

# Hydroxylation of CYP11A1-Derived Products of Vitamin D3 Metabolism by Human and Mouse CYP27B1

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Received January 12, 2013; accepted February 28, 2013

## ABSTRACT

CYP11A1 can hydroxylate vitamin D3 at carbons 17, 20, 22, and 23, producing a range of secosteroids which are biologically active with respect to their ability to inhibit proliferation and stimulate differentiation of various cell types, including cancer cells. As 1 $\alpha$ -hydroxylation of the primary metabolite of CYP11A1 action, 20S-hydroxyvitamin D3 [20(OH)D3], greatly influences its properties, we examined the ability of both human and mouse CYP27B1 to 1 $\alpha$ -hydroxylate six secosteroids generated by CYP11A1. Based on their  $k_{cat}/K_m$  values, all CYP11A1-derived metabolites are poor substrates for CYP27B1 from both species compared with 25-hydroxyvitamin D3. No hydroxylation of metabolites with a 17 $\alpha$ -hydroxyl group was observed. 17 $\alpha$ ,20-Dihydroxyvitamin D3 acted as an inhibitor on human CYP27B1 but not the mouse enzyme. We also tested CYP27B1 activity on 20,24-, 20,25-, and 20,26-dihydroxyvitamin D3, which are products of CYP24A1 or

CYP27A1 activity on 20(OH)D3. All three compounds were metabolized with higher catalytic efficiency ( $k_{cat}/K_m$ ) by both mouse and human CYP27B1 than 25-hydroxyvitamin D3. CYP27B1 action on these new dihydroxy derivatives was confirmed to be 1 $\alpha$ -hydroxylation by mass spectrometry and nuclear magnetic resonance analyses. Both 1,20,25- and 1,20,26- trihydroxyvitamin D3 were tested for their ability to inhibit melanoma (SKMEL-188) colony formation, and were significantly more active than 20(OH)D3. This study shows that CYP11A1-derived secosteroids are 1 $\alpha$ -hydroxylated by both human and mouse CYP27B1 with low catalytic efficiency, and that the presence of a 17 $\alpha$ -hydroxyl group completely blocks 1 $\alpha$ -hydroxylation. In contrast, the secondary metabolites produced by subsequent hydroxylation of 20(OH)D3 at C24, C25, or C26 are very good substrates for CYP27B1.

## Introduction

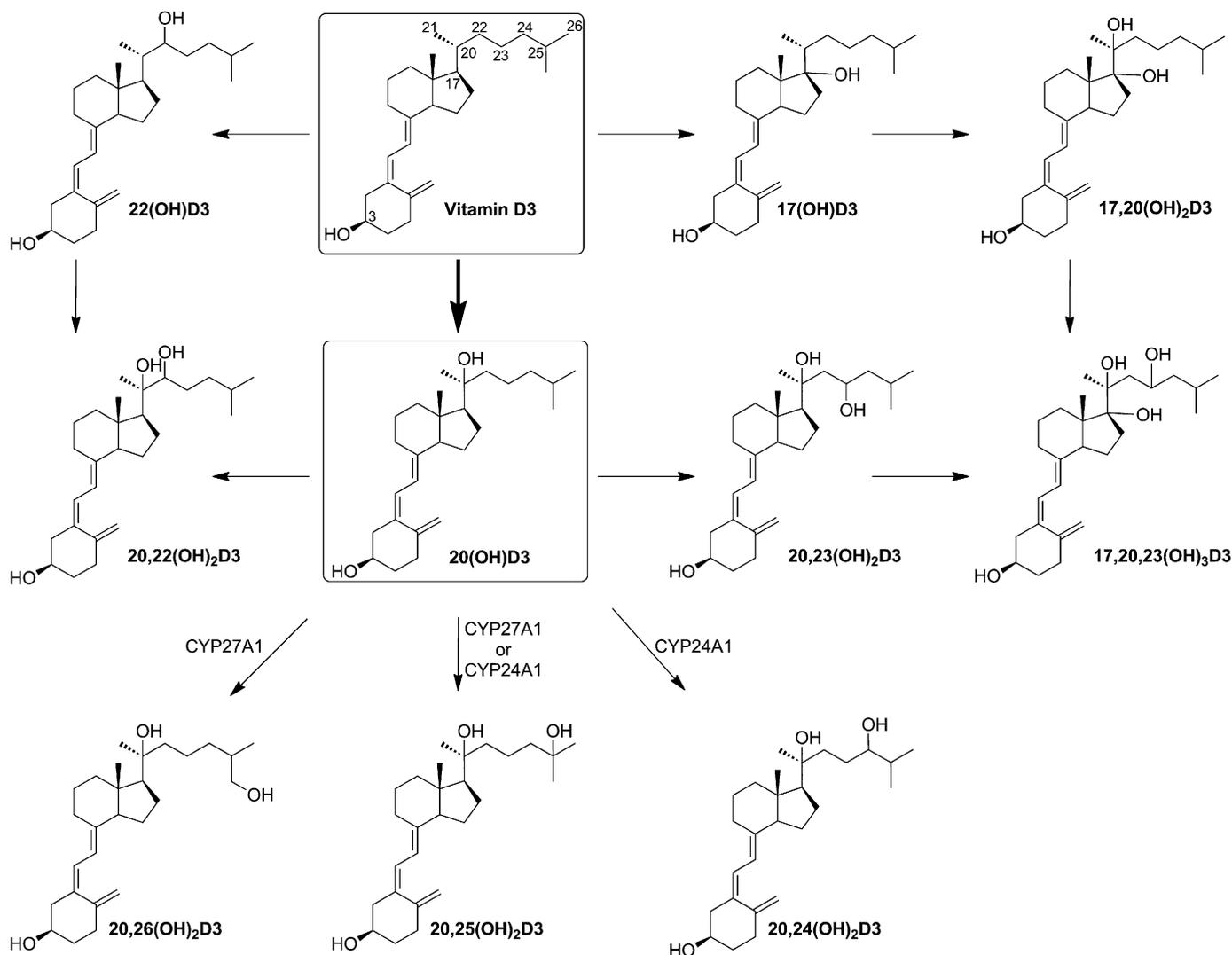
CYP27B1 is the mitochondrial cytochrome P450 that catalyzes the final step of vitamin D activation, the 1 $\alpha$ -hydroxylation of 25-hydroxyvitamin D3 [25(OH)D3], to give 1,25-dihydroxyvitamin D3 [1,25(OH)<sub>2</sub>D3]. 1,25(OH)<sub>2</sub>D3 maintains calcium and phosphorus homeostasis, plays an important role in regulating the immune system and displays antiproliferative and prodifferentiative properties on many different cell types (Prosser and Jones, 2004; Bouillon et al., 2006; Masuda and Jones, 2006; Takahashi and Morikawa, 2006; Holick, 2007; Bikle, 2009). Its potential for the treatment of cancer and immune disorders is limited, however, due largely to the toxic hypercalcemia that occurs at the supraphysiological doses required.

This work was supported by the National Institutes of Health [Grant R01AR052190] (to A.T.S.) and [Grant 1S10RR026377-01]; the University of Western Australia; and the College of Pharmacy at the University of Tennessee Health Science Center.

dx.doi.org/10.1124/dmd.113.050955.

This has led to the search for new vitamin D analogs that retain the antiproliferative and prodifferentiative properties of 1,25(OH)<sub>2</sub>D3 but are not calcemic (Masuda and Jones, 2006; Takahashi and Morikawa, 2006; Lee et al., 2008; Slominski et al., 2010). Vitamin D analogs that have such properties are produced by the action of cytochrome P450scc (CYP11A1) on vitamin D (Slominski et al., 2010, 2011; Wang et al., 2012), and several of them show antimelanoma activity in cell culture models (Janjetovic et al., 2011; Slominski et al., 2012a, 2013b). CYP11A1 can act on vitamin D3, producing at least eight different mono-, di-, and trihydroxyvitamin D derivatives, with the major products being 20S-hydroxyvitamin D3 [20(OH)D3] and 20,23-dihydroxyvitamin D3 [20,23(OH)<sub>2</sub>D3] (Fig. 1) (Guryev et al., 2003; Slominski et al., 2005, 2012b; Tuckey et al., 2008a, 2011). Several of these secosteroids appear to be produced in vivo, with 20(OH)D3, 22(OH)D3, 20,23(OH)<sub>2</sub>D3, and 17,20,23(OH)<sub>3</sub>D3 being detected in cultured keratinocytes without the addition of exogenous vitamin D3 as a substrate (Slominski et al., 2012b). To date, 20(OH)D3 and 20,23(OH)<sub>2</sub>D3, as well as 20-hydroxyvitamin D2 [20(OH)D2], the

**ABBREVIATIONS:** *E. coli*, *Escherichia coli*; HPLC, high-performance liquid chromatography; HSQC, heteronuclear single-quantum correlation; NMR, nuclear magnetic resonance; 17,20(OH)<sub>2</sub>D2, 17 $\alpha$ ,20-dihydroxyvitamin D2; 17,20(OH)<sub>2</sub>D3, 17 $\alpha$ ,20-dihydroxyvitamin D3; 17,20,23(OH)<sub>3</sub>D3, 17 $\alpha$ ,20,23-trihydroxyvitamin D3; 20(OH)D2, 20-hydroxyvitamin D2; 20(OH)D3, 20S-hydroxyvitamin D3; 20,23(OH)<sub>2</sub>D3, 20,23-dihydroxyvitamin D3; 20,24(OH)<sub>2</sub>D3, 20,24-dihydroxyvitamin D3; 20,25(OH)<sub>2</sub>D3, 20,25-dihydroxyvitamin D3; 20,26(OH)<sub>2</sub>D3, 20,26-dihydroxyvitamin D3; 25(OH)D3, 25-hydroxyvitamin D3; 1,20(OH)<sub>2</sub>D3, 1 $\alpha$ ,20-dihydroxyvitamin D3; 1,25(OH)<sub>2</sub>D3, 1 $\alpha$ ,25-dihydroxyvitamin D3; 1,20,23(OH)<sub>3</sub>D3, 1 $\alpha$ ,20,23-trihydroxyvitamin D3; 1,20,24(OH)<sub>3</sub>D3, 1 $\alpha$ ,20,24-trihydroxyvitamin D3; 1,20,25(OH)<sub>3</sub>D3, 1 $\alpha$ ,20,25-trihydroxyvitamin D3; 1,20,26(OH)<sub>3</sub>D3, 1 $\alpha$ ,20,26-trihydroxyvitamin D3; TOCSY, total correlation spectroscopy.



**Fig. 1.** Pathways for the enzymatic synthesis of vitamin D analogs tested as substrates for CYP27B1. The substrate, vitamin D<sub>3</sub>, and the major product of CYP11A1 action on it, 20(OH)D<sub>3</sub>, are enclosed in boxes. All reactions shown by arrows without a label are catalyzed by CYP11A1. CYP27A1 and CYP24A1 also hydroxylate 20(OH)D<sub>3</sub>, as shown in the lower part of the figure.

major product of CYP11A1 activity on vitamin D<sub>2</sub> (Slominski et al., 2006; Nguyen et al., 2009), are the most extensively studied for their biologic effects. Via binding to the vitamin D receptor, these compounds can inhibit keratinocyte, melanoma, leukemia, breast, and liver cancer cell proliferation; promote differentiation; and display anti-inflammatory activity by decreasing nuclear factor- $\kappa$ B activity (Zbytek et al., 2008; Janjetovic et al., 2009, 2010, 2011; Li et al., 2010; Slominski et al., 2010, 2011, 2012a; Tang et al., 2010a; Tuckey et al., 2011; Kim et al., 2012; Lu et al., 2012; Wang et al., 2012). Importantly, all three of these derivatives lack calcemic activity in rodents, even at relatively high doses (Slominski et al., 2010, 2013a; Wang et al., 2012). However, the addition of a hydroxyl group to 20(OH)D<sub>3</sub> at the  $1\alpha$ -position (producing  $1\alpha,20$ -dihydroxyvitamin D<sub>3</sub> [1,20(OH)<sub>2</sub>D<sub>3</sub>]) causes the appearance of some calcemic activity, although less than that for 1,25(OH)<sub>2</sub>D<sub>3</sub> (Slominski et al., 2010). The therapeutic potential of the noncalcemic, CYP11A1-derived secosteroids is illustrated by the marked reduction in collagen synthesis and clinical signs of bleomycin-induced scleroderma in mice following 20(OH)D<sub>3</sub> treatment (Slominski et al., 2013a).

Recently, we have shown that 20(OH)D<sub>3</sub> can be hydroxylated by cytochrome P450s that are involved in the preliminary activation

(25-hydroxylase; CYP27A1) and inactivation (24-hydroxylase; CYP24A1) of vitamin D<sub>3</sub> (Tieu et al., 2012a,b). Specifically, CYP27A1 hydroxylates 20(OH)D<sub>3</sub> at either C25 or C26, producing 20,25-dihydroxyvitamin D<sub>3</sub> [20,25(OH)<sub>2</sub>D<sub>3</sub>] and 20,26-dihydroxyvitamin D<sub>3</sub> [20,26(OH)<sub>2</sub>D<sub>3</sub>] (Tieu et al., 2011). CYP24A1 also hydroxylates 20(OH)D<sub>3</sub> at C25, producing 20,25(OH)<sub>2</sub>D<sub>3</sub>. Additionally, it hydroxylates at the expected C24 position, producing 20,24-dihydroxyvitamin D<sub>3</sub> [20,24(OH)<sub>2</sub>D<sub>3</sub>] (Fig. 1) (Tieu et al., 2012b). All three of these products are more potent than the parent, 20(OH)D<sub>3</sub>, at inhibiting colony formation by melanoma cells in soft agar (Tieu et al., 2012b).

Previously, we reported that purified mouse CYP27B1 can  $1\alpha$ -hydroxylate the two major products of CYP11A1 action on vitamin D<sub>3</sub>, 20(OH)D<sub>3</sub> and 20,23(OH)<sub>2</sub>D<sub>3</sub>, and one of the major products of CYP11A1 action on vitamin D<sub>2</sub>, 20(OH)D<sub>2</sub>, with the products displaying altered biological activity compared with the parent secosteroids (Tang et al., 2010a,b, 2012; Slominski et al., 2011). Recently, we successfully expressed human CYP27B1 in *Escherichia coli* (*E. coli*), extracted and partially purified the enzyme, and carried out a detailed characterization of its catalytic activity on its classic substrates (Tang et al., 2012). To further characterize CYP27B1, especially the human isoform, and to further characterize the ability of

the CYP11A1-derived secosteroids to be metabolized by cytochromes P450, we examined the ability of both human and mouse CYP27B1 to metabolize these secosteroids, including those further hydroxylated by CYP27A1 and CYP24A1 (Fig. 1).

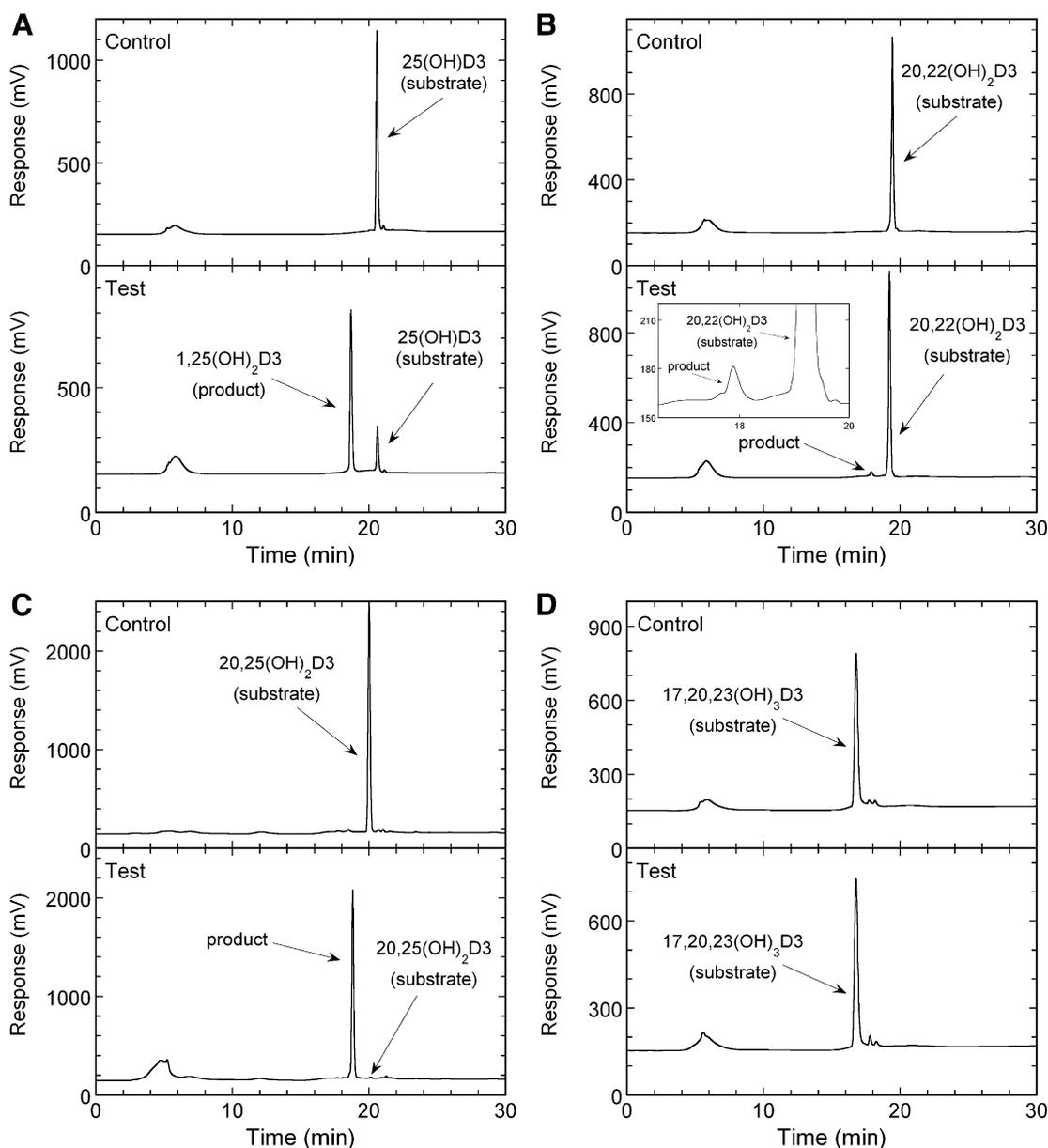
### Materials and Methods

**Preparation of Enzymes and Vitamin D Analogs.** Mouse and human adrenodoxin and human adrenodoxin reductase were expressed in *E. coli* and purified as before (Woods et al., 1998; Tuckey and Sadleir, 1999; Tang et al., 2010b, 2012). Mouse and human CYP27B1 were coexpressed in *E. coli* with the chaperonins, GroEL/ES, to facilitate correct protein folding, as described before (Tang et al., 2010b, 2012). The cDNA constructs for mouse and human CYP27B1 encoded a 4- and 6-histidine tag, respectively, at the C terminus, and the N-terminal mitochondrial-targeting sequences were removed. The expressed mouse and human CYP27B1 were extracted using CHAPS detergent

(3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate) and purified by nickel affinity and octyl Sepharose chromatography (Tang et al., 2010b, 2012).

20(OH)D<sub>3</sub>, 22-hydroxyvitamin D<sub>3</sub> [22(OH)D<sub>3</sub>], 20,22-dihydroxyvitamin D<sub>3</sub> [20,22(OH)<sub>2</sub>D<sub>3</sub>], 20,23(OH)<sub>2</sub>D<sub>3</sub>, 17 $\alpha$ ,20-dihydroxyvitamin D<sub>3</sub> [17,20(OH)<sub>2</sub>D<sub>3</sub>], and 17,20,23(OH)<sub>3</sub>D<sub>3</sub> were generated by the action of CYP11A1 on vitamin D<sub>3</sub> solubilized in 2-hydroxypropyl- $\beta$ -cyclodextrin and purified by thin-layer chromatography and high-performance liquid chromatography (HPLC) (Guryev et al., 2003; Tuckey et al., 2008a, 2011). 20,24(OH)<sub>2</sub>D<sub>3</sub> and 20,25(OH)<sub>2</sub>D<sub>3</sub> were produced by the action of rat CYP24A1 on 20(OH)D<sub>3</sub> (Tieu et al., 2012b), whereas 20,26(OH)<sub>2</sub>D<sub>3</sub> was generated by the action of human CYP27A1 on 20(OH)D<sub>3</sub> (Tieu et al., 2012b).

**Measurement of Human CYP27B1 Activity in Phospholipid Vesicles.** Phospholipid vesicles comprising dioleoyl phosphatidylcholine (Sigma-Aldrich, St. Louis, MO), bovine heart cardiolipin (Sigma-Aldrich), and the vitamin D<sub>3</sub> analogs under study were prepared by sonication in a bath-type sonicator as described in detail before (Lambeth et al., 1982; Tuckey and Kamin, 1982; Tuckey et al., 2008b). The incubation mixture comprised vesicles



**Fig. 2.** Chromatograms showing metabolism of 25(OH)D<sub>3</sub> (A), 20,22(OH)<sub>2</sub>D<sub>3</sub> (B), 20,25(OH)<sub>2</sub>D<sub>3</sub> (C), and 17,20,23(OH)<sub>3</sub>D<sub>3</sub> (D) incorporated into phospholipid vesicles by human CYP27B1. Small unilamellar phospholipid vesicles were prepared with 85% dioleoyl phosphatidylcholine (DOPC) and 15% cardiolipin with the substrates at a ratio of 0.02 mol/mol phospholipid. CYP27B1 (0.1  $\mu$ M) was added to the vesicles, and reactions were carried out in the presence of human adrenodoxin reductase (0.4  $\mu$ M) and adrenodoxin (15  $\mu$ M) for 1 hour at 37°C. The secosteroids were extracted with dichloromethane and analyzed by reverse-phase HPLC on a C18 column. In control incubations, adrenodoxin was omitted (top panels).

(510  $\mu\text{M}$  phospholipid), CYP27B1 (5–800 nM), adrenodoxin (15  $\mu\text{M}$ ), adrenodoxin reductase (0.4  $\mu\text{M}$ ), glucose 6-phosphate (2 mM), glucose-6-phosphate dehydrogenase (2 U/ml), and NADPH (50  $\mu\text{M}$ ) in the same buffer used for vesicle preparation (20 mM HEPES, pH 7.4, 100 mM NaCl, 0.1 mM dithiothreitol, 0.1 mM EDTA). Samples (typically 0.25–2.0 ml) were pre-incubated for 4 minutes at 37°C, and then the reaction was initiated by the addition of adrenodoxin. Samples were incubated at 37°C with shaking for 2 minutes (unless otherwise stated), and then reactions were terminated by the addition of 2 volumes of ice-cold dichloromethane and vortexing (Guryev et al., 2003). After phase separation by centrifugation, the lower organic phase was retained, and the upper aqueous phase was extracted twice more with two volumes of dichloromethane. The dichloromethane was subsequently removed under nitrogen, and the residual sample was dissolved in 64 or 75% methanol in water or 100% ethanol for analysis. Product formation was measured by reverse-phase HPLC using either a PerkinElmer Biocompatible Binary Pump 250/TurboChrom (PerkinElmer, Waltham, MA) or HP Agilent 1050/ChemStation system with a GraceSmart C18 column (4.6  $\times$  150 mm, particle size 5  $\mu\text{m}$ ; Agilent Technologies, Palo Alto, CA) as described before (Tang et al., 2010b).

**Analysis of Kinetics Data.** The experimental data were fitted to the Michaelis-Menten equation. If substrate inhibition was observed, data were also fitted to the inhibition model equation that we have previously described (Tang et al., 2010b), using Kaleidagraph 4.1 (Abelbeck Synergy Software, Reading, PA). This equation is derived from a model where two molecules of substrate bind to the enzyme substrate complex simultaneously with equal  $K_i$  values to form an ES<sub>3</sub> (enzyme bound to 3 molecules of substrate) complex that is catalytically inactive. As well as the  $K_m$  and  $k_{cat}$  values, this equation also provides the inhibitor constant for substrate ( $K_i$ ).

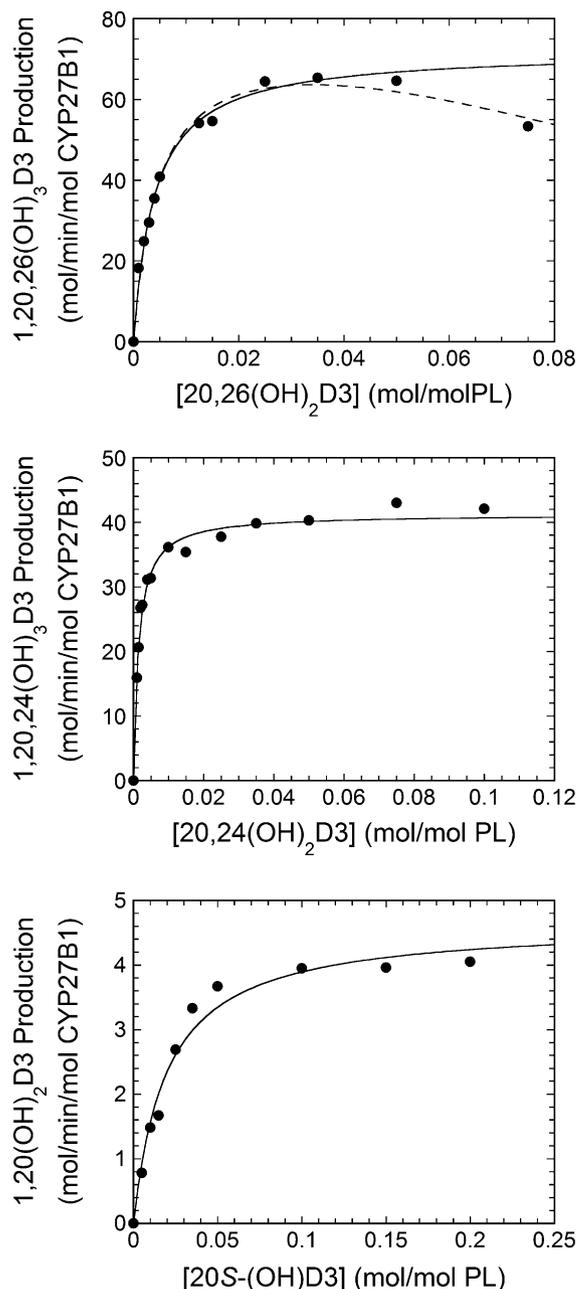
**Large-Scale Preparation of 1 $\alpha$ -Hydroxylated Metabolites.** To produce metabolites on a scale that permitted structure determination by nuclear magnetic resonance (NMR) (more than 30  $\mu\text{g}$ ), mouse CYP27B1 (0.3  $\mu\text{M}$ ) was incubated with 20,24(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, or 20,26(OH)<sub>2</sub>D<sub>3</sub> incorporated into phospholipid vesicles (0.025 or 0.03 mol/mol phospholipid) in 10–25-ml incubations comprising vesicle preparation buffer (as described earlier), 15  $\mu\text{M}$  mouse adrenodoxin, 0.4  $\mu\text{M}$  human adrenodoxin reductase, 2 mM glucose-6-phosphate, 2 U/ml glucose-6-phosphate dehydrogenase, and 50  $\mu\text{M}$  NADPH. The reactions were performed for 1 hour at 25°C with shaking. Reactions were stopped with 2 volumes of ice-cold dichloromethane, and products were extracted in a scaled-up version of that described earlier. The products were purified by reverse-phase HPLC using a GraceSmart column (PerkinElmer Biocompatible Binary Pump/TurboChrom system) with a gradient of 64% methanol in water at a flow rate of 0.5 ml/min for 10 minutes (Guryev et al., 2003; Tuckey et al., 2008a). The yield of products, subsequently shown to be the expected 1,20,24-trihydroxyvitamin D<sub>3</sub> [1,20,24(OH)<sub>3</sub>D<sub>3</sub>], 1,20,25-trihydroxyvitamin D<sub>3</sub> [1,20,25(OH)<sub>3</sub>D<sub>3</sub>], and 1,20,26-trihydroxyvitamin D<sub>3</sub> [1,20,26(OH)<sub>3</sub>D<sub>3</sub>] (see *Results*), was determined spectrophotometrically at 263 nm using an extinction coefficient of 18,000  $\text{M}^{-1}\text{cm}^{-1}$  (Hiwatashi et al., 1982).

**Mass Spectroscopy.** Mass spectra were acquired in a Bruker Esquire-Liquid Chromatography/Mass Spectrometry system (Bruker Daltonics, Billerica, MA), using the ionization source of electrospray ionization with nitrogen as the nebulizing gas. Data were collected and processed by an ACD mass processor (Advanced Chemistry Development, Toronto, ON, Canada).

**NMR Spectroscopy.** NMR measurements were performed using an inverse triple-resonance 3-mm probe on a Varian Unity Inova 500 MHz spectrometer (Agilent Technologies). The sample was dissolved in methanol- $d_4$  and transferred to a 3-mm Shigemi NMR tube (Shigemi Inc., Allison Park, PA). Temperature was regulated at 22°C, and was controlled with an accuracy of  $\pm 0.1^\circ\text{C}$ . Chemical shifts were referenced to residual solvent peaks for CD<sub>3</sub>OD (3.31 ppm for proton and 49.15 ppm for carbon). Standard two-dimensional NMR experiments [<sup>1</sup>H-<sup>1</sup>H correlation spectroscopy, <sup>1</sup>H-<sup>1</sup>H total correlation spectroscopy (TOCSY; mixing time = 80 milliseconds), <sup>1</sup>H-<sup>13</sup>C heteronuclear single-quantum correlation spectroscopy (HSQC), and <sup>1</sup>H-<sup>13</sup>C heteronuclear multiple-bond correlation spectroscopy] were conducted to fully elucidate the structures of the metabolites. All data were processed using ACD software, with zero filling in the direct dimension and linear prediction in the indirect dimension.

**Measurement of Cell Proliferation in Soft Agar.** The effect of the secosteroids hydroxylated by CYP27B1 on the tumorigenicity of SKMEL-188 melanoma cells was determined by assaying their ability to form colonies in

soft agar, as described in detail before (Slominski et al., 2012a; Tieu et al., 2012b). Secosteroids were added in 100  $\mu\text{l}$  of media to final concentrations of 0.1 or 10 nM. Each condition was tested in quadruplicates. Cells were grown at 37°C with 5% CO<sub>2</sub> over 2 weeks, with secosteroids in fresh media (100  $\mu\text{l}$ ) being added after every 72 hours. Colonies were then scored and stained with methylthiazol-tetrazolium reagent (Promega, Madison, WI), and then counted under the microscope. Data were analyzed with Student's *t* test (for two groups) using Prism 4.0 (GraphPad Software, San Diego, CA).



**Fig. 3.** The effect of substrate concentration on the rate of hydroxylation of different substrates by human CYP27B1. Reaction rates were determined for 20,26(OH)<sub>2</sub>D<sub>3</sub> (top), 20,24(OH)<sub>2</sub>D<sub>3</sub> (middle), and 20(OH)D<sub>3</sub> (bottom) using substrates incorporated into phospholipid (PL) vesicles prepared from dioleoyl phosphatidylcholine (DOPC) and cardiolipin. CYP27B1 was incubated with the vesicle-associated substrates for 2 minutes in the presence of adrenodoxin and adrenodoxin reductase. Substrates and products were extracted and analyzed by reverse-phase HPLC. Data for high substrate concentration where inhibition was observed were excluded from the Michaelis-Menten curve fits (solid lines). Data for 20,26(OH)<sub>2</sub>D<sub>3</sub> were also fitted to a model describing substrate inhibition as described in *Materials and Methods* (dashed line).

TABLE 1

Kinetic parameters for metabolism of various substrates incorporated into phospholipid vesicles by mouse and human CYP27B1

Activity of human and mouse CYP27B1 was determined toward various substrates incorporated into small unilamellar phospholipid (PL) vesicles comprising 15 mol% cardiolipin and 85 mol% dioleoyl phosphatidylcholine (DOPC). Data are the mean  $\pm$  S.E. of the curve fit for representative experiments. Kinetic parameters were determined from fitting the Michaelis-Menten equation to the data.

Substrate	Mouse			Human		
	$K_m$	$k_{cat}$	$k_{cat}/K_m$	$K_m$	$k_{cat}$	$k_{cat}/K_m$
	mmol/mol PL	min <sup>-1</sup>		mmol/mol PL	min <sup>-1</sup>	
25(OH)D3 <sup>a,b</sup>	5.9 $\pm$ 1.2	41 $\pm$ 3	6.9 $\pm$ 1.9	5.4 $\pm$ 0.8	55.4 $\pm$ 4.4	10.3 $\pm$ 2.3
20(OH)D3 <sup>b</sup>	49 $\pm$ 21	8.5 $\pm$ 1.7	0.17 $\pm$ 0.11	19.5 $\pm$ 3.0	4.7 $\pm$ 0.2	0.24 $\pm$ 0.05
22(OH)D3		Low activity detected			Low activity detected	
17,20(OH) <sub>2</sub> D3		No activity detected			No activity detected	
20,22(OH) <sub>2</sub> D3 <sup>c</sup>	53.2 $\pm$ 8.4	1.8 $\pm$ 0.2	0.03 $\pm$ 0.01		Low activity detected	
20,23(OH) <sub>2</sub> D3	12.1 $\pm$ 1.6	8.1 $\pm$ 0.3	0.67 $\pm$ 0.12	9.7 $\pm$ 1.8	4.1 $\pm$ 0.2	0.43 $\pm$ 0.10
17,20,23(OH) <sub>3</sub> D3		No activity detected			No activity detected	
20,24(OH) <sub>2</sub> D3 <sup>c</sup>	1.4 $\pm$ 0.2	39.5 $\pm$ 1.3	28.7 $\pm$ 5.1	1.4 $\pm$ 0.1	41.3 $\pm$ 0.7	29.1 $\pm$ 2.8
20,25(OH) <sub>2</sub> D3 <sup>c</sup>	3.4 $\pm$ 0.3	63.9 $\pm$ 1.6	18.6 $\pm$ 2.1	2.2 $\pm$ 0.4	63.7 $\pm$ 3.0	29.6 $\pm$ 6.5
20,26(OH) <sub>2</sub> D3 <sup>c</sup>	2.9 $\pm$ 0.6	60.7 $\pm$ 3.2	20.9 $\pm$ 5.4	3.9 $\pm$ 0.3	72.1 $\pm$ 1.8	18.3 $\pm$ 1.9

<sup>a</sup> Compounds where substrate inhibition was observed for mouse and human CYP27B1.

<sup>b</sup> Kinetic parameters from previously published data (Tang et al., 2010b, 2012), except for human CYP27B1 with 20(OH)D3.

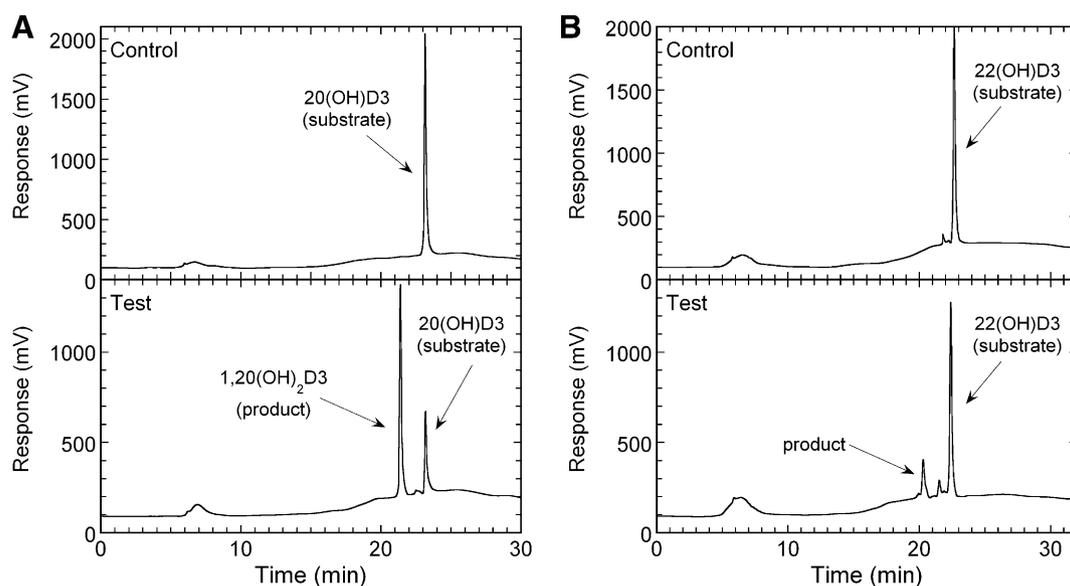
<sup>c</sup> Compounds where substrate inhibition was observed for mouse CYP27B1 only.

**Other Procedures.** The concentration of CYP27B1 was determined from the CO-reduced minus reduced difference spectrum using an extinction coefficient of 91,000 M<sup>-1</sup>cm<sup>-1</sup> for the absorbance difference between 450 and 490 nm (Omura and Sato, 1964). The concentrations of all hydroxyvitamin D3 stock solutions were determined using an extinction coefficient of 18,000 M<sup>-1</sup>cm<sup>-1</sup> at 263 nm (Hiwatashi et al., 1982).

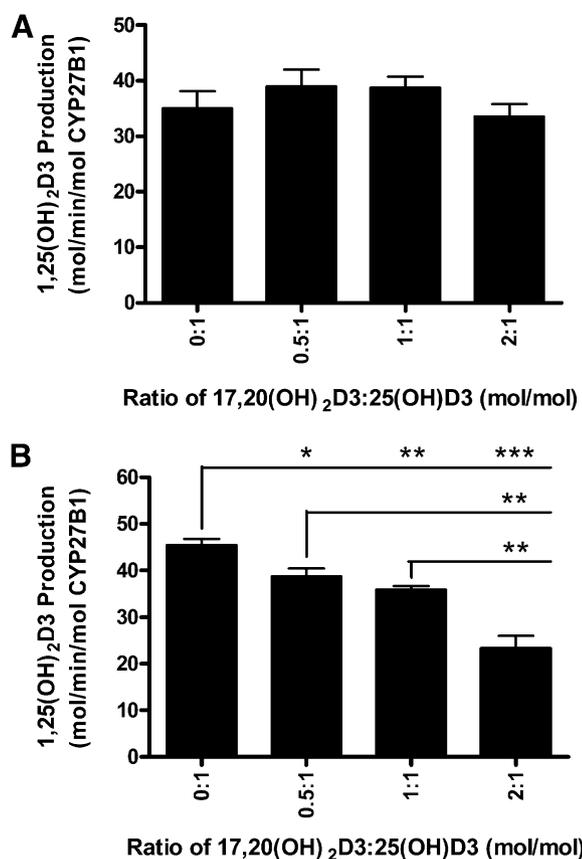
## Results

**Kinetics of the Metabolism of CYP11A1-Derived Vitamin D3 Analogs by CYP27B1.** CYP27B1 activity was measured with substrates incorporated into phospholipid vesicles, a system that mimics the native environment of the cytochrome in the inner mitochondrial membrane, which we have used previously with CYP27B1 (Tang et al., 2010b, 2012). Substrates tested included the primary products of CYP11A1 action of vitamin D3 [20(OH)D3, 22(OH)D3, 20,22(OH)<sub>2</sub>D3, 20,23(OH)<sub>2</sub>D3, 17,20(OH)<sub>2</sub>D3, and 17,20,23(OH)<sub>3</sub>D3], as well as secondary products generated by the action of CYP24A1

or CYP27A1 on 20(OH)D3 [20,24(OH)<sub>2</sub>D3, 20,25(OH)<sub>2</sub>D3, and 20,26(OH)<sub>2</sub>D3]. Products were identified from their retention times compared with authentic standards in the case of 1,20(OH)<sub>2</sub>D3 and 1 $\alpha$ ,20,23-trihydroxyvitamin D3 [1,20,23(OH)<sub>3</sub>D3] (Tuckey et al., 2008a; Tang et al., 2010a), and by mass spectrometry and NMR in the case of the products from 20,24(OH)<sub>2</sub>D3, 20,25(OH)<sub>2</sub>D3, and 20,26(OH)<sub>2</sub>D3 (described below). For 22(OH)D3 and 20,22(OH)<sub>2</sub>D3, where limited substrate was available and the hydroxylation rate was low, insufficient product was generated by CYP27B1 to identify their structures by NMR, but it is presumed that they are the 1 $\alpha$ -hydroxy derivatives. For all substrates where the metabolite was identified, there was a single 1 $\alpha$ -hydroxylated product with both the mouse and human enzymes. This is illustrated in Fig. 2 for 25(OH)D3, 20,22(OH)<sub>2</sub>D3, and 20,25(OH)<sub>2</sub>D3. No products were detected for 17,20(OH)<sub>2</sub>D3 or 17,20,23(OH)<sub>3</sub>D3 (Fig. 2D), indicating that the presence of the 17 $\alpha$ -hydroxyl group prevented hydroxylation by CYP27B1. This was also the case for the metabolism of 17 $\alpha$ ,20-dihydroxyvitamin



**Fig. 4.** Chromatograms illustrating the difference in CYP27B1 activity toward 20(OH)D3 (A) and 22(OH)D3 (B). Substrates were incorporated into phospholipid vesicles at a ratio of 0.025 mol/mol phospholipid and incubated with mouse CYP27B1 (0.8  $\mu$ M) in the presence of adrenodoxin reductase and mouse adrenodoxin for 1 hour at 37°C. The secosteroids were extracted with dichloromethane and analyzed by reverse-phase HPLC on a C18 column. Control incubations where adrenodoxin was omitted are also shown (top panels).



**Fig. 5.** Analysis of the ability of 17,20(OH)<sub>2</sub>D<sub>3</sub> to inhibit CYP27B1 activity. (A) Mouse CYP27B1 or (B) human CYP27B1 was incubated with 25(OH)D<sub>3</sub> at a concentration of 0.025 mol/mol phospholipid in vesicles for 2 minutes with 17,20(OH)<sub>2</sub>D<sub>3</sub> present at the ratios indicated. Data represent the means  $\pm$  S.E.M. ( $n = 3$ ), and were analyzed using the Student's  $t$  test on GraphPad Prism. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

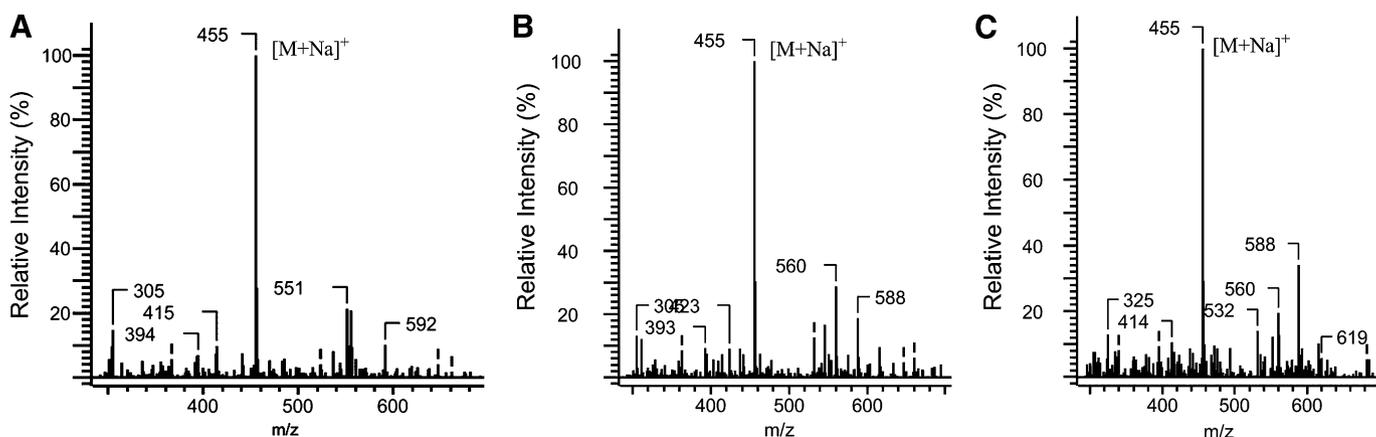
D<sub>2</sub> [17,20(OH)<sub>2</sub>D<sub>2</sub>] by CYP27B1, where in contrast to 20(OH)D<sub>2</sub> (Tang et al., 2010b), no hydroxylation was observed (unpublished data).

We have previously reported that inhibition of both mouse and human CYP27B1 occurs with high concentrations of 25(OH)D<sub>3</sub> (Tang et al., 2010b, 2012). In the current study, no inhibition of mouse or human CYP27B1 activity was observed at high concentrations of 20(OH)D<sub>3</sub> or 20,23(OH)<sub>2</sub>D<sub>3</sub>, but weak substrate inhibition was

observed for 20,26(OH)<sub>2</sub>D<sub>3</sub> (as shown in Fig. 3 for the human enzyme), giving a  $K_i$  value of 133 mmol/mol phospholipid, which is 30 times higher than its  $K_m$ . High concentrations of 20,24(OH)<sub>2</sub>D<sub>3</sub> inhibited the mouse enzyme, giving a  $K_i$  value of 241 mmol/mol phospholipid, which is 166-fold higher than its  $K_m$ . 20,22(OH)<sub>2</sub>D<sub>3</sub> also showed substrate inhibition with mouse CYP27B1 ( $K_i = 37.4$  mmol/mol phospholipid), but due to low activity, kinetic parameters were not measured for the human enzyme. In each case where inhibition was observed, the data fit well to a one catalytic site/two inhibitory site model (see *Materials and Methods*). Since the  $K_i$  values were very high relative to the  $K_m$  values, and the  $K_m$  and  $k_{cat}$  values calculated from a Michaelis-Menten curve fit (using data at lower substrate concentrations) were indistinguishable from those determined with the inhibition model, only the Michaelis-Menten data are shown (Table 1). 20(OH)D<sub>3</sub> was metabolized relatively poorly by human and mouse CYP27B1, displaying higher  $K_m$  and lower  $k_{cat}$  values than for 25(OH)D<sub>3</sub>. 22(OH)D<sub>3</sub> was an even poorer substrate for both mouse and human CYP27B1, as shown in a 1-hour incubation of mouse CYP27B1 with this secosteroid compared with 20(OH)D<sub>3</sub> (Fig. 4). The low activity with 22(OH)D<sub>3</sub> and the limited amount of this secosteroid available prevented a full kinetic analysis from being carried out.

When the vitamin D<sub>3</sub> side chain contained both 20- and 22-hydroxyl groups [as in 20,22(OH)<sub>2</sub>D<sub>3</sub>], activity was also low, with the  $k_{cat}$  being 5-fold lower than that for 20(OH)D<sub>3</sub> for the mouse enzyme, and too low to quantitate for the human enzyme. Movement of the second hydroxyl group on the side chain to the 23 position [20,23(OH)<sub>2</sub>D<sub>3</sub>] markedly increased the  $k_{cat}/K_m$  for both the mouse and human enzymes, with values being higher than those for 20(OH)D<sub>3</sub>. Shifting the second hydroxyl group to C24, C25, or C26 dramatically improved the ability of CYP27B1 to 1 $\alpha$ -hydroxylate the analog, with  $K_m$  values being similar to or lower than those for 25(OH)D<sub>3</sub> and the  $k_{cat}/K_m$  values being higher. The highest  $k_{cat}/K_m$  value for any of the substrates analyzed with the mouse enzyme was for 20,24(OH)<sub>2</sub>D<sub>3</sub>, and for the human enzyme was equal for 20,24(OH)<sub>2</sub>D<sub>3</sub> and 20,25(OH)<sub>2</sub>D<sub>3</sub>. Generally, values for both  $k_{cat}$  and  $K_m$  were reasonably similar between the mouse and human enzymes, with the largest differences being for 20(OH)D<sub>3</sub>, where the human enzyme showed both  $k_{cat}$  and  $K_m$  values approximately half those of the mouse enzyme.

**17,20(OH)<sub>2</sub>D<sub>3</sub> Is a Weak Inhibitor of Human CYP27B1 Activity.** Our results show that both human and mouse CYP27B1 are inactive toward 17,20(OH)<sub>2</sub>D<sub>3</sub> and other 17 $\alpha$ -hydroxylated secosteroids (Table 1). This could be due to very poor binding of the analogs to



**Fig. 6.** Mass spectra with electrospray ionization of product from CYP27B1 action on 20,24(OH)<sub>2</sub>D<sub>3</sub> (A), 20,25(OH)<sub>2</sub>D<sub>3</sub> (B), and 20,26(OH)<sub>2</sub>D<sub>3</sub> (C).

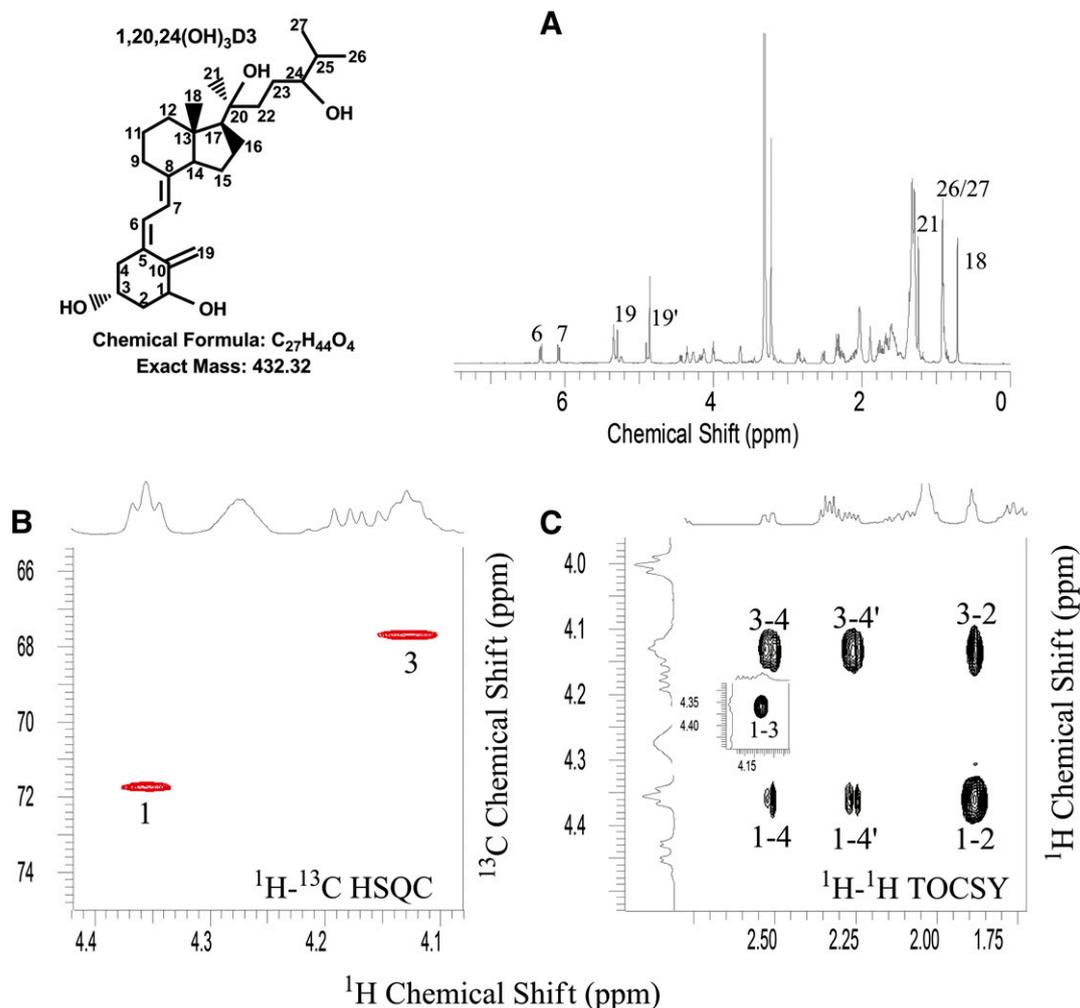


Fig. 7. NMR spectra of 1,20,24(OH)<sub>3</sub>D<sub>3</sub>. (A) One-dimensional proton, (B) <sup>1</sup>H-<sup>13</sup>C HSQC, and (C) <sup>1</sup>H-<sup>1</sup>H TOCSY.

the active site of CYP27B1, or binding in a position that prevents hydroxylation. To distinguish between these possibilities, we tested the ability of 17,20(OH)<sub>2</sub>D<sub>3</sub> to inhibit the 1 $\alpha$ -hydroxylation of 25(OH)D<sub>3</sub>. Significant inhibition of human CYP27B1 activity was seen at 17,20(OH)<sub>2</sub>D<sub>3</sub>:25(OH)D<sub>3</sub> ratios of 0.5:1, 1:1, and 2:1, but no inhibition was observed for the mouse enzyme at these ratios (Fig. 5). This suggests that some binding of 17,20(OH)<sub>2</sub>D<sub>3</sub> to the human enzyme occurs, permitting it to act as an inhibitor, presumably competitive. Insufficient 17,20(OH)<sub>2</sub>D<sub>3</sub> was available to permit a full analysis of the type of inhibition or the  $K_i$ .

**Synthesis of CYP27B1 Metabolites of 20,24(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, and 20,26(OH)<sub>2</sub>D<sub>3</sub> for Structure Determination.** Mouse CYP27B1 was used to scale up reactions to produce sufficient products from hydroxylation of 20,24(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, and 20,26(OH)<sub>2</sub>D<sub>3</sub> to enable structure determination by mass spectrometry and NMR. Complete conversion of 20,24(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, and 20,26(OH)<sub>2</sub>D<sub>3</sub> was achieved when 0.3  $\mu$ M mouse CYP27B1 was incubated with the substrates in phospholipid vesicles at a concentration of 0.025 or 0.03 mol/mol phospholipid in a 1-hour incubation. The major limitation in producing the metabolites was the availability of substrates which required enzymatic synthesis of 20(OH)D<sub>3</sub> by CYP11A1 and its subsequent hydroxylation by purified CYP27A1 or CYP24A1 (see *Materials and Methods*). Overall, 30–60  $\mu$ g of purified products were prepared for each compound, which was adequate for structure determination.

**Determination of the Structure of Products Resulting from the Action of CYP27B1 on 20,24(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, and 20,26(OH)<sub>2</sub>D<sub>3</sub>.** Mass spectrometry confirmed that the metabolites generated by CYP27B1 action on 20,24(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, and 20,26(OH)<sub>2</sub>D<sub>3</sub> were all trihydroxy derivatives. The observed molecular ion for each compound had a mass of 455 [M + Na]<sup>+</sup>, giving a molecular weight of 432 (Fig. 6). The site of hydroxylation on the three compounds by CYP27B1 was unambiguously assigned to be at the 1 $\alpha$ -position based on the NMR spectra for these metabolites. First, for the product of 20,24(OH)<sub>2</sub>D<sub>3</sub> and 20,25(OH)<sub>2</sub>D<sub>3</sub>, none of the four methyl groups (18, 21, 26, and 27) are hydroxylated based on <sup>1</sup>H NMR (Figs. 7A and 8A). <sup>1</sup>H-<sup>13</sup>C HSQC revealed the presence of a new methine group at 4.35 or 4.36 ppm (<sup>13</sup>C at 71.3 ppm; Figs. 7B and 8B). <sup>1</sup>H-<sup>1</sup>H TOCSY (Figs. 7C and 8C) demonstrated that the new methine at 4.35 or 4.36 ppm is in the same spin system as that of 2-CH<sub>2</sub> (<sup>1</sup>H at 1.89 ppm), 3-CH (<sup>1</sup>H at 4.13 ppm), and 4-CH<sub>2</sub> (2.53 and 2.27 ppm), indicating the hydroxylations occurred in the 1 position.

With 20,26(OH)<sub>2</sub>D<sub>3</sub>, similar to the product of the other two dihydroxy derivatives, C18, C21, and C27 were not hydroxylated based on <sup>1</sup>H NMR (Fig. 9B). <sup>1</sup>H-<sup>13</sup>C HSQC revealed the presence of a new methine group at 4.36 ppm (<sup>13</sup>C at 71.3 ppm; Fig. 9B). <sup>1</sup>H-<sup>1</sup>H TOCSY (Fig. 9C) showed that the methine at 4.36 ppm has correlations to 2-CH<sub>2</sub> (<sup>1</sup>H at 1.89 ppm), 3-CH (<sup>1</sup>H at 4.13 ppm), and 4-CH<sub>2</sub> (2.52 and 2.26 ppm), indicating the hydroxylation occurred in the 1 position. Overall, the previous analysis shows that the

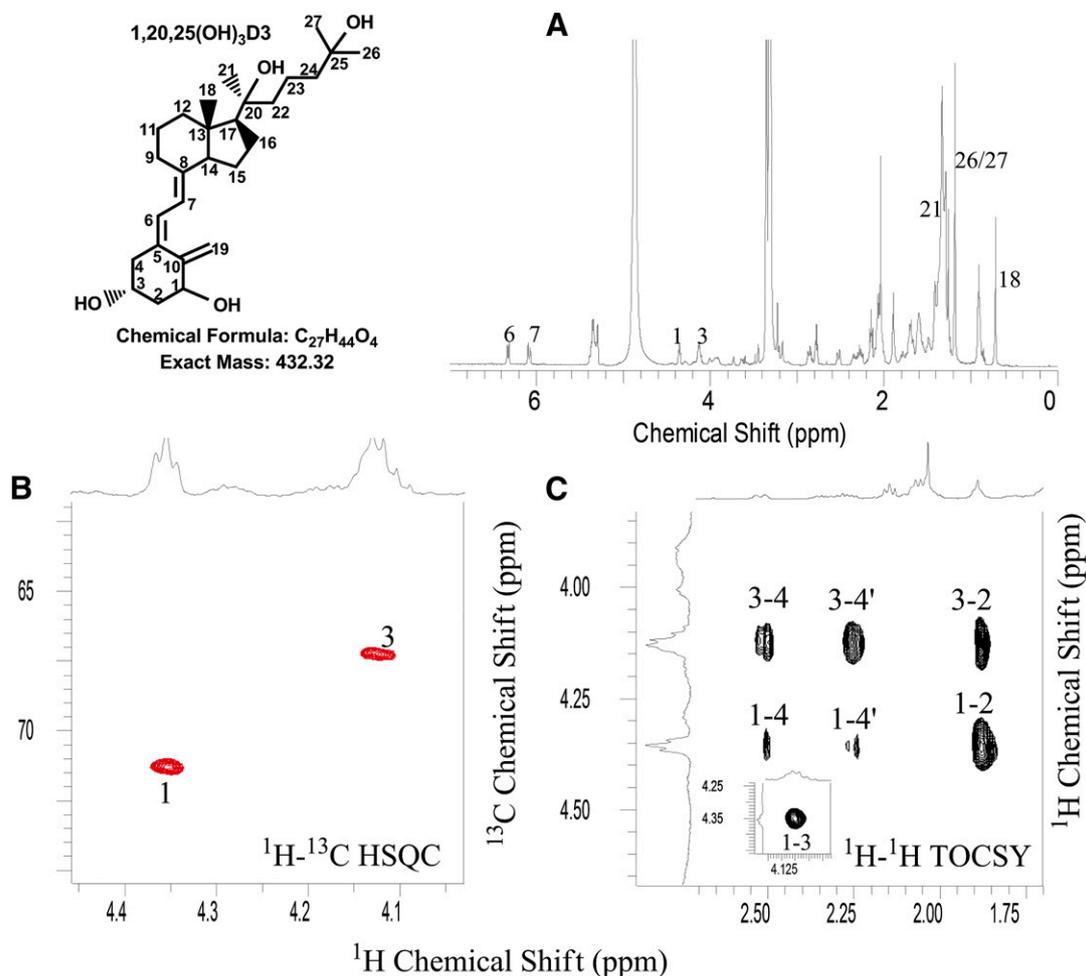


Fig. 8. NMR spectra of 1,20,25(OH)<sub>3</sub>D<sub>3</sub>. (A) 1D proton, (B) <sup>1</sup>H-<sup>13</sup>C HSQC, and (C) <sup>1</sup>H-<sup>1</sup>H TOCSY.

hydroxylation sites can be assigned to the 1 position unambiguously. The full assignments for the CYP27B1-hydroxylated metabolites and the dihydroxy derivatives are summarized in Tables 2–4.

The new hydroxyl group at C1 is in the 1 $\alpha$  configuration (i.e., the remaining proton attached to C1 has 1 $\beta$  configuration) for all three CYP27B1-derived metabolites based on the analysis of the chemical shift and <sup>1</sup>H-<sup>1</sup>H coupling constants between protons attached to C1 and C2, as described in the following text using 1,20,26(OH)<sub>3</sub>D<sub>3</sub> as an example. First, the proton chemical shift (4.35 ppm) at C1 (Fig. 10) is very similar to that of the 1 $\beta$ -H (4.29 ppm) in 1,25(OH)<sub>2</sub>D<sub>3</sub> measured in the same solvent (CD<sub>3</sub>OD) (Eguchi and Ikekawa, 1990). Second, the vicinal <sup>1</sup>H-<sup>1</sup>H coupling constant ( $^3J_{H-1, H-2\alpha} = ^3J_{H-1, H-2\beta} = 5.9$  Hz) between the proton at C1 and the two protons at C2 of this metabolite is essentially the same as that in 1,25(OH)<sub>2</sub>D<sub>3</sub> ( $^3J_{H-1\beta, H-2\alpha} = ^3J_{H-1\beta, H-2\beta} = 6.0$  Hz) (Eguchi and Ikekawa, 1990). This pseudo-triplet splitting pattern is only possible when the proton directly attached to C1 is in the 1 $\beta$  configuration due to similar vicinal coupling constants between H-1 $\beta$  and H-2 $\alpha$  ( $^3J_{\text{vic}}$ ) or H-2 $\beta$  ( $^3J_{\text{vic}}$ ). If the proton directly attached to C1 was in the 1 $\alpha$  configuration, the values of the two vicinal coupling constants would be very different ( $^3J_{\text{aa}} \approx 11\text{ Hz}$ ,  $^3J_{\text{ac}} \approx 6\text{ Hz}$ ), resulting in a clear doublet of doublet instead of a pseudo-triplet as observed. Taken together, the previous analysis shows that the hydroxyl group at C1 must be in the 1 $\alpha$  configuration for all three CYP27B1-derived products.

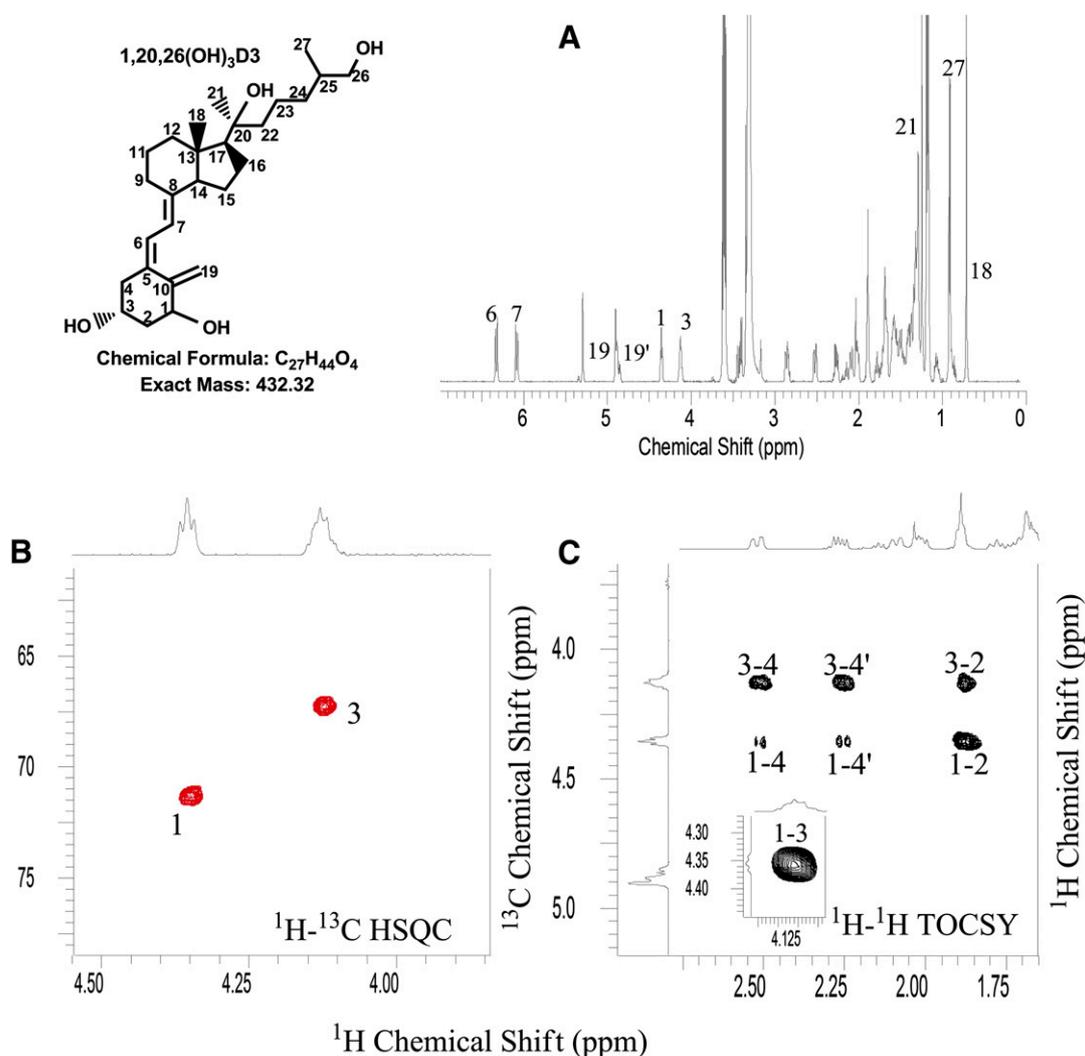
**The Effect of 1 $\alpha$ -hydroxylation on Antimelanoma Activity.** Targeting of the vitamin D receptor represents a promising strategy for treatment of melanoma (Pinczewski and Slominski, 2010; Brożyna

et al., 2011; Szyszka et al., 2012), a deadly disease for which therapy is still unsatisfactory (Hauschild et al., 2012). Therefore, we tested whether the 1 $\alpha$ -hydroxylation of 20,25(OH)<sub>2</sub>D<sub>3</sub> and 20,26(OH)<sub>2</sub>D<sub>3</sub>, both good substrates for CYP27B1, altered their ability to inhibit melanoma colony formation in soft agar. This assay was previously used to characterize the parent compounds and is a good measure of tumorigenicity. Although 20,24(OH)<sub>2</sub>D<sub>3</sub> is also an excellent substrate for CYP27B1, this had not been synthesized at the time of testing.

The parent compounds and their 1 $\alpha$ -hydroxy derivatives at 10 nM significantly inhibited colony formation compared with the vehicle control ( $P < 0.0002$ ). Excluding 1,20,26(OH)<sub>3</sub>D<sub>3</sub>, they also inhibited colony formation significantly more than 20(OH)D<sub>3</sub> (Tieu et al., 2012b). These significant differences were also seen when the secosteroid concentration was decreased to 0.1 nM (Fig. 11). At 0.1 nM, 1,20,25(OH)<sub>3</sub>D<sub>3</sub> caused significantly greater inhibition of colony formation than 20,25(OH)<sub>2</sub>D<sub>3</sub>, whereas at 10 nM, 1,20,26(OH)<sub>3</sub>D<sub>3</sub> caused significantly less inhibition than 20,26(OH)<sub>2</sub>D<sub>3</sub>.

## Discussion

The ability of CYP11A1 to hydroxylate the vitamin D<sub>3</sub> side chain at C20 and C22 (Tuckey et al., 2011) has enabled us to look at the effect of the position of the side chain hydroxyl group on CYP27B1 activity. The current study shows that when the hydroxyl group on the vitamin D side chain is moved from C25 to C20, the catalytic



**Fig. 9.** NMR spectra of 1,20,26(OH)<sub>3</sub>D<sub>3</sub>. (A) 1D proton, (B) <sup>1</sup>H-<sup>13</sup>C HSQC, and (C) <sup>1</sup>H-<sup>1</sup>H TOCSY.

efficiency ( $k_{\text{cat}}/K_m$ ) of the 1 $\alpha$ -hydroxylase decreases by 40- to 45-fold, due to both an increase in  $K_m$  and a decrease in  $k_{\text{cat}}$ . When the side chain hydroxyl group is at C22, rather than at C20, the activity is even lower, not permitting measurement of its kinetic parameters.

Recently, we reported that 20(OH)D<sub>3</sub> can be hydroxylated by two vitamin D-metabolizing enzymes, human CYP27A1 (Tieu et al., 2012a) and rat CYP24A1 (Tieu et al., 2012b). The main products of CYP24A1 action on 20(OH)D<sub>3</sub> are 20,24(OH)<sub>2</sub>D<sub>3</sub> and 20,25(OH)<sub>2</sub>D<sub>3</sub>, and for CYP27A1 are 20,25(OH)<sub>2</sub>D<sub>3</sub> and 20,26(OH)<sub>2</sub>D<sub>3</sub>. Along with the primary products of CYP11A1 action on vitamin D<sub>3</sub>, 17,20(OH)<sub>2</sub>D<sub>3</sub>, 20,22(OH)<sub>2</sub>D<sub>3</sub>, and 20,23(OH)<sub>2</sub>D<sub>3</sub>, we have a unique set of derivatives all containing a 20-hydroxyl group plus one additional hydroxyl group at every position on the side chain, except C21 (note that C26 and C27 are equivalent). All of these 20-hydroxy derivatives have been shown to be biologically active (Zbytek et al., 2008; Janjetovic et al., 2009, 2010, 2011; Li et al., 2010; Slominski et al., 2010, 2011, 2012a; Tang et al., 2010a; Tuckey et al., 2011; Kim et al., 2012; Lu et al., 2012; Wang et al., 2012). The low catalytic efficiency of CYP27B1 toward 20(OH)D<sub>3</sub> is greatly enhanced when the second hydroxyl group is added toward the end of the side chain, as seen for 24,20(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, and 20,26(OH)<sub>2</sub>D<sub>3</sub>. All of these compounds displayed higher  $k_{\text{cat}}/K_m$  values than that for 25(OH)D<sub>3</sub>, primarily due to their lower  $K_m$  values. Conversely, the

addition of a second hydroxyl group on the side chain close to the “D” ring, for example, at C22, gave a very low  $k_{\text{cat}}/K_m$  value for the mouse enzyme, lower than that for 20(OH)D<sub>3</sub>, and too low to measure for the human enzyme. When the second hydroxyl group is in the 17 $\alpha$  position of the D ring [17,20(OH)<sub>2</sub>D<sub>3</sub>], no activity was observed even at high CYP27B1 concentrations. The ability of the 17 $\alpha$ -hydroxyl group to completely inhibit CYP27B1 activity was also seen with the trihydroxy derivative, 17,20,23(OH)<sub>3</sub>D<sub>3</sub>, and the vitamin D<sub>2</sub> derivative, 17,20(OH)<sub>2</sub>D<sub>2</sub>. We observed that 17,20(OH)<sub>2</sub>D<sub>3</sub> could inhibit 25(OH)D<sub>3</sub> metabolism by human CYP27B1, but not the mouse enzyme, suggesting that it can bind to the active site of the human enzyme, but in an orientation that precludes hydroxylation.

Overall, the kinetic data presented in this study indicate that the position of one or more hydroxyl groups on the vitamin D side chain dramatically influences the ability of both mouse and human CYP27B1 to hydroxylate the derivative. Yamamoto et al. (2004, 2005) prepared a modeled structure of CYP27B1 and used mutagenesis to conclude that Thr409 of the human CYP27B1 hydrogen bonds to the 25-hydroxyl group of 25(OH)D<sub>3</sub>, playing a critical role in substrate binding. Since we observed that the  $K_m$  values for 20,24(OH)<sub>2</sub>D<sub>3</sub>, 20,25(OH)<sub>2</sub>D<sub>3</sub>, and 20,26(OH)<sub>2</sub>D<sub>3</sub> were comparable to or lower than that for 25(OH)D<sub>3</sub>, it would seem likely that a hydroxyl group at C24 or C26, such as at C25, can also hydrogen bond to

TABLE 2

NMR chemical shift assignments for 1,20,24(OH)<sub>3</sub>D<sub>3</sub> analysis of its two-dimensional NMR (solvent: methanol-d<sub>4</sub>)

The assignments are compared with that for its dihydroxy derivative prior to hydroxylation by CYP27B1.

C Atom	20,24(OH) <sub>2</sub> D <sub>3</sub>		1,20,24(OH) <sub>3</sub> D <sub>3</sub>		Structure
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C	
1	2.11 $\alpha$ , 2.41 $\beta$	33.5	4.36	71.7	
2	1.96 $\alpha$ , 1.53 $\beta$	36.5	1.89	44	
3	3.76	70.4	4.13	67.7	
4	2.52 $\alpha$ , 2.18 $\beta$	46.8	2.52 $\alpha$ , 2.27 $\beta$	46.4	
5	NA	136.4	NA	136.4	
6	6.22	122.5	6.32	125.2	
7	6.03	119.3	6.08	119.6	
8	NA	141.1	NA	NI	
9	1.68 $\alpha$ , 2.85 $\beta$	28.4	1.69 $\alpha$ , 2.86 $\beta$	30.1	
10	NA	146.2	NA	NI	
11	1.55 $\alpha$ , 1.69 $\beta$	24.2	1.56 $\alpha$ , 1.67 $\beta$	24.8	
12	1.36 $\alpha$ , 2.10 $\beta$	42.3	1.38 $\alpha$ , 2.11 $\beta$	42.6	
13	NA	45.4	NA	47.3	
14	2.01	57.6	2.01	58.2	
15	1.77	23.1	1.49 $\alpha$ , 1.77 $\beta$	23.3	
16	1.68 $\alpha$ , 1.77 $\beta$	23	1.68	24.6	
17	1.69	60.4	1.68	60.8	
18	0.7	14.1	0.72	14.4	
19	4.75, 5.04	112.4	4.90, 5.29	112.3	
20	NA	74.7	NA	76.3	
21	1.24	25.9	1.24	26.2	
22	1.36	39.6	1.71, 1.38	41.4	
23	1.74, 1.38	41	1.33	30.5	
24	3.22	78.2	3.21	78.6	
25	1.62	33.5	1.63	35.3	
26	0.93	19.3	0.91	19.6	
27	0.92	18.5	0.92	18.3	

NA, not applicable (ternary carbons); NI, not identifiable (due to low resolution).

Thr409. The higher  $K_m$  values observed with 20(OH)D<sub>3</sub> and 20,22(OH)<sub>2</sub>D<sub>3</sub> suggest that their side chain hydroxyl groups are too far from Thr409 to form this hydrogen bond. Differences in the observed  $k_{cat}$  values between different substrates are likely due to the distance of C1 from the activated oxygen bound to the heme group, or steric effects of the substrate on the optimal conformation of the enzyme for catalysis.

The present work shows that the position and number of hydroxyl groups on the side chain of the vitamin D analog do not alter the site of hydroxylation by CYP27B1, which is always the C1 $\alpha$  position. This suggests that the A ring of vitamin D analogs must bind to the enzyme active site in a specific orientation so that only the 1 $\alpha$ -hydroxy derivative is produced. Thus, CYP27B1 is highly specific for 1 $\alpha$ -hydroxylation, consistent with the limited literature available where there are no reports of this enzyme hydroxylating at the C1 $\beta$  position or at adjacent carbon atoms. However, low 25-hydroxylase activity of CYP27B1 has been observed when 1 $\alpha$ -hydroxyvitamin D<sub>3</sub> is used as substrate, requiring binding of the secosteroid to the active site of the enzyme in the opposite orientation to that for 1 $\alpha$ -hydroxylation (Uchida et al., 2004; Tang et al., 2010b).

It has been documented that the CYP11A1-derived vitamin D<sub>3</sub> analogs, 20(OH)D<sub>3</sub> and 20,23(OH)<sub>2</sub>D<sub>3</sub>, display similar properties to 1,25(OH)<sub>2</sub>D<sub>3</sub>, such as inhibiting cell proliferation, promoting differentiation of keratinocytes and leukemia cells, and suppressing tumorigenicity of melanoma (Zbytek et al., 2008; Janjetovic et al., 2009, 2011; Li et al., 2010; Slominski et al., 2010, 2012a; Tuckey et al., 2011; Kim et al., 2012; Lu et al., 2012; Wang et al., 2012).

Importantly in terms of their potential for therapeutic applications, such as in the treatment of scleroderma (Slominski et al., 2013a), it has been shown that, unlike 1,25(OH)<sub>2</sub>D<sub>3</sub>, 20(OH)D<sub>3</sub> lacks calcemic activity in rodents at doses up to 30  $\mu$ g/kg, whereas severe hypercalcemia was detected with 1,25(OH)<sub>2</sub>D<sub>3</sub> at a dose of only 2  $\mu$ g/kg (Wang et al., 2012). However, when a 1 $\alpha$ -hydroxyl group was added to 20(OH)D<sub>3</sub> to produce 1,20(OH)<sub>2</sub>D<sub>3</sub>, moderate calcemic activity was observed (Slominski et al., 2010). This suggests that the presence of a 1 $\alpha$ -hydroxyl group is necessary for the vitamin D analog to regulate serum calcium levels. Thus, the relatively poor ability of CYP27B1 to hydroxylate CYP11A1-derived hydroxyvitamin D<sub>3</sub> analogs may be important in vivo for preventing them from causing hypercalcemia. It has to be noted that both 20(OH)D<sub>3</sub> and 20(OH)D<sub>2</sub> do not require hydroxylation to express phenotypic activity (Slominski et al., 2011). Nevertheless, there is evidence for at least some 1 $\alpha$ -hydroxylation of 20(OH)D<sub>3</sub> and 20,23(OH)<sub>2</sub>D<sub>3</sub> in vivo, as their 1 $\alpha$ -hydroxylated products have been detected in placental explants incubated with vitamin D<sub>3</sub> and in cultured keratinocytes (Slominski et al., 2012b). The CYP11A1-derived secosteroids, 17,20(OH)<sub>2</sub>D<sub>2</sub>, 17,20(OH)<sub>2</sub>D<sub>3</sub>, and 17,20,23(OH)<sub>3</sub>D<sub>3</sub>, may provide some therapeutic advantage over the other CYP11A1-derived secosteroids because of the inability of CYP27B1 to hydroxylate them and, therefore, alter their properties.

In this study, we performed an initial investigation of the anti-proliferative activity of two of the new trihydroxy derivatives generated with high catalytic efficiency by CYP27B1, 1,20,25(OH)<sub>3</sub>D<sub>3</sub> and 1,20,26(OH)<sub>3</sub>D<sub>3</sub>, and compared this to the activity of their parent

TABLE 3

NMR chemical shift assignments for 1,20,25(OH)<sub>3</sub>D<sub>3</sub> analysis of its two-dimensional NMR (solvent: methanol-d<sub>4</sub>)

The assignments are compared with that for its dihydroxy derivative prior to hydroxylation by CYP27B1.

C Atom	20,25(OH) <sub>2</sub> D <sub>3</sub>		1,20,25(OH) <sub>3</sub> D <sub>3</sub>		Structure
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C	
1	2.12 $\alpha$ , 2.41 $\beta$	33.5	4.35	71.3	
2	1.97 $\alpha$ , 1.54 $\beta$	36.6	1.89	43.6	
3	3.76	70.5	4.13	67.2	
4	2.54 $\alpha$ , 2.19 $\beta$	47	2.53 $\alpha$ , 2.27 $\beta$	46	
5	NA	136	NA	135.4	
6	6.22	122.6	6.33	124.8	
7	6.03	119.4	6.08	119.2	
8	NA	141.1	NA	NI	
9	1.69 $\alpha$ , 2.86 $\beta$	29.7	1.69 $\alpha$ , 2.87 $\beta$	29.8	
10	NA	145.9	NA	149.4	
11	1.54 $\alpha$ , 1.68 $\beta$	22.7	1.58 $\alpha$ , 1.69 $\beta$	24.2	
12	1.39 $\alpha$ , 2.10 $\beta$	42.2	1.38 $\alpha$ , 2.11 $\beta$	42	
13	NA	45.4	NA	46.9	
14	2.02	57.8	2	57.7	
15	1.78	22.9	1.77	22.8	
16	1.68 $\alpha$ , 1.56 $\beta$	24.4	1.70, 1.48	22.7	
17	1.7	59.9	1.7	59.8	
18	0.7	14	0.72	14	
19	4.74, 5.04	112.64	4.90, 5.30	111.8	
20	NA	74.4	NA	75.9	
21	1.26	26.1	1.26	25.9	
22	1.48, 1.35	45.4	1.47, 1.37	45.1	
23	1.4	19.9	1.41	19.8	
24	1.41	45.5	1.41	45.2	
25	NA	70	NA	71.1	
26	1.19	29.2	1.18	29.1	
27	1.19	29.2	1.18	29.1	

NA, not applicable (ternary carbons); NI, not identifiable (due to low resolution).

secosteroids, 20,25(OH)<sub>2</sub>D<sub>3</sub> and 20,26(OH)<sub>2</sub>D<sub>3</sub>. As reported before (Tieu et al., 2012b), the parent secosteroids displayed a significantly higher inhibitory effect on colony formation by melanoma cells grown in soft agar than either 20(OH)D<sub>3</sub> or 1,25(OH)<sub>2</sub>D<sub>3</sub> at concentrations of both 0.1 and 10 nM. Interestingly, the antiproliferative activity of 1,20,25(OH)<sub>3</sub>D<sub>3</sub> and 1,20,26(OH)<sub>3</sub>D<sub>3</sub> on melanoma cells (SKMEL-188) was found to be significantly different from that of their parent compounds, with a greater effect seen for 1,20,25(OH)<sub>3</sub>D<sub>3</sub> and a lesser effect for 1,20,26(OH)<sub>3</sub>D<sub>3</sub> [although still significantly more than for 20(OH)D<sub>3</sub>]. The increased potency resulting from the 1 $\alpha$ -hydroxylation of 20,25(OH)<sub>2</sub>D<sub>3</sub>, with the product completely inhibiting colony formation at 10 nM and inhibiting by 66% at 0.1 nM, suggests that it is a good candidate for further testing of its antimelanoma activity. The previous data are also consistent with recent clinicopathologic analyses that clearly demonstrate a decrease in CYP27B1 expression during progression of melanoma, as well as with the positive correlation between shorter survival of patients and low CYP27B1 expression in melanoma specimens (Brożyna et al., 2013). Thus, the current and previous studies suggest that CYP27A1 and CYP24A1 can increase the antimelanoma potency of 20(OH)D<sub>3</sub>, with further modification by CYP27B1. Based on our previous studies (Kim et al., 2012; Slominski et al., 2012a), we expect that these effects are mediated through the activation of the vitamin D receptor. However, the final proof would require additional testing using models described previously (Kim et al., 2012), and an extended panel of melanoma lines available in our laboratories, and comparison with normal melanocytes (Brożyna et al., 2012; Slominski et al., 2012a,

2013a,b). This will be complemented by testing the toxicity of these modified compounds.

In conclusion, this study clearly defines the kinetic parameters for the action of CYP27B1 on a range of secosteroids produced by CYP11A1 in a vesicle-reconstituted system that resembles the inner mitochondrial membrane. All of the secosteroids produced directly by CYP11A1 action on vitamin D<sub>3</sub> are relatively poor substrates for CYP27B1. In contrast, the secondary metabolites produced by the action of CYP27A1 or CYP24A1 on 20(OH)D<sub>3</sub>, which have a hydroxyl group close to the end of the side chain, are very good substrates for CYP27B1.

#### Acknowledgments

The authors thank Alex Hua for help in preparing the figures.

#### Authorship Contributions

Participated in research design: Tang, Tuckey, Slominski, Li.

Conducted experiments: Tang, Janjetovic, Chen.

Contributed new reagents or analytic tools: Tieu.

Performed data analysis: Tang, Janjetovic, Chen, Tuckey, Slominski, Li.

Wrote or contributed to the writing of the manuscript: Tang, Tuckey, Slominski, Chen, Li.

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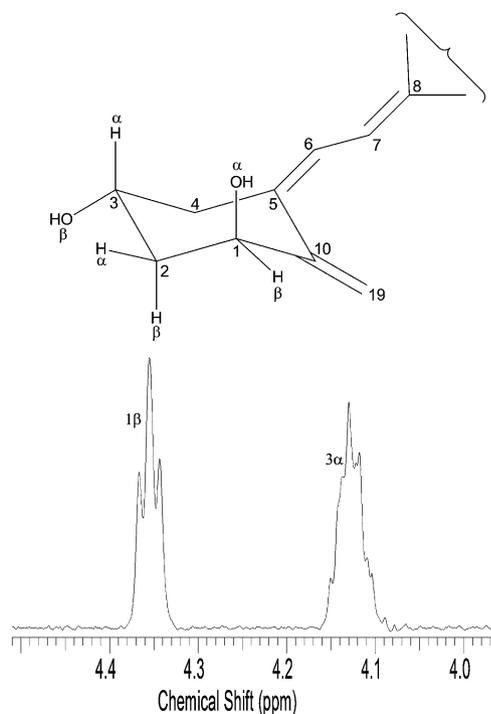
TABLE 4

NMR chemical shift assignments for 1,20,26(OH)<sub>3</sub>D<sub>3</sub> analysis of its two-dimensional NMR (solvent: methanol-d<sub>4</sub>)

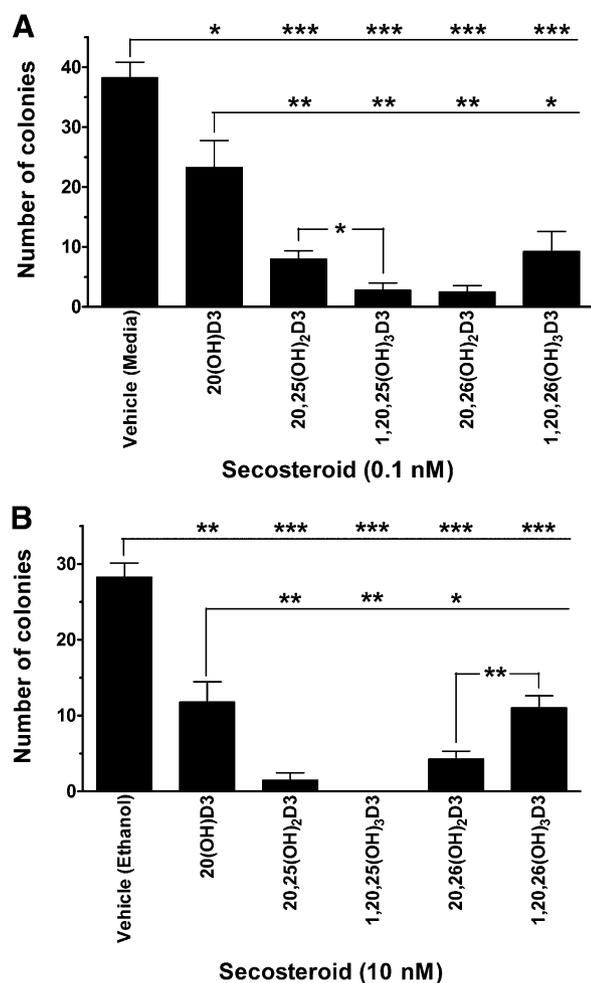
The assignments are compared with that for its dihydroxy derivative prior to hydroxylation by CYP27B1.

C Atom	20,26(OH) <sub>2</sub> D <sub>3</sub>		1,20,26(OH) <sub>3</sub> D <sub>3</sub>		Structure
	<sup>1</sup> H	<sup>13</sup> C	<sup>1</sup> H	<sup>13</sup> C	
1	2.12 $\alpha$ , 2.42 $\beta$	33.4	4.36	71.3	
2	1.97 $\alpha$ , 1.53 $\beta$	36.3	1.89	43.6	
3	3.77	70.5	4.13	67.4	
4	2.54 $\alpha$ , 2.20 $\beta$	46.8	2.52 $\alpha$ , 2.26 $\beta$	46.2	
5	NA	136	NA	135.9	
6	6.22	122.6	6.32	124.8	
7	6.03	119.3	6.08	119.2	
8	NA	140.4	NA	NI	
9	1.69 $\alpha$ , 2.86 $\beta$	29.6	1.69 $\alpha$ , 2.87 $\beta$	29.8	
10	NA	145.7	NA	149.6	
11	1.56 $\alpha$ , 1.67 $\beta$	24.3	1.55 $\alpha$ , 1.67 $\beta$	23.2	
12	1.38 $\alpha$ , 2.10 $\beta$	42.1	1.37 $\alpha$ , 2.07 $\beta$	42.1	
13	NA	45.5	NA	46.8	
14	2.01	57.7	2.01	57.7	
15	1.5	22.7	1.47	23.2	
16	1.72 $\alpha$ , 1.79 $\beta$	22.7	1.68 $\alpha$ , 1.78 $\beta$	23.2	
17	1.68	59.9	1.68	59.9	
18	0.7	13.9	0.71	13.8	
19	4.75, 5.05	112.5	4.90, 5.29	111.8	
20	NA	74.5	NA	76	
21	1.25	26	1.24	25.9	
22	1.34, 1.51	44.9	1.50, 1.33	45.1	
23	1.43, 1.29	22.3	NA*	NI	
24	1.41, 1.07	34.9	1.40, 1.08	34.8	
25	1.59	36.7	1.58	36.6	
26	3.36	68.4	3.36	68.2	
27	0.91	16.8	0.91	16.8	

NA, not applicable (ternary carbons); NI, not identifiable (due to low resolution).

Fig. 10. Segment of <sup>1</sup>H NMR spectrum for 1,20,26(OH)<sub>3</sub>D<sub>3</sub>.

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**Fig. 11.** The new secosteroids, 1,20,25(OH)<sub>3</sub>D<sub>3</sub> and 1,20,26(OH)<sub>3</sub>D<sub>3</sub>, inhibit colony formation by SKMEL-188 cells. Colony formation was determined using the soft agar assay, and colonies of greater than 0.2 mm were counted. Data represent means ± S.E.M. ( $n = 4$ ), and were analyzed using the Student's  $t$  test on GraphPad Prism. \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

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