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1-(HYDROXYALKYL)-25-HYDROXYVITAMIN D_3 ANALOGS OF CALCITRIOL. 2. PRELIMINARY BIOLOGICAL EVALUATION

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Abstract 1α-Hydroxy**methyl**-25-hydroxyvitamin D_3 homolog (–)-**2** shows: (1) increased expression of early and late murine epidermal differentiation markers; (2) weak induction of 24-hydroxylase activity in cultured porcine kidney epithelial cells; (3) strong resistance to metabolism in human leukemic cells; and (4) no significant antiproliferative (antimitogenic) activity in human leukemic cells. Surprisingly, 1-(hydroxy**ethyl**) diastereomer (+)-**5** is easily metabolized in human leukemic cells, and this 1β-homolog (+)-**5** is several-fold less active than calcitriol but unexpectedly much more potent than 1α-homolog (–)-**4** in inhibiting proliferation of murine keratinocytes.

We report here preliminary biological testing of ring-A modified 1-(hydroxyalkyl)-25-hydroxyvitamin D_3 analogs **2-5**, the structures of which are shown in the preceding publication in this issue.[1]

24-Hydroxylase Studies.[2] Calcitriol induced 24-hydroxylase (24OHase) activity in a dose-dependent manner in LLC-PK_1 cells. The maximal effect was with 100 nM calcitriol, showing a 10-fold stimulation over control (1025 ± 27%), and half maximal effect at 30 nM. Higher calcitriol concentrations caused declining activity. Diastereomer (–)-**2** also induced 24OHase in a dose-dependent fashion. The maximal effect was not reached at concentrations soluble in culture media (10 μM); however, extrapolation from the dose-response curve indicates the maximal effect would be at 100 μM. We found a small but significant increase of 24OHase activity after exposure with 100 nM of diastereomer (–)-**2** (180 ± 15%), and a further increase with 1 μM of diastereomer (–)-**2** (417 ± 3.4%). These data show that the dose needed to achieve the half maximal effect of (–)-**2** is about 100 times higher than that of calcitriol for inducing 24OHase activity. The normal physiological role of the increase of 24OHase activity after calcitriol exposure is to facilitate metabolism of calcitriol in cells and tissues. The weak induction of 24OHase activity by (–)-**2** predicts that this analog is more stable than calcitriol in cells.[3]

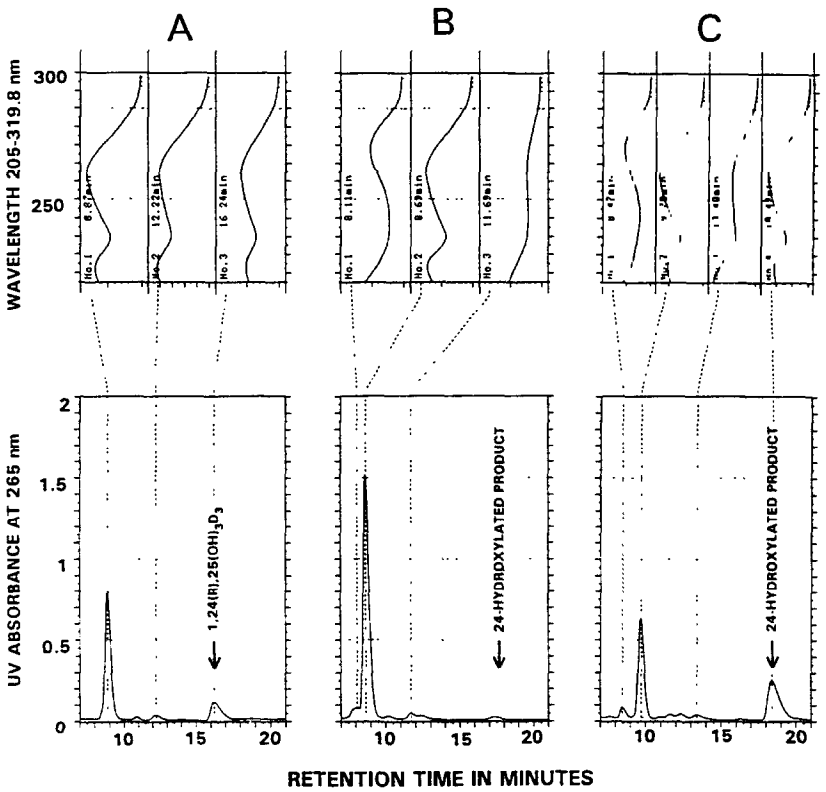
The very weak binding of (–)-**2** to the VDR *in vitro* and the severely compromised induction of 24OHase activity in cultured cells suggest that (–)-**2** is a weak agonist of the VDR. Diastereomer (–)-**2** induces 24OHase with the potency of the natural $25OHD_3$. However (–)-**2** was shown to have antimitogenic activity indistinguishable from that of calcitriol,[4] which is 500 times greater than the potency of $25OHD_3$. **This suggests a dissociation in specificity of calcitriol analog (–)-2 for various genomic functions of VDR.** A similar dissociation of 24OHase induction and antimitogenic activity for a skin fibroblast cell line with a genetic defect in the VDR functions has been reported.[5] That cell line was from a patient with a hereditary resistance to calcitriol. The selective retention of a VDR function, but loss of another were evident *in vivo*: calcitriol treatment resulted in hypoparathyroidism without any increase in serum calcium or in intestinal calcium absorption.[6] In the cells of this patient the antimitogenic activity of calcitriol was normal, while induction of 24OHase or mitogenic effect was undetectable.[5] Immunocytology showed a very small number of normally reorganizing receptors in these cells.[7] This suggested mediation of the antiproliferative effect of calcitriol by such small numbers of VDR or with such low affinity that they are insufficient to mediate other VDR functions. It is possible that the antiproliferative effect of diastereomer (–)-**2** is mediated by the same subpopulation of VDRs, which remained intact in that mutant cell line, or through a second, not yet characterized receptor. Future development of radiolabeled (–)-**2** may help to identify this putative receptor.

Metabolism Studies. From both *in vitro* and *in vivo* studies of various target tissues including rat kidney and human leukemic cells,[8] it appears that calcitriol is metabolized through two major pathways. The first pathway involves 24-hydroxylation of calcitriol into $1,24(R),25(OH)_3D_3$ that is then further metabolized and is finally inactivated by conversion into calcitroic acid. The second pathway involves 23-hydroxylation of calcitriol into $1,23(S),25(OH)_3D_3$ that is then further metabolized into calcitriol lactone, the function of which is still not understood thoroughly. Under physiological conditions, it appears that the classical target organs for vitamin D (i.e., kidney, bone, and intestine) have inherent basal calcitriol metabolizing ability, whereas this property seems to be absent in other non-classical target tissues such as leukemic cells and skin fibroblasts.[8a,9] The ability to metabolize calcitriol, however, can be induced in non-classical target tissues by pretreating these tissues with calcitriol itself, for example as shown here with the LLC-PK_1 cell line.

The chronic myelogenous leukemia cell line has been established as a model cell line to study the metabolism of calcitriol and its analogs.[10] As these leukemic cells do not have any inherent ability to metabolize calcitriol, these cells are routinely pretreated with 50 nM of calcitriol for a period of 3 days, at which time the cells are found to have maximal metabolizing ability. As radiolabeled synthetic analogs are not presently available, we have used the scanning photodiode array detector to detect and quantitate the various vitamin D metabolites because of their unique UV spectral properties. As shown in Figure 1A, calcitriol is converted into a major polar metabolite that was identified as $1,24(R),25(OH)_3D_3$ based on its HPLC co-migration with an authentic synthetic standard.[11] Even though there are other metabolite peaks between the calcitriol and the $1,24(R),25(OH)_3D_3$ peaks, under the experimental conditions used [cell number, volume of media, and amount of calcitriol (0.5 μM)] we repeatedly found that roughly the same amount of $1,24(R),25(OH)_3D_3$ is produced in each incubation study, and $1,24(R),25(OH)_3D_3$ is the single major metabolite formed, indicating the importance of the 24-hydroxylation pathway. As the amount of $1,24(R),25(OH)_3D_3$ produced in leukemic cells has been very consistent, this cell line can be used with confidence to understand how newly synthesized vitamin D analogs differ from calcitriol in their pathways of metabolism. In the present study, we concentrated our efforts at understanding the metabolic fate of the two ring-A modified vitamin D

analogs (–)-2 and (+)-5. As shown in Figure 1B, 1α-hydroxymethyl diastereomer (–)-2 appears to be metabolized into its daughter metabolites rather slowly when compared to calcitriol (Figure 1A), as indicated by the amount of unmetabolized substrate recovered and by the the absence of significant daughter metabolite peaks. One of the major metabolites can be identified tentatively as the 24-hydroxylated product by its HPLC migration properties. In contrast, as seen in Figure 1C, 1-hydroxyethyl diastereomer (+)-5 is readily metabolized into a polar metabolite that is identified as the corresponding 24-hydroxylated product based on the following information. First, from the characteristic UV spectrum of the peak shown in Figure 1C, it can be concluded that the major polar daughter metabolite contains an intact 5,6-*cis*-triene chromophore. After purification of this metabolite by two different HPLC systems, mass spectra (70 eV) were obtained on a Hewlett-Packard 5985B mass spectrometer. The metabolites (1μg each) were introduced into the ion source maintained at 200°C via a direct-insertion probe. The mass spectra of the parent compound and of the daughter metabolite contain common peaks at m/z 315, 297, 180, and 162 which indicate that the daughter metabolite is formed as a result of a change occurring specifically on the side-chain of its parent compound. The molecular ion at m/z 460 in the mass spectrum of the daughter metabolite indicates that this metabolite possesses an additional hydroxyl group. Furthermore, this metabolite's susceptibility to chemical oxidative cleavage by sodium periodate establishes that the additional hydroxyl group is *vicinal* to the original 25-hydroxyl group. With these findings, we are now able to identify the daughter metabolite specifically as the 24-hydroxylated product.

Figure 1. HPLC and UV analyses of lipid-soluble metabolites of calcitriol (panel A) and of diastereomers (–)-2 (panel B) and (+)-5 (panel C) produced in human leukemic RWLeu-4 cells.



From the chromatographic profiles in Figure 1, it is clear that 1-hydroxy**methyl** diastereomer (–)-**2** is metabolized to a much smaller extent than calcitriol, whereas 1-hydroxy**ethyl** diasteromer (+)-**5** is metabolized to a larger extent than calcitriol. Thus, it can be concluded that structural modifications on ring-A can result in changes in side-chain metabolism and therefore also changes in final deactivation of vitamin D compounds. Whereas it is well established that synthetic analogs of calcitriol with modified side-chains may follow different metabolic paths and/or undergo metabolism at different rates, **it has not been known until this study that modification of ring-A may also cause differences in side-chain metabolism.**

Antiproliferation and Differentiation Studies. A. Murine Keratinocytes. We recently published the antiproliferative data for 1-hydroxy**methyl** diastereomers (–)-**2** and (+)-**3**.[4] The antiproliferative effects of calcitriol [(+)-**1**] and of the two 1-(hydroxy**ethyl**) diastereomers (–)-**4** and (+)-**5** are shown in Figure 2. Treatment with all three compounds reduced the growth rates of the keratinocyte cell line PE over a 96 h period. When evaluated at a concentration of 1μM, calcitriol and the 1β-hydroxyethyl analog (+)-**5** were equally effective at inhibiting keratinocyte growth. The 1α-hydroxymethyl analog (–)-**4** was a less effective inhibitor, although a substantial reduction in cell number relative to vehicle-treated cells was also observed with this diastereomer. The activity of all of these compounds was due to cytostatic rather than cytotoxic effects, as cell viability was unchanged in the treated groups as determined by dye exclusion assay. The growth inhibitory potency of the more active 1-hydroxyethyl diastereomer was determined and compared to that of calcitriol. As shown in Figure 3, calcitriol was several-fold more potent than 1β-hydroxyethyl homolog (+)-**5**; the concentrations required for 50 percent inhibition of cell growth were approximately 100 nM and 500 nM, respectively.

Figure 2. Growth inhibition of murine keratinocyte cell line PE by calcitriol and 1-(hydroxyetheyl) homologs at 1 μM. Values represent the mean from 12 wells ± SD. Arrows indicate administration of fresh medium into which the compounds dissolved in DMSO had been added. Control cells were treated with DMSO alone (0.1 % in culture medium). The treated values are significantly different from the solvent control at 72 and 96 h ($p < 0.001$. Student's *t* test).

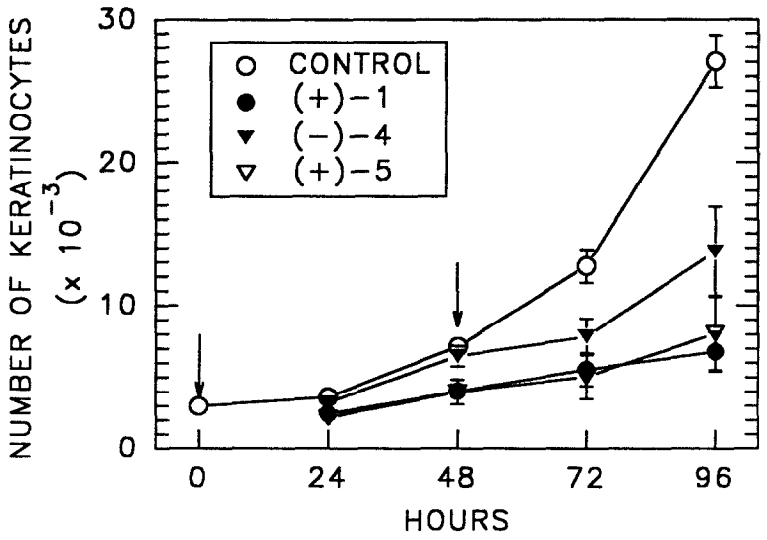
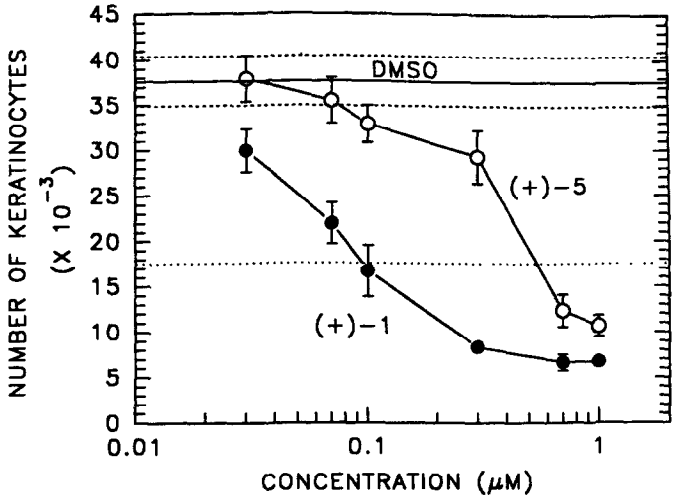


Figure 3. Concentration-dependent inhibitory effects of calcitriol, (+)-**1**, and the 1-(hydroxyethyl) homolog (+)-**5** on growth of murine keratinocyte cell line PE. Cells were treated with the indicated concentrations of compounds as described in the legend to Figure 2 and assayed at 96 h. Values represent the mean from 12 wells ± SD. *Solid line* indicates mean cell number for DMSO-treated cultures ± SD (*dashed line*). *Dotted line*, 50 percent inhibition level.



Diastereomer (–)-**2** induced epidermal differentiation of mouse primary keratinocyte cells *in vitro*. Differentiation was characterized by increased expression of early and late murine epidermal differentiation markers as determined by Western blot analysis. The minimal concentration of (–)-**2** needed to induce expression of early marker protein keratin 1 (K1) but not keratin 10 (K10) and to enhance expression of late granular protein markers filaggrin and loricrin (a cornified envelope precursor) was 1 μM in low (.05 mM) Ca^{2+} medium. The degree of expression of the markers induced by 1 μM (–)-**2** was nearly equivalent to that induced by .12 mM extracellular Ca^{2+}, one of the most effective means of stimulating epidermal differentiation *in vitro*.[12]

B. Human Leukemic Cells. In contrast to the results with murine keratinocytes, all of the analogs **2-5** were significantly less active than calcitriol in inhibiting proliferation or inducing differentiation in the human chronic myelogenous leukemia cell line RWLeu4. Analog (–)-**4** had no activity up to 1 X 10^{-6} M, analogs (–)-**2** and (+)-**5** had activity only in the 1 X 10^{-6} M range, and analog (+)-**3** had limited activity at 1 X 10^{-8} M and significant activity only at 1 X 10^{-7} M. Morphological evidence of differentiation (adherence to plastic, clumping, and cytoplasmic projections) occurred only at doses that were growth inhibitory. The control IC_{50} of calcitriol for these cells was 6 X 10^{-9} M.

Conclusions: The most important observations and generalizations resulting from this collaborative project are as follows: (1) 1β-hydroxy**ethyl** analog (+)-**5** is a potent inhibitor of cell proliferation in cultured murine keratinocytes, but neither (–)-**4** nor (+)-**5** [nor 1α-hydroxymethyl analogs (–)-**2** nor (+)-**3**] has significant antiproliferative activity in human leukemic cells; (2) 1α–hydroxy**methyl** analog (–)-**2** selectively enhances expression of late differentiation markers in cultured murine keratinocyte cells; 3) although 1α-hydroxy**methyl** analog (–)-**2** is resistant to metabolism in human leukemic cells under conditions in which calcitriol is easily metabolized, 1β-hydroxy**ethyl** analog (+)-**5** is easily metabolized into a 24-hydroxylated product that has been identified by mass spectrometry; **this is a dramatic effect of a remote ring-A structural change on side-chain metabolism**; and (4) the low but measurable 24-hydroxylase genomic activity of 1α-hydroxy**methyl** analog (–)-**2** in porcine kidney epithelial cells is not due to

formation of calcitriol or other active metabolites. The very low genomic activity and very slow rate of metabolism of 1α-hydroxymethyl analog (–)-**2** plus its high activity in inhibiting proliferation of murine keratinocytes (but not human leukemic cells) and in enhancing expression of late differentiation markers make this ring-A analog of calcitriol a promising candidate for study as a selective chemotherapeutic agent for skin diseases like psoriasis. **The unusual selectivity of biological function of such 1-(hydroxyalkyl)-25-hydroxyvitamin D_3 derivatives makes this class of ring-A analogs worthy of more detailed chemical and biochemical investigations with the aims of more thoroughly understanding their biological mechanism of action and of developing therapeutically useful new drugs.**

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Measurement of 24OHase activity: The enzyme activity was quantitated by measuring the conversion of [^{3}H]-25OHD$_3$ to [^{3}H]-24,25(OH)$_2$D$_3$ during 1 hour incubation at 37°C as described earlier.[13] Calciferols were extracted once with methanol:chloroform, and then twice with chloroform. Vitamin D metabolites were separated by HPLC on a Bondesil Silica Column (5 μm) with hexane:isopropanol (96:4) elution solvent. Radioactivity was measured in Packard TriCarb 2000 liquid scintillation analyzer. Purity and amount of (–)-**2** were tested before experiments by HPLC. Hormone effect was measured in three different experiments, each with triplicate data points. Data are expressed as the percent of conversion in hormone treated samples over the conversion in samples treated with vehicle alone. Statistical analysis of data was with t-test; data are presented as mean ± S.D.
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