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Vitamin D

Removal of C-ring from the CD-ring skeleton of 1α,25-dihydroxyvitamin D_3 does not alter its target tissue metabolism significantly

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Abstract

It is now well established that 1α,25$(OH)_2D_3$ is metabolized in its target tissues through the modifications of both side chain and A-ring. The C-24 oxidation pathway is the side chain modification pathway through which 1α,25$(OH)_2D_3$ is metabolized into calcitroic acid. The C-3 epimerization pathway is the A-ring modification pathway through which 1α,25$(OH)_2D_3$ is metabolized into 1α,25$(OH)_2$-3-epi-D_3. During the past two decades, a great number of vitamin D analogs were synthesized by altering the structure of both side chain and A-ring of 1α,25$(OH)_2D_3$ with the aim to generate novel vitamin D compounds that inhibit proliferation and induce differentiation of various types of normal and cancer cells without causing significant hypercalcemia. Previously, we used some of these analogs as molecular probes to examine how changes in 1α,25$(OH)_2D_3$ structure would affect its target tissue metabolism. Recently, several nonsteroidal analogs of 1α,25$(OH)_2D_3$ with unique biological activity profiles were synthesized. Two of the analogs, SL 117 and WU 515 lack the C-ring of the CD-ring skeleton of 1α,25$(OH)_2D_3$. SL 117 contains the same side chain as that of 1α,25$(OH)_2D_3$, while WU 515 contains an altered side chain with a 23-yne modification combined with hexafluorination at C-26 and C-27. Presently, it is unknown how the removal of C-ring from the CD-ring skeleton of 1α,25$(OH)_2D_3$ would affect its target tissue metabolism. In the present study, we compared the metabolic fate of SL 117 and WU 515 with that of 1α,25$(OH)_2D_3$ in both the isolated perfused rat kidney, which expresses only the C-24 oxidation pathway and rat osteosarcoma cells (UMR 106), which express both the C-24 oxidation and C-3 epimerization pathways. The results of our present study indicate that SL 117 is metabolized like 1α,25$(OH)_2D_3$, into polar metabolites via the C-24 oxidation pathway in both rat kidney and UMR 106 cells. As expected, WU 515 with altered side chain structure is not metabolized via the C-24 oxidation pathway. Unlike in rat kidney, both SL 117 and WU 515 are also metabolized into less polar metabolites in UMR 106 cells. These metabolites displayed GC and MS characteristics consistent with A-ring epimerization and were putatively assigned as C-3 epimers of SL 117 and WU 515. In summary, we report that removal of the C-ring from the CD-ring skeleton of 1α,25$(OH)_2D_3$ does not alter its target tissue metabolism significantly.

Keywords: Vitamin D; Metabolism; Nonsteroidal vitamin D analogs; SL 117; WU 515; 1α,25$(OH)_2D_3$; D-ring analogs; C-24 oxidation pathway; C-3 epimerization pathway

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The discovery that structural modifications of the seco-steroid hormone, $1\alpha,25(OH)_2D_3$[1] can result in the dissociation of the hormone's action on regulating cell growth and differentiation from its calcemic actions, has led to the synthesis of numerous vitamin D analogs with a wide spectrum of biological actions. For a comprehensive understanding of this topic, the reader is referred to a few excellent reviews [1–5]. Recently, several nonsteroidal analogs of $1\alpha,25(OH)_2D_3$ with unique biological activity prNSofiles were synthesized. These novel nonsteroidal vitamin D analogs, lack either the full five membered D-ring (C-ring analogs) or the full six membered C-ring (D-ring analogs) or both C- and D-rings (E-ring analogs) of the CD-ring skeleton of $1\alpha,25(OH)_2D_3$ [6,7] We selected two of the D-ring analogs (SL 117 and WU 515) for our present study. SL 117, the basic D-ring analog contains the same side chain as that of $1\alpha,25(OH)_2D_3$ (Fig. 1). This analog is equipotent to $1\alpha,25(OH)_2D_3$ in modulating cell growth and differentiation *in vitro* but generates only minimal calcemic actions *in vivo* (Table 1). Another D-ring analog, WU 515 contains an altered side chain with a 23-yne modification combined with hexafluorination at C-26 and C-27 (Fig. 1). This analog is also less calcemic like SL 117 but is several fold more potent than $1\alpha,25(OH)_2D_3$ in modulating cell growth and differentiation of a variety of cell types (Table 1). Thus, removal of the C-ring from the CD-ring skeleton of $1\alpha,25(OH)_2D_3$ generates novel analogs with potent effect on cell growth and differentiation, while displaying low calcemic activity *in vivo*.

At present, the mode of action of the various synthetic vitamin D analogs at the molecular level is not fully understood. However, in our previous studies we successfully used some of these analogs as molecular probes to examine how the changes in $1\alpha,25(OH)_2D_3$ structure would affect its target tissue metabolism. It is now well established that $1\alpha,25(OH)_2D_3$ is metabolized in its various target tissues via modifications of both the side chain and the A-ring. The C-24 oxidation pathway, initiated by hydroxylation at C-24 is the major side chain modification pathway and is responsible for the conversion of $1\alpha,25(OH)_2D_3$ into the final metabolite, calcitroic acid [8,9]. The C-3 epimerization pathway is the minor A-ring modification pathway through which $1\alpha,25(OH)_2D_3$ is metabolized into 1α,25-dihydroxy-3-epi-vitamin D_3 [$1\alpha,25(OH)_2$-3-epi-D_3] [10–12]. For a comprehensive understanding of the target tissue metabolism of $1\alpha,25(OH)_2D_3$, the reader is referred to a recent review [13]. In our previous studies, we investigated the target tissue metabolism differences between $1\alpha,25(OH)_2D_3$ and two novel vitamin D analogs namely $1\alpha,25(OH)_2$-16-ene-D_3 and $1\alpha,25(OH)_2$-20-epi-D_3. The results of these studies indicated that minor changes such as inclusion of 16-ene and 20-epi modifications to $1\alpha,25(OH)_2D_3$ structure affect its target tissue metabolism significantly [14–16]. Now, with the availability of D-ring analogs, we asked the question how a major change in the structure of $1\alpha,25(OH)_2D_3$ such as the removal of C-ring from its CD-ring skeleton would affect its target tissue metabolism. To answer this question, we compared the metabolic fate of two D-ring analogs (SL 117 and WU 515), with that of $1\alpha,25(OH)_2D_3$ in both isolated perfused rat kidney and rat osteosarcoma cells (UMR 106). The results of these studies are reported in the present paper.

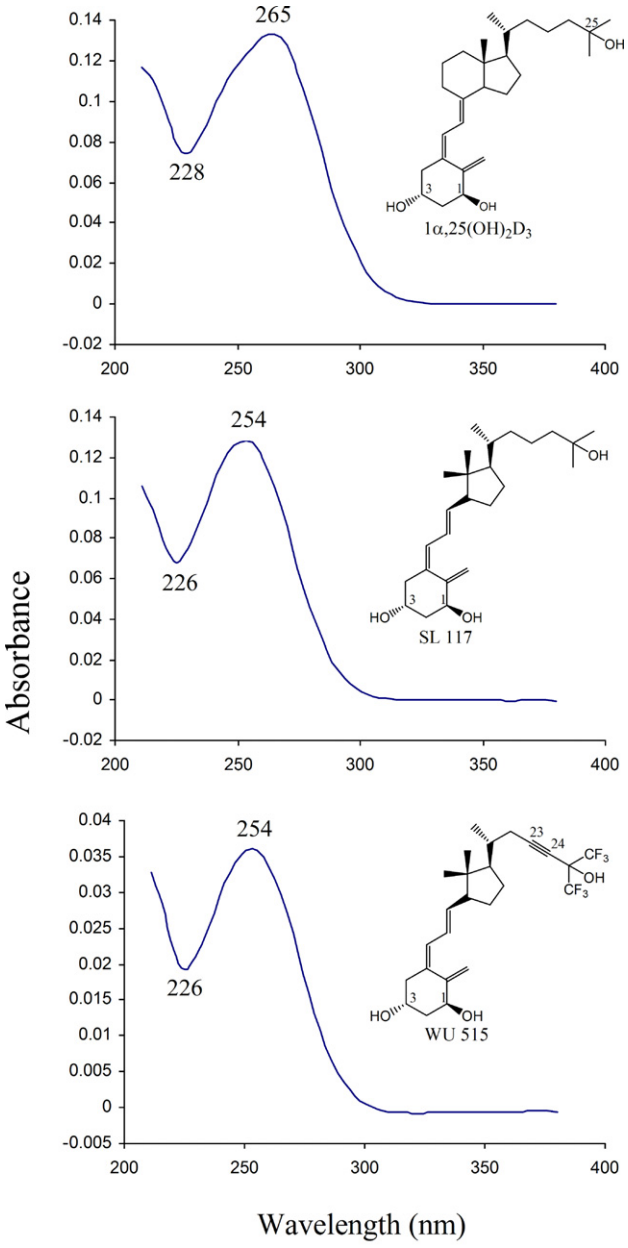

Fig. 1. Chemical structures and UV spectra of $1\alpha,25(OH)_2D_3$ and the two nonsteroidal analogs SL 117 and WU 515.

[1] *Abbreviations used:* $1\alpha,25(OH)_2D_3$, 1α, 25-dihydroxyvitamin D_3; $1\alpha,24(R),25(OH)_3D_3$, 1α,24(R),25-tryhydroxyvitamin D_3; $1\alpha,25(OH)_2$-24-oxo-D_3, 1α,25-dihydroxy-24-oxovitamin D_3; $1\alpha,23,(S)25(OH)_3$-24-oxo-D_3, 1α,23(S),25-trihydroxy-24-oxovitamin D_3; $1\alpha,23(OH)_2$-24,25,26,27-tetranor D_3 or C-23 alcohol, 1α,23-dihydroxy-24,25,26,27-tetranorvitamin D_3; $1\alpha,25(OH)_2$-3-epi-D_3, 1α,25-dihydroxy-3-epi-vitamin D_3; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; VDR, vitamin D receptor; hDBP, human D-binding protein; VDRE, Vitamin D response element.

Table 1
Summary of the biological effects of D-ring analogs SL117 and WU515 on (1) VDR and hDBP binding (2) HL-60 and MG-63 differentiation (3) MCF-7, keratinocytes and peripheral blood mononuclear cell (PMBC) proliferation and (4) calcium levels in serum and urine of vitamin D replete normal mice

Compound	Binding studies		*In vitro* studies					*In vivo* studies	
	VDR (%)	DBP (%)	HL-60 (%)	MG-63 (%)	MCF-7 (%)	Kerat (%)	PBMC s (%)	Ca in serum (mouse) (%)	Ca in urine (mouse) (%)
$1\alpha,25(OH)_2D_3$	100	100	100	100	100	100	100	100	100
SL 117	80	10	85	30	85	90	80	0.3	2
WU 515	70	3	1000	1000	5000	3000	150	6	13

Antiproliferative activity was assessed by [^{3}H] thymidine incorporation. Prodifferentiating effects were determined on HL-60 cells by NBT reducing assay and on MG-63 by osteocalcin measurements. Results are expressed as % activity (at 50% dose-response) as compared to $1\alpha,25(OH)_2D_3$ (= 100% activity). *In vivo* effects were tested on vitamin D replete normal NMRI mice by daily intraperitoneal injections for 7 consecutive days. Calcium levels were determined in urine and serum, and the dose-response curve for D-ring analogs was compared with that of $1\alpha,25(OH)_2D_3$ (= 100% activity). (Data summarized from Ref. [7].)

Materials and methods

Vitamin D compounds

Crystalline $1\alpha,25(OH)_2D_3$ was synthesized at Hoffmann-La Roche, Nutley, NY, USA. All the known natural metabolites of $1\alpha,25(OH)_2D_3$ which include $1\alpha,24(R),25$-trihydroxyvitamin D_3 [$1\alpha,24(R),25(OH)_3D_3$], $1\alpha,25$-dihydroxy-24-oxovitamin D_3 [$1\alpha,25(OH)_2$-24-oxo-D_3], $1\alpha,23(S)$, 25-trihydroxy-24-oxovitamin D_3 [$1\alpha,23(S),25(OH)_3$-24-oxo-D_3], and $1\alpha,23$-dihydroxy-24,25,26,27-tetranorvitamin D_3 [$1\alpha,23(OH)_2$-24,25,26,27-tetranor- D_3 or C-23 alcohol], were biologically synthesized in the rat kidney perfusion system as previously described [8,17]. The D-ring analogs of $1\alpha,25(OH)_2D_3$ (SL 117 and WU 515) were synthesized at one of our laboratories (Universiteit Gent, Gent, Belgium) [18].

Solvents

All solvents were from Burdick and Jackson Laboratories, Muskegan, MI.

High performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS)

HPLC analysis of the lipid extracts from the kidney perfusate and from cells and media was performed with a Waters System Controller (Millennium 32) equipped with a photodiode array detector (Model PDA 996) to monitor the UV absorbing material at 265 nm.

GC/MS analysis was performed using a Hewlett-Packard GC-MSD system which is equipped with a 6890 GC, 5973 mass-selective detector and 7683 autosampler (Hewlett-Packard, Wilmington, DE). Each vitamin D compound was prepared as a 5–15 ng/μL solution in a 1:1 mixture of acetonitrile and derivatizing reagent (Power SIL Prep, Alltech Associates, Deerfield, IL) and incubated at 70 °C for 15 min. 1–3 μL of each solution containing the trimethylsilyl ether derivative was injected onto a DB-5MS low-bleed capillary column (30 m × 0.25 mm × 0.25 μm film thickness)(J & W Scientific, Folsom, CA). The amount of each analyte injected varied slightly depending on MS signal response. UHP helium carrier gas was used at a flow rate of 0.8 mL/min with a temperature program ranging from 140–300 °C (20 °C/min ramp). Full-scan electron impact mass spectra were acquired for each experiment.

Animals

The Animals Ethics and Care Committee of the Women and Infants Hospital, Providence, RI approved the animal experiments performed in this study. Male Sprague-Dawley rats (about 350 g) purchased from Taconic Laboratories (Germantown, NY) were fed a regular rodent diet sufficient in calcium, phosphorus and vitamin D. In our previous studies we demonstrated that the increase in activity of the enzymes involved in further metabolism of $1\alpha,25(OH)_2D_3$ can be induced in kidneys by pretreating rats with $1\alpha,25(OH)_2D_3$ [17,19]. Therefore, in an analogous fashion in this study the rats were given an intracardiac injection of 1 μg of $1\alpha,25(OH)_2D_3$ in 50 μL of ethanol 6 h prior to isolation of the kidney from the animal to increase the enzymatic activity required for further metabolism of $1\alpha,25(OH)_2D_3$ and the D-ring analogs SL 117 and WU 515.

Study of the metabolism of the D-ring analogs (SL 117 and WU 515) and $1\alpha,25(OH)_2D_3$ in rat kidney

The metabolism of the D-ring analogs, SL 117 and WU 515, was compared to $1\alpha,25(OH)_2D_3$ in rat kidney using the isolated kidney perfusion technique as described before in detail [8,17,19]. Each kidney was perfused with 5 μM concentration of $1\alpha,25(OH)_2D_3$ or one of its analogs (SL 117 and WU 515) in 100 mL of perfusate and perfusions were continued for 8 h. The lipid extracts of the kidney perfusate samples were analyzed for the various further metabolites of $1\alpha,25(OH)_2D_3$ or its analogs (SL 117 and WU 515) using the technique of HPLC described later.

Control perfusions containing only perfusate and the Vitamin D compounds were performed in the absence of a kidney. The control studies indicated that the Vitamin D compounds did not undergo any change or breakdown either during the 8h perfusion period or during the extraction period.

Study of the metabolism of the D-ring analogs (SL 117 and WU 515) and $1\alpha,25(OH)_2D_3$ in UMR 106 cells

UMR 106 cells were maintained by McCoy's culture media supplemented with a 10% fetal calf serum (FCS) and antibiotics (penicillin 100 IU/mL and streptomycin 100 μg/mL). Cell culture medium was changed every 3–4 days. The cells were subcultured when approximately 80% confluent and were not subcultured beyond five passages. For the metabolism studies, 3×10^6 cells were seeded in T150 tissue culture bottles and grown to confluence. The incubations were carried out at 37 °C in a humidified atmosphere under 5% CO_2. Confluent UMR 106 cells were incubated with 1 μM concentration of either $1\alpha,25(OH)_2D_3$ or its analogs (SL 117 or WU 515) in 50 mL of media containing 10% FCS. The incubations were stopped after 24 h, with 10 mL of methanol. The lipid extracts of both cells and media were analyzed for the various further metabolites of $1\alpha,25(OH)_2D_3$ or its analogs (SL 117 and WU 515) using the technique of HPLC described later.

Control incubations without cells containing only media and the Vitamin D compounds were performed. The control studies indicated that the Vitamin D compounds did not undergo any change or breakdown either during the 24 h perfusion period or during the extraction period.

Lipid extraction

Lipid extraction from both kidney perfusate and cells and media was performed according to the procedure of Bligh and Dyer [20] except that methylene chloride was substituted for chloroform. Prior to the lipid

extraction, the kidney perfusate samples and the UMR 106 cells with media were spiked with 5 μg of $25OHD_3$, which was used as an internal standard. The recovery of the internal standard was used to assess the extraction efficiencies of the various lipid-soluble vitamin D metabolites.

Isolation and purification of the metabolites of SL 117, WU 515 and 1α,25(OH)$_2$D$_3$ produced by the rat kidney and UMR 106 cells

Lipid extracts obtained from both kidney perfusate samples and cells and media were separately subjected directly to HPLC using a Zorbax-SIL column (9.4 × 250 mm) eluted with three different solvent mixtures at a flow rate of 2 mL/min. HPLC analysis of the lipid extracts was first conducted using a solvent mixture consisting of 10% isopropanol in hexane (HPLC system-I). Subsequently, a second solvent mixture consisting of 6% isopropanol in methylene chloride (HPLC system-II) was used to obtain a better resolution of the metabolites. Final purification of the metabolites for mass spectrometric analysis was obtained using a Zorbax-SIL column eluted with 15% isopropanol in hexane (HPLC system-III).

Sodium metaperiodate ($NaIO_4$) oxidation

A total of 1 μg of the test vitamin D compound was dissolved in 15 μL of methanol and was allowed to react with 10 μL of 5% aqueous $NaIO_4$. After 30 min at 25 °C, the reaction product was dried under nitrogen gas and subjected to HPLC using a Zorbax-SIL column eluted with hexane:isopropanol (90:10) at a flow rate of 2 mL/min.

Sodium borohydride ($NaBH_4$) reduction

A total of 1 μg of the test vitamin D compound was dissolved in 50 μL of ethanol containing 1 mg of $NaBH_4$. After 30 min at 25 °C, the reaction product was dried under nitrogen and dissolved in 2 mL of hexane:isopropanol mixture (96:4). The sample was filtered through a syringe fitted with a Swinney filter holder containing a 0.45 μm Teflon filter (Millipore) and was concentrated under nitrogen to a volume of 100 μL. Then the sample was subjected to HPLC using a Zorbax-SIL column eluted with hexane:isopropanol (96:4) at a flow rate of 2 mL/min.

Results

Metabolites of 1α,25(OH)$_2$D$_3$ and the analogs SL 117 and WU 515 produced by rat kidney

The HPLC profiles of the lipid extracts of the final perfusate samples of three kidney perfusions were shown in Fig. 2. Rat kidneys were perfused with either 1α,25(OH)$_2$D$_3$ (panel A) or the analogs SL 117 (panel B) or WU 515 (panel C). As reported previously, 1α,25(OH)$_2$D$_3$ was metabolized in rat kidney into the well known polar metabolites derived via the C-24 oxidation pathway [8,9]. SL 117 with the same side chain as that of 1α,25(OH)$_2$D$_3$ was also metabolized into three polar metabolites labeled A1, A2 and A3. We tentatively assigned metabolites A3 and A1 as C-24 OH SL 117 and C-24 oxo SL 117, respectively, based on their chromatographic mobility compared with the corresponding natural metabolites of the hormone 1α,25(OH)$_2$D$_3$. Metabolites A3 and A1 were purified further using HPLC systems II and III for their structure identification by GC/MS described later. The minor metabolite A2 was not identified in this study. Unlike SL 117, WU 515 with altered side chain was not metabolized into polar metabolites. This finding indicates that WU 515 resists its metabolism through C-24 oxidation pathway.

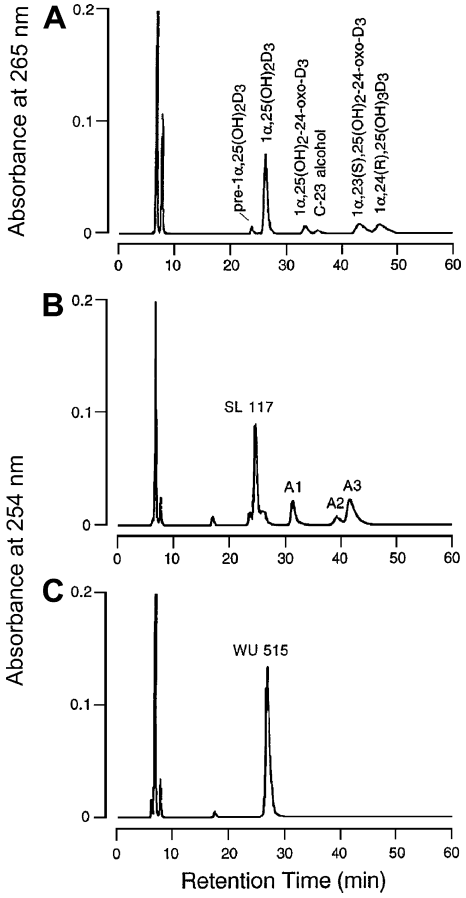

Fig. 2. The HPLC profiles of the lipid extracts of the final perfusate samples of three kidney perfusions using 1α,25(OH)$_2$D$_3$ and its analogs (SL 117 and WU 515) as the substrates. Each kidney was perfused for 8 h with 5 μM concentration of each substrate in 100 mL of perfusate. Lipid extract of final kidney perfusate (10 mL) from each kidney perfusion was subjected to HPLC analysis using a Zorbax-SIL column (9.4 mm × 250 mm) eluted with 10% isopropanol in hexane at a flow rate of 2 mL/min. The substrate and the metabolites of each vitamin D compound were traced by their typical UV spectra shown in Fig. 1. Panel A: HPLC profile of 1α,25(OH)$_2$D$_3$ and its polar metabolites derived via the C-24 oxidation pathway. The peak eluting before 1α,25(OH)$_2$D$_3$ is pre 1α,25(OH)$_2$D$_3$. Panel B: HPLC profile of SL 117 and its polar metabolites (peaks A1, A2, and A3). Panel C: HPLC profile of WU 515, which is not metabolized into polar metabolites.. Metabolites A3 and A1 were identified as C-24 OH SL 117 and C-24 oxo SL 117, respectively. The minor metabolite A2 was not identified in this study.

Identification of metabolites A3 and A1 as C-24 OH SL 117 and C-24 oxo SL 117, respectively

The mass spectral characteristics of the trimethylsilylated metabolite A3 are shown in Fig. 3 Panel A. The molecular ion was detected at *m*/*z* 694 indicating the presence of an additional hydroxyl group when compared to its parent, SL 117 (Fig. 5, panel B). Fragments at *m*/*z* 604 and 514 represent sequential losses of one and two trimethylsilylated hydroxyl groups. The loss of

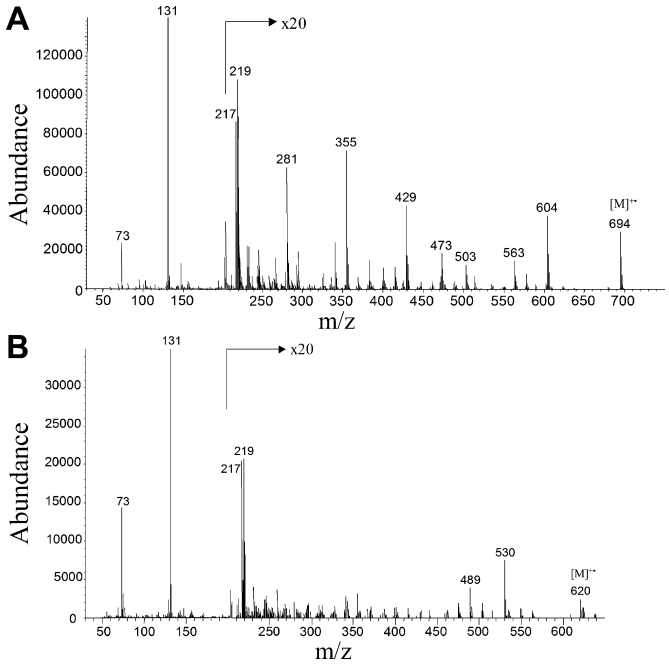

Fig. 3. Mass spectra of trimethylsilylated derivatives of metabolites of analog SL117 (A3 and A1) produced by the isolated perfused rat kidney. Panel A: Metabolite A3, GC retention time 16.34 min. Panel B: Metabolite A1, GC retention time 16.13 min.

131 Da from the A-ring (*m/z* 563) and the detection of an ion fragment at *m/z* 217, arising from A-ring cleavage, denote the presence of intact hydroxyl groups at C-1 and C-3 on the A-ring. The fragment ion at *m/z* 131 representing the cleavage across the C-24/25 bond on the sidechain indicates the presence of intact C-25 hydroxyl group. All these masses are consistent with those expected for the C-24 hydroxylated metabolite of SL 117. Masses of *m/z*, 281, 355 and 429 are background peaks arising from column bleed. The metabolite A3 was converted into a less polar metabolite when it was subjected to sodium periodate oxidation (data not shown). The susceptibility of metabolite A3 to $NaIO_4$ oxidation provided further evidence that an additional hydroxyl group on the side chain is vicinal to the original C-25 hydroxyl group of SL 117. Furthermore, the fragment at *m/z* 131 indicated that metabolite A3 not only contains an intact C-25 hydroxyl group but also that no hydroxylation has occurred on either C-26 or C-27. Thus, based on HPLC retention and MS fragmentation data, metabolite A3 was identified as C-24 OH SL 117.

The mass spectral characteristics of the trimethylsilylated metabolite A1 shown in Fig. 3 Panel B are as follows: *m/z* 620 $[M]^{+\cdot}$, 530 $[M–TMSOH]^{+\cdot}$, 440 $[M–2TMSOH]^{+\cdot}$, 489 $[M\text{-}131]^{+}$, 217 [A-ring fragment]$^{+}$, and 131 [side chain fragment]$^{+}$. These masses are identical to the masses expected for C-24 oxo SL 117. Sodium borohydride reduction of metabolite A1 yielded a compound which comigrated with C-24 OH SL 117 on a straight phase HPLC system (data not shown). Based on these findings, metabolite A1 was identified as C-24 oxo SL 117.

Metabolites of 1α,25(OH)$_2$D$_3$ and the analogs SL 117 and WU 515 produced by UMR 106 cells

Our studies comparing the metabolism of SL 117 and WU 515 with that of 1α,25(OH)$_2$D$_3$ in the isolated perfused rat kidney indicated that SL 117, like 1α,25(OH)$_2$D$_3$, is metabolized via the same C-24 oxidation pathway, while WU 515 (with altered side chain) resists metabolism via this pathway. However, it was not possible to obtain information about the metabolism of these two analogs via C-3 epimerization as rat kidney does not express this pathway. Therefore, the metabolism of SL 117 and WU 515 was also examined in UMR 106 cells, which are known to express both C-24 oxidation and C-3 epimerization pathways [10].

The HPLC profiles of the lipid extracts of media and the cells of UMR 106 cells incubated for 24 h with either 1α,25(OH)$_2$D$_3$ (panel A), SL 117 (panel B) or WU 515 (panel C) are shown in Fig. 4. As reported previously, 1α,25(OH)$_2$D$_3$ was metabolized in UMR 106 cells not only into the polar metabolites derived via the C-24 oxidation pathway, but also into a less polar metabolite, 1α,25(OH)$_2$-3-epi-D$_3$, derived via the C-3 epimerization pathway (Fig. 4, panel A). SL 117, like 1α,25(OH)$_2$D$_3$ is also metabolized into polar metabolite peaks B1, B2 and B3 but also into a less polar metabolite peak X (Fig. 4, panel B). All of the metabolites exhibited UV spectral characteristics identical to that of SL 117 shown in Fig. 1. Metabolites B3 and B1 were tentatively designated as C-24 OH SL 117 and C-24 oxo SL 117, respectively, based on chromatographic retention. Metabolites B3 and B1 were further purified using HPLC systems II and III for their structure identification. GC/MS analyses of metabolites B3 and B1 produced data almost identical to those obtained from metabolites A3 (C-24 OH SL 117) and A1 (C-24 oxo SL 117) produced by rat kidney (data not shown). Therefore, metabolites B3 and B1 were identified as C-24 OH SL 117 and C-24 oxo SL 117, respectively. The minor metabolite B2 was not identified in this study. The less polar metabolite X was also further purified using HPLC systems II and III for its structure identification. The analog WU 515 resisted its metabolism via the C-24 oxidation pathway in UMR 106 cells like in rat kidney and thus no polar metabolites were produced. On the contrary, the same analog unlike in rat kidney is metabolized into a less polar peak Y (Fig. 4, panel C).

Identification of metabolites X and Y as the C-3 epimers of SL 117 and WU 515, respectively

Unlike in rat kidney, the metabolic profiles obtained in UMR 106 cells for both SL 117 and WU 515 did, however, show that both compounds were converted into less polar metabolites X (Fig. 4, panel B) and Y (Fig. 4, panel C). Metabolites X and Y were further purified using HPLC systems II and III for GC/MS analysis. Fig. 5 shows the mass spectrum of metabolite X (panel A) and standard SL 117 (panel B). While the mass spectrum of the metabolite yielded the same general ion fragment profile, there were

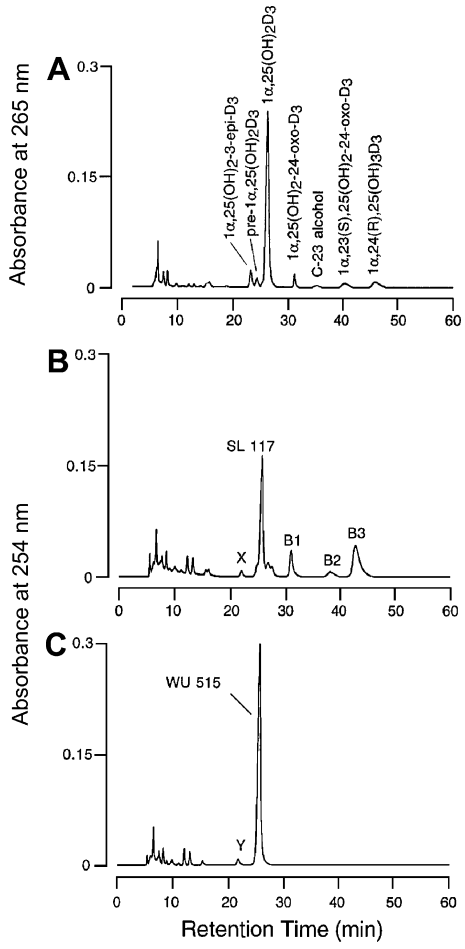

Fig. 4. The HPLC profiles of the lipid extracts of media and the cells of UMR 106 cells incubated for 24 h with 1 μM concentration of either $1\alpha,25(OH)_2D_3$ or the analogs (SL 117 or WU 515). HPLC analysis of the lipid extracts of both cells and media was performed using the same chromatographic conditions described in the legend to Fig. 2. The substrate and the metabolites of each vitamin D compound were traced by their typical UV spectra shown in Fig. 1. Panel A: HPLC profile of the substrate $1\alpha,25(OH)_2D_3$; the less polar metabolite $1\alpha,25(OH)_2$-3-epi-D_3, derived via the C-3 epimerization pathway and the polar metabolites derived via the C-24 oxidation pathway. Panel B: HPLC profile of the substrate SL 117 and its less polar metabolite (Peak X) and polar metabolites (peaks B1, B2 and B3). Panel C: HPLC profile of the substrate WU 515 and its less polar metabolite (Peak Y) Metabolites B3 and B1 were identified as C-24 OH SL 117 and C-24 oxo SL 117, respectively. The minor metabolite B2 was not identified in this study. Metabolites X and Y were identified as putative C-3 epimers of SL 117 and WU 515, respectively.

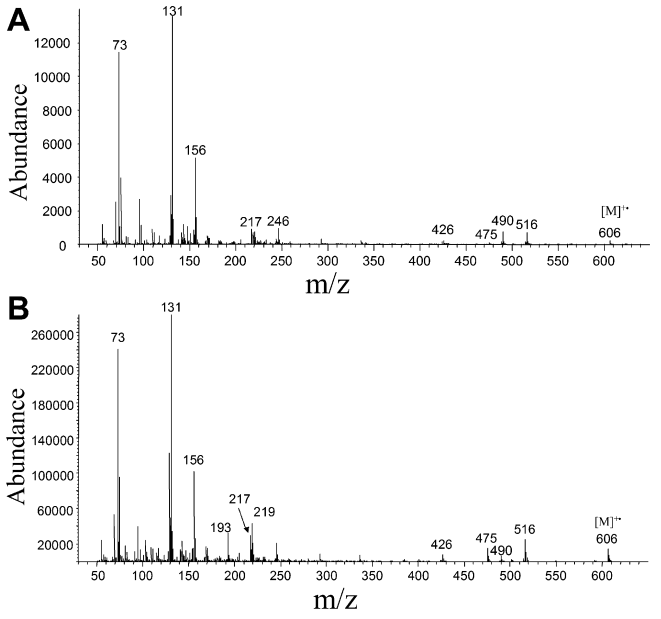

Fig. 5. Mass spectra of trimethylsilyl derivatives of the analog SL 117 and its metabolite X produced by UMR 106 cells. Panel A: less polar metabolite X (GC retention time 15.60 min). Panel B: Synthetic standard SL 117 (GC retention time 15.23 min).

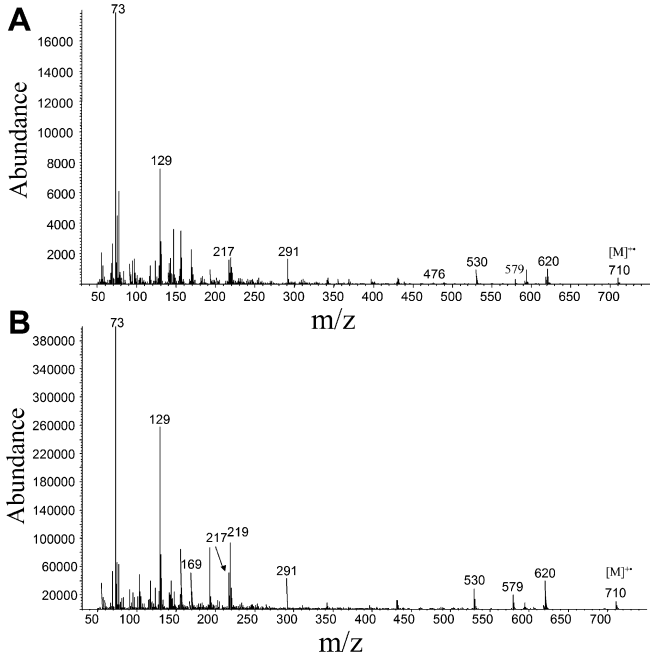

Fig. 6. Mass spectra of trimethylsilyl derivatives of the analog WU 515 and its metabolite Y produced by UMR 106 cells. Panel A: less polar metabolite Y (GC retention time 14.06 min). Panel B: Synthetic standard WU 515 (GC retention time 13.86 min).

significant differences in the relative intensity of certain fragments. Most notable differences were observed in the relative intensities of certain fragments such as *m/z* 475 $[M-131]^+$, *m/z* 490 $[M–TMSOH–CH_3–H]^{+\cdot}$ and *m/z* 217 [ion fragment comprised of the 3- and 1-trimethylsilyloxy groups along with the A-ring carbons 1,2 and 3.] Similar behavior has been observed by comparing mass spectral characteristics of other vitamin D analogs with their corresponding synthetic C-3 epimers (unpublished observations). In addition, the GC retention time of metabolite X (15.6 min) is significantly different when compared to that of its parent (15.2 min). Therefore, metabolite X was assigned a putative structure of 3-epi-SL 117 based on retention behavior and mass spectral characteristics.

The mass spectra of WU 515 metabolite Y (panel A) and standard WU 515 (panel B) are shown in Fig. 6. These mass spectra, in combination with GC retention times, illustrated

a similar phenomenon as seen with metabolite X. Therefore, metabolite Y was assigned a putative structure of 3-epi-WU 515 based on retention behavior and mass spectral characteristics.

Discussion

Several recent studies have indicated that certain minor structural modifications of the side chain of 1α,25(OH)$_2$D$_3$ can alter significantly its metabolism via both the C-24 oxidation and C-3 epimerization pathways and this in turn has a significant impact on its biological activity [21–26]. For example, we compared the metabolism of 1α,25(OH)$_2$D$_3$ with that of a simple analog, 1α,25(OH)$_2$-16-ene-D$_3$ in an isolated perfused rat kidney and found that both compounds are metabolized through the same C-24 oxidation pathway, forming their respective 24-hydroxy and 24-oxo metabolites. As expected 1α,25(OH)$_2$-24-oxo-D$_3$, the metabolite of 1α,25(OH)$_2$D$_3$, is further metabolized into 1α,23(S),25(OH)$_3$-24-oxo-D$_3$ at a rapid rate. On the contrary, 1α,25(OH)$_2$-24-oxo-16-ene-D$_3$, the metabolite of the analog, resisted C-23 hydroxylation and as a result, 1α,25(OH)$_2$-24-oxo-16-ene-D$_3$ accumulates. Thus, we reported that a simple modification, such as insertion of a double bond between C-16 and C-17 in the D-ring of 1α,25(OH)$_2$D$_3$ produces a partial block in its metabolism via C-24 oxidation pathway [14,15]. Another analog, 1α,25(OH)$_2$-20-epi-D$_3$, differs from 1α,25(OH)$_2$D$_3$ only by a simple modification of a change in stereochemistry of the methyl group at C-20 to an unnatural orientation. Like in the case of 1α,25(OH)$_2$-16-ene-D$_3$ a partial block in the metabolism of 1α,25(OH)$_2$-20-epi-D$_3$ via the C-24 oxidation pathway was also noted [16,27]. Furthermore, the stable 24-oxo metabolites of both 16-ene and 20-epi analogs were found to be almost as active as their corresponding parent analog in inhibiting proliferation and inducing differentiation of cancer cells, transactivating a VDRE reporter construct *in vitro* and in exerting immunosuppressive activity *in vivo* without causing hypercalcemia [15,16,28,29]. Thus, the incorporation of either 16-ene or 20-epi modification into 1α,25(OH)$_2$D$_3$ molecule decreases the rate of its further metabolism and allows the accumulation of the corresponding bioactive C-24 oxo metabolite. This phenomenon of altered pattern of metabolism in case of both 16-ene and 20-epi analogs has been proposed as one of the possible explanations for their unique biological actions. Furthermore, we also reported that addition of a 23-yne modification to 1α,25(OH)$_2$-16-ene-D$_3$ (1α,25(OH)$_2$-16-ene-23-yne-D$_3$) completely prevents its metabolism via the C-24 oxidation pathway and as a result the activity of the analog was enhanced by several fold [21] Interestingly, further addition of a 20-epi modification to 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ analog enhanced its rate of C-3 epimerization by approximately 10-fold [30]. Thus, all the aforementioned studies indicate that minor structural modifications of the sidechain of 1α,25(OH)$_2$D$_3$ can produce a significant change not only in its biological activity but also in its target tissue metabolism through both C-24 oxidation and C-3 epimerization pathways.

In the present study, unlike our previous studies, we investigated how a major modification in 1α,25(OH)$_2$D$_3$ structure such as removal of the C-ring would alter its metabolism. We selected two novel D-ring analogs (SL 117 and WU 515) which lack the C-ring and studied their metabolism in both rat kidney and UMR 106 cells. Our results indicate that SL 117with the same side chain as that of 1α,25(OH)$_2$D$_3$ is metabolized into C-24 OH SL 117 and C-24 oxo SL 117 and these metabolites were identified by HPLC retention characteristics and mass spectrometry. This finding indicates that SL 117 is metabolized like 1α,25(OH)$_2$D$_3$ through the same C-24 oxidation pathway. On the contrary, the analog WU 515 with altered sidechain structure containing 23-yne modification combined with hexafluorination at C-26 and C-27, is not metabolized into side chain modified polar metabolites. This finding indicates that WU 515 resists its metabolism through the C-24 oxidation pathway. It was also found that both SL 117 and WU 515 were converted into less polar metabolites when incubated in UMR 106 cells, which are known to express the C-3-epimerization pathway. These metabolites also displayed GC and MS characteristics consistent with A-ring epimerization (identical mass, different retention and fragmentation pattern) and were putatively assigned as C-3 epimers of SL 117 and WU 515. Thus, we report that removal of the C-ring from 1α,25(OH)$_2$D$_3$ does not alter its target tissue metabolism significantly.

Similar to our observations obtained from the metabolism studies, the biological activity studies of SL 117 and WU 515 indicated that the C-ring in the CD-ring skeleton of 1α,25(OH)$_2$D$_3$ is not essential to generate vitamin D-like actions [7] Although SL 117 and WU 515 bind to the VDR with almost the same affinity (Table 1), SL 117 is equipotent to 1α,25(OH)$_2$D$_3$ while WU 515 is several fold more potent than 1α,25(OH)$_2$D$_3$ in modulating cell growth and differentiation and transactivating a VDRE reporter construct *in vitro* [7]. Also WU 515 was more potent than 1α,25(OH)$_2$D$_3$ in protecting the VDR against proteolytic degradation [7]. Thus, the absence of significant correlation between VDR affinity and VDR-mediated transcriptional activities of SL 117 and WU 515 can be explained in part by the metabolic stability of WU 515 when compared to SL 117.

In summary, our results indicate that (1) SL 117, like 1α,25(OH)$_2$D$_3$, is metabolized through the same C-24 oxidation pathway in both rat kidney and UMR 106 cells. (2) WU 515, unlike SL 117, is not metabolized through C-24 oxidation pathway. (3) In UMR 106 cells, both SL 117 and WU 515 are metabolized into less polar putative C-3 epimers which are probably produced through the C-3 epimerization pathway known to exist in these cells. Thus, we report that the removal of the C-ring from the CD-ring skeleton of 1α,25(OH)$_2$D$_3$ does not alter its target tissue metabolism significantly. Finally, by putting together our present and the previously published observations (Table 1),

it can be concluded that for optimal interaction of $1\alpha,25(OH)_2D_3$ and the analogs (SL 117 and WU 515) with VDR and the enzymes involved in their target tissue metabolism does not require a full CD-ring structure. On the contrary, the interaction of $1\alpha,25(OH)_2D_3$ and its analogs with DBP is significantly impaired when the CD-ring structure is disrupted.

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