1,25-Dihydroxy-19-nor-vitamin D₂, a Vitamin D Analog with Reduced Bone Resorbing Activity In Vitro

L. SHANNON HOLLIDAY,* STEPHEN L. GLUCK,* EDUARDO SLATOPOLSKY,† and ALEX J. BROWN†
*Department of Medicine, Division of Nephrology and Hypertension, and Department of Anatomy & Cell Biology, University of Florida College of Medicine, Gainesville, Florida; and †Renal Division, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri.

Abstract. 1,25-Dihydroxy-19-nor-vitamin D₂ (19-norD₂), a new analog of 1,25(OH)₂D₃, suppresses parathyroid hormone in renal failure patients and in uremic rats but has less calcemic activity than 1,25(OH)₂D₃. Although 19-norD₂ has high affinity for the vitamin D receptor and similar pharmacokinetics to those of 1,25(OH)₂D₃, it has much less bone resorbing activity in vivo. The intrinsic activity of 19-norD₂ on osteoclastogenesis and activation of bone resorption in mouse bone marrow cultures was examined to determine the mechanism involved. 19-norD₂, 1,25(OH)₂D₃ (10 nM) were equivalent in stimulating the formation and maintenance of large multinucleated, tartrate-resistant acid phosphatase–positive cells. However, the amount of bone resorbed by osteoclasts stimulated by 10 nM 19-norD₂, as measured by pit-forming assays, was reduced approximately 20% greater than 1,25(OH)₂D₃, not enough to account for the different effects on bone resorption. The VDR levels were identical in cultures that were treated with 19-norD₂ and 1,25(OH)₂D₃. In summary, 19-norD₂ is less effective than 1,25(OH)₂D₃ in stimulating mouse marrow osteoclasts to resorb bone. The reason for this difference is not clear but seems to involve the late maturation and/or activation of osteoclasts as the number of pits produced by each tartrate-resistant acid phosphatase–positive cell is reduced under stimulation by 19-norD₂ compared with 1,25(OH)₂D₃.

Development of secondary hyperparathyroidism is a common occurrence in patients with chronic renal failure. The pathogenesis of the hyperparathyroidism has been attributed to phosphate retention and the low levels of 1,25(OH)₂D₃ in these patients, both of which produce a tendency toward hypocalcemia. The parathyroid glands respond initially to the low calcium by increasing parathyroid hormone (PTH) secretion and synthesis, but with chronic hypocalcemia the parathyroid glands become hyperplastic (1). 1,25(OH)₂D₃ can also suppress PTH gene transcription (2), and therefore the low levels of 1,25(OH)₂D₃ also lead to overexpression of the PTH gene, further exacerbating the hyperparathyroidism.

Treatment of secondary hyperparathyroidism involves correction of the hyperphosphatemia, usually with calcium-based phosphate binders that retard intestinal absorption of dietary phosphate, and restoration of the 1,25(OH)₂D₃ levels by replacement therapy. However, as a result of its potent calcemic actions in the intestine and bone, 1,25(OH)₂D₃ often produces hypercalcemia in renal patients, especially in those who are receiving oral calcium. This limitation of 1,25(OH)₂D₃ therapy has led to the development of vitamin D analogs that retain the direct action of 1,25(OH)₂D₃ to suppress PTH gene expression but with less calcemic activity. Several analogs have been developed for the treatment of secondary hyperparathyroidism. These include 19-nor-1,25(OH)₂D₂ (19-norD₂) (3,4), 22-oxacalcitriol (OCT) (5,6), and 1α(OH)D₂ (7). 19-norD₂ and OCT have been shown to exert a selective action on PTH in animal models of renal failure, e.g., suppression of PTH levels with less hypercalcemia (3,5).

The mechanisms by which these analogs exert this selectivity on the parathyroid glands are under investigation. The selective action of OCT on PTH seems to be due to its altered pharmacokinetics (8,9). Its low affinity for the serum vitamin D binding protein (DBP) leads to rapid clearance but greater tissue accessibility. The transient appearance of OCT in target tissues after injection elicits only short-lived effects on intestinal calcium absorption and bone mobilization but a prolonged suppression of PTH gene expression (10).

The mechanism for the selectivity of 19-norD₂ is less clear but is not due to altered pharmacokinetics (11). Furthermore, the decreased intestinal calcium absorption and bone mobilization activities measured in vivo are due to an acquired resistance that is observed only after several days of chronic treatment (12). These observations led us to determine whether the low bone mobilizing activity of 19-norD₂ observed in vivo could be reproduced in the mouse bone marrow culture system (13). We report that 19-norD₂ is equipotent to 1,25(OH)₂D₃ in...
differentiating osteoclast precursors to a mature phenotype but has a decreased capacity to induce the mature osteoclasts to resorb bone.

**Materials and Methods**

**Materials**

Reagents were obtained from Sigma (St. Louis, MO) unless noted otherwise. 1,25-(OH)$_2$D$_3$ was a generous gift from Dr. Milan R. Uskokovic (Hoffman-LaRoche, Nutley, NJ). 1,25(OH)$_2$-19-nor-D$_2$ (19-norD$_2$) was provided by Abbott Laboratories (Chicago, IL). 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$-19-nor-D$_2$ were generously provided by Dr. Satya Reddy (Brown University, Providence, RI). Sperm whale teeth for the in vitro bone mobilization assay were obtained from the U.S. Department of Fisheries (San Diego, CA).

**In Vitro Bone Resorption Assay**

Resorption assays were performed as described previously (14) using sperm whale teeth sliced into 100-μm thick specimens with a surface area of 1 cm$^2$. The slices were washed by agitation in 50 mL of sterile PBS, transferred to 24-well plates, and soaked overnight in αMEM D10 before exposure to mouse marrow cultures. Mouse marrow was cultured for 5 d on tissue culture plates in αMEM D10 containing various concentrations of 1,25(OH)$_2$D$_3$ or 19-norD$_2$. Cultures were fed every other day by replacing half the media per plate and adding fresh vitamin D compounds. After 5 d in culture, osteoclasts appeared. These were detected by staining for tartrate-resistant acid phosphatase (TRAP) activity using a kit from Sigma. TRAP-positive (TRAP+) cells were segregated into two groups: multinuclear (2 to 10 nuclei) and giant (>10 nuclei), as described previously (15).

**Vitamin D Receptor Content**

The vitamin D receptor (VDR) content in the bone marrow cell cultures was determined by an intact cell assay previously described in detail (16). Briefly, mouse bone marrow cells were incubated as described above with radiolabeled 1,25(OH)$_2$D$_3$ or 19-norD$_2$ for 5 d. The cells were washed three times with PBS containing 1% bovine serum albumin (BSA) to remove the radiolabeled vitamin D compounds, and then incubated for 2 h at 37°C in serum-free medium containing 1 nM 1,25(OH)$_2$[26,27-3H]D$_3$ with or without 250 nM radiolabeled 1,25(OH)$_2$D$_3$. Under these conditions, vitamin D compounds bound to the VDR at the end of the 5-d incubation period were competed away by the excess of tritiated ligand. At the end of the incubation, the medium was aspirated and the cells were placed on ice. After the monolayers were washed once with cold PBS containing 1% BSA and twice with cold PBS, they were sonicated into TEDKI buffer (10 mM Tris-HCl, pH 7.4, 1.5 mM ethylenediaminetetraacetate, 5 mM dithiothreitol, 300 mM KCl, and 300 μM phenylmethylsulfonyl fluoride). Aliquots of the sonicate were removed for protein analysis, and the remainder was mixed with the charcoal-coated dextran. After 15 min on ice, the samples were centrifuged for 15 min at 2000 x g. Tritium in the supernatant was determined by scintillation counting. Specific binding was calculated as the difference between total binding (tritiated 1,25(OH)$_2$D$_3$ only) and nonspecific binding (tritiated 1,25(OH)$_2$D$_3$ plus radiolabeled 1,25(OH)$_2$D$_3$). VDR content is expressed as specific binding (fmol) per milligram of cell protein.

**Catabolism of 1,25(OH)$_2$D$_3$ and 19-norD$_2$**

The rates of catabolism of 1,25(OH)$_2$D$_3$ and 19-norD$_2$ by mouse bone marrow cultures were determined by plating the freshly isolated bone marrow cells in culture dishes in αMEM D10 medium containing 10 nM 1,25(OH)$_2$[26,27-3H]D$_3$ or 19-nor-[11-3H]D$_2$ at a specific activity of 0.05 μCi/μmol. Medium containing the tritiated vitamin D compounds was replaced after 48 h. Samples of the medium (100 μl) were taken on day 3 (immediately after the medium change), day 4, and day 5 to determine the amount of tritiated compound remaining. The medium aliquots were mixed with 100 μl of acetonitrile containing 500 ng of radioinert compound to act as carrier and to allow assessment of recovery. Two milliliters of acetonitrile:water (1:1) was added, and the samples were centrifuged at 2000 x g for 10 min. The supernatants were applied to C18-silica cartridges. The cartridges were washed with 5 mL of water, and the vitamin D compounds were eluted with 4 mL of acetonitrile and dried under a stream of nitrogen. The amount of the tritiated vitamin D compound remaining was determined by resolving the samples on normal phase HPLC using methylene chloride:isopropanol (97:3) as the running solvent. Fractions eluting near the 1,25(OH)$_2$D$_3$ or 19-norD$_2$ peaks were collected, dried, and quantified by scintillation counting. Recovery was assessed by measuring the UV absorbance of the peak of the carrier vitamin D compound.

**Statistical Analyses**

Results are expressed as mean ± SEM. Samples were compared by ANOVA using the program SigmaStat (Jandel, San Rafael, CA). P values < 0.05 were considered significant.
Results

Effects of 19-norD\(_2\) on Osteoclast Differentiation

The ability of 19-norD\(_2\) to differentiate osteoclast precursor cells to mature osteoclasts was assessed in primary cultures of mouse bone marrow grown on plastic dishes. Cultures were treated with 10 nM 1,25-(OH)\(_2\)D\(_3\) or 19-norD\(_2\) on days 1, 3, and 6 of culture. On day 7, the cells were fixed with 2.5% glutaraldehyde and stained for TRAP activity. TRAP+ multinuclear (2 to 10 nuclei/cell) and giant cells (>10 nuclei/cell) were counted. The numbers of TRAP+ cells from cultures stimulated with 19-norD\(_2\) were not significantly different from cultures stimulated with 1,25(OH)\(_2\)D\(_3\), and both stimulators generated many more TRAP+ cells than if no stimulation occurred (Figure 1).

The relative potencies of 19-norD\(_2\) and 1,25(OH)\(_2\)D\(_3\) to induce osteoclast maturation were determined by treating mouse bone marrow cultures for 5 d with various concentrations of the two compounds. The concentration-dependence curves for the formation of multinucleated TRAP+ cells (Figure 2A) and TRAP+ giant cells (Figure 2B) were biphasic and peaked at 10 nM with both vitamin D compounds. There was no significant difference in the responses to 1,25(OH)\(_2\)D\(_3\) and 19-norD\(_2\) at 1, 10, and 100 nM, indicating that the two compounds had similar potencies in stimulating osteoclastogenesis.

Effect of 19-norD\(_2\) on Osteoclastic Bone Resorption

Having found little difference in the ability of 1,25(OH)\(_2\)D\(_3\) or 19-norD\(_2\) to stimulate the formation of TRAP+ cells, we next tested whether there were differences in the capacity of the two compounds to stimulate bone resorption. Mouse marrow in tissue culture plates was stimulated for 5 d with 10 nM 19-norD\(_2\). On day 6, the cells were scraped and loaded onto dentine slices and then incubated in the absence or presence of 10 nM 1,25(OH)\(_2\)D\(_3\) or 19-norD\(_2\). After 5 d, the dentine slices were stripped of cells and the number of resorption pits, the size of the pits, and the total area resorbed were determined by scanning electron microscopy as described in the Materials and Methods section (Figure 3). In the absence of either vitamin D compound during the incubation with dentine slices, no pits were observed despite that cultures placed on the dentine slices initially contained mature osteoclasts. Conversely, substantial resorption was observed when either vitamin D compound was present during the resorption phase. However, the amount of total area resorbed was 63% less in the 19-norD\(_2\)-treated cultures than in those treated with 1,25(OH)\(_2\)D\(_3\) during the resorption phase. This seemed to be due to a fewer number of pits formed in the presence of 19-norD\(_2\) (Figure 3).

These findings suggest that 19-norD\(_2\) may be unable to activate mature osteoclasts to resorb bone. Therefore, mouse

![Figure 1](image-url)

![Figure 2](image-url)
bone marrow cultures were differentiated by treatment with either 19-nor-D2 or 1,25(OH)2D3 and then treated with 19-nor-D2 during the resorption phase. The results, shown in Figure 4, indicate that osteoclasts that formed in response to 1,25(OH)2D3 treatment during the differentiation phase were able to resorb bone in response to 19-nor-D2 added during the resorption phase. The amount of resorption under these conditions was not significantly different from that achieved with 1,25(OH)2D3 (Figure 4). Furthermore, osteoclasts that formed in response to 19-nor-D2 treatment during the differentiation phase resorbed bone at a high rate when treated with 1,25(OH)2D3 during the resorption phase. Thus, the reduced bone resorption was observed only when the cultures were treated with 19-nor-D2 during both the differentiation and resorption phases.

Concentration-dependence curves for activation of resorption were determined by first differentiating the osteoclast precursor by treatment with various amounts of 19-nor-D2 or 1,25(OH)2D3 and then incubating the differentiated cultures on dentine slices in the presence of the same concentrations of the same compound for 5 additional days. The highest resorption was obtained at 10 nM of both compounds, but the maximum amount achieved with 19-nor-D2 was approximately 30% of that achieved with 1,25(OH)2D3 (Figure 5A). The lower resorption by 19-nor-D2 was reflected in the reduced number of pits (Figure 5B); the area per pit was not different for the two compounds (Figure 5C).

To test possible mechanisms that may be responsible for the lower bone resorbing activity of 19-nor-D2, we measured the rates of catabolism of 1,25(OH)2D3 or 19-nor-D2 in the mouse marrow cultures. Relatively little degradation of either molecule was detected during the first 3 d of culture (data not shown). Fresh medium containing tritiated 19-nor-D2 or 1,25(OH)2D3 was added on day 3, and the amount of each compound remaining was determined by HPLC analysis as described in the Materials and Methods section. Significant catabolism of both 1,25(OH)2D3 and 19-nor-D2 occurred between days 3 and 5 (Figure 6). After 5 d, 78% of the 19-nor-D2 and 63% of the 1,25(OH)2D3 had been degraded. The slightly higher rate of degradation of 19-nor-D2 may contribute to its lower bone resorbing activity but is unlikely to be the primary factor responsible for the disparate effects of 19-nor-D2 and 1,25(OH)2D3 on bone resorption.

Active vitamin D compounds are known to stabilize the VDR in target cells, leading to VDR upregulation and potentiation of the activity of the ligand. Therefore, we determined whether the lower activity of 19-nor-D2 with time could be due to defective stabilization of the VDR. Bone marrow cultures treated for 5 d with 10 nM 19-nor-D2 or 1,25(OH)2D3 had the same VDR levels as determined by whole-cell binding assay (Figure 7). Thus, the disparate activities of 19-nor-D2 and 1,25(OH)2D3 could not be explained by differences in VDR levels.

To determine which structural differences in 19-nor-D2 are responsible for the decreased bone resorbing activity in vitro, we also tested the activities of 1,25(OH)2D3 and 19-nor-D3 in this system. Bone marrow cultures were treated with 10-nM concentrations of the compounds during both the differentiation and activation phases, and the area resorbed was determined. Although total resorption was lower in cultures that were treated with 19-nor-D3, the reduction was not significant (Figure 8). The data suggest that both the 19-nor modification.

**Figure 3. In vitro** bone resorption in response to 10 nM 1,25(OH)2D3 or 19-nor-D2. Mouse marrow cultures were treated for 5 d in culture dishes with 10 nM 19-nor-D2. The cultures were scraped, plated onto dentine slices, and treated with 10 nM 1,25(OH)2D3 (■) or 10 nM 19-nor-D2 (□) for 5 d. The dentine slices were then prepared for scanning electron microscopy, and the total area resorbed, the number of pits, and the area resorbed per pit were determined. The data are expressed as a percentage of the value for each parameter obtained by treatment with 1,25(OH)2D3. n = 4 per group. ∗P < 0.05 versus 1,25(OH)2D3-treated group.

**Figure 4.** Reduced bone resorption by 19-nor-D2 is dependent on time of exposure. Mouse marrow cultures were incubated on tissue culture dishes for 5 d with 10 nM 1,25(OH)2D3 or 10 nM 19-nor-D2. On day 6, the cells were scraped and loaded onto dentine slices. Cultures from each group were incubated with 10 nM 1,25(OH)2D3 or 10 nM 19-nor-D2 for 5 d. The dentine slices were then examined for resorption by scanning electron microscopy. The data are expressed as a percentage of the value for each parameter obtained by treatment with 1,25(OH)2D3. n = 4 to 5 per group. ∗P < 0.05 versus the group treated with 1,25(OH)2D3 in both the differentiation and activation phases.
and the $D_2$ side chain are necessary for the reduced activity of 19-nor$D_2$.

**Discussion**

Analogs of vitamin D that retain the PTH-suppressing activity of 1,25(OH)$_2$D$_3$ but have lower calcemic activity provide a safer means of correcting or possibly preventing the secondary hyperparathyroidism that develops in patients with chronic renal disease. 19-nor$D_2$ was shown to meet these criteria in the renal failure rat model (3) and, after successful clinical trials (4), is now available for treatment of patients. The mechanism(s) responsible for the selectivity of 19-nor$D_2$, specifically...
its decreased calcemic activity, is not well understood. However, we recently found that with chronic treatment, 19-norD₂ is less potent than 1,25(OH)₂D₃ in stimulating both intestinal calcium transport and bone mobilization in vivo and that these disparate effects of 19-norD₂ and 1,25(OH)₂D₃ do not seem to involve pharmacokinetic differences (12,17). This pointed toward an intrinsic defect in 19-norD₂ to stimulate calcium transport and bone resorption that may be reproducible in vitro.

In the present study, we showed that 19-norD₂ is dramatically less effective than 1,25(OH)₂D₃ in stimulating bone resorption in an in vitro mouse marrow culture system. In principle, this reduction in bone resorption could be accomplished by limiting the number of mature osteoclasts present or by limiting the bone resorptive activity per osteoclast. One way to limit the bone resorptive activity of osteoclasts is to reduce their survival. However, the number of surviving mature osteoclasts was not different in cultures treated with 19-norD₂ or 1,25(OH)₂D₃ (Figures 1 and 2). We believe, therefore, that 19-norD₂ is fully capable of inducing survival factors that are required by the osteoclasts.

It was more difficult to distinguish between potential effects on differentiation versus activation. By one measure of differentiation, the formation of multinucleated TRAP+ cells, we did not detect statistical differences in the number of cells that were differentiated by the 19-norD₂ and 1,25(OH)₂D₃, and we did not detect distinct morphologic differences. However, in resorption studies, we found that the reduced activity of 19-norD₂ was evident only when the analog was present during both the differentiation and activation phases. Cultures differentiated with 19-norD₂ could be activated by 1,25(OH)₂D₃ to resorb bone as well as cultures treated with 1,25(OH)₂D₃ in both phases, suggesting that the limitation in the action of 19-norD₂ could be rapidly reversed by 1,25(OH)₂D₃. However, cultures differentiated with 1,25(OH)₂D₃ could resorb bone normally when treated with 19-norD₂ during the activation phase, suggesting that the reduced activity of 19-norD₂ could not be manifested within the 5-d activation phase. At least one explanation for these findings is that the reduced resorption by 19-norD₂ requires a prolonged exposure to the analog. This would be consistent with our in vivo observation that calcium mobilization from bone within 24 h of injection is similar for 19-norD₂ and 1,25(OH)₂D₃, whereas after 7 daily injections, there is a clear difference in the calcemic response by the bone (12).

The dose-response curves for 1,25(OH)₂D₃ and 19-norD₂ for TRAP+ cell formation and bone resorption showed maximum stimulation for each compound at 10 nM. This indicated that the diminished action of 19-norD₂ on bone resorption probably was not due simply to a shift in the concentration-dependence curve. In fact, the VDR affinity of 19-norD₂ is only slightly lower (one third less) than that of 1,25(OH)₂D₃ (3).

The mechanism(s) responsible for the lower in vitro bone resorbing activity of 19-norD₂ is not clear. One possible explanation, a more rapid catabolism of 19-norD₂, was investigated. The analog was degraded slightly more rapidly than 1,25(OH)₂D₃, and although this may contribute to the lower activity of 19-norD₂, it is unlikely that this could explain fully the disparity in bone resorption elicited by the two compounds. The activities of vitamin D analogs can also be greatly influenced by their affinities for the serum DBP. This protein can significantly retard cellular uptake of vitamin D compounds that bind with high affinity. This mechanism cannot explain the low in vitro bone resorbing activity of 19-norD₂ because its DBP affinity is only slightly lower (one third less) than that of 1,25(OH)₂D₃.

There is also evidence that some vitamin D analogs bind to the VDR differently than 1,25(OH)₂D₃, producing a functionally altered conformation in the receptor that can influence its activity and/or the recruitment of coactivators (18–21). Conformational changes in the VDR upon binding of 19-norD₂ has not been examined.

The bone resorbing action of 1,25(OH)₂D₃ does not involve a direct stimulation of the osteoclast but is mediated indirectly by a factor released by the osteoblast. Recent studies have identified this osteoclast differentiation factor as osteoprotegerin ligand (OPGL) (22–24), a protein on the surface of the osteoblast that is induced by 1,25(OH)₂D₃ (25,26). Interaction of OPGL with a receptor (receptor activator of NF-κB [RANK]) on osteoclast precursors induces maturation. In addition, binding of OPGL to RANK on mature osteoclasts is involved in 1,25(OH)₂D₃-mediated activation of bone resorption. Other factors can also influence osteoclast formation and activity. One of these, osteoprotegerin, binds to osteoclast differentiation factor and prevents its interaction with the receptor on the osteoclast (27,28). OPG expression is downregulated by 1,25(OH)₂D₃. It is the ratio of OPGL/OPG that determines both the rate of maturation of osteoclast precursors and the degree of activation of osteoclastic bone resorption.

A potential mechanism to explain our results is that 19-norD₂ produces a lower OPGL/OPG ratio. This lower ratio may still produce a full effect on differentiation (at least cell fusion and induction of TRAP expression) but is unable to
activate fully the mature osteoclasts. In fact, Lacey et al. (22) found that maturation of osteoclast precursors requires lower levels of OPGL than are needed for activation of resorption. In the present study, when the cultures were switched from 1,25(OH)2D3 (in the differentiation phase) to 19-norD2 (during the activation phase), the OPGL/OPG ratio may have changed so slowly that the rate of bone resorption was not markedly less than with 1,25(OH)2D3 present during the activation phase. Only when the OPGL/OPG ratio was low at the onset of the activation phase, i.e., when 19-norD2 was present during the differentiation phase, and remained low was there a substantial reduction in bone resorption. This model also suggests that when cells were differentiated with 19-norD2 and then activated by 1,25(OH)2D3, the OPGL/OPG ratio increased rapidly enough to activate resorption nearly as effectively as when the cultures were treated with 1,25(OH)2D3 in both phases. This hypothesis, which is based on our current understanding of the mechanism by which vitamin D compounds mediate the maturation and activation of osteoclasts, remains to be tested.

In summary, we have demonstrated an impairment of bone resorption in vitro by 19-norD2. Although the mechanism for the disparate effects of the analog and 1,25(OH)2D3 are not known, the mouse bone marrow culture model will facilitate the determination of the molecular basis for the blunted calcemic actions of 19-norD2 on bone. In addition, structure/activity studies in this model may define modifications to vitamin D analogs that reduce their calcemic effect on bone and enhance their selectivity, providing safer, more effective therapeutic agents.

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References


