Novel, Selective Vitamin D Analog Suppresses Parathyroid Hormone in Uremic Animals and Postmenopausal Women

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Key Words

Secondary hyperparathyroidism · Renal failure · Vitamin D-based therapy

Abstract

Background: The use of 1 α -hydroxylated vitamin D therapy to control secondary hyperparathyroidism in renal failure patients has been a success story, culminating with the demonstration of increased life expectancy in patients treated with these compounds. However, hypercalcemic episodes have been a recurrent problem with these therapies and have resulted in the added use of calcium mimetics. Clearly there is good reason to search for improved vitamin D therapy. In our inventory of vitamin D compounds, 2-methylene-19-nor-(20S)-1 α ,25-dihydroxyvitamin D₃ (2MD) surfaced as a potential candidate. This was based on its preferential localization in the parathyroid gland and a clear suppression of serum parathyroid hormone (PTH) levels without a change in serum calcium in a clinical trial in postmenopausal women. **Methods:** 2MD has now been tested in the rat 5/6-nephrectomy model of renal failure, and in postmenopausal women to determine if it can suppress serum PTH at doses that do not elevate serum calcium and serum phosphorus concentrations. **Results:** Daily oral treatment of uremic rats on 2.5 ng/bw/day of 2MD dramatically suppressed PTH with-

out a change in serum calcium or serum phosphorus. Further, PTH was suppressed in postmenopausal women after only 3 daily oral doses of 2MD that continued for 4 weeks with no change in serum calcium or serum phosphorus. **Conclusion:** These results coupled with a pharmacokinetic half-life of ~24 h suggest that 2MD given either daily or at the time of dialysis may be a superior therapy for secondary hyperparathyroidism in chronic renal failure patients.

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Introduction

The treatment of renal osteodystrophy with a vitamin D compound was initiated by Stanbury's group before the discovery of the vitamin D endocrine system [1]. The discovery of 25-hydroxyvitamin D₃ (25-OH-D₃) as an active metabolite of vitamin D [2] led to its application in the treatment of bone diseases secondary to renal failure as the drug, Calderol[®] (Upjohn and Organon in the United States and Dedrogy[®] by Roussel Uclaf in Europe) [3, 4]. With radiolabeled 25-OH-D₃ came the realization that 25-OH-D₃ is rapidly metabolized to a highly potent metabolite that was isolated and identified as 1 α ,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) [5]. Fraser and Kodicek [6] further demonstrated that the

1 α -hydroxylation takes place in the kidney that was quickly confirmed [7]. Experiments demonstrating that 1,25-(OH)₂D₃ and not 25-OH-D₃ is the active form of vitamin D for calcium and phosphorus homeostasis brought about the realization that a lack of 1,25-(OH)₂D₃ might be responsible for renal osteodystrophy [8, 9]. As soon as synthetically produced 1,25-(OH)₂D₃ became available as Rocaltrol[®], it became the treatment of choice without fully realizing its mode of action [10, 11]. The discovery of the vitamin D receptor [12, 13] was soon followed by the finding that the parathyroid gland is a prime target of 1,25-(OH)₂D₃ regulating the production and secretion of parathyroid hormone (PTH) [14–16]. At last it became clear that the prime result of the vitamin D therapies in renal patients is the suppression of secondary hyperparathyroidism [15, 16]. A side effect of therapy with oral 1,25-(OH)₂D₃ is hypercalcemia resulting from increased intestinal calcium absorption and bone calcium mobilization [17]. Intravenous 1,25-(OH)₂D₃ (Calcijex[®]) was a clear improvement bypassing the ‘first-pass’ effect of oral 1,25-(OH)₂D₃ on intestine [17]. 1 α -Hydroxyvitamin D₂ and 1 α -hydroxyvitamin D₃ require 25-hydroxylation in the liver before becoming active. They also represent an improvement over Rocaltrol[®] by avoiding first-pass effect on calcium absorption from the intestine [18, 19]. Two analogs, 19-nor-1 α ,25-dihydroxyvitamin D₂ [20] and 22-oxa-1 α ,25-dihydroxyvitamin D₃ [21], were introduced with reduced calcemic activity that appeared to provide a wider therapeutic window. Vitamin D treatments in general and 19-nor-1 α ,25-dihydroxyvitamin D₂ (Zemlar[®]) in particular increase the life expectancy of chronic kidney disease (CKD) patients [22].

Another analog, 2-methylene-19-nor-(20S)-1 α ,25-dihydroxyvitamin D₃ (2MD) with bone anabolic activity was being developed for the treatment of osteoporosis [23]. This highly potent analog also possesses increased bone-mobilizing activity [23]. In postmenopausal women, this analog did not increase bone mass but increased both bone synthesis and bone resorption biomarkers [24]. Importantly, it markedly suppressed circulating PTH levels in these patients without changing serum calcium levels. Furthermore, distribution studies conducted in rats, showed a strong localization of 2MD to the thyroid/parathyroid gland. The possible usefulness of 2MD in the treatment of secondary hyperparathyroidism of renal failure seemed compelling. We have carried out a number of studies in the 5/6-nephrectomy rat model of CKD and find that 2MD is a potent suppressor of secondary hyperparathyroidism and appears to have a wide thera-

peutic window. In addition, clinical trials done in postmenopausal women provide encouraging pharmacodynamic and pharmacokinetic results indicating that 2MD will work as an oral therapy for patients with secondary hyperparathyroidism. This paper provides these results.

Methods

Whole-Body Autoradioluminography (WBAL) in Rats

The tissue distribution of 2MD was studied using tritium-labeled 2MD (one tritium incorporated at carbons 9 and 11 of the D ring). The specific activity was 54 Ci/mmol and radiochemical purity as assessed by HPLC was 98%. Male Long-Evans rats were given one single oral dose of radiolabeled 2MD (1.9 μ g/kg or 247 μ Ci/kg) mixed in Neobee M-5 oil. One rat was euthanized by CO₂ asphyxiation at 1, 2, 4, 8, 12, 24, 72, or 168 h after the dose was administered. Euthanized rats were prepared for WBAL by immersion in a hexanes/dry ice bath for 10 min. Quantification of the cryosection images was done as previously described [25, 26]. The lower limit of quantitation was 0.2 ng equivalent hormone/g tissue.

Uremic Rat Studies

Male weanling Sprague-Dawley rats were purchased from Harlan Laboratories (Indianapolis, Ind., USA). On arrival, animals were allowed access to water and standard rodent chow (LabDiet[®] Rat Diet 5012; LabDiet, St. Louis, Mo., USA) ad libitum. Throughout the study, the animals were maintained in temperature- (21.1–22.2°C) and humidity- (40–50%) controlled rooms under a 12 h light/12 h dark cycle. All work was conducted under protocols reviewed and approved by a University of Wisconsin-Madison Institutional Animal Care and Use Committee (Protocol No. A01192).

After 1 week of acclimatization, rats underwent a two-stage subtotal nephrectomy to produce a uremic state. The first surgery was done to remove two thirds of the renal mass of the left kidney by pole ligation. The second surgery was done 1 week later and a ligature was placed around the renal hilum of the right kidney and the entire renal mass was excised. Pain was managed with pre- and postoperative subcutaneous injections of buprenorphine (0.05 mg/kg).

Immediately after the second surgery, rats were switched to a purified diet containing 0.6% calcium and 0.9% phosphorus [20]. After 4 weeks, the rats had elevated parathyroid hormone levels and were randomized into treatment groups by their baseline iPTH values. Any rat with an iPTH <100 pg/ml was considered unsuccessful in developing secondary hyperparathyroidism and, therefore, excluded from the study. Dosing solutions were delivered to the back of the tongue using a Hamilton syringe fitted with a blunt-ended needle covered in Tygon tubing. Rats received vehicle or vitamin D analogs each morning for 8 weeks. In addition to baseline, blood collections were also performed after both 4 and 8 weeks of treatment.

All blood collections were performed within the first 6 h of the vivarium's light cycle. Rats were sedated with ether and blood was sampled from the tail artery. Blood draws that occurred during treatment with study drug were performed approximately 24 h after the previous dose. iPTH levels were measured in serum by ELISA (Immutopics, Inc., San Clemente, Calif., USA). Serum calcium levels were measured in 0.1% lanthanum chloride by atomic

Fig. 1. Tissue distribution of ^3H -2MD in Long-Evans rats. Each bar represents 1 rat. 2MD radioequivalents (ng Eq/g) were determined by averaging the concentration of radioactivity from multiple sections. NT = Not taken, i.e. an evaluable sample was not obtained.

absorption spectroscopy (Perkin-Elmer Spectrometer, Model 3110). Both serum phosphorus and serum creatinine were measured with a clinical chemistry benchtop analyzer (ABX Pentra 400; Horiba Medical) using reagents purchased from HORIBA ABX Diagnostics (Irvine, Calif., USA).

Postmortem whole-body bone mineral densitometry was performed using DEXA (Hologic QDR 80191).

The vehicle for all oral formulations was 5% ethanol in Neobee M-5 oil (Spectrum). Neobee M-5 oil was measured gravimetrically. The 2MD was determined to be >99% pure by HPLC analysis. 2MD concentrations in ethanol were measured by UV spectrometry using a molar extinction coefficient = 42,000 at 252 nm. Dosing solutions were formulated to be delivered at 0.5 ml/kg bw. The actual volume of dosing solution delivered to each rat was based on individual body weight and adjusted weekly.

Phase 1B Dose-Finding Study in Postmenopausal Women

A double-blind, placebo-controlled once-daily oral dose of 2MD in postmenopausal women study was conducted to ascertain safety and efficacy. This study was conducted at a single US site (Comprehensive NeuroScience, Inc., Fort Lauderdale, Fla., USA). Subjects were assigned to 5 cohorts with 12 subjects in each group (8 2MD, 2 calcitriol and 2 placebo subjects/cohort). After the first cohort received 7 doses, laboratory data were reviewed and the second cohort initiated based on the safety information from the first cohort. All subsequent cohorts were begun after the first week of laboratory results were available from the preceding cohort. 64 postmenopausal women were enrolled and randomized to treatment; 4 subjects withdrew from the study (1 each from the placebo and 0.05- μg 2MD groups and 2 from the 0.17- μg 2MD group) resulting in 60 healthy postmenopausal women completing the trial in one of seven dose groups – placebo, 0.5 μg calcitriol, 50, 110, 170, 220 or 440 ng 2MD ($n = 8$ for each 2MD group and $n = 10$ for calcitriol and placebo groups). Dose administration occurred once daily in the form of gelatin capsules for 28 consecutive days. Total serum calcium, serum phosphorus, and iPTH were analyzed at screening, and baseline and 1, 3, 7, 14, and 28 days postdose initiation and 2 weeks after the dose was stopped. Plasma was also collected for pharmacokinetic measurements using HPLC in conjunction with a cell-based assay. This assay involved solid-phase extraction followed by HPLC separation of 2MD from other endogenous forms of vitamin D and then quantitation using a re-

porter cell assay [27]. This trial has been registered at ClinicalTrials.gov (NCT01969656) was approved by the Independent Institutional Review Board (6738 West Sunrise Blvd, Suite 102, Plantation, FL 33313, USA), and was conducted in accordance with the Helsinki Declaration of 1975.

Statistical Analysis

All statistical analyses were done using the SAS mixed-model procedures with Dunnett's adjustment (SAS Institute, Inc., Cary, N.C., USA).

Results

It was unexpected to find that radioactive 2MD localized to the greatest extent in thyroid/parathyroid glands (fig. 1). As expected, intestine and bone, both well-known target organs of vitamin D action, did show localization of radioactive 2MD, while a non-target tissue (e.g. heart) did not. Previously using a highly specific antibody, no VDR could be detected in either heart or skeletal muscle [28]. The low or basal level of radioactivity seen in the heart muscle (fig. 1) is found in all tissues and likely resulted from retained blood that contains ca. 10 pg/ml of 2MD. By 72 h postdose, the radioactivity had essentially disappeared from all but three tissues.

The localization in the thyroid/parathyroid gland led us to consider that 2MD might have preferential action in the parathyroid gland to suppress synthesis and secretion of PTH. As with previous vitamin D compounds, the 5/6-nephrectomy rat model was used to test this hypothesis. A representation of several such experiments is shown in figure 2. A dose-dependent suppression of serum PTH was observed after both 4 and 8 weeks of dosing with 2MD. A daily oral dose of 1 ng/kg bw/day suppressed serum PTH by at least 35% without changing serum cal-

Fig. 2. Serum PTH, calcium, and phosphorus levels in uremic rats treated either with 2MD (**a–c**) or Zemlar® (**d–f**). Each bar represents the mean $\pm$ SEM of 8–13 nephrectomized rats or 5 sham-

operated rats. Asterisks indicate statistically significant differences compared to vehicle controls and plus signs are compared to sham controls. $p < 0.001$.

Fig. 3. Bone mineral density (BMD) of uremic rats treated with 2MD. Rats described in figure 2 were used to determine BMD. Asterisks indicate statistically significant differences from vehicle controls and plus signs are significant differences from sham controls. $p < 0.001$.

cium and phosphorus levels for as long as 8 weeks (fig. 2a). A dose of 2.5 ng/kg bw/day suppressed PTH maximally and within 4 weeks of dose initiation. Serum calcium levels remained unchanged until a dose of 5 ng 2MD/kg bw/day was administered (fig. 2b). 2MD had little effect on serum phosphorus (fig. 2c), although a trend to lower values was noted in one study.

We tested Zemplar® (paricalcitol) in an identical or side-by-side comparison (fig. 2d–f). Significant PTH suppression occurred with a daily dose of 100 ng/kg bw/day and was accompanied by significant elevation of serum calcium compared to sham controls (fig. 2e). Thus, the effective dose of 2MD was 40 times less than Zemplar® and without serum calcium elevation.

Based on 2MD's strong potency in other models of bone disease [23], BMD was measured in the uremic rats at termination (8 weeks of daily oral 2MD administration) (fig. 3). As predicted from all rat models tested previously [23], 2MD not only prevented the loss of bone mineral density as a result of the nephrectomies but actually increased BMD above sham levels.

The effectiveness of 2MD in suppressing PTH in the human population was demonstrated in a previous study of postmenopausal women in which a dose of 220 ng/day

for 6 months caused a significant reduction of PTH [24]. In this population, three consecutive daily doses (110–440 ng) of 2MD caused a significant decrease in PTH (fig. 4a). PTH suppression is also apparent, although not statistically significant, 2 weeks after the last dose of 2MD. This rapid and sustained effect is consistent with the pharmacokinetic profile observed in this same population of women (table 1). Although the plasma concentration-time data did not allow calculation of a terminal half-life in this study, a half-life of ~24 h was estimated. No significant increases in serum calcium or phosphorus were noted at any dose level in this trial (fig. 4b, c). In this study, plasma 2MD was measured as a function of time following the last dose of 2MD (fig. 5). Because of its long half-life (table 1), plasma levels remained constant for 24 h after the last dose.

Discussion

The value of vitamin D-based therapy in dialysis patients is very clear from their increased survival [22] and the fact that the major, if not sole, source of 1,25-(OH)₂D₃, the active form of vitamin D is the kidney. Certainly the development of vitamin D therapy has progressed from the use of larger doses of vitamin D itself [1], to its first metabolite, 25-hydroxyvitamin D (3,4), and to the first use of oral 1,25-(OH)₂D₃ [10, 11]. Unfortunately in chronically orally dosed animals, 1,25-(OH)₂D₃ loses its effectiveness because 1,25-(OH)₂D₃ induces CYP24A1 [29–31] in the intestine that then destroys 1,25-(OH)₂D₃ as it is absorbed [32], limiting its impact on internal targets such as the parathyroid gland. By providing vitamin D compounds that are 1-hydroxylated but lack the 25-hydroxyl, orally administered analogs are unable to induce CYP24A1 in the intestine as it is absorbed. Once in the circulation, these analogs are 25-hydroxylated in the liver, giving rise to the hormonal form that can then act to suppress the parathyroid gland. Thus, 1α-hydroxyvitamin D₂ and 1α-hydroxyvitamin D₃ have been successfully applied in the USA as Hectorol® and elsewhere as Alfarol® and 1Alpha, etc. [18, 19], resulting in a definitive improvement over oral 1,25-(OH)₂D₃ (Rocaltrol®). The provision of 1,25-(OH)₂D₃ in an injectable form (Calcijex®) was also an improvement by avoiding stimulating CYP24A1 in the intestine and first-pass effect in intestinal absorption of calcium and phosphate [32]. However, the dose range before unwanted hypercalcemia remained small, giving nephrologists the difficult task of adjusting the dose [17]. The introduction of two analogs with reduced calcemic activity (i.e. 22-oxa (OCT) and Zem-

Fig. 4. Serum PTH (a), serum calcium (b), and serum phosphorus (c) levels in postmenopausal women after 28 days of once-daily 2MD administration and 2 weeks after discontinuation of treatment. Each data point represents the mean $\pm$ SD of 8–10 women. All but the lowest dose showed a statistically significant suppression after 3 days ($p < 0.05$). The highest dose group, 440 ng 2MD, showed statistically lower values at all time points except at day 42. $p < 0.01$.

plar[®]) was a further improvement in dialysis therapy [20, 21]. Because of their short half-lives in vivo, the 3 times/week doses needed were fairly high, and the range of effectiveness prior to hypercalcemia remained narrow. The introduction of calcium mimetics seemed to provide the

needed safety margin to the current vitamin D therapies [33], but with a considerable increase in expense and further complication of adjusting the dose of two drugs and an increased risk of side effects. Further, the calcium mimetics did not appear to increase survival of dialysis pa-

Fig. 5. Time course of plasma 2MD concentrations after 28 days of once-daily oral administration in postmenopausal women. Each bar represents the mean $\pm$ SD of 8–10 women.

Table 1. Mean pharmacokinetic parameters following multiple (28 consecutive) once-daily oral doses of 2MD^a

Dose level ng/day	C _{max} pg/ml	T _{max} h	C _{min} ^b pg/ml	AUC _{0–24} pg·h/ml
50	0.66 $\pm$ 0.2	12 (2–24)	0.47 $\pm$ 0.1 ^c	11.8 $\pm$ 3.8
110	2.6 $\pm$ 0.3	3 (2–12)	1.4 $\pm$ 0.9	41.2 $\pm$ 18
170	3.7 $\pm$ 1.2	2.5 (1–12)	1.7 $\pm$ 0.8	57.0 $\pm$ 14
220	4.3 $\pm$ 1.7	3 (1–12)	2.5 $\pm$ 0.9	76.9 $\pm$ 29
440	12.5 $\pm$ 2.3	2.5 (2–12)	7.9 $\pm$ 1.7	236 $\pm$ 35

C_{max} = Maximum observed plasma concentration; T_{max} = time of C_{max}; C_{min} = minimum observed plasma concentration; AUC_{0–24} = area under the plasma concentration curve hours 0–24.

^a Values are mean values $\pm$ SD except for T_{max}, which is median (range); n = 8; ^b C_{min} defined as plasma 2MD concentration at time = 24 h on day 28; ^c n = 7 due to insufficient sample volume from 1 subject at time = 24 h.

tients [34]. It is clear that there is a need for improved therapies for osteodystrophy and secondary hyperparathyroidism in dialysis patients.

In our continuing search for analogs that have selective activity, we prepared an analog that differs from 1,25-(OH)₂D₃ in three ways. Firstly, we introduced an inversion in stereochemistry at the 20-position; secondly, we eliminated the 10,19-methylene group, and thirdly, we introduced a methylene group on carbon-2 [35]. The result, 2MD proved to be the most potent form of vitamin D but most importantly, selective for bone by 30- to 100-fold [23]. Unexpectedly, it is also selective for the parathyroid gland (fig. 2) and potently suppresses serum PTH

[24]. This property and its slow rate of metabolism by CYP24A1 [36] immediately suggested it as a candidate for the treatment of secondary hyperparathyroidism of renal failure.

The entry preclinical bioassay for candidate drugs to control secondary hyperparathyroidism is the 5/6-nephrectomy rat model that successfully predicted the value of Calcijex[®], 22-Oxa and Zemplar[®] [17, 20, 21, 37]. The result of this bioassay for 2MD clearly demonstrates its potency and shows a wider therapeutic window than one of the market leaders, Zemplar[®] (i.e. target efficacy without hypercalcemia). It also acts rapidly and is a osteoanabolic agent, which suggests it is likely to improve the skeleton of dialysis patients. Although the present results suggest excellent promise, its value in dialysis patients must await results from phase 2 trials currently in progress and beyond.

Acknowledgements

This work was supported in part by a fund from the Wisconsin Alumni Research Foundation and a grant from Deltanoid Pharmaceuticals, Inc. The WBAL study and PK analyses were conducted by Pfizer, Inc., Department of Pharmacokinetics, Dynamics and Metabolism, Groton, Conn., USA. Many thanks to all the technical staff for the preparation and care of the uremic rats, including Xiahong Ma, Shinobu Miyasaki, Jennifer Vaughan, William Blaser.

Disclosure Statement

J.B. Zella, L.A. Plum, M. Clagett-Dame, and H.F. DeLuca have a financial interest in Deltanoid Pharmaceuticals, Inc. that is developing 2MD as a pharmaceutical.

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