 cells.

Effect of 1,25-(OH)₂D₃ and 19-Nor-1,25-(OH)₂D₂ on VDR Content

VDR protein was measured in whole-cell extracts from MG-63 cells that had been treated for 48 h with 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ at a concentration of 10⁻⁷ M. The top panel of Figure 1 shows a typical Western blot for the VDR. Recombinant human VDR was used as a positive control to assess for the specificity of the antibody used in these studies. This antibody, 9A7, is a rat monoclonal antibody that recognizes the DNA binding domain of the VDR molecule. The double bands, one co-migrating with recombinant human VDR, may represent differentially phosphorylated VDR isoforms. The bottom panel of Figure 1 shows the corresponding densitometric analysis. 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ treatment induced a marked increase in VDR protein compared with cells that were treated with vehicle alone (1,25-(OH)₂D₃, 4423 ± 194 integrated optical density [IOD]; 19-nor-1,25-(OH)₂D₂, 4073 ± 242 IOD *versus* vehicle, 2991 ± 160 IOD). The compilation of results from two independent experiments indicated an increase in VDR of 71.2 and 42.9% by 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂, respectively.

Effect of 1,25-(OH)₂D₃ and 19-Nor-1,25-(OH)₂D₂ on Cell Proliferation

The effect of various concentrations of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ on total DNA is shown in Figure 2. ANOVA analysis showed that treatment of MG-63 cells for 72 h with concentrations of 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ ranging from 10⁻¹¹ to 10⁻⁶ M resulted in a significant dose-dependent reduction in DNA content for both sterols

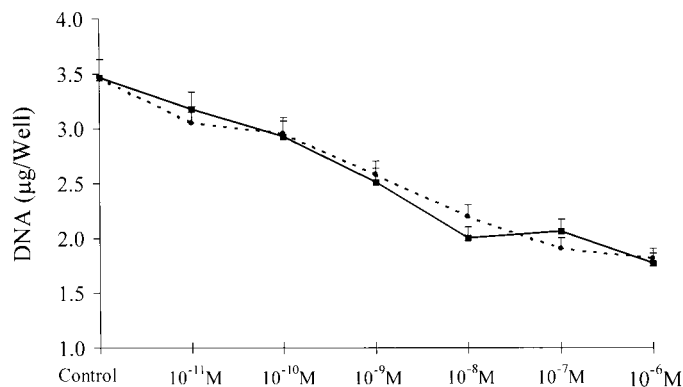


Figure 2. Comparative effects of increasing doses of 1,25-(OH)₂D₃ (solid line) and 19-nor-1,25-(OH)₂D₂ (broken line) on DNA content. MG-63 cells were treated with concentrations from 10⁻¹¹ M to 10⁻⁷ M 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ for 72 h. DNA is expressed as µg/well. Data points and error bars represent mean ± SEM (n = 7).

(1,25-(OH)₂D₃, P < 0.0001; 19-nor-1,25-(OH)₂D₂, P < 0.0001). The dose-response curves for both compounds were virtually identical. Bonferroni comparison revealed that the decrease in DNA induced by both sterols reached statistical significance at 10⁻¹⁰ M. At a dose of 10⁻⁶ M, DNA decreased from 3.46 ± 0.19 µg/well in control cells to 1.77 ± 0.03 µg/well (↓ 48.8%, P < 0.001) in 1,25-(OH)₂D₃-treated cells and to 1.81 ± 0.02 µg/well (↓ 47.7%, P < 0.001) after 19-nor-1,25-(OH)₂D₂ treatment.

On the basis of the reduction in DNA observed after 72 h of treatment with 10⁻⁷ M of 1,25-(OH)₂D₃ or 19-nor,1,25-

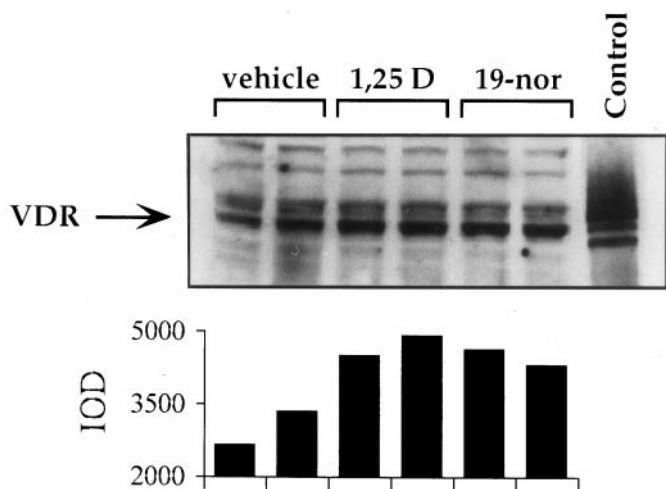


Figure 1. The top panel is a representative enhanced chemiluminescence (ECL) Western blot analysis of the VDR of 40 µg of total protein from whole-cell extracts from MG-63 cells treated for 48 h with vehicle or 10⁻⁷ M of either 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂. The bottom panel represents the densitometric analysis of VDR content using Gel-Pro Analyzer. The results are two experiments done in duplicate (n = 4) and are expressed as integrated optical density/area.

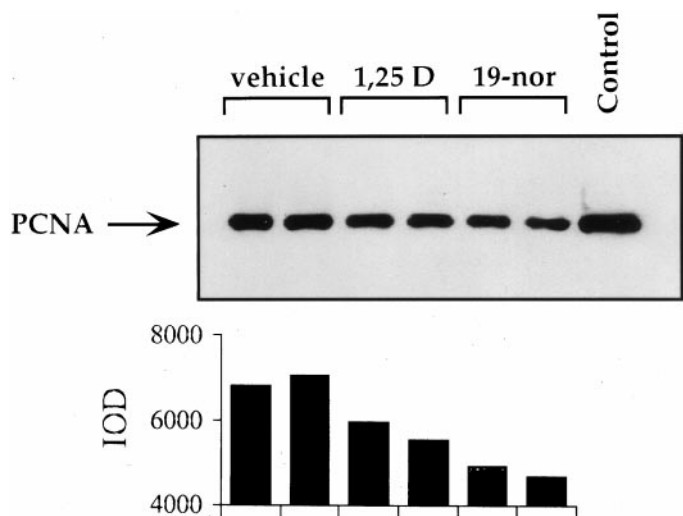


Figure 3. The top panel is a representative ECL Western blot analysis of PCNA of 40 µg of total protein from whole-cell extracts from MG-63 cells treated for 48 h with vehicle or 10⁻⁷ M of either 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂. The bottom panel represents the densitometric analysis of proliferating cell nuclear antigen (PCNA) content using Gel-Pro Analyzer. The results are two experiments done in duplicate (n = 4) and are expressed as integrated optical density/area.

(OH)₂D₂, we examined the expression of PCNA in whole-cell extracts from MG-63 cells 48 h after treatment with either sterol because a halt in proliferation rate precedes an actual reduction in DNA content. The top panel of Figure 3 shows a typical Western blot for PCNA, and the bottom panel depicts the corresponding densitometric analysis. 1,25-(OH)₂D₃ treatment decreased PCNA from 6919 ± 127 IOD in vehicle-treated cells to 5750 ± 199 IOD. 19-Nor-1,25-(OH)₂D₂ decreased PCNA to 4813 ± 124 IOD. Compilation of results from two independent experiments indicates a differential reduction in PCNA expression by 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ of 19.8% and 31.3%, respectively.

Effect of 1,25-(OH)₂D₃ and 19-Nor-1,25-(OH)₂D₂ on Bone-Forming Activity

Osteocalcin. Media OC levels were measured in confluent cultures of MG-63 cells. The effect of increasing doses of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ on OC secretion by MG-63 cells is shown in Figure 4A. While undetectable in control cells, ANOVA analysis demonstrated a dose-dependent increase in OC in cells that were treated for 72 h with various concentrations of 1,25-(OH)₂D₃ (*P* < 0.0001) or 19-nor-1,25-(OH)₂D₂ (*P* < 0.0001). Bonferroni comparison revealed that OC increased significantly from 0.107 ± 0.058 ng/μg DNA with 10⁻¹¹ M 1,25-(OH)₂D₃ to 8.65 ± 0.49 ng/μg DNA (*P* < 0.001) when cells were treated with 10⁻⁷ M 1,25-(OH)₂D₃. A similar increase was seen with 19-nor-1,25-(OH)₂D₂ (10⁻¹¹ M, 0.096 ± 0.065 ng/μg DNA; 10⁻⁷ M, 9.19 ± 0.27 ng/μg

DNA; *P* < 0.001). This increase in OC with both compounds reached significance at a concentration of 10⁻¹⁰ M (*P* < 0.05). Unpaired *t*-test analysis indicated that 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ increased OC levels to the same degree at every concentration tested.

The time course for the effect of these two compounds on OC secretion is shown in Figure 4B. OC was measured at 24, 48, and 72 h in media from confluent MG-63 cells that were treated with 10⁻⁷ M 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂. ANOVA analysis demonstrated a significant increase in OC over time in cells that were treated with 1,25-(OH)₂D₃ (*P* < 0.0001) or 19-nor-1,25-(OH)₂D₂ (*P* < 0.0001). OC was undetectable in control cells at all time points and was barely detectable after 24 h of treatment with either 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂. Bonferroni comparison revealed a significant increase in OC at 48 h (1,25-(OH)₂D₃, 2.37 ± 0.14 [*P* < 0.01]; 19-nor-1,25-(OH)₂D₂, 2.31 ± 0.04 ng/μg DNA [*P* < 0.01]) and 72 h (1,25-(OH)₂D₃, 6.17 ± 0.48 [*P* < 0.001]; 19-nor-1,25-(OH)₂D₂, 6.45 ± 0.31 ng/μg DNA [*P* < 0.001]) with either sterol. Both compounds had identical potency on OC secretion at each time point tested.

Alkaline Phosphatase. The demonstration that 1,25-(OH)₂D₃-induced ALP activity in MG-63 cells is greatly enhanced by co-treatment with TGF-β (21) led us to study the effects of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ in confluent cultures of MG-63 cells that were co-treated with either sterol

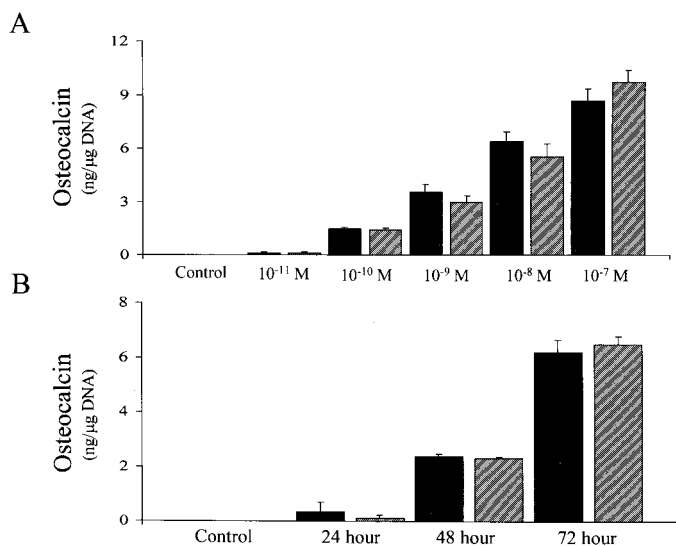


Figure 4. (A) Comparative effects of increasing doses of 1,25-(OH)₂D₃ (■) and 19-nor-1,25-(OH)₂D₂ (▨) on media osteocalcin (OC). MG-63 cells were treated with concentrations from 10⁻¹¹ M to 10⁻⁷ M of 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ for 72 h. Media osteocalcin is expressed as ng/μg DNA. Bars and error bars represent mean ± SEM (*n* = 6). (B) Time course for the effects of increasing doses of 1,25-(OH)₂D₃ (■) and 19-nor-1,25-(OH)₂D₂ (▨) on media OC. MG-63 cells were treated with 10⁻⁷ M 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ for 0 to 72 h. Media OC levels are expressed as ng/μg DNA. Bars and error bars represent mean ± SEM (*n* = 6).

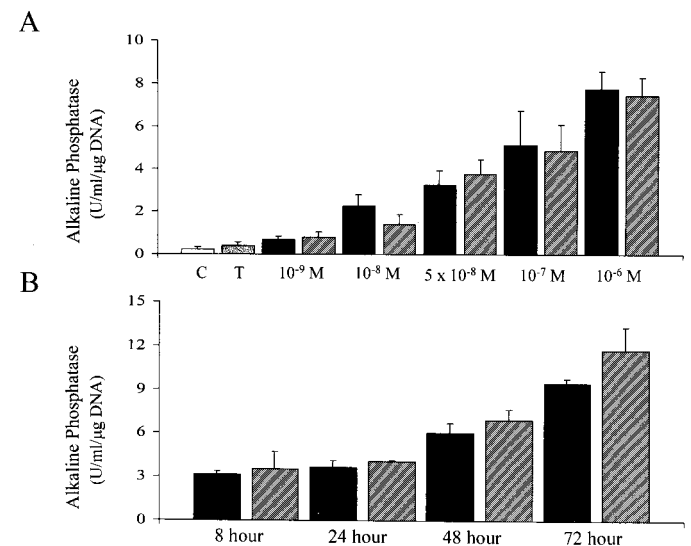


Figure 5. (A) Comparative effects of increasing doses of 1,25-(OH)₂D₃ (■) and 19-nor-1,25-(OH)₂D₂ (▨) on alkaline phosphatase (ALP) activity. MG-63 cells were treated with various concentrations (10⁻⁹ to 10⁻⁶ M) of 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ for 72 h. ALP activity is expressed as U/ml.μg DNA. The second bar (T) represents the effect of transforming growth factor-β alone. Bars and error bars represent mean ± SEM (*n* = 6). (B) Time course for the effects of increasing doses of 1,25-(OH)₂D₃ (■) and 19-nor-1,25-(OH)₂D₂ (▨) on ALP activity. MG-63 cells were treated with 10⁻⁷ M of 1,25-(OH)₂D₃ or 19-nor-1,25-(OH)₂D₂ for 8 to 72 h. ALP activity is expressed as U/ml.μg DNA. Bars and error bars represent mean ± SEM (*n* = 6).

and 10 ng/ml TGF- β . The effect of increasing doses of 1,25-(OH) $_2$ D $_3$ and 19-nor-1,25-(OH) $_2$ D $_2$ on ALP activity in MG-63 cells after a 72-h incubation period is shown in Figure 5A. Treatment with TGF- β alone had no effect on ALP activity compared with control cells (TGF- β , 0.22 ± 0.09 U/ml. μ g DNA; control, 0.40 ± 0.18 U/ml. μ g DNA). ANOVA analysis showed a significant dose-dependent increase in ALP activity by 1,25-(OH) $_2$ D $_3$ or 19-nor-1,25-(OH) $_2$ D $_2$ at doses ranging from 10^{-9} M to 10^{-6} M ($P < 0.0001$ for both compounds). ALP activity rose in a dose-dependent manner, increasing to 7.73 ± 0.84 U/ml. μ g DNA with 10^{-6} M 1,25-(OH) $_2$ D $_3$ ($P < 0.001$) and to 7.43 ± 0.83 U/ml. μ g DNA with 10^{-6} M 19-nor-1,25-(OH) $_2$ D $_2$ ($P < 0.001$). The increase in ALP activity reached significance at 10^{-7} M for both 1,25-(OH) $_2$ D $_3$ ($P < 0.05$) and 19-nor-1,25-(OH) $_2$ D $_2$ ($P < 0.01$). The profiles for both dose-response curves were the same. Unpaired *t*-test analysis showed no statistically significant difference between either compound at any dose tested.

The time course for the effect of these two compounds on ALP activity is shown in Figure 5B. Confluent cells were treated with 10^{-7} M 1,25-(OH) $_2$ D $_3$ or 19-nor-1,25-(OH) $_2$ D $_2$, and ALP activity was measured at various times from 8 to 72 h. ANOVA analysis demonstrated a significant increase in ALP over time in cells that were treated with 1,25-(OH) $_2$ D $_3$ ($P < 0.0001$) or 19-nor-1,25-(OH) $_2$ D $_2$ ($P < 0.0001$). Treatment with TGF- β alone had no effect on ALP activity at any time point tested (data not shown). ALP activity was increased by both 1,25-(OH) $_2$ D $_3$ and 19-nor-1,25-(OH) $_2$ D $_2$ in as little as 8 h (1,25-(OH) $_2$ D $_3$, 3.13 ± 0.98 U/ml. μ g DNA; 19-nor-1,25-(OH) $_2$ D $_2$, 3.50 ± 1.19 U/ml. μ g DNA) and continued to increase in a similar manner for up to 72 h (1,25-(OH) $_2$ D $_3$, 9.39 ± 0.353 [$P < 0.001$]; 19-nor-1,25-(OH) $_2$ D $_2$, 11.71 ± 1.57 U/ml. μ g DNA [$P < 0.01$]). Unpaired *t* test revealed no significant differences in ALP activity between the two compounds at any time point tested.

Discussion

The role of 1,25-(OH) $_2$ D $_3$ on bone remodeling is complex because of the dual effects of the sterol, increasing both bone formation and resorption. Although osteoclasts are responsible for resorbing bone, both actions of 1,25-(OH) $_2$ D $_3$ on bone remodeling are mediated through the osteoblast (22–24). Previous studies from our laboratory showed the bone-resorbing activity of 19-nor-1,25-(OH) $_2$ D $_2$ to be approximately 10 times less than that of 1,25-(OH) $_2$ D $_3$ (4), suggesting the possibility of an impaired action of 19-nor-1,25-(OH) $_2$ D $_2$ on the osteoblast. The importance of maintaining a balance between bone formation and resorption raised serious concerns about possible deleterious effects of long-term 19-nor-1,25-(OH) $_2$ D $_2$ therapy on bone formation. The present study assessed directly the effects of 19-nor-1,25-(OH) $_2$ D $_2$ on osteoblast bone-forming activity.

Because 1,25-(OH) $_2$ D $_3$ promotes bone formation by inhibiting the proliferation of osteoblast precursors and promoting their differentiation into a more mature phenotype (5,6), we compared the effects of 19-nor-1,25-(OH) $_2$ D $_2$ and 1,25-

(OH) $_2$ D $_3$ on cell proliferation and two markers of bone formation in the human osteoblast-like cell line, MG-63.

VDR content and intracellular levels of vitamin D metabolites are the main determinants of the magnitude of the response to vitamin D therapy by a target tissue (25,26). Studies in uremic rats that were treated with 1,25-(OH) $_2$ D $_3$ or 19-nor-1,25-(OH) $_2$ D $_2$ for 8 wk revealed 19-nor-1,25-(OH) $_2$ D $_2$ to be less potent than 1,25-(OH) $_2$ D $_3$ in upregulating the intestinal VDR (3). In short-term studies, however, 1,25-(OH) $_2$ D $_3$ and 19-nor-1,25-(OH) $_2$ D $_2$ had similar effects on the VDR content (27). These opposing effects of 19-nor-1,25-(OH) $_2$ D $_2$ on regulating intestinal VDR levels led us to compare the effectiveness of 1,25-(OH) $_2$ D $_3$ and 19-nor-1,25-(OH) $_2$ D $_2$ regulation of the VDR protein in MG-63 cells before assessing the relative potency of both sterols on osteoblast function. Both sterols were equally effective in increasing VDR content in this cell line.

1,25-(OH) $_2$ D $_3$ and 19-nor-1,25-(OH) $_2$ D $_2$ also are equally potent in the control of osteoblast proliferation as evidenced by similar decreases in DNA content induced by both sterols in MG-63 cells. However, when PCNA expression was used to assess mitotic activity, 19-nor-1,25-(OH) $_2$ D $_2$ was more potent than 1,25-(OH) $_2$ D $_3$. The cause of this discrepancy was not investigated further. Although PCNA expression correlates with mitotic activity, growth arrest could occur despite high PCNA if PCNA activity is blocked by the cyclin-dependent kinase inhibitor p21. In addition to its intrinsic ability to inhibit mitosis, p21 binds PCNA, preventing DNA replication (28). The demonstration that 1,25-(OH) $_2$ D $_3$ induces *p21* gene transcription (29) leads us to postulate that a differential ability of 1,25-(OH) $_2$ D $_3$ and 19-nor-1,25-(OH) $_2$ D $_2$ to induce p21 expression in MG-63 cells could render both sterols equally effective in controlling proliferation despite different PCNA content.

1,25-(OH) $_2$ D $_3$ induces MG-63 cells to differentiate into a more osteoblastic phenotype. A certain order of gene expression exists during the differentiation of osteoblasts (21). ALP expression is elevated mainly after the decrease in proliferation, whereas OC expression is a marker of the most differentiated osteoblast. Co-treatment of MG-63 cells with 1,25-(OH) $_2$ D $_3$ and TGF- β is thought to promote the differentiation of these cells to a more intermediate stage of differentiation and retard their further differentiation (11,30). In these studies, 19-nor-1,25-(OH) $_2$ D $_2$ and 1,25-(OH) $_2$ D $_3$ were equally potent in increasing both markers of bone formation, ALP and OC, in a time- and dose-dependent manner, indicating that both of these compounds can induce equally the differentiation of MG-63 cells to a mature osteoblastic phenotype.

These *in vitro* results suggest that 19-nor-1,25-(OH) $_2$ D $_2$ may be as active as 1,25-(OH) $_2$ D $_3$ in stimulating bone formation *in vivo*. This is not surprising considering that the affinity of 19-nor-1,25-(OH) $_2$ D $_2$ for the VDR is only three times less than that of 1,25-(OH) $_2$ D $_3$ (H. F. DeLuca, University of Wisconsin, Madison, WI, personal communication, July 1999) and our findings in the present study showing that in MG-63 cells, 19-nor-1,25-(OH) $_2$ D $_2$ can mimic 1,25-(OH) $_2$ D $_3$ in its ability to upregulate VDR content.

In contrast to the findings in the present study showing

identical potencies of both vitamin D compounds in stimulating the expression of markers of bone formation in osteoblasts, we previously showed 19-nor-1,25-(OH)₂D₂ to be approximately 10 times less active in promoting bone resorption than 1,25-(OH)₂D₃ (4). *In vitro* studies of mouse bone marrow cultures revealed 19-nor-1,25-(OH)₂D₂ to be 70% less bone resorptive than 1,25-(OH)₂D₃ (31). Because 1,25-(OH)₂D₃-induced bone resorption also is mediated through the osteoblastic VDR, the differential effects of 1,25-(OH)₂D₃ and 19-nor-1,25-(OH)₂D₂ on bone formation and resorption seem to be gene specific and not cell specific.

Potential mechanisms to explain the disparity between 19-nor-1,25-(OH)₂D₂ and 1,25-(OH)₂D₃ regulation of genes involved in bone formation and resorption include intrinsic properties of the D₂ and D₃ moieties (32–36) or the 19-nor modification, either of which might lead to a different rate of catabolism for 19-nor-1,25-(OH)₂D₂ compared with 1,25-(OH)₂D₃ in osteoblasts and altered VDR binding to nuclear co-activators. More rapid catabolism of 19-nor-1,25-(OH)₂D₂ compared with 1,25-(OH)₂D₃ could explain the observed differences between bone formation and resorption. There is evidence that the analog 22-oxa-calcitriol, which is cleared rapidly from circulation, produces a transient increase in calcium transport but a prolonged suppression of PTH (37). Although the rate of degradation of 19-nor-1,25-(OH)₂D₂ in osteoblasts is not known, a faster rate of catabolism, while having little effect on genes that need only a short exposure to the 19-nor-1,25-(OH)₂D₂-VDR complex, could decrease significantly the transcription of genes that require a longer exposure.

There also is evidence in the literature that vitamin D analogs can bind differently than 1,25-(OH)₂D₃ to the VDR, producing a conformational change in the VDR molecule that favors the selective recruitment of nuclear transcriptional co-activators and resulting in a variable degree of VDR-mediated transcription (38–41). The binding of 19-nor-1,25-(OH)₂D₂ to the VDR could prevent the recruitment of nuclear co-activators required for bone resorption while still being capable of bringing to the transcription initiation complex those co-activators required for genes involved in bone formation.

In summary, these *in vitro* studies demonstrate that 19-nor-1,25-(OH)₂D₂ has the same potency as 1,25-(OH)₂D₃ not only in upregulating the VDR and in controlling osteoblastic growth but also in inducing osteoblastic OC levels and ALP activity. This makes it unlikely that this analog would have deleterious effects on bone remodeling.

The results from this *in vitro* study, however, should not be completely equated to those expected *in vivo*. The prolonged administration of vitamin D or its analogs to patients with secondary hyperparathyroidism could result in an excessive decrease in PTH levels and a subsequent reduction in bone resorption and formation leading to adynamic bone disease. In addition, there are instances of adynamic bone disease occurring in patients with elevated PTH values (42) that could result from a resistance to vitamin D therapy. Clearly, an assessment of the effects of prolonged 19-nor-1,25-(OH)₂D₂ therapy on bone in these patients is mandatory.

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