An Evaluation of 1,25-Dihydroxyvitamin D_3 analogues on the proliferation and differentiation of cultured human keratinocytes, calcium metabolism and the differentiation of human HL-60 cells

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Synthetic analogues of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) were examined for their biological activities in four assay systems: (a) inhibition of proliferation and stimulation of terminal differentiation in cultured normal human keratinocytes, (b) intestinal calcium absorption and bone calcium mobilization in vitamin D-deficient rats, (c) competitive binding to the rat intestinal 1,25$(OH)_2D_3$ receptor, and (d) induction of differentiation of human HL-60 leukemia cells. Six analogues were found to have minimal activity in enhancing intestinal calcium absorption and bone calcium mobilization while retaining at least the same activity as 1,25$(OH)_2D_3$ in inhibiting proliferation and inducing terminal differentiation of cultured keratinocytes. Evidence suggests that it may be possible to dissociate antiproliferative activity from differentiation-inducing activity and calcium metabolism by specific modifications of the 1,25$(OH)_2D_3$ molecule. The uncoupling of these activities could potentially create an ideal analogue to treat psoriasis that should have potent antiproliferative activity with minimum effects on differentiation and on calcium metabolism.

Keywords: 1,25-dihydroxyvitamin D_3; proliferation; differentiation; keratinocyte; calcium; HL-60

Introduction

During the past decade, there was abundant evidence discovered indicating that 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) has other functions that are not related to calcium and phosphorus metabolism.[1,2] Stumpf et al., using an autoradiographic technique, first reported the specific nuclear localization of ^{3}H-1,25$(OH)_2D_3$ in a variety of tissues that were not directly associated with mineral metabolism.[3,4] These included the pituitary gland, pancreas, parathyroid glands, gonads, stomach, and skin. Later, receptor-like macromolecules specific for 1,25$(OH)_2D_3$ were characterized in many of those tissues.[5,6] Cultured human and mouse keratinocytes and fibroblasts, and malignant melanoma cells all possess nuclear receptors for 1,25$(OH)_2D_3$, which strongly suggested that the skin was a target organ for 1,25$(OH)_2D_3$ in addition to being the site of vitamin D_3 synthesis.[6-11] Incubation of cultured normal human and murine fibroblasts and keratinocytes with 1,25$(OH)_2D_3$ resulted in time- and dose-dependent decreases in their proliferation.[6,12,13] 1,25$(OH)_2D_3$ also stimulated terminal differentiation in cultured keratinocytes as evidenced by a dose-dependent increase in the proportion of the differentiated desquamated cells

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in the culture.[12,13] In addition, the hormone promoted time- and dose-dependent increases in the intracellular calcium concentration,[14] in transglutaminase activity and in cornified envelope formation.[7,12-13] The observation that $1,25(OH)_2D_3$ inhibited proliferation and induced terminal differentiation of cultured human keratinocytes prompted the use of this hormone in a clinical trial for the treatment of the common hyperproliferative epidermal disorder, psoriasis. Numerous reports have indicated that topically or orally administered $1,25(OH)_2D_3$ and its analogues are effective in alleviating many of the manifestations of psoriasis.[15-20] However, because $1,25(OH)_2D_3$ affects calcium metabolism, it could be potentially toxic when administered orally in therapeutic doses to patients with psoriasis. There continues to be a need to develop analogs of this hormone that have potent antiproliferative activity on epidermal cells while having minimal activity on calcium metabolism. In this report, chemically synthesized analogs of $1,25(OH)_2D_3$ were examined for their biological activities in a variety of assays including: (a) inhibition of proliferation and stimulation of terminal differentiation in cultured normal human keratinocytes, (b) intestinal calcium absorption and bone calcium mobilization in vitamin-D deficient rats, (c) competitive binding to the rat intestinal $1,25(OH)_2D_3$ receptor, and (d) induction of differentiation of human HL-60 leukemia cells.

Materials and methods

Keratinocyte culture

Keratinocytes were grown in culture using a modification of the method of Rheinwald and Green[21,22] as previously described.[12] Briefly, keratinocytes were obtained from neonatal foreskin after overnight trypsinization at 4° C. Keratinocytes were plated on the lethally irradiated 3T3 feeder cells in serum-free Dulbecco's modified Eagle's medium (DMEM) with 1.8 mmol/L calcium and growth factors: epidermal growth factor (25 ng/mL); hydrocortisone (200 ng/mL); insulin (5 μg/mL); transferrin (5 μg/mL); prostaglandin E_1 (50 ng/mL); and cholera toxin (0.1 μg/mL) and incubated at 37° C. The medium was changed three times per week. When primary culture reached 50–80% confluency, cells were subcultured to 35 mm dishes and cultured at 37° C in the same medium except that cholera toxin and hydrocortisone were removed from the medium.

Quantitation of morphologic changes during keratinocyte differentiation

Beginning at 1 week in culture, groups of triplicate plates of keratinocytes were incubated with fresh medium containing $1,25(OH)_2D_3$ (10^{-10} M–10^{-6} M), its analogues (10^{-10} M–10^{-6} M) or vehicle alone. Cells were fed and dosed three times per week. After 1 week of dosing, the medium was removed from each culture. The attached cells were then trypsinized for 30–40 minutes with 0.1% EDTA and 0.1% trypsin and then neutralized with medium. The keratinocytes were spun down and resuspended in a known volume of medium. Triplicate aliquots were taken for counting the basal (small, rounded) and squamous (larger, irregular-shaped, flattened) cells. The remaining cells were spun down and treated with 10 mmol/L Tris-HCl (pH 7.4) with 1% β-mercaptoethanol and 1% sodium dodecyl sulfate (SDS) at room temperature for 10 minutes.[23] Only cells with cornified envelopes were present after this treatment. The concentrations of analogues and $1,25(OH)_2D_3$ that caused 50% of the maximal activity (ED_{50}) were obtained from the dose-response curves performed in the same experiment and were used to calculate the percent relative potencies of the analogues as compared with $1,25(OH)_2D_3$ which was designated as 100%.

Intestinal calcium absorption and bone calcium mobilization

Intestinal calcium absorption and bone calcium mobilization were determined according to the methods described previously.[24] Weanling rats were fed a diet adequate in calcium and phosphorus, but deficient in vitamin D, for 2 weeks and were then switched to a diet deficient in vitamin D and low in calcium (0.02%) for an additional 2 weeks. Groups of six rats receiving intravenously either 95% ethanol alone or 30 pmol of $1,25(OH)_2D_3$ or analogues in ethanol were sacrificed 18 hours after the administration and their duodena and blood were collected. Intestinal calcium transport activity using ^{45}Ca was measured by the everted gut sac technique.[25] One hundred μL of samples from both the inside and outside of the duodenal sac were counted as described[24] to obtain ^{45}Ca-serosal (inside)/^{45}Ca-mucosal (outside). The ratio is considered to be a measurement for calcium transport activity.

The blood from the rats described above was centrifuged, and 0.1 mL of serum was mixed with 0.1% $LaCl_3$ solution. Serum calcium concentration was determined with a Perkin-Elmer atomic absorption spectrophotomer, model 303 as described.[24] The percent relative potencies of analogues as compared to $1,25(OH)_2D_3$ were calculated by dividing the analogue-induced changes in intestinal calcium transport or serum calcium concentration over control animals by that induced by $1,25(OH)_2D_3$ and multiplied these ratios by 100.

Determination of the $1,25(OH)_2D_3$ receptor binding activity

The binding of ^3H-$1,25(OH)_2D_3$ to its rat intestinal receptor was carried out as previously described.[26] Analogues of $1,25(OH)_2D_3$ were examined at multiple concentrations and their ability to compete with the ^3H-$1,25(OH)_2D_3$ for rat intestinal receptor binding was assessed. A standard curve for nonradioactive $1,25(OH)_2D_3$ was generated with each assay and the concentration that caused 50% displacement of the radioactive $1,25(OH)_2D_3$ was obtained. Similarly, the concentration of an analogue that caused 50% displacement was determined and compared with that of $1,25(OH)_2D_3$ to calculate the percent relative potency of the analogue.

Differentiation of human HL-60 cells

Differentiation of human HL-60 leukemia cells was determined by phagocytic activity as described.[27] Cells obtained from control and $1,25(OH)_2D_3$ (0.1 nmol/L to 100 nmol/L)– or analogue (0.01 nmol/L to 1 μmol/L–treated HL-60 cultures were suspended at a concentration of 1×10^6 cells/mL in RPMI 1640 medium (Gibco, Grand Island, NY USA) supplemented with heat-inactivated 10% fetal calf serum. Candida albicans were washed with saline and added to the cell suspension at a final concentration of 4×10^6 fungi/mL. The suspension was incubated at 37° C for 30 minutes and the percentage of cells that had phagocytosed at least one fungus was determined with a hemocytometer. The ED_{50} for

1,25(OH)$_2$D$_3$ and its analogues obtained from their dose-response curves were used to calculate their relative potencies. For example, if an analogue had an ED_{50} of 1 nmol/L and the ED_{50} for 1,25(OH)$_2$D$_3$ was 10 nmol/L, the analogue was therefore 10-fold more active than 1,25(OH)$_2$D$_3$.

Results

A comparison of the biologic activity of 1,25(OH)$_2$D$_3$ and its analogs with an additional hydroxyl group or a double bond in the side chain of the molecule is shown in *Figure 1*. 1,24R,25-Trihydroxyvitamin D$_3$ (RO 21-7729) was equally active as 1,25(OH)$_2$D$_3$ in inhibiting proliferation and inducing differentiation of human normal keratinocytes. Although this analogue competed poorly against 1,25(OH)$_2$D$_3$ for the rat intestinal 1,25(OH)$_2$D$_3$ receptor, it had comparable intestinal calcium absorption activity as 1,25(OH)$_2$D$_3$. However, its bone calcium mobilization activity was only one-sixth of that found for 1,25(OH)$_2$D$_3$. The compound was also 80% less active than 1,25(OH)$_2$D$_3$ in inducing HL-60 leukemia cell differentiation. Similarly, 1,23R,25 trihydroxyvitamin D$_3$ (RO23-5112) and its S epimer (RO23-2019) were found to be as potent as 1,25(OH)$_2$D$_3$ in inducing cornified envelope formation and in the antiproliferative assay. Interestingly, neither of them showed any significant activity in enhancing intestinal calcium absorption and bone calcium mobilization. Furthermore, the two trihydroxylated analogues competed poorly against 1,25(OH)$_2$D$_3$ for binding to rat intestinal 1,25(OH)$_2$D$_3$ receptor. Thus, additional hydroxylation at C23 or C24 did not significantly alter the hormone's ability to inhibit cell growth and to promote differentiation of cultured keratinocytes. However, an additional hydroxylation at either of these two positions decreased their ability to induce the differentiation of HL-60 cells 50–86% as compared with 1,25(OH)$_2$D$_3$.

The effects of placing a double bond at different positions of the side chain were also studied (*Figure 1*). 22-Dehydro-1,25-dihydroxyvitamin D$_3$ (RO23-6710) had much less anti-proliferative and differentiation-inducing activities than 1,25(OH)$_2$D$_3$ in the keratinocyte cell cultures. However, the compound was almost

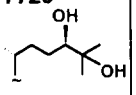
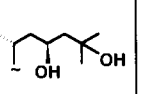
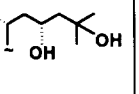
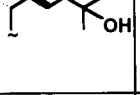
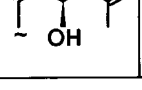
	% OF 1,25(OH)$_2$D$_3$ EFFECT					
	HUMAN KERATINOCYTES IN CULTURE		RAT		COMPETITIVE BINDING TO 1,25(OH)$_2$D$_3$ RAT INTESTINE RECEPTORS	DIFFERENTIATION OF HUMAN HL-60 LEUKEMIA CELLS
	INHIBITION OF PROLIFERATION	INDUCTION OF CORNIFIED ENVELOPES	INTESTINAL CALCIUM ABSORPTION	BONE CALCIUM MOBILIZATION		
R021-7729 R= 	100	100	93	17	2	20
R023-2019 R= 	100	100	0	0	-	14
R023-5112 R= 	100	100	6	0	1	50
R023-6710 R= 	10	1	92	133	122	125
R023-5084 R= 	100	0.1	-	-	0.048	-

Figure 1 The biological activities of 1,25(OH)$_2$D$_3$ analogues with an additional hydroxy group or a double bond in the side chain of the 1,25(OH)$_2$D$_3$ molecule.

as active as $1,25(OH)_2D_3$ in stimulating intestinal calcium absorption and bone calcium mobilization. In spite of a 99% decrease in the induction of keratinocyte differentiation, as compared with $1,25(OH)_2D_3$, RO23-6710 had activity similar to the natural hormone in the induction of HL-60 cell differentiation. Modification of the side chain of $1,25(OH)_2D_3$ by the introduction of a double bond between C25 and C26, and the hydroxylation at C23 produced RO23-5084 (*Figure 1*), which had the same antiproliferative activity as $1,25(OH)_2D_3$, whereas its differentiation-inducing activity was reduced to 0.1% of the hormone's activity.

1α-Hydroxy-25-fluorovitamin D_3 was initially synthesized to study the importance of the 25-OH function in the biological activity of $1,25(OH)_2D_3$ because of the stable carbon-fluoride bond and similar atomic dimension between hydrogen and fluorine. Several analogues of $1,25(OH)_2D_3$ with fluorine substitution on the side chain or on the A-ring were synthesized and tested for their biological activities and possible therapeutic applications.[28-30] Along this line, we examined the biological activities of seven fluorinated vitamin D compounds in cultured keratinocytes (*Figure 2*). The results indicate that fluorine substitution at C24, C26, or C27 position (RO23-0233,RO22-9343,RO23-6889, and RO23-4194) did not significantly alter the hormone's antiproliferative and differentiation-inducing activities. However, the presence of a double bond between C22 and C23 and a total substitution of the hydrogen atoms by fluorine at C26 and C27 (RO23-6536) greatly reduced its differentiation-inducing ability without affecting its antiproliferative activity. The dissociation of the two activities was also found when a fluorine was substituted for the 25-OH group of $1,24(R)25(OH)_3D_3$ (RO21-7729). The resulting fluoro-analogue (RO22-4913) had the same antiproliferative activity as $1,25(OH)_2D_3$ and its differentiation-inducing activity was reduced to 1% of the activity of $1,25(OH)_2D_3$. In most, but not all cases, fluorination at the side chain increased its ability to absorb calcium from the intestine, to mobilize calcium from bones, and to bind to the intestinal $1,25(OH)_2D_3$ receptor

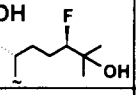
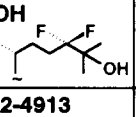
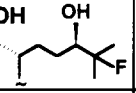
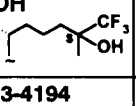
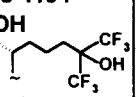
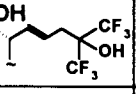
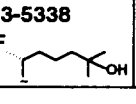
	% OF $1,25(OH)_2D_3$ EFFECT					
	HUMAN KERATINOCYTES IN CULTURE		RAT		COMPETITIVE BINDING TO $1,25(OH)_2D_3$ RAT INTESTINE RECEPTORS	DIFFERENTIATION OF HUMAN HL-60 LEUKEMIA CELLS
	INHIBITION OF PROLIFERATION	INDUCTION OF CORNIFIED ENVELOPES	INTESTINAL CALCIUM ABSORPTION	BONE CALCIUM MOBILIZATION		
R023-0233 X=OH R= 	100	100	65	40	115	30
R022-9343 X=OH R= 	100	100	235	58	229	1000
R022-4913 X=OH R= 	100	1	-	-	0.165	-
R023-6889 X=OH R= 	100	100	197	163	199	300
R023-4194 X=OH R= 	100	50	229	137	82	2000
R023-6536 X=OH R= 	100	0.1	221	110	62	3000
R023-5338 X=F R= 	100	100	0	0	-	-

Figure 2 The biological activities of $1,25(OH)_2D_3$ analogues with fluorine substitution in the side chain and ring A of the $1,25(OH)_2D_3$ molecule.

(*Figure 2*). On the other hand, substitution of the 1α-OH group by fluorine produced an analogue (RO23-5338) that was as active as 1,25(OH)$_2$D$_3$ in inhibiting keratinocyte proliferation and in promoting differentiation. Most interestingly, this analogue had no effect on intestinal calcium absorption and bone mineral mobilization. The effects of fluorine substitution on HL-60 cell differentiation were more variable, ranging from a 66% decrease to a 30-fold increase in activity. In the case of RO23-6536, the induction of cornified envelope formation was decreased to 0.1% of the 1,25(OH)$_2$D$_3$ activity, and the induction of HL-60 cell differentiation was increased 30 fold.

Introduction of a double bond to ring D at the C16-17 position of 1,25(OH)$_2$D$_3$ structure decreased the antiproliferative activity 90%. On the other hand, it did not alter the analogue's differentiation-inducing activity (RO24-2637, *Figure 3*). Its binding to the 1,25(OH)$_2$D$_3$ rat intestinal receptor was increased 240% as compared with 1,25(OH)$_2$D$_3$. Further addition of a double bond at C23-24 position (RO24-2201), however, restored the antiproliferative activity to the same level as 1,25(OH)$_2$D$_3$. Interestingly, the binding of RO24-2201 to the rat intestinal receptor was reduced to 80% of 1,25(OH)$_2$D$_3$ activity. Removal of 1α-hydroxyl group from this series of analogues (RO24-2287, RO24-2648, and RO24-2090) did not affect their ability to induce differentiation of cultured keratinocytes. However, the effects on keratinocyte proliferation were more variable. There was a 90% decrease for RO24-2648; whereas, no changes were observed for RO24-2287 and RO24-2090. As expected, the removal of 1α-hydroxyl group greatly reduced the analog's ability to bind to 1,25(OH)$_2$D$_3$ receptors (*Figure 3*). In the absence of the 1α-OH group, the addition of a triple bond between C23 and C24 to this Δ^{16} analog did not have any impact on both activities examined in the keratinocyte assay systems (RO24-2090) (*Figure 3*). The two activities were enhanced 100 fold, however, when both the 1α-OH group and the triple bond were present (R023-7553) (*Figure 3*). The elimination of the double bond from the D-ring of RO23-7553 drastically reduced the antiproliferative and differentiation inducing activities of the resulting analog (RO23-7498) to

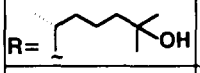
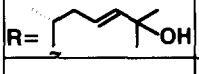
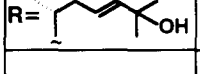
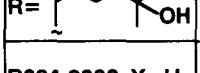
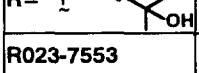
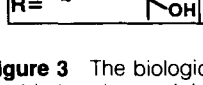
	% OF 1,25(OH)$_2$D$_3$ EFFECT					
	HUMAN KERATINOCYTES IN CULTURE		RAT		COMPETITIVE BINDING TO 1,25(OH)$_2$D$_3$ RAT INTESTINE RECEPTORS	DIFFERENTIATION OF HUMAN HL-60 LEUKEMIA CELLS
	INHIBITION OF PROLIFERATION	INDUCTION OF CORNIFIED ENVELOPES	INTESTINAL CALCIUM ABSORPTION	BONE CALCIUM MOBILIZATION		
R024-2637 X=OH R= 	10	100	-	-	240	500
R024-2201 X=OH R= 	100	100	-	-	80	300
R024-2287 X=H R= 	100	100	-	-	0.12	0.5
R024-2648 X=H R= 	10	100	-	-	0.23	3
R024-2090 X=H R= 	100	100	72	0	0.07	1
R023-7553 X=OH R= 	10000	10000	53	0	51	1000

Figure 3 The biological activities of 1,25(OH)$_2$D$_3$ analogues with the addition of a double bond in ring-D and with or without an additional double bond or a triple bond in the side chain of the 1,25(OH)$_2$D$_3$ or 25-OH-D$_3$ molecule.

the level of $1,25(OH)_2D_3$ (*Figure 4*). Further removal of the 1α-OH group from RO23-7498 did not cause any additional reduction of the two activities examined (R023-9375) (*Figure 4*). RO23-7553, RO23-7498, RO24-2090, and RO23-9375 exhibited very low to no bone calcium mobilization ability while retaining more than half of its intestinal calcium absorption activity and no more than 51% of its receptor binding activity, as compared with $1,25(OH)_2D_3$.

The biological activities of two deuterated compounds were examined (*Figure 4*). RO21-5535/2 and RO21-5535/3 had the same activities as $1,25(OH)_2D_3$ in inducing the differentiation of human HL-60 leukemia cell line, in the cultured human keratinocyte, in the binding to rat intestinal $1,25(OH)_2D_3$ receptor preparation and in rat intestinal calcium absorption tests. In the bone calcium mobilization experiment however, RO21-5535/2 showed about 66% of the $1,25(OH)_2D_3$ activity, while RO21-5535/3 was 50% more active than the natural hormone (*Figure 4*). Although the analogues (except RO23-7553) shown in *Figures 3 and 4* had the same activity as $1,25(OH)_2D_3$ in inducing keratinocyte differentiation, they differed greatly in their ability to induce HL-60 leukemia cell differentiation; three analogues had a three- to five-fold increase, two had no change and four had only 0.5–3% of the $1,25(OH)_2D_3$ activity.

Discussion

Several interesting results have emerged from this study. First, six analogues of $1,25(OH)_2D_3$ (RO23-2019, RO23-5112, RO23-5338, RO23-0233, RO23-7553, and RO23-9375) were found to be at least as potent as the natural hormone in inhibiting proliferation and inducing terminal differentiation of cultured human keratinocytes, while having minimal activity in enhancing intestinal calcium absorption or bone calcium mobilization. These analogues could be candidates as antipsoriatic drugs that would not alter calcium metabolism. One of the most promising compounds is RO23-7553, which was found to be about 100 times more potent than $1,25(OH)_2D_3$ in inhibiting keratinocyte proliferation, while having only 50% of its activity in intestinal calcium transport.

Specific side chain modifications by the introduction of a double bond and an additional hydroxyl group (e.g., RO23-5084) and by fluorine substitution of either the 25-OH group (e.g., RO22-4913) or the hydrogen atoms at C26 and C27 (e.g., RO23-6536) greatly re-

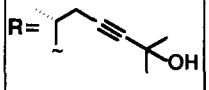
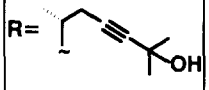
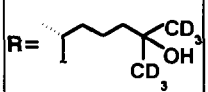
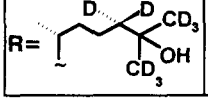
	% OF $1,25(OH)_2D_3$ EFFECT					
	HUMAN KERATINOCYTES IN CULTURE		RAT		COMPETITIVE BINDING TO $1,25(OH)_2D_3$ RAT INTESTINE RECEPTORS	DIFFERENTIATION OF HUMAN HL-60 LEUKEMIA CELLS
	INHIBITION OF PROLIFERATION	INDUCTION OF CORNIFIED ENVELOPES	INTESTINAL CALCIUM ABSORPTION	BONE CALCIUM MOBILIZATION		
R023-7498 X=OH 	100	100	95	15	39	300
R023-9375 X=H 	100	100	71	0	0.062	1
R021-5535/2 X=OH 	100	100	92	64	100	100
R021-5535/3 X=OH 	100	100	106	147	100	100

Figure 4 The biological activities of $1,25(OH)_2D_3$ analogues with the addition of a triple bond to the side chain of $1,25(OH)_2D_3$ or 25-OH-D_3 molecule, or with deuterium substitution in the side chain of the $1,25(OH)_2D_3$ molecule.

duced the differentiation-inducing activity to 0.1–1% of the activity of 1,25(OH)$_2$D$_3$ without affecting antiproliferative potency (*Figure 1 and 2*). It may be possible to dissociate antiproliferative activity from differentiation-inducing activity by specific modifications of the 1,25(OH)$_2$D$_3$ molecule. The uncoupling of the two activities could potentially create an ideal analogue that could have potent antiproliferative activity with minimum effects on differentiation and on calcium metabolism. Among the three analogues that were shown to have minimal differentiation-inducing potency, RO23-6536 had high intestinal calcium absorption activity and therefore, the analogue would not offer any advantage over 1,25(OH)$_2$D$_3$ in treating psoriasis or other hyperproliferative disorders such as leukemia. The calcium transport and bone calcium mobilization activities of the other two analogues (RO22-4913 and RO23-5084) are not known at the present time. Thus, this report does not provide any ideal analogues that have only antiproliferative activity present. Experiments are underway to determine their activities on calcium metabolism.

A series of vitamin D compounds that contain A-ring, D-ring, and side chain modification of 1,25(OH)$_2$D$_3$ were studied for their abilities to bind nuclear receptors and to stimulate transmembrane calcium influx in ROS 17/2.8 cells.[31] It was found that while some analogs stimulated Ca^{2+} channel opening without significant nuclear receptor binding activity, other analogs displayed little or no activity in opening Ca^{2+} channels but bound very well to the nuclear receptors. Thus, it appears possible to modify 1,25(OH)$_2$D$_3$ so that it can preferentially affect rapid cytosolic activities, while having minimum binding to its nuclear receptor.

The 1α-hydroxy group may not be essential for affecting the differentiation and proliferation of the cultured human keratinocytes. This conclusion is derived from the finding that 1α-fluorinated analogue (RO23-5338, *Figure 2*), which can not be hydroxylated at the 1α position, had essentially the same potency as 1,25(OH)$_2$D$_3$.

MC903 and 1,25(OH)$_2$D$_3$ have been studied in the laboratory, as well as in clinical trials. Like 1,25(OH)$_2$D$_3$, these two analogues inhibit the proliferation of cultured human keratinocytes.[32,33] 1,24(OH)$_2$D$_3$ is equally effective as 1,25(OH)$_2$D$_3$ in inhibiting proliferation of cultured human keratinocytes, however, MC903 is about 100 times less effective. Clinical results are in good agreement with the in vitro observations.[20,34] Although 1,25(OH)$_2$D$_3$ and 1,24(OH)$_2$D$_3$ are effective in treating psoriasis, both agents also affect calcium metabolism and induce terminal differentiation of cultured human keratinocytes. MC903 also induces terminal differentiation, however it has minimal calcemic effect.[34] Is it possible that 1,25(OH)$_2$D$_3$ receptors in the skin are different from the intestine so that analogues such as MC903 work specifically in the skin while having no effect on the intestine? This possibility appears unlikely because there is strong evidence that the nuclear receptor for 1,25(OH)$_2$D$_3$ in all tissues examined are the same.[6,35,36] There is firm evidence that MC903 is rapidly degraded and therefore has less calcemic action in vivo. When we examined the effect of various analogues on the intestinal calcium absorption, we found that in many instances their calcium transport activity did not correspond to their binding ability to the intestinal 1,25(OH)$_2$D$_3$ receptor (RO21-7729, *Figure 1*; RO23-4194, RO23-6536, *Figure 2*; RO23-7498, *Figure 4*). A simple explanation for this discrepancy is that these analogues may have different clearance rates in vivo by being metabolized to more active metabolites or being degraded to inactive compounds and excreted. An alternative explanation is that some analogues may act more specifically on the plasma membrane than on nuclear receptor-mediated gene expression to induce calcium transport.[31] It has been demonstrated by several investigators that 1,25(OH)$_2$D$_3$ may cause intestinal calcium transport by changing the membrane lipid composition, and therefore, the membrane fluidity.[37,38] It should be noted that analogues, such as RO21-7729, RO23-7553, RO23-9375, and RO24-2090, possess greater intestinal calcium transport activity than bone calcium mobilization activity. This preferential action on the intestine has been demonstrated previously in the case of 5,6-trans-25-OH-D$_3$.[39] Thus, when judging different biologic activities of 1,25(OH)$_2$D$_3$ analogues, such as calcemic versus antiproliferative activity, it is imperative to evaluate these compounds parenterally to evaluate both effects in vivo. At the present time, all reports, including this one, that evaluated 1,25(OH)$_2$D$_3$ analogues for their antiproliferative activity, were conducted in vitro, while the calcemic effect was studied in vivo.

The ability of 1,25(OH)$_2$D$_3$ to stimulate cell differentiation was first demonstrated in human promyelocytic leukemia cells (HL-60) in 1982.[27] In this cell line, the cultured cells were differentiated into mature macrophages in the presence of 1,25(OH)$_2$D$_3$. Since then, this assay system has been used as a method to determine the differentiation-inducing activity of various 1,25(OH)$_2$D$_3$ analogues. The promotion of cornified envelope formation by 1,25(OH)$_2$D$_3$ in cultured human and mouse keratinocytes[12,13] provided a new and reliable assay system to study cellular differentiation activity of 1,25(OH)$_2$D$_3$ analogues. In this report we compared the differentiation-inducing activity of nineteen synthetic 1,25(OH)$_2$D$_3$ analogues in these two different cell culture systems. Among them, only two analogues (RO21-5535/2, RO21-5535/3, *Figure 4*) showed consistent potency in both assay systems as compared with 1,25(OH)$_2$D$_3$. The activity of the other 17 analogues when assayed in HL-60 cells were very different from that obtained by counting cornified envelopes in cultured keratinocytes. For example, RO23-4194, RO23-6536, *(Figure 2)*, and RO23-6710 *(Figure 1)* were less active than 1,25(OH)$_2$D$_3$ in inducing keratinocyte differentiation, while they were more active than the natural hormone in inducing HL-60 differentiation. Because HL-60 cells were cultured in the presence of 10% heat inactivated fetal bovine serum

in RPMI, and keratinocytes were grown on serum-free DMEM, it is possible that the difference in culture conditions may affect the differentiation activity of $1,25(OH)_2D_3$ and its analogues. Nevertheless, the discrepancy strongly suggests that the mechanism of $1,25(OH)_2D_3$ and its analogue-induced differentiation in these two cultured cell systems may be different.

It is well established that cultured keratinocytes are sensitive to calcium in the medium and will differentiate in the presence of a high calcium (e.g., 1.2 mmol/L), whereas, low calcium (e.g., 0.15 mmol/L) favors proliferation.[40,41] Recent evidence has indicated that $1,25(OH)_2D_3$ enhances a rapid hydrolysis of phosphatidyl inositol phosphate and consequently increases the intracellular concentrations of calcium in a variety of cultured cells including keratinocytes.[14,42] If this membrane effect is found to be related to the hormone's differentiating/antiproliferative activity, it may be possible to develop analogues that have selective membrane- and nuclear-related biologic activities. Studies are underway to determine the activity of some of the most promising analogues on intracellular calcium and phosphatidyl inositol metabolism.

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