Novel nonsecosteroidal vitamin D mimics exert VDR-modulating activities with less calcium mobilization than 1,25-dihydroxyvitamin D_3

Marcus F Boehm[1], Patrick Fitzgerald[2], Aihua Zou[2], Marc G Elgort[2], Eric D Bischoff[2], Lora Mere[3], Dale E Mais[4], Reid P Bissonnette[2], Richard A Heyman[2], Alex M Nadzan[1]*, Melvin Reichman[3] and Elizabeth A Allegretto[2]

Background: The secosteroid 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) acts through the vitamin D receptor (VDR) to elicit many activities that make it a promising drug candidate for the treatment of a number of diseases, including cancer and psoriasis. Clinical use of $1,25(OH)_2D_3$ has been limited by hypercalcemia elicited by pharmacologically effective doses. We hypothesized that structurally distinct, nonsecosteroidal mimics of $1,25(OH)_2D_3$ might have different activity profiles from vitamin D analogs, and set out to discover such compounds by screening small-molecule libraries.

Results: A *bis*-phenyl derivative was found to activate VDR in a transactivation screening assay. Additional related compounds were synthesized that mimicked various activities of $1,25(OH)_2D_3$, including growth inhibition of cancer cells and keratinocytes, as well as induction of leukemic cell differentiation. In contrast to $1,25(OH)_2D_3$, these synthetic compounds did not demonstrate appreciable binding to serum vitamin D binding protein, a property that is correlated with fewer calcium effects *in vivo*. Two mimics tested in mice showed greater induction of a VDR target gene with less elevation of serum calcium than $1,25(OH)_2D_3$.

Conclusions: These novel VDR modulators may have potential as therapeutics for cancer, leukemia and psoriasis with less calcium mobilization side effects than are associated with secosteroidal $1,25(OH)_2D_3$ analogs.

Addresses: Departments of [1]Medicinal Chemistry, [2]Retinoid Research, [3]New Leads Discovery and [4]Endocrine Research, Ligand Pharmaceuticals, Inc., 10255 Science Center Drive, San Diego, CA 92121, USA.

*Present address: Chugai Pharmaceuticals, Inc., 6275 Nancy Ridge Drive, San Diego, CA 92121, USA.

Correspondence: Elizabeth A Allegretto
E-mail: ballegretto@ligand.com

Key words: breast cancer, 1,25-dihydroxyvitamin D_3 mimic, leukemia, prostate cancer, vitamin D receptor

Received: **25 November 1998**
Revisions requested: **21 December 1998**
Revisions received: **25 January 1999**
Accepted: **26 January 1999**

Published: **6 April 1999**

Chemistry & Biology May 1999, **6**:265–275
http://biomednet.com/elecref/1074552100600265

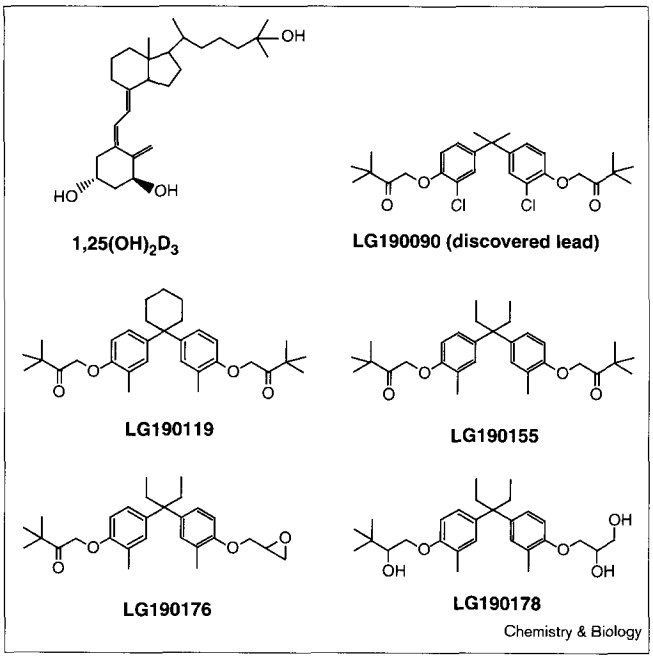
 ISSN 1074-5521

Introduction

The active form of vitamin D_3, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), is a secosteroid that has long been recognized as a major regulator of calcium homeostasis and bone development and remodelling [1,2]. Since the early 1980s $1,25(OH)_2D_3$ has been shown to be a potent differentiator of leukemic cells [3–5] and a growth inhibitor of various types of cancer cells, including those of the prostate, breast and colon [6–8]. $1,25(OH)_2D_3$ has also been shown to have immunosuppressive effects [9,10], some of which have been borne out in animal models of Lupus, autoimmune diabetes and multiple sclerosis [11–13]. The molecular mechanism of $1,25(OH)_2D_3$ is known to be through binding to its intracellular receptor, the vitamin D receptor (VDR). This binding results in heterodimer formation with retinoid X receptor (RXR), which enables high-affinity binding to vitamin D responsive element (VDRE) sequences within, and subsequent transcriptional activation of, vitamin D target genes such as the bone proteins osteocalcin and osteopontin, and a metabolic enzyme, 25-hydroxyvitamin D_3-24-hydroxylase (24(OH)ase) [14–15].

The activity of $1,25(OH)_2D_3$ as a modulator of growth and an inducer of differentiation of a variety of cancer cell types both *in vitro* [16–18] and in animal models [19–23] suggested a potential clinical application of this hormone in cancer, as well as for psoriasis [24], a disease that has both hyperproliferative and autoimmune components. However, the normal calcium-mobilization function of $1,25(OH)_2D_3$ is increased as the dosage is increased to pharmacological levels, leading to hypercalcemia (elevated serum calcium) in animal and in human subjects, which has limited the usefulness of $1,25(OH)_2D_3$ as a drug. Recently, however, chemical modification of $1,25(OH)_2D_3$ has yielded a number of secosteroidal analogs that have a greater ratio of desirable effects to unwanted calcium mobilization effects [25]. One of these analogs, MC903 or Dovonex®, is currently in use as a topical treatment for mild to moderate psoriasis [26]. Although a number of $1,25(OH)_2D_3$ analogs have shown some separation of antiproliferative activities from calcium-raising effects, this separation does not seem to be sufficient for long-term oral drug therapy of conditions such as cancers and severe psoriasis, and, therefore, there is a need for improved compounds. All compounds known to

Figure 1



Structures of vitamin D mimic compounds. LG190176 and LG190178 are mixtures of stereoisomers.

date that activate VDR are analogs of $1,25(OH)_2D_3$. We hypothesized that nonsecosteroidal VDR ligands might display different profiles of activity and metabolism than do secosteroidal $1,25(OH)_2D_3$ analogs, including less calcemic properties, which might render them attractive as both topical and oral pharmaceuticals for treating a variety of diseases.

This hypothesis was based in part on the success that nonsteroidal androgen receptor (AR) and estrogen receptor (ER) modulators have had as drugs. Although the natural AR and ER ligands are steroids (dihydrotestosterone and 17-β-estradiol, respectively), nonsteroidal compounds have been synthesized that modulate the activity of these receptors and show enhanced tissue selectivity in comparison to the steroids. The AR ligands flutamide (Eulexin®) and bicalutamide (Casodex®) are currently used to treat prostate cancer and the ER modulator tamoxifen (Nolvadex®) is the drug of choice for ER-positive breast cancer. Additionally, a promising new nonsteroidal-ER modulator drug, raloxifene (Evista®), is now available and several other selective nonsteroidal-ER ligands are in the drug pipeline for use in hormone replacement therapy and breast cancer.

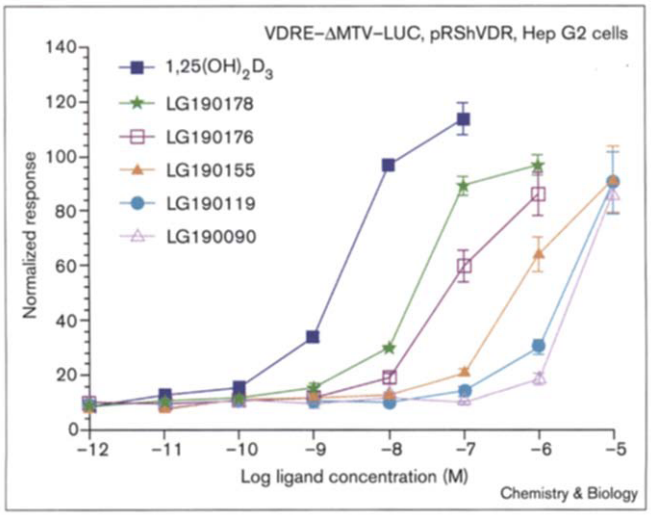
Described herein are novel nonsecosteroidal activators of VDR (Figure 1). These compounds mimicked various $1,25(OH)_2D_3$ activities *in vitro*, such as interaction with VDR, VDR-dependent transcriptional activation, inhibition of proliferation of human cancer cells and primary human keratinocytes and monocytic differentiation of HL60 leukemic cells. These compounds did not bind to serum vitamin D binding protein (DBP), a property that has been previously correlated with less calcemic potential *in vivo* [27]. Additionally, these compounds induced RNA levels of a VDR target gene, kidney 24(OH)ase, in rodents to a greater extent than $1,25(OH)_2D_3$ did, with less increase in serum calcium than exhibited by $1,25(OH)_2D_3$. Therefore, these structurally novel compounds might have promise as therapeutics for a wide variety of diseases with less severe calcium-related side effects than secosteroidal vitamin D analogs.

Results

Transactivational properties of $1,25(OH)_2D_3$ and vitamin D mimics

In an attempt to identify nonsecosteroidal activators of VDR, compound libraries were screened in a cotransfection–cotransactivation (CTF) assay in which Hep G2 cells were transfected with a luciferase reporter vector driven by one copy of a VDRE from the h24(OH)ase promoter (VDRE–ΔMTV–LUC [28]) with or without a VDR expression vector (pRShVDR). In this assay $1,25(OH)_2D_3$ induced luciferase activity in the presence of pRShVDR (Figure 2), but not without the expression plasmid (data not shown), indicating that the response was VDR-dependent. $1,25(OH)_2D_3$ elicited an approximately 10-fold induction of luciferase with a potency (EC_{50}) of 2–5 nM (Figure 2, Table 1). One compound, a *bis*-phenyl derivative designated LG190090 (Figure 1), induced luciferase activity ~ninefold in a VDR-dependent manner with an EC_{50} of ~2.5 μM (Figure 2). Several related analogs were synthesized around this hit, some of which are shown in Figure 1, that display increased potency in the CTF assay (Figure 2). Chemical modifications at the bridgehead of LG190090 to a cyclohexyl (LG190119) or diethyl (LG190155, LG190176, and LG190178) group led to increased potency in CTF assays (Table 1), and additional polar groups such as hydroxyl moieties (LG190178) also resulted in increased potency in CTF assays and increased binding affinity for VDR *in vitro* (see below and Table 1). Each of these compounds required the presence of VDR expression vector to induce VDRE-driven reporter activity (data not shown). Compound efficacies were 80–100% of $1,25(OH)_2D_3$ and potencies ranged from 2.2 μM to 40 nM (Figure 2, Table 1). None of these compounds showed cross-reactivity with various other intracellular receptors in cotransactivation assays utilizing reporters driven by their respective response elements, including RXRs and retinoic acid receptors (RARs; data not shown). These results indicated that these compounds stimulated the VDRE-driven reporter by activation of VDR and were henceforth termed vitamin D mimics, because they resembled $1,25(OH)_2D_3$ functionally without sharing its structure.

Figure 2



Vitamin D mimics induce transcription from a VDRE-driven reporter vector in a VDR-dependent manner in cotransfection–cotransactivation assays. pRShVDR was cotransfected along with VDRE–ΔMTV–LUC into Hep G2 cells and cells were subsequently treated with $1,25(OH)_2D_3$ or vitamin D mimics in various concentrations. Luciferase activity was measured and values were normalized for transfection efficiency with β-galactosidase values.

VDR-binding characteristics of vitamin D mimics

To determine if the vitamin D mimics bound directly to VDR *in vitro*, ligand-binding assays were performed. Unlabeled $1,25(OH)_2D_3$ competed with [^{3}H]-$1,25(OH)_2D_3$ for binding to yeast-expressed hVDR with an equilibrium dissociation constant (K_i) of 0.5 nM. Many of the vitamin D mimics that displayed lower potency (EC_{50} >500 nM) in Hep G2 cell cotransactivation assays did not compete with [^{3}H]-$1,25(OH)_2D_3$ in VDR-binding assays performed under equilibrium conditions *in vitro*, whereas the more potent mimics in the cell-based assay did demonstrate binding to VDR under these conditions (Table 1 and data not shown). For example, LG190178 (EC_{50} ~40 nM in the Hep G2 cell CTF assay) showed a K_i value of 150 nM in the VDR-binding assay (Table 1). This observation was true for most mimics synthesized; as compound potency increased in the Hep G2 cell cotransactivation assay, VDR binding was observed *in vitro*. Therefore, the VDR-dependent cell-based assay was more sensitive than the *in vitro* VDR equilibrium-binding assay in detection of VDR activators of this class. Additionally, none of the vitamin D mimics displayed any binding to RXRs or RARs (data not shown).

We hypothesized that detection of VDR binding of the less potent vitamin D mimic compounds under equilibrium conditions in a competition binding assay with [^{3}H]-$1,25(OH)_2D_3$ was precluded by the fast on-rate and slow off-rate of the high-affinity ligand $1,25(OH)_2D_3$. To test this, LG190119, LG190155 or LG190178 was preincubated with VDR overnight prior to incubation with [^{3}H]-$1,25(OH)_2D_3$ for various time periods between 2 min and 1 h to determine if the compounds would inhibit binding of [^{3}H]-$1,25(OH)_2D_3$ to VDR in comparison with preincubation of VDR with vehicle alone. LG190178, which showed demonstrable binding to VDR under equilibrium conditions (Table 1), also inhibited the binding of [^{3}H]-$1,25(OH)_2D_3$ to VDR in the preincubation assay (Table 2). Percent inhibition of [^{3}H]-$1,25(OH)_2D_3$ binding to VDR by LG190178 ranged from 94% at 2 min to 97% at equilibrium (20 h). Preincubation of VDR with LG190119 or LG190155 also resulted in decreased binding of [^{3}H]-$1,25(OH)_2D_3$ to VDR at short time intervals in comparison with preincubation of VDR without compound, and this percent inhibition decreased with increased incubation time with [^{3}H]-$1,25(OH)_2D_3$ (Table 2). At 2 min LG190119 elicited 26.5% inhibition of [^{3}H]-$1,25(OH)_2D_3$ binding to VDR and LG190155 preincubation led to 32% less binding. These percentages decreased at subsequent time points, showing 15–16% inhibition at 60 min, and essentially no inhibition

Table 1

Activity of selected vitamin D mimics in *in vitro* and cell-based assays.

Compound	CTF EC_{50} (nM)*	VDR Binding K_i (nM)†	LNCaP BrdU EC_{50} (nM)‡	HL60 NBT EC_{50} (nM)§	NHEK BrdU EC_{50} (nM)#	DBP Binding IC_{50} (nM)¶
LG190090	2500	>10000	3000	10000	ND	>10000
LG190119	2200	>10000	2000	2000	500	>10000
LG190155	600	>10000	300	800	500	>10000
LG190176	60	>10000	100	150	200	>10000
LG190178	40	150	20	30–50	30-50	>10000
$1,25(OH)_2D_3$	2–5	0.5	2	8–10	10–30	200 (h); 40 (r)

*EC_{50} values determined by cotransfection–transactivation assays using pRShVDR and VDRE(1)–ΔMTV–LUC in Hep G2 cells. †K_i (equilibrium dissociation constant) values determined by *in vitro* competition binding to yeast-expressed hVDR expressed versus [^{3}H]-$1,25(OH)_2D_3$. ‡EC_{50} values determined by growth inhibition of LNCaP human prostate cancer cells by BrdU incorporation. §EC_{50} values determined by nitroblue tetrazolium assay of differentiation of HL60 human leukemia cells. #EC_{50} values determined by growth inhibition of normal human epidermal keratinocytes by BrdU incorporation. ¶IC_{50} values determined by *in vitro* competition binding to human (h) or rat (r) serum vitamin D binding protein versus [^{3}H]-$25(OH)D_3$. ND, not determined.

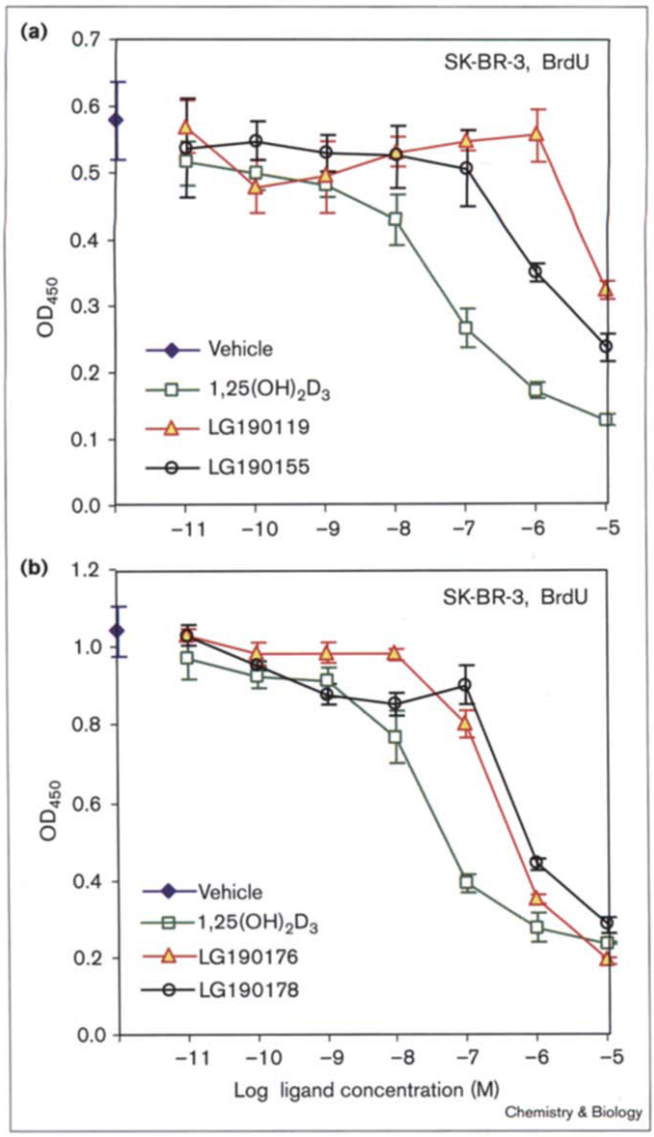
Table 2

Percent inhibition of [^{3}H]-1,25(OH)$_2$D$_3$ binding to VDR *in vitro* by preincubation with various compounds.

Compound	Compound preincubation concentration*	Time of incubation with [^{3}H]-1,25(OH)$_2$D$_3$				
		2 min	10 min	30 min	60 min	20 h
LG190119	10 μM	26.5 ± 3.5†	21 ± 1.4	16 ± 2.8	16.5 ± 6.4	1.3 ± 0.4
LG190155	10 μM	32 ± 2.8	18 ± 4.2	16 ± 2.8	15 ± 1.4	3 ± 1
LG190178	10 μM	94 ± 2.1	96 ± 1	99.5 ± 0.7	100 ± 0	97 ± 1.5
1,25(OH)$_2$D$_3$	1 μM	100	100	100	100	100

*Preincubation of yeast-expressed hVDR (2.5 μg total soluble protein extract) with compounds was for 20 h prior to addition of 0.34 nM [^{3}H]-1,25(OH)$_2$D$_3$. †Percent inhibition determined by the ratio of [^{3}H]-1,25(OH)$_2$D$_3$ binding to VDR in the presence of compound to [^{3}H]-1,25(OH)$_2$D$_3$ binding to VDR in the absence of compound relative to inhibition of [^{3}H]-1,25(OH)$_2$D$_3$ binding to VDR by unlabeled 1,25(OH)$_2$D$_3$ at each time point (average of two individual experiments with each point assayed in triplicate).

at 20 h (equilibrium conditions), as expected. Therefore, although direct binding of VDR by LG190119 and LG190155 was not detectable under equilibrium conditions, both compounds do interact with VDR, as demonstrated by their ability to inhibit [^{3}H]-1,25(OH)$_2$D$_3$ from binding to VDR during short time intervals.



Activity of vitamin D mimics in SK-BR-3 breast cancer cell and LNCaP prostate cancer cell growth assays

Growth of SK-BR-3 human breast cancer cells has been shown previously to be inhibited by 1,25(OH)$_2$D$_3$ [29]. Using BrdU incorporation assays, 1,25(OH)$_2$D$_3$ had an EC$_{50}$ of 30 nM and ~90% efficacy in inhibiting DNA replication in these cells (Figure 3). The vitamin D mimics also inhibited the growth of these cells, displaying 50–100% efficacy (Figure 3). The rank order of potencies of the mimics in inhibiting DNA replication in SK-BR-3 cells agreed with those observed in the cotransactivation assay (Table 1). The SK-BR-3 cell number also decreased upon treatment with 1,25(OH)$_2$D$_3$ or vitamin D mimics (data not shown).

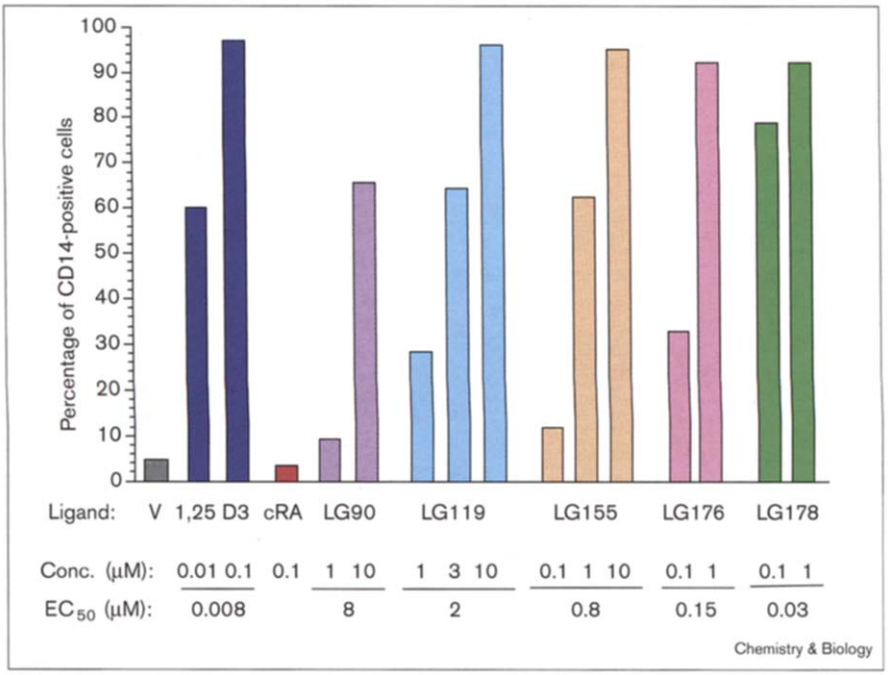
Growth of LNCaP human prostate cancer cells has also been shown to be inhibited by 1,25(OH)$_2$D$_3$ [8,18], and these cells were also tested for their response to the vitamin D mimics. 1,25(OH)$_2$D$_3$ was a potent growth inhibitor of these cells with an EC$_{50}$ of 2 nM and ~95% efficacy in the BrdU assay (Table 1). The vitamin D mimics inhibited BrdU incorporation in these cells with 60–100% efficacy and showed a rank order of potency values that were similar to those obtained in the Hep G2 cell CTF assays and SK-BR-3 cell growth assays (Table 1, Figure 3). Therefore, the vitamin D mimics, like 1,25(OH)$_2$D$_3$, were able to inhibit the proliferation of two human cancer cell lines in culture.

Figure 3

Vitamin D mimics inhibit the growth of SK-BR-3 human breast cancer cells in culture. SK-BR-3 cells were plated at 3000 cells per well in 96-well plates. **(a)** 1,25(OH)$_2$D$_3$, LG190119 or LG190155, or **(b)** 1,25(OH)$_2$D$_3$, LG190176 or LG190178 were added in ethanol vehicle in media at 7 concentrations between 10^{-5} and 10^{-11} M. After 3 days, BrdU incorporation ELISA were performed and the colorimetric reaction was stopped and absorbance was measured at 450 nm. Vehicle average is denoted by a solid diamond on the y axis.

Figure 4

Vitamin D mimics induce differentiation of HL60 human leukemic cells to macrophages. HL60 cells were grown and plated as described in the Materials and methods section. Ligands at various concentrations were added to the cells in ethanol vehicle and incubation proceeded for 5 days. Cells were harvested and CD14 analyses via FACS were performed. V, vehicle; $1,25D_3$, $1,25(OH)_2D_3$; cRA, 9-*cis* retinoic acid; LG90, LG190090; LG119, LG190119; LG155, LG190155; LG176, LG190176; LG178, LG190178.



Differentiation of HL60 cells into macrophages by vitamin D mimics

$1,25(OH)_2D_3$ is a known differentiator of HL60 human leukemia cells into macrophages/monocytes [5]. Using nitroblue tetrazolium (NBT) [5,30] and CD14 (a specific marker for macrophages/monocytes) FACS [31] assays, $1,25(OH)_2D_3$ induced differentiation of HL60 cells with an EC_{50} of 8–10 nM, and ~100% efficacy was achieved with 100 nM $1,25(OH)_2D_3$ (Figure 4). Even the least potent vitamin D mimics were efficient inducers of HL60 cell differentiation as assessed by both NBT reduction (Table 1) and CD14 induction (Figure 4). LG190090 had an EC_{50} of 8 μM in these assays (Table 1, Figure 4), with an efficacy of ~70% differentiation at 10 μM (Figure 4). The more potent mimics differentiated the cells with EC_{50} values of 30–150 nM and >90% efficacy (Table 1, Figure 4). Retinoids are also known to induce differentiation of HL60 cells, however, this differentiation is to granulocytes, which do not express CD14. As shown in Figure 4, 100 nM 9-*cis* retinoic acid (cRA) did not induce CD14 expression in these cells, although NBT was reduced (data not shown). Therefore, the vitamin D mimics differentiated HL60 cells down the macrophage pathway, indicating a VDR-dependent mechanism.

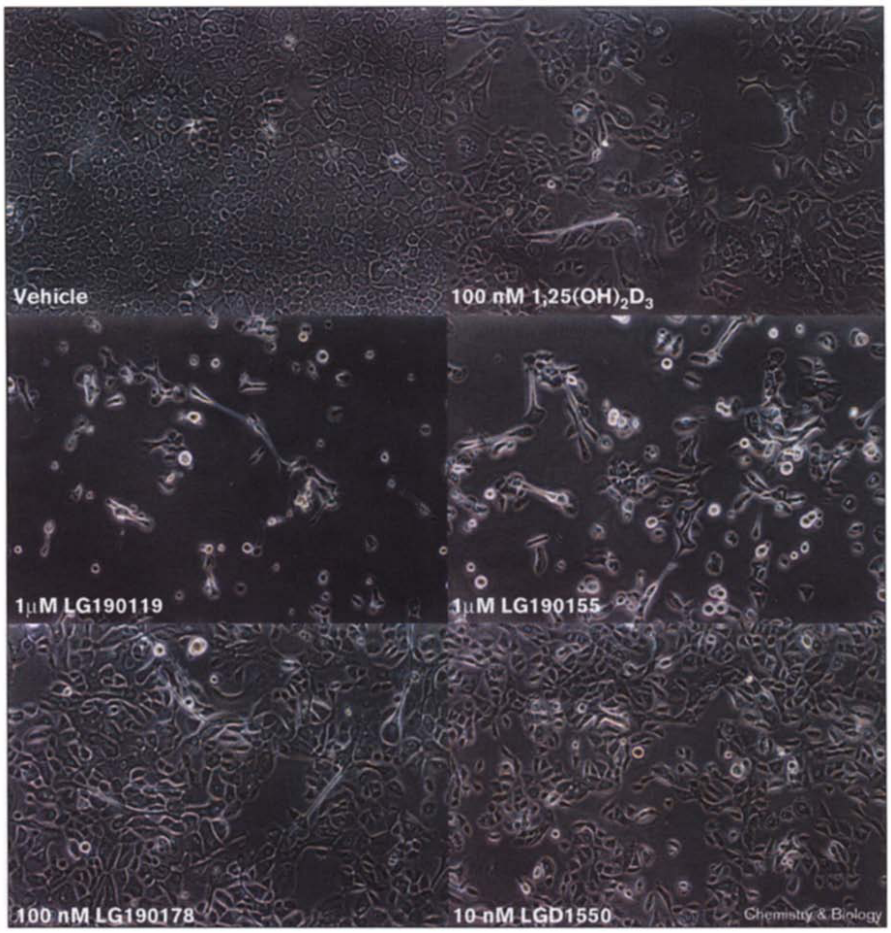
Vitamin D mimics growth inhibit and change the morphology of primary human keratinocytes

Treatment of normal human epidermal keratinocytes for 3 days with $1,25(OH)_2D_3$ resulted in inhibition of BrdU incorporation with an EC_{50} value of ~30 nM (Table 1), in agreement with previous work [32], and treatment for 7 days markedly changed the morphology of the cells (Figure 5), as reported previously [33]. The vitamin D mimics also inhibited the growth of the keratinocytes (Table 1) with rank order of potencies in agreement with those from other assays (Table 1, Figures 2–4) and elicited morphological changes, including the formation of spindle-like structures (Figure 5) that have been previously demonstrated with $1,25(OH)_2D_3$ [33]. LGD1550, a potent RAR-selective ligand also inhibited the growth of keratinocytes (data not shown); however, the spindle-like structures were not observed (Figure 5). The vitamin D mimics described herein, therefore, share many activities and specificities of $1,25(OH)_2D_3$ in a variety of *in vitro* cell-based assays.

Vitamin D mimics do not bind to rat or human serum DBP

The ability of various $1,25(OH)_2D_3$ analogs to bind DBP has been associated with their ability to raise serum calcium *in vivo*, whereas analogs with lower affinities for DBP showed lowered calcium potential in animal models [27]. To examine the DBP-binding characteristics of these vitamin D mimics, rat or human serum was incubated with $[^3H]$-$25(OH)D_3$ with and without unlabeled competitor ligands. IC_{50} values of $25(OH)D_3$ (the endogenous precursor of $1,25(OH)_2D_3$) for rat and human DBP were 1 nM and 2 nM, respectively; IC_{50} values of $1,25(OH)_2D_3$ for rat and human DBP were 30–50 nM and 200 nM, respectively (in agreement with values published previously [27,34]). Using this DBP-binding assay, none of the vitamin D mimics tested bound appreciably to either rat or human

Figure 5



Vitamin D mimics induce morphological changes in normal human epidermal keratinocytes. Keratinocytes were grown and treated as described in the Materials and methods section. Cells were incubated with ligands (as denoted in the figure) for 7 days, with media change and compound replenishment on days 2 and 4.

DBP, showing IC_{50} values greater than 10 μM (Table 1). Therefore the DBP-binding properties of the vitamin D mimics implied that they might be less calcemic *in vivo* than $1,25(OH)_2D_3$.

Vitamin D mimics induce a VDR target gene *in vivo*, with less serum calcium increase than $1,25(OH)_2D_3$

In order to test the oral bioavailability and activity of the vitamin D mimics *in vivo*, LG190119 and LG190155 were administered in sesame oil to mice by gavage for 3 or 5 consecutive days at various concentrations. $1,25(OH)_2D_3$ was tested at 2, 5 and 15 μg/kg, LG190119 was tested at 10 and 30 mg/kg, and LG190155 was tested at 0.1 and 0.5 mg/kg. $1,25(OH)_2D_3$ induced kidney 24(OH)ase RNA, as assessed by Northern analysis, 5.7-fold after 3 days in one experiment and 20- to 25-fold after 5 days in a second assay (Table 3). LG190119 induced kidney 24(OH)ase RNA 10- to 13-fold after 3 days and 30- to 54-fold after 5 days treatment, whereas LG190155 induced 24(OH)ase 6-fold after 3 days treatment (Table 3). Although the dose of 15 μg/kg $1,25(OH)_2D_3$ caused significant hypercalcemia after 3 days, the 2 μg/kg dose and 5 μg/kg dose slightly elevated calcium after 5 days, with the 5 μg/kg dose being significantly different from vehicle. In-depth toxicity studies were not carried out, however, weight loss was observed in each group of animals treated with $1,25(OH)_2D_3$ for both 3 and 5 days, and with the higher dose of LG190119 after 5 days of treatment (Table 3). The ratio of target gene induction to serum calcium concentration was greater with LG190119 or LG190155 than it was with $1,25(OH)_2D_3$ (Table 3), indicating greater separation of these two *in vivo* activities with the vitamin D mimics than with $1,25(OH)_2D_3$. These data indicate that compounds of this structural class exhibit some of the same desirable biological actions of $1,25(OH)_2D_3$, with less calcemic activity, and, therefore, might represent a new class of VDR modulators with potential clinical utility for treating a variety of diseases.

Discussion

The VDR is a nuclear receptor that has been known to be activated only by its endogenous secosteroidal ligand, $1,25(OH)_2D_3$, or synthetic secosteroidal analogs that are structurally very similar to $1,25(OH)_2D_3$. In an attempt to identify structurally distinct, nonsecosteroidal activators

Table 3

Analysis of VDR target gene regulation and calcemic potential of vitamin D mimics in mice.

Compound	Dose	Kidney 24(OH)ase*	Serum Ca^{2+}†	24(OH)ase/ Ca^{2+}	24(OH)ase/ Ca^{2+} Increase	% Weight change‡
3-day treatment (female BALB/c)						
Vehicle			8.3 ± 0.5			+0.7
$1,25(OH)_2D_3$	15 μg/kg	5.7	>15§	0.38	0.85	−12.0§
LG190119	10 mg/kg	13.5	9.1 ± 0.5	1.48	16.9	−1.6
LG190119	30 mg/kg	10.6	9.0 ± 0.6	1.18	15.1	−2.9
LG190155	0.1 mg/kg	6.0	9.6 ± 0.5	0.63	5.17	+1.7
LG190155	0.5 mg/kg	6.0	11.5 ± 0.9§	0.52	1.88	−1.5
5-day treatment (male BALB/c)						
Vehicle			8.6 ± 0.4			−1.5
$1,25(OH)_2D_3$	2 μg/kg	20.8	9.7 ± 0.6	2.14	18.9	−9.9§
$1,25(OH)_2D_3$	5 μg/kg	24.9	10.5 ± 0.6§	2.37	13.1	−12.8§
LG190119	10 mg/kg	30.8	9.1 ± 0.2	3.38	61.6	−5.1
LG190119	30 mg/kg	53.7	10.1 ± 0.5	5.31	35.8	−11.1§

*Mean fold induction versus vehicle corrected with GAPDH; error within groups was 15% (Northern analysis). †Normal mouse serum calcium range is 7.9–10.5 mg/dl. ‡Mean of the percent weight change per animal per group over time of study. §Statistically different than vehicle (ANOVA, $p<0.01$).

of VDR, we utilized a high-throughput cotransfection–cotransactivation (CTF) assay to screen a small-molecule library. Using this assay, a compound was discovered that elicited VDR-dependent stimulation of a VDRE-driven luciferase reporter vector. This compound, a symmetrical *bis*-phenyl derivative designated LG190090, was structurally unrelated to $1,25(OH)_2D_3$ (see Figure 1). Although LG190090 was much less potent in CTF assays (EC_{50} ~2.5 μM) than $1,25(OH)_2D_3$ (EC_{50} ~2–5 nM), it showed almost equal efficacy. Additional compounds structurally related to LG190090 were synthesized and many had activity in the CTF assay, some of which displayed increased potency compared with the lead molecule (Table 1). Chemistry efforts (synthesis of related analogs) increased the potencies of the vitamin D mimic compounds more than 100-fold. For example, one of the more potent compounds synthesized, LG190178, displayed an EC_{50} of ~40 nM in the CTF assay, only ten times less potent than $1,25(OH)_2D_3$ but ~100 times more potent than the original lead compound.

Most of the vitamin D mimics that had relatively weak potency in the CTF assay (EC_{50} >500 nM) did not compete with [^{3}H]-$1,25(OH)_2D_3$ in VDR ligand-binding *in vitro* assays under equilibrium conditions. However, compounds with EC_{50} values in CTF assays of <500 nM did bind directly to VDR *in vitro* under these conditions (Table 1 and data not shown). For example, LG190178 had a K_i value of 150 nM in this binding assay. Therefore, the cell-based CTF assay is a more sensitive assay than the binding assay for detection of compounds of this series, an observation that has been made for other intracellular receptor screening assays (data not shown). Although the less potent vitamin D mimics did not bind to VDR under equilibrium conditions in competition with [^{3}H]-$1,25(OH)_2D_3$, preincubation of VDR with LG190119 or LG190155 resulted in a decreased amount of [^{3}H]-$1,25(OH)_2D_3$ that bound to VDR at short time periods (Table 2), indicating that these compounds do interact with VDR. All of the compounds of this series showed lower EC_{50} values in cell-based assays than K_i values in VDR equilibrium ligand-binding *in vitro* assays. This is in contrast to $1,25(OH)_2D_3$, which has a K_i of 0.5 nM for VDR and an EC_{50} of 2–5 nM in the Hep G2 cell CTF assay. This series of nonsecosteroidal vitamin D mimic compounds' ratios of CTF activity to VDR-binding activity *in vitro* is similar to secosteroidal vitamin D analogs, which do not bind well to serum DBP. Compounds that do not bind DBP show greater potency in cell-based assays (which utilize serum in the growth media) than compounds that do bind DBP, because more of the former type of compound may enter the cell because it bypasses DBP. Therefore, the ratios of CTF potencies to VDR equilibrium binding affinities implied that the vitamin D mimics do not bind DBP well. This was indeed the case, determined by direct measurement using an *in vitro* DBP-binding assay. DBP- binding ability of $1,25(OH)_2D_3$ analogs has been correlated with their increased ability to raise calcium levels *in vivo* [27].

The vitamin D mimics described herein exert many of the actions of $1,25(OH)_2D_3$. They inhibit the growth of cancer cells, including those of LNCaP prostate cancer and SK-BR-3 breast cancer. The mimics induce differentiation of HL60 leukemic cells to macrophages, a VDR-dependent pathway. The vitamin D mimics also inhibit the growth of and induce morphological changes in human keratinocytes, predictive assays for the utility of compounds in the treatment of psoriasis. The results of these cell-based *in vitro* assays imply that the vitamin D mimic compounds

might have therapeutic utility in diseases such as cancer, leukemia and psoriasis. The lack of appreciable DBP-binding by the mimics predicted that these compounds might be less calcemic than $1,25(OH)_2D_3$ *in vivo* and thereby not be limited in their usefulness in the clinic by hypercalcemia side effects.

To test this hypothesis, the vitamin D mimics LG190119 and LG190155 were administered to mice orally and their ability to regulate a VDR target gene and increase serum calcium levels were assessed. Kidney 24(OH)ase RNA was induced at each concentration of $1,25(OH)_2D_3$ tested. Calcium was elevated within the normal range with the lower doses of $1,25(OH)_2D_3$ tested and increased beyond the normal range with the highest dose of $1,25(OH)_2D_3$. LG190119 and LG190155 were orally bioavailable in mice as indicated by their ability to induce kidney 24(OH)ase RNA at both doses tested. LG190119 and LG190155 induced higher levels of 24(OH)ase RNA compared with $1,25(OH)_2D_3$, while raising serum calcium to a lesser extent. LG190119 and LG190155 therefore show some dissociation of biological actions from calcium potentiation side effects *in vivo* and show promise as potential therapeutic agents.

This series of nonsecosteroidal vitamin D mimics are the first known compounds, other than $1,25(OH)_2D_3$ analogs, that activate VDR. These compounds share various activities with $1,25(OH)_2D_3$, both in cell-based assays and *in vivo*, as discussed here. These activities are measures of genomic activites of $1,25(OH)_2D_3$, that is, activities that occur through VDR in cell nuclei to modulate gene transcription. It will be interesting to test these compounds for their ability to elicit fast actions of $1,25(OH)_2D_3$, activities that are postulated to be VDR-independent. Although many activities of $1,25(OH)_2D_3$ were also observed to occur with the vitamin D mimics, one characteristic that the vitamin D mimics did not share with $1,25(OH)_2D_3$ was the ability to bind to serum DBP. Lowered affinity for DBP has been correlated with lowered calcium-mobilization effects in animals [25,27] and may therefore predict separation of desirable effects from the calcium-mobilization activity that has limited the usefulness of calcitriol as a drug. Additionally, $1,25(OH)_2D_3$ and various analogs thereof have been shown to be metabolized by certain cells into compounds that are growth stimulatory (M.J. Campbell *et al.*, and G. Brown (1998). Pro-proliferating effects of vitamin D_3 metabolites during differentiation of HL60 cells [abstract]. *Proc. Am. Assoc. Cancer Res.* **39**, 276). Because nonsecosteroidal vitamin D mimics are not expected to be substrates for 24(OH)ase and other $1,25(OH)_2D_3$ metabolic enzymes, these types of undesirable effects would be less likely with the mimics. Therefore, these structurally novel nonsecosteroidal vitamin D mimic compounds might have utility in the treatment of a variety of diseases that $1,25(OH)_2D_3$ has been shown or postulated to be beneficial in, including cancers of the breast, colon and prostate, psoriasis, autoimmune disorders and renal osteodystrophy, without some of the liabilities of $1,25(OH)_2D_3$ or its structurally related analogs. Efficacy studies in rodent models of cancer and other diseases are currently underway to further explore the potential of this class of compounds as drugs.

Significance

We report here on the discovery of novel nonsecosteroidal activators of the vitamin D receptor (VDR). We hypothesized that structurally distinct activators of VDR would have different activity profiles than its endogenous secosteroidal ligand, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$). This has been successfully demonstrated for nonsteroidal modulators of the androgen receptor (bicalutamide and flutamide) and the estrogen receptor (tamoxifen and raloxifene), which are currently marketed for therapeutic use in prostate cancer, breast cancer and hormone replacement therapy. These nonsecosteroidal VDR modulators mimic many of the activities of $1,25(OH)_2D_3$. They interact with VDR *in vitro*, stimulate transcription from a VDR-response element in cotransfection assays in a VDR-dependent manner, inhibit the proliferation of human breast cancer cells (SK-BR-3), prostate cancer cells (LNCaP) and primary keratinocytes, and induce monocytic differentiation of HL60 leukemic cells. Although these properties of $1,25(OH)_2D_3$ make it a promising therapeutic for human cancer, leukemia, psoriasis and other diseases, its use in the clinic has been limited because it causes hypercalcemia as pharmacologically effective doses are reached. Unlike $1,25(OH)_2D_3$, the vitamin D mimic compounds described here do not bind to serum vitamin D binding protein (DBP), a property that has been previously correlated with less calcemic potential *in vivo*. Additionally, these compounds induced a VDR target gene in rodents to a greater extent than $1,25(OH)_2D_3$ and with less increase in serum calcium than exhibited by $1,25(OH)_2D_3$. Therefore, these structurally novel compounds might have promise as therapeutics for a wide variety of diseases.

Materials and methods

Chemical synthesis

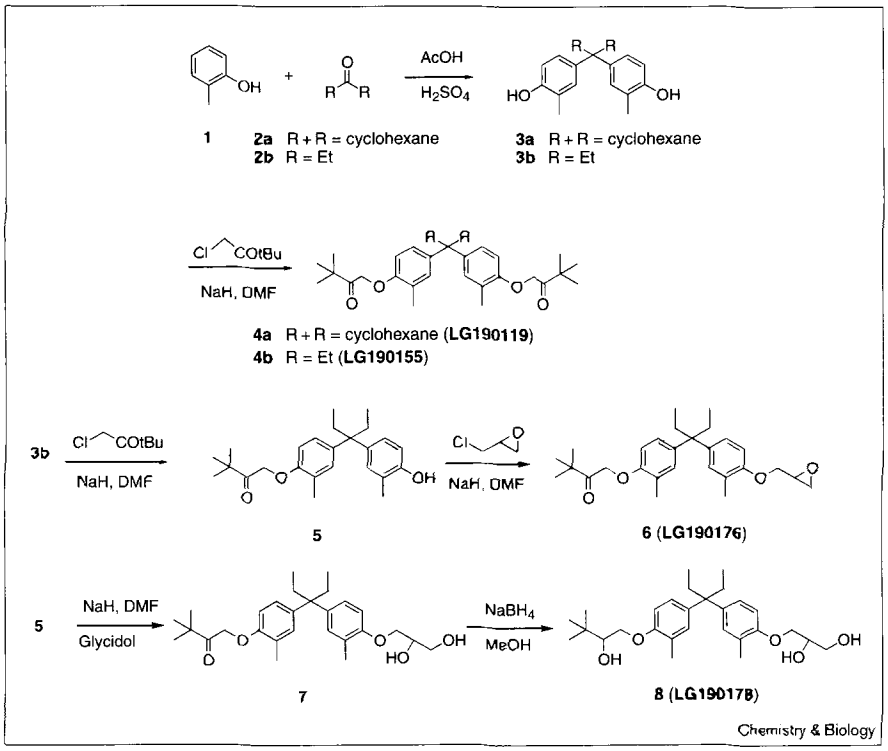
The synthesis of analogs **4a**, **4b**, **6** and **8** is shown in Figure 6. Phenols **3a** and **3b** were synthesized by acid-catalyzed condensation of *o*-cresol with the appropriate ketones **2a** and **2b**. Alkyl ethers were prepared using a Williamson-type condensation between the phenols and the appropriate alkyl halides to give compounds **4a**, **4b**, **5** and **6** (**6** is a racemic mixture). Treatment of phenol **5** with NaH and glycidol followed by hydride reduction of the ketone gave triol **8** as a mixture of diastereomers.

*1,1-*Bis*[4-(2-oxo-3,3-dimethylbutoxy)-3-methylphenyl)]cyclohexane* **4a** *(LG190119)*

To 5.0 g (46.3 mmol) of *o*-cresol **1** and 2.0 g of cyclohexanone **2a** in 20 ml of acetic acid was added 4 ml concentrated H_2SO_4. The reaction was stirred at room temperature for 3 days and poured over ice, followed by addition of 1:5 EtOAc:hexanes. The organic layer was separated and washed (H_2O, brine), dried ($MgSO_4$) and concentrated.

Figure 6

Synthesis of vitamin D mimics.



Purification by silica gel chromatography (5% EtOAc/95% hexanes) resulted in 1.8 g (6.3 mmol) of phenol **3a** (14% yield). ^{1}HNMR $CDCl_3$ d 6.98 (d, J = 2.0 Hz, 2H, Ar-H), 6.95 (dd, J = 2 Hz, J = 8.6 Hz, 2H, Ar-H), 6.64 (d, J = 8.6 Hz, 2H, Ar-H), 2.19 (s, 6H, Ar-CH_3), 2.19 (m, 4H, 2(-CH_2-)), 1.27-1.52 (m, 6H, -CH2-CH_2-CH_2-).

To 1.0 g (3.37 mmol) of diol **3a** in 10 ml of DMF was added 306 mg (8.00 mmol) of NaH. After stirring for 5 min, 1.07 g (8.00 mmol) of chloropinacolone was added. The reaction mixture was stirred for a further 30 min followed by addition of H_2O and EtOAc. The organic layer was separated and washed (H_2O, brine), dried ($MgSO_4$) and concentrated. Purification by silica gel chromatography (10% EtOAc/90% hexanes) resulted in 820 mg (1.66 mmol) of **4a** as a white powder (49% yield). ^{1}HNMR $CDCl_3$ d 7.09 (d, J = 2.0 Hz, 2H, Ar-H), 6.96 (dd, J = 2.0 Hz, J = 8.6 Hz, 2H, Ar-H), 6.51 (d, J = 8.6 Hz, 2H, Ar-H), 4.82 (s, 4H, -CH_2O-), 2.25 (s, 6H, Ar-CH_3), 2.19 (m, 4H, 2(-CH_2-)), 1.27-1.52 (m, 6H, -CH2-CH2-CH2-), 1.25 (s, 18H, t-but).

3,3-Bis[4-(2-oxo-3,3-dimethylbutoxy)-3-methylphenyl)]pentane **4b** *(LG190155)*

Compound **4b** was prepared (as a white powder) in a similar manner as compound **4a** except that 3-pentanone was used instead of cyclohexanone. ^{1}HNMR $CDCl_3$ d 6.89 (m, 4H, Ar-H), 6.49 (d, J = 8.3 Hz, 2H, Ar-H), 4.82 (s, 4H, -CH_2-), 2.23 (s, 6H, Ar-CH_3), 2.00 (q, J = 7.3 Hz, 4H, -CH_2CH_3), 1.23 (s, 18H,(t-but), 0.58 (t, J = 7.3 Hz, 6H, -CH_2CH_3).

1′-[4-(2-Oxo-3,3-dimethylbutoxy)-3-methylphenyl]-1′-[4-(2,3-epoxypropoxy)-3-methylphenyl]pentane **6** *(LG190176)*

To 1.0 g (3.5 mmol) of the diol **3b** in 10 ml dimethyl formamide (DMF) was added 8.4 mg (3.5 mmol) of NaH. After stirring for 5 min, 471 mg (3.5 mmol) of chloropinacolone was added. The reaction mixture was stirred for a further 30 min followed by addition of H_2O and then EtOAc. The organic layer was separated and washed (H_2O, brine), dried ($MgSO_4$), and concentrated. Three spots were observed by TLC and the desired product **5** corresponded to the middle spot. Purification by silica gel chromatography (10% EtOAc-90% hexanes) resulted in 250 mg (0.57 mmol) of **5** (16% yield).

To 80 mg (0.21 mmol) of **5** in 1 ml of dry DMF was added 5.5 mg (0.23 mmol) of NaH. The mixture was stirred at room temperature for 30 min followed by addition of 22.0 mg (0.23 mmol) of epichlorohydrin. The reaction was heated at 120°C for 1 h or until complete by TLC (20% EtOAc 80% hexanes). Water was added followed by EtOAc. The organic layer was separated and washed (H_2O, brine), dried ($MgSO_4$), and concentrated. Purification by silica gel chromatography (10% EtOAc/90% hexanes) resulted in 30 mg (0.07 mmol) of **6** as a clear oil (33% yield). ^{1}H NMR $CDCl_3$ d 6.89 (m, 4H, Ar-H), 6.66 (d, J = 8.3 Hz, 1H, Ar-H), 6.49 (d, J = 8.3 Hz, 1H, Ar-H), 4.83 (s, 2H, -CH_2-), 4.18 (dd, J = 11.3, J = 3.3 Hz, 1H, -CH2-), 3.95 (dd, J = 11.3, J = 3.3 Hz, 1H, -CH2-), 3.35 (m, 1H, -CH-), 2.90-2.76 (m, 2H, CH_2), 2.23 (s, 3H, Ar-CH_3), 2.18 (s, 3H, Ar-CH_3), 2.00 (q, J = 7.3 Hz, 4H, -CH_2CH_3), 1.24 (s, 18H,(t-but), 0.59 (t, J = 7.3 Hz, 6H, -CH_2CH_3).

2′-[4-(2-Hydroxy-3,3-dimethylbutoxy)-3-methylphenyl]-2′-[4-(2,3-dihydroxypropoxy)-3-methylphenyl]pentane **8** *(LG190178)*

To 200 mg (0.52 mmol) of **5** in 4 ml of dry DMF was added 14.0 mg (0.58 mmol) of NaH and the mixture was stirred for 20 min. Glycidol (43.0 mg, 0.58 mmol) was added and the reaction was warmed to 80°C for 1 h. The reaction was poured into 1:1 EtOAc:hexane and the organic layer was separated, washed (H_2O, brine), dried ($MgSO_4$) and concentrated. Purification by silica gel chromatography (10% EtOAc-90% hexanes) resulted in 117 mg (0.26 mmol) of **7** (50% yield).

To 70 mg (0.15 mmol) of **7** in 2.5 ml of MeOH at 0°C was added 5.7 mg (0.15 mmol) of $NaBH_4$. After stirring for 20 min, the reaction was quenched with sat. NH_4Cl and the product was extracted with EtOAc. The organic layer was washed with H_2O, then brine, dried ($MgSO_4$), concentrated and purified by silica gel chromatography to give 23 mg (0.05 mmol) of **8** as a clear oil (33% yield). ^{1}H NMR $CDCl_3$ d 6.94 (m, 4H, Ar-H), 6.69 (d, J = 8.3 Hz, 2H, Ar-H), 4.12-4.02 (m, 4H), 3.88-3.67

(m, 4H), 2.17 (s, 3H, Ar-CH_3), 2.16 (s, 3H, Ar-CH_3), 2.04 (q, J = 7.3 Hz, 4H, -CH_2CH_3), 1.00 (s, 9H, (t-but), 0.59 (t, J = 7.3 Hz, 6H, -CH_2CH_3).

Cotransactivation assays

Hep G2 (human hepatoma, ATCC) cells were transfected with and without a human VDR expression vector (pRShVDR [28,35]) along with a reporter plasmid encoding luciferase driven by one copy of a VDRE sequence from the h24(OH)ase promoter (VDRE(1)–ΔMTV–LUC [28]). Transfections were performed in triplicate in 96-well gelatin-coated plates with 20 μg DNA (0.5 μg receptor vector, 5 μg reporter plasmid, 5 μg β-galactosidase plasmid and pGEM carrier DNA to 20 μg) per ml of $CaPO_4$–Hepes buffer. Six hours later, 1,25$(OH)_2D_3$ (Solvay DuPhar) or various compounds were added to cells in concentrations from 10^{-12}–10^{-5} M and incubation was for 40 h. Cells were lysed and luciferase activities were normalized with β-galactosidase values to control for variable transfection efficiencies, as previously described [28].

VDR in vitro *ligand-binding assays*

Human VDR [35] was constructed into a yeast expression plasmid as described previously [36]. Crude yeast cell protein extracts containing recombinantly-expressed hVDR (4 μg per tube) were utilized for competition ligand-binding assays using [^{3}H]-1,25$(OH)_2D_3$ (Amersham). For equilibrium assays, incubation of protein extracts and ligands was for 16 h at 4°C. For nonequilibrium assays, VDR-containing yeast extract (2.5 μg per tube) was incubated with various compounds for 20 h at 4°C prior to addition of 0.34 nM [^{3}H]-1,25$(OH)_2D_3$ for time periods ranging from 2–60 min at 4°C. Separation of bound ligand from unbound ligand was achieved by use of hydroxylapatite resin as described previously [37]. Equilibrium competition analyses utilized [^{3}H]-1,25$(OH)_2D_3$ at 2 nM and concentrations of invented compounds from 0.1 nM to 10 μM. Binding buffer for both types of assays contained 10 mM Tris, pH 7.5, 1 mM DTT and 0.4 M KCl and wash buffer contained 10 mM Tris, 7.5, 0.1 M KCl and 10 mM CHAPS.

Cancer cell growth inhibition assays and analysis

SK-BR-3 human breast cancer cells (ATCC) and LNCaP human prostate cancer cells (ATCC) were plated in 96-well flat-bottomed plates in 50 μl serum-containing media/well. All cell growth assays were carried out in media containing 5–7.5% FBS. Cell plating numbers (cells per well) were as follows: SK-BR-3–3000 and LNCaP–1000, and allowed to adhere for 24 h prior to compound addition. Tenfold serial dilutions of compounds were performed with changing of pipette tips between each dilution and then were added to cells (50 μl per well) to final concentrations of 1×10^{-5} M to 1×10^{-11} M, (1% ethanol), in triplicate. Cells and ligands were incubated at 37°C for 3 (SK-BR-3) or 4 (LNCaP) days. BrdU (5-bromo-2′-deoxyuridine) incorporation was analyzed as a measure of DNA replication (Cell Proliferation ELISA kit, Boehringer) and absorbance was measured at 450 nm. One row of wells contained media only and these absorbance values were averaged as background and subtracted from absorbance values from each well containing cells. Efficacy (% growth inhibition) was determined as [1–(O.D. at maximal response with compound/O.D. of average vehicle) × 100]. Potency (EC_{50}) values were determined as the concentration of compound that elicited a half maximal response.

Leukemic cell differentiation

HL60 human promyelocytic leukemia cells (ATCC) were grown in media containing 15% FBS and plated at 100,000 cells/ml in 10 cm dishes or in six-well plates. Ligands were added in ethanol vehicle (1%) at various concentrations for 5 days without changing the media. On the fifth day, cells were harvested and assayed for markers of differentiation. Cells that bioreduced nitroblue tetrazolium (NBT) turned blue–black and represented differentiated cells [30], whereas undifferentiated cells were colorless. Cells were counted and percent differentiated cells were determined from the two numbers. The macrophage-specific cell surface protein marker, CD14, was assayed for by use of a fluorescent dye tagged anti-CD14 antibody (Becton Dickinson) and FACS analyses [31]. EC_{50} values represent the concentration of ligand that induced 50% of the cells to be NBT formazan-positive or CD14-positive.

Keratinocyte assays

Normal human epidermal keratinocytes (NHEK) were grown according to vendor specifications (Clonetics, San Diego). BrdU assays were performed as above except that cells were plated at 200 cells per well (96-well plates) and treatment with ligands (10^{-5}–10^{-11} M) was for 3 days. For cell staining, 30,000 cells per well were plated in six well plates. Fresh media with ligands was added on day 0, day 2 and day 4. On day 7, cells were washed with PBS, fixed and photographed.

DBP-binding assay

DBP competition binding assays were done as performed previously [27,34] using human serum (Scantibodies) or rat serum (Gibco-BRL) as sources of DBP diluted 1:10,000 in sodium phosphate buffer (pH 7.4). Sera were incubated with 2 nM [^{3}H]-25$(OH)D_3$ (Amersham) with and without unlabeled 25$(OH)D_3$ or 1,25$(OH)_2D_3$ in concentrations ranging from 0.01 nM to 1 μM, or unlabeled vitamin D mimic compounds ranging from 10 nM to 10 μM, for 2 h at 4°C. Separation of bound from unbound ligand was achieved by the use of Dextran-coated charcoal in gelatin phosphate buffer.

In vivo *gene regulation and calcium potential*

Balb/c mice (Harlan Sprague-Dawley; female, 10 weeks, ~19 g (3 day study), male, 8 weeks, ~22 g (5 day study)) were housed under normal lighting and received a vitamin-D-deficient, calcium-replete diet (Purina Mills #5826-C-L) and water *ad libitum* for 7 days prior to initiation of treatment with compounds. Mice were housed in a U.S. Department of Agriculture-registered facility in accordance with NIH guidelines for the care and use of laboratory animals. Compounds were administered in sesame oil vehicle with 4% ethanol by oral gavage (dose volume 0.1 ml). Dosing was everyday for 3–5 days (3–4 animals per dosing group). On the final day of dosing, 4–6 h after the last dose, animals were euthanized, blood and kidneys were removed. Serum was prepared and calcium determinations were done by use of a colorimetric assay (Sigma). 15–20 μg total kidney RNA was utilized in Northern blot analysis using a rat 24(OH)ase probe; a GAPDH probe was used to normalize for RNA loading, as described previously [38]. Quantitation was by PhosphorImager analysis.

Acknowledgements

The authors thank N. Weigel for helpful discussions and suggestions and critical review of the manuscript. We thank D. Robertson and L. Fish for support of this project and helpful discussions. We thank P. Koeffler and S. Thacher for discussions on HL60 cell and keratinocyte assays, respectively. The assistance of A. McAuliffe, J. Knight, M. Garcia, L. Tehrani, K. Nguyen, K. Flatten, K. Ogilvie and C.H. Jin is appreciated.

References

1. Haussler, M.R. & McCain, T.A. (1977). Basic and clinical concepts related to vitamin D metabolism and action. *N. Eng. J. Med.* **297**, 974-983, 1041-1050.
2. DeLuca, H.F. (1988). The vitamin D story: a collaborative effort of basic science and clinical medicine. *FASEB J.* **2**, 224-236.
3. Abe, E., *et al.*, & Suda, T. (1981). Differentiation of mouse myeloid leukemia cells induced by 1 alpha,25-dihydroxyvitamin D_3. *Proc. Natl Acad. Sci. USA* **76**, 4990-4994.
4. Bar-Shavitz, D., *et al.*, & Kahn, A.J. (1983). Induction of monocytic differentiation and bone resorption by 1,25-dihydroxyvitamin D_3. *Proc. Natl Acad. Sci. USA* **80**, 5907-5911.
5. Mangelsdorf, D.J., Koeffler, H.P., Donaldson, C.A., Pike, J.W., & Haussler, M.R. (1984). 1,25-Dihydroxyvitamin D_3-induced differentiation in a human promyelocytic leukemia cell line (HL-60): receptor-mediated maturation to macrophage-like cells. *J. Cell Biol.* **98**, 391-398.
6. Colston, K., Colston, M.J. & Feldman, D. (1981). 1,25-dihydroxyvitamin D_3 and malignant melanoma: the presence of receptors and inhibition of cell growth in culture. *Endocrinology* **108**, 1083-1086.
7. Dokoh, S., Donaldson, C.A., & Haussler, M.R. (1984). Influence of 1,25-dihydroxyvitamin D_3 on cultured osteogenic sarcoma cells: correlation with the 1,25-dihydroxyvitamin D_3 receptor. *Cancer Res.* **44**, 2103-2109.

8. Skowronski, R.J., Peehl, D.M. & Feldman, D. (1993). Vitamin D and prostate cancer: 1,25 dihydroxyvitamin D_3 receptors and actions in human prostate cancer cell lines. *Endocrinology* **132**, 1952-1960.
9. Tsoukas, D.D., Provvedini, D.M. & Manolagas, S.C. (1984). 1,25-dihydroxyvitamin D_3: a novel immunoregulatory hormone. *Science* **224**, 1438-1440.
10. Manolagas, S.C., Provvedini, D.M. & Tsoukas, C.D. (1985). Interactions of 1,25-dihydroxyvitamin D_3 and the immune system. *Mol. Cell. Endocrinol.* **43**, 113-122.
11. Mathieu, C., Waer, M., Casteels, K., Laureys, J. & Bouillon, R. (1995). Prevention of type I diabetes in NOD mice by nonhypercalcemic doses of a new structural analog of 1,25-dihydroxyvitamin D_3, KH1060. *Endocrinology* **136**, 866-872.
12. Lemire, J.M., Ince, A. & Takashima, M. (1992). 1,25-Dihydroxyvitamin D_3 attenuates the expression of experimental murine lupus of MRL/l mice. *Autoimmunity* **12**, 143-148.
13. Cantorna, M.T., Hayes, C.E. & DeLuca, H.F. (1996). 1,25-Dihydroxyvitamin D_3 reversibly blocks the progression of relapsing encephalomyelitis, a model of multiple sclerosis *Proc. Natl Acad. Sci. USA* **93**, 7861-7864.
14. Ozono, K., Sone, T. & Pike, J.W. (1991). The genomic mechanism of action of 1,25-dihydroxyvitamin D_3. *J. Bone Min. Res.* **6**, 1021-1027.
15. Haussler, M.R., *et al.*, & Jurutka, P.W. (1998). The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J. Bone Miner. Res.* **13**, 325-349.
16. Zugmaier, G., *et al.*, & Knabbe, C. (1996). Growth-inhibitory effects of vitamin D analogs and retinoids on human pancreatic cancer cells. *Br. J. Cancer* **73**, 1341-1346.
17. Higashimoto, Y., *et al.*, & Saijo, N. (1996). 1-alpha,25-dihydroxyvitamin D_3 and all-*trans*-retinoic acid inhibit the growth of a lung cancer cell line. *Anticancer Res.* **16**, 2653-2660.
18. Blutt, S.E., Allegretto, E.A., Pike, J.W. & Weigel, N.L. (1997). 1,25 Dihydroxyvitamin D_3 and 9-*cis* retinoic acid act synergistically to inhibit the growth of LNCaP prostate cells and cause accumulation of cells in G_1. *Endocrinology* **138**, 1491-1497.
19. Honma, Y., *et al.*, & Suda, T. (1983). 1 alpha,25-Dihydroxyvitamin D_3 and 1 alpha-hydroxyvitamin D_3 prolong survival time of mice inoculated with myeloid leukemia cells. *Proc. Natl Acad. Sci. USA* **80**, 201-204.
20. Potter, G.K., *et al.*, & Moore, M.A. (1985). Action of 1,25-$(OH)_2D_3$ in nude mice bearing transplantable human myelogenous leukemic cell lines. *Exp. Hematol.* **13**, 722-732.
21. Colston, K.W., Chander, S.K., Mackay, A.G. & Coombes, R.C. (1992). EB1089: a new vitamin D analogue that inhibits the growth of breast cancer cells *in vivo* and *in vitro*. *Biochem. Pharmacol.* **44**, 693-702.
22. Eisman, J.A., Barkla, D.H. & Tutton, P.J.M. (1987). Suppression of *in vivo* growth of human cancer solid tumor xenografts by 1,25-dihydroxyvitamin D_3. *Cancer Res.* **47**, 21-25.
23. Belleli, A., Levy, J. Guberman, R., & Lamprecht, S.A. (1992). A protective role of 1,25-dihydroxyvitamin D_3 in chemically induced rat colon carcinogenesis. *Carcinogenesis* **13**, 2293-2298.
24. Smith, E.L., Pincus, S.H., Donovan, L. & Holick, M.F. (1988). A novel approach for the evaluation and treatment of psoriasis. Oral or topical use of 1,25-dihydroxyvitamin D_3 can be a safe and effective therapy for psoriasis. *J. Amer. Acad. Dermatol.* **19**, 516-528.
25. Bouillon, R., Okamura, W.H. & Norman, A.W. (1995). Structure-function relationships in the vitamin D endocrine system. *Endocrine Rev.* **16**, 200-257.
26. Kragballe, K., Beck, H.I. & Sogaard, H. (1988). Improvement of psoriasis by a topical vitamin D_3 analogue (MC 903) in a double-blind study. *Br. J. Dermatol.* **119**, 223-230.
27. Dusso, A.S., *et al.*, & Brown A.J. (1991). On the mechanisms for the selective action of vitamin D analogs. *Endocrinology* **128**, 1687-1692.
28. Zou, A., Elgort, M.G. & Allegretto, E.A. (1997). RXR ligands activate the human 25-hydroxyvitamin D_3-24 hydroxylase promoter via RXR heterodimer binding to two VDREs and elicit additive effects with 1,25 dihydroxyvitamin D_3. *J. Biol. Chem.* **272**:19027-19034.
29. Elstner, E., *et al.*, &. Koeffler, H.P. (1995). 20-Epi-vitamin D_3 analogs: a novel class of potent inhibitors of proliferation and inducers of differentiation of human breast cancer cell lines. *Cancer Res.* **55**, 2822-2830.
30. Collins, S.J., Ruscetti, F.W., Gallagher, R.E. & Gallo, R.C. (1979). Normal functional characteristics of cultured human promyelocytic leukemia cells (HL-60) after induction of differentiation by dimethylsulfoxide. *J. Exp. Med.* **149**, 969-974.
31. Oberg, F., Botling, J. & Nilsson, K. (1993). Functional antagonism between vitamin D_3 and retinoic acid in the regulation of CD14 and CD23 expression during monocytic differentiation of U-937 cells. *J. Immunol.* **150**, 3487-3495.
32. Gniadecki, R. (1997). Effects of 1,25-dihydroxyvitamin D_3 and its 20-epi analogs (MC 1288, MC 1301, KH 1060), on clonal keratinocyte growth: evidence for differentiation of keratinocyte stem cells and analysis of the modulatory effects of cytokines. *Br. J. Pharmacol.* **120**, 1119-1127.
33. Jones, K.T. & Sharpe, G.R. (1994). Intracellular free calcium and growth changes in single human keratinocytes in response to vitamin D and five 20-epi-analogs. *Arch. Dermatol. Res.* **286**, 123-129.
34. Bouillon, R., van Baelan, H. & de Moor, P. (1980). Comparative study of the affinity of the serum vitamin D-binding protein. *J. Steroid Biochem.* **13**, 1029-1034.
35. Baker, A.R., *et al.*, & O'Malley, B.W. (1988). Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc. Natl Acad. Sci. USA* **85**, 3294-3298.
36. McDonnell, D.P., Pike, J.W., Drutz, D.J., Butt, T.R. & O'Malley, B.W. (1989). Reconstitution of the vitamin D-responsive osteocalcin transcription unit in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **9**, 3517-3523.
37. Allegretto, E.A., *et al.*, & Heyman, R.A. (1993). Characterization of hormone-binding and transactivation properties of retinoic acid and retinoid X receptors. *J. Biol. Chem.* **268**, 26625-26633.
38. Allegretto, E.A., *et al.*, & Pike, J.W. (1995). Retinoid X receptor functions as a hormone receptor *in vivo* to induce a key metabolic enzyme for 1,25-dihydroxyvitamin D_3. *J. Biol. Chem.* **270**, 23906-23909.

Because *Chemistry & Biology* operates a 'Continuous Publication System' for Research Papers, this paper has been published via the internet before being printed. The paper can be accessed from http://biomednet.com/cbiology/cmb – for further information, see the explanation on the contents pages.