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# Screening of Selective Inhibitors of 1α,25-Dihydroxyvitamin $D_3$ 24-Hydroxylase Using Recombinant Human Enzyme Expressed in *Escherichia coli*†

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ABSTRACT: High-level heterologous expression of human 1α,25-dihydroxyvitamin $D_3$ 24-hydroxylase (CYP24A1) in *Escherichia coli* was attained via a fusion construct by appending the mature CYP24A1 without the leader sequence to the maltose binding protein (MBP). Facile purification was achieved efficiently through affinity chromatography and afforded fully functional enzyme of near homogeneity, with a $k_{cat}$ of 0.12 $min^{-1}$ and a $K_M$ of 0.19 μM toward 1α,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$]. A convenient and reliable cell-free assay was established and used to screen vitamin D analogues with potential inhibitory properties toward CYP24A1. Some of the compounds exhibited potent inhibition with $K_I$ values as low as 0.021 μM. Furthermore, TS17 and CPA1 exhibited superior specificity toward CYP24A1 over 25-hydroxyvitamin $D_3$ 1α-hydroxylase (CYP27B1), with selectivities of 39 and 80, respectively. Addition of TS17 or CPA1 to a mouse osteoblast culture sustained the level of 1,25$(OH)_2D_3$ in the medium. Their activities in vitamin D receptor (VDR) binding, CYP24A1 transcription, and HL-60 cell differentiation were evaluated as well.

Since the discovery of vitamin D nearly a century ago, the benefit of vitamin D in preventing rickets and maintaining bone health has been long recognized and appreciated. In addition to its classic role in calcium and phosphate homeostasis, a growing body of research and emerging evidence are continuously unveiling the significance of vitamin D in a diverse array of physiological functions, including cell proliferation, differentiation and apoptosis, and immune response. Consequently, vitamin D deficiency has been associated with a number of diseases such as cancers, autoimmune dysfunctions, cardiovascular diseases, and infections (*1*−*3*).

Vitamin $D_3$, the natural form of vitamin D produced in the skin through UV exposure, is biologically inert. Activation of vitamin $D_3$ is initiated in the liver to produce 25-hydroxyvitamin $D_3$ [25$(OH)D_3$],[1] which is further converted to 1α,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$], the hormonally active secosteroid, by 25$(OH)D_3$ 1α-hydroxylase (CYP27B1) mainly in the kidney. Degradation of 1,25$(OH)_2D_3$ leads to hormone inactivation and is conducted by 1,25$(OH)_2D_3$ 24-hydroxylase (CYP24A1) (*4*).

CYP24A1 is a member of the cytochrome P450 superfamily and conserved across vertebrate species ranging from fish to humans (*4*). Almost all cells express CYP24A1, with the highest activity observed in the kidney (*5*). CYP24A1 resides on the periphery of the inner mitochondrial membrane via an N-terminal target sequence destined for its subcellular localization. Its activity requires a multicomponent electron transfer system that delivers the reducing equivalents of NADPH to ferrodoxin reductase and ferrodoxin, and subsequently to the heme iron center in the active site of CYP24A1 (*4*, *5*). CYP24A1 is a multifunctional enzyme and catalyzes multiple steps of monooxygenation reactions, leading to two major catabolic pathways of 1,25$(OH)_2D_3$ and producing calcitroic acid (C24 hydroxylation) and 1,25$(OH)_2D_3$-26,23-lactone (C23 hydroxylation), respectively, as the end products (*6*−*9*). Despite sequence conservation of CYP24A1 among various organisms, the C24 and C23 hydroxylation pathways are highly species-dependent. The C24 hydroxylation is predominant in rat and mouse (*8*, *10*), while the C23 pathway is preferentially expressed in guinea pig (*11*). Humans exhibit a ∼4:1 ratio of C24 to C23 hydroxylation (*7*, *9*). The cellular expression of CYP24A1 is modulated by a broad range of factors. The most influential and the principal induction mechanism is the vitamin D receptor (VDR)-mediated transcriptional upregulation induced by 1,25$(OH)_2D_3$ via two tandem vitamin D response elements in the CYP24A1 promoter, which can elevate the enzyme level substantially by up to 100-fold, resulting in autoregulatory attenuation of 1,25$(OH)_2D_3$.

In light of prospective therapeutic applications, a number of vitamin D analogues have been developed for various disorders (*12*, *13*). Although many analogues have shown promising outcomes, the frequently occurring hypercalcemic effect remains the major drawback and has limited their use. CYP24A1, as the main catabolizing enzyme of 1,25$(OH)_2D_3$, can also break down vitamin D analogues in a similar catabolic process and is partially responsible for the high and toxic efficacious dosages. CYP24A1 inhibitors, on the other hand, would hinder hormone degradation, thus extending the half-life and bioavailability of active hormonal signals and alleviating systemic side effects by lowering drug intake. The potential clinical implications would make them a novel strategy in the area of vitamin D intervention.

Ketoconazole and liarozole, two nonspecific CYP24A1 inhibitors (*14*−*16*), are both imidazole derivatives, in which the azole

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[1]Abbreviations: 25$(OH)D_3$, 25-hydroxyvitamin $D_3$; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin $D_3$; CYP27B1, 25-hydroxyvitamin $D_3$ 1α-hydroxylase; CYP24A1, 1α,25-dihydroxyvitamin $D_3$ 24-hydroxylase; VDR, vitamin D receptor; MBP, maltose binding protein; 2MD, 2-methylene-19-nor-(20*S*)-1,25$(OH)_2D_3$; IPTG, isopropyl β-D-thiogalactoside; δ-ALA, 5-aminolevulinic acid; Adx, adrenodoxin; AdR, adrenodoxin reductase; (20*R*)-2MD, 2-methylene-19-nor-(20*R*)-1,25$(OH)_2D_3$.


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moiety can replace the heme-coordinating cysteine and bind to iron, thereby eliminating the cofactor from the holoenzyme. Because there are 57 P450 genes in the human genome (*17*), this mechanism of inhibition also accounts for the low selectivity, which is particularly important in this case because it is crucial not to impair the synthesis of $1,25(OH)_2D_3$ catalyzed by CYP27B1, a P450 enzyme closely related to CYP24A1. The first selective CYP24A1 inhibitors were reported by Schuster et al. (*18*, *19*) as a result of laborious effort in synthesizing and screening hundreds of azole compounds. Simons' group reported CYP24A1 inhibitory activity of tetralone derivatives and *N*-[2-(1*H*-imidazol-1-yl)-2-phenylethyl]arylamides, but their selectivity has not been assessed (*20*, *21*). Another group of CYP24A1 inhibitors is based on the structure of $1,25(OH)_2D_3$ with modifications on the side chain, where C24 is replaced with a sulfone or a sulfoximine functional group (*22*, *23*). However, the current assay methods for CYP24A1 inhibition rely on the use of primary human keratinocyte culture, recombinant hamster cell line, or rat kidney mitochondria (*18*−*23*). Because of the disparity in cell growth conditions (e.g., the nutrient in the media and the growth stage of the cells), or different preparation procedures and sources of kidney mitochondria, these assays tend to have experimental variability in enzyme content and the presence of physiological partners and other interacting factors. It may be difficult to compare the result generated from different laboratories as $IC_{50}$ values are often influenced by experimental conditions. Maintaining the culture is also inconvenient and time-consuming. In addition, they involve the use of radioactively labeled substrates for detection. A simple, clean, and sensitive enzymatic system would be ideal for CYP24A1 inhibitory studies. However, the prerequisite of such an assay would call for highly purified CYP24A1 in a large quantity suitable for biochemical characterization. So far, only rat CYP24A1 has been successfully overexpressed in *Escherichia coli* and purified to homogeneity (*24*). Although rat and human CYP24A1 are 83% identical in amino acid sequence, they do exhibit differences in kinetics, catalytic pathways, and structural details (*6*−*9*, *25*), which will in turn affect inhibitory properties. Expression and purification of the human enzyme would be highly desirable.

In this work, variants of human CYP24A1 proteins, with a C-terminal His tag or an N-terminal fusion to maltose binding protein (MBP), were overexpressed in *E. coli* and purified to apparent homogeneity. The hydroxylase activity was reconstituted in vitro, and the cell-free assay system was applied in the screening of vitamin D analogues for inhibition of CYP24A1. Two compounds, TS17 and CPA1, were identified as potent and selective CYP24A1 inhibitors.

## MATERIALS AND METHODS

*Materials*. Oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases, expression vector pMAL-c4x, and amylose resin were purchased from New England Biolabs (Beverly, MA). Vector pET-29b(+) was from Novagen (Madison, WI). Talon resin was from Clontech Laboratories (Mountain View, CA). $1,25(OH)_2D_3$, $25(OH)D_3$, and 2-methylene-19-nor-(20*S*)-$1,25(OH)_2D_3$ (2MD) were purchased from SAFC-Pharma (Madison, WI). 26,27-[$^3$H]-$1,25-(OH)_2D_3$ was provided by Perkin-Elmer (Boston, MA). All other vitamin D compounds were synthesized in the laboratory according to published procedures or procedures that will be published elsewhere.

*DNA Constructs*. The gene encoding human CYP24A1 protein (*cyp24a1*) was amplified via polymerase chain reaction (PCR) from its cDNA clone in the pGEM-3z plasmid (*26*) using primers 5′-GGAATTCCATATGCCTCAACCTAGAGAAG-TGCCAGTCTGCCC-3′ and 5′-CCGGAATTCTCGAGTCG-CTGGCAAAACG-3′. Silent mutations (underlined) were introduced to enrich the A/T content at the N-terminus of the sequence for optimal expression in *E. coli* (*9*). The PCR product was digested with restriction endonucleases *Nde*I and *Xho*I and subcloned into expression vector pET-29b(+). The resulting construct, pET29b-hCYP24, carried a deletion of the first 35 residues of CYP24A1, which constitute the signal peptide, and a six-histidine tag incorporated at the C-terminus. To generate construct pMAL-hCYP24, in which MBP was fused to the N-terminus, primer 5′-CTAGTCTAGATTATCGCTGGCAAAACGC-3′ was used as the 3′ PCR primer and the gene was cloned into the *Eco*RI- and *Xba*I-digested pMAL-c4x vector. The identity of each construct was confirmed by DNA sequencing and coincided with the reported sequence (*9*).

*CYP24A1 Expression and Purification*. *E. coli* BL21-Gold(DE3) cells (2 L) carrying plasmids pGro7 (Takara) and pET29b-hCYP24 were grown in LB medium supplemented with 40 mg/L kanamycin and 20 mg/L chloramphenicol at 37 °C. The cells were induced at an $OD_{600}$ of 0.7−0.8 at 25 °C by the addition of 3 mg/mL L-(+)-arabinose, 0.5 mM isopropyl $\beta$-D-thiogalactoside (IPTG), and 0.8 mM 5-aminolevulinic acid ($\delta$-ALA) and grown for an additional 6 h. Cells were harvested by centrifugation and resuspended in 100 mL of lysis buffer containing 20 mM Tris (pH 7.9), 400 mM NaCl, 5 mM imidazole (binding buffer), 0.75% (v/v) Triton X-100, 0.4% (w/v) protamine sulfate, 0.1× EDTA-free Halt protease inhibitor cocktail (Pierce), 1 mM $\beta$-mercaptoethanol, and 0.2 mg/mL chicken egg white lysozyme. The cells were lysed by being stirred at 4 °C for 20 min followed by brief sonication and centrifugation. The supernatant was loaded on a Talon metal affinity column (2.5 cm × 3 cm) equilibrated in binding buffer. The column was washed with 80 mL of the binding buffer, and the protein was eluted with 60 mM imidazole in the same buffer. Fractions containing pure CYP24A1-His (~55 kDa) as analyzed by 12% SDS−PAGE were combined and concentrated in an Amicon centrifugal filter (Millipore) and applied to a PD-10 desalting column (GE Healthcare) to remove imidazole. The protein was adjusted to 20% (v/v) glycerol, flash-frozen in liquid $N_2$, and stored at −80 °C. For the purification of MBP-CYP24A1, *E. coli* Rosetta 2(DE3) cells carrying plasmid pMAL-hCYP24 were grown in LB medium supplemented with 50 mg/L ampicillin and 20 mg/L chloramphenicol and induced with 0.5 mM IPTG and 0.8 mM $\delta$-ALA. Cells were lysed as described above except that the binding buffer contained 20 mM Tris (pH 7.7) and 125 mM NaCl. The lysate supernatant was loaded on an amylose resin column (2.5 cm × 4 cm). The column was washed with 100 mL of binding buffer, and the protein was eluted with 10 mM maltose in the same buffer. For the purification of CYP24A1 without an MBP fusion, MBP-CYP24A1 was incubated with factor Xa at 20 °C for 1 h and passed through the amylose column twice to remove free MBP and undigested fusion protein. Protein concentrations were determined by the Bradford method using bovine serum albumin as a standard.

*Determination of Heme Content*. The $N_2$-degassed CYP24A1 solution was reduced with sodium dithionite and transferred into two cuvettes. One of the samples was saturated with CO by bubbling the gas for ~1 min. The reduced CO

difference spectra were recorded in a Unicam (Cambridge, U.K.) UV1 spectrometer. The P450 content was determined on the basis of the absorption difference between 450 and 490 nm ($\Delta\varepsilon_{450-490}$ = 91 $mM^{-1}$ $cm^{-1}$) (*27*). The heme content was confirmed by using a Triton−methanol assay (*28*). CYP24A1 was diluted in 0.1 M NaOH, and various aliquots were mixed in 2.5% Triton X-100 in methanol. The absorbance of the resulting solution was measured at 405 nm, and the heme concentration was calculated using bovine hemin as a standard ($\varepsilon_{405}$ = 120 $mM^{-1}$ $cm^{-1}$).

*Purification of CYP27B1, Adrenodoxin, and Adrenodoxin Reductase*. The expression plasmids for mouse CYP27B1, pKCHis-m1α (*29*), and chaperones GroEL/ES, pGro12 (*30*), were kindly provided by T. Sakaki (Toyama Prefectural University, Toyama, Japan). Expression and purification of CYP27B1 were conducted as described by Uchida et al. (*31*) with slight modifications. A Talon column was used, and the CHAPS concentration was reduced to 0.5%. The expression plasmids for bovine adrenodoxin (Adx), pBA1159 (*32*), and adrenodoxin reductase (AdR), pBAR1607 (*33*), were kindly provided by M. Waterman (Vanderbilt University, Nashville, TN). The recombinant Adx and AdR were expressed in *E. coli* DH5α and Rosetta 2(DE3), respectively. Adx was purified using a modified method of Kimura et al. (*34*) from a DE53 anion exchange column (Whatman) followed by a Sephadex G-75 column (Amersham Biosciences). The Adx concentration was determined on the basis of the absorption at 414 nm ($\varepsilon_{414}$ = 9.8 $mM^{-1}$ $cm^{-1}$) (*35*). AdR was purified by affinity chromatography using CNBr-activated Sepharose 4B (GE Healthcare) immobilized with Adx (*33*, *36*). Adx was coupled to the medium following the manufacturer's instructions. The AdR concentration was determined according to FAD absorption at 450 nm ($\varepsilon_{450}$ = 11.3 $mM^{-1}$ $cm^{-1}$) (*37*).

*CYP24A1 Activity Assay*. The reconstituted CYP24A1 reaction mixture (0.5−2 mL) contained 20 mM Tris (pH 7.5), 125 mM NaCl, 0.1 μM Adx, 0.1 μM AdR, 0.075 μM CYP24A1, and 0−2.5 μM substrate. The vitamin $D_3$ substrates were prepared in ethanol, and the concentrations were measured on the basis of UV absorption at 265 nm ($\varepsilon_{265}$ = 18.2 $mM^{-1}$ $cm^{-1}$) (*38*) or 252 nm ($\varepsilon_{252}$ = 42.0 $mM^{-1}$ $cm^{-1}$) (*39*). The reaction was initiated by the addition of NADPH at a final concentration of 0.5 mM and the mixture incubated at 37 °C for 5−30 min to keep substrate conversion at ≤20%. The reaction was quenched and the mixture extracted with 4 volumes of dichloromethane twice. The organic layer was collected and dried under blowing $N_2$. After being redissolved in 5% 2-propanol [for 25(OH)$D_3$ reaction] or 15% 2-propanol (for all other reactions) in hexane, the reaction mixture was subjected to HPLC on a silica column (ZORBAX RX-SIL, 9.4 mm × 250 mm, Agilent) run in the same solvent monitored at 265 nm. The initial rates calculated from product formation were fitted to the Michaelis−Menten equation [$V = k_{cat}[E]_0[S]/(K_M + [S])$] using GraphPad Prism 5 to obtain the $k_{cat}$ and $K_M$ values.

*CYP24A1 Inhibition Assay*. Assay reactions were performed in a buffer containing 20 mM Tris (pH 7.5), 125 mM NaCl, 0.1 μM Adx, 0.1 μM AdR, 0.075 μM CYP24A1, varying concentrations of 1,25$(OH)_2D_3$ (1.25−2.50 μM) and inhibitors, and 0.5 mM NADPH. The reactions were conducted and analyzed by HPLC as described above. The $K_I$ values were determined by fitting the relative activity ($V/V_0$) against the inhibitor concentration [I] using the equation $V/V_0 = (K_M + [S])/[K_M(1 + [I]/K_I) + [S]]$, where $V$ and $V_0$ are the reaction rates in the presence and absence of inhibitors, respectively.

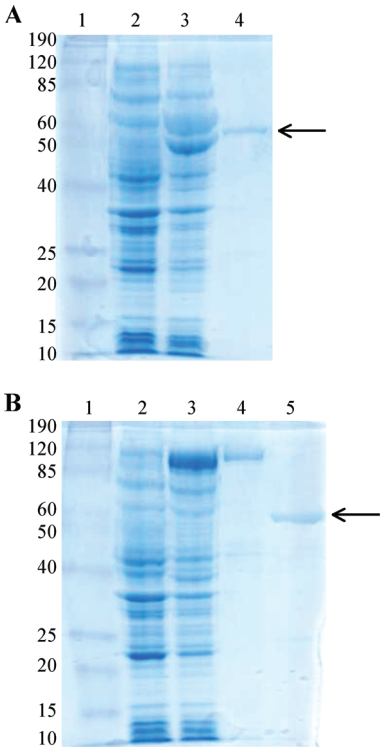


FIGURE 1: SDS−PAGE (12%) of CYP24A1-His and MBP-CYP24A1. (A) BL21-Gold(DE3) cells carrying plasmids pGro7 and pET29b-hCYP24: lane 1, marker; lane 2, lysate from uninduced cells; lane 3, lysate from induced cells; lane 4, purified CYP24A1-His. (B) Rosetta 2(DE3) cells carrying plasmid pMAL-hCYP24: lane 1, marker; lane 2, lysate from uninduced cells; lane 3, lysate from induced cells; lane 4, purified MBP-CYP24A1; lane 5, CYP24A1 free of MBP.

*CYP27B1 Inhibition Assay*. Inhibition of CYP27B1 was assayed in a manner similar to that of CYP24A1 as described above except that the buffer contained 20 mM Tris (pH 7.5), 125 mM NaCl, 0.1% CHAPS, 2 μM Adx, 0.2 μM AdR, 0.025 μM CYP27B1, various amounts of 25(OH)$D_3$ (1.25−2.50 μM) and inhibitors, and 1.0 mM NADPH. The HPLC mobile phase was in 7% 2-propanol in hexane.

*Determination of the 1,25(OH)$_2$D$_3$ Half-Life*. Primary mouse osteoblasts were isolated from fetal calvaria and cultured to 70% confluency in αMEM containing 10% FBS in 24-well plates (1 mL/well). Two days later, the culture was treated with 2.5 nM or 2.5 pM 1,25$(OH)_2D_3$ and a trace amount of [$^3$H]-1,25$(OH)_2D_3$ in the presence and absence of 1 μM ketoconazole, 1 μM TS17, or 1 μM CPA1. At various time points (0, 8, 24, and 48 h), 200 μL of medium was withdrawn and extracted with 800 μL of dichloromethane twice. The organic phase was dried down and separated via HPLC in 15% 2-propanol in hexane as described above. The residual 1,25$(OH)_2D_3$ in the medium over a 48 h period was quantitated from tritium count.

*Other Biological Studies of TS17 and CPA1*. VDR binding, CYP24A1 transcription, and HL-60 differentiation assays were performed as previously described (*40*).

## RESULTS

*Expression and Purification of Human CYP24A1*. Expression of human CYP24A1 in *E. coli* has been reported by Sakaki et al. (*9*). However, because of its low level of expression and poor solubility and stability, the protein has never been purified to homogeneity. To obtain the enzyme in sufficient

quantity for biochemical characterization, we constructed the expression plasmid encoding the mature protein with a C-terminal six-histidine tag to facilitate its purification by metal affinity chromatography. Coexpression with molecular chaperones GroEL/ES assisted proper folding of the protein, and protein extraction from the membrane was achieved by using 0.75% Triton X-100. Together, these measurements allowed easy and quick enrichment of pure CYP24A1-His (Figure 1). To further improve the yield of the protein, the second construct was made to express an N-terminally MBP-fused CYP24A1 variant, MBP-CYP24A1, which not only resulted in a significant boost in the level of expression and considerable enhancement of the solubility but also took advantage of the MBP fusion tag for single-step purification from the amylose column (Figure 1). Moreover, the use of the Rosetta 2(DE3) strain, which overcomes codon bias in *E. coli*, led to an additional increase in the level of expression. As a result, the yield of MBP-CYP24A1 reached 15 mg/L of culture, while expression of CYP24A1-His afforded a yield of only 1 mg/L of culture. CYP24A1 free of MBP fusion was obtained following factor Xa cleavage (Figures 1 and 2). All proteins had a heme content of 77−85% from multiple batches of purification.

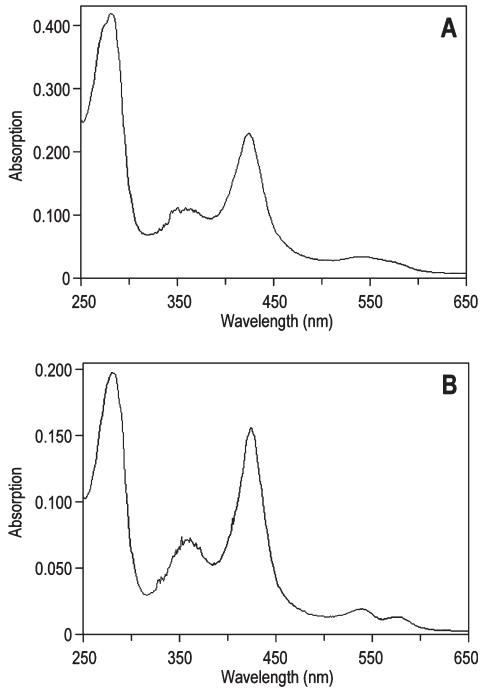


FIGURE 2: UV−vis spectra of MBP-CYP24A1 (A) and CYP24A1 (B). CYP24A1 was cleaved from MBP-CYP24A1 with factor Xa and purified on the amylose column. The ratio of absorption ($A_{280}/A_{418}$) was decreased from 2.0:1 to 1.2:1.

*Catalytic Properties of CYP24A1.* CYP24A1 activity toward the naturally occurring vitamin $D_3$ compounds, 1,25$(OH)_2D_3$ and 25$(OH)D_3$, as well as two synthetic vitamin $D_3$ analogues, 2MD and 2-methylene-19-nor-(20*R*)-1,25$(OH)_2D_3$ [(20*R*)-2MD], was determined (Figure 3). All three CYP24A1 variants, CYP24A1-His, MBP-CYP24A1, and CYP24A1, had essentially the same activity and exhibited saturation kinetics toward 1,25-$(OH)_2D_3$, suggesting that the C-terminal His tag or N-terminal MBP fusion does not interfere with CYP24A1 activity and CYP24A1 produced from factor Xa-cleaved MBP-CYP24A1 retains its catalytic properties. The reaction kinetics toward 1,25$(OH)_2D_3$ and 25$(OH)D_3$ were quite similar, with $k_{cat}$ values of 0.118−0.120 $min^{-1}$ and $K_M$ values of 0.19−0.33 $\mu$M (Table 1). The reaction activity toward 2MD, a VDR agonist and osteogenic agent for rats (*41*, *42*), exhibited an 8-fold lower $k_{cat}$ (0.015 $min^{-1}$), but the $k_{cat}$ value toward (20*R*)-2MD (0.100 $min^{-1}$), in which the stereochemistry at the C20 position was the same as that of the

Table 1: Catalytic Properties of CYP24A1 Variants

| substrate | $k_{cat}$ ($min^{-1}$) | $K_M$ ($\mu$M) | $k_{cat}/K_M$ ($M^{-1}$ $s^{-1}$) |
|---|---|---|---|
| 1,25$(OH)_2D_3$[a] | 0.118 ± 0.005 | 0.19 ± 0.03 | $1.0 \times 10^4$ |
| 1,25$(OH)_2D_3$[b] | 0.093 ± 0.015 | 0.15 ± 0.05 | $1.0 \times 10^4$ |
| 1,25$(OH)_2D_3$[c] | 0.105 ± 0.018 | 0.17 ± 0.05 | $1.0 \times 10^4$ |
| 25$(OH)D_3$[a] | 0.120 ± 0.012 | 0.33 ± 0.04 | $6.1 \times 10^3$ |
| 2MD[a] | 0.015 ± 0.004 | 0.14 ± 0.03 | $1.8 \times 10^3$ |
| (20*R*)-2MD[a] | 0.100 ± 0.008 | 0.34 ± 0.07 | $4.9 \times 10^3$ |

[a]Activity of MBP-CYP24A1. [b]Activity of CYP24A1-His. [c]Activity of CYP24A1.

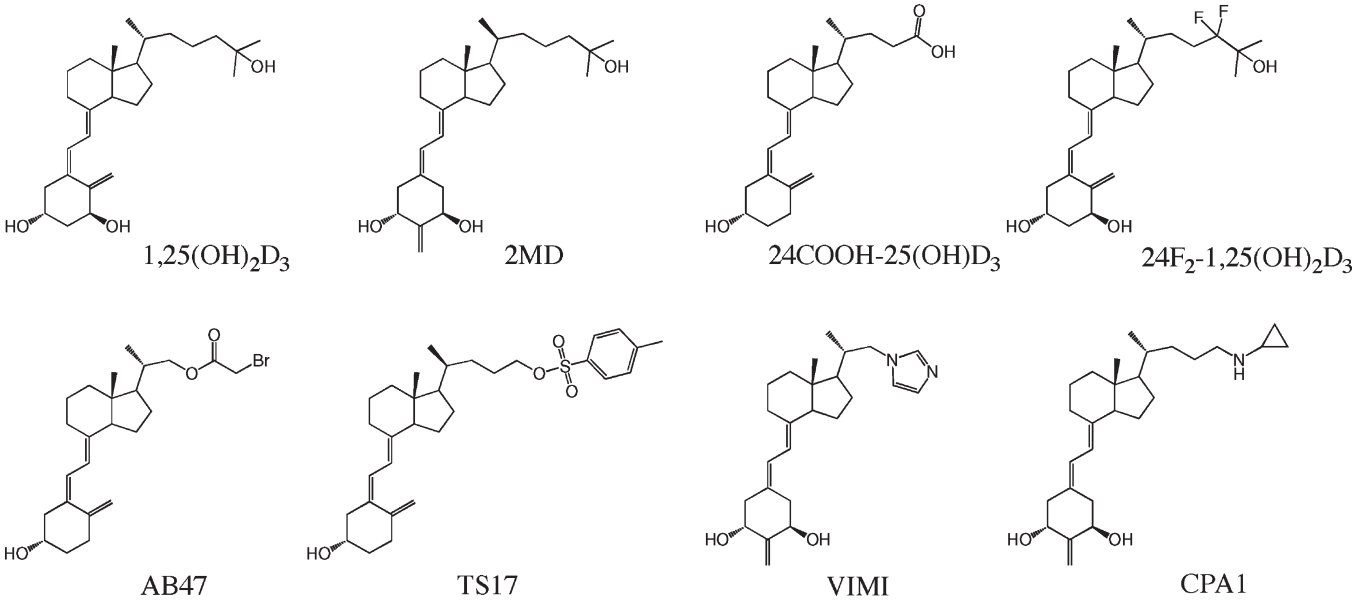


FIGURE 3: Structures of CYP24A1 substrates and inhibitors.

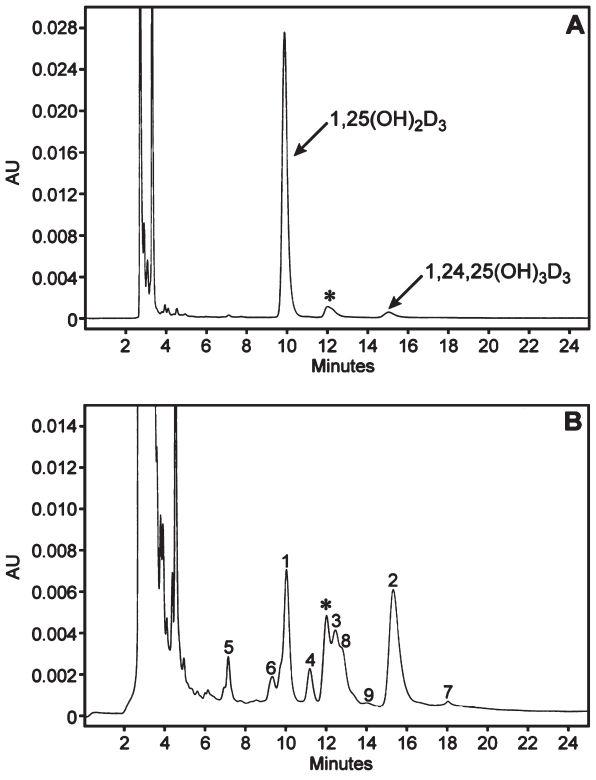


FIGURE 4: HPLC chromatogram of $1,25(OH)_2D_3$ degradation catalyzed by CYP24A1 under kinetic assay conditions (A) and with full catalytic potential (B). Peaks were numbered according to the order of appearance: 1, substrate $1,25(OH)_2D_3$; 2, $1,24,25(OH)_3D_3$ and $1,23,25(OH)_3D_3$; 3, 24-oxo-$1,25(OH)_2D_3$; 4−9, compounds yet to be identified or confirmed. The asterisk denotes a feature present in all HPLC traces, including that without CYP24A1.

natural substrates, was comparable to those toward $1,25(OH)_2D_3$ and $25(OH)D_3$. This drastic change in activity was also observed with several other vitamin D analogues with alterations in only the 20*S* or 20*R* configuration (data not shown), suggesting that the configuration at the C20 position of the substrate is critical to CYP24A1 activity. Because MBP-CYP24A1 acts as a fully functional enzyme, it was used in the majority of the work conducted here for its high yield and convenient preparation.

In the kinetic assays, the concentrations of Adx and AdR, the electron-donating partners in the P450-containing system, were kept low to maintain C24 hydroxylation as the major reaction and minimize sequential oxidation and/or hydroxylation catalyzed by CYP24A1 (*6*, *9*). As a result, no additional metabolite was observed in the HPLC chromatogram under such conditions, including the 23-hydroxylated product (Figure 4a). This is probably because the C23 hydroxylation pathway accounts for only 20% (*9*) and substrate conversion was no more than 20%; therefore, this minor product fell below the detection limit. When the CYP24A1 enzyme was incubated with substrate for longer periods of time with high concentrations of Adx and AdR, all the metabolites reported previously (*6*−*9*, *43*) were produced (Figure 4b). These products, either identified or unknown, confirmed CYP24A1 as a multicatalytic enzyme and confirmed that the enzymes purified in this study gave identical products.

*Inhibition of CYP24A1 and CYP27B1*. We tested a number of vitamin D analogues synthesized in the laboratory, with variations on the A ring and the C−D ring and, mostly, with extensive modifications on the side chain, as potential CYP24A1 inhibitors. Almost all of the compounds did not show noticeable turnover under experimental conditions in the inhibition assay. Among them, six compounds along with ketoconazole exhibited potent inhibition of CYP24A1, with $K_I$ values ranging from 0.021 to 0.090 $\mu$M, and were subjected to additional assays to measure their inhibitory activities toward CYP27B1 (Figure 3 and Table 2). Ketoconazole, which had a $K_I$ value of 0.032 $\mu$M toward CYP24A1, was equally effective at suppressing CYP27B1 activity, with a $K_I$ value of 0.053 $\mu$M. This was also true with four of the six compounds, which showed moderate selectivity of < 20 for inhibition of CYP24A1 and CYP27B1. TS17 and CPA1, however, exhibited specific inhibition of CYP24A1, with substantial selectivity of 39 and 80 over CYP27B1, respectively. Further analysis revealed that they were competitive inhibitors of both enzymes (Figure 5) and that prolonged incubation with the enzymes did not exert extra inhibitory activities, suggesting that inhibition occurred instantly without conformational changes in the proteins.

Table 2: Inhibition Constants of Vitamin D Analogues toward CYP24A1 and CYP27B1

| | $K_I$ ($\mu$M) | | |
|---|---|---|---|
| inhibitor | CYP24A1 | CYP27B1 | selectivity |
| AB47 | 0.021 ± 0.007 | 0.068 ± 0.011 | 3.2 |
| VIMI | 0.021 ± 0.006 | 0.090 ± 0.017 | 4.3 |
| $24F_2$-$1,25(OH)_2D_3$ | 0.024 ± 0.005 | 0.092 ± 0.015 | 3.8 |
| 24COOH-$25(OH)D_3$ | 0.090 ± 0.010 | 1.70 ± 0.28 | 19 |
| TS17 | 0.039 ± 0.002 | 1.52 ± 0.28 | 39 |
| CPA1 | 0.042 ± 0.014 | 3.34 ± 0.38 | 80 |
| ketoconazole | 0.032 ± 0.005 | 0.053 ± 0.010 | 1.6 |

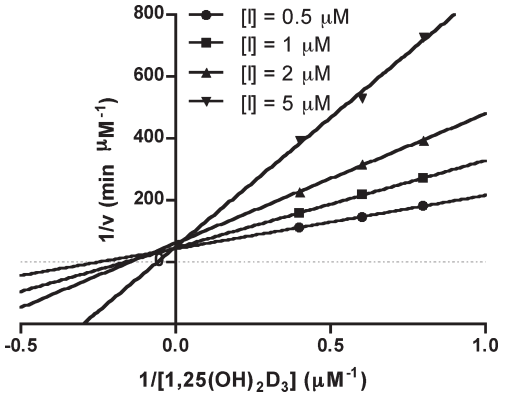


FIGURE 5: Lineweaver−Burke plot showing competitive inhibition of CYP24A1 by CPA1.

*Effect of CYP24A1 Inhibitors on the $1,25(OH)_2D_3$ Half-Life*. To confirm the inhibitory effect of TS17 and CPA1 toward CYP24A1 in vitamin D responsive cells, we determined the half-life of $1,25(OH)_2D_3$ in mouse osteoblast culture with and without a supplement of inhibitors. At high doses of $1,25(OH)_2D_3$ treatment (2.5 nM), as the pool of residual $1,25(OH)_2D_3$ continuously decreased in the medium and eventually reached near depletion in 48 h, it remained stable in the presence of TS17 or CPA1 over the entire period (Figure 6). To a lesser extent, addition of ketoconazole extended the half-life of $1,25(OH)_2D_3$ from 24 to 42 h under the same conditions. With a small dose of $1,25(OH)_2D_3$ (2.5 pM), however, no change in $1,25(OH)_2D_3$ level was observed, and there was therefore no effect from inhibitors (data not shown). The discrepancy of $1,25(OH)_2D_3$ stability at

two concentrations most likely lies in the fact that CYP24A1 expression was probably induced only at a large $1,25(OH)_2D_3$ dose (2.5 nM). As a control, the $1,25(OH)_2D_3$ level remained constant in the cell-free medium (data not shown). The ability of TS17 and CPA1 to sustain $1,25(OH)_2D_3$ in the mouse osteoblast culture further demonstrated their effectiveness in CYP24A1 inhibition.

*Other Biological Evaluations of TS17 and CPA1*. To determine the binding affinity of TS17 and CPA1 toward VDR, a competition assay (*44*) was conducted using recombinant rat VDR. TS17 did not seem to bind VDR even at high concentrations (up to 1 $\mu$M), and CPA1 turned out to be a much weaker ligand for VDR, with 107 times lower binding affinity than the natural hormone $1,25(OH)_2D_3$ (Table 3). The ability of TS17 and CPA1 to induce transcription of vitamin D responsive genes was examined in a luciferase reporter system driven by the CYP24A1 promoter (*45*). Consistent with the reduced VDR binding affinity, the VDR-mediated transcriptional activity of CPA1 was decreased by 18-fold and no activity was observed for TS17 (Table 3). Finally, the cellular activity of CPA1 to induce differentiation of human promyelocytic leukemia HL-60 cells into monocytes (*46*) was comparable with respect to that of $1,25(OH)_2D_3$ (Table 3). TS17, in sharp contrast, did not induce HL-60 differentiation and reached toxicity at 1 $\mu$M.

## DISCUSSION

Heterologous expression of CYP24A1 has been attempted in COS cells (*26*, *47*) and baculovirus-infected *Spodoptera frugiperda* cells (*7*, *26*), but the mammalian and insect systems could offer only transient expression that was also not sufficient for extensive biochemical investigations. Although expression in *E. coli* (JM109 and DH5α), which lacks endogenous P450 proteins and thus has no interference, has proven to be successful in larger-scale production of CYP24A1 from various species, including chickens, rats, and humans (*6*, *9*, *24*, *48*), purification of the homogeneous protein has been reported for only rat CYP24A1 (*24*). This was in part due to the disparity in codon usage between eukaryotic and prokaryotic organisms and the nature of CYP24A1 being a membrane protein, which limited the expression level and ability to fold properly. Our use of the Rosetta 2(DE3) cells bypassed the codon bias issue and increased the level of expression by 5-fold compared to that of the commonly used DH5α cells (data not shown). To support correct folding and/or repair mistakes, molecular chaperones such as DnaK/DnaJ/GrpE, GroEL/ES, and trigger factor, either as an individual set or in conjunction with each other, were coexpressed with CYP24A1. GroEL/ES seemed to be more helpful than others that had little to no effect (data not shown). As useful as it might be, the chaperone approach by no means surpassed the striking benefit of creating an MBP fusion, which functions as a pseudochaperone in the fusion construct, promoting solubility and expression (*49*). To solubilize the overexpressed human CYP24A1 variants from *E. coli* lysate, a series of ionic, nonionic, and zwitterionic detergents that have been used to isolate CYP24A1 and other P450s were examined, including cholate, Lubrol, Emulgen 911, digitonin, Tergitol NP-10, Tween 20, Triton X-100, and CHAPS. All of them were able to solubilize CYP24A1 to certain extent, with Tween 20 being the least efficient. Triton X-100 and CHAPS were superior to the others in terms of both solubilizing power and the ability to reconstitute CYP24A1 activity. The enzyme extracted with Triton X-100 and CHAPS exhibited the same catalytic activity, but for the purification of MBP-CYP24A1, CHAPS caused minor precipitation, likely because of its incompatibility with MBP.

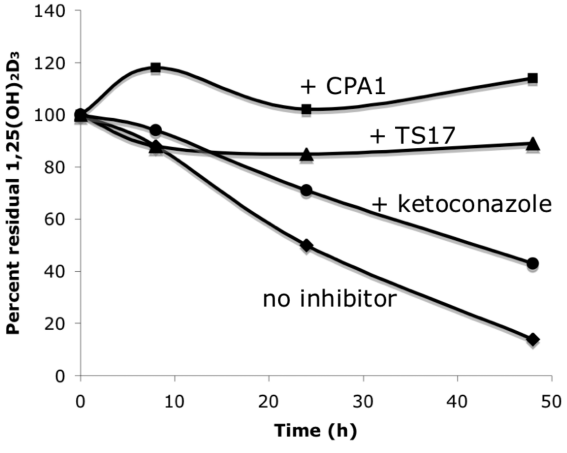


FIGURE 6: Time course of residual $1,25(OH)_2D_3$ in the medium of the mouse osteoblast culture in the presence and absence of CYP24A1 inhibitors.

The overall activity ($k_{cat}/K_M$) of human CYP24A1 is consistent with the previously reported value, although the individual parameters, particularly $K_M$, did not agree as well (*9*). This was probably because the membrane fraction of *E. coli* containing CYP24A1 as well as other constituents was used in the study instead of the purified protein, which created disturbance in the kinetic assay especially when the $K_M$ value is in the submicromolar range. In contrast to the scarce studies on human CYP24A1 incurred by handling difficulties (availability of specimen, low level of expression, and poor stability), investigations of the rat enzyme have been conducted more frequently. However, there were vast discrepancies in the results; e.g., with $1,25(OH)_2D_3$ as the substrate, the $K_M$ varied from 0.23 $\mu$M to as high as 20.9 $\mu$M (*6*, *50*), which added to the controversy about the preferred substrate of CYP24A1 [$1,25(OH)_2D_3$ vs $25(OH)D_3$]. We believe the disagreement derived at least partially from the preparation of the enzyme and the use of highly purified

Table 3: VDR Binding Properties, Transcriptional Activities, and HL-60 Differentiation Activities of TS17 and CPA1 in Comparison to Those of $1,25(OH)_2D_3$

| | VDR binding | | CYP24A1 transcription | | HL-60 differentiation | |
|---|---|---|---|---|---|---|
| | $EC_{50}$ (M) | ratio | $EC_{50}$ (M) | ratio | $EC_{50}$ (M) | ratio |
| $1,25(OH)_2D_3$ | $1.4 \times 10^{-10}$ | 1 | $1.8 \times 10^{-10}$ | 1 | $3.4 \times 10^{-9}$ | 1 |
| CPA1 | $1.5 \times 10^{-8}$ | 107 | $3.4 \times 10^{-9}$ | 18 | $8.6 \times 10^{-9}$ | 2.5 |
| TS17 | NA[a] | NA[a] | NA[a] | NA[a] | NA[a] | NA[a] |

[a]Not available.

protein would resolve the ambiguity. The fact that CYP24A1 is capable of catalyzing multiple oxidation and/or hydroxylation reactions and the fact that the human enzyme can use both C24 and C23 pathways suggest a wide substrate binding pocket in CYP24A1 for recognizing a variety of metabolites. Nonetheless, the stereochemical preference at the C20 position is clearly more important than modifications on the A ring [comparing activity toward $1,25(OH)_2D_3$ and (20*R*)-2MD] because changes in the C20 configuration would alter the orientation, vicinity, and, hence, accessibility of the side chain relative to the active site residues involved in catalysis while the A ring is distant from the heme prosthetic group in the substrate access channel as seen in the docking models (*25*, *51*−*54*).

Using purified CYP24A1 and CYP27B1, our inhibition assays eliminated variations and interference intrinsic to the cell- or tissue-based system and provided more reliable and comparable data. For example, large margins of deviation in the reported $IC_{50}$ values for the inhibition of CYP24A1 by ketoconazole [126 nM in human keratinocytes (*18*) and 20 $\mu$M using rat kidney mitochondria (*20*)] can be avoided. Despite the limited number of compounds we have tested, points could still be taken to serve as guidance for future development of potent and selective inhibitors of CYP24A1. (a) Modification on the A ring is less important, which is consistent with previous observations (*23*). (b) The stereochemistry at C20 is important to catalysis but may not play a critical role in inhibitory activities. (c) Longer and bulkier side chains tend to deliver stronger inhibition, likely as a result of improved affinity. Since the report of cyclopropylamines as suicide inhibitors of P450s (*55*), compounds containing this moiety have been explored in the inhibition of a number of P450s as well as other oxidative enzymes (*56*−*62*), but unlike that described in many cases of cyclopropylamine-based inhibitors, time-dependent and irreversible inactivation was not observed for CPA1 that had cyclopropylamine incorporated at the end of the vitamin D side chain. Instead, it acted as a competitive inhibitor as did most other inhibitors tested in this work. As shown in the recently determined crystal structure of rat CYP24A1 (*54*), the open cleft conformation displays a broad substrate binding cavity, and although the aromatic cluster blocking access to the catalytic center confers some degree of specificity, the inhibitors can easily pass the barrier and achieve efficient binding because they are all structural analogues of $1,25(OH)_2D_3$. However, because of the modifications on the side chain, they will not be properly juxtaposed to induce the conformational change to reach the ultimate productive, closed state of the enzyme needed for catalysis but simply compete with the natural substrate for the binding pocket. To gain insight into the structure−activity relationship, X-ray crystallographic work of CYP24A1 bound with selective inhibitors is underway.

Elevated CYP24A1 levels have been seen in many types of human cancers (*63*), immune dysfunctions, and secondary hyperparathyroidism. To make things more complicated, intervention with vitamin D analogues often stimulates the expression of CYP24A1 via the same VDR-mediated mechanism as that employed by $1,25(OH)_2D_3$, resulting in rapid elimination of the active metabolites, which drastically counters the effectiveness of the treatment. Selective CYP24A1 inhibitors would solve the dilemma by serving as auxiliary drugs to potentiate the current treatment options, but without jeopardizing the normal biosynthesis of $1,25(OH)_2D_3$. Studies with cell models, either by small molecule inhibitors or epigenetic silencing, have already demonstrated the effect of CYP24A1 inhibition on enhancing $1,25(OH)_2D_3$ efficacy (*14*−*16*, *64*−*68*). The concept has recently been evaluated in some early phase clinical trials and shed light on the treatment of vitamin D-related diseases (*12*).

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