Biochemical Pharmacology, Vol. 52, pp. 133–140, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00173-6

Anti-proliferative Activity and Target Cell Catabolism of the Vitamin D Analog 1α,24(S)-$(OH)_2D_2$ in Normal and Immortalized Human Epidermal Cells

Glenville Jones,† Valarie Byford,**
Hugh L. J. Makin,‡ Richard Kremer,§ Robert H. Rice,‖
Lea Ann deGraffenried,‖ Joyce C. Knutson¶ and Charles W. Bishop¶

*Department of Biochemistry, Queen's University, Kingston, ON, Canada K7L 3N6; ‡Department of Chemical Pathology, London Hospital Medical College, University of London, London, E1 2AD, U.K.; §Department of Medicine, McGill University, Montreal, PQ, Canada H3A 1A1; ‖Department of Environmental Toxicology, University of California-Davis, Davis, CA 95616; and ¶Lunar Corporation, Madison, WI 53713, U.S.A.

ABSTRACT. Vitamin D analogs represent valuable new agents for the suppression of proliferation of a variety of cell types, including those of the skin. One such analog is the vitamin D_2 metabolite, 1α,24(S)-dihydroxyvitamin D_2, which binds strongly to the vitamin D receptor and induces vitamin D-dependent gene expression *in vitro*. In the work described here, we studied the anti-proliferative activity and target cell metabolism of 1α,24(S)-dihydroxyvitamin D_2 in cells of human epidermal origin. We found this analog to be equally potent in its anti-proliferative effect to the hormone 1α,25-dihydroxyvitamin D_3. Furthermore, 1α,24(S)-dihydroxyvitamin D_2 was metabolized by the human keratinocyte cell line HPK1A-*ras* at a slower rate than either 1α,25-dihydroxyvitamin D_3 or calcipotriol, a drug used effectively in the treatment of psoriasis. We characterized the metabolic products of 1α,24(S)-dihydroxyvitamin D_2 as a mixture of side-chain truncated and hydroxylated products. The main product was identified by GC–MS and NMR techniques as 1α,24(S),26-trihydroxyvitamin D_2. The biological activity of this main product was determined in a vitamin D-dependent, growth-hormone reporter gene expression system to be lower than that of the parent molecule. We conclude from these data that 1α,24(S)-dihydroxyvitamin D_2 is a valuable new anti-proliferative agent with a slower rate of catabolism by cells of epidermal origin. Preliminary evidence suggests that the parent molecule, and not its products, is responsible for this biological activity *in vitro*. BIOCHEM PHARMACOL 52;1:133–140, 1996.

KEY WORDS. vitamin D analogs; 1α,25-dihydroxyvitamin D_3; 1α,24(S)-dihydroxyvitamin D_2; human epidermal cells; human keratinocyte, HPK1A-*ras*; anti-proliferative activity; GC–MS; NMR

The demonstration of a duality of functions for the hormone calcitriol [1α,25-$(OH)_2D_3$]** in calcium homeostasis and cell differentiation has led to the development of vitamin D analogs for use in hyperproliferative disorders such as in dermatology [1]. Many of these dermatologically useful analogs are termed "non-calcemic" because they do not cause an increase in plasma calcium when they are administered topically at concentrations that cause improvement of the hyperproliferative condition (e.g. psoriasis) [2]. One compound, calcipotriol [3], has been shown to be an effective treatment for psoriasis [4], and is now licensed for use in the U.S.A., Europe, and worldwide. Several other vitamin D analogs are under development for use in psoriasis, osteoporosis, breast cancer, and hyperparathyroidism [1, 5]. One of these compounds is a vitamin D_2 derivative, 1α,24(S)-$(OH)_2D_2$, which can be formed as a natural product when large doses of vitamin D_2 are administered to animals *in vivo* [6] or from 1α-OH-D_2 when incubated with the hepatoma Hep3B *in vitro* [7]. This analog possesses a 2-fold lower affinity than calcitriol for the VDR; has a 10-fold lower affinity than calcitriol for the plasma DBP, and is able to induce vitamin D-dependent gene expression in VDRE-mediated GH- and CAT-reporter transcriptional activity models [7]. With a possible anti-psoriatic application in mind, we describe here studies in which we set out to measure the anti-proliferative activity of 1α,24(S)-

† Corresponding author. Tel. (613) 545-2498; FAX (613) 545-2987.

** *Abbreviations:* 1α,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 1α,24(S)-$(OH)_2D_2$, 1α24(S)-dihydroxyvitamin D_2; DBP, vitamin D binding protein; VDR, vitamin D receptor; VDRE, vitamin D response element; hGH, human growth hormone; DPPD, 1,2-dianilinoethane; HIM, hexane–isopropanol–methanol; and FBS, fetal bovine serum.

Received 7 September 1995; accepted 19 January 1996.

$(OH)_2D_2$ in the appropriate cell type, namely normal human epidermal cells.

In previous work, we have demonstrated the ability of vitamin D-target cells to catabolize the hormone calcitriol via a calcitriol-inducible-C24-oxidation pathway to less active catabolites including the C23 truncated compound calcitroic acid [8, 9]. We have postulated that such a catabolic pathway would inactivate calcitriol, thereby desensitizing the target cell to further hormonal stimulation [8, 9]. Our additional goal in the current studies was thus to study the nature and rate of catabolism of 1α,24(S)-$(OH)_2D_2$ in an appropriate target cell, in this case the keratinocyte cell line HPK1A-*ras*. We recently used this cell line to successfully study the destruction of calcitriol and calcipotriol to less active products [10]. Parts of this work have been published previously in a preliminary form [11].

MATERIALS AND METHODS

Vitamin D Compounds and Cell Lines Used

1α,24(S)-$(OH)_2D_2$ and 1α,24(*R*)-$(OH)_2D_2$ were synthesized as previously described [7]. 1α,25-$(OH)_2D_3$ was a gift of Dr. Milan Uskokovic, Hoffmann-LaRoche (Nutley, NJ, U.S.A.).

The human keratinocyte cell line HPK1A [12] transformed with the *ras* oncogene, HPK1A-*ras* [13], was developed previously in collaboration with Dr. Johng Rhim (National Cancer Institute). The SV-40 transformed African Green Monkey kidney cell line COS-1 was purchased from the American Tissue Culture Collection (Rockville, MD, U.S.A.). The two vectors used in transfection experiments were gifts from Dr. M. Haussler and Dr. K. Whitfield (University of Arizona, Tucson, AZ, U.S.A.): pSG5-hVDR1/3 plasmid [14], which has the hVDR1/3 DNA inserted into the *Eco*RI site of pSG5 vector, and $(CT4)^4$TKGH plasmid, which has four copies of the CT4 synthetic rat osteocalcin VDRE ligated and annealed into the pTKGH vector [15] that contains a thymidine promoter linked to the hGH gene. hGH radioimmunoassay kits were purchased from Nichols Diagnostics (San Juan Capistrano, CA, U.S.A.). Trypsin, penicillin G, gentamycin, fungizone and Dulbecco's modified Eagle's medium were purchased from Gibco (Grand Island, NY, U.S.A.). *N*-Trimethylsilylimidazole (TSIM) was purchased from the Pierce Chemical Co. (Rockford, IL, U.S.A.). Organic solvents of HPLC grade were obtained from Caledon Laboratories (Georgetown, Ontario, Canada). FBS and BSA were from ICN (Costa Mesa, CA, U.S.A.). DEAE-dextran was obtained from Pharmacia (Uppsala, Sweden). Other reagents used for transfection: chloroquine, DMSO, and the antioxidants DPPD and butylated hydroxytoluene (BHT) were supplied by the Sigma Chemical Co. (St. Louis, MO, U.S.A.). All solvents used were of HPLC grade and were obtained from Caledon Laboratories.

Anti-proliferative Activity Assay

Normal human epidermal cells were passaged routinely with 3T3 feeder layer support in a DMEM-F12 blend containing 5% FBS [16]. For treatment with vitamin D analogs, they were transferred to commercial low calcium (0.15 mM Ca^{2+}) serum-free medium (Clonetics, San Diego, CA, U.S.A.) that was changed at 2- to 3-day intervals. Since only a small minority of the cells from feeder layer cultures (the basal-like ones) attach in the low calcium medium, 30–100 × 10^5 cells were inoculated in 6-cm dishes. Dissolved in ethanol, the vitamin D analogs were added on day 4, at which time a baseline cell number was determined, and at each subsequent medium change. The final ethanol concentration was 0.1%. Cultures were harvested 7–10 days later, and the cell number was measured electronically with a Coulter counter. Occasional experiments using the SIK line of spontaneously immortalized human epidermal cells [17] were performed without feeder layer support in a 3:1 mixture of the above commercial low calcium medium and the DMEM-F12 blend supplemented with 10% FBS.

Generation of Metabolites of 1α,24(S)-$(OH)_2D_2$ using HPK1A and HPK1A-ras Cells

Metabolism was studied using HPK1A-*ras* or HPK1A cells as described previously [10, 18]. Cells were maintained in 150-mm plates using Dulbecco's culture medium (Dulbecco's modified Eagle's medium). Near confluence, cells were treated with 1α,25-$(OH)_2D_3$ (10 nM) to induce transcription of catabolic enzymes. After 18 hr, the medium was replaced by Dulbecco's modified Eagle's medium supplemented with 1% BSA and 100 μM DPPD. Cells were then incubated for 48 hr in the presence of vehicle (0.01% EtOH) or a 10 μM concentration of analog in vehicle.

Purification of Metabolites

Cells and medium were extracted as described previously [18, 19]. HPLC separation of metabolites was achieved using a Zorbax-SIL column (0.62 × 8 cm; 3 μm) eluted with HIM (91:7:2) at a flow rate of 1 mL/min. Metabolites were identified based on their characteristic vitamin D chromophore (UV_{max} = 265 nm, UV_{min} = 228 nm, UV_{max}/UV_{min} = 1.75). Further HPLC steps were performed on the crude peaks isolated from the first step of purification, using a Zorbax-CN (0.46 × 25 cm; 6 μm) column and an HIM (91:7:2) solvent system at a flow rate of 1 mL/min. Two rounds of HPLC yielded metabolites that gave single homogeneous peaks.

Chemical Modification of Metabolites

Purified metabolites were subjected to chemical modification using sodium metaperiodate or sodium borohydride as described previously [18]. Modified metabolites were subjected to HPLC analysis using the system described above.

GC–MS

Purified metabolites were derivatized to pertrimethylsilyl ethers and then analyzed by GC–MS as described previ-

ously [18]. Injection of metabolites of analogs described in this paper into the high temperature injection zone of the GC causes B ring closure producing pyro- and isopyro-isomers, but, for simplicity, in discussion of fragmentation and in figures illustrating spectra obtained, the uncyclized metabolite structure is used rather than that of the correct pyro-isomer. Mass spectra were obtained by averaging each pyro-peak and subtracting the background.

NMR

One-dimensional and two-dimensional magnitude COSY spectra were obtained using a Bruker AMX-600 (Bruker Instruments, Coventry, U.K.) operated by the University of London Inter-Collegiate Research Services (ULIRS) NMR Facility based at Queen Mary & Westfield College, University of London, U.K.

The sample of peak 11 [1α,24(S),26-$(OH)_3D_2$] (60 μg) was dissolved in 0.5 mL deuterated chloroform ($^2ChCl_3$, 99.8%; Goss Scientific Instruments, London, U.K.), which provided both a field/frequency lock and an internal chemical shift reference (residual $CHCl_3$, δ = 7.27 ppm). The spectrometer was operating at 600 MHz with a probe temperature of 300°K.

The parameters for the one-dimensional spectrum were: 1024 transients collected using a spectral band width of 7692.31 Hz and 32,768 data points, giving an acquisition time of 2.13 sec. A pulse angle of 53° and a relaxation delay of 2 sec were employed.

The two-dimensional shift-correlated (COSY) spectrum was acquired in magnitude mode with 2048 data points in the t2 dimension, 502 increments of t1, a relaxation delay of 2 sec, and 48 transients. A sine-bell function was employed in both dimensions during processing.

Transfection and GH Assays

The DEAE-dextran method of Sambrook *et al.* [20] was used for the cotransfection of the two vectors pSG5-hVDR1/3 and $(CT4)^4$TKGH into COS-1 cells, which were about 80% confluent. Each plasmid containing 5 μg of total DNA was added to a 150-mm culture plate of COS-1 cells. Following a 30-min exposure to the DNA–DEAE–dextran mixture, cells were treated with 80 μM chloroquine for 4.5 hr. Cells were subjected to DMSO shock for 2.5 min, and then incubated overnight in DMEM containing 10% FBS. The cells were trypsinized and subcultured at a 1:54 pass ratio into 12-well plates in duplicate and incubated for 5 hr. The cells were treated with vehicle (ethanol) or different concentrations of 1α,25-$(OH)_2D_3$, 1α,24(S)-$(OH)_2D_2$, and its principal metabolite, peak 11, later identified as 1α,24(S),26-$(OH)_3D_2$. After 48 hr, 40-μL aliquots of medium were removed from each well, and transcriptional activity was quantitated by radioimmunoassay of hGH using the procedure supplied by the kit manufacturers.

RESULTS

Anti-proliferative Activity of 1α24(S)-$(OH)_2D_2$

Addition of vitamin D analogs reduced the growth of normal human epidermal cells in culture. In the experiment illustrated in Fig. 1, each vitamin D analog produced a moderate reduction in growth at 0.1 μM and a complete cessation at 1 μM. Although minor differences in potency among the three vitamin D analogs were observed in such experiments, differences were not clear and reproducible. In two lots of the calcium commercial medium in which cell multiplication was considerably less than that shown, the analogs stimulated growth slightly at concentrations in the range of 0.1 μM. In other experiments employing the SIK line of spontaneously immortalized keratinocytes in serum-containing medium, the agents were essentially indistinguishable in potency, although the observed degree of growth inhibition was substantially less than illustrated for the normal epidermal cells.

Rate of Metabolism of 1α,24(S)-$(OH)_2D_2$ by HPK1A and HPK1A-ras Cells

We found that 1α,24(S)-$(OH)_2D_2$ was metabolized similarly by both HPK1A and HPK1A-*ras* cells, each cell line giving qualitatively the same pattern of products on HPLC. However, HPK1A-*ras* cells produced quantitatively more metabolic products than HPK1A (data not shown). When we used HPK1A-*ras* to compare the rate of metabolism of 1α,24(S)-$(OH)_2D_2$ to two other vitamin D analogs, 1α,25-$(OH)_2D_3$ and calcipotriol, we observed about half the rate

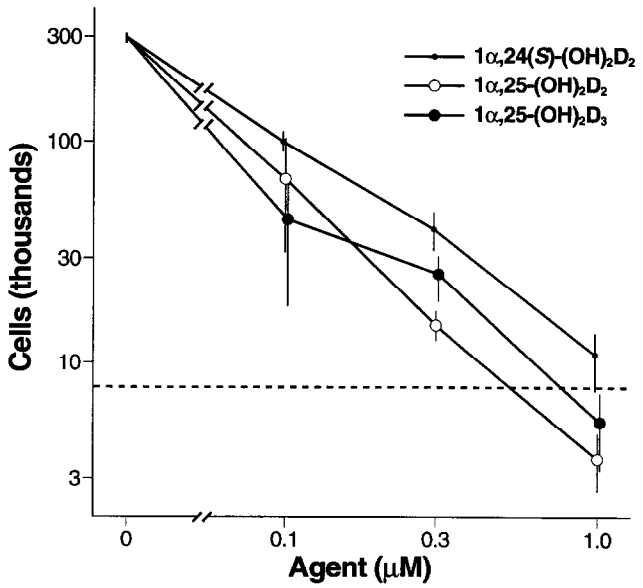

FIG. 1. Inhibition of human epidermal cell growth by vitamin D analogs. Cultures were treated with 1α,24(S)-$(OH)_2D_2$, 1α,25-$(OH)_2D_2$, or 1α,25-$(OH)_2D_3$ at the concentrations indicated and harvested 10 days later. Values are the means ± SD of triplicate cultures. The dashed line shows the baseline cell number when the analogs were first added to cultures.

of product formation for 1α,24(S)-$(OH)_2D_2$ as compared with control incubations (Fig. 2). In the experiment depicted in Fig. 2, the rate of disappearance of 1α,24(S)-$(OH)_2D_2$ from the incubation medium was not significantly different from that of the other two vitamin D compounds. This is due, in part, to the greater variability inherent in measuring small changes in the substrate concentration. Furthermore, in other experiments where cells reached a higher cell density or were more metabolically active, the catabolism of 1α,24(S)-$(OH)_2D_2$ approached 30–40% of substrate and became significantly different from the other vitamin D analogs where it reached in excess of 60% of substrate. In these experiments, the appearance of lipid-soluble, UV-absorbing metabolic products from 1α,24(S)-$(OH)_2D_2$ was always about 50% slower than in control incubations.

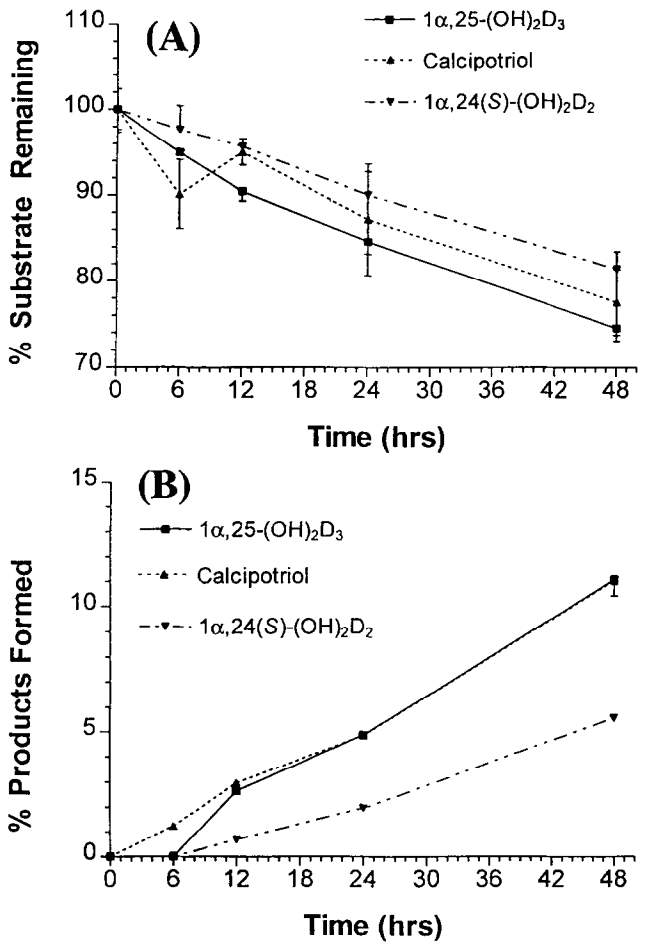

FIG. 2. Comparison of the rate of metabolism of 1α,24(S)-$(OH)_2D_2$ with that of other vitamin D analogs, 1α,25-$(OH)_2D_3$ and calcipotriol, in HPK1A-*ras* cells. The rate of disappearance of each substrate (A) was followed, as well as the rate of appearance of the metabolic products (B) of each (as the sum of all recovered lipid-soluble products) over a 48-hr incubation period. 1α-OH-D_3 was added as an internal standard to assess recovery during extraction and HPLC. Each data point represents the mean ± SEM of triplicate determinations.

Identification of the Metabolic Products of 1α,24(S)-$(OH)_2D_2$

Since the pattern of metabolites was qualitatively identical for both HPK1A and HPK1A-*ras* cells, our efforts were focussed on the identification of the products of the latter more metabolically active cell line. A typical HPLC pattern from incubating 10 μM 1α,24(S)-$(OH)_2D_2$ with HPK1A-*ras* cells is shown in Fig. 3. Several UV peaks possessing the vitamin D chromophore can be discerned, and these were collected as peaks 4–11. Aside from the unchanged substrate, **peak 5,** and the main product, **peak 11,** the remaining peaks were only partially purified and thus are discussed only briefly below:

Peak 4 (at 7.6 min in Fig. 3) was identified tentatively as either 1α,24(S)-$(OH)_2$-previtamin D_2 or the 3-epi-1α,24(S)-$(OH)_2D_2$.

Peak 5 contained unchanged substrate.

Peaks 6 and 7 contained multiple components of which three were run on GC–MS giving molecular ions of *m/z* 530, 602, and 604. The size of these metabolites is consistent with them being molecules truncated between carbons 24 and 25 of the side chain.

Peaks 9 and 10 possessed the vitamin D chromophore but were present in too small a quantity to purify and subject to GC–MS. They remain unidentified.

Peak 11 proved to be the most polar of the metabolites collected and when chromatographed on Zorbax-CN, it ran as a single component at 17.34 min. We obtained 4.225 μg of which 2 μg was used for GC–MS. This compound was converted to a pertrimethylsilylated derivative that ran with a retention time of 18.305 min on GC, suggesting a molecule larger than 1α,24(S)-$(OH)_2D_2$. The mass spectrum peak 11 (Fig. 4) showed a molecular ion of *m/z* 732 and fragments at *m/z* 642 (M^+-90); 601 (M^+-131 due to the loss of C_2, C_3, and C_4); 511 (M^+-131-90); 421 (M^+-131-90-90); and 331 (M^+-131-90-90-90). Peak 11 is thus a mono-hydroxylated derivative of 1α,24(S)-$(OH)_2D_2$, but the exact position of the extra hydroxyl was not revealed by the GC–MS analysis. Again the fragmentation pattern included as a prominent ion *m/z* 143, which is derived from a molecule with unchanged C22 through C24 of the side chain of 1α,24(S)-$(OH)_2D_2$. Based upon the GC–MS data, particularly the retention of this *m/z* 143 fragment and the lack of a *m/z* 131 found in all 25 hydroxylated vitamin D metabolites, the extra hydroxyl group is probably at C-26(27).

As expected, peak 11 was *not* cleaved by sodium *m*-periodate treatment, confirming that the extra hydroxyl was not vicinal to 24(S) at C-25 or C-28 in the side chain of the metabolite. Although the positive control, 1α,24(*R*),25-$(OH)_3D_3$, *was* cleaved successfully under these conditions, steric hindrance is sometimes observed in the side chain of vitamin D_2 compounds, and this cannot be ruled out as a reason for the negative periodate reaction with peak 11. Nevertheless, based upon the combined

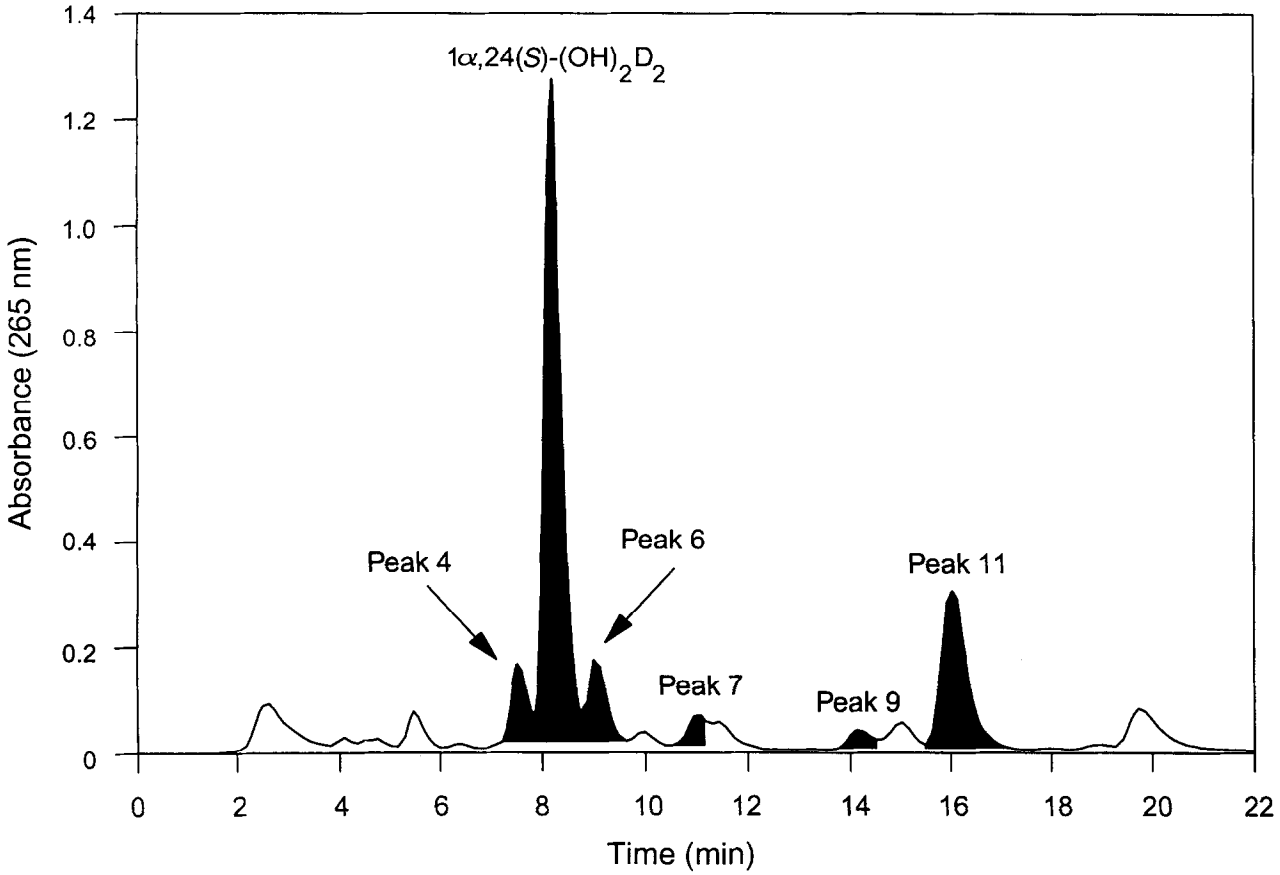

FIG. 3. HPLC profile of lipid extract of 1α,24(S)-$(OH)_2D_2$ incubated with HPK1A-*ras* cells. HPLC conditions: Zorbax-SIL (0.62 × 8 cm; 3 μm); HIM, 91:7:2; 1 mL/min. Peaks possessing the vitamin D chromophore are shown in black. See text for details of subsequent analysis.

HPLC, GC–MS and periodate data, we concluded that peak 11 was probably 1α,24(S)-26-$(OH)_3D_2$.

NMR studies confirmed our interpretation. A total of 72 μg of purified peak 11 was obtained from roller culture incubation with HPK1A-*ras* cells, 60 μg of which was used for NMR studies that revealed the location of the extra hydroxyl function and confirmed the identity of peak 11 as 1α,24(S),26-$(OH)_3D_2$. The 600 MHz 1-D proton NMR (Fig. 5) showed the typical features of a 1α-hydroxylated vitamin D_2 [21]. These assignments are: 6.38 (d, 1H, 6-H); 6.0 (d, 1H 7-H); 5.4–5.56 (m, 24, 22-H, and 23-H); 5.33 [m (sharp), 1H, 19(Z)-H]; 5.0 [m (sharp), 1H, 19(E)-H]; 4.43 (m, 1H, 1-H); 4.23 (m, 1H, 3-H); 1.31 (s, 3H, 28-CH_3); 1.06 (d, 3H, 21-CH_3); 0.83 (d, 3H, 27-CH_3), all of which were also observed in the spectrum of 1α,24(S)-$(OH)_2D_2$ reported previously [7]. Additional features included the addition of a multiplet at 3.6–3.75, which can be attributed to the addition of a 26 hydroxyl group. Consistent with this interpretation was the disappearance of the doublet at 0.85–0.90 corresponding to the unsubstituted C-26 methyl group present in the spectrum of 1α,24(S)-$(OH)_2D_2$. Similar observations were made in the NMR spectrum of 24,26-$(OH)_2D_2$ by Koszewski *et al.* [22], and these conclusions were substantiated by two-dimensional COSY spectra obtained in the present study (data not shown), which showed coupling of C-26 protons to the C-25 proton. We have, therefore, conclusively identified peak 11 as 1α,24(S),26-$(OH)_3D_2$.

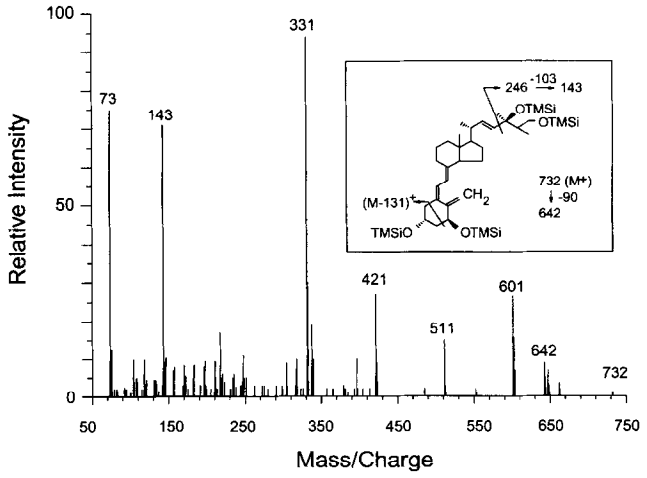

FIG. 4. EI mass spectrum of the pertrimethylsilyl ether of peak 11 collected from the HPLC shown in Fig. 3. Inset is the putative fragmentation pattern of the metabolite.

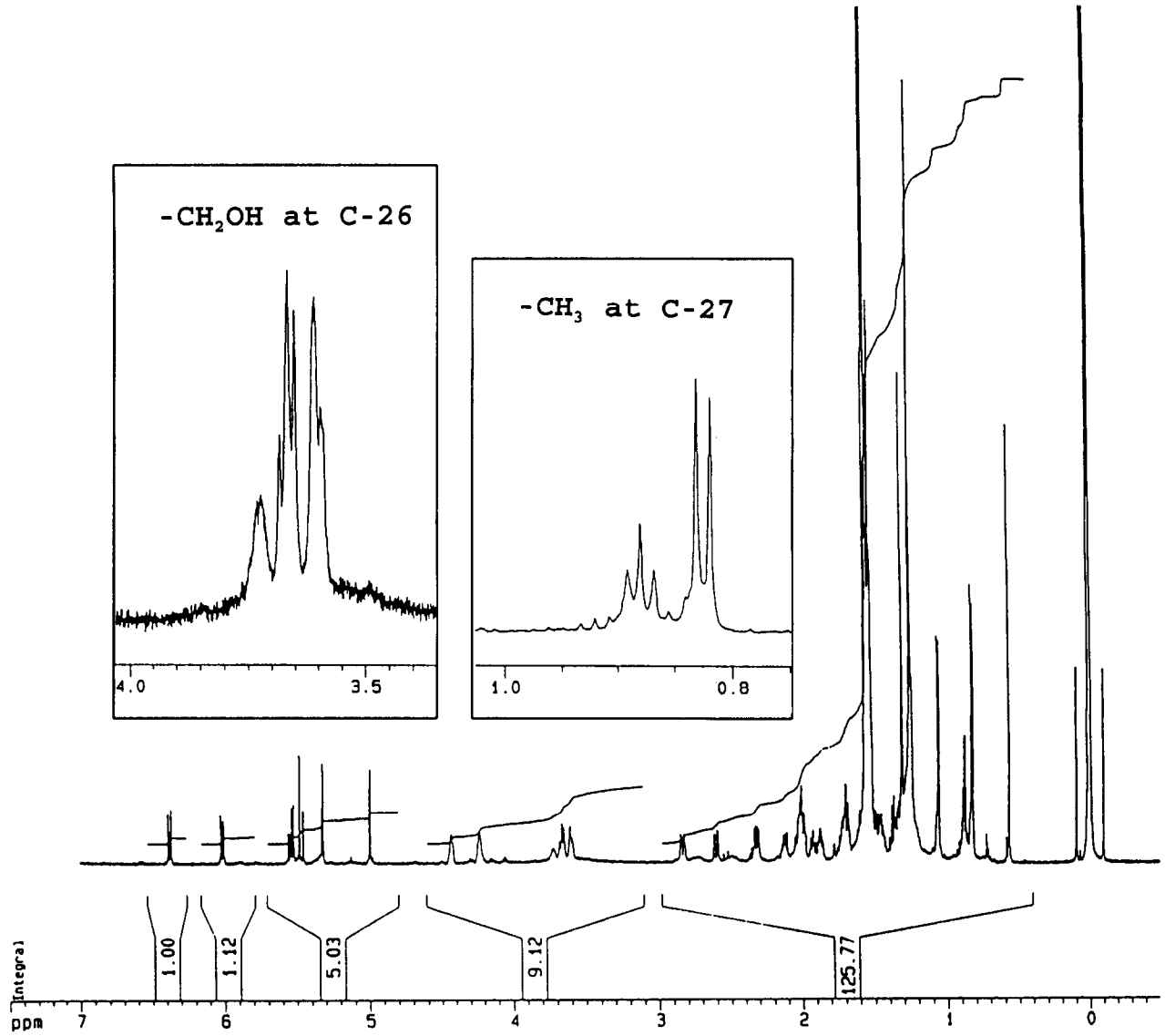

FIG. 5. Six hundred MHz ^{1}H-NMR of peak 11, later identified as 1α,24(S),26-$(OH)_3D_2$. Shown in the two insets are magnifications of important regions of the spectrum that showed differences to spectra reported for 1α,24(S)-$(OH)_2D_2$. See text for interpretation of the spectrum.

Biological Activity of Peak 11 *in hGH-Reporter Assays*

Peak 11 was assayed at concentrations ranging from 10^{-7} to 10^{-9} M, whereas control compounds 1α,24(S)-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$ were assayed over a slightly lower range (Fig. 6). Both of the control vitamin D analogs were effective at concentrations between 10^{-10} and 10^{-9} M, whereas peak 11 at this concentration gave an activity only just above that of the uninduced basal promoter, as measured in the ethanol-stimulated blank. We estimate peak 11 to be about 5–10 times less effective than 1α,25-$(OH)_2D_3$ or 1α,24(S)-$(OH)_2D_2$ in this assay.

DISCUSSION

This paper demonstrates the potent anti-proliferative activity of a novel vitamin D analog, 1α,24(S)-$(OH)_2D_2$, in human epidermal cells *in vitro*. The new compound was essentially indistinguishable from 1α,25-$(OH)_2D_3$ for this biological parameter, a finding that is consistent with previous results for 1α,24(S)-$(OH)_2D_2$ in VDRE-driven reporter gene assays in monkey kidney cells and rat osteosarcoma cells (ROSCO) [7]. In addition, we have shown here that, like 1α,25-$(OH)_2D_3$, 1α,24(S)-$(OH)_2D_2$ is susceptible to catabolism in target cells but at a reduced rate. The principal product of 1α,24(S)-$(OH)_2D_2$ was identified as 1α,24(S),26-$(OH)_3D_2$, which implies a different pathway of metabolism from the C-24 oxidation pathway observed for calcitriol. This catabolite, 1α,24(S),26-$(OH)_3D_2$, which appeared to be around 10 times less active than its parent compound, was accompanied by side-chain cleaved molecules that had lost carbons 25, 26, and 27, and in which the C22=C23 double bond had been reduced. Similar truncated metabolites with a reduced C22=C23 double bond

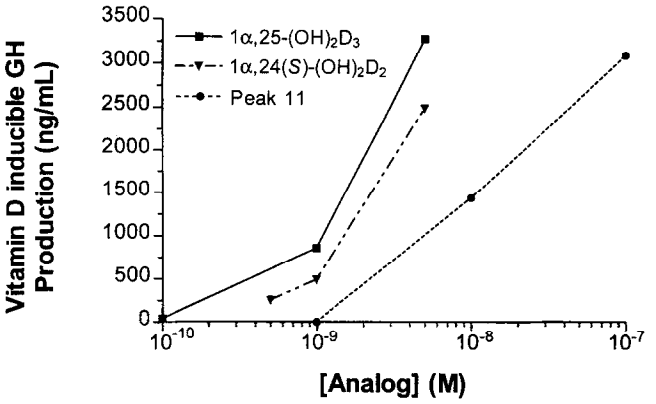

FIG. 6. hGH transcriptional activation by vitamin D metabolites including purified peak 11 [1α,24(S),26-$(OH)_3D_2$]. Key to symbols: 1α,24(S)-$(OH)_2D_2$, (▼); 1α,25-$(OH)_2D_3$ (■); and peak 11 [1α,24(S),26-$(OH)_3D_2$] (●).

have been reported to be formed *in vitro* from calcipotriol [10]. Taken together these findings suggest that 1α,24(S)-$(OH)_2D_2$ represents a new, anti-proliferative analog of vitamin D with potential for use in skin tissues which offers the further advantage that it is metabolized in target cells at one-half the rate of the natural hormone, 1α,25-$(OH)_2D_3$.

The identification of the principal catabolite as 1α,24(S),26-$(OH)_3D_2$ suggests the presence of a 26-hydroxylase enzyme inside target cells. Others have documented 26-hydroxylated vitamin D_2 compounds *in vivo* [22], but the enzymes responsible for their formation have never been characterized. There are two likely sources of this enzyme activity, namely the 26-hydroxylase of 25-OH-D_3 which is involved in 26,23-lactone formation [23], and the liver 25-hydroxylase which has been shown to be capable of 26(27)-hydroxylation of vitamin D_3 and bile acids. The cytochrome P450 for this latter enzyme is purported to be CYP27, and in transfection experiments, this enzyme has been shown to be capable of 24-, 25-, or 26(27)-hydroxylation of a number of vitamin D compounds [24]. Indeed, if CYP27 is present in HPK1A-*ras* cells, and thus far this has not been proven, then it could be responsible for the 26-hydroxylation observed. However, this would make CYP27 responsible for not only the synthesis of 1α,24(S)-$(OH)_2D_2$ (in the liver) but also its destruction in the target cell (and presumably also in the liver).

Our choice of the transformed human keratinocyte cell line, HPK1A-*ras*, for the metabolism experiments was dictated by the lack of a suitably labelled radioactive 1α,24(S)-$(OH)_2D_2$ substrate. Radioactive substrates allow the use of lower concentrations of substrate and smaller numbers of cells. This, in turn, makes it possible to study vitamin D metabolism in cell types that grow more slowly, such as in primary keratinocyte cultures [25–27]. Nevertheless, there is no evidence that primary keratinocytes or keratinocyte cell lines, HPK1A or HPK1A-*ras*, metabolize vitamin D qualitatively any differently [10]. Certainly, the qualitative picture obtained here for 1α,24(S)-$(OH)_2D_2$ metabolism is no different between the cell line HPK1A-*ras* and its precursor, HPK1A. Furthermore, since several vitamin D analogs, including 1α,24(S)-$(OH)_2D_2$, are being developed for use in the treatment of psoriasis and in the absence of good *in vitro* models for psoriatic keratinocytes, it would seem prudent to study effects and metabolism of vitamin D in as many of keratinocytes as possible. Of course, as with any *in vitro* model, validation of these results will await the effect of the pharmacological agent *in vivo*.

The reduced rate of product formation from 1α,24(S)-$(OH)_2D_2$ as compared with the hormone 1α,25-$(OH)_2D_3$ suggests that the new analog is catabolized more slowly. Though the rate of disappearance of 1α,24(S)-$(OH)_2D_2$ does not exactly match the rate of appearance of its metabolic products, this disappearance of 1α,24(S)-$(OH)_2D_2$ does become significantly different from that of 1α,25-$(OH)_2D_3$ when we allow metabolism to be more extensive. We interpret this result to mean that small differences in the disappearance of different substrates are masked by the large variations in the measurement of the remaining material. Another interpretation is that there are no differences in substrate recovered, and the small but significant differences in products recovered are due to the *loss* of other (lipid-soluble or water-soluble?) products during the extraction procedure. Though we cannot categorically rule out such a possibility, thus far we have failed to detect water-soluble metabolites of 1α,24(S)-$(OH)_2D_2$, and furthermore, we used an internal standard to correct for recovery of lipid-soluble products. If one accepts our case for differences in the rate of vitamin D-target cell catabolism of 1α,24(S)-$(OH)_2D_2$ in the context of its other properties, namely, similar VDR binding and lower DBP binding than 1α,25-$(OH)_2D_3$, then this constitutes a novel spectrum of biological activities. Such properties give 1α,24(S)-$(OH)_2D_2$ potential to be used as a drug [1] since the molecule probably circulates bound to a non-specific plasma protein rather than to DBP [28, 29] as do other natural vitamin D compounds, since it binds avidly to the VDR [30], and since it resists target metabolism via the C-24 oxidation pathway [8, 9]. This set of activities probably explains the differences in metabolic clearance observed for 1α,24(S)-$(OH)_2D_2$ as compared with 1α,25-$(OH)_2D_2$ and 1α,25-$(OH)_2D_3$ in the rat [31].

It is not the first time that the technique of proton NMR has been applied for the definitive identification of small amounts of biologically generated vitamin D metabolites [22, 32]. However, we believe that this is the first use of a 600 MHz instrument, with its higher resolving power, in this context. Usually GC–MS techniques provide definitive proof of metabolite identity but, in rare cases such as this, the data contained in the mass spectrum (in Fig. 4) are inconclusive. Koszewski *et al.* [22] reached the same conclusion in their work on 24,26-$(OH)_2D_2$, and they analyzed 5 μg of material using a 300 MHz NMR instrument in order to obtain definitive proof of identity. We obtained a strong spectrum that was devoid of artifactual peaks from 60 μg of pure material here, and the resolution with the 600 MHz instrument was excellent. It is clear that high field strength

proton NMR may have increasing value in confirming the structures of vitamin D metabolites assigned on the basis of mass spectral data.

This work was supported by a joint University–Industry grant (UI-11884) from the Medical Research Council of Canada. We thank Dr. Harold Toms for operation of the NMR instrument used in these studies.

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