Identification of a Unique Subset of 2-Methylene-19-Nor Analogs of Vitamin D with Comedolytic Activity in the Rhino Mouse

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The active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), and a series of 2-methylene-19-nor analogs of 1,25(OH)₂D₃ were evaluated for their ability to reduce the size of utricles (comedolytic activity) in a rhino mouse model of acne. All analogs tested, as well as the native hormone, increased the skin epidermal thickness. In contrast, only a subset of analogs that lacked a full side chain and 25-hydroxyl group were found to possess comedolytic activity. A reduction in comedone area could be achieved without adversely affecting serum calcium levels. Although all compounds that contained a side chain ranging from 2 to 5 carbons in length had similar potency as comedolytic agents, increasing the length of the side chain resulted in a progressive increase in calcemic liability. Dose-response studies of the comedolytic analogs showed that an increase in epidermal thickness was achieved at a lower dose than that needed to induce comedolysis. Thus, we have identified a unique subset of vitamin D analogs that produce comedolysis in the absence of hypercalcemia. Further, the activity of vitamin D analogs in causing epidermal hyperproliferation has been distinguished from that resulting in a reduction in utricle size.

Journal of Investigative Dermatology (2010) 130, 2359-2367; doi:10.1038/jid.2010.142; published online 10 June 2010

INTRODUCTION
Acne is a disease of the pilosebaceous unit of the skin. The pathogenesis of this disease involves hyperkeratinization of the upper region of the follicle, increased sebum production by the sebaceous gland, colonization of the follicle by Propionobacterium acnes, and induction of an inflammatory response. Acne therapy is directed towards these pathogenic causes with localized topical treatment used most often, whereas systemic therapy is reserved for the most severe cases (Gollnick, 2003). Topical treatments primarily include retinoid therapy, antibiotics, benzoyl peroxide, and combinations of these agents (Degitz and Ochsendorf, 2008; Kimball, 2008). These treatments are all effective at controlling acne but also induce some unwanted side effects such as photosensitivity and skin irritation. Alternative topical therapeutic agents for acne that do not possess these side effects, or have a wider therapeutic window would be of great advantage for patients with this condition.

Animal models that simulate a subset of disease characteristics have been developed, but none of them recapitulate all aspects of human acne, which is a multifaceted condition. The rhino mouse has been useful in assessing the comedolytic activity of compounds, including retinoids (Van Scott, 1972; Kligman and Kligman, 1979; Ashton et al., 1984; Mezick et al., 1984, 1985; Bouclier et al., 1990, 1991; Bernerd et al., 1991a, b; Zheng et al., 1993; Fort-Lacoste et al., 1999; Sakuta and Kanayama, 2005; Mirshahpanah and Maibach, 2007). The rhino (hrhr⁴) phenotype is due to an autosomal recessive mutation in the hairless (hr) gene. In the rhino mouse, utriculi are derived from the infundibular zone of the initial follicular units, and are histologically similar to comedones (Mann, 1971).

The active hormonal form of vitamin D, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), modulates the differentiation and proliferation of keratinocytes in the skin. In cultured keratinocytes, 1,25(OH)₂D₃ induces differentiation and exerts antiproliferative activity (Hosomi et al., 1983; Holick, 1994; Bikle, 2005). This is believed to account for the positive effects of 1,25(OH)₂D₃ and analogs in the treatment of psoriasis. However, topical application of 1,25(OH)₂D₃ and analogs to normal skin has been shown to induce keratinocyte proliferation and epidermal hyperplasia, both in mice (Gniadecki and Serup, 1995; Gniadecki et al., 1995;
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Fujimura et al., 2000) and in humans (Levy et al., 1994). 1,25(OH)₂D₃ can either stimulate or inhibit DNA synthesis in cultured human keratinocytes, depending on the composition of the culture media (Gniadecki, 1996). Thus, the ability of 1,25(OH)₂D₃ to promote or inhibit proliferation or differentiation may depend on the physiological context, raising the possibility that vitamin D and its analogs could be useful as therapeutic agents for other disorders of keratinization. Recently, maxacalcitol, an analog of vitamin D₃ used in the treatment of psoriasis, was evaluated in the rhino mouse and was found to have comedolytic activity (Hayashi et al., 2006). This observation suggests that 1,25(OH)₂D₃ and its analogs may also have utility in the treatment of comedonal acne.

In addition to its effects on cellular differentiation, the best-described physiological function of the vitamin D hormone is to maintain circulating levels of calcium and phosphorus (Jones et al., 1998). However, the ability of 1,25(OH)₂D₃ to increase blood calcium level constitutes the major side effect in the pharmacological use of 1,25(OH)₂D₃ and its analogs. The recent generation of 19-nor-1,25(OH)₂D₃ analogs with modifications in the 2-carbon position has yielded compounds with physiological selectivity and in some cases, analogs with lower calcemic activity (DeLuca et al., 2007). In this study, we examine the activity of a series of 2-methylene-19-nor analogs of 1α,25-dihydroxyvitamin D₃ in the skin of the rhino mouse. Herein, we show that, although all analogs tested induce epidermal thickening, only a subset exert comedolytic activity.

RESULTS

The ability of 1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) and a number of structurally modified 2-methylene-19-nor derivatives of 1,25(OH)₂D₃ and D₂ to induce comedolysis and/or epidermal thickening in the rhino mouse was evaluated as described in Figure 1. Two general groups of analogs were studied: those containing a full side chain (containing carbons 20–25; Table 1) and those in which the side chain was shortened and the 25-hydroxyl group eliminated. Figure 2a shows the structure of 1,25(OH)₂D₃ and the structure common to 2-methylene-19-nor analogs. Previous studies of 19-nor analogs containing a full side chain revealed a selective activity profile including a higher potency in inducing cellular differentiation than the parent hormone. Shortened side chain analogs were also previously shown to be biologically active in vivo and nearly devoid of calcemic liability (Plum et al., 2004).

1,25(OH)₂D₃ does not induce comedolysis

Mice treated with 1,25(OH)₂D₃ did not show a reduction in comedone size at either of the two doses tested (6 or 19 nmol kg⁻¹BW⁻¹), compared with vehicle control (Figure 2b–d). In fact, comedone area was significantly increased in both the groups receiving 1,25(OH)₂D₃ (Figure 2e). When the effect of 1,25(OH)₂D₃ on epidermal thickness was assessed, only a modest increase (33% above vehicle) was noted at the 19 nmol kg⁻¹BW⁻¹ dose (Figure 2f) and there was no visual skin inflammation or influx of inflammatory cells (neutrophils, data not shown). Animals in both hormone-treated groups were exposed to drug, as evidenced by a significant increase in total serum calcium levels (11.6 and 12.0 mg per 100 ml, in the 6 and 19 nmol kg⁻¹BW⁻¹ groups, respectively, compared with 9.5 and 8.7 mg per 100 ml in the vehicle groups (Figure 2g). Thus, 1,25(OH)₂D₃ was ineffective in inducing comedolysis and only the highest dose of the native hormone was able to modestly increase the epidermal thickness.

2-Methylene-19-nor analogs of vitamin D with a full side chain are not comedolytic

The structure and activities of 19-nor analogs of 1,25(OH)₂D₃ and D₂ containing a 25-hydroxylated side chain and modified to contain a methylene group in the 2-carbon position are shown in Table 1. All of them bind with high affinity to the vitamin D receptor (VDR) in vitro, activate transcription, and induce bone calcium mobilization, and intestinal calcium transport in vivo (Sicinski et al., 2002; Barycki et al., 2009; see the patent information described in the section Chemicals under Materials and Methods). The dose of drug used to test for activity in skin was based on in vivo potency, with the goal of using a dose that would produce a 1.0–3.0 mg per 100 ml increase in serum calcium level to ensure sufficient drug exposure. For some analogs, a noncalcemic dose was also examined. After 3 weeks of treatment, all 2-methylene-19-nor analogs shown in Table 1 were active in increasing epidermal thickness, whereas none were effective in reducing the comedone area. Epidermal
thickness was increased the most (nearly 3 times that of vehicle) by the highly potent analog, 2-methylene-19-nor-(20S)-26,27-dimethylene-1α,25(OH)2D3 (CAGE-3); thickening was observed at a dose of CAGE-3 as low as 0.025 nmol kg⁻¹ BW¹ and was maximal at a 3.2-fold higher dose (0.079 nmol kg⁻¹ BW¹; Supplementary Figure S1 online). An increase in cellularity in the dermis comprised largely of mononuclear cells and neutrophils was also noted in hematoxylin and eosin sections, and a doubling of neutrophil number occurred at the same dose that produced a significant increase in epidermal thickness (data not shown). However, there was no evidence for an increase in the number of eosinophils when examined by histological staining (Luna) in the highest CAGE-3-dose group. Interestingly, an increase in sebaceous remnant area but not number was observed with increasing doses of the compound starting at 0.079 nmol kg⁻¹ BW¹ (Supplementary Figure S1f online), although it is not known whether the amount or composition of sebaceous lipid was altered. The ability of compounds to increase epidermal thickness was not due to elevated serum calcium levels, as the analogs 2-methylene-(20R,25S)-19,26-dinor-1α,25-dihydroxyvitamin D3 and CAGE-3 when tested at noncalcemic doses still produced significant skin thickening (Table 1; Supplementary Figure S1g online).

2-Methylene-19-nor-(20S)-1α-hydroxybishomopregnacalciferol (2MbisP) induces both comedolysis and epidermal thickening

As none of the 19-nor analogs with a full side chain showed comedolytic activity, the ability of 2MbisP, a low-calcemic 19-nor analog that lacks a 25-hydroxyl group and most of the vitamin D side chain, was tested. Remarkably, 2MbisP not only increased epidermal thickness but also reduced the size of comedones in a dose-dependent manner. As shown in Figure 3, a dose of 69 nmol kg⁻¹ BW¹ was ineffective in comedolysis, but produced a significant increase in epidermal thickness and some neutrophil infiltration (approximately a fourfold increase in cell number over vehicle; data not shown). The effect of 2MbisP on epidermal thickening was

<table>
<thead>
<tr>
<th>Table 1. Biological activity of 19-nor 1,25(OH)2 –D2 and -D3 analogs containing a 25-hydroxylated side chain</th>
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<td><strong>Chemical name</strong></td>
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<tr>
<td>2-Methylene-1α,25-dihydroxy-(17E)-17(20)-dehydro-19-norvitamin D3 (Vit-III 17-20E)</td>
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<tr>
<td>2-Methylene-(20R, 25S)-19,26-dinor-1α,25-dihydroxyvitamin D3 (NEL)</td>
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<tr>
<td>2-Methylene-19-nor-(20S)-26,27-dimethylene-1α,25-dihydroxyvitamin D3 (CAGE-3)</td>
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<tr>
<td>2-Methylene-18,19-dinor-(20S)-1α,25-dihydroxyvitamin D3 (VD-03)</td>
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<tr>
<td>2-Methylene-19-nor-24-epi-1α,25-dihydroxyvitamin D3 (20R-2MD-24-epi)</td>
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The activities of compounds in binding to the receptor (kD; nM), and in inducing HL60 cell differentiation (IC50; nM) are as follows: 2-methylene-1α,25-dihydroxy-(17E)-17(20)-dehydro-19-norvitamin D3 (Vitamin-III 17–20E) (0.4, 20; US 7,241,752 B2); 2-methylene-(20R,25S)-19,26-dinor-1α,25-dihydroxyvitamin D3 (NEL) (0.04, 1; US 7,528,122 B2); 2-methylene-19-nor-(20S)-26,27-dimethylene-1α,25-dihydroxyvitamin D3 (CAGE-3) (0.03, 0.04; Sicinski et al., 2002); 2-methylene-18,19-dinor-(20S)-1α,25-dihydroxyvitamin D3 (VD-03) (0.02, 0.4; Barycki et al., 2009); 2-methylene-19-nor-24-epi-1α,25-dihydroxyvitamin D3 (20R-2MD-24-epi) (0.04, 0.1; US 7,232,810 B2). Values for 1,25(OH)2D3 are given for comparison (0.07, 4). Significant differences from vehicle are indicated by an asterisk, *P<0.05.
maximal at a dose 0.5 log units higher (218 nmol kg\(^{-1}\)BW), and an increase in sebaceous gland area was also seen, but there was no further increase in neutrophil number. At this dose, a significant reduction in comedone area was observed (44%), with a maximal effect observed at 690 nmol kg\(^{-1}\)BW. Higher doses of compound (up to 2,180 nmol kg\(^{-1}\)BW) did not improve the extent of comedolysis, and produced a significant increase in serum calcium level (2.1 ± 0.2 mg per 100 ml above vehicle; data not shown). No redness or swelling of the skin was noted after 21 days of treatment, although in some cases exfoliation was evident (data not shown).

The activity of 2MbisP in decreasing comedone area and increasing epidermal thickness did not change when compared at 3 and 6 weeks, indicating that the effects were maximal at the earlier time point (Supplementary Figure S2 online). Groups treated topically with 2MbisP (218 nmol kg\(^{-1}\)BW) for 3 or 6 weeks showed a 43% reduction in comedone area, as well as a 230% increase in epidermal thickness compared with the vehicle groups. The ability of 2MbisP to increase epidermal thickness was the result of an increase in cell proliferation as assessed by an increase in bromodeoxyuridine incorporation in the basal cell layer as a result of this treatment (Supplementary Figure S3 online).

The effect of side chain length on activity
In the next experiment, the importance of side chain length to comedolytic activity was explored in more detail after 3 weeks of topical drug application. 2-methylene-19-nor-1α-hydroxy-pregnacalciferol (2MPregna) and 2-methylene-19-nor-1α-hydroxy-homopregnacalciferol (2MP), each with a further reduction in side chain length compared with 2MbisP (two and one carbon(s), respectively), as well as a compound with an additional carbon compared with 2MbisP,
2-methylene-19-nor-(20S)-trishomo-1α-hydroxypregnacalciferol (2MtrisP), were studied. All of the abbreviated side chain 2-methylene-19-nor analogs, when administered at 690 nmol kg\(^{-1}\)BW, showed significant comedolytic activity compared with the vehicle control (Figure 4a). At the lower dose tested (218 nmol kg\(^{-1}\)BW), all of the analogs, with the exception of 2MP, produced a slight reduction in comedone area. All analogs tested also caused a significant increase in epidermal thickness at both doses (Figure 4b). Although small changes in the side chain length did not alter comedolytic activity, increasing the length had a pronounced effect on the total serum calcium level (Figure 4c). Serum calcium levels at day 21 remained unchanged for both 2-methylene-19-nor-1α-hydroxy-pregnacalciferol, and 2MP at each dose, and for 2MtrisP at the lower dose. However, at the highest dose of 2MtrisP and both doses of 2MtrisP, serum calcium levels were significantly increased to above that of the vehicle group. When the side chain of 2MtrisP was further extended by two carbons, the comedolytic potential of the analog could not be tested because of the severe hypercalcemia that was apparent by day 4 of the experiment (data not shown). Therefore, 19-nor-1α,25-(OH)\(_2\)D\(_3\) analogs that significantly reduce the comedone area at doses that do not cause hypercalcemia were identified; however, increasing the length of the side chain increased calcemic liability. Furthermore, this work shows that a terminal hydroxyl group is not necessary for 2-methylene-19-nor analogs of 1,25(OH)\(_2\)D\(_3\) to show comedolytic activity.
2-Methylene-19-nor-(20R)-1α-hydroxy-bishomopregnacalciferol (20R-2MbisP) is an effective comedolytic agent but is slightly less potent than the 20S isomer. The importance of the configuration of the methyl group at carbon 20 within the 2MbisP structure in comedolytic activity was further explored. At 218 nmol kg\(^{-1}\)bw, 20R-2MbisP was ineffective in reducing the comedone area (Figure 5), whereas a higher dose of 690 nmol kg\(^{-1}\)bw was similar in efficacy to the 218 nmol kg\(^{-1}\)bw dose of 20S-2MbisP (see Figure 3). Furthermore, 20R-2MbisP was able to produce a 55% reduction in comedone size without an accompanying increase in serum calcium levels. Thus, the 20R isomer of 2MbisP seemed to be approximately 0.5 log units less active in comedolytic activity than the 20S configuration. However, both compounds were efficacious at doses that did not increase blood calcium levels.

**DISCUSSION**

From these studies, a specific class of vitamin D analogs that alters the size and shape of utricles and thickness of the epidermis in the skin of the rhino mouse in a dose-dependent manner was identified. Whereas 1,25(OH)\(_2\)D\(_3\) and all 2-methylene-19-nor analogs tested caused an increase in epidermal thickness, only those that lack the 25-hydroxyl group and have a side chain less than five carbons in length exhibit comedolytic activity.

1,25(OH)\(_2\)D\(_3\) functions by binding to the nuclear VDR, and this ligand-receptor complex regulates the transcription of target genes (DeLuca, 2008; Haussler et al., 2008). The VDR is expressed in a number of cell types in the skin, including the keratinocyte (Stumpf et al., 1979; Hosomi et al., 1983; Pillai et al., 1988; Reichrath et al., 1997). All of the 2-methylene-19-nor-1,25(OH)\(_2\)D\(_3\) and D\(_2\) analogs tested bind with high affinity to the VDR, and have been shown to be transcriptionally active in reporter cell systems (Scinscki et al., 2002; Plum et al., 2004; Barycki et al., 2009; see Table 1 footnote). However, the compounds that are most effective in inducing comedolysis do not have the highest affinity to the VDR. Therefore, there is a specificity of action in comedolysis that cannot be explained merely on the basis of the binding affinity to the VDR. It is possible that tissue and/or cell-specific factors present in the skin are responsible for the ability of some compounds to produce both a hyperplastic epidermis and a reduction in utricle size. It has been shown that hormone binding alters the conformation of the C-terminus of the VDR, creating a binding surface for the productive interaction with coactivator proteins involved in modulating chromatin structure and in interacting with the basal transcriptional machinery (Rochel et al., 2000; Tocchini-Valentini et al., 2001; Vanhooke et al., 2004, 2007). Multiple coactivator proteins have been described, and thus it is possible that the shortened side chain analogs influence the nature of this interaction in a unique manner.

The VDR has been shown to interact with the hairless gene (Hsieh et al., 2003). There are some similarities with respect to defects in hair follicle cycling in VDR knockout and hairless mutant mice (Li et al., 1997; Yoshizawa et al., 1997; Zarach et al., 2004); however, it should be noted that the function of the VDR in this process is independent of the VDR ligand (Skorija et al., 2005). Cell transfection studies suggest that Hairless (Hr) may alter the responsiveness of the VDR to its ligand, but evidence for this *in vivo* is currently lacking. Whether the rhino mouse serves as an accurate predictor of all vitamin D analog-related effects in humans remains to be established.

The shortened side chain 2-methylene-19-nor analogs show many similarities to the effects of retinoids in the rhino model, including hyperproliferation of the epidermis and reversion of utricles to follicles, or in some cases, their complete involution (Kligman and Kligman, 1979). It has been proposed that the ability of retinoids to induce epidermal proliferation is key to their ability to reduce the size of the utricle diameter (Ashton et al., 1984). However, our studies clearly show that vitamin D analogs that increase the thickness of the epidermis do not always have comedolytic activity. Furthermore, analogs that possess comedolytic activity induce hyperplasia at doses at which they are not yet active as comedolytic agents. Only one vitamin D analog has previously been reported to have comedolytic activity in the rhino mouse and it was suggested that the resultant comedolysis was due to an induction of hyperplasia (Hayashi et al., 2006). Our work supports the conclusion that, at least for vitamin D analogs, activity in increasing epidermal proliferation or thickness, *per se*, is not sufficient to induce comedolysis.

Vitamin D analogs are used to treat psoriasis and they cause remission of psoriatic plaques by inhibiting keratinocyte hyperproliferation and inducing differentiation (Reichrath et al., 1997). In this study, however, an increase in epidermal thickness was observed in response to topical application of all 2-methylene-19-nor analogs. In normal human skin, a vitamin D analog used in the treatment of psoriasis, calcipotriol, has been shown to produce a significant increase in skin thickness after 3 weeks of topical application *in vivo* (Levy et al., 1994). In addition to epidermal thickening, the size of sebaceous gland remnants increased with the comedolytic compound 2MbisP, as well as with the noncomedolytic analog CAGE-3. The increase in the sebaceous area and thickening of the epidermis both seem to result from increased cell proliferation (Supplementary Figure S3 online). Interestingly, enlargement of sebaceous glands was also reported after topical treatment of the rhino mouse with retinoid (Kligman and Kligman, 1979; Zheng et al., 1993). *In vitro* studies on sebaceous gland cells have shown that 1,25(OH)\(_2\)D\(_3\) exerts a biphasic effect on sebocyte proliferation that is dependent on dose and cell culture conditions (Krämer et al., 2009). Thus, the effect of vitamin D compounds on skin seems to differ depending on the physiological or pathological state of the organism at the time of application. This highlights the potential of vitamin D analogs as treatment for keratinization disorders such as psoriasis and acne, as well as in conditions in which an induction of epidermal thickening is desired, such as adjunct therapy to corticosteroid drug use.
The major limitation in the use of 1,25(OH)₂D₃ and its analogs in clinical medicine is the potent effect that these compounds have on calcium metabolism. Pharmacological administration of the native hormone by both oral and topical routes can lead to hypercalcemia (Langner et al., 1993; Osborn et al., 1995). A recent major advance has been the development of vitamin D analogs with lesser calcemic liability. The 2MbisP, 2MP, and 2-methylene-19-nor-1α,25(OH)₂D₃ analogs given intraperitoneally have been shown to be very active in inducing the 24-hydroxylase enzyme in skin and suppressing the secretion of parathyroid hormone into the bloodstream in vivo, while having very little effect, if any, on intestinal calcium absorption or calcium mobilization from bone (Plum et al., 2004; DeLuca et al., 2007). These compounds, as well as the isomer 2OR-2MbisP, also function as comedolytic agents when applied topically to the rhino mouse at doses that cause no increase in serum calcium levels. Increasing the vitamin D side chain up to five carbons in length does not alter the comedolytic potential of the molecule, but it does cause an increase in calcemic liability. Thus, efficacy can be obtained with specific analogs in the absence of perturbing blood calcium.

In conclusion, we have shown that a subset of 2-methylene-19-nor-1,25(OH)₂D₃ analogs exert potent comedolytic activity in rhino mouse skin, whereas all analogs tested, as well as the native hormone, increase the epidermal thickness. Thus, the low-calcemic shortened side chain 2-methylene-19-nor-1,25(OH)₂D₃ analogs described herein represent a class of vitamin D analogs with potential for use in skin disorders including acne.

MATERIALS AND METHODS

Chemicals

1,25(OH)₂D₃, 2MbisP, 2MP, and 2-methylene-19-nor-1α,25-hydroxy-pregnacalciferol were synthesized and purified by SAFC (Madison, WI, USA) using previously described methods and as modified by SAFC (Sicinski et al., 1998; Plum et al., 2004). 2OR-2MD-24-epi (DeLuca HF, Sicinski RR, Gowlugari S, inventors; Wisconsin Alumni Research Foundation Madison, WI, USA assignee. 2-Methylene-19-nor-Vitamin D Compounds. United States patent US 7,232,810 B2, 19 June 2007), Vit-III 17-20E (DeLuca HF, Tadi BP, Plum LA, Clagett-Dame M, inventors; Wisconsin Alumni Research Foundation Madison, WI, USA assignee. 17,20(E)-dehydro vitamin D analogs and their uses. United States patent US 7,241,752 B2, 10 July 2007), 2-methylene-(20R,25S)-19,26-dinor-1α,25-dihydroxy D₃ (DeLuca HF, Chiellini G, Gryzwacz P, Plum LA, Clagett-Dame M, inventors; Wisconsin Alumni Research Foundation Madison, WI, USA assignee. Vitamin D analog-2-methylene-(20R,25S)-19,26-dinor-1α,25-dihydroxy D₃. Methods and uses thereof. United States patent US 7,528,122 B2, 5 May 2009), CAGE-3 (Sicinski et al., 2002), VD-03 (Barycki et al., 2009), 2OR-2MbisP (DeLuca HF, Plum LA, Clagett-Dame M, inventors; Wisconsin Alumni Research Foundation Madison, WI, USA assignee. 2-Methylene-19-nor-(20R)-1α-hydroxy-bishomopregnacalciferol. United States patent US 2006/0135799 A1, 22 June 2006), and 2MtrisP (DeLuca HF, Plum LA, Clagett-Dame M, inventors; Wisconsin Alumni Research Foundation Madison, WI, USA assignee. 2-Methylene-19-nor-(20S)-1α-hydroxy-trishomopregnacalciferol. United States patent US 2006/0135798 A1, 22 June 2006) were prepared as described. The compounds were determined by HPLC and nuclear magnetic resonance to be >98% pure.

Compounds were dissolved in ethanol and their concentration was determined by a UV spectrophotometer using a molar extinction coefficient of 42,000 at a wavelength of 252 nm for the 2-methylene-19-nor analogs and 18,200 at 265 nm for 1,25(OH)₂D₃. Dosing solutions were prepared in an ethanol:propylene glycol vehicle (70:30, v/v) so that the dose administered to a 30 g mouse was delivered in a maximum volume of 100 µl.

Animals

All procedures involving animals were reviewed and approved by the University Committee on Use and Care of Animals at the University of Wisconsin Madison, WI, USA, which is a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. clip mice (C3H/HeJ), heterozygote female (h₁⁺h₂) and homozygote male (h₁h₂) pairs, were obtained from Jackson Laboratory (Bar Harbor, ME). Homozygote rhino mice were obtained from an in-house breeding program. Homozygote rhino mice were assigned to study groups at 6-8 weeks of age, with three males and three females per group (n = 6 per group) with an average weight of 21.3 ± 0.1 g.

Compounds were applied on the entire dorsal (back) area of the mouse once a day for 21 consecutive days. Animals were weighed three times per week, and the dose of drug administered was adjusted weekly on the basis of body weight. Blood was taken 24 hours after the seventh and twenty-first dose by maxillary bleed for determination of serum calcium levels (see Supplementary Materials and Methods for details). Skin biopsy samples were collected 3 days after the last application of compounds.

Skin histology

Skin biopsy samples for histology (approximately 6 mm in length) were laid flat on wooden sticks and fixed overnight in 4% paraformaldehyde, dehydrated with increasing percentages of methanol, and embedded in paraffin. Five 10 µm sections were prepared from each biopsy sample at 150 µm intervals. Slices were stained with Gill’s hematoxylin and eosin, imaged, and analyzed using MetaMorph Software (Molecular Devices, Downington, PA). The area of every comedone in each of five slices was calculated and the average comedone area was determined for each mouse. Similarly, epidermal thickness was determined by measuring the thickness of the epidermis using MetaMorph Software. The area of sebaceous glands was measured in a similar manner in a subset of experiments. From these data, mean was determined for the vehicle group and for each group of animals, the percent of vehicle was determined and expressed as mean (percent of vehicle) ± SEM. The average comedone area in vehicle-treated mice in these studies was 6615 ± 345 µm² and the thickness of the epidermis was 21.4 ± 0.3 µm. Neutrophils were counted in random fields (five per animal; 400-fold magnification) for the 1,25(OH)₂D₃ (19 mmol kg⁻¹), 2MbisP (69, 218, 690 mmol kg⁻¹) and CAGE-3 (0.0079, 0.025, 0.079, 0.25 mmol kg⁻¹) dosing groups. Statistical analysis of variance was performed as described in Supplementary Materials and Methods.
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CONFLICT OF INTEREST
The authors are listed as inventors on patents assigned to the Wisconsin Alumni Research Foundation that relate to a number of compounds described in this report. LAP, HFD, and MCD are officers in Deltanoid Pharmaceuticals.

ACKNOWLEDGMENTS
We thank Shimobu Miyasaki, Mary Kaiser, and Wendy Hellwig of the Department of Biochemistry for their technical assistance. We also thank Laura Vanderploeg of the Biochemistry Media Lab for the artwork and Nicholas S Keuler of the College of Agriculture and Life Sciences statistics group for help in statistical analysis. We also thank Ruth Sullivan, DVM, PhD for assistance in evaluation of hematoxylin and eosin sections. Nina Nieves received support from an Advanced Opportunity Fellowship from the University of Wisconsin-Madison Graduate School. This work was also supported in part by a research grant to the University of Wisconsin-Madison from Deltanoid Pharmaceuticals.

SUPPLEMENTARY MATERIAL
Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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