Identification and characterization of noncalcemic, tissue-selective, nonsecosteroidal vitamin D receptor modulators

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Vitamin D receptor (VDR) ligands are therapeutic agents for the treatment of psoriasis, osteoporosis, and secondary hyperparathyroidism. VDR ligands also show immense potential as therapeutic agents for autoimmune diseases and cancers of skin, prostate, colon, and breast as well as leukemia. However, the major side effect of VDR ligands that limits their expanded use and clinical development is hypercalcemia that develops as a result of the action of these compounds mainly on intestine. In order to discover VDR ligands with less hypercalcemia liability, we sought to identify tissue-selective VDR modulators (VDRMs) that act as agonists in some cell types and lack activity in others. Here, we describe LY2108491 and LY2109866 as nonsecosteroidal VDRMs that function as potent agonists in keratinocytes, osteoblasts, and peripheral blood mononuclear cells but show poor activity in intestinal cells. Finally, these nonsecosteroidal VDRMs were less calcemic in vivo, and LY2108491 exhibited more than 270-fold improved therapeutic index over the naturally occurring VDR ligand 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] in an in vivo preclinical surrogate model of psoriasis.

Introduction

The biologically active form of vitamin D, 1,25-dihydroxyvitamin D_3 [calcitriol, 1,25-$(OH)_2D_3$], plays an important role in cell differentiation, proliferation, and immunomodulation and mineral homeostasis. 1,25-$(OH)_2D_3$ and its synthetic analogs exert these effects by binding to the vitamin D receptor (VDR) that belongs to the steroid/thyroid hormone nuclear receptor superfamily (1). VDR, a ligand-dependent transcription factor, functions as a heterodimer with another nuclear receptor, namely, retinoid X receptor (RXR). Upon ligand binding, VDR undergoes a conformational change that promotes RXR-VDR heterodimerization (2, 3). Liganded RXR-VDR heterodimer translocates to the nucleus, binds to the vitamin D response elements (VDREs) present in the promoter regions of responsive genes, and recruits chromatin-modifying enzymatic activities through interaction with coactivators and VDR-interacting protein (DRIP) complex, which ultimately leads to the initiation of transcription (4). Pleiotropic actions of VDR ligands on various cells types have prompted the testing of their therapeutic activities in several human diseases as well as in various animal models of these diseases. These efforts have resulted in the development of VDR ligands for the treatment of psoriasis, osteoporosis, and secondary hyperparathyroidism (5, 6). 1,25-$(OH)_2D_3$ and its synthetic analogs have also shown efficacy in various in vitro and in vivo models of autoimmune diseases (arthritis, multiple sclerosis, and inflammatory bowel disease) and cancers of head and neck, prostate, colon, and breast as well as leukemia (6–9). However, the major hurdle facing the translation of basic and applied research to therapeutic ligands is hypercalcemia associated with currently available VDR ligands. Although calcitriol and calcipotriol (a synthetic VDR ligand) have been approved as topical agents for the treatment of mild to moderate psoriasis, their use (especially oral) has at least in some cases been associated with an increased incidence of hypercalcemia/hypercalciuria (10–14), and they exhibit poor efficacy when compared with biologics (15). In the clinic, the therapeutic efficacy of calcitriol and calcipotriol is increased by their use in combination with topical steroids (16, 17). Therefore, there is an unmet clinical need for the identification of a new generation of VDR ligands that exhibit an improved therapeutic index (TI).

1,25-$(OH)_2D_3$ is a secosteroidal compound (Figure 1A), and most of the VDR ligands that have been described to date, including calcipotriol, have a secosteroidal backbone (18). Administration of VDR ligands results in hypercalcemia by increasing calcium absorption from the intestine. Consistent with this view, VDR-null mice display marked hypocalcemia (19). 1,25-$(OH)_2D_3$ action on duodenal enterocytes induces calcium transport protein 1 (CaT1) expression, which channels calcium from the

Nonstandard abbreviations used: AIB, amplified in breast cancer; CaT1, calcium transport protein 1; EC_{50}, concentration of compound required to induce 50% of maximum activity; KerTr, transformed keratinocyte; LBD, ligand-binding domain; MOG_{35-55}, myelin oligodendrocyte glycoprotein; OCN, osteocalcin; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; PGC-1α, peroxisome proliferative activated receptor γ, coactivator 1α; PHA, phytohemagglutinin; RXR, retinoid X receptor; SRC, steroid receptor coactivator; TI, therapeutic index; TIF, transcription intermediary factor; TMED, threshold minimum effective dose; TPA, 12-O-tetradecanoyl phorbol-13-acetate; TR, thyroid hormone receptor; VDR, vitamin D receptor; VDRE, vitamin D response element; VDRM, VDR modulator.

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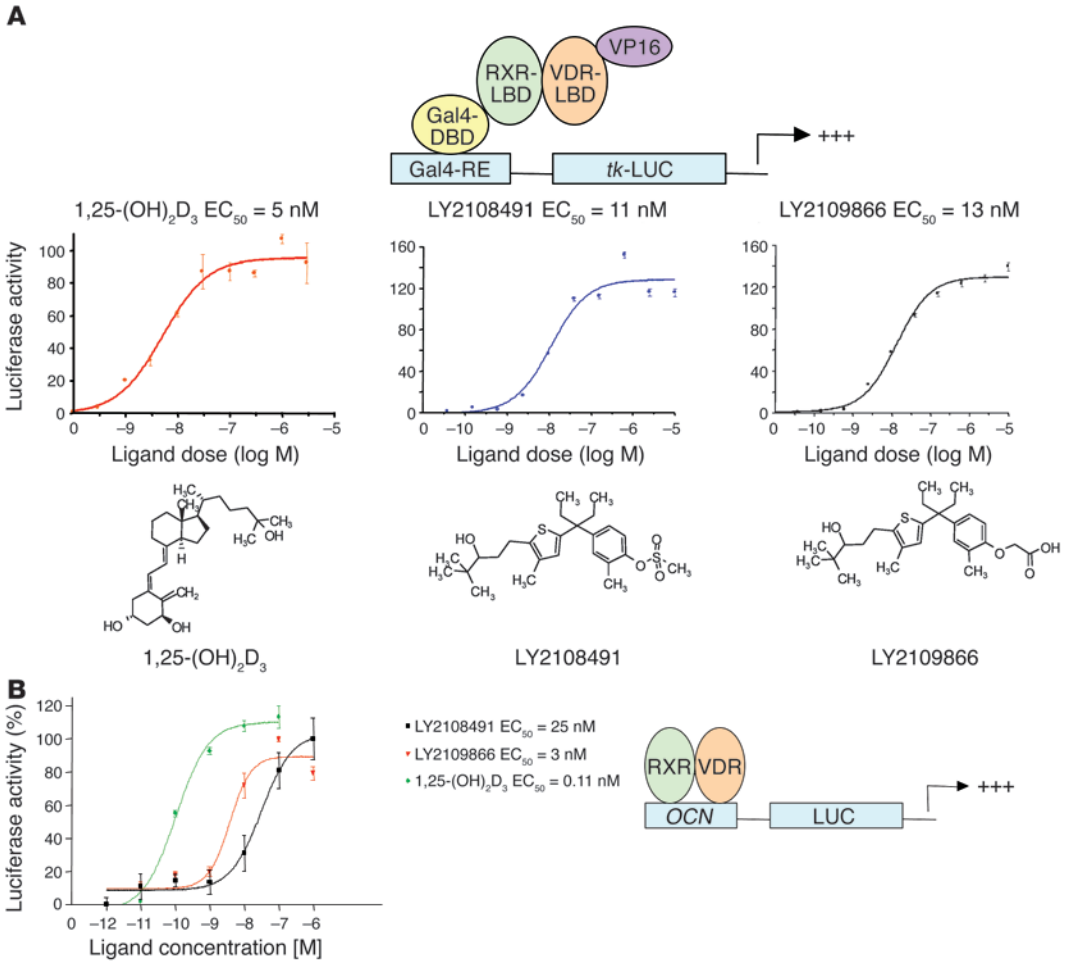

Figure 1
LY2108491 and LY2109866 are nonsecosteroidal VDR ligands. (**A**) Nonsecosteroidal compounds are potent agonists in a VDR-based ligand-sensing assay. SaOS-2 cells were cotransfected with expression vectors encoding Gal4-RXRα-LBD and VP16-VDR-LBD along with a Gal4-responsive luciferase reporter. After transfection, cells were treated with vehicle or various concentrations of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866, and the reporter activity was expressed as light units ± SEM. A schematic of the ligand-sensing assay and the chemical structures of 1,25-$(OH)_2D_3$, LY2108491, and LY2109866 are also presented. *tk*-LUC, thymidine kinase–LUC. (**B**) Nonsecosteroidal VDR ligands induce VDRE-dependent expression of the rat osteocalcin promoter in osteoblasts. ROS17/2.8 cells stably transfected with rat osteocalcin reporter (OCN-LUC) were treated with various concentrations of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866. Results are shown in percentage of the luciferase activity obtained by treating the cells with 1 μM 1,25-$(OH)_2D_3$. All the transfections were performed in triplicate.

intestinal lumen into the cell (20). VDR ligands also induce the expression of an EF-hand–containing carrier protein, calbindin 9k, that ferries the bound calcium from the apical to the basolateral membrane (20). Therefore, a tissue-selective/cell-context–dependent VDR ligand that is transcriptionally less active in intestinal cells but a potent agonist in other cell types (e.g., keratinocytes and lymphocytes) may exhibit reduced hypercalcemia liability. Such a tissue-selective VDR modulator (VDRM) may exhibit a better TI, which is required for the treatment of dermatological indications, including psoriasis, actinic keratosis, and squamous cell carcinoma of the skin. Since nonsteroidal structures have provided tissue-selective estrogen receptor modulators (SERMs) that are agonist in bone and antagonist or transcriptionally inactive in breast and uterine cells (21, 22), we synthesized nonsecosteroidal analogs of vitamin D and show here the identification and characterization of LY2108491 and LY2109866, the nonsecosteroidal VDRMs. We demonstrate that LY2108491 and LY2109866 function as potent and efficacious agonists in keratinocytes, human PBMCs, and osteoblasts but exhibit attenuated transcriptional activity in intestinal cells. This cell context–dependent activity translated in vivo into reduced hypercalcemic liability of these compounds. We also demonstrate that in a surrogate in vivo preclinical model of psoriasis, LY2108491 and LY2109866 show significantly improved therapeutic indices relative to 1,25-$(OH)_2D_3$.

Results

Identification of a nonsecosteroidal VDR agonist. One of the fundamental properties of 1,25-$(OH)_2D_3$ and its synthetic analogs is that they induce heterodimerization of VDR with RXR, resulting in the formation of an RXR-VDR heterodimer, the functional unit of vitamin D action (2, 3). RXR-VDR heterodimerization was used in a ligand-sensing assay since VDR ligands and not RXR ligands drive heterodimerization between RXR and VDR (3). Therefore,

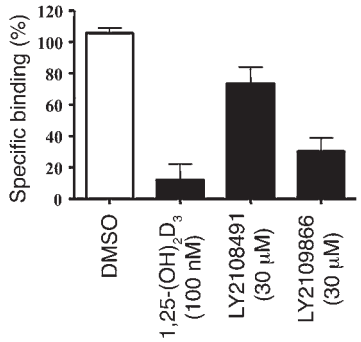

Figure 2
Binding of nonsecosteroidal VDR ligands to VDR-LBD in a classical biochemical ligand-binding assay. Inhibition of [^{3}H]-1,25-$(OH)_2D_3$ binding to VDR by cold 1,25-$(OH)_2D_3$ and its nonsecosteroidal analogs is shown. VDR ligand-binding activity was determined by using purified His-tagged VDR-LBD along with [^{3}H]-1,25-$(OH)_2D_3$ (1 nM). Concentration of each of the cold competitor ligands is shown.

nonsecosteroidal VDR ligands LY2108491 and LY2109866 (Figure 1A) were analyzed for their ability to promote RXR-VDR heterodimerization in a mammalian 2-hybrid system. A ligand-sensing assay was performed by cotransfecting SaOS-2 cells with Gal4-RXRα-LBD (LBD, ligand-binding domain) and VP16-VDR-LBD expression vectors, along with a Gal4-responsive reporter. 1,25-$(OH)_2D_3$ induced RXR-VDR heterodimerization with an EC_{50} (concentration of the ligand required to induce 50% of the maximum activity) value of 5 nM (Figure 1A). LY2108491 and LY2109866, nonsecosteroidal vitamin D analogs, promoted RXR-VDR heterodimerization with EC_{50} values of 11 nM and 13 nM, respectively (Figure 1A). Therefore, LY2108491 and LY2109866 exhibit similar potency to 1,25-$(OH)_2D_3$ in a ligand-sensing assay. The receptor specificity of nonsecosteroidal VDR ligands was confirmed by transfecting SaOS-2 cells with the Gal4-DNA–binding domain chimeras of various nuclear receptor–LBD constructs, along with a Gal4-responsive luciferase reporter. LY2108491 and LY2109866 induced the expression of the Gal4-dependent reporter only through Gal4-VDR-LBD and not through Gal4–thyroid

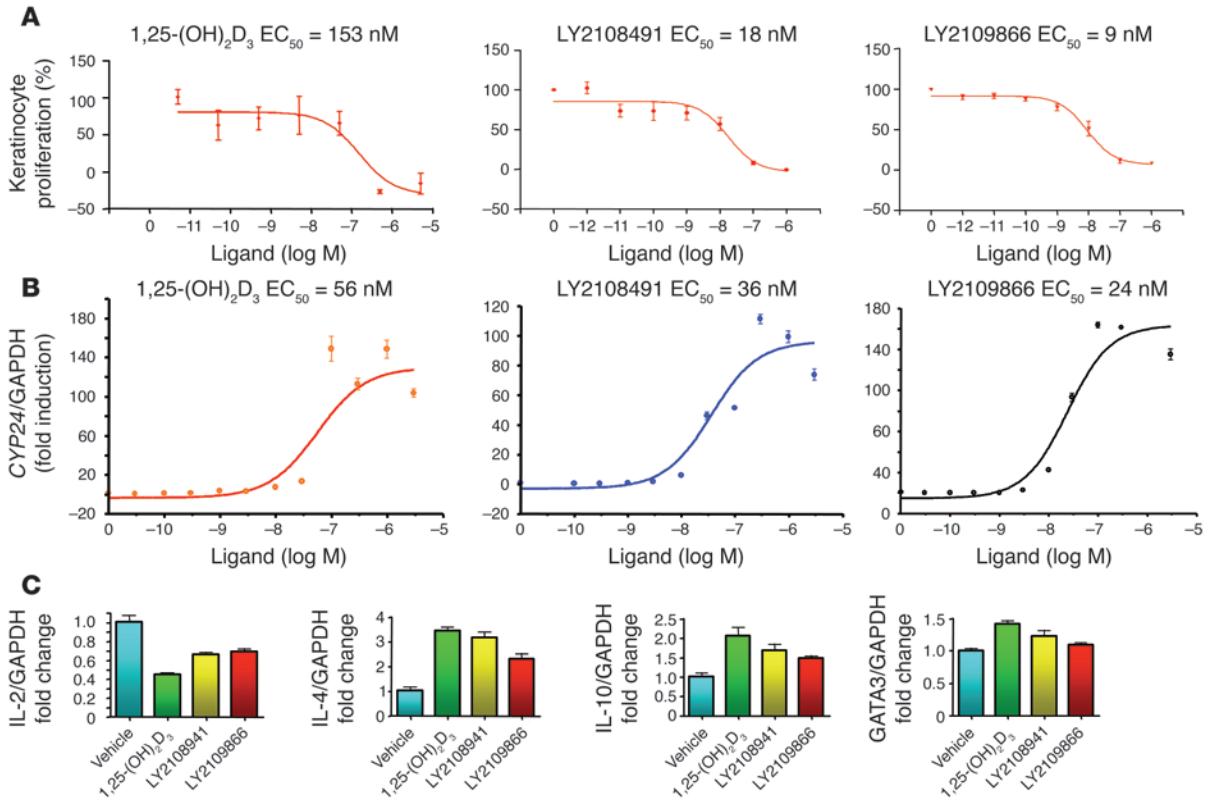

Figure 3
Nonsecosteroidal VDR ligands are potent agonists in keratinocytes and PBMCs. (**A**) LY2108491 and LY2109866 are potent inhibitors of keratinocyte proliferation. KerTr cells plated in 96-well plates were dosed with various concentrations of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 for 72 hours at 37°C before BrdU incorporation into DNA was analyzed as a measure of cell proliferation. Results (mean ± SEM) of experiments performed in triplicate are shown. (**B**) Nonsecosteroidal VDR ligands are potent inducers of *CYP24* gene expression in keratinocytes. TaqMan quantitative RT-PCR (Q-PCR) was performed on total RNA prepared from KerTr cells treated with various concentrations of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 for 24 hours. Levels of GAPDH mRNA were measured in all the samples, and the results were normalized and presented as fold induction (± SEM) compared with normalized *CYP24* levels in vehicle-treated cells. (**C**) Nonsecosteroidal VDR ligands are efficacious in TPA- and PHA-activated PBMCs. Primary cells isolated from donors were stimulated with TPA (100 ng/ml) and PHA (25 μl/ml) and treated with vehicle or 100 nM each of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 for 24 hours. TaqMan Q-PCR was performed on RNA obtained from vehicle-treated or VDR ligand–treated samples, using primer pairs and probes for IL-2, IL-4, IL-10, GATA3, and GAPDH. The amount of IL-2, IL-4, IL-10, and GATA3 transcripts relative to GAPDH transcripts is shown as mean ± SEM of quadruplicate experiments.

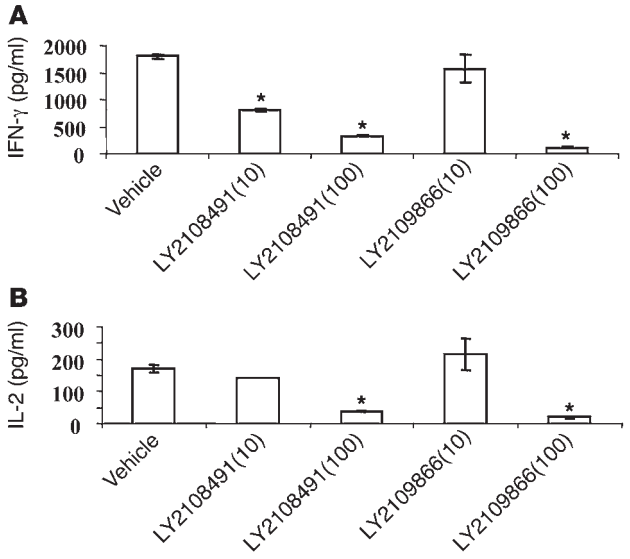

Figure 4
LY2108491 and LY2109866 decrease Th1 cytokine response in vivo. Immunized mice were treated with LY2108491 (10 and 100 μg/kg), LY2109866 (10 and 100 μg/kg), or vehicle for 10 days. Splenocytes were stimulated ex vivo with 10 μg/ml $MOG_{35–55}$ peptide. Supernatants were collected 72 hours after stimulation, and IFN-γ (**A**) and IL-2 (**B**) levels were determined by Luminex. The values shown represent the mean of triplicate determinations, and error bars represent SEM. *$P < 0.05$.

hormone receptor α (Gal4-TRα), Gal4-TRβ, Gal4–retinoic acid receptor α (Gal4-RARα), Gal4-RARβ, Gal4-RARγ, Gal4-PPARα, Gal4-PPARδ, Gal4-PPARγ, Gal4-RXRα, Gal4–RAR-related orphan receptor α (Gal4-RORα), or Gal4-liver receptor homolog 1 (Gal4-LRH1) LBDs (data not shown). However, LY2108491 but not LY2109866 also induced the expression of a Gal4-responsive reporter through Gal4–pregnane X receptor expression vector. LY2108491, LY2109866, and the positive control rifampicin, each at a concentration of 200 nM, induced the expression of the Gal4-responsive reporter in Huh7 cells by 22-, 1-, and 5-fold, respectively. Moreover, in a biochemical cofactor recruitment assay, LY2108491 and LY2109866 induced the recruitment of steroid receptor coactivator 1 (SRC-1) to purified VDR-LBD protein but not to TRα, TRβ, estrogen receptor α (ERα), LXRα, liver X receptor β (LXRβ), RXRα, and LRH1 LBD proteins (data not shown). These results demonstrate the receptor specificity of these nonsecosteroidal ligands.

To determine whether LY2108491 and LY2109866 also induce VDRE-dependent gene expression, we used ROS17/2.8 rat osteosarcoma cells that were permanently transfected with a rat osteocalcin promoter luciferase reporter, osteocalcin-LUC (OCN-LUC) (23). 1,25-$(OH)_2D_3$ induced rat OCN-LUC gene expression with an EC_{50} value of 0.11 nM (Figure 1B). LY2108491 and LY2109866 induced the expression of the osteocalcin VDRE-based luciferase reporter with EC_{50} values of 26 nM and 2.5 nM, respectively (Figure 1B). Therefore, LY2108491 and LY2109866 are nonsecosteroidal VDR agonists.

In an in vitro ligand-binding assay, LY2108491 and LY2109866 were less potent than 1,25-$(OH)_2D_3$. Unlabeled 1,25-$(OH)_2D_3$ (100 nM) competed with [^{3}H]-1,25-$(OH)_2D_3$ for binding to baculovirus-expressed human VDR-LBD whereas LY2108491 and LY2109866 were less efficacious even at a concentration of 30 μM in the competition assay (Figure 2). This observation was true for almost all the nonsecosteroidal VDR ligands synthesized. Therefore, VDR-dependent cell-based assays were more sensitive than the in vitro VDR equilibrium ligand-binding assay in identifying nonsecosteroidal VDR ligands. A plausible explanation for the failure of nonsecosteroidal ligands of this class to compete in ligand-binding assays might be the fast on-rate and slow off-rate of the high-affinity ligand 1,25-$(OH)_2D_3$. Similarly, biphenyl class of nonsecosteroidal VDR ligands also did not efficiently compete with [^{3}H]-1,25-$(OH)_2D_3$ when the VDR binding assays were performed under equilibrium conditions (24). Further, crystal structure of LY2108491-complexed VDR-LBD revealed that the VDR ligand bound in the same space that is normally occupied by 1,25-$(OH)_2D_3$ (data not shown).

LY2108491 and LY2109866 are potent agonists in keratinocytes. 1,25-$(OH)_2D_3$ and synthetic vitamin D analogs inhibit the proliferation of keratinocytes and induce their differentiation (25). To compare the potencies of LY2108491 and LY2109866 with that of 1,25-$(OH)_2D_3$ in inhibiting the proliferation of cultured keratinocytes, human immortalized transformed keratinocyte (KerTr) cells were treated with the VDR ligands for 4 days, and cell proliferation was quantitated by BrdU incorporation into the DNA. 1,25-$(OH)_2D_3$ inhibited the proliferation of KerTr cells with an IC_{50} value of 150 nM (Figure 3A). However, LY2108491 and LY2109866 were more potent than 1,25-$(OH)_2D_3$ in inhibiting the proliferation of KerTr cells and showed IC_{50} values of 18 nM and 9 nM, respectively (Figure 3A). Therefore, LY2108491 and LY2109866 appear to be better agonists than the secosteroidal analog 1,25-$(OH)_2D_3$ in inhibiting the growth of proliferating keratinocytes. We also compared the activities of 1,25-$(OH)_2D_3$, LY2108491 and

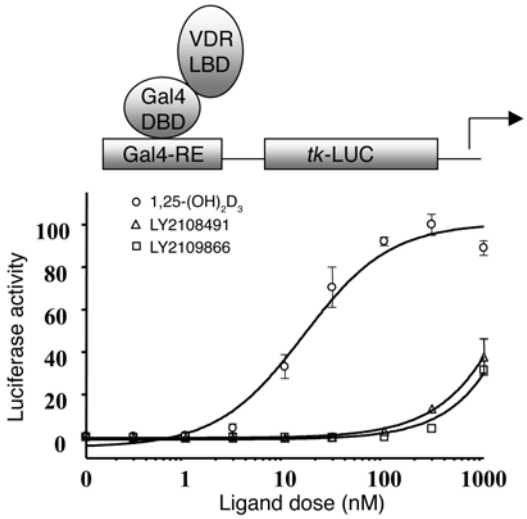

Figure 5
LY2108491 and LY2109866 are less potent in inducing VDR-dependent gene expression in intestinal cells. Luciferase activity (± SEM) of Caco-2 cells transfected with Gal4-VDR-LBD in a mammalian 1-hybrid setting in the presence of vehicle or various concentrations of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 are shown. Results are in arbitrary light units obtained from experiments performed in triplicate.

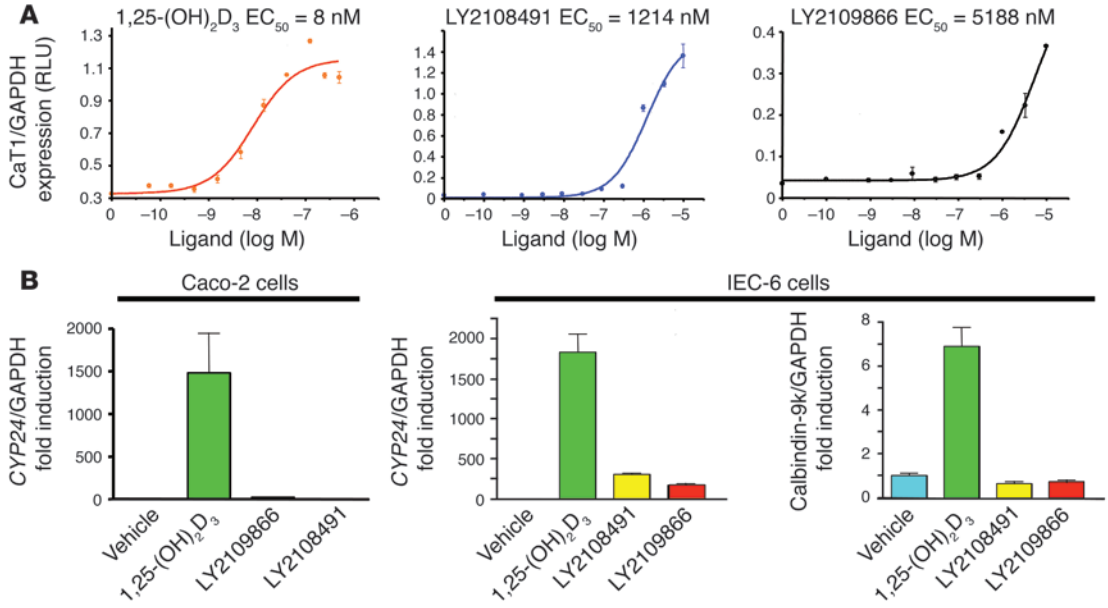

Figure 6

LY2108491 and LY2109866 are less potent and efficacious in inducing the expression of vitamin D–responsive genes in intestinal cells. (**A**) Nonsecosteroidal VDR ligands show weak potencies in inducing the expression of endogenous CaT1 genes in differentiated Caco-2 cells. TaqMan Q-PCR was performed on total RNA prepared from differentiated Caco-2 cells treated with various concentrations of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 for 24 hours. Levels of GAPDH mRNA were measured in all the samples, and the results were normalized and presented (± SEM) as RLU after normalization with the GAPDH transcript levels. (**B**) LY2108491 and LY2109866 are poor inducers of endogenous vitamin D–responsive genes in intestinal cells. TaqMan Q-PCR was performed on total RNA prepared from differentiated Caco-2 or rat duodenal IEC-6 cells treated with vehicle or 100 nM each of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 for 24 hours. The amount of *CYP24* and calbindin-9k transcripts relative to GAPDH transcripts is shown as mean ± SEM of quadruplicate experiments.

LY2109866 in inducing the expression of an endogenous VDRE-dependent gene, *CYP24* (24-hydroxylase) in immortalized keratinocytes. 1,25-$(OH)_2D_3$, LY2108491, and LY2109866 induced the expression of the endogenous *CYP24* gene with EC_{50} values of 56, 36, and 24 nM, respectively (Figure 3B). Thus, nonsecosteroidal VDR ligands are as potent as 1,25-$(OH)_2D_3$ in inducing the expression of *CYP24* gene in human keratinocytes.

LY2108491 and LY2109866 are efficacious agonists in PBMCs. Apart from keratinocyte hyperproliferation, the second hallmark of psoriasis pathology involves T cells. Psoriasis is considered to be a Th1-mediated disease since the lesions show an increased expression of proinflammatory cytokines (IL-2 and IFN-γ) and decreased expression of Th2 antiinflammatory cytokines IL-4 and IL-10 (26). 1,25-$(OH)_2D_3$ affects the Th1-Th2 balance, and it has been shown to augment Th2 cell development, which is accompanied by decreased IL-2 and increased production of IL-4 and IL-10 cytokines in vitro and in vivo (27,28). Further, 1,25-$(OH)_2D_3$ also augments the expression of GATA-binding protein 3 (GATA3) (27), a master regulator of Th2 differentiation (29). As expected, 1,25-$(OH)_2D_3$ reduced IL-2 and induced IL-4, IL-10, and GATA3 expression in phytohemagglutinin/12-O-tetradecanoyl phorbol-13-acetate–activated (PHA/TPA-activated) PBMCs (Figure 3C). LY2108491 and LY2109866 also inhibited IL-2 and induced IL-4, IL-10, and GATA3 expression (Figure 3C). Fold changes in the expression levels of IL and GATA3 genes were statistically significant ($P < 0.05$) when compared with the vehicle treatment. The only exception was GATA3 expression, which followed the trend but did not exhibit statistical significance between LY2109866 and vehicle treatments. Interestingly, LY2108491 and 1,25-$(OH)_2D_3$ did not display significantly different efficacies in enhancing T cell–mediated expression of IL-4 and GATA3 (Figure 3C). Therefore, LY2108491 and LY2109866 are efficacious VDR agonists in T cells.

Nonsecosteroidal VDR ligands also showed inhibition of Th1 response in a T cell recall model in vivo. Compared with the vehicle-treated mice, splenocytes obtained from LY2108491- and LY2109866-treated animals produced significantly less IFN-γ in response to myelin oligodendrocyte glycoprotein ($MOG_{35–55}$) stimulation (Figure 4A). Both VDR ligands showed a dose-responsive inhibition of IFN-γ production, and LY2108491 was more potent in inhibition of IFN-γ elaboration than LY2109866. Both LY2108491 and LY2109866 also significantly inhibited IL-2 in response to $MOG_{35–55}$ stimulation (Figure 4B).

LY2108491 and LY2109866 are cell context–dependent VDRMs. Although LY2108491 and LY2109866 were at least as potent as 1,25-$(OH)_2D_3$ in inducing RXR-VDR heterodimerization in SaOS-2 osteosarcoma cells and in inducing the expression of the endogenous VDRE-dependent gene *CYP24* in keratinocytes, they were more potent than the secosteroidal analog in inhibiting the proliferation of keratinocytes. However, LY2108491 and LY2109866 were less potent than 1,25-$(OH)_2D_3$ in inducing the transactivation of a Gal4-luciferase reporter with the Gal4-VDR-LBD expression vector in a mammalian 1-hybrid assay in Caco-2 human intestinal cells. The EC_{50} values for 1,25-$(OH)_2D_3$, LY2109481, and LY2109866 were 14, greater than 1000, and greater than 1000 nM, respectively, in Caco-2 cells (Figure 5). Of note, even at 1000 nM concentration, LY2108491 and LY2109866 were 60% less efficacious than 1,25-$(OH)_2D_3$ (Figure 5).

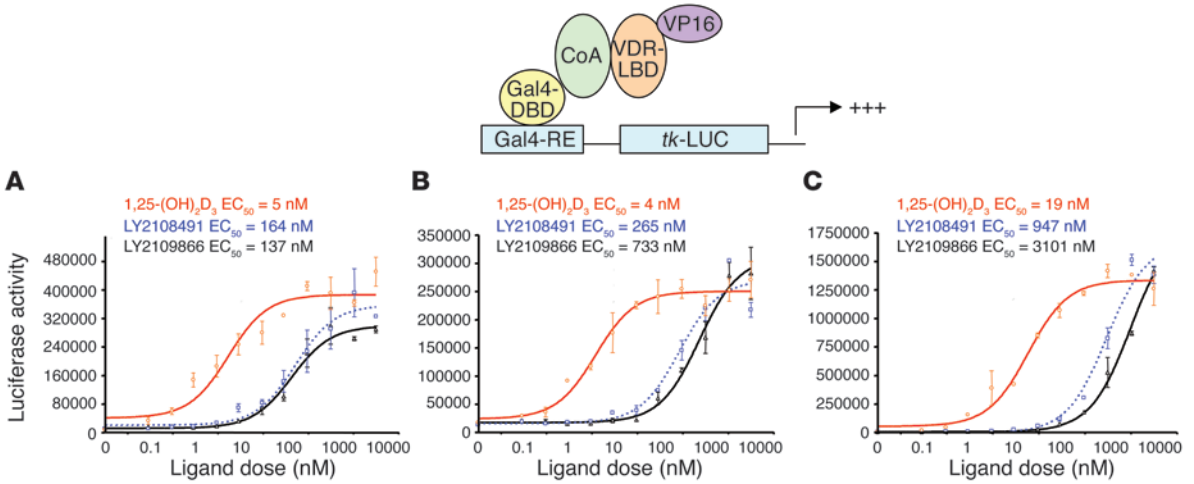

Figure 7
Nonsecosteroidal VDRMs are less potent than 1,25-$(OH)_2D_3$ in recruiting p160 family members to VDR. Luciferase activity (± SEM) of Caco-2 cells transfected with Gal4–SRC-1 (**A**), Gal4-TIF2 (**B**), or Gal4-AIB1 (**C**) and VP16-VDR-LBD in a mammalian 2-hybrid setting in the presence of vehicle or various concentrations of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 is shown. Luciferase activity is shown as arbitrary light units obtained from experiments performed in triplicate.

These results indicate the cell type selectivity of LY2108491 and LY2109866 since these compounds were less potent than 1,25-$(OH)_2D_3$ in Caco-2 cells.

We next examined the expression of an endogenous vitamin D–responsive gene, CaT1, in differentiated Caco-2 cells. CaT1, whose expression is obligatory for 1,25-$(OH)_2D_3$–mediated calcium absorption from the gut, is a vitamin D–responsive gene in vitro and in vivo, and its expression is drastically reduced in VDR-knockout animals (19, 30). Although Caco-2 cells are colon cancer cells, upon density-dependent differentiation (6–14 days of culture), they differentiate into small intestinal-like cells that express many of the markers of small intestinal luminal cells, including CaT1, which is normally expressed in duodenal cells (30). Upon differentiation, these cells also acquire the molecular machinery required for vitamin D–dependent transepithelial calcium transport (apical to basolateral membrane) analogous to that of enterocytes (31). 1,25-$(OH)_2D_3$ (EC_{50} = 8 nM) was a potent inducer of CaT1 mRNA in differentiated Caco-2 cells (Figure 6A). In contrast, LY2108491 (EC_{50} = 1214 nM) and LY2109866 (EC_{50} = 5188 nM) showed very low potency in inducing the expression of the endogenous CaT1 gene in differentiated Caco-2 cells (Figure 6A). LY2108491 and LY2109866 were also significantly less efficacious than 1,25-$(OH)_2D_3$ in inducing *CYP24* expression in Caco-2 cells. 1,25-$(OH)_2D_3$ (100 nM) induced *CYP24* gene expression by approximately 1,500-fold whereas LY2108491 and LY2109866 (100 nM each) failed to increase *CYP24* in differentiated Caco-2 cells (Figure 6B). We also compared LY2108491 and LY2109866 with 1,25-$(OH)_2D_3$ for their effect on the expression of 2 VDRE-dependent genes, namely *CYP24* and calbindin-9k in IEC-6 (rat duodenal crypt cell line) cells. Treatment of IEC-6 cells with 1,25-$(OH)_2D_3$ (100 nM) for 24 hours resulted in a robust induction of *CYP24* and calbindin-9k gene expression (Figure 6B). In contrast, LY2108491 and LY2109866 were significantly less efficacious than 1,25-$(OH)_2D_3$ in inducing the expression of endogenous *CYP24* in these cells (Figure 6B). 1,25-$(OH)_2D_3$ induced calbindin-9k expression by 7-fold after 24 hours of treatment (Figure 6B). However, both LY2108491 and LY2109866 failed to induce calbindin-9k gene expression in IEC-6 cells (Figure 6B). All of these observations further support the notion that LY2108491 and LY2109866 are cell context–dependent VDRMs.

VDR-cofactor interaction. It has been suggested that selective nuclear receptor modulators induce novel conformational changes within the LBD that lead to selective cofactor recruitment.

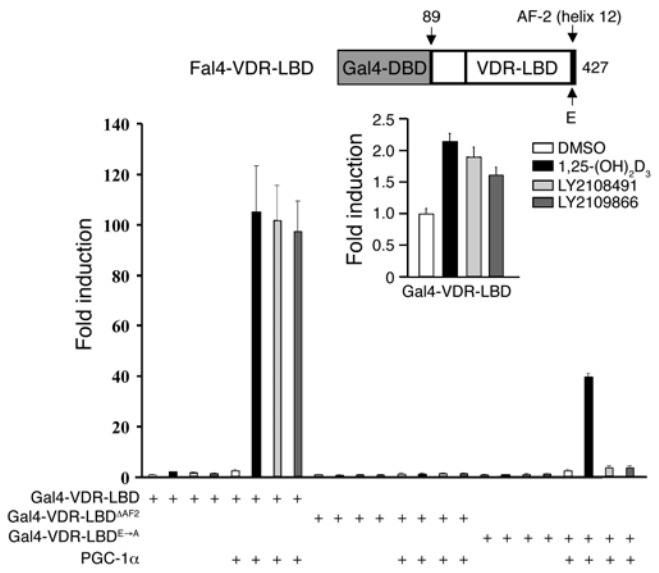

Figure 8
Differential interaction of PGC-1α–receptor interaction with VDR ligands. Luciferase activity (± SEM) of HEK 293 cells transfected with Gal4-VDR-LBD, Gal4-VDR-LBD$^{\Delta AF-2}$, or Gal4-VDR-LBD^{E420A} with or without PGC-1α expression vector in the presence of vehicle or 1 μM each of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 is shown. Luciferase activity is shown as arbitrary light units obtained from experiments performed in triplicate.

Table 1
Nonsecosteroidal VDRMs exhibit better therapeutic indices than $1,25\text{-}(OH)_2D_3$

VDR ligand	Hypercalcemia TMED (nmol/100 μl/4 cm²)	Epidermal proliferation TMED (nmol/100 μl/4 cm²)	TI
$1,25\text{-}(OH)_2D_3$	<1	3	<0.3
LY2108491	>729	9	>81
LY2109866	>243	9	>27

TMEDs of $1,25\text{-}(OH)_2D_3$, LY2108491, and LY2109866 required for epidermal proliferation and hypercalcemia after topical application on the backs of hairless mice are presented. A comparison of the therapeutic indices of the VDR ligands is also shown.

Ligand-selective cofactor recruitment may underlie the novel pharmacological properties of the modulators. In order to investigate this, nonsecosteroidal VDR ligands were also compared with $1,25\text{-}(OH)_2D_3$ for their effect on the interaction of VDR with SRC/p160 coactivator family of proteins. The recruitment of SRC-1, SRC-2 (TIF2, transcription intermediary factor 2), and SRC-3 (AIB1, amplified in breast cancer 1) to VDR was monitored by a mammalian 2-hybrid system after transfection of Caco-2 cells with VP16-VDR-LBD, Gal4 DNA–binding domain chimeras of 1 of the cofactor expression vectors (Gal4–SRC-1, Gal4-TIF2, or Gal4-AIB1) and a Gal4 reporter luciferase plasmid. $1,25\text{-}(OH)_2D_3$ was equipotent in inducing the interaction of VDR with SRC-1, TIF2, and AIB1 with EC_{50} values of 5, 4, and 19 nM, respectively, for VDR–SRC-1, VDR-TIF2, and VDR-AIB1 interactions (Figure 7, A–C). However, LY2108491 and LY2109866 were less potent than $1,25\text{-}(OH)_2D_3$ in inducing VDR-cofactor interactions. LY2108491 augmented VDR–SRC-1, VDR-TIF2, and VDR-AIB1 interactions with EC_{50} values of 164, 265, and 947 nM, respectively (Figure 7, A–C). For LY2109866, the EC_{50} values for VDR–SRC-1, VDR-TIF2, and VDR-AIB1 interactions were 137, 733, and 3101 nM (Figure 7, A–C). In particular, LY2108491 and LY2109866 were considerably less potent in inducing VDR-TIF2 and VDR-AIB1 interactions when compared with $1,25\text{-}(OH)_2D_3$. These results also indicate differential cofactor recruitment by LY2108491 and LY2109866 since they were 6- and 23-fold, respectively, more potent in recruiting SRC-1 than AIB1 to VDR. The attenuated ability of LY2108491 and LY2109866 in recruiting coactivator proteins (particularly AIB1) to VDR may suggest that these nonsecosteroidal VDRMs induce distinct conformations within the LBD that may explain the cell-context dependent activity of these ligands.

To demonstrate further that the occupancy of VDR-LBD by the secosteroidal and nonsecosteroidal ligands indeed induces differential conformations with pharmacological significance, we used peroxisome proliferative activated receptor γ, coactivator 1α (PGC-1α) coactivator to augment Gal4-VDR-LBD–dependent transactivation of a Gal4-responsive reporter in a mammalian 1-hybrid assay. Treatment of Gal4-VDR-LBD–transfected cells with 1 μM $1,25\text{-}(OH)_2D_3$, LY2108491, or LY2109866 resulted in a 2-fold increase in the expression of the Gal4-responsive luciferase reporter in HEK 293 cells (Figure 8). Overexpression of PGC-1α resulted in a 50-fold enhancement of transcriptional activity of the reporter in the presence of $1,25\text{-}(OH)_2D_3$, LY2108491, or LY2109866 (Figure 8). As expected, deletion of helix 12 (Gal4-VDR-LBD$^{\Delta AF\text{-}2}$) abolished the transcriptional activity in response to both the ligands and also to overexpression of PGC-1α. Previous studies have demonstrated the "charge clamp" glutamic acid residue in helix 12 (E420) as playing an important role in ligand-dependent VDR-mediated transcription and coactivator interaction. Further, mutation studies have shown that the VDR E420 does not play any role in either ligand binding or heterodimerization with RXR (32). It has previously been demonstrated that the requirement of the charge clamp residue of PPARγ and TRβ for their coactivation by PGC-1α is determined by the identity of the ligand (33, 34). Therefore, to ascertain whether this charge clamp E420 residue of VDR played any role in the ability of VDR ligands to differentially influence the interaction of VDR with PGC-1α, we mutated the glutamic acid to alanine and performed cotransfection assays utilizing the E420A mutant receptor in the presence of $1,25\text{-}(OH)_2D_3$, LY2108491, or

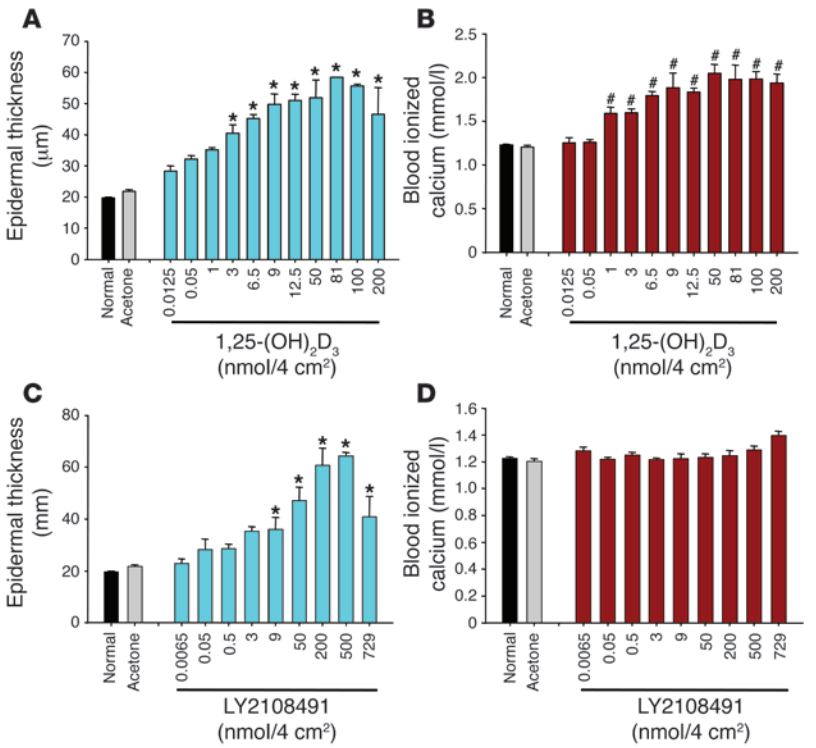

Figure 9
VDRMs are less calcemic in vivo and show efficacy in a surrogate animal model of psoriasis. (**A**) Histological evaluation of hairless mouse skin after topical treatment with $1,25\text{-}(OH)_2D_3$ at indicated doses. (**B**) Effect of various concentrations of topical $1,25\text{-}(OH)_2D_3$ treatment on blood ionized calcium levels in hairless mice. (**C**) Histological evaluation of hairless mouse skin after topical treatment with LY2108491 at indicated doses. (**D**) Effect of various concentrations of topical LY2108491 treatment on blood ionized calcium levels in hairless mice. Error bars represent SEM of the mean. *$P < 0.05$. #Hypercalcemia above the normal blood ionized calcium level of 1.34 mM.

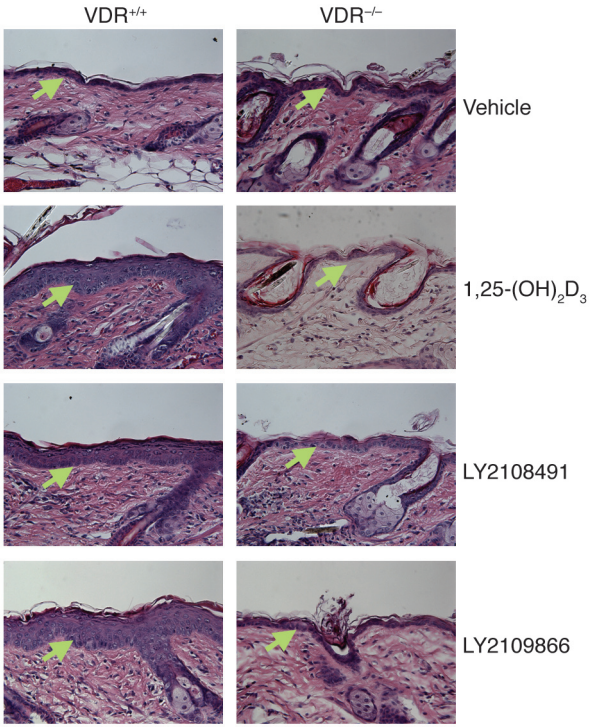

Figure 10
VDRMs do not induce epidermal proliferation in VDR-knockout mice. Histological evaluation of $VDR^{+/+}$ or $VDR^{-/-}$ mouse skin after topical treatment with vehicle, 1,25-$(OH)_2D_3$ (10 nmol), LY2108491 (50 nmol), or LY2109866 (50 nmol) is presented. Mice were sacrificed at 72 hours after dosing, and skin samples were collected. Epidermal thickness was examined by H&E staining of paraffin sections. Epidermis is shown with green arrows.

LY2109866. VDR ligands did not show any activation of the Gal4-reponsive reporter through the Gal4-VDR-LBD^{E420A} expression vector (Figure 8). Interestingly, overexpression of PGC-1α with the E420A mutant receptor resulted in a 15-fold enhancement of the transcriptional activity in the presence of 1,25-$(OH)_2D_3$ but only a 1.5-fold enhancement in the presence of either LY2108491 or LY2109866 (Figure 8). These results suggest that the nonsecosteroidal VDRMs are capable of inducing different VDR and/or RXR-VDR conformation than that engendered by the occupancy of the VDR-LBD with 1,25-$(OH)_2D_3$.

LY2108491 and LY2109866 show better therapeutic indices than calcitriol in vivo. Topical calcitriol is used in clinic for the treatment of psoriasis (35). In order to compare the therapeutic indices of LY2108491 and LY2109866 with that of calcitriol in vivo, a hairless mouse model of epidermal proliferation after topical application of VDR ligands was used. This epidermal proliferation mouse model is regarded as a surrogate in vivo preclinical model of psoriasis since compounds (VDR ligands [calcitriol and calcipotriol] and retinoids [etretinate, acitretin, isotretinoin and tazarotene]) that inhibit keratinocyte proliferation in psoriatic lesions, in fact, induce epidermal proliferation when applied topically in normal skin (36, 37). This apparent paradox is also shared by psoralen and UV A as well as UV B therapies that are also therapeutically effective in psoriasis (36). In this model, serum ionized calcium was also measured at 24 hours after the dosing, and the animals were sacrificed 72 hours after dosing to collect skin for histological evaluation of epidermal proliferation. The TI is the ratio between the threshold minimum effective dose (TMED) that does not result in hypercalcemia and the TMED that results in keratinocyte proliferation (an increase of 1 keratinocyte layer thickness in the epidermis) (Table 1). TMEDs for hypercalcemia and epidermal proliferation, respectively, are thus the measures of the potencies of VDR ligands for side effects and efficacy. Calcitriol [1,25-$(OH)_2D_3$] showed an epidermal response with a TMED of 3 nmol/100 μl/4 cm^2, and in the same animal it produced hypercalcemia when applied topically at a dose of 1 nmol but not at 0.05 nmol/100 μl/4 cm^2 (Figure 9, A and B, and Table 1). Therefore, the TI of calcitriol in this in vivo model was less than 0.3 since the noncalcemic dose would be less than 1 nmol/100 μl/4 cm^2. Like calcitriol, LY2108491 also stimulated keratinocyte proliferation and thus increased epidermal thickness in vivo in hairless mice (Figure 9, C and D). However, the TI for LY2108491 was found to be greater than 81 (Table 1) since its TMED for epidermal proliferation was 9 nmol/100 μl/4 cm^2 and its TMED for hypercalcemia was greater than 729 nmol/100 μl/4 cm^2 (Figure 9, C and D, and Table 1). Note that even at a dose of 729 nmol, it did not raise ionized blood calcium above the normal levels. Similarly, the TI for LY2109866 was more than 27 since its noncalcemic dose was more than 243 nmol/100 μl/4 cm^2 and its TMED for epidermal proliferation was 9 nmol/100 μl/4 cm^2 (Table 1). Therefore, LY2108491 and LY2109866 were greater than 270- and greater than 90-fold, respectively, better than calcitriol in terms of their TI in vivo in a topical surrogate model of psoriasis.

Nonsecosteroidal VDRMs induce epidermal proliferation in vivo through VDR signaling pathway. In order to rule out the possibility that the VDR ligands induced epidermal proliferation in vivo by acting upon other nuclear receptors present in the skin or by pathways other than nuclear vitamin D signaling, wild-type ($VDR^{+/+}$) and VDR knockout ($VDR^{-/-}$) mice were treated topically with vehicle, 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 on their shaved backs (Figure 10). Topical treatment of $VDR^{+/+}$ mice with 1,25-$(OH)_2D_3$ (10 nmol/100 μl/4 cm^2) or one of the VDRMs (50 nmol/100 μl/4 cm^2 each) for 72 hours

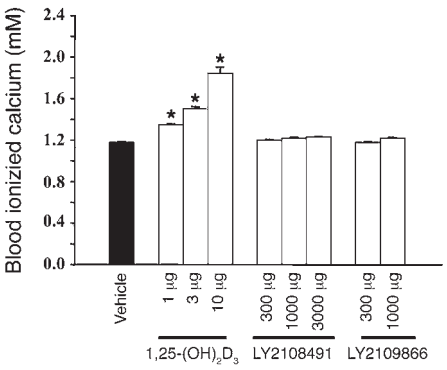

Figure 11
LY2108491 and LY2109866 are noncalcemic in vivo when administered orally in a short-term mouse model of hypercalcemia. 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 were administered in sesame seed oil at indicated doses to mice by gavage for 6 consecutive days, and blood ionized calcium was measured 6 hours after the last dose. Error bars represent SEM. *$P < 0.05$.

resulted in epidermal proliferation when examined histologically by H&E staining in paraffin-embedded sections (Figure 10). However, all the VDR ligands did not show any increase in epidermal thickness when applied to the shaved backs of $VDR^{-/-}$ animals. Epidermal thickness in control vehicle-treated $VDR^{+/+}$ and $VDR^{-/-}$ animals is also presented (Figure 10).

LY2108491 and LY2109866 are less calcemic in vivo. We hypothesized that the decreased VDR-mediated transcriptional activity of LY2108491 and LY2109866 in intestinal cells and the reduced hypercalcemia liability of these VDRMs through the topical route may also translate into reduced intestinal calcium absorption and hypercalcemic action when administered orally. To examine this hypothesis, 1,25-$(OH)_2D_3$, LY2108491, and LY2109866 were analyzed for their hypercalcemia liability in vivo after oral administration. Mice were treated for 6 days with the VDR ligands, and ionized calcium was measured in the blood 6 hours after the last dosing. 1,25-$(OH)_2D_3$ resulted in statistically significant increases in blood ionized calcium levels in mice at doses as small as 1 μg/kg/day, with frank hypercalcemia observed (above the normal range) at 3 μg/kg/day (Figure 11). In contrast, LY2108491 did not result in hypercalcemia even at 3000 μg/kg/day, and LY2109866 did not result in hypercalcemia in mice at a dose of 1000 μg/kg/day (Figure 11). Therefore, LY2108491 and LY2109866 were significantly less likely (>1,000 and >300 times, respectively) to induce hypercalcemia than 1,25-$(OH)_2D_3$ in vivo through the oral route. These results confirm that LY2108491 and LY2109866 are less calcemic in vivo. Therefore, these nonsecosteroidal VDRMs may exhibit less hypercalcemia liability when administered orally and also when applied topically in a therapeutic setting.

Discussion

The clinical success of the nonsteroidal selective estrogen receptor modulators (SERMs) in reducing the estrogenic action in breast and uterus while retaining the estrogenic agonist action in bone has provided a rationale for the development of nonsteroidal nuclear receptor ligands in order to achieve tissue-selective action (21). As a result, efforts are ongoing to obtain tissue-selective modulators of estrogen, glucocorticoid, androgen, progesterone, mineralocorticoid, and PPARs for various indications (21, 22). In this manuscript, we report the first identification to our knowledge of nonsecosteroidal tissue-selective VDRMs that function as agonists in keratinocytes and PBMCs (T cells) but show extremely reduced transcriptional activity in intestinal cells. This cell context–selective action translated into reduced hypercalcemia in vivo in comparison with 1,25-$(OH)_2D_3$ when mice were treated orally or topically with the VDRMs LY2108491 and LY2109866 (Figures 9 and 11 and Table 1). Although nonsecosteroidal and nonsteroidal VDR ligands have been described (24, 38, 39), they have not been shown to act as a modulator in a cell context–dependent manner. Recently, a tissue- and cell type–selective secosteroidal VDRM (Ro-26-9228) has been described (40). However, Ro-26-9228 was less tissue-selective and showed VDR-dependent transcriptional activity in Caco-2 cells with an EC_{50} value of 120 nM (40). It showed only 17- to 27-fold better TI than 1,25-$(OH)_2D_3$ in an ovariectomized rat model of osteoporosis (40). Further, it showed a potency of 149 nM in inducing the expression of the endogenous vitamin D–responsive gene CaT1 in Caco-2 cells whereas corresponding EC_{50} values for LY2108491 and LY2109866 were 1214 and 5188 nM, respectively (data not shown).

The VDRMs LY2108491 and LY2109866 displayed many of the biological actions of 1,25-$(OH)_2D_3$. They induced RXR-VDR heterodimerization (Figure 1A); upregulated the expression of a VDRE-dependent gene, osteocalcin in osteoblasts (Figure 1B); inhibited the growth of proliferating keratinocytes (Figure 4A); and inhibited IL-2 while augmenting IL-4, IL-10, and GATA3 expression in human PBMCs (Figure 4C). They also inhibited Th1 response in a T cell recall model in vivo. However, these nonsecosteroidal VDRMs were more than 71 times less potent and 60% less efficacious than 1,25-$(OH)_2D_3$ in inducing VDR-LBD–dependent transactivation in intestinal cells (Figure 5). They were also 200 to more than 1000 times less potent than their secosteroidal counterpart in inducing the expression of CaT1, a vitamin D–responsive gene in differentiated Caco-2 cells (Figure 6A). In addition, LY2108491 and LY2109866 (100 nM) did not significantly induce the expression of another vitamin D–dependent gene, *CYP24* (Figure 6B). This cell context–dependent activity was further extended to rat IEC-6 duodenal cells, in which LY2108491 and LY2109866 (100 nM), unlike 1,25-$(OH)_2D_3$, failed to induce the expression of endogenous calbindin-9k gene (Figure 6B). Further, they were significantly less efficacious than 1,25-$(OH)_2D_3$ in enhancing the expression of endogenous *CYP24* gene in IEC-6 cells (Figure 6B). These results and the knowledge that the promoter regions of *CYP24*, calbindin-9k, and CaT1 genes contain functional VDREs (6, 19, 30) indicate a global attenuation or unresponsiveness of the VDR signaling pathway to these nonsecosteroidal VDRMs in intestinal/duodenal cells. At the same time, the vitamin D signaling pathway still responds to 1,25-$(OH)_2D_3$–complexed VDR for the expression of the above-mentioned genes in Caco-2 and IEC-6 duodenal cells (Figure 6, A and B). Our results are also in accordance with the observations that the secosteroidal VDRM, Ro-26-9228, showed weaker potency than 1,25-$(OH)_2D_3$ in inducing the expression of vitamin D–responsive genes calbindin-9k and *CYP24* in Caco-2 cells, and it did not induce the expression of vitamin D–responsive genes *CYP24*, calbindin-9k, and PMCA-1 in rat duodenum (40, 41).

Studies with VDR-null animals have indicated duodenal CaT1 to be a major mediator of 1,25-$(OH)_2D_3$–mediated calcium absorption from intestine and hypercalcemia (19). Therefore, the weak agonist activity of nonsecosteroidal VDRMs in human and rat intestinal cells predicted that these ligands might be less calcemic than 1,25-$(OH)_2D_3$ in vivo. In order to test this hypothesis, LY2108491 and LY2109866 were administered orally and topically to mice, and their effect on blood ionized calcium was examined. Both nonsecosteroidal VDRMs showed less potency in inducing hypercalcemia by either route (Figures 9 and 11 and Table 1). The reduced calcemic liability of these VDRMs prompted us to test these compounds in an in vivo surrogate model of psoriasis. The paradox in vitamin D biology is that VDR ligands inhibit the proliferation of keratinocytes in psoriatic lesions, but they also induce the proliferation of keratinocytes in normal skin (36, 37). In addition to VDR ligands, retinoids, which are also therapeutically active in psoriasis, display a similar phenotype (36). Therefore, epidermal proliferation in mouse skin has been regarded as a surrogate model of psoriasis for the identification of topically active VDR ligands. All the VDR ligands, namely calcitriol, calcipotriol, and tacalcitol, which are used in clinic for the topical treatment of psoriasis, stimulate epidermal proliferation when applied topically to mice (36, 37). In the same animal model, LY2108491 and LY2109866 exhibited a dermal effect at a dose of 9 nmol/100 μl/cm^2 dose. Interestingly, LY2108491 and LY2109866 did not raise blood calcium levels above the normal range even at the highest tested

doses of 729 and 243 nmol/100 μl/cm², respectively (Figure 9D and Table 1). Therefore, nonsecosteroidal VDRMs show striking dissociation of biological efficacy from calcium liability in vivo. Both LY2108491 and LY2109866 showed more than 270- and more than 900-fold, respectively, better therapeutic indices than calcitriol in the surrogate model of psoriasis (Figure 9 and Table 1). The skin effect of the VDRM was mediated through VDR since the epidermal proliferation effect was not observed in VDR-null animals (Figure 10). These results also demonstrate the receptor specificity of the VDRMs described herein.

Psoriasis is characterized by hyperproliferation and abnormal differentiation of keratinocytes as well as epidermal infiltration of activated T cells (15). Therefore, an ideal therapeutic for psoriasis ought to be efficacious in both keratinocytes and immune cells for maximum clinical benefit and to satisfy the current unmet medical need. The nonsecosteroidal VDRMs also showed efficacy in skewing the balance of helper T cells from pathologic Th1 to antiinflammatory Th2 cells, as evidenced by their ability to inhibit IL-2 and augment IL-4 and IL-10 expression in PHA/TPA-activated PBMCs (Figure 3C). Both IL-4 and IL-10 possess antiinflammatory properties and can suppress T cell–mediated autoimmune processes (26, 28). Further, IL-10 promotes Th2 response by regulating Th1-Th2 balance through antigen-presenting cells (26, 28). The regulation of these cytokines assumes significance because an IL-2 diphtheria immunotoxin (42) and protein biologics for IL-4 (43) as well as IL-10 (44) have been successfully used in clinic for the treatment of plaque-type psoriasis. LY2108491 and LY2109866 also induced the expression of GATA3 (Figure 3C), a transcription factor that is obligatory for the development of Th2 cells (29).

It has been postulated that the tissue-selective activity of the nuclear receptor modulators is due to their ability to induce distinct conformational changes in the receptor, which may result in differential recruitment of cofactors required for the regulation of target gene expression. Our results indicate that LY2108491 and LY2109866 may impart different receptor conformations relative to 1,25-$(OH)_2D_3$ since they were less potent in facilitating the recruitment of cofactors to VDR in Caco-2 cells (Figure 7). It was particularly true for VDR-AIB1 interaction, where the EC_{50} values were 947 and 3101 nM, respectively, for LY2108491 and LY2109866, whereas for 1,25-$(OH)_2D_3$, the corresponding EC_{50} value was 18 nM in Caco-2 cells (Figure 7C). Further, the differential recruitment of PGC-1α to E420A mutant of the receptor by the VDR ligands (Figure 8) indicates that the nonsecosteroidal VDRMs described herein impart different conformational change to the receptor than that conferred by 1,25-$(OH)_2D_3$. Interestingly, Takeyama et al. reported the first selective interaction of VDR with cofactors, using a secosteroidal vitamin D analog, OCT (45). OCT was found to be less potent than 1,25-$(OH)_2D_3$ in inducing VDR–SRC-1 and VDR-AIB1 interactions (45). Similarly, 1,25-$(OH)_2D_3$, and not the secosteroidal VDRM, Ro-26-9228, complexed with VDR obtained from Caco-2 cells and interacted with glutathione-S-transferase–TIF2 in vitro in a glutathione-S-transferase–pulldown assay (41). These results also suggest that the binding of synthetic ligands with a spectrum of variations in their cellular activities may induce slight differences in VDR conformations in a ligand structure–dependent manner, which may result in differential recruitment of coactivators. Therefore, tissue selectivity may arise as a result of differential cofactor recruitment by VDRMs. Although cocrystallization of VDR-LBD with various synthetic ligands does not support this notion (46), it should be borne in mind that the crystallization efforts, although extremely important in understanding VDR-ligand interactions, did not involve wild-type VDR-LBD, RXR, and coactivators.

In summary, we report here the synthesis and characterization of novel nonsecosteroidal VDRMs that function in a cell and tissue context–dependent manner. Nonsteroidal modulators of estrogen receptor, tamoxifen and raloxifene, are approved drugs for the treatment of breast cancer and prevention/treatment of postmenopausal osteoporosis, respectively. Similarly, nonsteroidal modulators of androgen receptor (bicalutamide and flutamide) are approved drugs for the treatment of prostate cancer. Steroidal analogs of nuclear receptors, in general, are traditionally limited by their undesirable side effects, which in the case of VDR ligands include hypercalcemia. Our results clearly show that nonsecosteroidal VDRMs are more tissue selective in action and as a result are less calcemic in vivo. LY2108491 and LY2109866 also display superior therapeutic indices in vivo in a surrogate model of psoriasis. Therefore, these nonsecosteroidal VDRMs might have therapeutic utility in dermal indications, such as psoriasis, actinic keratosis, and skin cancers. The identification of VDRMs further extends the recurring theme of tissue-selective action in nuclear receptor biology to VDR and demonstrates that nonsecosteroidal ligands have the potential to elicit cell- and tissue-selective effects that may lead to improved therapeutic indices. Our observations, along with the molecular tools described in this manuscript, also open the potential for the discovery and identification of bone-selective, prostate-selective, or promyelocyte-selective VDRMs as future therapeutics for various indications. The structurally novel nonsecosteroidal tissue-selective VDRMs belonging to the phenylthiophene class described herein might have utility not only in the treatment of other dermal indications, but also in those that are nondermal.

Methods

Cell culture and transfections. For the RXR-VDR heterodimerization assay, SaOS-2 cells maintained in DMEM supplemented with 10% FBS were plated at 5000 cells per well in a 96-well plate. The next day, cells were transfected using 0.5 μl of FuGENE (Roche Diagnostics Corp.), 100 ng of luciferase reporter vector pFR-LUC (Stratagene), and 10 ng each of pVP16-VDR-LBD and pGal4-RXRα-LBD expression vectors per well. For the Caco-2 1-hybrid mammalian transactivation assay, Caco-2 cells, maintained in DMEM supplemented with 10% FBS, were plated at 5000 cells per well in a 96-well plate. Cells were transfected using 0.5 μl of FuGENE (Roche Diagnostics Corp.), 100 ng of luciferase reporter vector pFR-LUC (Stratagene), and 10 ng of pGal4-VDR-LBD expression vectors per well. For the VDR-cofactor interaction assays, Caco-2 cells maintained in DMEM supplemented with 10% FBS were plated at 5000 cells per well in a 96-well plate. The next day, cells were transfected using 0.5 μl of FuGENE, 100 ng of luciferase reporter vector pFR-LUC, and 10 ng each of pVP16-VDR-LBD and 1 of the Gal4-cofactor (pGal4–SRC-1, pGal4-TIF2 or pGal4-AIB1) expression vectors per well. Total DNA amount was kept constant by adding empty vector DNA as needed. Cells were treated with the ligand 24 hours after transfection, and luciferase activity was quantitated the next day using Steady-Glo luciferase detection reagent (Promega). HEK 293 cells were maintained in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. Prior to transfections, cells were plated into 96-well plates at a density of 25,000 cells per well in the medium supplemented with 10% dextran charcoal–stripped FBS. Transfections were performed using FuGENE 6 (Roche Molecular Biochemicals). Each transfection contained 25 ng of luciferase reporter plasmid and various combinations of other expression plasmids as described in the legend to Figure 8. Twenty-four hours after transfection, fresh media containing vehicle, 1,25-$(OH)_2D_3$

(100 nM), LY2108491 (1 μM), or LY2109866 (1 μM) were added. Luciferase activity was quantitated the next day using Steady-Glo luciferase detection reagent (Promega).

Rat OCN-LUC assay. The activation of osteocalcin VDRE by VDR ligands was evaluated in a rat osteoblast–like cell line (ROS17/2.8) stably expressing rat osteocalcin promoter (1.154 kb) fused with luciferase reporter gene. The development of the stably transfected ROS17/2.8 cell line (RG-15) containing OCN-LUC has been described (23). Confluent RG-15 cells maintained in DMEM/F-12 medium (3:1) containing 5% FBS and 300 μg/ml G418 at 37°C were trypsinized (0.25% trypsin) and plated into white opaque 96-well cell culture plates (25000 cells per well). After 24 hours, cells (in DMEM/F-12 medium containing 2% FBS) were treated with the indicated concentrations of the compounds. After 48 hours of treatment, the medium was removed, and cells were lysed with 50 μl of lysis buffer (from Luciferase Reporter Assay System, Roche Diagnostics Corp.) and assayed for luciferase activity, using the Luciferase Reporter Gene Assay kit from Boehringer Mannheim. Aliquots (20 μl) of cell lysates were pipetted into wells of white opaque microtiter plates (DYNEX Technologies) and placed in an automated injection MLX microtiter plate luminometer. The luciferase reaction mix (100 μl) was injected sequentially into the wells. The light signals generated in the reactions were integrated over an interval of 2 seconds, and the resulting luminescence values were used as a measure of luciferase activity (relative units).

VDR ligand binding. Determination of VDR ligand–binding activity was performed using purified baculovirus-produced His-tagged VDR-LBD protein. Assays were performed in 96-well Optiplates (PerkinElmer). Each binding reaction was performed in binding assay buffer (50 mM Tris-HCl, pH 7.5, 1.5 mM EDTA, 5 mM DTT, 300 mM KCl, and 0.01% Tween 20) using 1 μg of VDR protein and 1 nM 1,25-dihydroxy[23,24(n)-^{3}H]cholecalciferol (102 Ci/mmol) (Amersham Biosciences) with varying concentrations of competitor compounds in 150 μl total volume for 15 minutes at room temperature. After 15 minutes incubation, 50 μl of polylysine-coated yttrium silicate SPA beads (0.125 mg/50 μl in 30% glycerol/binding assay buffer) (Amersham Biosciences) was added to each binding reaction followed by shaking for 30 minutes. Nonspecific binding was determined by incubation with 1,000-fold excess of cold 1,25-$(OH)_2D_3$. Plates were read using Packard TopCount Scintillation Counter (PerkinElmer).

Keratinocyte proliferation inhibition assay. KerTr cells (human transformed skin keratinocyte, obtained from ATCC) were plated in 96-well flat-bottom plates (3000 cells per well) in 100 μl keratinocyte serum-free medium supplemented with bovine pituitary extract in the absence of EGF (Invitrogen Corp.) and incubated at 37°C for 2 days. The cells were treated with various concentrations of VDR ligands in triplicate, dissolved in 100 μl keratinocyte serum-free medium supplemented with bovine pituitary extract in the absence of EGF, and incubated at 37°C for 72 hours. BrdU (5-bromo-2′-deoxyuridine) incorporation was analyzed (Cell Proliferation ELISA kit; Roche Diagnostics Corp.), and absorbance was measured at 405 nm. Potency values (IC_{50}) values were determined as the concentration (nM) of compound that elicited a half-maximal response.

Quantitative RT-PCR assays. Human colon carcinoma, Caco-2 cells, maintained in DMEM (high glucose with 25 mM HEPES buffer; Invitrogen Corp.) supplemented with 10% FBS (Invitrogen Corp.) were plated at 5500 cells per well in a 96-well plate in a total volume of 100 μl/well. The cells were kept in the 96-well plate for 6 days to differentiate them into small intestinal cells that express the calcium transporter CaT1. On day 3 after plating, spent media were removed and replaced with fresh media (150 μl/well). On day 6, the spent media were again removed, and the cells were maintained in treatment media (180 μl/well) (DMEM [low glucose, without phenol red; Invitrogen Corp.] containing 10% charcoal-stripped FBS [HyClone]). The cells were treated with various concentrations of VDR ligands prepared in treatment media (20 μl/well).

Rat IEC-6 duodenal cells maintained in DMEM (high glucose with 25 mM HEPES buffer; Invitrogen Corp.) supplemented with 10% FBS (Invitrogen Corp.) were plated in T25 flasks. On the day of treatment with the VDR ligands, spent media were removed, and the cells were kept in treatment media (DMEM [low glucose, without phenol red; Invitrogen Corp.] containing 10% charcoal-stripped FBS [HyClone]). The cells were treated with vehicle, 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 (100 nM each) prepared in treatment media.

PBMCs were isolated from normal human donors by sedimentation on Ficoll-Hypaque. Informed consent was obtained from human donors. Cells were resuspended in RPMI-1640 supplemented with charcoal-treated FBS (2%). PBMCs (25×10^6/T75 flask) were activated with PHA (10 μg/ml) and TPA (100 ng/ml). The cells were cultured in the presence of 1,25-$(OH)_2D_3$, LY2108491, or LY2109866 (100 nM each) prepared in RPMI-1640 containing 2% charcoal-treated FBS.

Twenty hours after treatment, total RNA was prepared by the RNeasy 96 method, as described by the manufacturer (QIAGEN). The RNA was reverse transcribed and amplified for various human and rat (human CaT1, *CYP24*, and GAPDH for Caco-2 cells; human IL-2, IL-4, IL-10, GATA3, and GAPDH for PBMCs; and rat *CYP24*, calbindin-9k, and GAPDH for IEC-6 cells) mRNAs by quantitative RT-PCR using the ABI PRISM 7900HT Sequence Detection System (Applied Biosystems). Optimized primer pairs and probes for human (CaT1, *CYP24*, and GAPDH) and rat (*CYP24*, calbindin-9k, and GAPDH) genes were obtained commercially (Applied Biosystems). Each 20 μl quantitative RT-PCR reaction in a 384-well TaqMan PCR plate consisted of forward and reverse primers (900 nM), TaqMan probe (200 nM), total RNA (4 μl for each well of the 96-well culture plate), and 10 μl of TaqMan Universal PCR Master Mix, (Roche Diagnostics Corp.). Reactions were incubated at 48°C for 30 minutes, followed by 10 minutes at 95°C, and subjected to 40 cycles of PCR (95°C for 15 seconds followed by 60°C for 1 minute).

Antigen-specific T cell recall assay. This study was approved by the Animal Committee of Eli Lilly and Company to ensure compliance with NIH guidelines. Female C57BL/6 mice purchased from Taconic were immunized s.c. at 2 sites on the back with 300 μg $MOG_{35–55}$ (MEVGWYRSPFSRVVH-LYRNGK, Peptides International Inc.) emulsified in a total of 200 μl CFA (BD Diagnostics) containing 500 μg *M. tuberculosis* H37 Ra (BD Diagnostics). Immunized mice were treated with vehicle (sesame oil), LY2108491 (10 and 100 μg/kg), or LY2109866 (10 and 100 μg/kg). Ten days later, splenocytes were isolated by homogenizing spleens between frosted glass slides (Fisher Scientific International), and rbcs were removed with ACK lysing buffer (BioWhittaker Inc.). Pooled splenocytes of 5 individual mice from the same group were plated in triplicate in a 96-well round-bottom plate at 2×10^5 cells per well in 200 μl complete RPMI 1640 medium (Invitrogen Corp.) supplemented with 2 mM L-glutamine, 25 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 5.5×10^{-5} M 2-mercaptoethanol, and 5% FCS (all supplements from Invitrogen Corp.) containing 10 μg/ml $MOG_{35–55}$ (Peptides International Inc.) or medium, and cultured at 37°C, 5% CO_2 for 72 hours. Cytokine levels produced from cultured splenocytes were analyzed with R&D Systems MAP kit (R&D Systems) (LUM000) with IL-2 (LUM402) and IFN-γ (LUM485) beads. Comparisons of cytokine levels among various groups were analyzed by ANOVA test, and P values of less than 0.05 were considered significant.

Vector construction. Gal4-DBD chimeras of SRC-1, TIF2, or AIB1 have been reported previously (45). Expression vectors pVP16-VDR-LBD, pGal4-RXRα-LBD, and pGal4-VDR-LBD have also been described (2). Gal4-VDR-LBD$^{\Delta AF\text{-}2}$ was constructed from Gal4-VDR-LBD (pM-VDR-LBD; encompassing amino acids 89–427 of VDR) (2) by PCR amplification and subcloned into the *Eco*RI and *Xba*I sites of the pM vector (Clontech Laboratories Inc.). Full-length VDR and VDR activation function–2

(AF-2) fused to the Gal4-DBD were constructed by PCR amplification and cloned into the pM vector between the *Eco*RI and *Xba*I sites. The E420A mutant in Gal4-VDR-LBD was generated using the QuikChange site-directed mutagenesis kit (Stratagene). The expression plasmid for PGC-1α has been described (34).

In vivo hypercalcemia assay. Female, 6- to 7-week-old DBF1 mice weighing approximately 25 g were purchased from Harlan. Mice were housed with ad libitum access to food (TD 5001 with 0.95% calcium and 0.67% phosphorus, vitamin D3, 4500 IU/kg; Harlan Teklad) and water. Compounds were given orally via gavage daily for 6 days. Dosing volume was 100 μl/mouse with 4 mice in each group. Serum ionized calcium was examined at 6 hours after last dosing using a Ciba-Corning 634 Ca^{++}/pH Analyzer (Chiron Corp.).

In vivo epidermal proliferation model. Female, 40- to 42-day-old, SKH1-hrBR (CRl) hairless mice (Charles River Laboratories) weighing approximately 20 g were housed with ad libitum access to food (TD 5001 with 0.95% calcium and 0.67% phosphorus, vitamin D3 4500 IU/kg; Harlan Teklad) and water. Solutions for topical treatment were prepared in acetone, and a single topical application of various doses of the compounds was administered to a preset surface area of 2 × 2 cm^2 on the back waist area. Dosing volume was 100 μl/mouse with 4 mice in each group. Serum ionized calcium was examined at 24 hours after dosing using a Ciba-Corning 634 Ca^{++}/pH Analyzer (Chiron Corp.). Mice were sacrificed at 72 hours after dosing, and skin samples were collected. Epidermal cell height was quantitated on 8-μm thickness of H&E-stained paraffin sections using an image probe system (Image-Pro Plus version 3.0; MediaCybernetics). Special attention was paid to ensure that the sections were cut perpendicular to the skin surface.

Epidermal proliferation in $VDR^{+/+}$ and $VDR^{-/-}$ mice. $VDR^{-/-}$ mice were generated by gene targeting as described previously (47). $VDR^{+/+}$ and $VDR^{-/-}$ mice were weaned at 3 weeks of age and housed with ad libitum access to food (CE-2 with 1.03% calcium and 0.97% phosphorus; CLEA Japan Inc.) and water. Female, 40- to 42-day-old, back-shaved $VDR^{+/+}$ and $VDR^{-/-}$ mice weighing 15–20 g were used in this study. $VDR^{+/+}$ mice (n = 3) and $VDR^{-/-}$ mice (n = 3) were treated topically as described above with vehicle, 1,25-$(OH)_2D_3$ (10 nmol), LY2108491 (50 nmol), or LY2109866 (50 nmol). Mice were sacrificed at 72 hours after dosing, and skin samples were collected. Epidermal thickness was assessed using H&E-stained paraffin sections

Statistics. Statistical significance versus control was defined as $P < 0.05$ in the Dunnett's test. Compound potency for hypercalcemia and epidermal proliferation was defined as the TMED, which is the lowest dose achieving a mean response greater than a predetermined level of serum calcium or cell proliferation that is also statistically significant in the Dunnett's test. In vitro concentration-response curves were fit using the sigmoidal/variable slope fitting option in GraphPad Prism (version 4; GraphPad Software).

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