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SHORT COMMUNICATION

New Vitamin D Receptor Agonists with Decreased Metabolic Stability

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ABSTRACT. The aim of the study was the development of vitamin D receptor agonists with decreased metabolic stability for the topical treatment of psoriasis and related hyperproliferative skin diseases. Calcitriol analogues 1, 2, 3, all of which contain modifications in the side chain, were synthesized. The obtained analogues were full agonists when the induction of CD14 expression in HL-60 cells, the induction of 5-lipoxygenase activity in Mono Mac 6 cells, and the inhibition of phytohemagglutinin (PHA)-stimulated lymphocyte proliferation were studied. The EC_{50} value of the most active compound 1 was 1.2 nM in the CD14 assay and 1 nM in the 5-lipoxygenase assay, whereas calcitriol gave EC_{50} values in these assays of 3.7 and 9 nM, respectively. In the lymphocyte proliferation assay, compound 1 and calcitriol had IC_{50} values of 0.3 and 2.8 nM, respectively. All three compounds had receptor binding affinities similar to that of calcitriol. The compounds showed a decreased metabolic stability in rat liver homogenates and had a 50-fold lower affinity for the vitamin D-binding protein than calcitriol, which suggests that calcitriol analogues are metabolized more rapidly after systemic uptake or application. When injected into rats, the analogues displayed an approximately 100-fold lower hypercalcemic effect than calcitriol. In summary, our study presents three new and potent vitamin D receptor agonists with interesting profiles for development as antipsoriatic drugs. BIOCHEM PHARMACOL **59**;12: 1597–1601, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. vitamin D; calcitriol; differentiation; psoriasis; analogue

Calcitriol, the active metabolite of vitamin D_3, has profound effects on cellular differentiation and proliferation as well as on calcium homeostasis [1]. In addition, it has been shown that calcitriol has prominent immunomodulatory properties [2]. The effects of the hormone are mediated by the vitamin D receptor, which belongs to the family of nuclear receptors. Binding of calcitriol to its receptor and subsequent interaction of the ligand–receptor complex with DNA induces changes in gene expression of many genes in a great variety of cell types [3], which seems to be the molecular basis for many calcitriol actions. The biological profile of calcitriol suggests that vitamin D receptor agonists have a therapeutic potential in a variety of autoimmune and hyperproliferative diseases, e.g. psoriasis [4, 5]. However, the systemic application or the extensive topical use of calcitriol itself in the treatment of psoriasis is limited because of the hypercalcemic side effects occurring after resorption of the hormone through the skin. A strategy to decrease the hypercalcemic effects of topically applied vitamin D analogues is the development of compounds with increased metabolic lability, e.g. calcipotriol (MC 903), which are rapidly cleared after resorption [6].

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In this study, vitamin D analogues were synthesized and their biological profile was determined. Induction of both CD14 expression in HL-60 cells and of 5-lipoxygenase activity was used to assess effects of the analogues on cellular differentiation and functional maturation, respectively [7, 8]. Furthermore, binding affinity to the vitamin D receptor and the vitamin D-binding protein was determined. Metabolic stability of the compounds was analyzed in rat liver homogenates, and hypercalcemic activity was determined in rats *in vivo*. Herein, we report on a series of vitamin D receptor agonists with similar intrinsic activity and receptor binding affinity as calcitriol, but with decreased metabolic stability and hypercalcemic activity.

MATERIALS AND METHODS

Cell Culture

Cell culture was performed as described previously [8]. Vitamin D analogues were synthesized in the Department of Medicinal Chemistry at Schering AG (patent DE 4221961).

Induction of CD14 Expression in HL-60 Cells

HL-60 cells (2.5×10^4 cells/well) were incubated in 24-well plates in RPMI-1640 with 10% fetal bovine serum and incubated with increasing concentrations of calcitriol or vitamin D analogues for 96 hr at 37°. Monocytic cell

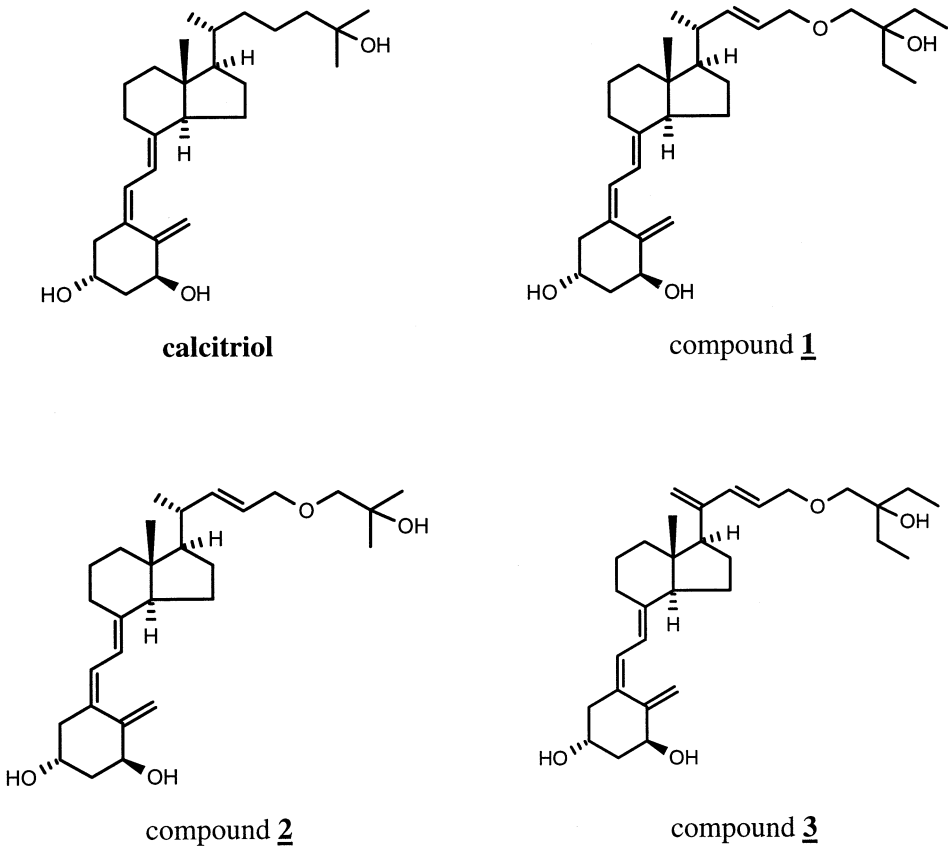

FIG. 1. Structures of vitamin D analogues.

differentiation was assessed by flow cytometry as the percentage of CD14-expressing cells after staining with a fluorescein isothiocyanate (FITC)-labeled monoclonal anti-CD14 antibody (clone RHO 52, Dianova).

Determination of 5-Lipoxygenase Activity in Mono Mac 6 Cells

Mono Mac 6 cells were differentiated with transforming growth factor-beta1 (2 ng/mL) and the indicated concentrations of vitamin D analogues for 4 days. Then, 5-lipoxygenase activity was determined as described [8]. Intact cells (in 1 mL PBS) were stimulated by addition of arachidonic acid and ionophore A23187 (40 and 10 μM final concentrations, respectively). When cell homogenates were assayed, the cells were sonicated and the homogenates stimulated by the addition of Ca^{2+} and arachidonic acid (2 mM and 40 μM final concentrations, respectively) [8]. The 5-lipoxygenase products formed were extracted by solid-phase extraction columns and analyzed by HPLC as described earlier [9]. 5-Lipoxygenase activity was expressed as ng of 5-lipoxygenase products per 10^6 cells, which included leukotriene B_4, the all-*trans* isomers of leukotriene B_4, and 5-hydroxyeicosatetraenoic acid.

Determination of Inhibition of PHA-Induced Lymphocyte Proliferation

Mononuclear blood cells were isolated by density gradient centrifugation. Cells (2.5×10^5/mL) were incubated for 96 hr in a microtiter plate in RPMI-1640 supplemented with 10% fetal bovine serum in the presence of 5 μg/mL of PHA* and the presence or absence of the test compounds. [^{3}H]Thymidine (0.2 μCi/well) was added during the last 4 hr of the incubation. The cells were transferred onto glass fiber filters, and the incorporated radioactivity was determined with a scintillation counter.

Vitamin D Receptor Binding Affinity

A receptor protein preparation was made from the mucosa of normal pig intestine as described previously [8]. Receptor binding affinity was assessed by displacement of [^{3}H]-1,25-$(OH)_2$-vitamin D_3 from the receptor preparation. Samples of 250 μL (1 mg/mL protein) were incubated with 10 μL (0.025 μCi) of [^{3}H]-1,25-$(OH)_2$-vitamin D_3, and increasing concentrations of vitamin D analogues were added. After incubation for 60 min at 4°, bound [^{3}H]-1,25-$(OH)_2$-vitamin D_3 was determined after removal of free tracer by addition of 250 μL 1% dextran-coated charcoal, 20-min incubation at 4°, and centrifugation. Relative binding affinity (RBA) was calculated from the displacement curves according to the equation RBA = IC_{50} [^{3}H]-1,25-$(OH)_2$-vitamin $D_3 \times 100/IC_{50}$ vitamin D analogues, IC_{50} being the

* *Abbreviations:* PHA, phytohemagglutinin; EMR, effective molar ratio; and IC_{50}, drug concentration leading to 50% inhibition.

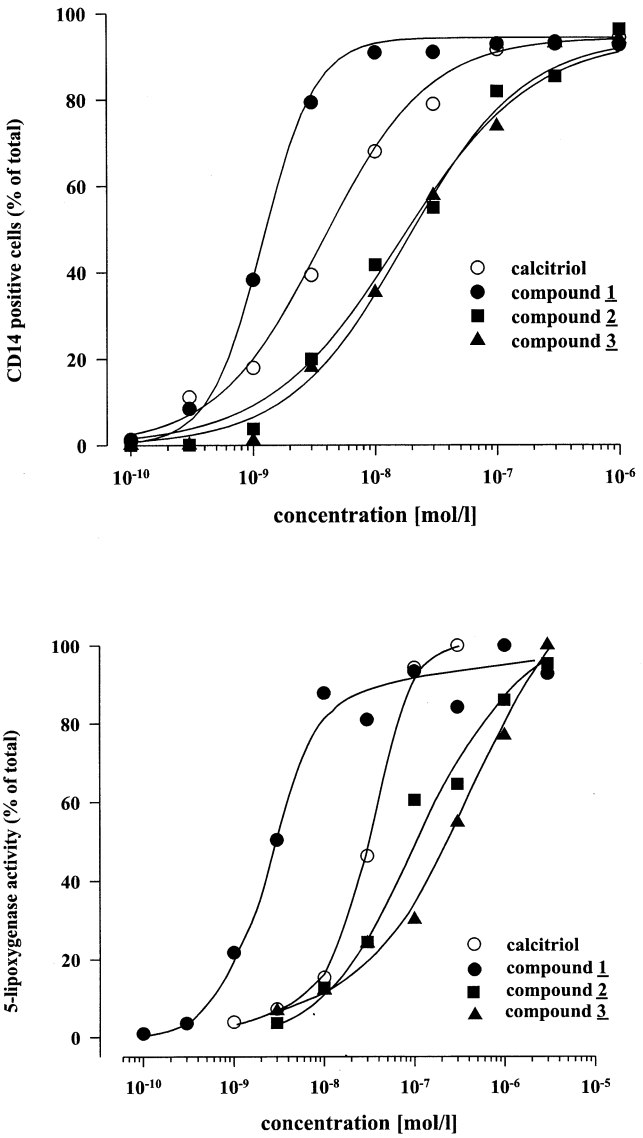

FIG. 2. Dose–response curves of the induction of CD14 expression in HL-60 cells (N = 4) (upper panel) and the induction of 5-lipoxygenase activity in Mono Mac 6 cells (N ≥ 2) (lower panel) by vitamin D analogues.

concentration at which 50% displacement of the tracer was achieved [10].

Determination of Affinity for Vitamin D-Binding Protein

Affinity of the test compounds for purified human vitamin D-binding protein (DBP) was assessed by displacement of [^{3}H]-1,25-$(OH)_2$-vitamin D_3. DBP (1.25 mg/mL) was diluted in PBS containing 1% ovalbumin, [^{3}H]-1,25-$(OH)_2$-vitamin D_3, and the test compounds. After incubation for 60 min at 4°, dextran-coated charcoal was added and the solution was incubated for 40 min at 4°. Then, bound and free [^{3}H]-1,25-$(OH)_2$-vitamin D_3 was separated by centrifugation. Relative binding of the test compounds is expressed in ratio to calcitriol.

TABLE 1. Induction of CD14 expression by vitamin D analogues

	CD14 detection in HL-60 cells		
Compound	Intrinsic activity (% at 1 μM)	EC_{50} (nM)	EMR
Calcitriol	100	3.7	1
1	100	1.2	0.3
2	100	16	4
3	100	18	5

EMR: effective molar ratio (EC_{50} substance/EC_{50} calcitriol).

Determination of Metabolic Stability

Metabolic stability of vitamin D analogues (1×10^{-5} M) was analyzed *in vitro* using crude rat liver homogenates ($1000 \times g$ supernatant, 1.5 mg protein/mL). Incubations of vitamin D analogues were carried out at 37° in the presence of the NADPH-regenerating system using an incubation mixture of 500 μL each of liver supernatant and of a 50 mM HEPES buffer (pH 7.4), containing 154 mM KCl, 5 mM $MgSO_4$, 0.75 mM $NADP^+$, 7.5 mM isocitrate, and 0.5 U/mL isocitrate dehydrogenase. Vitamin D analogues were added in an ethanolic solution (10 μL). Reactions were stopped after 0, 10, 60, and 120 min by adding 3 mL diethyl ether. Lipid extracts were dried down under a stream of nitrogen and the extraction was repeated three times. The residues were dissolved in acetonitrile and analyzed on reverse-phase HPLC (Nucleosil C18 column eluted with acetonitrile/H_2O/trifluoroacetic acid [60/40/0.015 v/v/v], flow rate 1 mL/min.). Quantification of unchanged compound was based on UV detection at 262 nm, using calcipotriol as internal standard.

Hypercalcemic Activity In Vivo

Calcitriol (2 μg/kg) or the test compounds were injected subcutaneously (single administration) to fasting female Wistar rats (150 to 170 g body weight). After 22 hr, the animals were killed by decapitation and blood was collected. Calcium concentration in serum was determined by flame photometry. Five rats were used for each concentration of the test compounds.

RESULTS AND DISCUSSION

Induction of cell differentiation of HL-60 cells along the monocytic pathway by vitamin D analogues was assessed by the induction of CD14 expression. Induction of gene expression and functional cell maturation by the test compounds was evaluated with 5-lipoxygenase, which represents both a primary and secondary vitamin D response gene in Mono Mac 6 cells, where the enzyme activity correlates with the functional maturation of the cells [8, 11–13]. Calcitriol analogues containing modifications in the side chain where oxygen was inserted in order to reduce the metabolic stability of the compounds were synthesized

TABLE 2. Induction of 5-lipoxygenase activity in Mono Mac 6 cells and inhibition of lymphocyte proliferation

	Induction of 5-lipoxygenase			Inhibition of lymphocyte proliferation	
Compound	Intrinsic activity (% at 3 μM)	EC_{50} (nM)	EMR	IC_{50} (nM)	EMR
Calcitriol intact cells	100	33	1	2.8	1
homogenates	100	9	1		
1 intact cells	100	2	0.06	0.3	0.1
homogenates	100	1	0.1		
2 intact cells	100	95	2.8	1.9	0.7
homogenates	100	45	5.0		
3 intact cells	100	220	6.6	2.4	0.9
homogenates	100	200	22		

EMR: effective molar ratio (EC_{50} or IC_{50} substance/EC_{50} or IC_{50} calcitriol).

(Fig. 1). All the test compounds were full agonists in both assay systems and the intrinsic activity was comparable with calcitriol, although higher (3, 2) or lower (1) concentrations were required in the two biological test systems to achieve a maximal response (Fig. 2). In the case of compound 2, the changes in the side chain resulted in a 4-fold reduction in receptor affinity and in a concomitant loss of activity, as indicated by the EMR, in the induction of CD14 and 5-lipoxygenase expression (Tables 1 and 2). Replacement of the two terminal methyl groups by ethyl moieties led to the development of compound 1 (Fig. 1). This structural modification gave an approximate 2-fold increase in receptor affinity and 13- and 50-fold increases in activity in the CD14 and 5-lipoxygenase assays. Compared with calcitriol, the receptor binding affinity of compound 1 was 50% lower, but this compound was about 3- and 10-fold more active in the CD14 and 5-lipoxygenase assays, respectively, as indicated by the EMR (Tables 1 and 2). Such discrepancies between receptor binding and biological activity were also reported for a number of 20-epi analogues of calcitriol such as KH 1060 and were attributed to changes in vitamin D receptor conformation, differential interaction with coactivators, and increased half-life times of vitamin D receptor–ligand complexes [14–16]. Alteration of the geometry at C-20 in compound 1 by introduction of a double bond gave compound 3 (Fig. 1), which had a similar vitamin D receptor affinity to compound 1 but a 17-fold and 110- to 200-fold lower biological activity in the CD14 and 5-lipoxygenase assays, respectively.

In order to study the immunomodulating effects of the vitamin D analogues, inhibition of PHA-induced lymphocyte proliferation was investigated (Table 2). Compounds 2, 3, and calcitriol are virtually equipotent in this test system, which differs from the results obtained in the CD14 and 5-lipoxygenase assays, where both compounds were significantly less active as indicated by the respective EMR values (Tables 1 and 2). Compound 1 was more potent than calcitriol in all three assays, giving EMR values of 0.3, 0.1/0.06, and 0.1 in the CD14, 5-lipoxygenase, and lymphocyte proliferation assay, respectively (Tables 1 and 2 and Fig. 3). Thus, compared with calcitriol, compound 1 is 10-fold more active and seems to have an identical profile regarding induction of cell differentiation and maturation and lymphocyte proliferation, whereas compounds 2 and 3 have a lower capability to induce cell differentiation and maturation, but an equal capacity to inhibit lymphocyte proliferation.

In an attempt to characterize the metabolic stability of the compounds, affinity for the vitamin D-binding protein and stability of the compounds in rat liver homogenates were determined. Interestingly, all three analogues were metabolized significantly more rapidly in rat liver homogenates than calcitriol (Table 3). Furthermore, compared with calcitriol, an approximately 50-fold lower affinity for the vitamin D-binding protein was found for all three compounds in the vitamin D-binding protein assay (Table 3). The IC_{50} values for displacement of [^{3}H]-1,25-$(OH)_2$-vitamin D_3 were about 100 μM for the analogues, whereas

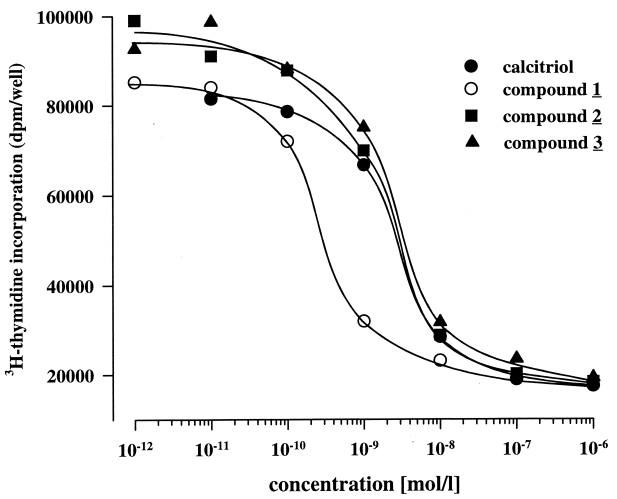

FIG. 3. Dose–response curves of the inhibition of PHA-induced lymphocyte proliferation by calcitriol and compounds 1, 2, and 3 (N = 4).

TABLE 3. Affinity of the vitamin D analogues to the vitamin D receptor and to vitamin D-binding protein, and metabolic stability and hypercalcemic effects of the compounds

Compound	Vitamin D receptor affinity RBA (%) (N = 4)	Affinity for vitamin D-binding protein		Metabolic stability t½ (min) (N = 6)	Hypercalcemic effects DR
		IC_{50} (μM)	RBA (%) (N = 4)		
Calcitriol	100	2.2	100	>120	
Compound 1	50	120	1.8	60	~100
Compound 2	23	100	2.2	60	~100
Compound 3	45	130	1.7	20	~100

RBA: relative binding affinity (reference calcitriol = 100%). t½: metabolic stability in rat liver homogenate, time until 50% of compound disappeared. DR ~ 100 means that 200 μg/kg of the test compound has about the same hypercalcemic effect as 2 μg/kg calcitriol.

calcitriol and 25-OH-vitamin D_3 had IC_{50} values of 2.2 μM and 24 nM, respectively. All three compounds displayed a strongly reduced hypercalcemic activity. When injected into rats, compounds 1 and 3 at 200 μg/kg body weight were equally or slightly more hypercalcemic than calcitriol at 2 μg/kg, leading to an increase in serum calcium concentration of 2.9 to 3.2 mM (both calcitriol and compound 1) and to 3.3 mM (compound 3). However, compound 2 at 200 μg/kg was slightly less hypercalcemic (3.0 mM) than calcitriol at 2 μg/kg, demonstrating that all compounds have an approximately 100-fold lower hypercalcemic activity than calcitriol (Table 3). The metabolism data from rat liver and the vitamin D-binding protein data suggest that the low hypercalcemic activity is probably due to the metabolic instability of the compounds and to the decreased affinity for the vitamin D-binding protein, leading to the rapid clearance of the drugs after systemic application.

In summary, we present three new, potent vitamin D receptor agonists with powerful immunomodulatory and cell differentiating activities, decreased metabolic stability, and low hypercalcemic activity. Due to their biological profiles, they represent interesting candidates for further development as antipsoriatic drugs.

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