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Potent Antiproliferative Effects of 25-Hydroxy-16-ene-23-yne-vitamin D_3 That Resists the Catalytic Activity of Both CYP27B1 and CYP24A1

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ABSTRACT

The potency of 25-hydroxyvitamin D_3 (25(OH)D_3) is increased by several fold through its metabolism into 1α,25-dihydroxyvitamin D_3 (1α,25(OH)$_2D_3$) by cytochrome P450 27B1 (CYP27B1). Thus, the pivotal role of 1α-hydroxylation in the activation of vitamin D compounds is well known. Here, we examined the metabolism of 25-hydroxy-16-ene-23-yne-vitamin D_3 (25(OH)-16-ene-23-yne-D_3), a synthetic analog of 25(OH)D_3 in a cell-free system and demonstrated that 25(OH)-16-ene-23-yne-D_3 is neither activated by CYP27B1 nor inactivated by cytochrome P450 24A1 (CYP24A1). These findings were also confirmed in immortalized normal human prostate epithelial cells (PZ-HPV-7) which are known to express both CYP27B1 and CYP24A1, indicating that the structural modifications featured in 25(OH)-16-ene-23-yne-D_3 enable the analog to resist the actions of both CYP27B1 and CYP24A1. To provide intelligible structure-function information, we also performed molecular docking analysis between the analog and CYP27B1. Furthermore, 25(OH)-16-ene-23-yne-D_3 was found to suppress the growth of PZ-HPV-7 cells with a potency equivalent to 1α,25(OH)$_2D_3$. The antiproliferative activity of 25(OH)-16-ene-23-yne-D_3 was found to be vitamin D receptor (VDR)-dependent as it failed to inhibit the growth of mammary tumor cells derived from VDR-knockout mice. Furthermore, stable introduction of VDR into VDR-knockout cells restored the growth inhibition by 25(OH)-16-ene-23-yne-D_3. Thus, we identified 25-hydroxy-16-ene-23-yne-vitamin D_3 as a novel non-1α-hydroxylated vitamin D analog which is equipotent to 1α,25(OH)$_2D_3$ in its antiproliferative activity. We now propose that the low potency of the intrinsic VDR-mediated activities of 25(OH)D_3 can be augmented to the level of 1α,25(OH)$_2D_3$ without its activation through 1α-hydroxylation by CYP27B1, but by simply preventing its inactivation by CYP24A1. J. Cell. Biochem. 115: 1392–1402, 2014.

KEY WORDS: VITAMIN D; 16-ene-23-yne; CYP27B1; CYP24A1; ANTIPROLIFERATIVE ACTIVITY

Vitamin D_3, either produced in the skin upon UV exposure or received in the diet as a supplement, is metabolized into 25-hydroxyvitamin D_3 (25(OH)D_3) mainly in the liver by a specific microsomal cytochrome P450 2R1 (CYP2R1) [Zhu et al., 2013] and other non-specific CYPs such as CYP27A1 [Strushkevich et al., 2008; Zhu and DeLuca, 2012]. 25(OH)D_3 is further metabolized

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in the kidney by a specific mitochondrial CYP27B1 into 1α,25-dihydroxyvitamin D_3 (1α,25$(OH)_2D_3$), the hormonally active form of vitamin D_3 [Gray et al., 1972; Plum and DeLuca, 2010; Verstuyf et al., 2010; Schuster, 2011]. Along with the CYPs involved in the activation of vitamin D_3, a mitochondrial CYP24A1, which is induced by 1α,25$(OH)_2D_3$ itself, plays an important role in the inactivation of 1α,25$(OH)_2D_3$. CYP24A1 is responsible for multiple oxidations of the side chain [Akiyoshi-shibata et al., 1994; Beckman et al., 1996; Miyamoto et al., 1997; Sakaki et al., 1999a; Annalora et al., 2010; Schuster, 2011] through which 1α,25$(OH)_2D_3$ is metabolized into calcitroic acid. This process ultimately results in the loss of hormonal activity of 1α,25$(OH)_2D_3$. For more information on CYPs involved in the activation and inactivation of vitamin D_3, the reader is referred to recent reviews [Sakaki et al., 2005; Schuster, 2011; Jones et al., 2014].

Along with its main physiological function of regulating systemic calcium and phosphate metabolism, 1α,25$(OH)_2D_3$ is involved in a wide array of biological actions that are accomplished through genomic signal transduction, mediated by the nuclear vitamin D receptor (VDR) [Brumbaugh and Haussler, 1975; Plum and DeLuca, 2010; Verstuyf et al., 2010]. Such actions of 1α,25$(OH)_2D_3$, including the inhibition of growth and induction of differentiation of malignant cells, raised the possibility of using 1α,25$(OH)_2D_3$ as a drug for the treatment of a variety of clinical conditions including different cancers [Abe et al., 1981; Smith et al., 1986; Skowronski et al., 1993; Johnson et al., 2002; Krishnan et al., 2012; Mehta et al., 2012]. However, the administration of 1α,25$(OH)_2D_3$ at pharmacologically active doses is frequently accompanied by the side effect of hypercalcemia. As a result, less calcemic vitamin D analogs with the desired therapeutic effects of 1α,25$(OH)_2D_3$ have been developed. The reader is referred to excellent reviews on this topic [Bikle, 1992; Bouillon et al., 1995; Brown, 1998; Jones, 2012; Rosen et al., 2012; Christakos et al., 2013]. Furthermore, considerable attention has also been focused on a subgroup of vitamin D analogs, namely the synthetic vitamin D prodrugs such as 1α-hydroxyvitamin D_3 (alfacalcidol), 1α-hydroxyvitamin D_2 (doxercalciferol), and 1α-hydroxyvitamin D_5. In particular, 1α-hydroxyvitamin D_3 and 1α-hydroxyvitamin D_2 have been exploited for clinical use especially in patients with chronic kidney disease and osteoporosis as these prodrugs with their preexisting 1α-hydroxyl group are able to bypass the highly regulated 1α-hydroxylation catalyzed by CYP27B1 and depend on less stringently regulated 25-hydroxylation catalyzed by nonspecific enzymes such as CYP27A1 to be converted into their respective active forms [Tan, Jr. et al., 1997; Mehta et al., 2000; Mehta et al., 2003; Shoji et al., 2004; Hansen et al., 2011]. Thus, the inclusion of 1α-hydroxyl group as a key structural motif in the synthesis of vitamin D analogs for various therapeutic applications has become a paradigm.

During the past three decades, many promising 1α-hydroxylated analogs have been synthesized and a few of these analogs were approved as drugs for the treatment of various diseases. Only recently, a non-1α-hydroxylated vitamin D analog, namely 25-hydroxy-19-nor-vitamin D_3, was shown to possess VDR-mediated antiproliferatve activity in immortalized human prostate epithelial cells (PZ-HPV-7) with a potency equivalent to 1α,25$(OH)_2D_3$ [Urushino et al., 2007]. It was initially hypothesized that the potency of 25-hydroxy-19-nor-vitamin D_3 was due to its activation into 1α,25-dihydroxy-19-nor-vitamin D_3 through 1α-hydroxylation by CYP27B1. However, interestingly 25-hydroxy-19-nor-vitamin D_3 was found to resist 1α-hydroxylation by CYP27B1 both in a cell-free enzyme assay and in PZ-HPV-7 cells which are well-known to express CYP27B1. Thus, the result of this study for the first time challenged the original paradigm of including 1α-hydroxyl group as a key structural motif in the synthesis of vitamin D analogs. As a result, the possibility for the synthesis of a new generation of non-1α-hydroxylated vitamin D analogs with potent VDR-mediated biological activities has emerged.

We now report the identification of another non-1α-hydroxylated vitamin D analog, namely 25-hydroxy-16-ene-23-yne-vitamin D_3 (25(OH)-16-ene-23-yne-D_3) (Fig. 1), which displays VDR-mediated antiproliferative activity in PZ-HPV-7 cells with a potency equivalent to 1α,25$(OH)_2D_3$. This interesting analog, like 25-hydroxy-19-nor-vitamin D_3, also resists CYP27B1-catalyzed 1α-hydroxylation. Thus, it is intriguing to note that the addition of certain structural modifications to 25$(OH)D_3$ featured not only in the proximity of C-1 in A-ring as shown in 25-hydroxy-19-nor-vitamin D_3 but also away from A-ring as shown in 25(OH)-16-ene-23-yne-D_3 can impede CYP27B1-catalyzed 1α-hydroxylation. These two analogs, however, differ considerably in their further metabolism and final inactivation. Here, we present the unique properties of 25(OH)-16-ene-23-yne-D_3, a non-1α-hydroxylated vitamin D analog that resists both CYP27B1-catalyzed activation and CYP24A1-catalyzed inactivation.

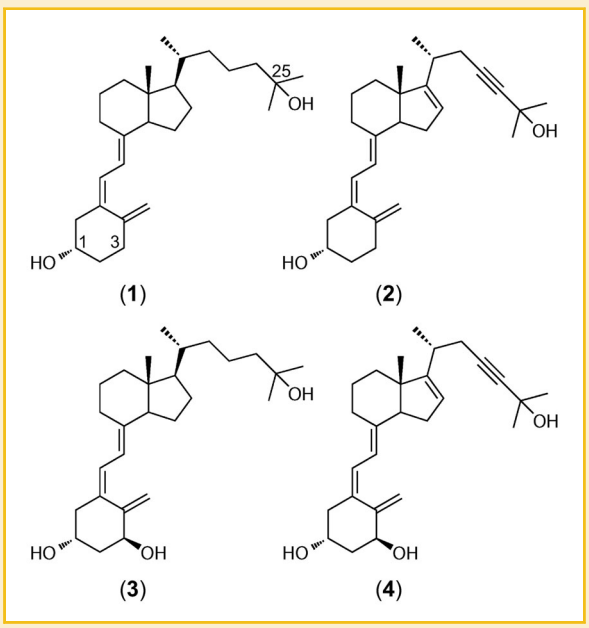

Fig. 1. Structures of 25$(OH)D_3$ (1), 25(OH)-16-ene-23-yne-D_3 (2), and their respective 1α-hydroxylated metabolites 1α,25$(OH)_2D_3$ (3), and 1α,25$(OH)_2$-16-ene-23-yne-D_3 (4).

MATERIALS AND METHODS

VITAMIN D COMPOUNDS AND CHEMICALS

Crystalline 25(OH)D_3, 1α,25$(OH)_2D_3$, 1α,25-dihydroxy-3-epi-vitamin D_3 (1α,25$(OH)_2$-3-epi-D_3), 24,25-dihydroxyvitamin-D_3 (24,25$(OH)_2D_3$), 25-hydroxyvitamin D_3-26,23-lactone (25(OH)D_3-26,23-lactone), 25 (OH)-16-ene-23-yne-D_3, 25-hydroxy-16-ene-23-ene-D_3, and 1α,25-dihydroxy-16-ene-23-yne-vitamin D_3 (1α,25$(OH)_2$-16-ene-23-yne-D_3) were synthesized at Hoffmann-La-Roche (Nutley, NJ). All known natural metabolites of 25(OH)D_3 and 1α,25$(OH)_2D_3$ which include 25-hydroxy-24-oxo-vitamin D_3 (25(OH)-24-oxo-D_3), 23,25-dihydroxy-24-oxo-vitamin D_3 (23,25$(OH)_2$-24-oxo-D_3), 23-hydroxy-24,25,26,27-tetranorvitamin D_3 (23(OH)-24,25,26,27-tetranor-D_3), 1α,25-dihydroxy-24-oxo-vitamin D_3 (1α,25$(OH)_2$-24-oxo-D_3), 1α,23,25-trihydroxy-24-oxo-vitamin D_3 (1α,23,25$(OH)_3$-24-oxo-D_3), and 1α,23-dihydroxy-24,25,26,27-tetranorvitamin D_3 (1α,23$(OH)_2$-24,25,26,27-tetranor-D_3) were biologically synthesized in the rat kidney perfusion system as described [Reddy and Tserng, 1989]. All other chemicals were purchased from Sigma–Aldrich (St. Louis, MO) and used without further purification.

EXPRESSION AND PURIFICATION OF CYP27B1

Mouse CYP27B1 was co-expressed with molecular chaperonins GroEL/ES in *Escherichia coli* (DH5α) and purified as described [Uchida et al., 2004; Rhieu et al., 2009]. Recombinant *E. coli* cells were grown in terrific broth containing 50 μg/mL ampicillin and 25 μg/mL kanamycin. When the cell density (OD_{660}) reached 0.8, the induction of CYP27B1 and GroEL/ES transcription under the *tac* promoter and the *araB* promoter were initiated by adding isopropyl-thio-β-D-galactopyranoside and L-arabinose at final concentration of 1 mM and 4 mg/mL, respectively. δ-aminolevulinic acid was supplemented at a final concentration of 1 mM. Cultures were further incubated at 26 °C for 24 h with shaking at 190 rpm. Cells were resuspended in buffer A (100 mM potassium phosphate buffer (pH 7.4) containing 1% Chaps, 0.2 mM PMSF, 500 mM NaCl, and 20% glycerol) and disrupted by sonication at 4 °C. The resulting cells were subjected to ultracentrifugation at 100,000 × *g* at 4 °C for 1 h. The supernatant was applied to a Ni^{2+}-NTA agarose column (Qiagen, Valencia, CA). The resulting column was washed with buffer B (100 mM potassium phosphate buffer (pH 7.4) containing 0.1% Chaps, 50 mM imidazole, 500 mM NaCl, and 20% glycerol) and the bound CYP27B1 was eluted with buffer B containing 200 mM imidazole at a flow rate of 0.5 mL/min. The eluted CYP27B1 was applied to a PD-10 desalting column (GE Healthcare, Piscataway, NJ) to remove imidazole. The purity and concentration of the purified CYP27B1 were determined by SDS-PAGE and the reduced CO-difference spectrum using a difference extinction coefficient at 446 and 490 nm of $\varepsilon_{446-490} = 91\ mM^{-1}\ cm^{-1}$ [Omura and Sato, 1964], respectively.

EXPRESSION AND PURIFICATION OF CYP24A1, ADRENODOXIN, AND ADRENODOXIN REDUCTASE

Recombinant rat CYP24A1 (WT, Δ2-32) was expressed in *E. coli* (DH5α-FIQ) and purified as described [Annalora et al., 2004]. Purified CYP24A1 samples with A_{417}/A_{280} ratio exceeding 1.0 were considered pure and its concentration was measured by the CO-difference spectrum as described above. The bovine adrenodoxin (ADX) clone pKK223-2 was expressed in *E. coli* and purified as described [Gnanaiah and Omdahl, 1986] with minor modifications. Step NaCl elutions (0.14, 0.16, 0.18, and 0.3 M) were used to wash the DEAE column and elute the bound ADX. Adrenodoxin reductase (ADR) expressed in *E. coli* was purified as described [Gnanaiah and Omdahl, 1986; Sagara et al., 1993] using an adrenodoxin-affinity column in a tandem DEAE:adrenodoxin-affinity column procedure. Spectral purity indexes of ADX (A_{414}/A_{276}) and ADR (A_{452}/A_{278}) used in the present study were 0.9 and 0.1, respectively [Kimura et al., 1978; Annalora et al., 2004]. All enzymes were stored at −80 °C prior to use.

METABOLISM OF VITAMIN D COMPOUNDS BY CYP27B1 AND CYP24A1 USING IN VITRO RECONSTITUTION ASSAY

The reconstituted CYP27B1 assay consisted of a mixture of substrates (0.5–2 μM), ADX (2.5 μM), ADR (0.5 μM), and CYP27B1 (0.5 μM) in 1 mL of 50 mM potassium phosphate buffer (pH 7.4). Reactions were initiated by the addition of NADPH at a final concentration of 1 mM. The assay was carried out at 37 °C for various time intervals (5–60 min) and terminated by adding 2 mL of methanol. The reactants were extracted by adding 4 mL of dichloromethane and subjected to HPLC analysis as described above. The reconstituted CYP24A1 assay was performed in a similar manner except that the mixture consisted of substrates (1–10 μM), ADX (0.1 μM), ADR (0.1 μM), and CYP24A1 (0.4 μM) in 1 mL of 50 mM potassium phosphate buffer containing 0.1% CHAPS (pH 7.4). The reaction was initiated by the addition of NADPH at a final concentration of 1 mM and the mixture incubated at 37 °C for various time periods (1–60 min). The reaction was quenched and extracted by adding 6 mL of methanol/dichloromethane (1:2, v/v) prior to HPLC analysis.

METABOLISM OF VITAMIN D COMPOUNDS IN PZ-HPV-7 CELLS

PZ-HPV-7 cells (American Type Culture Collection, Manassas, VA) were grown in T150 culture flasks (Corning, Corning, NY) and maintained on 50 mL of serum free-defined medium as described [Young et al., 2004]. At 80–90% confluence, cells were treated with 1 μM of 25(OH)D_3 or 25(OH)-16-ene-23-yne-D_3 for 2, 4, 6, and 24 h. The final concentration of ethanol (vehicle for all vitamin D compounds) was less than 0.1% (v/v) in all cases. The incubations were terminated with 10 mL of methanol and the lipids from both cells and culture medium were extracted for HPLC analysis. Control incubations containing only media and vitamin D compounds were performed to ensure that no metabolic conversion occurred in the absence of cells.

ANALYSIS OF VITAMIN D METABOLITES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

Straight-phase HPLC analysis of the lipid extracts from both the enzyme reaction mixture and cell culture was performed with a Waters System Controller (Millennium 3.2, Waters Associates, Milford, MA). Chromatography was accomplished on a Zorbax-SIL column (250 mm × 4.6 mm, Dupont, Wilmington, DE) with a hexane/isopropanol (8:2 or 9:1, v/v) isocratic system at a flow rate of 2 mL/min. A model 996 photodiode array detector (Waters

Associates, Milford, MA) was used to monitor lipids possessing the characteristic ultraviolet chromophore of vitamin D (λ_{max} at 265 nm; λ_{min} at 228 nm).

DETERMINATION OF SUBSTRATE-INDUCED DIFFERENCE SPECTRA

The substrate-induced difference spectra were measured in a Cary Model 500 double-beam spectrophotometer using quartz cuvettes of 10 mm path length and 1.2 mL capacity. After obtaining a baseline with the purified CYP27B1 (0.1 μM) prepared in both reference and sample cuvettes, 25(OH)-16-ene-23-yne-D_3 or 25(OH)D_3 (1 μM) was added to the sample cuvette while the equal volume of the vehicle solvent (absolute ethanol) was added to the reference cuvette. The final concentration of ethanol was less than 0.1% (v/v) in all cases.

MOLECULAR DOCKING STUDY

A three-dimensional model of mouse CYP27B1 was constructed as described [Urushino et al., 2006]. Surflex-Dock™ program was used to dock a substrate into the substrate-binding cavity. Of all possible poses, the one that directs C-1 position on A-ring to heme iron was extracted. Graphical manipulations were performed using SYBYL 8.1.1 (Tripos, St. Louis, MO).

ASSAY TO MEASURE CELL PROLIFERATION

Tritiated thymidine incorporation into DNA was used to determine the effect of 25(OH)-16-ene-23-yne-D_3 on cell proliferation in PZ-HPV-7 cells. Cells were cultured in a serum-free defined growth medium as described above and fed three times per week. PZ-HPV-7 cells were grown on 24-well plates to 70% confluence in the normal growth medium. Cells were then grown in the absence of growth factors for 24 h prior to the treatment with the vehicle solvent (ethanol), 1α,25$(OH)_2D_3$, or 25(OH)-16-ene-23-yne-D_3 at concentrations ranging from 0.1 to 100 nM for 18 h. At the end of incubation, the medium was replaced with 0.5 mL of fresh basal medium containing 0.1 μCi of [methyl-^{3}H] thymidine and incubated at 37 °C for 3 h. ^{3}H-thymidine incorporation into DNA was stopped by placing the 24-well plate on ice. Unincorporated ^{3}H-thymidine was removed and cells were washed three times with ice-cold PBS. DNA labeled with ^{3}H-thymidine was extracted by perchloric acid method as described [Smith et al., 1986].

REQUIREMENT OF VDR BY VITAMIN D COMPOUNDS TO EXPRESS THEIR ACTIVITY

Mammary tumor cell lines KO240 and KO288, which were derived from VDR-knockout (KO) mice [Zinser et al., 2003] were routinely grown in DMEM:F12 media containing 5% fetal bovine serum. To test whether 25(OH)-16-ene-23-yne-D_3 mediated effects via VDR, KO240 cells stably expressing human VDR (KO^{hVDR} cells) were generated using standard retroviral infection protocols and drug selection. Growth was assessed as adherent cell density as described [Zinser et al., 2003]. Briefly, equal numbers of cells were plated in 24 well plates and allowed to attach overnight. The following day cells were exposed to fresh media containing ethanol vehicle or vitamin D compounds at concentrations ranging from 0.1 to 100 nM. After 96 h, cells were fixed with glutaraldehyde or formaldehyde, stained with 0.1% crystal violet, and analyzed on a microplate reader for absorbance at 590 nm. Under these conditions, absorbance is proportional to adherent cell density. Data are reported as mean of four replicates per experiment and are representative of at least two independent experiments. To test VDR-mediated gene expression, RNA was isolated from KO^{hVDR} cells treated for 24 h with 100 nM of 25(OH)-16-ene-23-yne-D_3 or 100 nM of 1α,25$(OH)_2D_3$ and used for quantitation of *Cyp24a1* gene expression by qPCR. Data were calculated by the CT method, normalized to 18 S expression, and reported as fold change relative to vehicle treated control cells. qPCR data are presented as mean ± S.E. of at least three replicates. Means were considered statistically significant if $P < 0.05$ were obtained by one-way ANOVA followed by Dunnett's post-hoc test using Graph Pad Prism 5 software (San Diego, CA).

RESULTS

METABOLISM OF 25(OH)D_3 AND 25(OH)-16-ene-23-yne-D_3 BY MOUSE CYP27B1: 25(OH)-16-ene-23-yne-D_3 RESISTS CYP27B1-CATALYZED 1α-HYDROXYLATION

We performed a comparative metabolism study between 25(OH)D_3 and 25(OH)-16-ene-23-yne-D_3 in a reconstituted enzyme assay containing mouse CYP27B1. The lipid extracts were analyzed by HPLC and the profiles are shown in Figure 2. As expected, we observed that 25(OH)D_3 is metabolized into a polar metabolite (panel A1) which eluted at the same time as 1α,25$(OH)_2D_3$ standard shown in panel A2. Furthermore, the unequivocal structural identity of the polar metabolite produced from 25(OH)D_3 was determined as 1α,25$(OH)_2D_3$ by GC-MS analysis (data not shown). On the contrary, when we examined the metabolism of 25(OH)-16-ene-23-yne-D_3 by CYP27B1, we did not notice any production of the expected polar metabolite, 1α,25$(OH)_2$-16-ene-23-yne-D_3 (panel B1). The elution position of 1α,25$(OH)_2$-16-ene-23-yne-D_3 standard is shown in panel B2. These findings indicate that the addition of 16-ene and 23-yne modifications together to 25(OH)D_3 prevents its further metabolism by CYP27B1.

ANALYSIS OF SPECTRAL PERTURBATION INDUCED BY LIGAND BINDING

Binding of 25(OH)D_3 to CYP27B1 induced a type I spectral change (Fig. 3), indicating the spin state of heme iron of CYP27B1 is changed from low (S = 1/2) to high (S = 5/2). No spectral perturbation was observed in the presence of 25(OH)-16-ene-23-yne-D_3 (Fig. 3). Data suggest that a water molecule bound to the heme iron of CYP27B1 is not displaced by the ligand 25(OH)-16-ene-23-yne-D_3.

CONFORMATION OF 25(OH)-16-ene-23-yne-D_3 DOCKED IN CYP27B1

The modeled CYP27B1 possesses a bean shaped substrate binding cavity (Fig. 4). As a control, 25(OH)D_3 was accommodated in the cavity with a curved conformation, forming a hydrogen bond between 25-hydroxyl group and Ser408 (Fig. 4A). The position of C-1 of 25(OH)D_3 places the potential site of 1α-hydroxylation about 4.3 Å from the heme iron. On the other hand, no pose was obtainable for C-1 position of 25(OH)-16-ene-23-yne-D_3 to be oxidized. In the most favorite pose (i.e., linear conformation), the distance between C-1 of 25(OH)-16-ene-23-yne-D_3 and heme iron is 6.8 Å, suggesting

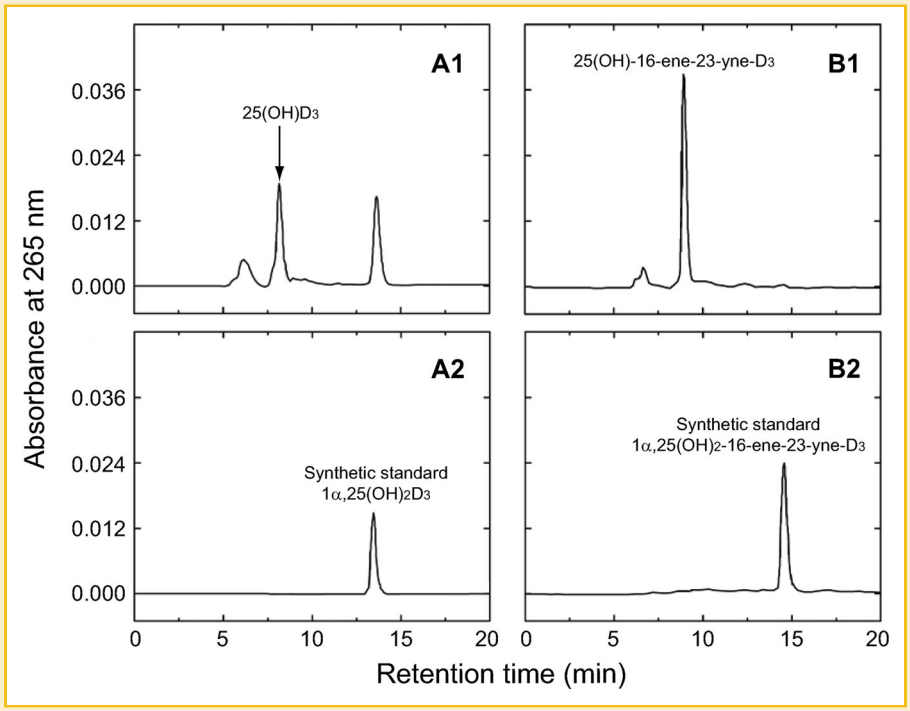

Fig. 2. HPLC profiles of metabolism of 25(OH)D_3 (panel A1) and 25(OH)-16-ene-23-yne-D_3 (panel B1) by mouse CYP27B1 in a cell free reconstituted system. HPLC analysis of lipid extracts of samples was performed using a Zorbax-SIL column (250 mm × 4.6 mm) eluted with 20% isopropanol in hexane at a flow rate of 2 mL/min. HPLC profiles of synthetic standards of 1α,25(OH)$_2D_3$ and 1α,25(OH)$_2$-16-ene-23-yne-D_3 are shown in panels A2 and B2, respectively.

that 25(OH)-16-ene-23-yne-D_3 is too distant for the 1α-hydroxylation to occur (Fig. 4B). This is consistent with spectroscopic analysis from the substrate-induced difference spectra, which detected no appreciable increase in the high spin content of CYP27B1 as 25(OH)-16-ene-23-yne-D_3 was added to displace the heme-bound water molecule (Fig. 3).

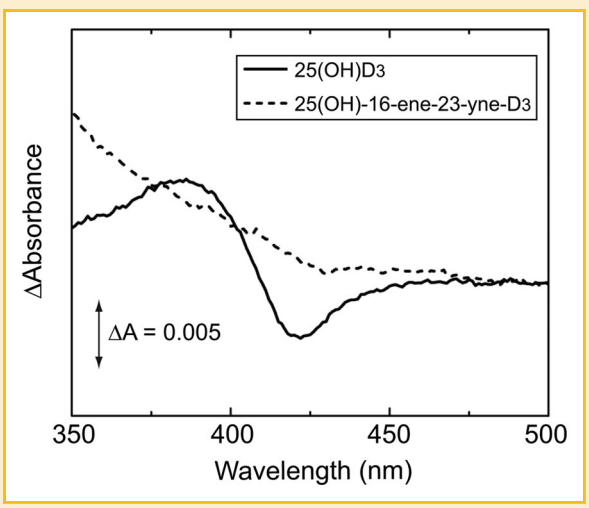

Fig. 3. Substrate-induced difference spectra of mouse CYP27B1 with 25(OH)D_3 and 25(OH)-16-ene-23-yne-D_3. The concentrations of enzyme and vitamin D compounds used were 0.1 and 1 μM, respectively.

METABOLISM OF 25(OH)-16-ene-23-yne-D_3 AND 25(OH)D_3 BY RAT CYP24A1: 25(OH)-16-ene-23-yne-D_3 RESISTS CYP24A1-CATALYZED INACTIVATION

We performed a comparative metabolism study between 25(OH)-16-ene-23-yne-D_3 and 25(OH)D_3 to understand how both compounds are inactivated by CYP24A1 using a reconstituted enzyme assay containing rat CYP24A1. For each compound, the assay was carried out for 15 and 60 min and the lipid extracts were analyzed by HPLC. As shown in Fig. 5A, 25(OH)-16-ene-23-yne-D_3 remained unmetabolized against the action of CYP24A1 up to 60 min. We recently reported that 1α,25(OH)$_2$-16-ene-23-yne-D_3, the 1α-hydroxylated form of 25(OH)-16-ene-23-yne-D_3, is metabolized by rat CYP24A1 into a single minor metabolite identified as 1α,25,26-trihydroxy-16-ene-23-yne-vitamin D_3 (1α,25,26(OH)$_3$-16-ene-23-yne-D_3) [Rhieu et al., 2011]. Based on this finding, we anticipated the metabolism of 25(OH)-16-ene-23-yne-D_3 into 25,26(OH)$_2$-16-ene-23-yne-D_3, a metabolite analogous to 1α,25,26(OH)$_3$-16-ene-23-yne-D_3. However, 25(OH)-16-ene-23-yne-D_3 unlike 1α,25(OH)$_2$-16-ene-23-yne-D_3 is not further metabolized and is fully resistant to CYP24A1-catalyzed inactivation.

Contrary to the above results obtained with 25(OH)-16-ene-23-yne-D_3, 25(OH)D_3 was readily metabolized by CYP24A1 into several polar metabolites during both 15 min (Fig. 5B) and 60 min (Fig. 5C) incubations. These polar lipid soluble metabolites of 25(OH)D_3 produced by rat CYP24A1 were indistinguishable to the metabolites produced by an isolated perfused rat kidney as reported in our previous study in which we provided for the first time full description of the metabolism of 25(OH)D_3 through both C-24 and C-23

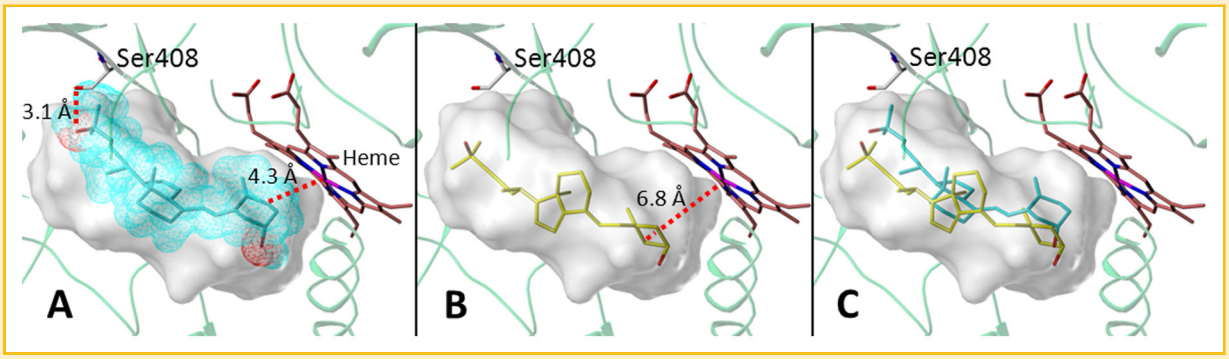

Fig. 4. Docking model of mouse CYP27B1 and vitamin D compounds. (A) Gray color blob shows substrate binding cavity generated by ProtoMol Docking model of 25(OH)D_3 in the active site of CYP27B1. Cyan stick and mesh show 25(OH)D_3 and its van der Waals surface, respectively. 25-Hydroxyl group forms a hydrogen bond with Ser408. (B) Docking model of 25(OH)-16-ene-23-yne-D_3 in the active site of CYP27B1. Yellow color shows docked 25(OH)-16-ene-23-yne-D_3. The C-1 position is 6.8 Å away from the heme iron, which is too distant to be oxidized. (C) The superimposed image of two docked ligands indicates that the shape of 25(OH)-16-ene-23-yne-D_3 is more linear than that of 25(OH)D_3 and that the position of 25(OH)-16-ene-23-yne-D_3 places more distant from both Ser408 and heme iron as compared to 25(OH)D_3.

oxidation pathways [Reddy et al., 2006]. Polar peaks 1, 2, 3, 4, and 5 shown in Figure 5B and C were identified as 25(OH)-24-oxo-D_3, 23(OH)-24,25,26,27-tetranor-D_3, 23,25$(OH)_2$-24-oxo-D_3, 24,25$(OH)_2D_3$, and 25(OH)D_3-26,23-lactone, respectively, by co-migration with known authentic standards produced from an isolated perfused rat kidney, reconfirming that 25(OH)D_3 is metabolized through both C-24 and C-23 oxidation pathways. In addition, the progression of CYP24A1-mediated inactivation of 25(OH)D_3 was evidenced by the accumulation of increasing amounts of 23(OH)-24,25,26,27-tetranor-D_3, the precursor of calcioic acid, the major inactive water-soluble metabolite of 25(OH)D_3 [Reddy et al., 2006].

METABOLISM OF 25(OH)-16-ene-23-yne-D_3 AND 25(OH)D_3 IN PZ-HPV-7 CELLS: 25-HYDROXY-16-ene-23-yne-VITAMIN D_3 RESISTS BOTH CYP27B1-CATALYZED 1α-HYDROXYLATION AND CYP24A1-CATALYZED INACTIVATION

To verify the findings obtained from the metabolism studies using cell-free reconstituted assays, we further examined the metabolism of 25(OH)-16-ene-23-yne-D_3 and 25(OH)D_3 in PZ-HPV-7 cells. The HPLC profile of 25(OH)-16-ene-23-yne-D_3 (Fig. 6A) shows no evidence of metabolism. A dashed arrow indicates the expected elution position of 1α,25$(OH)_2$-16-ene-23-yne-D_3. The HPLC profile of 25(OH)D_3 (Fig. 6B) shows several metabolites and the double asterisk denotes a mixture of two metabolites, 23,25$(OH)_2$-24-oxo-D_3 and 23(OH)-24,25,26,27-tetranor-D_3, which could not be separated by the HPLC system used for this specific analysis. Given the fact that PZ-HPV-7 cells express CYP27B1 [Wang et al., 2004], we also looked for the possibility of the conversion of 25(OH)D_3 into 1α,25$(OH)_2D_3$ in the same HPLC run. However, we did not observe any noticeable UV absorbing peak with typical UV characteristics (λ_{max} at 265 nm; λ_{min} at 228 nm) of vitamin D in the region of the expected elution position of 1α,25$(OH)_2D_3$ as shown by a dashed arrow (Fig. 6B). It is quite possible that even a minute amount of 1α,25$(OH)_2D_3$ produced in cells is sufficient enough to induce the expression of CYP24A1 that would rapidly metabolize 25(OH)D_3 as well as 1α,25$(OH)_2D_3$. It implies that the induction of CYP24A1 activity in cells prevents the accumulation of 1α,25$(OH)_2D_3$ in a quantity sufficient enough for its detection. We highlighted this important point in our previous study in which we investigated the metabolism of 25(OH)D_3 into 1α,25$(OH)_2D_3$ in human bone cells [Siu-Caldera et al., 1995]. With these observations in mind, we further examined the metabolism of 1α,25$(OH)_2D_3$ in order to provide direct evidence to show that the reason for our failure to detect 1α,25$(OH)_2D_3$ is due to its rapid metabolism by CYP24A1 in PZ-HPV-7 cells. As expected, the HPLC profile of 1α,25$(OH)_2D_3$ (Fig. 6C) shows several polar metabolites produced by CYP24A1. Each metabolite was identified based on the co-migration with known standards. The asterisk denotes a mixture of two metabolites, 1α,23,25-trihydroxy-24-oxo-vitamin D_3 (1α,23,25$(OH)_3$-24-oxo-D_3) and 1α,23-dihydroxy-24,25,26,27-tetranorvitamin D_3 (1α,23-$(OH)_2$-24,25,26,27-tetranor-D_3), which could not be separated by the HPLC system used for this specific analysis. Thus, we provided evidence to show that 1α,25$(OH)_2D_3$ is metabolized by CYP24A1 through C-24 oxidation pathway in PZ-HPV-7 cells. Furthermore, along with the polar metabolites produced by CYP24A1, we also noted a less polar metabolite identified as 1α,25$(OH)_2$-3-epi-D_3, a product of C-3 epimerization pathway [Reddy et al., 2001]. To the best of our knowledge, this is the first study to identify the production of 1α,25$(OH)_2$-3-epi-D_3 in PZ-HPV-7 cells, showing that 1α,25$(OH)_2D_3$ is metabolized in PZ-HPV-7 cells through both C-24 oxidation and C-3 epimerization pathways.

ANTIPROLIFERATIVE ACTIVITY OF 25(OH)-16-ene-23-yne-D_3 AGAINST PZ-HPV-7 CELLS

The antiproliferative activity of 25(OH)-16-ene-23-yne-D_3 was studied and compared to that of 1α,25$(OH)_2D_3$ in PZ-HPV-7 cells using ^{3}H-thymidine incorporation into DNA as described in the Method section. Similar to several previous studies using PZ-HPV-7 cells, 1α,25$(OH)_2D_3$ caused 96.6 ± 13.4, 88.9 ± 14.5, 60.7 ± 12.7, and 30.4 ± 5.3% inhibition at 0.1, 1, 10, and 100 nM, respectively, as compared to the controls. To our surprise, 25(OH)-16-ene-23-yne-D_3 showed similar potency as 1α,25$(OH)_2D_3$. The analog inhibited PZ-HPV-7 cell proliferation by 84.9 ± 11, 75.8 ± 8.3, 48.2 ± 8.3, and

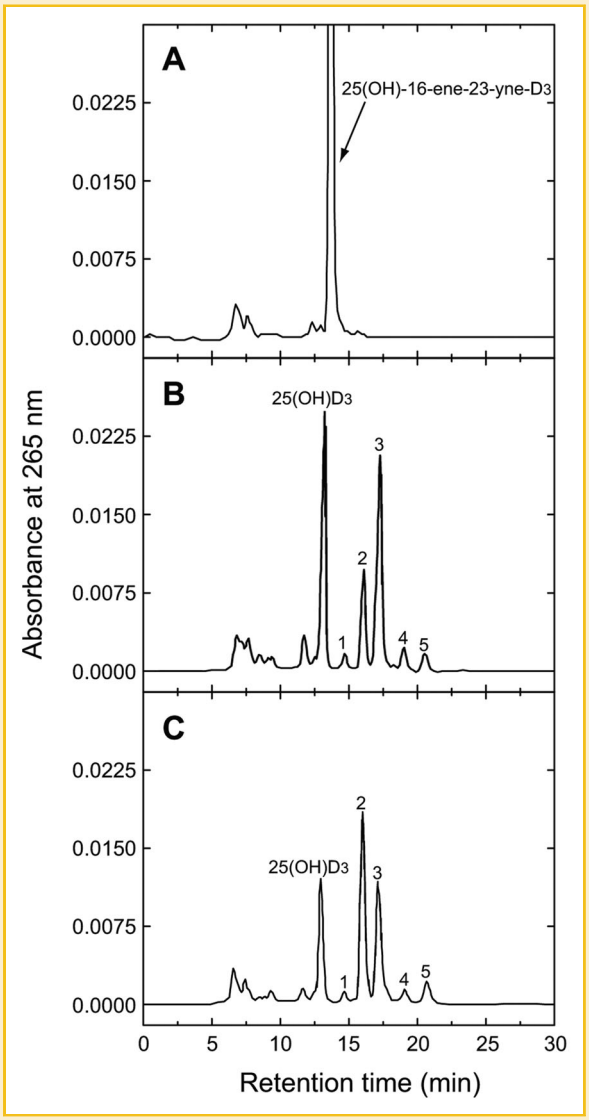

Fig. 5. HPLC profiles of metabolism of 25(OH)-16-ene-23-yne-D_3 and 25(OH)D_3 by rat CYP24A1 in a cell free reconstituted system. No metabolism of 25(OH)-16-ene-23-yne-D_3 by CYP24A1 was detected when incubated for 60 min (panel A). On the contrary, extensive metabolism of 25(OH)D_3 by CYP24A1 was detected when incubated for 15 min (panel B) and 60 min (panel C). The identity of each metabolite was confirmed by co-migration with known synthetic standards (1, 25(OH)-24-oxo-D_3; 2, 23(OH)-24,25,26,27-tetranor-D_3; 3, 23,25$(OH)_2$-24-oxo-D_3; 4, 24,25$(OH)_2D_3$; 5, 25(OH)D_3-26,23-lactone). The synthetic vitamin D analog, 1α,25,26-trihydroxy-16-ene-23-yne-vitamin D_3 (1 μg) was added as an internal standard to each sample prior to lipid extraction. HPLC analysis of lipid extracts of samples was performed using a Zorbax-SIL column (250 mm × 4.6 mm) eluted with 10% isopropanol in hexane at a flow rate of 2 mL/min.

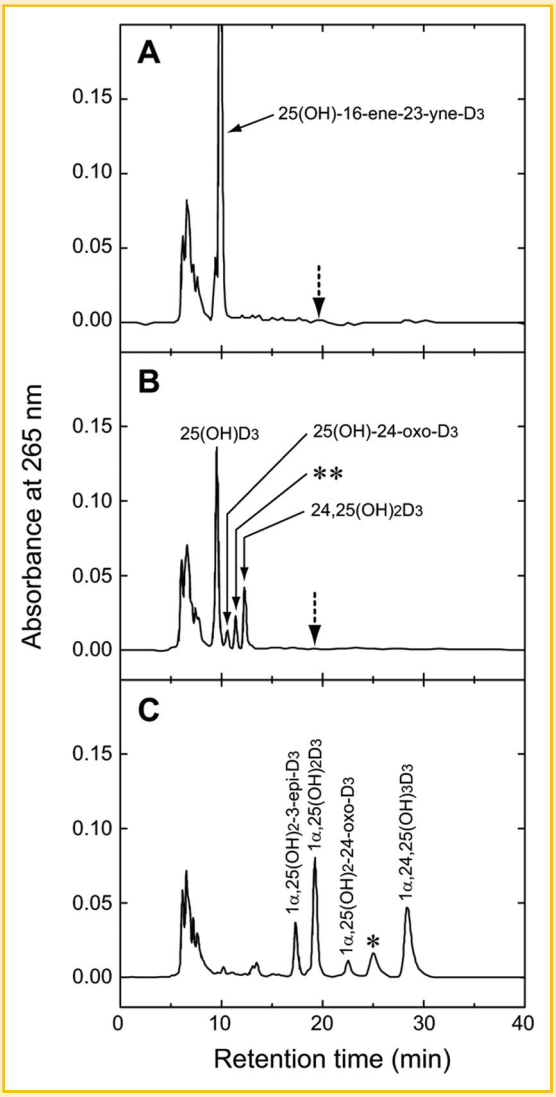

Fig. 6. HPLC profiles of metabolism of 25(OH)-16-ene-23-yne-D_3 (panel A), 25(OH)D_3 (panel B), and 1α,25$(OH)_2D_3$ (panel C) produced in PZ-HPV-7 cells. Each metabolite was identified based on co-migration with known standards. The asterisk (panel A) and double asterisks (panel B) denote a mixture of metabolites which cannot be separated in a given condition. The expected elution positions of 1α,25$(OH)_2D_3$ and 1α,25$(OH)_2$-16-ene-23-yne-D_3 are indicated by dashed arrows as shown in panels B and C, respectively. The synthetic vitamin D analog, 1α,25,26-trihydroxy-16-ene-23-yne-vitamin D_3 (1 μg) was added as an internal standard to each sample prior to lipid extraction. HPLC analysis of lipid extracts of samples was performed using a Zorbax-SIL column (250 mm × 4.6 mm) eluted with 15% isopropanol in hexane at a flow rate of 2 mL/min.

32.2 ± 4.9% at 0.1, 1, 10, and 100 nM, respectively, as compared to the controls (Fig. 7).

25(OH)-16-ene-23-yne-D_3 REQUIRES VDR TO EXPRESS ITS ANTIPROLIFERATIVE ACTIVITY

To determine whether 25(OH)-16-ene-23-yne-D_3 mediates its anti-proliferative effects through VDR, we utilized two tumor cell lines (KO288 and KO240) that were established from VDR-knockout mice. As shown in Figure 8A, 25(OH)-16-ene-23-yne-D_3 at concentrations as high as 100 nM had no effect on the growth of KO288 or KO240 cells. Similarly, 1α,25$(OH)_2D_3$ failed to alter growth of these VDR negative cells. We then tested the effects of both compounds on growth of KO240 cells that were engineered to stably express human VDR (KO^{hVDR} cells). As shown in Figure 8B, 25(OH)-16-ene-23-yne-

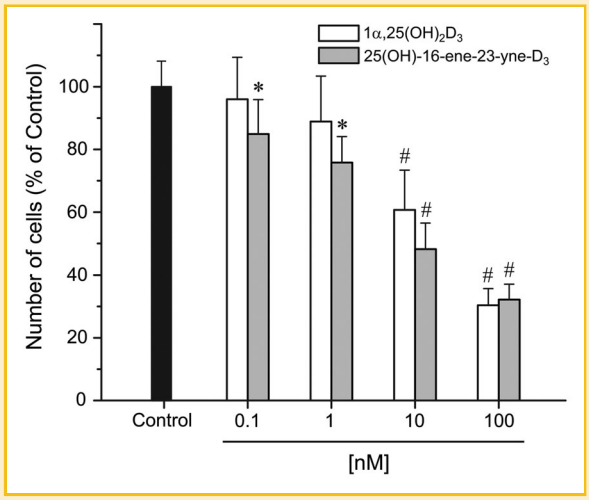

Fig. 7. Dose-dependent inhibition of ^{3}H-thymidine incorporation into DNA in PZ-HPV-7 cells induced by incubating with 0.1–100 nM of 1α,25(OH)$_2$D$_3$ or 25(OH)-16-ene-23-yne-D$_3$ for 18 h. Tritiated thymidine incorporation assay was performed on cells grown in 24-well plates as described in the Method section. Data represent mean ± S.D. of 5–8 determinations, $^*P < 0.05$, $^\#P < 0.01$ versus vehicle-treated controls.

D$_3$ inhibited growth of KO^{hVDR} cells over the range of 0.01–100 nM. Similar effects were observed in KO^{hVDR} cells treated with 1α,25(OH)$_2$D$_3$ in this dose range. The comparative effects of 25(OH)-16-ene-23-yne-D$_3$ and 1α,25(OH)$_2$D$_3$ on expression of the well characterized VDR target gene *Cyp24a1* were then evaluated in KO^{hVDR} cells. After 24 h incubation with 100 nM of 25(OH)-16-ene-23-yne-D$_3$, *Cyp24a1* was induced greater than 100-fold relative to vehicle treated cells (Fig. 8C). Induction of *Cyp24a1* by 1α,25(OH)$_2$D$_3$ at the same dose was over 3000-fold relative to control cells. Thus, 25(OH)-16-ene-23-yne-D$_3$ clearly activates VDR-mediated gene transcription but is approximately 30-fold less potent than 1α,25(OH)$_2$D$_3$ in this model cell system.

DISCUSSION

The importance of the addition of 1α-hydroxyl group to 25(OH)D$_3$ catalyzed by CYP27B1 has been clearly revealed by the fact that VDR binds to 1α,25(OH)$_2$D$_3$ with an affinity ~660-fold higher than 25(OH)D$_3$ and this in turn allows 1α,25(OH)$_2$D$_3$ to exert VDR-mediated calcemic as well as other noncalcemic biological activities [Bouillon et al., 1995]. Before the discovery of 1α,25(OH)$_2$D$_3$, it was noted that the patients with vitamin D-dependent rickets type I can be treated with pharmacologic doses of vitamin D$_3$. In these patients there is no possibility for the metabolism of 25(OH)D$_3$ into 1α,25(OH)$_2$D$_3$ as they have no CYP27B1 activity [Fraser et al., 1973]. The higher concentration of vitamin D$_3$ administered to these patients is readily metabolized into supraphysiological levels of 25(OH)D$_3$. The high circulating level of 25(OH)D$_3$ is believed to compensate for the poor binding affinity of 25(OH)D$_3$ to the VDR and thereby generates the same physiological effect as 1α,25(OH)$_2$D$_3$. These earlier observations indicated that 25(OH)D$_3$ by itself possesses low intrinsic VDR-mediated activity which can be substantially augmented by increasing the circulating levels of 25(OH)D$_3$ to a level that is twenty to thirty fold higher than the accepted physiological level.

Unequivocal evidence for the low intrinsic VDR-mediated activity of 25(OH)D$_3$ is provided more recently. For example, 25(OH)D$_3$ is found to suppress parathyroid hormone (PTH) secretion in bovine parathyroid cells where 25(OH)D$_3$ is converted into 1α,25(OH)$_2$D$_3$ by CYP27B1 [Ritter et al., 2006]. However, inhibition of CYP27B1 activity with clotrimazole, an inhibitor of CYPs, did not block 25(OH)D$_3$-mediated suppression of PTH, indicating that 25(OH)D$_3$ directly suppresses PTH secretion [Ritter et al., 2006]. In a later study, the same authors also reported the direct activity of 25(OH)D$_3$ on PTH secretion from thyroparathyroid explants developed from both wild-type and VDR-null mice [Ritter and Brown, 2011]. Inhibition of PTH secretion by 25(OH)D$_3$ was observed only in explants from wild-type mice but not in those from VDR-null mice. These findings provided direct proof to show that the suppressive action of 25(OH)D$_3$ on PTH secretion is indeed mediated by the VDR. In a different study, using CYP27B1-knockout cells and a CYP27B1-specific inhibitor, it was shown that 25(OH)D$_3$ has direct gene regulatory properties in different cellular systems including primary mouse kidney, skin, prostate cells, and human MCF-7 breast cancer cells [Lou et al., 2010]. More recently, DeLuca and colleagues demonstrated that oral administration of high doses of either vitamin D or 25(OH)D$_3$ in CYP27B1-knockout mice induced vitamin D intoxication evidenced by severe hypercalcemia [DeLuca et al., 2011]. Since CYP27B1-knockout mice are unable to produce 1α,25(OH)$_2$D$_3$, they concluded that high concentrations of 25(OH)D$_3$ can bind the VDR and induce gene transcription responsible for vitamin D toxicity. Based on the findings provided by the aforementioned studies, it is clear that 25(OH)D$_3$ possesses low intrinsic VDR-mediated biological activity without its activation into 1α,25(OH)$_2$D$_3$.

Here, we demonstrated how the low intrinsic VDR-mediated biological activities of 25(OH)D$_3$ can be further enhanced through the synthesis of novel analogs of 25(OH)D$_3$ such as 25(OH)-16-ene-23-yne-D$_3$. For example, 25(OH)-16-ene-23-yne-D$_3$ inhibited the growth of PZ-HPV-7 cells with a potency equal to 1α,25(OH)$_2$D$_3$ (Fig. 7). Most importantly, 25(OH)-16-ene-23-yne-D$_3$ resists CYP27B1 activity and does not undergo 1α-hydroxylation (Fig. 2). This finding precluded the possibility of the formation of even trace amounts of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$. Thus, the potent antiproliferative activity of 25(OH)-16-ene-23-yne-D$_3$ can be attributed to the analog itself without its further activation through 1α-hydroxylation into 1α,25(OH)$_2$-16-ene-23-yne-D$_3$. Historically, the 16-ene-23-yne modification was incorporated into 1α,25(OH)$_2$D$_3$ resulting in the synthesis of 1α,25(OH)$_2$-16-ene-23-yne-D$_3$. This prominent less calcemic vitamin D analog has been extensively studied for its many different biological activities [Schwartz et al., 1995; Light et al., 1997; Jain et al., 2011]. The reader is referred to a comprehensive review on the topic of vitamin D analogs with 16-ene-23-yne modification [Uskokovic et al., 2001]. Furthermore, we utilized two tumor cell lines established from VDR-knockout mice to confirm that the observed biological action of 25(OH)-16-ene-23-yne-D$_3$ is indeed mediated by the VDR (Fig. 8).

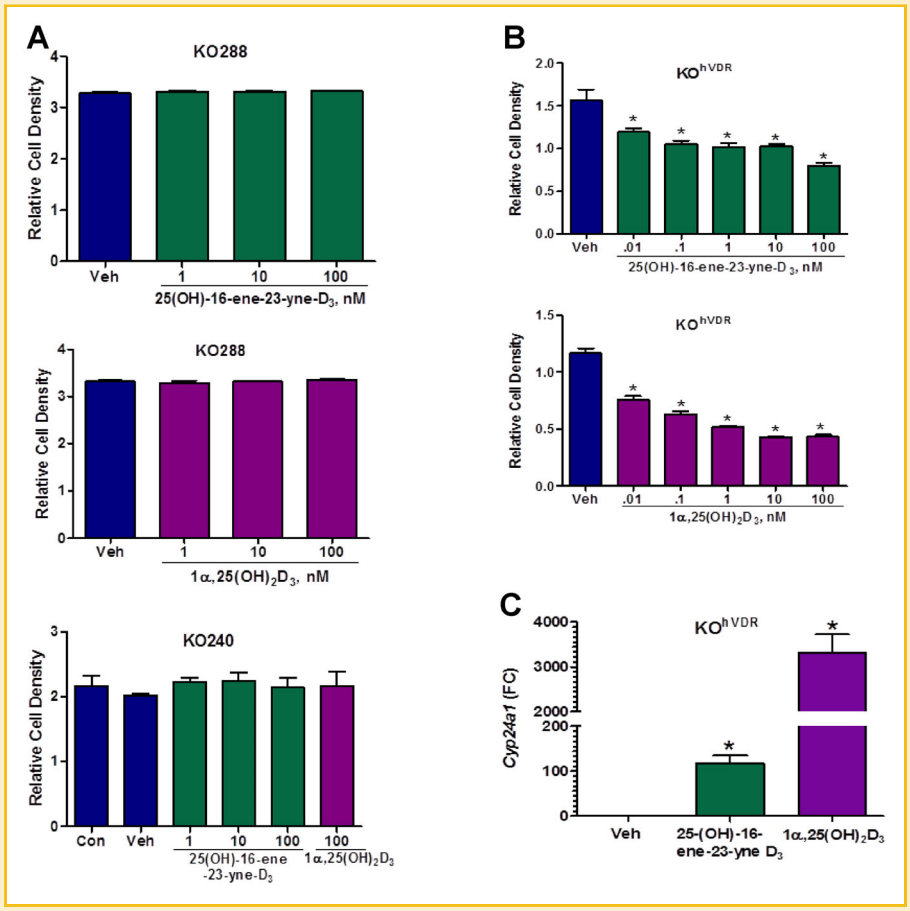

Fig. 8. 25(OH)-16-ene-23-yne-D_3 mediates its biological effects via VDR. (A) Effects of 96 h treatment with media alone (Con), ethanol vehicle (Veh), 25(OH)-16-ene-23-yne-D_3, or 1α,25$(OH)_2D_3$ on adherent growth of VDR negative KO288 and KO240 cell lines. Relative cell density reflects absorbance at 590 nm of crystal violet staining. (B) Effects of 96 h treatment with 25(OH)-16-ene-23-yne-D_3 or 1α,25$(OH)_2D_3$ on adherent growth of KO240 cells which stably express human VDR (KO^{hVDR}). Data are reported as mean of four replicates per experiment and are representative of at least two independent experiments. (C) *Cyp24a1* gene expression in $KO240^{hVDR}$ cells treated for 24 h with 100 nM 25(OH)-16-ene-23-yne-D_3 or 1α,25$(OH)_2D_3$. Data were obtained by qPCR, normalized to 18 S, and expressed as fold-change (FC) relative to control cells. Data represent mean ± S.E. of at least three replicates. *$P < 0.05$ as determined by one-way ANOVA and Dunnett's post test.

Despite the remarkably high specificity of CYP27B1 toward 25(OH)D_3 [Sakaki et al., 2005; Schuster, 2011; Jones et al., 2014], it is found that CYP27B1 has some degree of promiscuity toward a group of vitamin D compounds including 24,25$(OH)_2D_3$, 23,25-dihydroxyvitaminD$_3$, 24-oxo-25-hydroxyvitamin D_3, 24-oxo-23,25-dihydroxyvitamin D_3, 20-hydroxyvitamin D_3, 20,23-dihydroxyvitamin D_3, 20,24-dihydroxyvitamin D_3, 20,25-dihydroxyvitamin D_3, and 20,26-dihydroxyvitamin D_3 [Sakaki et al., 1999b; Urushino et al., 2007; Tang et al., 2010a; Tang et al., 2010b; Tang et al., 2012; Tang et al., 2013]. However, it is interesting to note that the 16-ene-23-yne modification to 25(OH)D_3 confers absolute resistance to the activity of CYP27B1. In support of our metabolism data, no spectral perturbation induced by 25(OH)-16-ene-23-yne-D_3 binding was observed (Fig. 3). Furthermore, the docking results suggest that the distance between the heme iron and a hydrogen atom at C-1 position of docked 25(OH)-16-ene-23-yne-D_3 is ~1.6-fold longer than the counterpart of 25(OH)D_3, thereby not being able to displace the iron-ligated water molecule (Fig. 4). We also obtained similar results when we examined the metabolism of another 25(OH)D_3 analog featuring the 16,23-diene modification that fully resisted the action of CYP27B1 (data not shown). This finding indicates that 25(OH)D_3 analogs featuring the unsaturated side chain (i.e., either double or triple bonds between C-23 and C-24) together with the 16-ene modification thwart enzymatic activity of CYP27B1. Whether such effects can be warranted solely by the 16-ene modification, however, awaits further study.

The mechanism underlying the VDR-mediated biological actions exerted by vitamin D compounds lacking the 1α-hydroxyl group has not been fully understood. Although both 25-hydroxy-19-nor-vitamin D_3 and 25(OH)-16-ene-23-yne-D_3 share the common property of resisting hydroxylation at C-1α position by CYP27B1 and displaying potent VDR-mediated antiproliferative activity in PZ-HPV-7 cells, the two analogs differ mainly in their interaction with CYP24A1. We noted that 25(OH)-16-ene-23-yne-D_3 completely resists CYP24A1-mediated inactivation (Figs. 5 and 6) while 25-hydroxy-19-nor-vitamin D_3 was reported to be readily metabolized by CYP24A1. Thus, the potent antiproliferative activity of 25(OH)-16-ene-23-yne-D_3, unlike 25-hydroxy-19-nor-vitamin D_3, appears to be due to its metabolic stability. As reported in our previous study [Rhieu et al., 2011], the presence of 16-ene-23-yne modification in

1α,25(OH)$_2$-16-ene-23-yne-D$_3$ renders the compound resistant to the action of CYP24A1. The same holds true for 25(OH)-16-ene-23-yne-D$_3$, which remains unmetabolized by CYP24A1 (Fig. 5), suggesting that the metabolic stability of 25(OH)-16-ene-23-yne-D$_3$ prolongs its antiproliferative activity in cells.

In summary, we identified 25(OH)-16-ene-23-yne-D$_3$ as a novel non-1α-hydroxylated vitamin D analog that exhibits antiproliferative activity against immortalized human prostate cells with a potency equal to 1α,25(OH)$_2$D$_3$. Despite the absence of the 1α-hydroxyl group, the observed biological action of 25(OH)-16-ene-23-yne-D$_3$ is mediated by the VDR. The enzymatic conversion of 25(OH)-16-ene-23-yne-D$_3$ to 1α,25(OH)$_2$-16-ene-23-yne-D$_3$ by CYP27B1 is completely impeded by the 16-ene-23-yne modification, which also enables 25(OH)-16-ene-23-yne-D$_3$ to gain metabolic stability against the action of CYP24A1, thereby prolonging its antiproliferative activity. We now propose that a significant increase in the potency of the intrinsic VDR-mediated activities of 25(OH)D$_3$ can be achieved by simply preventing its inactivation by CYP24A1 without the need for its activation through 1α-hydroxylation by CYP27B1. We now envision that the intensity of hypercalcemia exerted by the presence of 1α-hydroxyl group in 1α,25(OH)$_2$D$_3$ and its related analogs can be reduced substantially by the introduction of a new generation of non-1α-hydroxylated vitamin D analogs such as 25-hydroxy-19-nor-vitamin D$_3$ [Urushino et al., 2007] and 25(OH)-16-ene-23-yne-D$_3$.

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