Cytochrome P450 3A-mediated microsomal biotransformation of 1α,25-dihydroxyvitamin D₃ in mouse and human liver: drug related induction and inhibition of catabolism

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Running title: Modulation of CYP3A-mediated biotransformation of vitamin D₃

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Abbreviations: CYP, cytochrome P450; 1α,25(OH)₂D₃, 1α,25-dihydroxyvitamin D₃; 1α,23S,25(OH)₃D₃, 1α,23S,25-trihydroxyvitamin D₃; 1α,24R,25(OH)₃D₃, 1α,24R,25-trihydroxyvitamin D₃; VDR, vitamin D receptor; LC/MS, liquid chromatography/mass spectrometry; HLM, human liver microsomes; MLM, mouse liver microsomes; GR, glucocorticoid receptor; PXR, pregnane X receptor
ABSTRACT

The biological activities of vitamin D₃ are exerted through 1α,25(OH)₂D₃, the dihydroxy metabolite of vitamin D₃. Hepatic biotransformation of 1α,25(OH)₂D₃ by cytochrome P450 (CYP) enzymes could be an important determinant of bioavailability in serum and tissues. In the present study, we investigated the comparative biotransformation of 1α,25(OH)₂D₃ in mouse and human liver microsomes and determined the effects of commonly used drugs on the catabolism of 1α,25(OH)₂D₃. Severe symptoms of vitamin D-deficiency have historically been observed in patients receiving dexamethasone. To compare the effects of clinically important glucocorticoids on hepatic biotransformation of 1α,25(OH)₂D₃, adult male CD-1 mice were treated with either vehicle (50% ethanol), dexamethasone (80 mg/kg/day) or prednisone (80 mg/kg/day) for three consecutive days by intraperitoneal injection. Hydroxy metabolite formation pattern and the extent of substrate depletion were similar in mouse liver microsomes (MLM) from vehicle- or prednisone-treated mice, whereas treatment with dexamethasone led to emergence of additional metabolites and increased substrate depletion, as determined by liquid chromatography/mass spectrometry. The metabolite formation profile in vehicle-treated mice was different than that of human liver microsomes (HLM). Selective CYP chemical inhibitors have demonstrated that CYP3A isoforms are responsible for the microsomal biotransformation of 1α,25(OH)₂D₃ in MLM. Co-incubation of 1α,25(OH)₂D₃ with commonly used drugs led to approximately 60-100% inhibition of CYP3A4-mediated catabolism of 1α,25-(OH)₂D₃ in HLM. A species-based difference was identified between CYP3A-mediated hepatic microsomal metabolism of 1α,25(OH)₂D₃ in humans and mice. We have shown that clinically important glucocorticoids differentially modulate catabolism of active vitamin D₃ and that commonly used drugs could affect vitamin D homeostasis.
Introduction

Epidemiological studies have shown that lower serum levels of vitamin D3 are associated with increased risk of prostate, colon and breast cancer (Garland et al., 2009). The biological activities of vitamin D3 are attributed to 1\(\alpha\),25-dihydroxyvitamin D3 [1\(\alpha\),25(OH)\(_2\)D3], which acts primarily by binding to the vitamin D receptor (VDR) (Deeb et al., 2007). 1\(\alpha\),25(OH)\(_2\)D3 shows antiproliferative, pro-differentiation and antiangiogenic effects in both human prostate cancer cells in vitro and preclinical animal studies (Deeb et al., 2007). In addition, preliminary clinical investigations have reported a promising anti-tumor role of 1\(\alpha\),25(OH)\(_2\)D3. For example, co-treatment of advanced prostate cancer patients with taxanes (e.g. paclitaxel, docetaxel) and 1\(\alpha\),25(OH)\(_2\)D3 was shown to potentiate cytotoxic effects and/or increase the survival rates compared to treatment with only taxanes or placebo (Muindi et al., 2002; Beer et al., 2007). In addition, acquired immunodeficiency syndrome (AIDS) patients have lower vitamin D3 levels (Dao et al., 2011), which may render them susceptible to further lowering of immune function, bone diseases and increased risk of opportunistic infections. Vitamin D3 and its derivatives have been successfully used in the treatment of autoimmune skin diseases such as psoriasis and vitiligo (Sigmon et al., 2009).

In vertebrates, vitamin D3 is synthesized from 7-dehydrocholesterol in the skin through exposure to UVB radiation or can be obtained from dietary sources (Deeb et al., 2007). Serum proteins transport vitamin D3 to the liver, where multiple cytochrome P450 (CYP) enzymes in mitochondria (e.g. CYP27A1) and microsomes (e.g. CYP2J3, CYP2R1) are capable of hydroxylation it at the C-25 position (Prosser and Jones, 2004; Ingraham et al., 2008). The serum 25(OH)D3 has gained some acceptance as a measure of vitamin D3 in the body (Schuster, 2011). In response to low calcium levels, renal CYP27B1 catalyzes the 1\(\alpha\)-hydroxylation of 25(OH)D3.
to form $1\alpha,25(OH)_2D_3$, which is a small lipophilic molecule with superior cellular penetration and is the most potent endogenous agonist of VDR (Ingraham et al., 2008). Subsequently, $1\alpha,25(OH)_2D_3$ is converted to inactive metabolites by mitochondrial CYP24A1 in kidney (Schuster, 2011).

Epidemiological and clinical reports show that patients on certain classes of medications, such as anti-epileptics (e.g. carbamazepine, phenytoin) and protease inhibitors (e.g. efavirenz), experience lower vitamin D$_3$ levels, altered calcium metabolism and impaired bone health (Verrotti et al., 2009). Similarly, vitamin D-deficiency like bone-related problems (e.g. bone fracture and osteonecrosis) were observed in patients receiving dexamethasone, a commonly used anti-inflammatory glucocorticoid steroid, often used as a premedication for taxane-based chemotherapy of advanced cancers (Hussain et al., 2005; Beer et al., 2007) or as a single agent in acute lymphoblastic leukaemia (Inaba and Pui, 2010). Treatment of infant pigs with dexamethasone led to lower $1\alpha,25(OH)_2D_3$ levels, reduced calcium absorption and impaired bone metabolism (Weiler et al., 1995). In contrast, prednisone, which is also an anti-inflammatory glucocorticoid steroid with adverse effects on bone health (Reid and Heap, 1990), is less potent than dexamethasone in repressing markers for bone formation (e.g. osteocalcin) as well as overall bone health (e.g. bone fracture, osteopenia, osteonecrosis) (Dubois et al., 2003; Inaba and Pui, 2010). There is no experimental evidence to define the effects of glucocorticoids on biotransformation of biologically active $1\alpha,25(OH)_2D_3$. Also, a lack of studies present challenges for the direct comparison of dexamethasone- and prednisone-mediated negative effects on vitamin D$_3$ status and bone health.

$1\alpha,25(OH)_2D_3$ works both as a paracrine and an autocrine agent via VDR-mediated mechanisms (Morris and Anderson, 2010). Thus, catabolism of $1\alpha,25(OH)_2D_3$ in the liver could
have a significant impact on VDR response. CYP3A4 is the major hepatic phase I oxidative drug metabolizing enzyme with broad substrate specificity and is inducible following exposure to therapeutic, dietary and environmental agents (Thummel and Wilkinson, 1998). Gupta et al. (2004, 2005) reported that CYP3A4 mediates 24- and 25-hydroxylation activity of 1α(OH)D₃, but not vitamin D₃, in human liver microsomes (HLM). Hepatic biotransformation of 1α,25(OH)₂D₃ by CYP enzymes may influence its circulating serum and tissue levels. Xu et al. (2006) reported on the hepatic metabolism of 1α,25(OH)₂D₃ in humans, however, there is no report of 1α,25(OH)₂D₃ biotransformation in mice despite the fact that it’s a widely used animal model in vitamin D research. Our study therefore investigates the comparative biotransformation of 1α,25(OH)₂D₃ to its inactive hydroxy metabolites in mouse and human liver microsomes. In addition, we have sought to determine the effects of various medications, which are commonly used in the treatment regimens of patients with cancer (e.g. ketoconazole, tamoxifen, taxanes), AIDS (e.g. ritonavir, clarithromycin) as well as inflammatory disorders (e.g. dexamethasone, prednisone), on hepatic biotransformation of 1α,25(OH)₂D₃.
Materials and Methods

Chemicals

1α,25(OH)2D3, 25(OH)D3, dexamethasone sodium, prednisone, α-naphthoflavone, furafylline, sulfaphenazole, 8-methoxypsoralen, orphenadrine, quinine, SKF 525A, sodium diethyldithiocarbamate, docetaxel, paclitaxel, chloramphenicol and clarithromycin were obtained from Sigma-Aldrich Canada Ltd (Oakville, ON, Canada). Ritonavir and troleandomycin were purchased from Toronto Research Chemicals (North York, ON, Canada) and Enzo Life Sciences International, Inc. (Plymouth Meeting, PA), respectively. Ketoconazole and tamoxifen were obtained from MP Biomedical, LLC (Solon, OH). Pooled HLM of mixed gender were purchased from XenoTech, LLC (Lenexa, KS). Baculovirus-insect cell control microsomes (no cytochrome b5 or reductase), insect cells co-expressed with CYP reductase and cytochrome b5 and baculovirus insect cell microsomes containing human CYP3A4 enzyme co-expressed with CYP reductase and human cytochrome b5 (BD SUPERSOMES™ Enzymes) were purchased from BD Biosciences (Oakville, ON, Canada). Nicotinamide adenine dinucleotide phosphate (NADPH) regenerating solutions (A and B) were obtained from BD Gentest (Woburn, MA). Triazolam and its authentic metabolites were purchased from Cerilliant Corporation (Round Rock, TX). 5-Bromo-4-chloro-3-indolyl phosphate p-toluidine salt and nitro blue tetrazolium were purchased from Fisher Scientific (Ottawa, ON, Canada). Goat polyclonal anti-mouse CYP3A IgG and rabbit polyclonal anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) serum were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) and Novus Biologicals (Littleton, CO), respectively. Alkaline phosphatase conjugated swine anti-goat IgG and goat anti-rabbit IgG secondary antibodies were purchased from Cedarlane Labs.
(Burlington, ON, Canada) and Jackson Immunoresearch Lab (West Grove, PA), respectively. All other chemicals were obtained from commercial sources.

**Animal Treatment and Preparation of Liver Microsomes**

Adult male CD-1 mice were obtained from Harlan Laboratories Inc. (Montreal, QC, Canada). Mice were housed in polycarbonate cages with corn-cob bedding at a temperature of 20°C to 23°C and had a 12-h photoperiod. Mice were provided with commercial mouse diet and water *ad libitum*. Mice were cared for and treated in accordance with the principles and guidelines of the Canadian Council on Animal Care.

Mice were treated with dexamethasone or prednisone dissolved in 50% ethanol at a dosage of 80 mg/kg body weight (b.w.) or an equivalent volume of vehicle (three mice per group) by intraperitoneal (i.p) injection once daily, for three consecutive days. Mice were euthanised 24 h after the last dose and livers were immediately excised and were used for preparation of microsomes by differential ultracentrifugation. Microsomal pellets were suspended in 0.25 M sucrose and aliquots were stored at -80°C until needed. Total protein concentration in each microsomal sample was determined by the bicinchoninic acid (Thermo Scientific, Rockford, IL) assay using bovine serum albumin as the protein standard.

**1α,25(OH)₂D₃ Biotransformation Assay**

In vitro reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.4), mouse hepatic microsomal protein (0.15 mg/ml) or human hepatic microsomal protein (0.25 mg/ml) or human recombinant CYP3A4 (rCYP3A4) supersomes (15 pmole/ml), NADPH-regenerating system (solution A and B), and varying concentrations of 1α,25(OH)₂D₃ (2–40 μM)
in a final volume of 0.3 ml. A fixed concentration of 20 µM \(1\alpha,25(\text{OH})_2\text{D}_3\) was used to compare the treated samples and this concentration was also used in the inhibition studies. For the substrate depletion study, \(1\alpha,25(\text{OH})_2\text{D}_3\) was incubated with hepatic microsomes from vehicle-, dexamethasone- or prednisone-treated mice at a concentration of 1 µM. After an initial pre-incubation at 37°C for 10 min, the reaction was initiated by adding saturating concentrations of NADPH and was allowed to proceed for 30 min in a shaking water bath. The reaction was terminated with 900 µl of ice-cold acetonitrile. Protopanaxatriol (0.5 µg/ml) was used as an internal standard in the assay. Tubes were vortex-mixed for 1 min and centrifuged at 12,000 rpm for 5 min at 4°C. Supernatant was transferred to a clean eppendorf tube and dried down using a CentriVap centrifugal evaporation system (35°C). Samples were then reconstituted in 100 µl of methanol and an aliquot was injected into the LC/MS.

The assay conditions were optimized to ensure that product formation was linear with respect to amount of protein and incubation time. Appropriate controls, including samples without substrate, microsomes or NADPH, were routinely used in the assay. For the study using human rCYP3A4, control samples contained insect cell microsomes or CYP reductase (plus cytochrome b\(_5\)) microsomes instead of CYP3A4 protein.

A Waters Acquity Ultraperformance liquid chromatography (UPLC) system coupled to a Quattro Premier XE triple quadrupole mass spectrometer (MS) was used for analysis of hydroxy metabolite formation of \(1\alpha,25(\text{OH})_2\text{D}_3\) and substrate depletion. Chromatographic separations of hydroxy metabolites of \(1\alpha,25(\text{OH})_2\text{D}_3\) were carried out on a Waters Acquity™ UPLC BEH C\(_{18}\) column (2.1 X 100 mm, 1.7 µM) at 40°C with a flow rate maintained at 0.3 ml/min and total run time of 20 min. The mobile phase consisted of solvent A (water/2 mM ammonium acetate/0.1% formic acid) and solvent B (methanol/2 mM ammonium acetate/0.1% formic acid) with the
following conditions: 50% methanol (0-0.2 min), followed by a gradient of 50-100% methanol (0.2-15 min), isocratic elution with 100% methanol (12-20 min) and finally 50% methanol (17-20 min). The LC eluant was introduced into the MS and all data were collected in electrospray ionization positive (ESI+) mode with a capillary voltage of 3.2 kV. Source and desolvation temperatures were 120°C and 350°C, respectively, and N₂ gas flow was 1000 L/h. MASSLYNX version 4.1 software (Waters, Milford, MA) was used for data acquisition and Quantlynx analyses. The quantitative determination of 1α,25(OH)₂D₃ metabolites was performed by multiple reaction monitoring of the m/z 450 > 397 transition using cone voltage of 25 V and collision energy of 10 eV.

Data Analysis and Calculation of Enzyme Kinetic Parameters

Enzyme kinetic parameters (Kₘ and Vₘₐₓ) were determined using the SigmaPlot Enzyme Kinetics Module (version 1.3, Systat Software Inc., Richmond, CA). Nonlinear regression analysis was used to study the metabolite formation in response to a range of substrate concentrations. The Michaelis-Menten equation or Hill equation was used to calculate Kₘ and Vₘₐₓ values for the formation of hydroxylated metabolites of 1α,25(OH)₂D₃.

Chemical Inhibition Studies

CYP inhibitors were added into the incubation mixture containing 1α,25(OH)₂D₃ and hepatic microsomes from vehicle-, dexamethasone- or prednisone-treated mice containing an NADPH-regenerating system as described above. The CYP inhibitors and their concentrations were selected on the basis of previous reports and are as follows: ketoconazole (0.1, 1, 5, 10 μM), SKF 525A (1000 μM), furafylline (20 μM), α-naphthoflavone (5 μM), sulfaphenazole (10
µM), quinine (5 µM), diethyldithiocarbamate (25 µM), 8-methoxypsoralen (1 µM) and orphenadrine (200 µM) (Newton et al., 1995; Zhang et al., 2007). Reactions were initiated with NADPH after initial pre-incubation of mouse liver microsomes (MLM) with 1α,25(OH)₂D₃ and the CYP inhibitor for 10 min at 37°C. For the mechanism-based inhibitors (furafylline, 8-methoxypsoralen, orphenadrine, diethyldithiocarbamate and SKF525A) pre-incubation was carried out with all incubation constituents at 37°C for 15 min before the reaction was initiated by addition of 1α,25(OH)₂D₃.

Inhibition Study with Commonly Used Drugs and Determination of IC₅₀ Value

Study drugs: ketoconazole, tamoxifen, docetaxel, paclitaxel, ritonavir, clarithromycin, troleandomycin and cholaramphenicol were co-incubated with HLM and 1α,25(OH)₂D₃ and the reaction was allowed to proceed as described above. Drugs were pre-incubated with HLM and 1α,25(OH)₂D₃ for 10 min at 37°C followed by initiation of the reaction by adding NADPH. Ritonavir, tamoxifen, troleandomycin and clarithromycin (mechanism-based inhibitors) were pre-incubated at 37°C for 20 min with HLM and NADPH and the reaction was initiated by addition of 1α,25(OH)₂D₃. Varying concentrations of ketoconazole (0.05-10 µM), ritonavir (0.05-10 µM), tamoxifen (1-100 µM), docetaxel (0.1-1.24 µM) and clarithromycin (1-100 µM) were used to determine the half maximal inhibitory concentration (IC₅₀) values of these drugs for inhibition of 1α,25(OH)₂D₃ biotransformation in HLM. The CYP3A inhibitory potential of these drugs was evaluated by triazolam hydroxylation assay following co-incubation in HLM and human rCYP3A4 supersomes.
Gel Electrophoresis and Western Blot Analysis

CYP3A protein levels were measured in mouse hepatic microsomal samples by immunoblot analysis. GAPDH protein levels were measured in the same samples as a loading control. Microsomal proteins were resolved by 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were electrophoretically transferred onto nitrocellulose membranes. Membranes were incubated with goat polyclonal anti-mouse CYP3A IgG (1:100 dilution) or rabbit polyclonal anti-GAPDH serum (1:100 dilution) overnight at 4°C, followed by a 2 h incubation with alkaline phosphatase conjugated swine anti-goat IgG or with goat anti-rabbit IgG at a dilution of 1:3000. Immunoreactive CYP3A and GAPDH protein bands were visualized by alkaline phosphatase-based colorimetric detection and densitometric analyses were carried out with GeneTools Software (Syngene Inc., Frederick, MD). Relative CYP3A protein levels in microsomes are expressed as the ratio of the intensity of the CYP3A band to that of the GAPDH band.

Human and Mouse CYP3A Marker Activity Assay

Hydroxylation of triazolam to 1'-hydroxy triazolam and 4-hydroxy triazolam was used as a marker for the activity of mouse and human CYP3A enzymes. In vitro reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.4), mouse hepatic microsomal protein (0.3 mg/ml) or human hepatic microsomal protein (0.5 mg/ml), NADPH-regenerating system, and triazolam (243 µM for mouse microsomes or 50 µM for human microsomes) in a final volume of 0.150 ml incubated for 20 min at 37°C. The reaction was terminated with 450 µl of ice-cold acetonitrile. 1'-hydroxytriazolam-d4 (0.2 µg/ml) was used as an internal standard in the assay. Tubes were vortex-mixed for 1 min and centrifuged at 12,000 rpm for 5 min at 4°C. Supernatant
was transferred to a clean eppendorf tube and an aliquot was directly injected into the LC/MS for quantification of 1’-hydroxy triazolam and 4-hydroxy triazolam. Hydroxy metabolites of triazolam were separated on a Waters Acquity UPLC BEH C18 column (2.1 X 100 mm, 1.7 µM) at 35°C with a flow rate maintained at 0.3 ml/min and total run time of 5 min. The mobile phase consisted of solvent A (water/0.1% formic acid) and solvent B (acetonitrile/0.1% formic acid) with the following conditions: 50% acetonitrile (0-0.2 min), followed by a gradient of 50-100% acetonitrile (0.2-2 min), isocratic elution with 100% acetonitrile (2-3 min) and finally 50% acetonitrile (3-5 min). The LC eluant was introduced into the MS and all data were collected in electrospray ionization positive (ESI+) mode with a capillary voltage of 3 kV. Source and desolvation temperatures were 120°C and 300°C, respectively, and N2 gas flow was 900 L/h. MASSLYNX version 4.1 software (Waters, Milford, MA) was used for data acquisition and Quantlynx analyses. The quantitative determination of 1’-hydroxy triazolam and 4-hydroxy triazolam was performed by multiple reaction monitoring of the m/z 359 > 176 (cone voltage: 40 V and collision energy: 27 eV) and 359 > 314 (cone voltage: 35 V and collision energy: 27 eV) transitions, respectively.

**Statistical Analysis**

Parametric tests were used when the data passed the normality and equal variance test. Nonparametric tests were used when the data failed to pass the normality test and equal variance test (SigmaStat Statistical Software, Version 3.1, SPSS Inc., Chicago, IL, USA). Differences between mean values of two treatment groups were analyzed using the Student’s t-test (parametric) and when there were more than two treatment groups, the data were analyzed by one-way analysis of variance (parametric) or Kruskal-Wallis one-way analysis of variance test.
(nonparametric), followed by Student Newman Keuls multiple comparison test (SigmaStat™ Statistical Software, Version 3.1, SPSS Inc., Chicago, IL, USA). The level of significance was set \textit{a priori} at \( P < 0.05 \). IC\textsubscript{50} values were determined by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA). The IC\textsubscript{50} value data are expressed as means with associated 95\% confidence limits in parenthesis.
Results

Metabolism of 1α,25(OH)2D3 by Human and Mouse Liver Microsomes and Human Recombinant CYP3A4

An LC/MS-based in vitro biotransformation assay was optimized to study the metabolism of 1α,25(OH)2D3. Hydroxy metabolites of 1α,25(OH)2D3 were identified by comparing their retention times and mass to charge ratios (m/z) with those of authentic standards. Authentic metabolite standards of 1α,24R,25-trihydroxyvitamin D3 [1α,24R,25(OH)3D3] and 1α,23S,25-trihydroxyvitamin D3 [1α,23S,25(OH)3D3] were obtained as a kind gift from Dr. Toshiyuki Sakaki (Toyama Prefectural University, Toyama, Japan). Incubation of 1α,25(OH)2D3 with pooled HLM led to formation of four monohydroxy metabolites, M1-M4 (Fig. 1A). In the present experimental conditions, metabolites eluted between 8.5 to 9.7 min. The internal standard and substrate eluted at 8.8 min and 10.9 min, respectively. Metabolite peaks corresponding to 1α,23S,25(OH)3D3 (M1) and 1α,24R,25(OH)3D3 (M2) were eluted at 8.5 min and 8.8 min, respectively (Supplemental Fig. 1). M3 and M4 hydroxy metabolites resolved at 9 min and 9.7 min. From data showing relative abundance of ions, we can suggest that M4 was the major metabolite, whereas M1, M2 and M3 were formed in lesser amounts in HLM incubations. An identical metabolite formation pattern was observed following incubation of 1α,25(OH)2D3 with human rCYP3A4 supersomes. No metabolite formation was detected in reaction mixtures without substrate, NADPH or microsomes.

In contrast, incubation of 1α,25(OH)2D3 with hepatic microsomes from vehicle- or prednisone-treated mice yielded one major metabolite at 8.9 min (M5) in addition to 1α,23S,25(OH)3D3 (M1) and 1α,24R,25(OH)3D3 (M2) as minor metabolites (Fig. 1B). Interestingly, similar to the metabolite profile determined in HLM, dexamethasone-treated mice
yielded four metabolites, M1-M4. However, unlike HLM, M4 was a minor metabolite, whereas, M1, M2 and M3 were the major metabolites formed in the hepatic microsomes prepared from dexamethasone-treated mice.

Effect of Treatment with Glucocorticoids on 1α,25(OH)₂D₃ Metabolism

To investigate the effects of treatment with glucocorticoids, experiments were carried out with hepatic microsomes prepared from male CD-1 mice treated with vehicle, dexamethasone or prednisone. The formation of 1α,23S,25(OH)₃D₃ (M1) and 1α,24R,25(OH)₃D₃ (M2) was significantly stimulated (43- and 17-fold, respectively) in dexamethasone-treated mice compared to vehicle-treated group (Fig. 2A). Metabolites M3 and M4 were detectable only in hepatic microsomes from dexamethasone treated mice but not in vehicle- or prednisone-treated mice (Fig. 2B). In contrast, treatment with prednisone led to very little or no change in the formation of M1 and M2 metabolites (Fig. 2A) and a marginal increase in M5 formation compared to vehicle-treated mice (Fig. 2C). In the substrate depletion study, there was a 70% greater loss of 1α,25(OH)₂D₃ following incubation with hepatic microsomes from dexamethasone-treated mice compared to vehicle-treated mice. In contrast, the amount of 1α,25(OH)₂D₃ remained is same following incubation with hepatic microsomes either from prednisone- or vehicle-treated mice (Fig. 3).

Kinetic Analysis of 1α,25(OH)₂D₃ Metabolism

Kinetic analysis of metabolite formation from 1α,25(OH)₂D₃ was performed in pooled MLM and HLM over a range of substrate concentrations (2-40 µM). The metabolite formation data were fit to either the Michaelis Menten or Hill equation and kinetic parameters of
1α,25(OH)₂D₃ metabolites are shown in Table 1. To compare the metabolic efficiency of microsomes prepared from vehicle-, prednisone- or dexamethasone-treated mice, the rates of formation of the M1 and M2 were examined. The Vmax value for M2 formation was significantly higher in dexamethasone-treated mice and marginally increased in prednisone-treated mice compared to vehicle-treated mice (Table 1). In dexamethasone-treated mice, Km values for M1, M2 and M4 formation are low and comparable indicating that these products are predominantly formed at lower concentrations of 1α,25(OH)₂D₃. M4, the most abundant metabolite formed in HLM, has the lowest Km value (7.6 ± 0.2 µM). This suggests that even at lower concentrations of 1α,25(OH)₂D₃ formation of M4 will be preferred. Due to lack of availability of authentic standards Vmax values for M3, M4 and M5 could not be determined.

**CYP Chemical Inhibition Studies**

The contribution of individual CYP in 1α,25(OH)₂D₃ biotransformation was assessed using chemical inhibitors selectively targeting CYP isoforms. CYP inhibition studies were performed using pooled hepatic microsomes prepared from vehicle-, dexamethasone- or prednisone-treated mice. We used nine inhibitors of CYP enzymes: ketoconazole (CYP3A), SKF 525A (broad spectrum CYP inhibitor), furafylline (CYP1A2), α-naphthoflavone (CYP1A1), sulfaphenazole (CYP2C), quinine (CYP2D), diethylidithiocarbamate (CYP2E), 8-methoxypsoralen (CYP2A) and orphenadrine (CYP2B). The results of the chemical inhibition studies showed that ketoconazole at 1 µM inhibited the formation of hydroxy metabolites of 1α,25(OH)₂D₃ by ~90-100% in MLM (Fig. 4). Comparable inhibition was achieved following incubation with broad spectrum CYP inhibitor SKF 525A. Other CYP inhibitors showed little or no inhibition. Ketoconazole-mediated CYP3A inhibition was observed for the formation of M5
in vehicle- or prednisone-treated mice and for M1, M2 and M3 in dexamethasone-treated mice. The inhibitory effect of ketoconazole on 1α,25(OH)2D3 biotransformation in MLM was dose-dependent over a concentration range of 0.1-10 µM (data not shown).

**Induction of CYP3A Isoforms and Triazolam Hydroxylation Assay**

CYP3A protein levels and marker activity were measured in individual mouse hepatic microsomes. Hepatic CYP3A protein levels were significantly increased following intraperitoneal treatment of adult male CD-1 mice with dexamethasone. An antibody against mouse CYP3A isoforms (CYP3A11, CYP3A16, CYP3A41, CYP3A44 and, CYP3A25) was used to detect two protein bands in the immunoblot. The upper band, which was detected in control and treated samples, was used to determine the relative CYP3A protein level. Relative CYP3A protein levels were increased by approximately 53- and 2-fold in liver microsomes from dexamethasone- and prednisone-treated mice, respectively, compared to the vehicle-treated group (Fig. 5A). Similarly, the formation of 1-hydroxy triazolam and 4-hydroxy triazolam from triazolam, a CYP3A marker activity, was markedly increased in hepatic microsomes from dexamethasone-treated mice, compared to vehicle-treated and prednisone-treated mice (Fig. 5B).

**In Vitro Inhibition of 1α,25(OH)2D3 Catabolism by Drug Interactions**

Pooled HLM were co-incubated with 1α,25(OH)2D3 and the drugs listed below to evaluate their inhibitory potential to cause vitamin D3-drug interactions. Drugs used in the present study were those used as anti-cancer agents (ketoconazole, tamoxifen, docetaxel and paclitaxel), anti-virals (ritonavir) and anti-infectives (clarithromycin, chloramphenicol, troleandomycin). Results from the initial single concentration study suggest that ketoconazole (1
µM), docetaxel (1.24 µM) and ritonavir (10 µM) are the strongest inhibitors of 1α,25(OH)2D3 catabolism showing ~95-100% inhibition (Fig. 6A and 6B). Other drugs used in our study inhibited the formation of hydroxy metabolites by ~40-90%. The inhibitory effect of these drugs was further characterized by determining IC50 values using non-linear regression analysis. A summary of IC50 values for the formation of all the four metabolites of 1α,25(OH)2D3 in HLM are shown in Table 2. Analysis of IC50 values indicate that ritonavir is the most potent inhibitor of M1-M4 metabolite formation, followed by ketoconazole and docetaxel. Similar inhibitory effects of these drugs were also observed on triazolam hydroxylation in HLM (Fig. 6C) and human rCYP3A4 (data not shown).
Discussion

1α,25(OH)₂D₃, the active metabolite and the most potent natural analog of vitamin D₃, is an autocrine and paracrine regulator of multiple physiological functions including maintenance of calcium and phosphate levels, bone mineralization, cell proliferation and differentiation, inflammation and immunity (Holick, 2005). Thus, 1α,25(OH)₂D₃ is a potential candidate for the treatment and prevention of diseases such as rickets/osteomalacia, cancer and immune disorders (e.g. autoimmune diseases, AIDS-related opportunistic infections). Typically, in mammals CYP-mediated oxidative metabolism of 1α,25(OH)₂D₃ is considered as a vital step in its deactivation (Prosser and Jones, 2004). Although CYP24A1-mediated renal metabolism of 1α,25(OH)₂D₃ is well studied, hepatic biotransformation of 1α,25(OH)₂D₃ in humans and rodents is not well characterized. In the present study, we investigated the microsomal biotransformation of 1α,25(OH)₂D₃ in MLM and HLM and in human rCYP3A4 and determined the effects of commonly used drugs on the CYP3A-mediated metabolism of 1α,25(OH)₂D₃.

The results from our study show that 1α,25(OH)₂D₃ was metabolized to four metabolites in HLM and rCYP3A4 supersomes (Fig. 7). In contrast, in untreated MLM there was only one major metabolite unique from the ones found in humans, as was determined by LC/MS. However, the metabolite formation pattern in hepatic microsomes from dexamethasone-treated mice was comparable to that of HLM and rCYP3A4 supersomes. Metabolites M1 and M2 corresponded to 1α,23S,25(OH)₃D₃ and 1α,24R,25(OH)₃D₃ (M2), respectively. Based on the chromatographic and MS properties as well as literature reports, it’s likely that metabolite M3 is 1α,24S,25(OH)₃D₃ and M4 is 1α,23R,25(OH)₃D₃. Gupta et al. (2004, 2005) showed that 24- and 25-hydroxylation of 1α(OH)D₃, a clinically important analog of vitamin D₃, are catalyzed by CYP3A4 in a stereospecific manner. In kidney, CYP24A1-mediated metabolism of
1α,25(OH)2D3 yields both C-23 and C-24 hydroxylated products. From both careful examination of relative retention time and observation of additional multiple reaction monitoring transitions it is clear that M3 and M5 are two different metabolites with same molecular weight. While the chemical identity of the metabolite M5 of 1α,25(OH)2D3 could not be determined, potential candidates include a hydroxylation product of the aliphatic side chain at C-22 or hydroxylation of methyl group at C-21. For example, rCYP3A4 hydroxylates at the C-22 position of ursodeoxycholic acid, a steroidal structure with aliphatic side chain (Bodin et al., 2005).

Relative contributions of individual CYP enzymes to the hepatic biotransformation of 1α,25(OH)2D3 in mice was studied using a panel of selective CYP chemical inhibitors. Ketoconazole, used at low concentrations selective for CYP3A isoforms (Baldwin et al., 1995), inhibited the formation of all the five metabolites in MLM and HLM. It appears that metabolites M1-M4 emerged at the expense of M5 following treatment with dexamethasone. As there are six different CYP3A isoforms (CYP3A11, CYP3A13, CYP3A16, CYP3A25, CYP3A41 and CYP3A44) expressed in MLM, it’s plausible that treatment with dexamethasone led to induction of isoforms, which predominantly catalyze the formation of M1-M4 but not M5. In addition, identical metabolic profiles in rCYP3A4 supersomes and HLM indicate that CYP3A isoforms are primarily responsible for the hepatic microsomal biotransformation of 1α,25(OH)2D3. Previously, it has been shown that mitochondrial CYP24A1 is one of the major enzymes involved in the biotransformation of 1α,25(OH)2D3 in kidney (Prosser and Jones, 2004), however this does not apply to the liver and intestine. CYP3A4 enzyme constitutes approximately 40% and 80% of the total CYP enzymes in human liver and intestine, respectively (Paine et al., 2006), whereas mitochondrial CYP24A1 has low or undetectable hepatic and intestinal expression in humans and rodents (Komuro et al., 1999; Cheng et al., 2003; Xu et al., 2006). In contrast to
CYP3A, mitochondrial CYP24A1 is also less inducible by therapeutic or environmental agents (Zhou et al., 2006) and not many potent inhibitors are known. Thus, based on relative abundance of CYP24A1 and CYP3A4 in human liver and their induction or inhibition potential, it’s unlikely that CYP24A1 has any significant role in the hepatic biotransformation of 1α,25(OH)₂D₃.

Metabolite formation pattern in our study is similar to the one report we found of 1α,25(OH)₂D₃ biotransformation in HLM (Xu et al., 2006), which also concluded that hepatic metabolism of 1α,25(OH)₂D₃ in human liver is catalyzed by CYP3A4. We also found that human microsomal CYP3A4 hydroxylates 1α,25(OH)₂D₃ at C-23 position in the formation of 1α,23S,25(OH)₃D₃ (M1). However, unlike Xu et al. (2006) we identified 1α,24R,25(OH)₃D₃ (M2) as an additional product of 1α,25(OH)₂D₃ biotransformation following incubation with HLM. Rigorous analyses of the chromatograms from the Xu et al. (2006) study suggest that 1α,24R,25(OH)₃D₃ had also been produced in their incubations, albeit at a much lower extent than we observed and appeared as a shouldering peak to the adjacent 1α,24S,25(OH)₃D₃ peak. Possible explanations for the differences observed in the metabolite profiles and proportions could be incubation conditions (e.g. incubation time, amount of protein) and analytical methodologies (e.g. chromatographic and MS conditions). A species-based difference was also identified between CYP3A-mediated hepatic metabolism of 1α,25(OH)₂D₃ in humans and mice. In humans, M4 was the major metabolite, whereas M1, M2, M3 and M5 were the major metabolites in mice. Similarly, CYP24A1-mediated metabolism of 1α,25(OH)₂D₃ has been reported to be species-specific. In humans, both the C-23 and C-24 oxidation pathways are present (Beckman et al., 1996). However, rat and mouse CYP24A1 enzymes show a remarkable preference for C-24 to C-23 hydroxylation (Sakaki et al., 1999; Sakaki et al., 2000). To our
knowledge this is the first study of 1α,25(OH)2D3 biotransformation which identifies the CYP enzymes involved in MLM.

The effects of commonly used medications on the biotransformation of 1α,25(OH)2D3 have not previously been explored experimentally. Dexamethasone and prednisone are both clinically important drugs commonly used in cancer treatment regimens and as anti-inflammatory and immunosuppressant agents (Czock et al., 2005; Beer et al., 2007; Inaba and Pui, 2010). Results from our study have shown that administration of dexamethasone, but not prednisone, can greatly increase breakdown of 1α,25(OH)2D3 by CYP3A isoforms in mice which could potentially lead to diminished biological effects of vitamin D3. Although both dexamethasone and prednisone are harmful to bone health, our results could possibly explain the lower incidences of bone disorders following exposure to prednisone (Inaba and Pui, 2010). In contrast, drugs used to treat cancer (e.g. tamoxifen, taxanes), infectious diseases (e.g. clarithromycin, chloramphenicol) and AIDS (e.g. ritonavir) blocked the hepatic catabolism of the active vitamin D3 metabolite in vitro, potentially leading to additional chemopreventive effects and maintenance of bone health. Ketoconazole, a drug commonly used in fungal infections and in prostate cancer, potently inhibited 1α,25(OH)2D3 biotransformation in HLM. It has been observed previously that following administration of 200 mg ketoconazole in healthy subjects leads to a peak plasma concentration of ~4.5-7.5 µM (Heel et al., 1982; Gupta et al., 1994). Similarly, a single dose of 600 mg ritonavir produced a peak plasma level of 13.1 µg/ml or 18 µM in healthy volunteers (Ouellet et al., 1998). Likewise, anti-infective agents (clarithromycin, chloramphenicol) and anti-cancer agents (taxanes and tamoxifen) were able to inhibit the CYP3A4-mediated metabolism of 1α,25(OH)2D3. Thus, blockade of 1α,25(OH)2D3 catabolism
by these drugs can potentially enhance their therapeutic benefits by exacerbating the inherent anti-cancer and immunomodulatory effects of $1\alpha,25$(OH)$_2$D$_3$ in cancer and AIDS patients.

Dexamethasone induces CYP expression in human and mouse liver via involvement of nuclear receptors such as the pregnane X receptor (PXR) and glucocorticoid receptor (GR) (Rushmore and Kong, 2002). Although prednisone is a GR, but not PXR, agonist, there is no report of prednisone-mediated regulation of CYP3A enzymes. This could explain the differential effects of the two glucocorticoids. Moreover, Hashizume et al. (2008) have shown that PXR-regulated UGT1A isoforms are involved in the conjugation of $1\alpha,25$(OH)$_2$D$_3$ in human liver, which can also alter the $1\alpha,25$(OH)$_2$D$_3$ homeostasis. Thus, it’s critical to recognize that numerous commonly used drugs and herbal supplements are modulators of human and rodent PXR. For example, anti-epileptics (e.g. carbamazepine), anti-neoplastics (e.g. paclitaxel), protease inhibitors (e.g. efavirenz), anti-hypertensives (e.g. nifedipine) and herbal supplements (e.g. St. John’s wort, ginkgo biloba) are known activators of human PXR (Chang and Waxman, 2006; Yu et al., 2011). This suggests that exposure to these agents could contribute to the increased catabolism of $1\alpha,25$(OH)$_2$D$_3$, leading to altered vitamin D homeostasis. Another point of consideration, $1\alpha,25$(OH)$_2$D$_3$ also acts as an endogenous regulator of VDR-mediated CYP3A4 expression and therefore may have a role in its own homeostasis as well as could potentially affect biotransformation of CYP3A substrates.

In summary, this is the first report of hepatic biotransformation of $1\alpha,25$(OH)$_2$D$_3$ in mice. Species differences between metabolite profiles derived from mouse and human liver microsomes were observed in the biotransformation of $1\alpha,25$(OH)$_2$D$_3$. CYP3A isoforms were determined here to have a major role in the hepatic biotransformation of $1\alpha,25$(OH)$_2$D$_3$ in both the species. In addition, this is the first experimental report of stimulation or blockade of
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1α,25(OH)₂D₃ biotransformation in liver by therapeutic drugs. Dexamethasone, but not prednisone, augments CYP3A-mediated catabolism of 1α,25(OH)₂D₃ in mice. In contrast, medications, which are commonly used to treat cancer and AIDS patients, could lead to blockade of active vitamin D₃ catabolism. Overall, therefore, it is reasonable to deduce that treatment regimens used for cancer, AIDS patients and inflammatory diseases could affect vitamin D homeostasis.
Acknowledgments

The authors thank Dr. Toshiyuki Sakaki (Toyama Prefectural University, Toyama, Japan) for generously providing the authentic standards of \(1\alpha,24R,25(OH)_3D_3\) and \(1\alpha,23S,25(OH)_3D_3\).

Authorship Contributions

*Participated in research design:* Deb and Guns

*Conducted experiments:* Deb, Pandey and Adomat

*Contributed analytic tools:* Deb and Adomat

*Performed data analysis:* Deb

*Wrote or contributed to the writing of the manuscript:* Deb and Guns
References


Chang TK and Waxman DJ (2006) Synthetic drugs and natural products as modulators of constitutive androstane receptor (CAR) and pregnane X receptor (PXR). *Drug Metab Rev* **38**: 51-73.


DMD #41681


Footnote

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Figure Legends

FIG. 1. Representative LC/MS chromatogram showing hydroxylated metabolites ((M+NH₄)⁺, m/z 450) of 1α,25(OH)₂D₃ following biotransformation in MLM (0.15 mg/ml) and HLM (0.25 mg/ml) and in human rCYP3A4 supersomes (15 pmole/ml). The chromatograms were obtained from LC/MS analysis of incubations containing hepatic microsomes, 1α,25(OH)₂D₃ and NADPH-regenerating system for 30-min at 37ºC. A, hydroxylated metabolites of 1α,25(OH)₂D₃ in HLM and human recombinant CYP3A4 supersomes. B, hydroxylated metabolites of 1α,25(OH)₂D₃ in hepatic microsomes from vehicle (50% ethanol)-, prednisone- and dexamethasone-treated mice. Hydroxylated metabolites appeared between 8.5 to 9.7 min in the chromatogram. The multiple reaction monitoring transition of m/z 450 > 397 was used to identify the metabolites. The chromatograms labeled as no NADPH are negative control for the corresponding biotransformation experiments.

FIG. 2. Effect of treatment with dexamethasone and prednisone on hepatic biotransformation of 1α,25(OH)₂D₃ in mice. Microsomes were prepared from adult male CD-1 mice treated with dexamethasone (80 mg/kg b.w./day) or prednisone (80 mg/kg b.w./day) or an equivalent volume 50% ethanol (vehicle) for three consecutive days. Metabolite formation was measured after 30-min incubation of 1α,25(OH)₂D₃ (20 µM) with MLM (0.15 mg/ml) at 37ºC. Microsomal incubations were carried out in triplicate. Results are expressed as mean ± SD of three mice. ND, not detected. Differences between each treatment group and the control group were assessed by the Student’s t-test (parametric). When more than two groups were compared, data were analyzed using one-way analysis of variance (parametric), followed by the Student Newman Keuls multiple comparison test. *, Significantly different from the corresponding control.
(vehicle-treated) mice with a $P$ value $< 0.05$. #, Significantly different from the corresponding prednisone-treated mice with a $P$ value $< 0.05$.

**FIG. 3.** Dexamethasone-mediated stimulation of $1\alpha,25$(OH)$_2$D$_3$ depletion in MLM. $1\alpha,25$(OH)$_2$D$_3$ (1 µM) was incubated with hepatic microsomes (0.15 mg/ml) from vehicle (50% ethanol)-, prednisone- and dexamethasone-treated mice. Microsomal incubations were carried out in triplicate. Results are expressed as mean (% $1\alpha,25$(OH)$_2$D$_3$ remaining compared to vehicle) ± SD of three mice. Differences between treatment groups were compared using one-way analysis of variance (parametric), followed by the Student Newman Keuls multiple comparison test. *, Significantly different from the control (vehicle-treated) mice with a $P$ value $< 0.05$. #, Significantly different from the prednisone-treated mice with a $P$ value $< 0.05$.

**FIG. 4.** Effect of chemical inhibitors on biotransformation of $1\alpha,25$(OH)$_2$D$_3$ in pooled hepatic microsomes from (A) vehicle-, (B) dexamethasone- (C) and prednisone-treated mice. Metabolite formation was measured after preincubation of individual inhibitors with microsomes followed by 30-min incubation of $1\alpha,25$(OH)$_2$D$_3$ (20 µM) at 37ºC as described in the Methods section. Results are expressed as mean (% of control activity) ± SD of three different incubations. Control activity was measured in the presence of vehicle (methanol) without any inhibitor. Differences between treatment groups were compared using one-way analysis of variance (parametric), followed by the Student Newman Keuls multiple comparison test. *, Significantly different from the microsomes incubated with methanol (vehicle control) with a $P$ value $< 0.05$. #, Significantly different from the prednisone-treated mice with a $P$ value $< 0.05$. 

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FIG. 5. Effect of treatment with dexamethasone and prednisone on (A) CYP3A protein levels and (B) triazolam hydroxylation, a CYP3A marker activity. For panel A, hepatic microsomes from vehicle-, dexamethasone- and prednisone-treated mice were applied to the gel at 100, 10 and 25 μg microsomal protein per lane, respectively. Lanes 1-3, 4-6 and 7-9 correspond to the same treatments (left to right) as on the histogram. Blots were incubated with goat polyclonal anti-mouse CYP3A IgG (1:100 dilution) or rabbit polyclonal anti-GAPDH serum (1:100 dilution), as described in the Methods section. Relative CYP3A protein levels were determined by densitometric analysis of the immunostained bands and are expressed as a ratio of the intensity of the CYP3A band to that of the GAPDH band. For panel B, hydroxylation of triazolam was measured in the mouse hepatic microsomes. Hydroxylated metabolites of triazolam (1’-hydroxy triazolam and 4- hydroxy triazolam) were quantified using authentic metabolite standards in the LC/MS study. Results are expressed as mean ± SD of three mice. Differences between treatment groups were assessed by one-way analysis of variance (parametric) or by Kruskal-Wallis one-way analysis of variance test (nonparametric) followed by the Student Newman Keuls multiple comparison test. *, Significantly different from the corresponding control (vehicle-treated) mice with a P value < 0.05. #, Significantly different from the corresponding prednisone-treated mice with a P value < 0.05.

FIG. 6. Blockade of 1α,25(OH)2D3 catabolism in HLM in vitro by commonly used drugs. Metabolite formation was measured after preincubation of individual drugs with hepatic microsomes (0.25 mg/ml) followed by 30-min incubation of 1α,25(OH)2D3 (20 μM) at 37°C as described in the Methods section. Results are expressed as mean (activity) ± SD of three different incubations. Control activity was measured in the presence of vehicle (methanol).
without any drug. Differences between treatment groups were compared using one-way analysis of variance (parametric), followed by the Student Newman Keuls multiple comparison test. *, Significantly different from the microsomes incubated with methanol (vehicle control) with a $P$ value < 0.05.

**FIG. 7.** Schematic representation of biotransformation of $1\alpha,25$(OH)$_2$D$_3$, the active vitamin D$_3$, by human and mouse liver. $1\alpha,25$(OH)$_2$D$_3$ is converted to its hydroxylated metabolites, which are inactive, by hepatic CYP3A enzymes. Modulators of CYP3A isoforms can play a vital role in maintaining the active form of vitamin D$_3$. This summary has been compiled from the findings of our study and from Xu et al. (2006).
### TABLE 1: Kinetic parameters for the formation of hydroxy metabolites of 1α,25-(OH)₂D₃ in MLM, HLM and human rCYP3A4 supersomes. Values represent the mean ± standard error of triplicate determinations.

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
<th>M5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
</tr>
<tr>
<td>Control MLM &lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>&lt;LOQ&lt;sup&gt;a&lt;/sup&gt;</td>
<td>&lt;LOQ</td>
<td>2.3±0.2</td>
<td>4.2±0.6</td>
<td>NA&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Dex MLM &lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>1037.2±40.1</td>
<td>6.6±0.3</td>
<td>222.9±8.0&lt;sup&gt;#&lt;/sup&gt;</td>
<td>6.0±0.3&lt;sup&gt;*&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>Pred MLM &lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>3.6±0.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>8.5±1.7&lt;sup&gt;*&lt;/sup&gt;</td>
<td>NA</td>
</tr>
<tr>
<td>HLM &lt;sup&gt;c,d&lt;/sup&gt;</td>
<td>19.1±0.6</td>
<td>12.4±0.5</td>
<td>9.3±0.2</td>
<td>8.9±0.3</td>
<td>NA</td>
</tr>
<tr>
<td>rCYP3A4 &lt;sup&gt;d,e&lt;/sup&gt;</td>
<td>15.2±0.6</td>
<td>7.1±0.3</td>
<td>8.8±0.4</td>
<td>5.9±0.3</td>
<td>NA</td>
</tr>
</tbody>
</table>

<sup>a</sup> Below limit of quantitation (LOQ)
<sup>b</sup> NA- Not applicable
<sup>c</sup> V<sub>max</sub> is expressed as picomoles per minute per milligram of protein
<sup>d</sup> K<sub>m</sub> is expressed in µM
<sup>e</sup> V<sub>max</sub> is expressed as picomoles per minute per micromole of protein

<sup>*</sup>, Significantly different from the corresponding control (vehicle-treated) mice with a P value < 0.05
<sup>#</sup>, Significantly different from the corresponding prednisone-treated mice with a P value < 0.05
TABLE 2: Estimated IC$_{50}$ (µM) values of commonly used drugs for hydroxylation of 1α,25-(OH)$_2$D$_3$ in HLM.

<table>
<thead>
<tr>
<th></th>
<th>M1</th>
<th>M2</th>
<th>M3</th>
<th>M4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ritonavir</td>
<td>0.013 (0.001-0.154)</td>
<td>0.028 (0.003-0.227)</td>
<td>0.008 (0.000-0.113)</td>
<td>0.016 (0.002-0.151)</td>
</tr>
<tr>
<td>Ketoconazole</td>
<td>0.040 (0.024-0.065)</td>
<td>0.042 (0.027-0.067)</td>
<td>0.565 (0.162-1.971)</td>
<td>0.032 (0.021-0.050)</td>
</tr>
<tr>
<td>Clarithromycin</td>
<td>≥30</td>
<td>≥30</td>
<td>≥30</td>
<td>≥30</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>≥40</td>
<td>≥40</td>
<td>≥40</td>
<td>≥40</td>
</tr>
<tr>
<td>Docetaxel</td>
<td>0.038 (0.007-0.203)</td>
<td>0.077 (0.018-0.338)</td>
<td>0.012</td>
<td>0.038 (0.012-0.120)</td>
</tr>
</tbody>
</table>

a Values represent means (95% confidence interval) of triplicate determinations.
FIG. 3

![Bar chart showing the percentage of 1α,25-(OH)₂-D₃ remaining for different treatments.](chart.png)

- **Vehicle**: 100%
- **Dexamethasone**: 25%
- **Prednisone**: 100%

* and # symbols indicate statistical significance.

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FIG. 4C

- **Vehicle**
- **1 μM Ketoconazole**
- **1000 μM SKF525A**
- **20 μM Furofylline**
- **5 μM α-Naphthoflavone**
- **10 μM Sulfaphenazole**
- **5 μM Quinine**
- **25 μM Diethyldithiocarbamate**
- **1 μM 8-Methoxypsoralen**
- **200 μM Orphenadrine**

**M5 formation (% of control activity)**

* *
FIG. 5A

Relative CYP3A protein level

Vehicle
Dexamethasone
Prednisone

FIG. 5B

pmol/min/mg protein

Vehicle
Dexamethasone
Prednisone

1-OH TRZ
4-OH TRZ
FIG. 6C

The figure shows the effect of various compounds on the metabolism of 1-OH TRZ and 4-OH TRZ. The x-axis represents different concentrations of compounds such as 1 μM Ketoconazole, 100 μM Tamoxifen, 1.24 μM Docetaxel, 10 μM Paclitaxel, 10 μM Ritonavir, 100 μM Clarithromycin, 100 μM Troleandomycin, and 100 μM Chloramphenicol. The y-axis represents the metabolism rate in pmol/min/mg protein.

- **1-OH TRZ**: The bars indicate the metabolism rate for 1-OH TRZ.
- **4-OH TRZ**: The filled bars indicate the metabolism rate for 4-OH TRZ.

Significance is indicated by asterisks (*) above the bars, indicating a statistically significant difference.
FIG. 7

1α,23S,25(OH)3D3 (M1)
1α,23R,25(OH)3D3 (M4)
1α,24S,25(OH)3D3 (M3)
1α,24R,25(OH)3D3 (M2)
M5 (C-22 hydroxylation or C-21 methyl hydroxylation)?