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INCREASED BIOLOGICAL ACTIVITY OF 20-EPI-1,25-DIHYDROXYVITAMIN D_3 IS DUE TO REDUCED CATABOLISM AND ALTERED PROTEIN BINDING*

F. JEFFREY DILWORTH,† MARTIN J. CALVERLEY,‡ HUGH L. J. MAKIN§ and GLENVILLE JONES†‖

†Department of Biochemistry, Queen's University, Botterell Hall, Kingston, Ontario, Canada K7L 3N6; ‡Chemical Research Department, Leo Pharmaceutical Products, Ballerup, Denmark; and §Department of Chemical Pathology, London Hospital Medical College, University of London, London, U.K.

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Abstract—The 20-epi series of vitamin D_3 analogs has been shown to be made up of more potent inducers of cell differentiation than calcitriol *in vitro*. Using 20-epi-1α,25-dihydroxyvitamin D_3 (MC 1288), we attempted to rationalize this increased biological activity by examining several parameters including the binding affinity of the analog for the plasma binding globulin (DBP) and the target cell vitamin D receptor (VDR), as well as attempting to measure the rate at which MC 1288 is metabolized. MC 1288 was found to be metabolized 36 times more slowly than its epimer 1,25-dihydroxy vitamin D_3 (1,25-$(OH)_2D_3$), forming several metabolites which were analogous to metabolites of 1,25-$(OH)_2D_3$ formed in the side chain oxidation pathway. Bovine thymus VDR bound MC 1288 with five times greater affinity than calcitriol, while rat plasma DBP did not bind MC 1288 even at a concentration of 50 μM, 5000 times the B_{50} of 25-OH-D_3, the ligand used in the assay. Using a vitamin D-inducible growth hormone gene reporter system we were able to demonstrate that MC 1288 induces human growth hormone (hGH) activity 30-fold more efficiently than 1,25-$(OH)_2D_3$ in the presence of fetal calf serum (FCS), while the analog is only 10 times more efficient than 1,25-$(OH)_2D_3$ in the absence of FCS. We therefore conclude that MC 1288 is more biologically active than calcitriol *in vitro* due to a combination of factors: the increased VDR binding affinity, the decreased DBP binding affinity, and the decreased rate of metabolism. As with other analogs of vitamin D, the altered protein binding and decreased catabolism of MC 1288 may be important in pharmaceutical applications such as a topical treatment for psoriasis or skin cancer.

Key words: 1,25-dihydroxyvitamin D_3; vitamin D metabolism; vitamin D receptor; vitamin D analogs; vitamin D binding globulin; vitamin D dependent gene expression

The active form of vitamin D_3, 1,25-$(OH)_2D_3$¶, has been shown to be a potent inducer of cell differentiation [1]. This cell differentiation effect is modulated through a binding interaction between VDR and calcitriol which allows the complex to induce transcription of genes with a VDRE in their promoter [2]. However, the clinical usefulness of calcitriol or its analogs as cell differentiation agents is limited by the potent calcium regulatory properties of the molecules. This has prompted researchers to search for vitamin D analogs which would possess anti-tumor effects without calcemic activity.

Much attention has been paid to modification of the side chain of vitamin D since biological activity can often be retained or even increased by this change. Another stimulus for this attention to the side chain is that 1,25-$(OH)_2D_3$ is subject to hydroxylations at carbons 23, 24 and/or 26 [3]. Several side chain-modified analogs which have increased biological activity *in vitro* have already been synthesized, including calcipotriol [4], and 22-oxa-calcitriol [5].

One group of vitamin D analogs with side-chain modification that have been developed is the 20-epi series in which the stereochemistry at C-20 is inverted [6]. Most of the analogs with the 20-*S* configuration have been shown to be more biologically active in cell differentiation models *in vitro* while their calcemic activity was only slightly elevated *in vivo* [7].

In this study, the simplest molecule in the series, 20-epi-1,25-$(OH)_2D_3$ (MC 1288) (Fig. 1), was used to examine several parameters which may affect the activity of the analogs including: (1) how well the molecule binds to VDR; (2) how well the molecule binds to the DBP; (3) how the molecule is metabolized in the cell; and (4) the rate at which the molecule is metabolized. Using this approach we hoped to reveal those parameters which might be important for expression of the increased cell differentiating activity *in vitro*, and to extrapolate the effects of these parameters to an *in vivo* scenario.

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‖ Corresponding author.

¶ Abbreviations: 1,25-$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; DBP, vitamin D binding globulin; VDR, vitamin D receptor; VDRE, vitamin D response element; DPPD, *N*,*N*′-diphenylethylenediamine; HIM, hexane/isopropyl alcohol/methanol, FCS, fetal calf serum, GH, growth hormone.

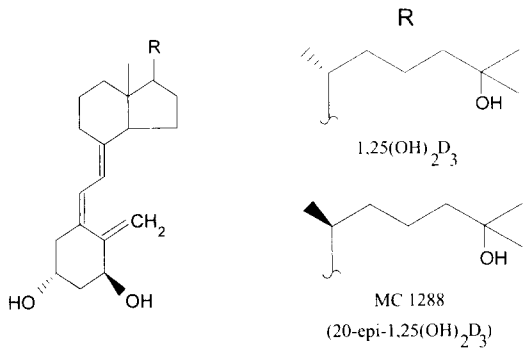

Fig. 1. The structure of MC 1288 and 1,25-$(OH)_2D_3$.

MATERIALS AND METHODS

25-hydroxyvitamin D_3 (25-OH-D_3) and 1,25-$(OH)_2D_3$ were gifts from Dr M. Uskokovic (Hoffmann LaRoche, Nutley, NJ, U.S.A.). 25-hydroxy-[26,27-methyl-^{3}H]-vitamin D_3 ([^{3}H]-25-OH-D_3) (28 Ci/mmole) was purchased from Amersham (Amersham, U.K.). [1β-^{3}H]-1,25-dihydroxyvitamin D_3 ([^{3}H]-1,25-$(OH)_2D_3$) (3 Ci/mmol) was synthesized by a method described previously [3]. The human keratinocyte cell line [8] transfected with the ras protooncogene [9], HPK1A-ras was the generous gift of Dr R. Kremer (Royal Victoria Hospital, McGill University, Montreal, PQ, Canada). The rat osteosarcoma cell line, UMR-106 [10] was obtained from Dr J. N. M. Heersche (University of Toronto, Toronto, ON, Canada). The SV40 transformed African Green Monkey kidney cell line, COS-1 was obtained from the American Tissue Culture Collection (Rockville, MD, U.S.A.). Male Wistar rats (average 300 g) purchased from Charles River (Canada) Inc. (St Constant, PQ, Canada) were used to prepare plasma for the DBP assay. All solvents used were of HPLC grade and were obtained from Caledon (Toronto, ON, Canada). The vectors pSG5-hVDR1/3 [11] and p(CT4)^{4}TKGH [12] used in the transfection experiments were the generous gift of Drs Mark Haussler and Kerr Whitfield (University of Arizona, Tuscon, AZ, U.S.A.).

Synthesis of (1S,3R,5Z,7E,20S)-9,10-*secocholesta*-5,7,10(19)-*triene*-1,3,25-*triol*[20-*epi*-1,25-$(OH)_2D_3$, *MC* 1288]. The synthesis of 20-epi-1,25-$(OH)_2D_3$ (hereafter referred to as MC 1288) was carried out as previously outlined [6]. The vitamin D analog was purified to chromatographic homogeneity on silica gel using ethyl acetate as eluant and obtained as an oil. A stock solution prepared in propan-2-ol was quantified by UV spectroscopic analysis (diluting an aliquot with 96% ethanol), assuming an extinction coefficient (λ_{265} nm) 17,500 dm^3/mol/cm for MC 1288.

Transfection and human GH assay. The vectors pSG5-hVDR1/3 and p(CT4)^{4}TKGH were co-transfected into COS-1 cells as described previously by Qaw *et al.* [13]. In serum free medium, 5 μg of each plasmid were added to 150-mm culture dishes of COS-1 cells. Cells were treated, in triplicate, with vehicle (0.15% ethanol) or different concentrations of 1,25-$(OH)_2D_3$ or MC 1288 in the presence of 10% FCS or 1% BSA. After 48 hr, a 100 μL aliquot of medium was removed from each well. Aliquots were assayed for GH using a human GH radioimmunoassay kit (Nichols Diagnostics) as outlined in the protocol provided.

VDR binding study. The ability of MC 1288 and 1,25-$(OH)_2D_3$ to compete with [26,27-$^3H_2$]-1,25-$(OH)_2D_3$ for binding bovine thymus VDR was measured (commercially available kit, INCSTAR, Stillwater, MN, U.S.A.) using the accompanying protocol. Non-radioactive steroid, at concentrations ranging from 25 to 400 pg/tube, was incubated in the presence of VDR and a fixed concentration of [^{3}H]-1,25-OH-D_3. Charcoal/dextran was used to separate bound and unbound label. Radioactivity in supernatant fluids was measured using a scintillation counter.

DBP binding study. The displacement of [^{3}H]-25-OH-D_3 from 1:10,000 diluted rat plasma in Tris–HCl buffer, pH 8.6 was measured following the addition of non-radioactive 25-OH-D_3 or MC 1288 as described previously [14, 15]. Radioactivity in the supernatant was determined using a scintillation counter.

Generation of metabolites of MC 1288 *using HPK1A-ras cells.* HPK1A-ras cells were grown in Dulbecco's culture medium [Dulbecco's modified Eagle's medium (MEM) supplemented with penicillin G (100 μg/mL), gentamycin (5 μg/mL), fungizone (300 ng/mL) containing 5% FCS (all purchased from Gibco, Grand Island, NY, U.S.A.)]. Cells were maintained in 150-mm plates at 37° in a humidified atmosphere of 5% CO_2 in air until confluence. Cells were then trypsinized and sub-cultured into 850-cm^2 roller culture bottles. Cells were maintained by replacing the medium and blowing mixed gases into the bottles every second day. Near confluence, 1,25-$(OH)_2D_3$ (10 nM) was added to the culture medium to induce transcription of catabolic enzymes. After 18 hr, the medium was removed and replaced by Dulbecco's culture medium supplemented with 100 μM DPPD (Sigma Chemical Co., St Louis, MO, U.S.A.) and 1% BSA (w/v) (Boehringer Mannheim, West Germany). The cells were then incubated for 48 hr in the presence of vehicle (0.01% EtOH) or 1.5 μM MC 1288 in vehicle.

Lipid extraction. Cells and medium were extracted using a modification of the method of Bligh and Dyer [16], in which the reagent chloroform was replaced by methylene chloride. The methylene chloride layer was evaporated to dryness under a stream of nitrogen, and redissolved in HIM (91:7:2 by vol.).

Purification of metabolites. Analytical HPLC of MC 1288 metabolites from HPK1Aras cells was performed on a modular system consisting of a model 590 pump, a U6K manual injector, a model 440 fixed wavelength detector (254 nm), and a model 990 photodiode array detector (Waters Scientific, Milford, MA, U.S.A.). Separation of metabolites was obtained on a 3 μm Zorbax-SIL (0.62 × 8 cm) column eluted with HIM 91:7:2 at a flow rate of 1 mL/min. Peaks showing a characteristic vitamin D_3 UV spectrum (UV_{max} = 265, UV_{min} = 228,

$UV_{max}/UV_{min} = 1.75$) were collected manually in glass Reacti-vials (Pierce, Rockville, MD, U.S.A.).

The peaks obtained from the analytical chromatography were purified on the same HPLC system using a Zorbax-CN (0.46 × 25 cm) column with HIM 91:7:2 at a flow rate of 1 mL/min. Again, metabolites were collected manually into glass Reacti-vials. This purification step was repeated once to obtain pure metabolite peaks for mass spectrometry.

GC–MS. GC–MS was used to obtain the mass spectra of the pertrimethylsilyl ethers of all the metabolites (except metabolites C and D) of MC 1288 as described by Qaw *et al.* [13]. Mass spectra were obtained by averaging each peak and subtracting the background.

Sodium periodate oxidation. Periodate cleavage was performed on all putative metabolites as previously reported [17]. Purified metabolites (200 ng) were dissolved in 40 μL of ethanol to which 20 μL of sodium metaperiodate (5% w/v in H_2O) was added. The residue was then redissolved in HIM (91:7:2 or 88:10:2 by vol.) for analysis by HPLC.

Sodium borohydride reduction. Putative carbonyl containing metabolites (peaks A, C and D) were subject to reduction by $NaBH_4$ as described previously [18]. Metabolites (approximately 200 ng) were dissolved in a solution of 200 μL of methanol and 10 μL of H_2O. $NaBH_4$ was added to the metabolite in large molar excess, and allowed to react for 1 hr at 0°. The residue was redissolved in HIM (91:7:2 or 88:10:2 by vol.) for analysis by HPLC.

Metabolism assay. The ability of MC 1288 and 1,25-$(OH)_2D_3$ to compete with [^{3}H]-1,25-$(OH)_2D_3$ for the enzymes of the side chain oxidation pathway was measured using modification of the calcitriol catabolism assay described previously [19]. A confluent 150-mm plate of UMR-106 cells was trypsinized and subcultured at a 1:3 ratio on 6-well plates. UMR-106 cells were maintained in McCoy's culture medium [McCoy's MEM (Gibco) supplemented with penicillin G (100 μg/mL), gentamycin (5 μg/mL), fungizone (300 ng/mL)] with 5% (v/v) FCS in a humidified atmosphere of 5% CO_2 in air. After 24 hr, cells were treated with 10 nM 1,25-$(OH)_2D_3$ to induce transcription of the catabolic enzymes. The medium was removed after 18 hr and replaced by McCoy's culture medium supplemented with 100 μM DPPD and 0.1% (w/v) BSA. Cells were then incubated, in triplicate, with 32 nM [^{3}H]-1,25-$(OH)_2D_3$ and varying amounts of MC 1288 or 1,25-$(OH)_2D_3$ for 3 hr. Cells and medium were extracted as described above. Three 500 μL aliquots of the aqueous fraction were transferred to scintillation vials and counted using a scintillation counter.

RESULTS

Biological activity studies

As shown in Table 1, *in vitro* binding affinity of bovine thymus VDR for MC 1288 was found to be five times greater than that of 1,25-$(OH)_2D_3$. Conversely, MC 1288 was shown to be a poor ligand for the plasma globulin DBP (Fig. 2), as it was ineffective at displacing radiolabeled 25-OH-D_3 even at concentrations as high as 1 μg/tube, which is 5000 times the B_{50} of the assay. The binding affinity of MC 1288 for DBP is far less than that of its epimer 1,25-$(OH)_2D_3$ which has previously been shown to bind with 350 times less affinity than 25-OH-D_3 [13].

Table 1. Biological activity of MC 1288 and 1,25-$(OH)_2D_3$

	VDR binding B_{50} (pg/tube)	GH transcriptional activation +DBP* ED_{50} (M)	GH transcriptional activation −DBP† ED_{50} (M)
MC 1288	1.5	5.3×10^{-11}	6.4×10^{-11}
1,25-$(OH)_2D_3$	7.5	1.6×10^{-9}	6.6×10^{-10}

Conditions for both VDR binding study and transcriptional cultivation study are outlined in the Materials and Methods.

Note: ED_{50} is the dose required to induce 50% of maximal induction of human GH transcription.

* Induction of transcription performed in the presence of 10% FCS.

† Induction of transcription performed in the presence of 1% BSA.

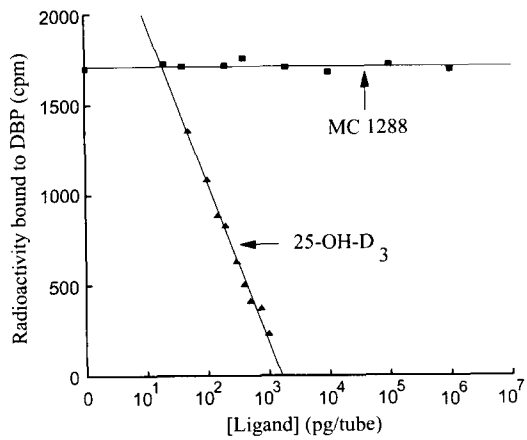

Fig. 2. Displacement of 25-OH[26,27-methyl-^{3}H]-D_3 from DBP using non-radiolabeled MC 1288 or 25-OH-D_3. Rat DBP was incubated with 25-OH-[^{3}H]-D_3 followed by varying amounts of MC 1288 (■) or 25-OH-D_3 (▲) as described in the Materials and Methods. Each point in the figures is the mean of two tubes.

COS-1 cells co-transfected with a VDR-expressing plasmid pSG5-hVDR1/3 and a GH-expressing plasmid p(CT4)^{4}TKGH were used to measure the ability of MC 1288 and 1,25-$(OH)_2D_3$ to induce the production of human GH *in vitro*. The ED_{50} of MC 1288 in this assay (Table 1), defined as the dose required to give a 50% maximal induction of human GH transcription, was found to be approximately 30 times greater than that of 1,25-$(OH)_2D_3$ in the presence of DBP. In the absence of DBP, the ED_{50} of MC 1288 was found to be only 10 times greater than 1,25-$(OH)_2D_3$.

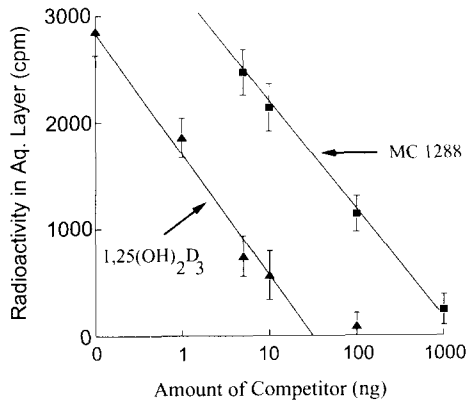

Fig. 3. Competitive inhibition of [1β-^{3}H]calcitroic acid production using non-radioactive MC 1288 and 1,25-$(OH)_2D_3$ in UMR-106 cells. [1β-^{3}H]-1,25-$(OH)_2D_3$ was incubated in UMR-106 cells in the presence of vehicle alone (EtOH) or varying concentrations of MC 1288 (■) or 1,25-$(OH)_2D_3$ (▲). Incubations were performed as described in Materials and Methods. Each point in the figure is the mean ± SE of three flasks counted in triplicate.

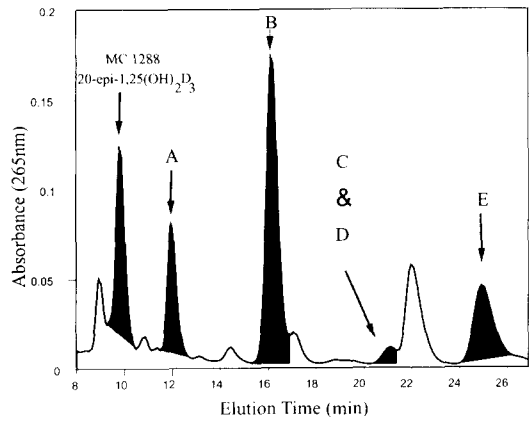

Fig. 4. Chromatogram of lipid extract from HPK1A-ras cells incubated with MC 1288. Metabolite peaks were identified based on their characteristic vitamin D chromophore (UV_{max} = 265 nm, UV_{min} = 228 nm, UV_{max}/UV_{min} = 1.75) and have been shaded. Metabolites were separated on a Zorbax-SIL column using the solvent HIM 91/7/2 at a flow rate of 1 mL/min. Only the 8–27 min region of the chromatogram has been shown.

Metabolism studies

The rate of catabolism of the epimers, MC 1288 and 1,25-$(OH)_2D_3$, were compared in UMR-106 cells (Fig. 3). Measuring the ability of a non-radioactive analog to compete with [1β-^{3}H]-1,25-$(OH)_2D_3$ for metabolic enzymes in cultured cells by assaying radioactivity in the form of aqueous soluble metabolites, we found that MC 1288 was metabolized 36 times slower than 1,25-$(OH)_2D_3$.

Quantitatively, we studied the chemical nature of metabolites of MC 1288 using HPK1A-ras cells which we have shown in other studies to be a rich source of target cell vitamin D side chain hydroxylases. Metabolites were identified based on their characteristic vitamin D_3 spectrum obtained on diode array spectrophotometry (UV_{min} = 228 nm, UV_{max} = 265 nm, UV_{max}/UV_{min} = 1.75). HPK1A-ras cells generated five metabolites of MC 1288, as shown in Fig. 4. These metabolites were not present when MC 1288 was incubated with medium in the absence of cells or when cells were incubated with medium alone (data not shown). Fractions corresponding to the five metabolites were collected manually and further purified on normal phase HPLC using a Zorbax-CN column for further analysis.

Characterisation of metabolites of MC 1288

Peak A. Peak A was the least polar of the five metabolites. To identify this metabolite, the purified peak was derivatized to form a pertrimethylsilyl derivative and analysis by mass spectrometry. The mass spectrum (Table 2) featured a molecular ion of *m/z* 646 which suggested the addition of a ketone group to the base compound. The fragment at *m/z* 515 indicates C_{24}–C_{25} fragility, suggesting that the ketone group is on C_{24}. Other fragments present in the mass spectrum include: *m/z* 556 (646-90) and 446 (646-90-90) which are the result of sequential losses of TMSiOH, 541 (646-90-15) due to the loss of TMSiOH and a methyl group, and 131 representing a fragment (C25, 26, 27-OTMSi) due to cleavage across the C2A-C25 band.

To confirm that peak A was a 24-oxo metabolite, the purified metabolite was treated with $NaBH_4$. Reduction of this metabolite resulted in a product which co-migrated with untreated peak B, the putative 20-epi-1,24,25-$(OH)_3D_3$ (Table 3). Peak A was shown to be insensitive to periodate cleavage (Table 3). These results strongly suggest the identity of Peak A is 20-epi-24-oxo-1,25-$(OH)_2D_3$.

Peak B. GC–MS of the pertrimethylsilylated version of peak B revealed a molecular ion of *m/z* 720 (Table 2). This suggests the addition of a single hydroxyl group to MC 1288. The fragment *m/z* 589 (720-131) indicates cleavage of the A-ring of the molecular ion, while the further fragments at *m/z* 499 (720-131-90) and 409 (720-131-90-90) represent the sequential loss of TMSiOH from this fragment. Fragments were also seen at *m/z* 630 (720-90) and 540 (720-90-90) due to the sequential loss of TMSiOH from the molecular ions; 615 (720-90-15) due to the loss of TMSiOH and CH_3; and 131 representing a fragment C25,26,27-OTMSi) due to cleavage across the C24-C25 bond.

Peak B was found to be sensitive to periodate oxidation (Table 3) indicating the presence of vicinal hydroxyl groups. These results would suggest that peak B is 20-epi,1,24,25-$(OH)_3D_3$.

Peaks C and D. Peaks C and D could not be resolved by HPLC as the metabolites co-migrated on both the Zorbax-SIL (Fig. 3) and Zorbax-CN systems. Insufficient quantities of these metabolites were generated to perform mass spectrometry. Evidence for the co-existence of two metabolites

Table 2. Mass spectrum data of MC 1288 derived metabolites

	Molecular ion	Other important fragments
Peak A	646	556 (646-90), 541 (646-90-15), 515 (646-131), 131
Peak B	720	630 (720-90), 615 (720-90-15), 589 (720-131), 499 (720-90-131), 409 (720-90-90-131), 131
Peak E	808*	718 (808-90), 703 (808-90-15), 628 (808-90-90), 613 (808-90-90-15)

GC–MS conditions: pertrimethylsilyl ethers derivatives of the metabolites were separated on a HP-1 cross-linked methyl silicone gum column with helium as a carrier gas at a flow rate of 1 mL/min.

* Molecular ion was not detected as spectrometer has a detection limit of *m/z* 750.

Table 3. Chromatography of MC 1288 metabolites generated in HPK1A-ras cells

		Retention time (min)		
Metabolite	Identity	Untreated	Periodate treated	$NaBD_4$ treated
*MC 1288	20-epi-1,25-$(OH)_2D_3$	9.02	9.03	9.02
*Peak A	20-epi-24-oxo-1,25-$(OH)_2D_3$	12.21	12.19	17.15
*Peak B	20-epi-1,24,25-$(OH)_3D_3$	17.14	10.24	—
†Peak C	20-epi-1,23,24,25-$(OH)_4D_3$	13.96	9.40 (8.91)	14.13
†Peak D	20-epi-1,24,25,26-$(OH)_4D_3$	13.96	10.43 (10.21)	14.13
†Peak E	20-epi-1,23,25,26-$(OH)_4D_3$	18.46	9.26 (7.36)	ND

ND = Not detected.

HPLC conditions: 3 μM Zorbax-SIL column (0.62 × 8 cm) with: *-solvent 91:7:2 HIM, 1 mL/min, †-solvent 88:10:2 HIM, 1 mL/min.

Note: values in brackets are retention times under conditions*.

under this HPLC peak rested with derivatization studies. Treatment of the peak with sodium metaperiodate produced two cleavage products which suggests that the HPLC peak is a mixture of two metabolites arbitrarily labeled peaks C and D (Table 3). The more polar of the two cleavage products, derived from peak D, co-migrated with the cleavage product of peak B suggesting that peaks B and D give the same product on periodate cleavage. Since peak B is 24-hydroxylated this would suggest that peak D is also hydroxylated at the C_{24} position, while a further modification in the side chain more distal to this group also exists probably at the C_{26} position. The insensitivity of peak D to borohydride reduction suggests that the modification at C_{26} is more likely a hydroxyl group rather than an aldehyde function. We propose that peak D could be 20-epi-1,24,25,26-$(OH)_4D_3$.

The other component of the HPLC peak, arbitrarily labeled peak C, yielded a product which was less polar than the cleavage product obtained from peak D when treated with sodium metaperiodate (Table 3). As was the case with peak D, this metabolite was also insensitive to treatment with $NaBH_4$. The limited results obtained from peak C suggest that this metabolite might be 20-epi-1,23,24,25-$(OH)_4D_3$.

Peak E. The mass spectrum of the per-trimethylsilylated peak E, the most polar of the five metabolites, did not give a detectable molecular ion as the mass of the derivatized molecule is presumably greater than the detection limit of the detector (~*m/z* 750). As shown in Table 2, fragments were seen at *m/z* 718 (808-90), and 628 (808-90-90) which correspond to sequential losses of TMSiOH groups from the putative molecular ion, and also 703 (808-90-15) and 613 (808-90-90-15) due to the loss of TMSiOH and CH_3 groups. This suggests that peak E has a molecular ion of *m/z* 808 which would correspond to a vitamin D_3 compound with five hydroxyl groups (including the C_3 position). The lack of a fragment at *m/z* 131 indicates that peak E must be modified in some way at the C_{26} position. The mass spectrum of peak E lacked distinctive fragments to allow for definitive identification.

Peak E was cleaved due to periodate oxidation although the product was chromatographically different from that of peaks C and D. Peak E appeared to be destroyed by treatment with $NaBH_4$ since we were unable to detect a product (Table 3). These results suggest that peak E may be 20-epi-1,23,25,26-$(OH)_4D_3$.

DISCUSSION

We have shown here that, a simple modification

of calcitriol such as altering its stereochemistry at C_{20} is sufficient to significantly increase its ability to bind VDR, decrease its ability to bind DBP, as well as decrease its rate of catabolism. Taken together, these altered properties give rise to a molecule with much greater biological activity than the parent molecule when used in a growth hormone expression system *in vitro*. These studies therefore confirm the findings of Binderup *et al.* [17] that the 20-epi series of calcitriol analogs may be useful to pursue pharmacologically while also revealing potential reasons why these molecules have altered function.

The binding affinity of bovine VDR for the two epimers was examined *in vitro*, showing MC 1288 to be a superior ligand for the nuclear receptor than calcitriol. Previous studies [7] have shown that rachitic chicken intestine VDR has comparable binding affinity for MC 1288 and 1,25-$(OH)_2D_3$. Similar variation between avian and mammalian VDR binding affinity has been seen previously for vitamin D_2 compounds [14] and for the vitamin D analog, calcipotriol [20], and probably represents evolutionary divergence of the proteins.

A more likely possibility for the increased biological activity of MC 1288 *in vitro* may be the altered catabolism of the analog. Catabolic studies indicated that MC 1288 is rendered inactive through sequential hydroxylation in the side chain. Five metabolites were biologically generated by culturing cells in the presence of saturating amounts of MC 1288. Two of the metabolites seen, 20-epi-1,24,25-$(OH)_3D_3$ (peak B) and 20-epi-24-oxo-1,25-$(OH)_2D_3$ (peak A) represent the products of the first two steps in the side chain cleavage pathway [21]. There was no detectable production of 20-epi-24-oxo-1,23,25-$(OH)_3D_3$, the third metabolite expected in this pathway [22–24]. This would indicate that 20-epi-24-oxo-1,25-$(OH)_2D_3$ is a very poor substrate for the putative 23 hydroxylase, or that the first two steps of the pathway occur at a reduced rate. The metabolite 20-epi-1,24,25-$(OH)_3D_3$ appears to be a better substrate for the 23-hydroxylase as we were able to generate what we believe to be small quantities of 20-epi-1,23,24,25-$(OH)_4D_3$. Other metabolites generated are believed to include 20-epi-1,23,25,26-$(OH)_4D_3$ and 20-epi-1,24,25,26-$(OH)_4D_3$. These tetrahydroxylated vitamin D analogs may represent intermediates in the lactone deactivation pathway [25–27], though no 20-epi-26,23-lactone or -lactol compounds were detected.

At physiological concentrations, MC 1288 was found to be catabolized at a significantly slower rate than calcitriol. This may be the most significant parameter contributing to the increased biological activity of MC 1288. The decreased catabolism of MC 1288 would allow the analog much more time to initiate transcription of vitamin D inducible genes than its epimer, calcitriol. This decreased rate of metabolism through the 24-oxidation pathway may account for the increased levels of metabolites from the lactone deactivation pathway.

The ability of MC 1288 to enter target cells was examined by comparing the DBP binding affinity for MC 1288 and calcitriol. MC 1288 has a very low affinity for DBP compared to 1,25-$(OH)_2D_3$. This low binding affinity would be expected to increase the biological activity of MC 1288 *in vitro* relative to calcitriol as it would be free to enter the cell whereas 1,25-$(OH)_2D_3$ which binds to DBP would be inhibited from entering the cell to a greater extent. This was examined using a GH-gene expression system where it was found that the biological activity of MC 1288 relative to 1,25-$(OH)_2D_3$ was decreased when DBP was not present.

As was previously demonstrated by Bouillon *et al.* [15], the inability of MC 1288 to bind DBP would probably have a greater significance *in vivo*. MC 1288 is most likely to be transported in the blood attached to non-specific proteins (i.e. albumin) which would direct the analog to the liver for degradation. This would lead to increased metabolic clearance of MC 1288 and thus decrease the amount of "free" MC 1288 that would be available to enter target cells in the body, thereby lowering its biological activity *in vivo*. The may account for the low calcemic effect of MC 1288 compared to the high cell differentiating effect *in vitro* observed by Binderup *et al.* [7].

In summary, the increased biological activity of MC 1288 *in vitro* appears to be in part due to the decreased rate of metabolism of the analog and in part to a lesser extent, the increased affinity of the VDR, and decreased affinity of DBP for the compound. The decreased affinity of DBP for MC 1288 may play a more significant role in controlling the distribution of the compound *in vivo* and thus make this compound a useful topical anti-tumor agent. The decreased metabolism of MC 1288 would allow this compound to exert its differentiating effects in the cell for a longer period of time without being degraded. Upon entry of this compound into the blood, it would be bound non-specifically to proteins in the blood, and be cleared more rapidly. Therefore, the 20-epi series of vitamin D analogs show potential for use in the treatment of psoriasis and skin cancer.

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