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Selective induction of intestinal CYP3A23 by 1α, 25-dihydroxyvitamin D_3 in rats

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Abbreviations:
CYP, cytochrome P450
VDR, vitamin D receptor
GAPDH, glyceraldehyde-3-phosphate dehydrogenase
$1,25(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3
$1α\text{-}(OH)D_3$, 1α-hydroxyvitamin D_3
i.p., intraperitoneal
PCR, polymerase chain reaction
NS, not significant

ABSTRACT

Enhancement of CYP3A transcription in both the small intestine and liver of the mouse by activation of a VDR signaling pathway was shown recently by Makishima et al. (Science, 2002). However, in humans and rats, hepatic VDR content is much lower than that found in small intestine, suggesting the possibility of tissue-selective responses to $1,25(OH)_2D_3$. The purpose of this study was to determine the effect of $1,25(OH)_2D_3$ on intestinal and hepatic CYP3A expression in the rat. We found that an acute intraperitoneal treatment (every 48 h) in adult male rats with $1,25(OH)_2D_3$ induced CYP3A transcription selectively in small intestine, but not in liver. At a dose of 100 ng, there was a 6.6-fold increase in intestinal CYP3A23 mRNA after the third treatment ($p < 0.05$). There were concordant effects of $1,25(OH)_2D_3$ treatment on intestinal CYP3A23 protein levels; 2.2-fold ($p < 0.05$), 3.5-fold ($p < 0.05$) and 4.8-fold ($p < 0.01$) increase following 1–3 doses of 100 ng $1,25(OH)_2D_3$, respectively. In contrast, there was no significant change of CYP3A23 protein content in liver at the $1,25(OH)_2D_3$ doses tested. In support of these findings, there was a 366-fold and 77-fold higher level of VDR mRNA expression in the respective rat and human jejunal mucosa, compared to the liver. These data suggest that the human liver will be less sensitive than the intestine to the transcriptional effects of $1,25(OH)_2D_3$ and that this regulatory pathway may contribute to inter-individual variability in constitutive intestinal CYP3A4 expression.

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1. Introduction

Members of the cytochrome P450 (CYP) family of hemoproteins catalyze the metabolism of structurally diverse xenobiotics and endogenous substances [1]. To date, the published literature indicates that the CYP3A subfamily is the most versatile catalyst of P450-dependent drug metabolism in humans. The major adult CYP3A enzyme, CYP3A4, is found predominantly in the liver and the mucosal epithelium (enterocytes) of the small intestine [2,3], where it can profoundly affect the bioavailability and efficacy of orally administered drugs [4,5]. One of the most poorly understood aspects of CYP3A-dependent drug metabolism is the source of its wide inter-individual variability in both the liver and intestine; greater than 10-fold differences in constitutive CYP3A4 content and catalytic activity have been observed [2,3]. Variation in CYP3A4 activity is particularly important for substrates with a narrow therapeutic index, such as cancer chemotherapeutics and the immunosuppressive drugs cyclosporine A and tacrolimus, as it can result in clinically significant differences in drug response and toxicity risk.

Unlike other important and polymorphic human P450 enzymes (CYP2C9, CYP2C19, CYP2D6 and CYP3A5) the large variability in constitutive CYP3A4 expression and function in vivo has not yet been attributed directly to a specific genetic variant [6] and may well be the result of inter-individual differences in hepatic and intestinal *CYP3A4* gene regulation (mediated by either secondary genetic or environmental factors). Indeed, hepatic and intestinal CYP3A4 can be induced by the exposure of humans to a number of structurally diverse agents, including glucocorticoids (e.g. dexamethasone), carbamazepine, phenobarbital, rifampin and hyperforin [7], an effect mediated by the nuclear hormone receptor, hPXR (pregnane X receptor) or SXR (steroid and xenobiotic receptor) [7–10]. However, a physiologically relevant ligand for human PXR/SXR that could mediate constitutive CYP3A4 expression has not yet been identified.

Another nuclear hormone receptor, the vitamin D receptor (VDR), found abundantly in the rat and human intestine [11–13], has been shown to affect CYP3A4 transcription in vitro in response to treatment with its natural ligand 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) [14–17]. After binding $1,25(OH)_2D_3$, VDR forms a heterodimer with the retinoid X receptor (RXR) that complexes with and activates vitamin D receptor-responsive elements (VDREs) present in the regulatory region of target genes [18]. In the classical vitamin D-responsive organs, including the intestine, bone, kidney, and parathyroid gland, vitamin D_3-activated VDR plays a central role in the regulation of calcium and phosphate homeostasis, bone mineralization and resorption, inhibition of cell growth, and parathyroid hormone synthesis [19]. In the case of *CYP3A4*, VDR mediates its transcription through cross-talk with the proximal hPXR response element (PXRE) (i.e. ER-6) [15]. In addition, VDR most likely activates a distal VDRE (i.e. DR-3), contributing to a maximum transcriptional enhancement.

Although it was shown recently that VDR ligands can also induce CYP3A in hepatocytes of humans and mice [20,21], in addition to enterocytes, the preferential expression of VDR in the human intestine [13] suggests that any *CYP3A* regulatory effects mediated by the hormone in vivo may be tissue-selective. Thus, in this study, our objective was to test whether an acute treatment with low-dose $1,25(OH)_2D_3$ selectively induces the VDR target gene *CYP3A23* in small intestine and not liver of the rat, which also exhibits marked tissue-selective VDR expression.

2. Materials and methods

2.1. Materials

Testosterone, trypsin inhibitor (from soybean), phenylmethylsulfonyl fluoride (PMSF), NADPH and alkaline phosphatase-conjugated secondary antibody were purchased from Sigma–Aldrich (St. Louis, MO). Protease inhibitor cocktail tablets were obtained from Roche Applied Science (Indianapolis, IN). 6β-Hydroxytestosterone and 11α-progesterone was obtained from Steraloids (Newport, RI) and $1,25(OH)_2D_3$ was obtained from Calbiochem (La Jolla, CA). A polyclonal anti-rat CYP3A23 antibody was purchased from Chemicon International (Temecula, CA). Rat CYP3A23 protein was a gift from Dr. Erin G. Schuetz (St. Jude Children's Research Hospital, Memphis, TN). CYP3A2 and CYP3A9 proteins were expressed and isolated from a baculovirus-insect cell expression system. All other reagents were of analytical grade. Nitrocellulose was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). BCIP/NBT reagent was purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD). PCR primers and probes were synthesized by IDT. NuPage 4–12% gradient gels were purchased from Invitrogen (Carlsbad, CA).

2.2. Animals

All animal experimentation protocols were approved by the institutional animal care and use committee at the University of Washington. Male Sprague-Dawley rats (~200 g; purchased from B & K Universal, Kent, WA) were housed under a 12 h light–dark cycle for 7 days prior to use. Ethanol:water = 1:1 was used as the solvent to dissolve $1,25(OH)_2D_3$. Animals were given either control vehicle (i.e. solvent), 10 or 100 ng $1,25(OH)_2D_3$ treatment (in 200 μl) by intraperitoneal (i.p.) injections every other day for a total of 1–3 doses. In addition, another group of rats that did not receive any treatment were designated as baseline controls. The high dose of $1,25(OH)_2D_3$ that we employed (100 ng) was selected based on the published work of Brown et al. [22], demonstrating that this amount was sufficient to activate the transcription of other known VDR gene targets, such as calbindin-D9K, in the rat small intestine. The lower 10 ng dose selected is similar on a per kg body weight basis (40 ng/kg) to the doses of $1,25(OH)_2D_3$ administered intravenously (20–60 ng/kg) to patients with hypocalcemia due to renal failure.

A total of four to six animals were included in each group. Approximately 24 h after the respective last dose, animals were sacrificed in a CO_2 gas chamber. The liver was removed and perfused with 30 ml (10 ml each, three times) ice-cold saline solution (0.9% NaCl). It was then cut into small pieces and snap frozen in liquid nitrogen. The lumen of the small intestine was flushed with 20 ml cold buffer (saline solution + 1 mM EDTA + 0.5 mg/ml trypsin inhibitor + protease

inhibitor cocktail (1 tablet per 50 ml) + 0.25 mM PMSF) and a 30 cm length of the upper small intestine was removed. The intestinal section was opened longitudinally and mucus was removed from the luminal surface by gentle wiping with Kimwipes. The mucosal epithelium was removed by gentle scraping using the edge of a glass slide. Mucosal scrapings were collected and frozen in liquid nitrogen. All tissues were kept at −80 °C until analysis. Liver microsomes and intestinal homogenate were prepared according to Paine et al. [3].

2.3. Human tissue samples

Paired (same donor) human liver and jejunal samples from nine Caucasian donors were obtained from the University of Washington, School of Pharmacy Human Tissue Bank (Seattle, WA). All tissue procurements were made under protocols approved by the University of Washington Institutional Review Board.

2.4. Isolation of total RNA from rat and human tissues

Total RNA was isolated from liver and intestinal mucosal tissues using TRIzol reagent (Invitrogen, Carlsbad, CA). There was no evidence of significant mRNA degradation in the samples.

2.5. Real-time PCR quantification of rat CYP3A and rat and human VDR mRNA

PCR primers and dual-labeled probes for rat genes were designed using the primer design software Primer Express (Applied Biosystems) and synthesized by IDT Inc. (Coralville, IA). Nucleotide sequences for primers and dual-labeled probes specific for quantifying rat CYP3A23, CYP3A9, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA were as follows: *CYP3A23*: forward primer 5′-CCTTTTTTTGGCACTGTGCTGAA-3′, reverse primer 5′-CGTGATGGCAAACAGAGGCA-3′, and probe 5′-CTATATGGGTTTATGGAAATTCGATGTGGAGTGCC-3′; *CYP3A9*: forward primer 5′-CTTTCTTGGGGACGATTCTTGCT-3′, reverse primer 5′-TGTCTGGATCCGTGATCGCTAG-3′, and probe 5′-CTGTCGACCATCGTACAACCCCCATAATTTCC-3′; GAPDH: forward primer 5′-TCCTGCACCACCAACTGCTT-3′, reverse primer 5′-GAGGGGCCATCCACAGTCTT-3′, and probe 5′-CTCATGACCACAGTCCATGCCATCAC-3′. Quantitative real time PCR for rat VDR mRNA was performed using the following specific primers and the SYBR green PCR kit (Applied Biosystems): forward primer 5′-TGC CCG ACC CTG GTG ACT TT-3′ and reverse primer 5′-CGC CTG AAG AAA CCT TTG CA-3′.

For human VDR and ubiquitin C genes, Assays-on-Demand™ products from Applied Biosystems (containing appropriate gene-specific primers and probe) were used. The primers and probe sequences for human GAPDH were: GAGGGGCCATCCACAGTCTT (reverse), GATCATCAGCAATGCCTCCT (forward), 6FAM-ACTCATGACAGTCCATGCCATCAC-TAMRA (probe). GAPDH, shown to be one of the most stably expressed control genes among different tissues [23], was chosen as the internal control gene to compare the gene expression levels between different organs (i.e. liver and intestine for this study). Another internal control gene, UBC (ubiquitin C) was used for validation of results. One intestine sample with relatively high expression of the target genes was selected to make standard curves after serial dilutions.

Reverse transcription and real-time quantitative PCR were carried out as follows. Briefly, for each liver or intestinal RNA sample, 2 μg total RNA was treated with 0.25 units of DNase I (Roche Diagnostics, Mannheim, Germany) at 37 °C for 30 min. DNase was inactivated by heating at 70 °C for 5 min. Reverse transcription was performed on RNA samples using random hexamers, according to the manufacturer's instructions for the Superscript First-Strand Synthesis RT-PCR System (Invitrogen). The only exception was that the resulting cDNA was not treated with RNase H. cDNA from liver and intestinal RNA samples were diluted with nuclease-free water to a final volume of 100 μl.

The liver or intestinal PCR mixture (24 μl final volume) consisted of 4 μl of cDNA, the appropriate forward and reverse primers (400 nM each), 100 nM TaqMan probe, and 1× TaqMan Universal PCR Master Mix (Applied Biosystems). Amplification and detection were performed using the ABI 7700 system with the following PCR reaction profile: 95 °C for 10 min, and 40 cycles of 95 °C for 20 s and 62 °C for 1 min. Reported *CYP3A23*, *CYP3A9*, and rat and human VDR mRNA data were normalized to GAPDH mRNA.

2.6. Western blot analysis

Total protein concentration for the liver microsomes and intestinal homogenate was determined by the method of Lowry et al. [24], using BSA as a reference protein.

Immunoquantitation of CYP3A23, CYP3A9, and CYP3A2 was performed as described by Paine et al. [3], with minor modifications. Briefly, liver microsomal protein (4 μg) and intestinal homogenates (6 μg) were resolved by electrophoresis on 4–12% acrylamide gradient gels (Invitrogen). After electrophoretic transfer, the nitrocellulose sheets were incubated with an anti-CYP3A23 IgG antibody (cross-reacted with other CYP3A isoforms). An Integrated Optical Density (IOD) for each BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) developed protein band was generated using a Bio-Rad ChemiDoc and Quantity One program (Hercules, CA).

2.7. Testosterone 6β-hydroxylation activity assay

All incubations (1 ml final volume) were performed in duplicate. Appropriate control experiments, to confirm linear product formation, were conducted. Liver microsomal samples (100 μg) or intestinal homogenate (500 μg) were preincubated with 250 μM testosterone in 0.1 M potassium phosphate, pH 7.4 at 37 °C for 5 min. NADPH (1 mM final concentration) was added to initiate the reaction. Reactions were terminated after 15 min (liver microsome) or 30 min (intestinal homogenate) with the addition of 5 ml of ethyl acetate. Samples were spiked with 11α-progesterone (internal standard), vortexed, centrifuged at 2000 × *g*, and the organic layer was removed and evaporated to dryness under a nitrogen stream. The residues were reconstituted in 70 μl of methanol:water (1:1) and analyzed by HPLC. For liver microsomal incubations, aliquots of 20 μl were injected onto a

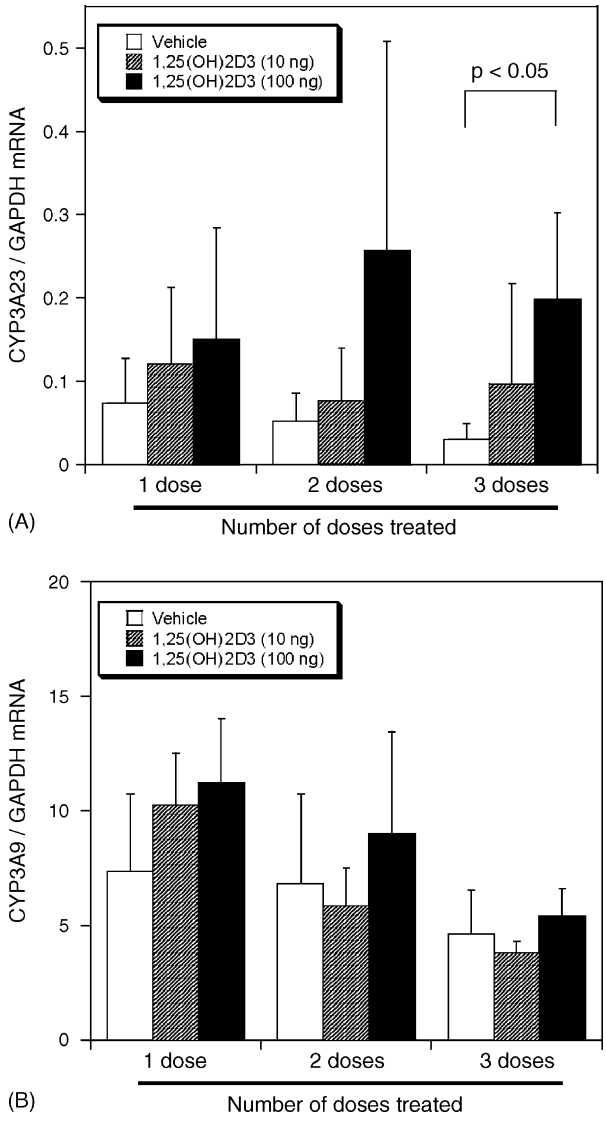

Fig. 1 – Rat intestinal CYP3A23 (A) or CYP3A9 (B) mRNA level normalized to GAPDH mRNA following different periods of treatment (24, 72 or 120 h, i.e. 1, 2, or 3 doses) with vehicle, 10 or 100 ng 1,25(OH)$_2$D$_3$. Vertical bars represent the mean (S.D.) of data from different rats for the respective vehicle, 10 or 100 ng 1,25(OH)$_2$D$_3$ treatment groups. The number of animal per treatment group for both (A) and (B) was as follows: 1 dose, n = 6, 4, 4; 2 doses, n = 6, 4, 6; 3 doses, n = 6, 4, 4.

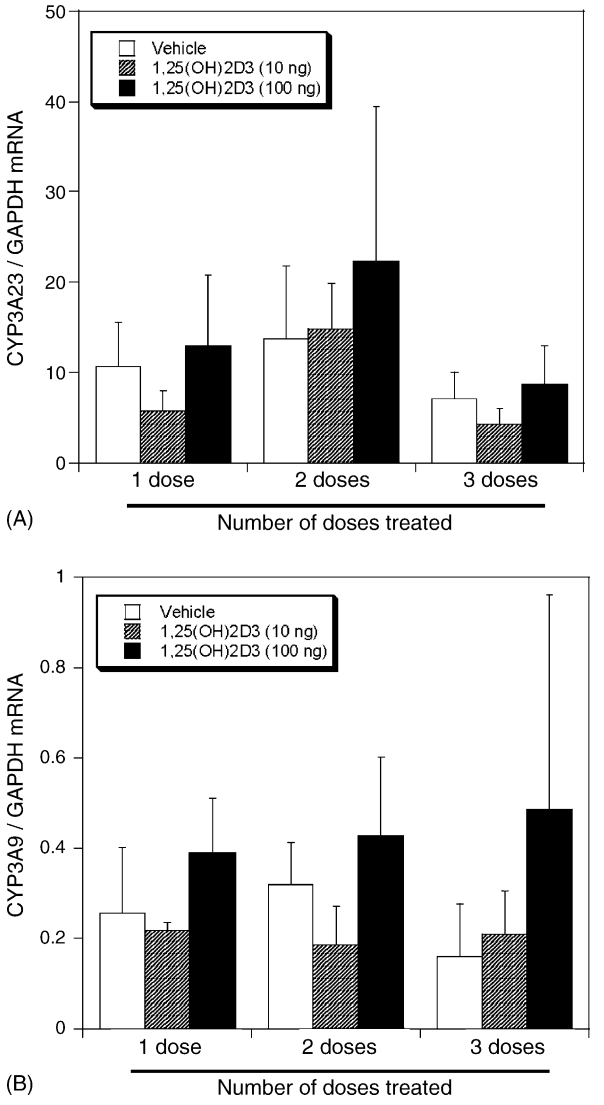

Fig. 2 – Rat hepatic CYP3A23 (A) or CYP3A9 (B) mRNA level normalized to GAPDH mRNA following different periods of treatment (24, 72 or 120 h, i.e. 1, 2, or 3 doses) with vehicle, 10 or 100 ng 1,25(OH)$_2$D$_3$. Vertical bars represent the mean (S.D.) of data from different rats for the respective vehicle, 10 or 100 ng 1,25(OH)$_2$D$_3$ treatment groups. The number of animal per treatment group for both (A) and (B) was as follows: 1 dose, n = 6, 4, 4; 2 doses, n = 6, 4, 6; 3 doses, n = 6, 4, 4.

Nomura C18 column (4.6 mm × 100 mm, 5 μm) and the analytes were eluted with methanol:water:acetonitrile gradient at a flow rate of 1.2 ml/min. Chromatographic peaks were monitored by UV detection at 254 nm. The retention times for 6β-hydroxytestosterone, 11α-progesterone and testosterone were 3.5, 8.2 and 10 min, respectively.

For intestinal homogenate incubations, the above chromatographic conditions did not result in satisfactory separation of 6β-hydroxytestosterone from other interfering peaks. Therefore, a new chromatographic separation method using a longer column was developed for better peak resolution. Aliquots of 20 μl were injected into a Alltech C18 column (4.6 mm × 200 mm, 5 μm) and eluted with a gradient of buffer A (80% methanol/5% acetonitrile/15% water) and buffer B (30% methanol/2% acetonitrile/68% water) at a flow rate of 1.0 ml/min. The retention times for 6β-hydroxytestosterone, 11α-progesterone and testosterone were 17.0, 25.1 and 28.9 min, respectively.

2.8. *Statistical analysis*

All data are reported as mean ± S.D. For each set of mRNA and protein data (hepatic and intestinal CYP3A23 and CYP3A9), a two-way analysis of variance (ANOVA) test was carried out

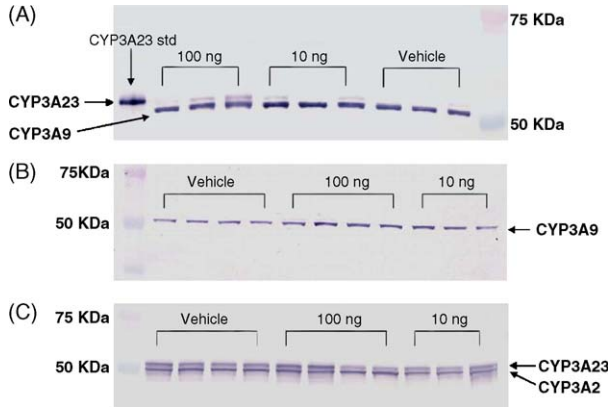

Fig. 3 – A representative Western blot of intestinal (A and B) and hepatic (C) protein levels of CYP3A isoforms after the second dose of 1,25(OH)$_2$D$_3$ treatment. Six micrograms of intestinal homogenate or 4 μg of liver microsomal protein was loaded into each lane. In (A), the blot was overdeveloped such that the CYP3A23 band could be detectable. In (B), the blot was modestly developed in order to quantify the CYP3A9 protein accurately. Note, in this case, the CYP3A23 band was not detectable. The treatments (i.e. vehicle, 10 or 100 ng 1,25(OH)$_2$D$_3$) given to the rats by intraperitoneal injections were labeled in the blots. CYP3A23 standard was loaded onto the gel in (A). Identification of each CYP3A isoform (i.e. CYP3A23, CYP3A9, and CYP3A2) on the blot was achieved by comparing the migration pattern of immunodetected tissue bands with that of recombinant CYP3A isoforms, before and after spiking with authentic protein (data not shown).

using the SAS program (Cary, NC). Individual data were log-transformed to satisfy ANOVA requirements for constant variance and normally distributed error terms. Dunnett's test was applied for pair-wise group comparisons. p-Values less than 0.05 were considered significant.

3. Results

3.1. *Transcriptional activation of rat intestinal and hepatic CYP3A23 and CYP3A9 by 1,25(OH)$_2$D$_3$*

Real-time RT-PCR quantitation of intestinal CYP3A23 and CYP3A9 mRNA is summarized in Fig. 1. Results from a two-way analysis of variance indicated a significant effect of 1,25(OH)$_2$D$_3$ treatment dose ($p = 0.02$) but not the number of doses (i.e. treatment duration) on CYP3A23 mRNA content (Fig. 1A). When Dunnett's test was applied for comparing each 1,25(OH)$_2$D$_3$ treatment group with the corresponding vehicle group, there was a statistically significant increase in mean CYP3A23 mRNA content at 24 h after the third (6.6-fold; $p = 0.03$), but not the first or second 100 ng dose. There were no significant differences between any of the 10 ng dose groups and corresponding vehicle groups, although the mean values following 1,25(OH)$_2$D$_3$ treatment were higher than that of the respective vehicle group at each of the treatment periods tested. There was no significant effect of treatment dose and duration on intestinal CYP3A9 mRNA content (Fig. 1B), and thus no pair-wise comparisons were conducted. In addition, by two-way ANOVA, there was no significant effect of either treatment dose or duration on hepatic CYP3A23 and CYP3A9 expression (Fig. 2), although the mean value for the 100 ng dose groups was always higher than that of the respective vehicle-treated control group.

Very similar results were obtained when 28S rRNA was used to normalize the intestinal and hepatic CYP3A data by a traditional PCR/gel-based method (data not shown).

3.2. *Effect of 1,25(OH)$_2$D$_3$ on rat CYP3A (CYP3A23, CYP3A9, and CYP3A2) protein levels in intestine and liver*

We developed a protocol to separate and quantify the individual CYP3A isoforms by Western blot analysis (Fig. 3). Identification of each CYP3A isoform (CYP3A23, CYP3A9, and CYP3A2) was achieved by comparing the migration pattern of immunodetected tissue bands with that of recombinant CYP3A isoforms, before and after spiking with authentic protein (not shown). Two-way analysis of variance of CYP3A protein content data revealed a significant effect of 1,25(OH)$_2$D$_3$ treatment dose ($p = 0.01$) but not the number of doses (i.e. treatment duration) on the intestinal CYP3A23 protein level (Table 1). When Dunnett's test was applied for comparing each 1,25(OH)$_2$D$_3$ treatment group with the corresponding vehicle group, there was a 2.2-fold ($p < 0.05$), 3.5-fold ($p < 0.05$), and 4.8-fold ($p < 0.01$) increase in mean

Table 1 – Western blot analysis of CYP3A23 and CYP3A9 in rat small intestine following different periods of treatment (24 h after 1, 2, or 3 doses; each dose was 48 h apart) with vehicle, 10 ng 1,25(OH)$_2$D$_3$ or 100 ng 1,25(OH)$_2$D$_3$

	Relative band IOD		
	Vehicle	10 ng 1, 25(OH)$_2$D$_3$	100 ng 1, 25(OH)$_2$D$_3$
CYP3A23			
Baseline[a]	1.0 ± 0.2[b]	–	–
1 dose	1.1 ± 0.3	1.1 ± 0.2	2.2 ± 0.5*
2 doses	1.1 ± 0.5	1.8 ± 0.6	3.5 ± 0.8*
3 doses	0.9 ± 0.1	1.4 ± 0.4	4.8 ± 0.4**
CYP3A9			
Baseline[a]	1.0 ± 0.1[b]	–	–
1 dose	1.0 ± 0.2	1.1 ± 0.2	1.3 ± 0.2
2 doses	1.1 ± 0.1	1.3 ± 0.3	1.5 ± 0.2
3 doses	0.9 ± 0.3	1.2 ± 0.3	1.1 ± 0.4

[a] No treatment.
[b] Values presented in each cell represent the mean ± S.D. of data from different rats for the respective vehicle, 10 or 100 ng 1,25(OH)$_2$D$_3$ treatment groups. For both CYP3A23 and CYP3A9, the number of animals per treatment groups was as follows: baseline, $n = 4$; 1 dose, $n = 6, 4, 4$; 2 doses, $n = 6, 4, 6$; 3 doses, $n = 6, 4, 4$.
* $p < 0.05$ comparing 100 ng 1,25(OH)$_2$D$_3$ and vehicle treatment groups.
** $p < 0.01$ comparing 100 ng 1,25(OH)$_2$D$_3$ and vehicle treatment groups.

Table 2 – Testosterone 6β-hydroxylation activity in rat intestine and liver following different periods of treatment (24 h after 1, 2, or 3 doses; each dose was 48 h apart) with vehicle, 10 ng 1,25(OH)$_2$D$_3$ or 100 ng 1,25(OH)$_2$D$_3$

	pmol/min/mg protein		
	Vehicle	10 ng 1, 25(OH)$_2$D$_3$	100 ng 1, 25(OH)$_2$D$_3$
Intestinal homogenate			
Baseline[a]	48.9 ± 22.5[b]	–	–
1 dose	34.7 ± 23.4	54.8 ± 15.5	57.2 ± 15.3
2 doses	42.5 ± 29.9	39.6 ± 9.5	67.7 ± 15.1
3 doses	28.3 ± 14.3	35.3 ± 10.0	39.0 ± 15.0
Liver microsomes			
Baseline[a]	6490 ± 365[b]	–	–
1 dose	8247 ± 1646	6666 ± 209	7991 ± 1089
2 doses	6763 ± 1895	9633 ± 1730	7423 ± 1136
3 doses	6098 ± 622	6574 ± 287	7090 ± 863

[a] No treatment.
[b] Values presented in each cell represent the mean ± S.D. of data from different rats for the respective vehicle, 10 or 100 ng 1,25(OH)$_2$D$_3$ treatment groups. For both intestine and liver, the number of animals per treatment group was as follows: baseline, $n = 4$; 1 dose, $n = 6, 4, 4$; 2 doses, $n = 6, 4, 6$; 3 doses, $n = 6, 4, 4$.

CYP3A23 protein content at 24 h after the respective first, second and third 100 ng 1,25(OH)$_2$D$_3$ doses. However, there were no significant differences between any of the 10 ng dose groups and corresponding vehicle groups. With regard to intestinal CYP3A9, there was no significant effect of treatment dose and time on protein content (Table 1), and thus no pairwise comparisons were conducted. In addition, by two-way ANOVA, there was no significant effect of either treatment dose or time on hepatic CYP3A23 and CYP3A2 protein content (data not shown).

3.3. *Effect of 1,25(OH)$_2$D$_3$ on testosterone 6β-hydroxylation activity in intestinal homogenate and liver microsomes*

Rat intestinal proteins are highly susceptible to protease-mediated degradation. Using a combination of protease inhibitor cocktail and trypsin inhibitor, we successfully preserved the CYP3A-mediated testosterone hydroxylation activity from both intestine and liver tissues. However, the 6β-hydroxylation activity of testosterone catalyzed by hepatic microsomes and intestinal homogenate (Table 2) was not enhanced by 1,25(OH)$_2$D$_3$ treatments.

3.4. *Comparison of hepatic and intestinal expression of VDR mRNA in rats and humans*

To help explain the observed differences in the inducibility of *CYP3A* genes in small intestine and liver by a 1,25(OH)$_2$D$_3$/VDR signaling pathway, we compared the relative expression of VDR in these tissues from rats and humans. VDR mRNA (normalized to GAPDH) in rat intestine was 366-fold higher than that seen in the rat liver (mean ± S.D.: 366 ± 46 versus 1.0 ± 0.5) (Fig. 4). Similarly, data collected from matched (same donor) human liver and jejunal tissues revealed that, relative to the liver, there was a ~77-fold higher VDR mRNA content in the jejunum (mean ± S.D.: 77 ± 58 versus 1.0 ± 1.1) (Fig. 4), using GAPDH as the reference control gene. In sharp contrast, another internal control housekeeping gene, ubiquitin C (UBC) exhibited roughly equivalent level of human hepatic and jejunal mRNA in these two tissues (mean ± S.D.: 1.5 ± 1.2).

4. Discussion

The regulation of *CYP3A4* gene expression by VDR and 1,25(OH)$_2$D$_3$ was first demonstrated in human intestinal Caco-2 and LS180 cell models [14,15]. In addition, experiments conducted in mice demonstrated a positive effect of VDR activation on intestinal and hepatic *CYP3A* transcription in vivo [21]. In that study, CYP3A11 mRNA levels in liver and intestine were both increased markedly in both tissues following treatment of PXR$^{-/-}$ and PXR$^{+/-}$ mice with a daily gavage dose of 1.5 μg 1α-(OH)D$_3$ (1α-hydroxyvitamin D$_3$) or 8 mg lithocholic acid (a secondary bile acid) for 3 days. Moreover, studies with cultured human hepatocytes have shown that CYP3A4 expression in these cells is increased following exposure to 1,25(OH)$_2$D$_3$ [20]. However, induction of human hepatic CYP3A expression in vivo by 1,25(OH)$_2$D$_3$ might not be as facile as that seen in the mouse, given that VDR expression in human hepatocytes is very low in comparison to mucosal epithelia of the small intestine [11,13]. To examine whether there can be organ selectivity in intestinal and hepatic VDR signaling, we measured the effect of an acute treatment with low-dose 1,25(OH)$_2$D$_3$ on CYP3A23 and CYP3A9 expression in rat small intestine and liver. The rat was selected as a model for humans because both species reportedly show marked preferential intestinal (compared to hepatic) VDR expression [11,13], which we confirmed (Fig. 4). *CYP3A9* was selected as a comparator gene because it has been shown to be less sensitive than *CYP3A23* to the effects of known CYP3A inducers (via PXR activation) in

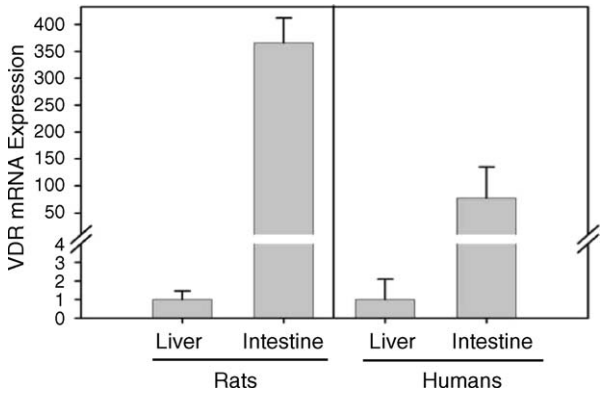

Fig. 4 – Comparison of the relative VDR mRNA expression (normalized to GAPDH mRNA) between the liver and small intestine tissues in rats and humans. Each value represents the mean ± S.D. of multiple samples ($n = 5$ for rats; $n = 9$ for humans). The mean expression levels of VDR mRNA in the liver of both rats and humans were normalized to be 1.

male rats [25,26] and, indeed, it appeared to be less responsive to 1,25(OH)$_2$D$_3$ treatment than *CYP3A23* (Fig. 1). These observations are consistent with a recent report [27] where intestinal CYP3A9 mRNA was induced ~1.7-fold 24 h after albeit a single higher dose (270 ng) than what we employed (100 ng).

As predicted, we observed a significant increase in intestinal but not hepatic CYP3A23 mRNA following 1,25(OH)$_2$D$_3$ treatment. The difference in experimental outcome, compared to that from Makishima et al. [21], may be due to the following: (1) Species difference in hepatic VDR expression. Theoretically, the level of nuclear receptors in different cell types could affect the sensitivity of cells to the treatment of a ligand. Rat and human hepatocytes exhibited relatively lower levels of nuclear VDR protein in comparison to mice hepatocytes [13]. Perhaps this difference is sufficient for a more robust response in mouse liver. (2) Species difference in hepatic 1,25(OH)$_2$D$_3$ distribution. In a recent paper published by Brown et al. [28], after giving radio-labeled 1,25(OH)$_2$D$_3$ to the rats, the liver exhibited a three to four-fold lower level of hormone exposure compared to the duodenum (reported as DPM/g tissue). This may have contributed to a relatively decreased inductive response in liver compared to small intestine. (3) A difference in the effective 1,25(OH)$_2$D$_3$ dose to the rat and mouse liver. In the study of Makishima et al. [21], the dose of 1α-(OH)D$_3$ (1.5 μg) given to mice was relatively high. 1α-(OH)D$_3$ is not as potent a ligand for VDR as is 1,25(OH)$_2$D$_3$ [29] and higher doses may be necessary to achieve comparable inductive responses. However, the rate-limiting step in synthesizing the fully active vitamin D$_3$ metabolite (1,25(OH)$_2$D$_3$) is 1α-hydroxylation; the 25-hydroxylation step is not tightly regulated [30]. Therefore, after giving a relatively high dose of 1α-(OH)D$_3$, one might anticipate a sharp increase in the concentration of the active hormone 1,25(OH)$_2$D$_3$ in serum and target cells (e.g. enterocytes and hepatocytes) in the treated mice. Thus, a higher exposure of 1,25(OH)$_2$D$_3$ could have resulted in more widespread activation of target genes, irrespective of the relative VDR expression level. In our study, we chose to give 1,25(OH)$_2$D$_3$ by intraperitoneal injection, instead of the oral/gavage route, to best mimic the systemic delivery of 1,25(OH)$_2$D$_3$ to enterocytes (or the liver), since our ultimate interest was to assess hormonal effects on human CYP3A4 expression in the constitutive state.

We (Fig. 4) and others [13] have shown that rats resemble humans closely in terms of their low expression level of VDR expression in hepatocytes, suggesting that the rat may represent a better model for studying quantitative effects of 1,25(OH)$_2$D$_3$ on intestinal and hepatic CYP3A than the mouse. Based on our data, we speculate that, under normal physiological concentrations of 1,25(OH)$_2$D$_3$, the expression of CYP3A4 in vivo in human hepatocytes may not be responsive in any significant way to the hormone because of low VDR content. Indeed, Gascon-Barre et al. have shown that the more abundant human and rat hepatocytes are relatively deficient in nuclear VDR protein in comparison to non-parenchymal cells such as endothelial, Kupffer and Stellate cells, and that increased hepatic CYP24 expression (a known VDR target gene) following in vivo treatment with 1,25(OH)$_2$D$_3$ occurs exclusively in these same non-parenchymal cells [13].

Regardless of possible species differences in hepatic CYP3A regulation by 1,25(OH)$_2$D$_3$, our data add to that of previous investigators showing the plausibility of a VDR-mediated pathway for regulation of intestinal CYP3A4 in humans. Although there are structural differences between the rat and human *CYP3A* genes, the respective inducible forms, *CYP3A23* and *CYP3A4*, share common transcription enhancer response elements (DR-3 and ER-6) that can mediate induction by 1,25(OH)$_2$D$_3$. The 100 ng 1,25(OH)$_2$D$_3$ intraperitoneal dose adopted for this study was selected based on the work of Brown et al. [22] who studied the regulation of calbindin-D9K by 1,25(OH)$_2$D$_3$ and VDR in rat intestine. Those investigators observed a six-fold increase in calbindin-D9K mRNA after seven daily doses of 100 ng 1,25(OH)$_2$D$_3$, with a magnitude similar to what we observed for the *CYP3A23* gene. Calbindin-D9K has been regarded as a VDR-specific intestinal marker gene for its sensitivity to the changes in VDR activation. Thus, based on results of this study, it is possible that *CYP3A23* in rat intestine is also a highly regulated target of VDR.

Measurement of each of the rat CYP3A isoforms of interest revealed a significant effect of 1,25(OH)$_2$D$_3$ treatment dose on intestinal CYP3A23 protein content. However, this response did not result in an increase in catalytic activity for the universal CYP3A probe substrate, testosterone. In retrospect, this observation is not too surprising, as the constitutive expression of intestinal CYP3A23 in rats is much lower than that of CYP3A9. Both of these CYP3A isoforms (and also intestinal CYP3A18) are expected to catalyze the testosterone 6β-hydroxylation reaction. Because the absolute level of CYP3A23 protein in the induced rat intestine was relatively low (and that the major intestinal CYP3A9 protein level was not affected), it was likely insufficient to produce a detectable change in total catalytic activity. However, one would expect a strong correlation between intestinal CYP3A4 protein induction and CYP3A catalytic activity in humans, because of its dominant metabolic role in this organ in both constitutive and induced states.

In summary, the induction of intestinal *CYP3A23* in rats by 1,25(OH)$_2$D$_3$ suggests that a VDR signaling pathway may play an important role in regulating constitutive intestinal CYP3A4 expression in humans. Variation in the intestinal VDR content and 1,25(OH)$_2$D$_3$ concentration (or any other VDR ligands such as certain bile acids) within enterocytes may be major determinants of inter-individual differences in intestinal CYP3A4 function and oral drug disposition. In contrast, the relatively low expression level of VDR in rat and human liver may preclude transcriptional modification of its gene targets, including *CYP3A4*, by normal constitutive levels of VDR ligands.

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