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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ABSTRACT:

The biological activities of vitamin D3 are exerted through the dihydroxy metabolite of vitamin D3 [1α,25(OH)2D3]. Hepatic biotransformation of 1α,25(OH)2D3 by cytochrome P450 (P450) 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 who received dexamethasone. To compare the effects of clinically important glucocorticoids with hepatic biotransformation of 1α,25(OH)2D3, adult male CD-1 mice were given intraperitoneal injections of either vehicle (50% ethanol), dexamethasone (80 mg/kg per day), or prednisone (80 mg/kg per day) for three consecutive days. 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 the 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 from that of human liver microsomes (HLM). Selective P450 chemical inhibitors have demonstrated that CYP3A isoforms are responsible for the microsomal biotransformation of 1α,25(OH)2D3 in MLM. Coincubation of 1α,25(OH)2D3 with commonly used drugs led to approximately 60 to 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 the clinical importance of glucocorticoids differentially modulates 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 1α,25-dihydroxyvitamin D3 [1α,25(OH)2D3], which acts primarily by binding to the vitamin D receptor (VDR) (Deeb et al., 2007). 1α,25(OH)2D3 shows antiproliferative, prodifferentiation, 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 antitumor role of 1α,25(OH)2D3. For example, cotreatment of advanced prostate cancer patients with taxanes (e.g., paclitaxel, docetaxel) and 1α,25(OH)2D3 was shown to potentiate cytotoxic effects and/or increase the survival rates compared with treatment with only taxanes or placebo (Muindi et al., 2002; Beer et al., 2007). In addition, 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 (P450) enzymes in mitochondria (e.g., CYP27A1) and microsomes (e.g., CYP2J3 and CYP2R1) are capable of hydroxylating 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 1α-hydroxylation of 25(OH)D$_3$ to form 1α,25(OH)$_2$D$_3$, which is a small lipophilic molecule with superior cellular penetration and is the most potent endogenous agonist of VDR (Ingraham et al., 2008). Subsequently, 1α,25(OH)$_2$D$_3$ 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 antiepileptics (e.g., carbamazepine and phenytoin) and protease inhibitors (e.g., efavirenz), experience lower vitamin D$_3$ levels, altered calcium metabolism, and impaired bone health (Verrotti et al., 2010). Likewise, vitamin D deficiency-likeative effects on vitamin D$_3$ status and bone health.

Vitamin D is also known to influence its circulating serum and tissue levels. Xu et al. (2006) reported that CYP3A4 mediates 24- and 25-hydroxylation activity of 1α,25(OH)$_2$D$_3$. In addition, 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.

1α,25(OH)$_2$D$_3$ works as a paracrine and as an autocrine agent via VDR-mediated mechanisms (Morris and Anderson, 2010). Thus, catalysis of 1α,25(OH)$_2$D$_3$ 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 after 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)D$_3$, but not vitamin D$_3$, in human liver microsomes (HLM). Hepatic biotransformation of 1α,25(OH)$_2$D$_3$ by P450 enzymes may influence its circulating serum and tissue levels. Xu et al. (2006) reported on the hepatic metabolism of 1α,25(OH)$_2$D$_3$ in humans; however, there is no report of 1α,25(OH)$_2$D$_3$ biotransformation in mice, despite the fact that it is a widely used animal model in vitamin D research. Our study investigates the comparative biotransformation of 1α,25(OH)$_2$D$_3$ to its inactive hydroxy metabolites in mouse liver microsomes (MLM) and HLM. 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)$_2$D$_3$.

Materials and Methods

Chemicals. 1α,25(OH)$_2$D$_3$, 25(OH)D$_3$, dexamethasone sodium, predni-
sone, α-naphthoflavone, furafylline, sulfaphenazole, 8-methoxypsoralen, or-
phenadrine, quinine, 2-diethylaminoloxy 2,2-diphenylvalerate hydrochloride
(SKF 525A), sodium diethyldithiocarbamate, doxetaxel, paclitaxel, chloram-
phenicol, 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 cytchrome b$_5$ or reductase), insect cells coexpressed with P450 reductase and cytochrome b$_5$, and baculo-
virus insect cell microsomes containing human CYP3A4 enzyme coexpressed
with P450 reductase and human cytchrome b$_5$ (BD Supersomes Enzymes) were purchased from BD Biosciences (Oakville, ON, Canada). 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 (Waltham, MA). Goat polyclonal anti-mouse CYP3A IgG and rabbit polyclonal anti-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 Laboratories (Burlington, ON, Canada) and Jackson ImmunoResearch Laboratories Inc. (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 20–23°C and had a 12-h photoperiod. Mice were given 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 b.wt. or an equivalent volume of vehicle (three mice per group) by intraperitoneal injection once a day, for three consecutive days. Mice were euthanized 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 Fisher Scientific, Waltham, MA) assay using bovine serum albumin as the protein standard.

1α,25(OH)$_2$D$_3$ Biotransformation Assay. In vitro reaction mixtures contained 100 mM potassium phosphate buffer (pH 7.4), mouse hepatic micro-
somal protein (0.15 mg/ml) or human hepatic microsomal protein (0.25 mg/ ml), or human recombinant CYP3A4 (rCYP3A4) Supersomes (15 pmol/ml),
NADPH-regenerating system (solution A and B), and varying concentrations
of 1α,25(OH)$_2$D$_3$ (2–40 nM) in a final volume of 0.3 ml. A fixed concentra-
tion of 20 μM 1α,25(OH)$_2$D$_3$ was used to compare the treated samples, and this concentration was also used in the inhibition studies. For the substrate depletion study, 1α,25(OH)$_2$D$_3$ was incubated with hepatic microsomes from vehicle-, dexamethasone-, or prednisone-treated mice at a concentration of 1 μM. After an initial preincubation 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, a clean Eppendorf tube and dried down using a CentriVap centrifugal evapora-
tion system (35°C). Samples were then reconstituted in 100 μl of methanol, and an aliquot was injected into the liquid chromatography/mass spectrometry
(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 P450 reductase (plus cytochrome b$_5$) microsomes instead of CYP3A4 protein.

A Waters Acuity ultrasphere liquid chromatography (UPLC) system (Waters, Milford, MA) coupled to a Quattro Premier XE (Waters) triple quadrupole mass spectrometer (MS) was used for analysis of hydroxy metabolite formation of 1α,25(OH)$_2$D$_3$ and substrate depletion. Chromatographic separations of hydroxy metabolites of 1α,25(OH)$_2$D$_3$ were performed on a Waters Acuity UPLC BEH C18 column (2.1 × 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 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) 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., San Jose, 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. P450 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 under Chemicals. The P450 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 preincubation of MLM with 1α,25(OH)₂D₃ and the P450 inhibitor for 10 min at 37°C. For the mechanism-based inhibitors (furafylline, 8-methoxypsoralen, orphenadrine, diethyldithiocarbamate, and SKF 525A), preincubulation was performed 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. Ketoconazole, tamoxifen, doxetaxel, paclitaxel, ritonavir, clarithromycin, troleandomycin, and chloramphenicol were coincubated with HLM and 1α,25(OH)₂D₃, and the reaction was allowed to proceed as described under 1α,25(OH)₂D₃ Biotransformation Assay. Drugs were preincubated with HLM and 1α,25(OH)₂D₃ for 10 min at 37°C followed by initiation of the reaction by adding NADPH. Ritonavir, tamoxifen, clarithromycin, and troleandomycin (mechanism-based inhibitors) were preincubated 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), doxetaxel (0.1–1.24 μM), and clarithromycin (1–100 μM) were used to determine the IC₅₀ values for 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 after coincubation in HLM and human cYP3A4 Supernatants.

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% SDS-polyacrylamide gel electrophoresis and were electrophoretically transferred onto nitrocellulose membranes. Membranes were incubated with goat polyclonal antibody to CYP3A1 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 performed 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 acetoniitrile. 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 × 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 (acetoniitrile/0.1% formic acid) with the following conditions: 50% acetoniitrile (0–0.2 min), followed by a gradient of 50 to 100% acetoniitrile (0.2–2 min), isocratic elution with 100% acetoniitrile (2–3 min), and finally 50% acetoniitrile (3–5 min). The LC eluant was introduced into the MS, and all data were collected in electrospray ionization positive 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 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; collision energy, 27 eV) and 359 > 314 (cone voltage, 35 V; 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). 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). The level of significance was set a priori at P < 0.05. IC₅₀ values were determined by nonlinear regression analysis using GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA). The IC₅₀ values are data 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 those of authentic standards. Authentic metabolite standards of 1α,24,25(OH)₃D₃ [1α,24R,25(OH)₂D₃] and 1α,23S,25(OH)₂D₃ were a kind gift from Dr. Toshiyuki Sakai (Toyama Prefectural University, Toyama, Japan). Incubation of 1α,25(OH)₂D₃ with pooled HLM led to formation of four monohydroxy metabolites, M1 to 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 and 10.9 min, respectively. Metabolite peaks corresponding to 1α,23S,25(OH)₂D₃ (M1) and 1α,24R,25(OH)₂D₃ (M2) were eluted at 8.5 and 8.8 min, respectively (Supplemental Fig. 1). M3 and M4 hydroxy metabolites resolved at 9 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 after 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)₂D₃ (M1) and 1α,24R,25(OH)₂D₃ (M2) as minor metabolites (Fig. 1B). Similar to
the metabolite profile determined in HLM, dexamethasone-treated mice yielded four metabolites, M1 to 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 performed 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 with the 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 with vehicle-treated mice (Fig. 2C). In the substrate-depletion study, there was a 70% greater loss of 1α,25(OH)₂D₃ after incubation with hepatic microsomes from dexamethasone-treated mice compared with vehicle-treated mice. In contrast, the amount of 1α,25(OH)₂D₃ remained the same after incubation with hepatic microsomes 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 with vehicle-treated mice (Table 1). In dexamethasone-treated mice, Kₘ values for M1, M2, and M4 formation are low and comparable, which indicates 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.

**P450 Chemical Inhibition Studies.** The contribution of individual P450 in 1α,25(OH)₂D₃ biotransformation was assessed using chemical inhibitors that selectively targeted P450 isoforms. P450 inhibition studies were performed using pooled hepatic microsomes prepared
from vehicle-, dexamethasone-, or prednisone-treated mice. We used nine inhibitors of P450 enzymes: ketoconazole (CYP3A), SKF 525A (broad spectrum P450 inhibitor), furafylline (CYP1A2), α-naphthoflavone (CYP1A1), sulfaphenazole (CYP2C), quinine (CYP2D), diethyldithiocarbamate (CYP2E), 8-methoxypsoralen (CYP2A), and orphenadrine (CYP2B). The results of the chemical inhibition studies showed that ketoconazole at 1/100 M inhibited the formation of hydroxy metabolites of 1α,25(OH)2D3 by 90 to 100% in MLM (Fig. 4). Comparable inhibition was achieved after incubation with broad-spectrum P450 inhibitor SKF 525A. Other P450 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 to 10 μM (data not shown).

Inhibition 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 after 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 with the vehicle-treated group (Fig. 5A). Likewise, the formation of 1α-hydroxy triazolam and 4-hydroxy triazolam from triazolam, a CYP3A marker activity, was markedly increased in hepatic microsomes from dexamethasone-treated mice, compared with vehicle-treated and prednisone-treated mice (Fig. 5B).

In Vitro Inhibition of 1α,25(OH)2D3 Catabolism by Drug Interactions. Pooled HLM were coincubated 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 anticancer agents (ketoconazole, tamoxifen, docetaxel, and paclitaxel), antivirals (ritonavir), and anti-infectives (clarithromycin, chloramphenicol, troleandomycin). Results from the initial single concentration study suggest that ketoconazole (1/100 M), docetaxel (1.24 μM), and ritonavir (10 μM) are the strongest inhibitors of 1α,25(OH)2D3 catabolism, showing 95 to 100% inhibition (Fig. 6, A and B). Other drugs used in our study inhibited the formation of hydroxy metabolites by 40 to 90%. The inhibitory effect of these drugs was further characterized by determining IC50 values using nonlinear regression analysis. A summary of IC50 values for the formation of all four metabolites of 1α,25(OH)2D3 in HLM are shown in Table 2. Analysis of IC50 values indicates that ritonavir is the most potent inhibitor of M1 to 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)_{2}D_{3}, the active metabolite and the most potent natural analog of vitamin D_{3}, is an autocrine and paracrine regulator of multiple physiological functions, which include maintenance of calcium and phosphate levels, bone mineralization, cell proliferation and differentiation, inflammation, and immunity (Holick, 2005). Thus, 1α,25(OH)_{2}D_{3} 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, P450-mediated oxidative metabolism of 1α,25(OH)_{2}D_{3} is considered as a vital step in its deactivation (Prosser and Jones, 2004). Although CYP24A1-mediated renal metabolism of 1α,25(OH)_{2}D_{3} is well studied, hepatic biotransformation of 1α,25(OH)_{2}D_{3} in humans and rodents is not well characterized. In the present study, we investigated the microsomal biotransformation of 1α,25(OH)_{2}D_{3} in MLM and HLM and in human rCYP3A4, and we determined the effects of commonly used drugs on the CYP3A-mediated metabolism of 1α,25(OH)_{2}D_{3}.

The results from our study show that 1α,25(OH)_{2}D_{3} was metabolized to four metabolites in HLM and rCYP3A4 Supersomes (Fig. 7).
position of ursodeoxycholic acid, a steroidal structure with aliphatic group at C-21. For example, rCYP3A4 hydroxylates at the C-22 position of the aliphatic side chain at C-22 or hydroxylation of methyl group at C-22. The possibility that neither metabolite could be determined, potential candidates include a hydroxylation product of the aliphatic side chain at C-22 or hydroxylation of methyl group at C-22. For example, rCYP3A4 hydroxylates at the C-22 position of ursodeoxycholic acid, a steroidal structure with aliphatic group at C-21. For example, rCYP3A4 hydroxylates at the C-22 position of the aliphatic side chain at C-22 or hydroxylation of methyl group at C-22. From both careful examination of relative retention time and observation of additional multiple reaction monitoring transitions, it is clear that M3 and M5 are unique from the ones found in humans, as was determined by LC/MS.

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 with that of HLM and rCYP3A4 Supersomes. Metabolites M1 and M2 corresponded toamin D3, are catalyzed by CYP3A4 in a stereospecific manner. 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 is 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 confirmed that hepatic metabolism of 1α,25(OH)2D3 yielded 25-hydroxylation 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 P450 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 is unlikely that CYP24A1 has any significant role in the hepatic biotransformation of 1α,25(OH)2D3.
FIG. 4. Effect of chemical inhibitors on biotransformation of 1α,25(OH)2D3 in pooled hepatic microsomes from vehicle- (A), dexamethasone- (B), and prednisone-treated (C) mice. Metabolite formation was measured after pre-incubation of individual inhibitors with microsomes followed by 30-min incubation of 1α,25(OH)2D3 (20 μM) at 37°C, as described under Materials and Methods. Results are expressed as mean (% of control activity) ± S.D. 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 P < 0.05.
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 after incubation with HLM. Rigorous analyses of the chromatograms from the Xu et al. (2006) study suggest that 1α,24S,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. Likewise, 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, 2000). To our knowledge, this is the first

![Graph showing effect of treatment with dexamethasone and prednisone on CYP3A protein levels and triazolam hydroxylation.](attachment:image.png)
FIG. 6. Blockade of 1α,25(OH)2D3 catabolism in HLM in vitro by commonly used drugs. Metabolite formation was measured (A–C) 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 under Materials and Methods. Results are expressed as mean (activity) ± S.D. 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 \( P < 0.05 \).
The effects of commonly used medications on the biotransformation of \(1\alpha,25\text{(OH)}_2\text{D}_3\) have not previously been explored experimentally. Dexamethasone and prednisone are both clinically important 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 \(1\alpha,25\text{(OH)}_2\text{D}_3\) by CYP3A isoforms in mice, which could potentially lead to diminished biological effects of vitamin D\(_3\). Although both dexamethasone and prednisone are harmful to bone health, our results could possibly explain the lower incidences of bone disorders after exposure to prednisone (Inaba and Pui, 2010). In contrast, drugs used to treat cancer (e.g., taxanes, infectious diseases (e.g., clarithromycin, chloramphenicol), and AIDS (e.g., ritonavir) blocked the hepatic catabolism of the active vitamin D\(_3\) metabolite in vitro, potentially leading to additional che-mopreventive effects and maintenance of bone health. Ketoconazole, a drug commonly used in fungal infections and in prostate cancer, potently inhibited \(1\alpha,25\text{(OH)}_2\text{D}_3\) biotransformation in HLM. It has been previously observed that oral administration of 200 mg of ketoconazole in healthy subjects leads to a peak plasma concentration of 4.5 to 7.5 \(\mu\text{M}\) (Heel et al., 1982; Gupta et al., 1994). Likewise, a single dose of ritonavir (600 mg) produced a peak plasma level of 13.1 \(\mu\text{g/ml}\) or 18 \(\mu\text{M}\) in healthy volunteers (Ouellet et al., 1998). Likewise, anti-infective agents (clarithromycin, chloramphenicol) and anticancer agents (taxanes and tamoxifen) were able to inhibit the CYP3A4-mediated metabolism of \(1\alpha,25\text{(OH)}_2\text{D}_3\). Thus, blockade of \(1\alpha,25\text{(OH)}_2\text{D}_3\) catabolism by these drugs can potentially enhance their therapeutic benefits by exacerbating the inherent anticancer and immunomodulatory effects of \(1\alpha,25\text{(OH)}_2\text{D}_3\) in patients who have cancer or AIDS.

Dexamethasone induces P450 expression in human and mouse liver via involvement of nuclear receptors such as the pregnane X receptor (PXR) and glucocorticoid receptor (Rushmore and Kong, 2002). Although prednisone is a glucocorticoid receptor (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 \(1\alpha,25\text{(OH)}_2\text{D}_3\) in human liver, which can also alter the \(1\alpha,25\text{(OH)}_2\text{D}_3\) metabolism.
homeostasis. Thus, it is critical to recognize that numerous commonly human and rodent PXR. For example, antiepileptics (e.g., carbamazepine), anti-neoplastics (e.g., paclitaxel), protease inhibitors (e.g., efavirenz), antihypertensives (e.g., nifedipine), and herbal supplements (e.g., St. John’s wort, gingko 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 1α,25(OH)2D3, which leads to altered vitamin D homeostasis. Another point of consideration is that 1α,25(OH)2D, also acts as an endogenous regulator of VDR-mediated CYP3A4 expression and therefore may have a role in its own homeostasis and could potentially affect biotransformation of CYP3A substrates.

In summary, this is the first report of hepatic biotransformation of 1α,25(OH)2D3 in mice. Species differences between metabolite profiles derived from mouse and human liver microsomes were observed in the biotransformation of 1α,25(OH)2D3. In the present study, CYP3A isoforms were determined to have a major role in the hepatic biotransformation of 1α,25(OH)2D3 in both species. In addition, this is the first experimental report of stimulation or blockade of 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 patients with cancer, AIDS, and inflammatory diseases could affect vitamin D homeostasis.

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Authorship Contributions
Conducted experiments: Deb and Guns.
Participated in research design: Deb and Adomat.
Contributed new reagents or analytic tools: Deb and Adomat.
Performed data analysis: Deb.
Wrote or contributed to the writing of the manuscript: Deb and Guns.

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