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Human PXR-mediated induction of intestinal CYP3A4 attenuates 1 α ,25-dihydroxyvitamin D₃ function in human colon adenocarcinoma LS180 cells

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Abstract

Oxidative catabolism of 1 α ,25-dihydroxyvitamin D₃ [1 α ,25(OH)₂D₃] is mediated by either CYP24A1 or CYP3A4. In this paper, we tested whether induction of CYP3A4 in the LS180 intestinal cell model enhances clearance of 1 α ,25(OH)₂D₃ and blunts its hormonal effect on expression of the apical membrane calcium transport protein, TRPV6. Treatment with the hPXR agonist rifampin significantly increased CYP3A4 mRNA content and catalytic activity, but had no effect on CYP24A1 or TRPV6 mRNA content. Pre-treating cells with rifampin for 48 hrs, prior to a 24 hr 1 α ,25(OH)₂D₃ treatment phase, was associated with a subsequent 48% increase in the elimination of 1 α ,25(OH)₂D₃ and a 35% reduction of peak TRPV6 mRNA. Introduction of the CYP3A4 inhibitor, 6',7'-dihydroxybergamottin, an active inhibitor in grapefruit juice, reversed the effects of rifampin on 1 α ,25(OH)₂D₃ clearance and TRPV6 expression. Over-expression of hPXR in LS180 cells greatly enhanced the CYP3A4 responsiveness to rifampin pretreatment, and elicited a greater relative suppression of TRPV6 expression and an increase in 1 α ,25(OH)₂D₃ disappearance rate, compared to vector expressed cells, following hormone administration. Together, these results suggest that induction of CYP3A4 in the intestinal epithelium by hPXR agonists can result in a greater metabolic clearance of 1 α ,25(OH)₂D₃ and reduced effects of the hormone on the intestinal calcium absorption, which may contribute to an increased risk of drug-induced osteomalacia/osteoporosis in patients receiving chronic therapy with potent hPXR agonists. Moreover, ingestion of grapefruit juice in the at-risk patients could potentially prevent this adverse drug effect.

Keywords

Cytochrome P450 3A4; TRPV6; 1 α ,25-Dihydroxyvitamin D₃; Human pregnane X receptor; Rifampin; Osteomalacia

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1. Introduction

Osteomalacia, characterized by defective bone mineralization, most commonly occurs in adults with vitamin D deficiency. It is often associated with a reduced dietary intake or synthesis of vitamin D, however osteomalacia has also been reported in patients receiving long-term therapy with certain drugs including rifampin, an antimicrobial agent [1], and the anti-epileptic drugs, carbamazepine, phenytoin and phenobarbital [2, 3]. Although the clinical manifestation and histology of drug-induced osteomalacia resembles that found in other adults with vitamin D deficiency [4], the molecular mechanism of this adverse drug effect is still unclear.

A primary function of vitamin D in maintaining calcium homeostasis and bone health is accomplished by stimulation of transcellular and paracellular calcium transport in the small intestine through vitamin D receptor (VDR)-dependent pathways [5]. One protein thought to regulate the rate and extent of intestinal calcium absorption is TRPV6. TRPV6 is a calcium channel protein expressed in the intestinal luminal epithelium and its synthesis and function is regulated in part by $1\alpha,25\text{-dihydroxyvitamin D}_3$ [$1\alpha,25(\text{OH})_2\text{D}_3$] through a VDR signaling pathway [5–8]. The basal duodenal TRPV6 expression is correlated with serum $1\alpha,25(\text{OH})_2\text{D}_3$ levels in men, as well as with VDR expression in both men and women, although TRPV6 expression in women is influenced predominantly by an age effect, not by vitamin D level [8]. In addition, TRPV6 transcription responds rapidly to $1\alpha,25(\text{OH})_2\text{D}_3$ in the human duodenum [9]. Moreover, studies with TRPV6 knockout mice demonstrate that transporting calcium by TRPV6 may be the rate-limiting step and gatekeeper of transcellular calcium flux [10]. Interestingly, $1\alpha,25(\text{OH})_2\text{D}_3$ might also mediate paracellular transport of calcium by a non-TRPV6 mediated mechanism [11].

Metabolic inactivation of $1\alpha,25(\text{OH})_2\text{D}_3$ constitutes a negative feedback mechanism for regulating its effect on calcium absorption in the small intestine [12]. Human cytochrome P450 24A1 (CYP24A1) and P450 3A4 (CYP3A4) contribute to the inactivation of $1\alpha,25(\text{OH})_2\text{D}_3$ [13–16]. However, the relative contributions of these two enzymes to vitamin D catabolism may be tissue-dependent. For example, in the healthy kidney, CYP24A1 activity likely dominates $1\alpha,25(\text{OH})_2\text{D}_3$ catabolism, because the expression of CYP3A4 is very low in that organ [17–19]. In contrast, in tissues such as the liver and small intestine, CYP3A4 could play a more important role in $1\alpha,25(\text{OH})_2\text{D}_3$ catabolism, because constitutive levels of CYP3A4 are high, whereas CYP24A1 expression is very low [17]. In addition, local production and hormonal effects of $1\alpha,25(\text{OH})_2\text{D}_3$ in the duodenum were indicated in previous studies [9]. Thus, $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated calcium absorption could be disrupted locally by drugs that alter CYP3A4 expression in the intestinal epithelium, independent of any change in the systemic vitamin D level [20].

In fact, it has been reported that treatment of healthy volunteers with the pregnane X receptor (hPXR) agonist rifampin causes preferential induction of human duodenal CYP3A4, but not CYP24A1, mRNA content [17]. Thus, acceleration of intestinal $1\alpha,25(\text{OH})_2\text{D}_3$ catabolism by stimulation of CYP3A4 synthesis may result in down-regulation of calcium transporters, such as TRPV6, and an impairment of calcium absorption. Long-term impairment of intestinal calcium absorption by chronic drug administration and CYP3A4 induction could result in osteomalacia [17]. Those drugs associated with osteomalacia share the capacity to activate the hPXR and constitutive androstane receptor (CAR) and thus up-regulate the expression of multiple cytochrome P450 enzymes, including CYP3A4 [21–23].

In this investigation, we tested the hypothesis that induction of CYP3A4 by hPXR agonists alters the intestinal cellular clearance of $1\alpha,25(\text{OH})_2\text{D}_3$, and thereby affects the transcription

of calcium transporters such as TRPV6, LS180 cell line was used as a model for intestinal enterocytes due to the robust expression of both VDR and hPXR [24, 25]. We report that hPXR agonists induce CYP3A4, but not CYP24A1 in LS180 cells, and under most conditions enhance $1\alpha,25(\text{OH})_2\text{D}_3$ catabolism and decrease the transcriptional response of TRPV6 to $1\alpha,25(\text{OH})_2\text{D}_3$. The findings presented here provide a viable molecular mechanism of drug-induced osteomalacia by hPXR activators.

2. Materials and Methods

2.1 Chemicals and Reagents

Rifampin, hyperforin, carbamazepine, and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Itasca, IL). 25-Hydroxyvitamin D_3 [$25(\text{OH})\text{D}_3$] and $1\alpha,25(\text{OH})_2\text{D}_3$ were obtained from Calbiochem (La Jolla, CA) and 24R,25-dihydroxyvitamin D_3 [$24\text{R},25(\text{OH})_2\text{D}_3$] was obtained from Sigma. Deuterated standards of vitamin D_3 metabolites, d_6 -25OHD $_3$ and d_6 - $1\alpha,25(\text{OH})_2\text{D}_3$ (containing six deuterium atoms at C-26 and C-27), were purchased from Medical Isotope Inc. (Pelham, NH). Levetiracetam was purchased from LKT Laboratories (St. Paul, MN). Midazolam (MDZ), 1'-hydroxymidazolam (1-OH MDZ), d_2 -1'-hydroxymidazolam (d_2 -1'-OH MDZ) and phenobarbital were purchased from Cerilliant (Round Rock, TX). Cell culture medium, penicillin-streptomycin and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Sodium pyruvate was purchased from Cellgro (Herndon, VA) and cell dissociation solution was purchased from Sigma. High capacity cDNA reverse transcription kits and PCR primers, SYBR green master mix and other PCR reagents were obtained from Applied Biosystems (ABI, Foster City, CA).

2.2 Cell Culture Conditions

The human intestinal epithelial cell line, LS180 and Caco-2 cell lines were obtained from ATCC (Manassas, VA). Cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% sodium pyruvate at 37 °C in 5% CO_2 . After reaching 80% confluence, the cells were de-attached by addition of dissociation solution, collected and isolated by centrifugation. The passaged cells were then seeded into 6-well plates and grown in MEM with 10% FBS until 60–70% confluence was reached. Twenty-four hours before the first experimental treatment, the medium was replaced with MEM containing 10% resin-charcoal-treated FBS. To initiate an experimental treatment, this medium was removed and the cells were washed twice with PBS and then treated with the drug or its vehicle (e.g., 0.1% DMSO for rifampin) in the same culture medium (2 mL final volume) for a specified period of time. For some experiments, sequential treatments or co-treatments were employed. Experiment-specific modifications of the general treatment regimen are described in more detail in the result sections, for clarity. At the end of each treatment period, cell culture medium was collected for quantification of 1-OH MDZ, $1\alpha,25(\text{OH})_2\text{D}_3$, or $24\text{R},25(\text{OH})_2\text{D}_3$ concentrations, depending on the study design. Cells were collected for measurement of intracellular $1\alpha,25(\text{OH})_2\text{D}_3$ and total protein, or mRNA, again depending on the study design. All samples were stored at –80 °C until analyzed.

2.3 Isolation of Total Cellular RNA and qRT-PCR Analysis

After experimental treatment, LS180 or Caco-2 cells collected from each culture well were homogenized in 1 mL TRIzol reagent and stored at –80 °C. Total cellular RNA was isolated according to the manufacturer-supplied protocol for TRIzol reagent. The isolated RNA was dissolved in DEPC-treated water and the concentration was determined using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE).

Reverse transcription was performed according to the manufacturer's instructions for the high capacity cDNA reverse transcription kit. For each reaction, 4 µg of isolated RNA was mixed with dNTP, random hexamer primers, and MultiScribe reverse transcriptase in reaction buffer in a total volume of 40 µL. The reverse transcription condition was set as 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 sec. qRT-PCR was performed using gene-specific primers and the SYBR green master mix with the ABI 7900 system (Applied Biosystems). The PCR mixture consisted of 1 µL of cDNA, gene-specific forward and reverse primers (25 pmol each), SYBR green master mix and distilled water, in a total volume of 25 µL for each reaction. The following program was used: a denaturation step at 94 °C for 10 min, 40 cycles of PCR (denaturation 94 °C for 30 sec; annealing 65 °C for 30 sec; and extension 72 °C for 30 sec), followed by 72 °C for 5 min and then a dissociation/melting step (95 °C for 15 sec, 65 °C for 15 sec, 95 °C for 15 sec, 25 °C for 5 min). All tested gene products were quantified using the comparative $\Delta\Delta C_t$ calculation for relative quantification of gene expression, normalized to GAPDH. The following primer sets (F/R) for human genes were used:

CYP3A4: (5'-GGCTTCATCCAATGGACTGCATAAAT-3' and 5'-TCCCAAGTATAACACTCTACACAGACAA-3');

CYP24A1: (5'-GGTGACATCTACGGCGTACAC-3' and 5'-CTTGAGACCCCCTTCCAGAG-3');

TRPV6: (5'-TGATGCGGCTCATCAGTGCCAGC-3' and 5'-GTAGAAGTGGCCTAGCTCCTCG-3');

GAPDH: (5'-GGCCTCCAAGGAGTAAGACC-3' and 5'-AGGGGAGATTCAAGTGTGGTG-3');

VDR: (5'-GACATCGGCATGATGAAGGAG-3' and 5'-GCGTCCAGCAGTATGGCAA-3');

PXR: (5'-TGGGTGACACCTCCGAGA-3' and 5'-TAGGGAGACAGGCCAGCA-3');

UGT1A1: (5'-TGCTCATTGCCTTTTCACAG-3' and 5'-GGGCCTAGGGTAATCCTTCA-3')

2.4 Midazolam 1'-Hydroxylation Activity Assay

To determine cellular CYP3A4 activity, LS180 cells were incubated for 30 min with an enzyme probe substrate, MDZ (8 µM), in 2 mL of culture media. The primary metabolite, 1'-OH MDZ, released into the cell culture medium was measured. A series of dilutions of 1'-OH MDZ standards were prepared in blank media. Briefly, after addition with the internal standard (d_2 -1'-OH MDZ) and an equal volume of sodium carbonate (0.1 M, pH = 11), the cell media or standard curve samples were extracted using 5 mL ethyl acetate. Following mixing and centrifugation, the organic phase was transferred to a clean tube and dried under a stream of nitrogen. The extracted samples were reconstituted in 100–150 µL of 50% methanol. LC-MS analysis was performed as previously described [24] using the Micromass Quattro II tandem quadrupole mass spectrometer (Micromass Ltd., Manchester, UK) coupled to a Shimadzu LD-10AD solvent delivery system and SIL-10AD autosampler (Shimadzu Scientific Instruments, Inc., Columbia, MD). Chromatographic separations were achieved with a XBD-C8, 5 µm, 2.1 × 50 mm column (Zorbax; Agilent Technologies, Palo Alto, CA) a mobile phase consisting of: water (A)-methanol (B) that contained 0.1 % acetic acid (gradient: 45% B for 0.5 min, increasing to 60% at 2 min, holding for 0.5 min, then increasing to 90% in 0.5 min, holding at 90% for another 1 min and back to the initial condition for 1 min. The total run time was 8.5 min. The column effluent was subjected to mass spectral analysis by positive electrospray ionization. Using selective ion monitoring,

target ions at m/z 342.2 (^{35}Cl -d₀-1'-OH MDZ) and 346.2 (^{37}Cl -d₂-1'-OH MDZ) respectively were monitored. Peak area ratios (1'-OH MDZ/d₂-1'-OH MDZ) of samples were compared with those in the standard curve and unknown concentrations were calculated.

2.5 Quantification of 1 α ,25(OH)₂D₃ and 24R,25(OH)₂D₃ in Cell Media

Measurement of the 1 α ,25(OH)₂D₃ concentration in the culture media was performed by liquid-liquid extraction, chemical derivatization and LC-MS/MS analysis as previously published [26]. Briefly, frozen media samples were thawed in the dark, mixed well, and then 2 mL was transferred for work-up and analysis. After spiking with 1 ng of d₆-1 α ,25(OH)₂D₃ as an internal standard and equilibrium in the dark for 30 min, acetonitrile (4 mL) was added to precipitate proteins. Following centrifugation, the supernatant was transferred to a clean glass tube and concentrated to ~ 2 mL under a nitrogen stream and then 5 mL ethyl acetate was added for analyte extraction. After centrifugation, the supernatant was transferred to a clean glass tube and dried again under a nitrogen stream. Remaining analytes were derivatized with 100 μL PTAD (1 mg/mL) in acetonitrile for 1 h at room temperature in the dark, and dried once more under a nitrogen stream [26–28]. The derivatized sample was then reconstituted in 100 μL acetonitrile, transferred to LC vials and stored at -20°C until analysis.

LC-MS/MS analysis was carried out under positive mode electrospray ionization on an Agilent 6410 QQQ equipped with HPLC1200 system (Agilent Technologies) [26]. Multiple Reaction Monitoring (MRM) for the transition from m/z 574 $\rightarrow$ 314 and 580 $\rightarrow$ 314 was used to detect 1 α ,25(OH)₂D₃ and d₆-1 α ,25(OH)₂D₃, respectively. HPLC was performed on a Hypersil Gold (2.1 $\times$ 100 mm, 1.9 μm) column (Thermo Scientific) using acetonitrile (B)-water (0.1% formic acid) (A) as a mobile phase. The flow rate was 0.2 mL/min with a gradient as follows: 45% B for 3 min, and then increased to 60% B linearly over 3 min, held at 60% B for 1 min, increased to 90% B in 1 min and then held for another 3 min, decreased back to 45% B over 1 min, followed by 8 min of re-equilibration time.

For some experiments, 24R,25(OH)₂D₃ concentrations in the Caco-2 cell medium after incubation with 25(OH)D₃ were measured using a method similar to that described for 1 α ,25(OH)₂D₃. In this case, MRM for the transition from m/z 574 $\rightarrow$ 298 was employed to detect 24R,25(OH)₂D₃. A standard curve was prepared consisting of 24R,25(OH)₂D₃ (0.1–1.6 ng/mL) and internal standard d₆-1 α ,25(OH)₂D₃ (1 ng) in 2 mL blank medium..

Because 1 α ,25(OH)₂D₃ and 24R,25(OH)₂D₃, are light sensitive and very lipophilic, the extraction procedures were conducted under low UV light, and protein in the medium was precipitated with organic solvent prior to liquid-liquid extraction. These two steps were critical for obtaining reliable data. The limit of detection and quantification for 1 α ,25(OH)₂D₃ was 3 pg/mL and 10 pg/mL, respectively. The limit of detection and quantification for 24R,25(OH)₂D₃ was 10 pg/mL and 50 pg/mL, respectively.

2.6 Quantification of Cell Lysate Protein

For those experiments in which intracellular 1 α ,25(OH)₂D₃ was to be measured, total protein concentration in each cell culture well was measured and used to control for variation in the number of cells in the well. At the end of each treatment, cells were collected in 2 mL cold PBS by centrifugation, and lysed using three freeze-thaw cycles. Half of the cell lysate volume was used for chemical quantification, e.g., 1 α ,25(OH)₂D₃, and the other half was diluted in PBS. Total protein in the diluted cell lysate was measured using the BCA assay (Pierce, Rockford, IL).

2.7 Quantification of $1\alpha,25(\text{OH})_2\text{D}_3$ in Cell Lysate

The amount of $1\alpha,25(\text{OH})_2\text{D}_3$ in cell lysates was determined using the same LC-MS/MS method described for analysis of the culture media, following the addition of internal standard, protein precipitation and liquid-liquid extraction of the cell lysate.

2.8 Statistical Analysis

All data are reported as mean $\pm$ SD. Statistical analyses were conducted using GraphPad Prism (Version 5.02, La Jolla, CA). A two-tailed, unpaired t-test was used to evaluate drug effects on LS180 cell gene expression. Statistical analysis on the half-lives of $1\alpha,25(\text{OH})_2\text{D}_3$ elimination was performed using a one-tailed, paired t-test. When drug effects were evaluated over time, ANOVA analysis was employed, followed by pairwise comparisons. A p value less than 0.05 was considered significant.

3. Results

3.1 Activation of Gene Expression by $1\alpha,25(\text{OH})_2\text{D}_3$ and Rifampin in LS180 Cells

Similar to our previous findings with human small intestine [17], the VDR target genes CYP24A1, CYP3A4 and TRPV6, were detected in LS180 cells and significantly induced (32- to 76-fold) after treatment with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$. Interestingly, UGT1A1, another human intestinal drug metabolizing enzyme, was also induced slightly (3.4-fold) by $1\alpha,25(\text{OH})_2\text{D}_3$. Treatment with 50 μM rifampin significantly increased (16- and 6-fold) the mRNA levels for known target genes, CYP3A4 and UGT1A1, but had no effect on CYP24A1 or TRPV6 expression (Supplemental Table 1). Human PXR and VDR expression were not affected by $1\alpha,25(\text{OH})_2\text{D}_3$ or rifampin in cultured LS180 cells. The concentrations of $1\alpha,25(\text{OH})_2\text{D}_3$ and rifampin employed in this experiment were chosen based on their proximity to either endogenous circulating concentrations {0.05 – 0.15 nM $1\alpha,25(\text{OH})_2\text{D}_3$ in plasma; [29, 30]} or therapeutic concentrations {5–10 μM rifampin; [31, 32]}. From these results, we concluded that the LS180 cell model was suitable for an investigation of the relationship between CYP3A4 or CYP24A1 induction and TRPV6 gene regulation by $1\alpha,25(\text{OH})_2\text{D}_3$.

1. Concentration-dependent effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and rifampin on CYP3A4, TRPV6 and CYP24A1 mRNA. LS180 cells were treated with 0, 0.1, 0.5 and 1.0 nM $1\alpha,25(\text{OH})_2\text{D}_3$ for 12 hrs, or with 0, 10, 20, and 50 μM rifampin for 48 hrs. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment caused a dose-dependent increase in the cellular CYP3A4, CYP24A1 and TRPV6 mRNA levels (Figure 1A). At the 0.5 nM dose, the increase was 40-, 13-, and 11-fold, respectively, relative to vehicle control. Rifampin also caused a dose-dependent increase in CYP3A4 mRNA levels, but had no effect on CYP24A1 and TRPV6 transcription (Figure 1B). At the highest dose tested (50 μM), there was an 18-fold increase in CYP3A4 mRNA, in comparison to vehicle control.
2. Time-dependent effects of $1\alpha,25(\text{OH})_2\text{D}_3$ and rifampin on CYP3A4, TRPV6 and CYP24A1 mRNA. LS-180 cells were treated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ or 50 μM rifampin to establish a time course of response profile. Cells were sampled at 0, 4, 8, 12, 24, 36, 48 hrs post-dose. $1\alpha,25(\text{OH})_2\text{D}_3$ treatment caused an apparent up-regulation in the transcription of all three VDR gene targets and achieved its maximum effect on mRNA levels at 24 hrs post dose, then declined thereafter (Figure 1C). In contrast, following rifampin treatment, CYP3A4 mRNA levels increased roughly linearly with time throughout the 48 hr treatment period, while TRPV6 and CYP24A1 mRNA levels were unchanged (Figure 1D).

3.2 Effect of Rifampin Pretreatment on $1\alpha,25(\text{OH})_2\text{D}_3$ -Stimulated Gene Expression

To evaluate the effects of rifampin pretreatment on target gene response to $1\alpha,25(\text{OH})_2\text{D}_3$, a two-stage sequential treatment design was adopted (Figure 2). This design attempts to mimic the pulsatile nature of oral rifampin (and other hPXR agonists) exposure in the small intestine following oral administration. Cells were first pretreated with 50 μM rifampin to induce CYP3A4 enzymatic activity or with 0.1% DMSO as control. After preincubation, rifampin was removed by washing with PBS twice, and cells were then incubated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ or the 0.1% ethanol vehicle for an additional period of time (0, 4, 8, 12, 16 and 24 hrs).

1. Effects on CYP3A4. CYP3A4 mRNA content was increased approximately 12–24 fold following 48 hrs of rifampin treatment (Figure 2A, $t = 0$). Removal of rifampin from the culture medium after the pretreatment period led to a decline in CYP3A4 mRNA content that approached baseline after 24 hrs. The administration of rifampin prior to $1\alpha,25(\text{OH})_2\text{D}_3$ treatment caused a 2-fold increase in CYP3A4 mRNA levels at its peak compared to that in cells getting $1\alpha,25(\text{OH})_2\text{D}_3$ and no rifampin pretreatment (86-fold vs 43-fold overall, Figure 2A). CYP3A (midazolam 1'-hydroxylation) activity was induced 4.6-fold following rifampin pretreatment (Figure 2B), and it declined to baseline over 24 hrs in the absence of $1\alpha,25(\text{OH})_2\text{D}_3$ treatment. Treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ alone caused a 4.3-fold maximal increase in CYP3A activity at 16 hrs. In contrast, there was a superinduction of CYP3A activity (20-fold overall) in cells receiving both rifampin pretreatment and $1\alpha,25(\text{OH})_2\text{D}_3$ treatment, with an apparent peak effect at 16 hrs after the hormone dose, and that effect persisted out to 24 hrs (Figure 2B).
2. Effects on CYP24A1 and TRPV6. In contrast to its effect on CYP3A4, the expression of CYP24A1 and TRPV6 was not changed by 50 μM rifampin pretreatment (Figures 2C and 2D; $t = 0$). Treatment with $1\alpha,25(\text{OH})_2\text{D}_3$ caused an expected time-dependent increase in CYP24A1 mRNA that peaked at 12–16 hrs and declined to near baseline levels by 24 hrs (Figure 2D). There was no significant difference in the maximal CYP24A1 response to $1\alpha,25(\text{OH})_2\text{D}_3$ between rifampin pretreated and vehicle pretreated cells, although the response appeared to peak earlier in the rifampin pretreated cells.

By comparing results from cells pretreated with rifampin versus with vehicle followed by $1\alpha,25(\text{OH})_2\text{D}_3$ treatment, we found that prior exposure to rifampin (with the accompanying CYP3A4 induction) blunted the maximal transcriptional activation of TRPV6 by approximately 35% (Figure 2C). The direction of this effect was the same for all replicate experiments at the 12, 16 and 24 hr time points, with significant differences observed at 16 and 24 hrs ($p = 0.0003$ and $p = 0.02$ respectively).

3.3 Effect of Pretreatment with Other PXR Agonists on CYP3A4 Activity and TRPV6 Expression

We next determined whether pretreatment with other hPXR activators could lead to a reduction in $1\alpha,25(\text{OH})_2\text{D}_3$ -induced TRPV6 expression similar to that caused by rifampin; the drugs phenobarbital, hyperforin, and carbamazepine were tested. The dosing concentration for each drug was selected based upon three factors: concentration for maximum CYP3A4 inductive effect *in vitro*, *in vivo* pharmacological relevance, and the absence of cellular toxicity. A similar two-stage pretreatment/treatment protocol to that described for rifampin (Figure 2) was performed. Rifampin was used as a positive control, while levetiracetam, a new generation anti-epileptic drug that does not activate hPXR, was used as a negative control.

Following 48 hrs of exposure to 50 μ M rifampin, 400 μ M phenobarbital or 500 nM hyperforin, CYP3A4 mRNA expression was enhanced by 16-, 5- and 4-fold ($p < 0.05$) and CYP3A4 enzymatic activity was increased by 2.1-, 2.0-, 1.3-fold ($p < 0.05$), respectively. In contrast, carbamazepine (a weak hPXR agonist) and levetiracetam (a non-hPXR agonist) caused no significant change in CYP3A4 mRNA content and CYP3A enzyme activity (Table 1). None of the drugs tested caused a direct change in TRPV6 expression (data not shown).

In comparison to vehicle control, pretreatment of LS180 cells with phenobarbital or hyperforin resulted in a decreased TRPV6 transcriptional response to low-dose $1\alpha, 25(\text{OH})_2\text{D}_3$ (Table 1). The magnitude of reduction in TRPV6 expression was approximately 29% ($p < 0.05$) and 15% ($p < 0.05$) for phenobarbital and hyperforin, respectively, compared to 32% ($p < 0.05$) for rifampin. In contrast, carbamazepine and levetiracetam had no significant effect on $1\alpha, 25(\text{OH})_2\text{D}_3$ -stimulated TRPV6 mRNA expression.

3.4 Cellular $1\alpha, 25(\text{OH})_2\text{D}_3$ Catabolism with Rifampin Pretreatment and Its Relationship to TRPV6 Expression

The depletion of $1\alpha, 25(\text{OH})_2\text{D}_3$ in cell medium (0.5 nM starting concentration) and cell lysate during a dose interval was measured in order to determine whether or not the apparent repressive effect of rifampin on $1\alpha, 25(\text{OH})_2\text{D}_3$ -dependent TRPV6 transcription could be attributed to enhanced $1\alpha, 25(\text{OH})_2\text{D}_3$ catabolism. In cell media, for both rifampin pretreated (50 μ M for 48 hrs) and control cells, no significant elimination of $1\alpha, 25(\text{OH})_2\text{D}_3$ was observed during the first 4 hrs period (Figure 3A). However, by 24 hrs post- $1\alpha, 25(\text{OH})_2\text{D}_3$ dose, 25% of the amount applied had been cleared from the media of control cells. In contrast, for cells pretreated with rifampin, 37% of the total $1\alpha, 25(\text{OH})_2\text{D}_3$ dose was lost within the same 24 hrs period; this represented a 48% relative change compared to cells without rifampin pretreatment ($p < 0.05$, Figure 3A). Fitting a first order elimination equation to the data for the entire $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment period indicated that there was a 48% increase in the disappearance rate of $1\alpha, 25(\text{OH})_2\text{D}_3$ in rifampin pretreated cells, compared to vehicle pretreated cells.

In the cell lysate, during the first 4 hrs, $1\alpha, 25(\text{OH})_2\text{D}_3$ was detectable but below the limitation of quantification. At 16 and 24 hrs post-dose, the amount of $1\alpha, 25(\text{OH})_2\text{D}_3$ in the lysate of cells pretreated with rifampin was lower (22–31%) than that found in cells that did not get rifampin pretreatment (mean change of two experiments), although there was little difference in the cellular amount between the 12–24 hr time-points, for both control and rifampin pretreatment groups (Figure 3B).

3.5 DHB Selectively Inhibited CYP3A4 Activity, Slowed $1\alpha, 25(\text{OH})_2\text{D}_3$ Elimination and Enhanced TRPV6 Expression

To evaluate the specific contribution of CYP3A4 to the rifampin enhanced depletion of $1\alpha, 25(\text{OH})_2\text{D}_3$, a selective CYP3A4 inhibitor, 6',7'-dihydroxybergamottin (DHB), was added to the LS180 cell culture media after 48 hrs of rifampin or DMSO pretreatment and continuing through the 24 hr period of $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment in order to knockdown CYP3A4 activity (Figure 4). The addition of 20 μ M DHB caused a 90% reduction in CYP3A4 activity (both basal and rifampin-induced midazolam 1'-hydroxylation), and that effect lasted throughout the period of $1\alpha, 25(\text{OH})_2\text{D}_3$ treatment (Figure 4A). Moreover, DHB treatment caused a 63% ($p < 0.05$) and 60% (NS) increase in TRPV6 mRNA levels at 16 hrs and 24 hrs after the $1\alpha, 25(\text{OH})_2\text{D}_3$ dose, respectively, in cells getting rifampin pretreatment prior to $1\alpha, 25(\text{OH})_2\text{D}_3$ (Figure 4B).

Pretreatment with rifampin caused an acceleration in the elimination of $1\alpha,25(\text{OH})_2\text{D}_3$ from the cell media (Table 2). An apparent half-life of $1\alpha,25(\text{OH})_2\text{D}_3$ calculated from the mean elimination data was lower (28 hrs) for cells pretreated with rifampin, compared to those getting the vehicle (44 hrs), corresponding to a 57% increase in the $1\alpha,25(\text{OH})_2\text{D}_3$ disappearance rate. DHB treatment effectively reversed the induction of $1\alpha,25(\text{OH})_2\text{D}_3$ elimination by rifampin pretreatment. The percentage of the $1\alpha,25(\text{OH})_2\text{D}_3$ dose remaining in cells pretreated with both rifampin and DHB at 16 and 24 hrs was 20% ($p < 0.05$) and 22% ($p < 0.05$) higher, respectively, than that found in cells pretreated with rifampin only (Table 2). This corresponded to a $1\alpha,25(\text{OH})_2\text{D}_3$ elimination half-life of 44 hrs for rifampin pretreated/DHB treated cells, compared to 28 hrs for cells getting rifampin pretreatment alone.

Another series of control experiments were performed to evaluate direct effects (if any) of DHB on gene expression in LS180 cells. There was no change in the cell content of CYP24A1, TRPV6, VDR and PXR mRNA after 24 hrs of exposure to 20 μM DHB, but a slight increase in CYP3A4 mRNA content. DHB was found not to be an antagonist, but a “weak” agonist of PXR function in a cell-based reporter assay (data not shown). In addition, because $1\alpha,25(\text{OH})_2\text{D}_3$ induced CYP24A1 transcription in the LS180 cell model (Figure 2D), which might lead to an increase in 24R-hydroxylase activity in the later part of the hormone treatment period, we sought to rule out a direct inhibitory effect of DHB on CYP24A1 activity and confirm its CYP3A4 selectivity. 24-Hydroxylation of $1\alpha,25(\text{OH})_2\text{D}_3$ and $25(\text{OH})\text{D}_3$ in LS180 cells was too slow to permit quantitation of product formation. However, CYP24A1 is highly induced in Caco-2 cells treated with $1\alpha,25(\text{OH})_2\text{D}_3$ [16, 33, 34]. Accordingly, Caco-cells were cultured with 250 nM $1\alpha,25(\text{OH})_2\text{D}_3$ for 2 weeks and then exposed to a single 50 ng/mL dose of $25(\text{OH})\text{D}_3$. The cell culture media was collected 24 hrs later and subjected to mass spectrometric analysis. Co-treatment with 20 μM DHB (4 hr prior to and during the $25(\text{OH})\text{D}_3$ incubation period) had no effect on 24R-hydroxylation activity (240 ± 20 vs 250 ± 15 pg/day/mg cell lysate protein; $p = 0.40$) in the cell system (Supplemental Figure 1).

3.6 Enhanced $1\alpha,25(\text{OH})_2\text{D}_3$ Elimination and Reduced TRPV6 Response in hPXR-overexpressed LS180 Cells Treated with Rifampin

In order to evaluate further the role of hPXR in the repression of TRPV6 expression following pretreatment with rifampin, we conducted a series of experiments using LS180 cells that had been genetically engineered to overexpress the hPXR.1 gene product [35]. In comparison to LS180 cells that were stably transfected with the gene vector alone (MOCK-LS180), cells stably transfected with the hPXR.1 gene (hPXR.1-LS180) showed an 8-fold higher level of PXR mRNA, a 2-fold higher level of CYP3A4 mRNA, a slightly lower (20%) level of TRPV6 mRNA, and a slightly reduced (20%) level of CYP24A1 mRNA (Supplemental Table 2). In addition, the VDR mRNA content in the hPXR.1-LS180 cells was 66% lower than that seen in the MOCK-LS180 cells. Accordingly, the relative effect of 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ alone on the expression of CYP3A4, TRPV6, and CYP24A1 in hPXR.1-LS180 cells was muted in comparison to that seen in MOCK-LS-180 cells treated with the hormone.

Examining the time-course of $1\alpha,25(\text{OH})_2\text{D}_3$ effect, CYP3A4, CYP24A1 and TRPV6 mRNA levels were increased 2-fold, 5-fold, and 4-fold after 24 hrs of hormone exposure in hPXR.1-LS180 cells, compared to a 15-fold, 30-fold and 8-fold inductive response in MOCK-LS-180 cells also treated with the hormone (Supplemental Figure 2).

1. Effect of Rifampin on Gene Expression in hPXR.1-LS180 Cells. As shown in Figure 5A, a 48 hr treatment with 50 μM rifampin alone resulted in a much greater increase in the level of CYP3A4 mRNA in hPXR.1-LS180 cells (120-fold),

compared to that seen in MOCK-LS180 cells (5-fold). Similarly the induction of CYP3A4 catalytic activity (midazolam 1'-hydroxylation) with rifampin treatment was much greater in hPXR.1-LS180 cells (45-fold), compared to that seen in MOCK-LS180 cells (2.5-fold), consistent with a higher level of receptor protein for agonist binding. Surprisingly, the CYP24A1 mRNA content was also much higher following rifampin treatment (200-fold) in hPXR.1-LS180 cells, whereas the drug had no effect in MOCK-LS180 cells.

2. Enhanced $1\alpha,25(\text{OH})_2\text{D}_3$ Elimination in hPXR.1-LS180 Cells Pretreated with Rifampin. The time-course and extent of $1\alpha,25(\text{OH})_2\text{D}_3$ elimination in hPXR.1-LS180 cells was compared to that occurring in MOCK-LS180 cells. The rate and extent of $1\alpha,25(\text{OH})_2\text{D}_3$ elimination was greater in hPXR.1-LS180 cells than that in MOCK-LS180 cells when they were pretreated with rifampin, but not different when they received vehicle pretreatment (Table 3). At the end of the 24 hr incubation period, 39% vs 54% of the $1\alpha,25(\text{OH})_2\text{D}_3$ dose was lost from the media of cells getting rifampin (50 μM) pretreatment in MOCK-LS180 cells vs hPXR.1-LS180 cells, respectively, which was significantly different from the respective losses in MOCK-LS180 (25%, $p = 0.05$) and hPXR.1-LS180 (31%, $p = 0.05$) cells pretreated with DMSO only, but not rifampin.

The apparent half-life of $1\alpha,25(\text{OH})_2\text{D}_3$ was 67 hr and 46 hr in vehicle pretreated MOCK-LS180 and hPXR.1-LS180 cells, respectively, whereas it was 33 hr and 22 hr in the same type of cells pretreated with rifampin. Thus, rifampin pretreatment caused a 110% increase in $1\alpha,25(\text{OH})_2\text{D}_3$ disappearance rate in hPXR.1-LS180 cells, and 100% in MOCK-LS180 cells. Moreover, the absolute clearance efficiency was about 51% higher in the hPXR.1-LS180 cells, in comparison to the MOCK-LS180 cells, following rifampin pretreatment.

3. Repression of $1\alpha,25(\text{OH})_2\text{D}_3$ -Mediated TRPV6 Induction by Rifampin and Reversal of that Effect by DHB. Looking specifically at TRPV6 expression following 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ administration, rifampin pretreatment was associated with a 60% reduction ($p < 0.05$) in the 12 hr transcriptional response of TRPV6 to $1\alpha,25(\text{OH})_2\text{D}_3$ in hPXR.1-LS180 cells (Figure 5B). This was in comparison to a 30% reduction ($p < 0.05$) in TRPV6 mRNA levels in MOCK-LS180 cells. This difference in response to rifampin between cell types was also significant ($p < 0.05$). Again, although $1\alpha,25(\text{OH})_2\text{D}_3$ elicited a weaker transcriptional response in hPXR.1-LS180 cells, compared to MOCK-LS180 cells (Figure 5B), the hPXR.1-LS180 cells were more sensitive to the effect of rifampin pretreatment on genes selectively induced by $1\alpha,25(\text{OH})_2\text{D}_3$ (e.g., TRPV6).

To test whether CYP3A4 was part of a causal pathway mediating the effect of rifampin pretreatment on $1\alpha,25(\text{OH})_2\text{D}_3$ elimination and TRPV6 expression in hPXR.1-LS180 cells, the CYP3A4 inhibitor DHB was included in the treatment protocol (as described above). As seen in Figure 5C, DHB treatment caused a 48% and 86% increase in TRPV6 mRNA levels in vehicle control and rifampin pretreated hPXR.1-LS180 cells, respectively ($p < 0.01$ and $p < 0.05$).

4. Discussion

In this investigation, we sought to test whether an increase in the expression of intestinal CYP3A4 by treatment with hPXR agonists, and enhanced catabolic elimination of $1\alpha,25(\text{OH})_2\text{D}_3$, can alter the expression of VDR gene targets encoding calcium transport proteins. CYP3A4 is capable of metabolizing $1\alpha,25(\text{OH})_2\text{D}_3$ to 23S- and 24R-trihydroxy vitamin D₃ products [16]. Thus, if an induction of CYP3A4 were to occur chronically *in vivo*, the increase in $1\alpha,25(\text{OH})_2\text{D}_3$ clearance could effectively lower the intestinal cellular

steady-state concentration of active hormone and suppress calcium transport protein synthesis (Figure 6). Results presented in this paper support that hypothesis.

In the current study, we found that induction of CYP3A4 in cultured LS180 cells by pretreatment with rifampin predisposed the cells to a higher clearance and shorter half-life of $1\alpha,25(\text{OH})_2\text{D}_3$ upon hormone treatment, as well as a lower intracellular concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ and a reduction in the transcriptional activation of the intestinal calcium transporter, TRPV6. A causal relationship between CYP3A4 induction and the reduction in TRPV6 transcription is supported by experiments showing that the loss of CYP3A activity following pretreatment with the suicide CYP3A4 inhibitor, DHB, reversed the effects of rifampin on both $1\alpha,25(\text{OH})_2\text{D}_3$ clearance and TRPV6 expression. While the magnitude of reduction in cellular TRPV6 mRNA content with rifampin pretreatment was modest (30–40%, Figure 2C), it was consistent with the modest (36%) decrease in the $1\alpha,25(\text{OH})_2\text{D}_3$ half-life, and with the moderate but significant differences in both $1\alpha,25(\text{OH})_2\text{D}_3$ concentration and cellular TRPV6 mRNA content seen 16 and 24 hrs after $1\alpha,25(\text{OH})_2\text{D}_3$ administration (Table 2).

Demonstration of this drug-hormone interaction required careful selection of $1\alpha,25(\text{OH})_2\text{D}_3$ and rifampin doses and the timing of drug administration. We chose a $1\alpha,25(\text{OH})_2\text{D}_3$ dosing concentration (0.5 nM) that was close to the endogenous circulating hormone concentration [30, 36, 37], but that could also exert sustained effects on gene transcription in the LS180 cells. We recognize that this represents an artificial, single-dose cell culture system. However, under this treatment condition, we were able to demonstrate enhanced clearance of $1\alpha,25(\text{OH})_2\text{D}_3$ and decreased cellular hormone exposure over the 24 hr dosing interval with rifampin pretreatment. This should have reduced net occupancy of the vitamin D receptor and caused the diminution of TRPV6 transcriptional activation, which we observed.

Another important feature of the interaction we describe was staggering of the rifampin and $1\alpha,25(\text{OH})_2\text{D}_3$ doses. Initial pilot experiments that involved co-administration of rifampin and $1\alpha,25(\text{OH})_2\text{D}_3$, resulted in a marked induction of CYP3A4 mRNA, but paradoxically a reduction in CYP3A4 catalytic activity (data not shown). This outcome was thought to be the result of competitive CYP3A4 inhibition by rifampin, a phenomenon we reported previously in experiments with primary human hepatocytes [38]. Reflecting on the phenomenon further, it seemed reasonable to adopt a sequential cell treatment strategy where rifampin was removed from the cell system after it had exerted its effects on CYP3A4 synthesis because this would mimic what might be encountered *in vivo*. Once a day rifampin dosing should elicit a transiently high inducer concentration in the intestinal mucosal epithelial barrier during the drug absorption period, followed by a relatively rapid clearance of rifampin by non-CYP3A4 clearance pathways ($t_{1/2} \sim 3$ hrs). This, it was anticipated, should lead to significant periods of enhanced intestinal CYP3A4 activity and $1\alpha,25(\text{OH})_2\text{D}_3$ clearance and a change in target gene transcription, which was observed.

Our results do not rule out the possibility that induction of CYP24A1 by $1\alpha,25(\text{OH})_2\text{D}_3$ contributed to the clearance of the hormone during the 24 hr hormone dosing period. Under the dosing conditions employed, CYP24A1 mRNA levels rose more than 50-fold within 12–16 hrs after addition of $1\alpha,25(\text{OH})_2\text{D}_3$. But the mRNA effect appeared transient, with near complete reversal by 24 hrs. This biphasic response of CYP24A1 to $1\alpha,25(\text{OH})_2\text{D}_3$ is similar what has been described *in vivo* in animals receiving repeated $1\alpha,25(\text{OH})_2\text{D}_3$ treatments [39, 40]. Although we were not able to measure 24R-hydroxylase activity in the LS180 cells, it could have had some importance during the 12–24 hr dose interval. However, we noted no significant difference in the time-course of CYP24A1 induction in rifampin pretreated, compared to control cells, suggesting that the effect of rifampin on TRPV6 transcription was the result of the increase in CYP3A4 catalytic activity (23R- and 24S-

hydroxylation) and not enhanced CYP24A1 activity (24R- and 23S-hydroxylation). The impact of DHB on CYP3A4 activity and TRPV6 expression lends further credence to this hypothesis, because the CYP3A4 inhibitor did not alter CYP24A1 activity in Caco-2 cell culture (Supplemental Figure 2) or in a reconstituted enzyme system (data not shown).

That the effect of rifampin pretreatment on TRPV6 expression was mediated by hPXR is supported by our finding that stable over-expression of the receptor caused a greater sensitivity of TRPV6 expression to pre-treatment with the hPXR agonist than that seen with native or MOCK-treated LS180 cells. First, there was a significant difference in the disappearance of $1\alpha,25(\text{OH})_2\text{D}_3$ in the hPXR-LS180 and MOCK-LS180 cells treated with rifampin (Table 3). Second, over-expression of hPXR was accompanied by a more pronounced induction of CYP3A4 activity and more rapid clearance of $1\alpha,25(\text{OH})_2\text{D}_3$ (shorter half-life) with rifampin pretreatment. Although CYP24A1 was once again induced by $1\alpha,25(\text{OH})_2\text{D}_3$ in the hPXR-overexpressed cells, as well as by rifampin pretreatment, the fact that co-treatment with DHB partially reversed the rifampin effect on both $1\alpha,25(\text{OH})_2\text{D}_3$ clearance and TRPV6 expression suggests an important role for CYP3A4 in the drug-hormone interaction, even with the artificial, high level of transcription factor expression and the anomalous effect of rifampin on CYP24A1 expression. In this regard, it should be stressed that rifampin and other hPXR agonists did not alter CYP24A1 expression in native or MOCK-transfected cells, which is consistent with our previous failure to observe any evidence of CYP24A1 induction in duodenal biopsies of healthy human volunteers receiving rifampin every 6 hrs for 2–14 days [17].

The cause of CYP24A1 induction by rifampin in the presence of artificially high levels of hPXR is unclear. One possible explanation is that the supra-physiological levels of hPXR bind to a reported weak response element found on the CYP24A1 gene [41]. We have shown previously that hPXR can suppress transcription of the CYP24A1 gene in response to $1\alpha,25(\text{OH})_2\text{D}_3$ in a transiently transfected cell culture system [17]. Thus, the observed effect of supra-physiologic hPXR may also be the result of secondary changes in gene regulation in the stably transfected cells that was not tested for, or possibly due to the marked reduction in VDR that accompanied stable hPXR transfection.

While pretreatment of LS180 cells with two other known hPXR agonists, phenobarbital and hyperforin, exerted similar effects as rifampin on $1\alpha,25(\text{OH})_2\text{D}_3$ clearance and TRPV6 expression, rifampin appear to be the most potent modulator tested. Interestingly, carbamazepine caused no significant changes in the TRPV6 response to $1\alpha,25(\text{OH})_2\text{D}_3$ and also had no effect on CYP3A4 transcription and midazolam hydroxylase activity. This difference in response may be attributed to the fact that the drug appears to be much better agonist for the hCAR receptor than hPXR [23] and that hCAR expression was undetectable in the LS180 cells (data not shown), or it could be due to the absence of critical drug-specific coactivators. Additional studies are needed to verify the applicability of our results with an isolated intestinal cell model to the *in vivo* human state.

Finally, the *in vitro* effects of DHB on the drug-vitamin D interaction suggest the possibility that regular consumption of grapefruit juice might help prevent drug-induced osteomalacia/osteoporosis. DHB is a major component of grapefruit juice and mediates the inactivation of intestinal CYP3A4 with periodic consumption of regular strength juice [42]. The dose of DHB (20 μM) in this study is comparable to the dietary level of normal strength grapefruit juice [42], which has been reported to efficiently inhibit the enteric CYP3A thereby altering bioavailability of CYP3A substrates; e.g. itraconazole [43], cyclosporine [44], tacrolimus [45], budesonide [46], oxycodone [47], and midazolam [48]. The inhibition of CYP3A4 is mainly localized in the gut and the net systemic effect is substrate dependent (a function of the intestinal first-pass drug extraction). In our study, *in vitro* evidence showed that DHB

diminished the enzymatic inactivation of $1\alpha,25(\text{OH})_2\text{D}_3$ by intestinal CYP3A4 and thereby increased the consequent transcriptional expression of TRPV6 (Table 2, Figure 4). If this is replicable in vivo, it could represent a simple preventative treatment for drug-induced vitamin D deficiency and osteopathy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Concentration- and time-dependent effects of rifampin and 1α,25(OH)₂D₃ on CYP3A4, CYP24A1 and TRPV6 expression in LS180 cells

Plated LS180 cells were treated with: A) 0, 0.1, 0.5 or 1 nM 1α,25(OH)₂D₃ for 12 hrs; B) 0, 10, 20 or 50 μM rifampin for 48 hrs; C) 0.5 nM 1α,25(OH)₂D₃ for 0 to 48 hrs; or D) 50 μM rifampin for 0 to 48 hrs. Levels of CYP3A4, CYP24A1 and TRPV6 mRNA were quantified by qRT-PCR analysis and normalized to the GAPDH mRNA content. Data in A) and B) are presented as a ratio of the measurements obtained with drug/hormone treatment compared to the vehicle (0.1% DMSO in A or 0.1% ethanol in B) treatment; mean of three replicate incubations ± SD are shown. Data in C) and D) are presented as a ratio of the measurements obtained at various time points to that obtained at t = 0 hrs; for each time point, the average of duplicate incubations is shown.

Figure 2. CYP3A4, CYP24A1 and TRPV6 expression and activity following sequential treatment with rifampin and $1\alpha,25(\text{OH})_2\text{D}_3$

Plated cells were pretreated with 50 μ M rifampin or 0.1% DMSO (vehicle) for 48 hrs. At the end of the pretreatment period, cells were washed with cold PBS to remove rifampin and then treated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ or 0.1% ethanol (vehicle) for the indicated period of time (0, 4, 8, 12, 16 and 24 hrs). qRT-PCR analysis of mRNA was performed for measurement of A) CYP3A4, C) TRPV6 and D) CYP24A1 mRNA content in the cell lysate. These data were normalized to the level of GAPDH mRNA and are presented as the ratio of the indicated drug treatment to sequential vehicle control (0.1% DMSO or 0.1% ethanol). B) At the indicated times, treated cells were also incubated for 30 min with 8 μ M MDZ for measurement of a 1'-OH MDZ formation rate. Observed rates for the different drug treatment conditions were normalized for the measured sequential vehicle control rates. For all time-points, the mean $\pm$ SD of 4 replicate experimental results are shown.

Figure 3. Effect of rifampin pretreatment on the elimination of $1\alpha,25(\text{OH})_2\text{D}_3$ from the LS180 cell culture media and cell lysate

Plated cells were pretreated with 50 μM rifampin or 0.1% DMSO (vehicle) for 48 hrs. At the end of the pretreatment period, cells were washed with cold PBS to remove rifampin and then treated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ or 0.1% ethanol (vehicle) for the indicated period of time (0, 4, 8, 12, 16 and 24 hrs). A) $1\alpha,25(\text{OH})_2\text{D}_3$ in cell media. Data are presented as a percentage of the amount detected at $t = 0$; mean $\pm$ SD of 5 replicate experiments are shown. B) $1\alpha,25(\text{OH})_2\text{D}_3$ in cell lysate. Data are presented as a mean of two independent experiments. The concentration of $1\alpha,25(\text{OH})_2\text{D}_3$ was normalized to total protein content per well as indicated.

Figure 4. Effect of DHB treatment on CYP3A4 activity and TRPV6 expression in LS180 cells

A) Plated LS180 cells were treated with 50 μ M RIF or 0.1% DMSO for 48 h, washed, and then treated with 20 μ M DHB or 0.1% ethanol for 4 hrs. Following that, cells were incubated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ + 20 μ M DHB or 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ alone for the indicated time, washed and then incubated with 8 μ M MDZ for 30 min; formation of 1'-OH MDZ was then measured. B) Cells were treated as described for part A), except that TRPV6 mRNA content in the cell lysate was measured by qRT-PCR at the indicated time. Data are presented as a fold-change in CYP3A4 activity or TRPV6 content, relative to baseline (vehicle control pretreatment); * indicates $p < 0.05$ for comparison of cells pretreated with rifampin + DHB vs. rifampin alone.

Figure 5. Effects of rifampin and $1\alpha,25(\text{OH})_2\text{D}_3$ in hPXR.1-LS180 and MOCK-LS180 cells

A) Plated hPXR.1-LS180 and MOCK-LS180 cells were treated with 50 μM rifampin or 0.1% DMSO for 48 hrs. CYP3A4 mRNA and MDZ 1'-hydroxylation activity was measured at the end of the pretreatment period. B) Plated hPXR.1-LS180 and MOCK-LS180 cells were treated with 50 μM rifampin or 0.1% DMSO for 48 hrs, washed and treated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ for 12 hrs. TRPV6 mRNA content in the cell lysate was measured at the end of the pretreatment period (for "control" and "rifampin") or at the end of the treatment period (for " $1\alpha,25(\text{OH})_2\text{D}_3$ " or "rifampin/ $1\alpha,25(\text{OH})_2\text{D}_3$ "). C) Plated hPXR.1-LS180 cells and MOCK-LS180 cells were treated with 50 μM RIF or 0.1% DMSO for 48 hrs, washed, and then treated with 20 μM DHB or 0.1% ethanol for 4 hrs. Following that, cells were incubated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ + 20 μM DHB or 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ alone for 12 hrs. TRPV6 mRNA content was compared. Data are presented as the fold-change with the drug treatment relative to vehicle control; mean $\pm$ SD of 3 replicate experiments are shown. *, $p < 0.05$ for indicated comparison in (B) and (C).

Figure 6.

A proposed mechanism for the regulation of $1\alpha,25(\text{OH})_2\text{D}_3$ -mediated TRPV6 expression by CYP3A4 induced by hPXR agonists.

Table 1
Effect of hPXR agonists on CYP3A4 mRNA and catalytic activity, and TRPV6 mRNA in response to 1 α ,25(OH) $_2$ D $_3$ in cultured LS180 cells

LS180 cells were pretreated with 50 μ M rifampin, 400 μ M phenobarbital, 500 nM hyperforin, 50 μ M carbamazepine, and 200 μ M levetiracetam or 0.1% DMSO (vehicle) for 48 hrs.

	hPXR Agonist ^a		Agonist/1 α ,25(OH) $_2$ D $_3$ ^b
	CYP3A4 mRNA	CYP3A4 Activity	TRPV6 mRNA
Control	1.0	1.0	1.0
Rifampin	16.3 $\pm$ 7.9 *	2.1 $\pm$ 0.3 *	0.67 $\pm$ 0.28 *
Phenobarbital	5.3 $\pm$ 2.8 *	2.0 $\pm$ 0.2 *	0.72 $\pm$ 0.18 *
Hyperforin	4.1 $\pm$ 1.0 *	1.3 $\pm$ 0.3 *	0.85 $\pm$ 0.20 *
Carbamazepine	1.2 $\pm$ 0.5	1.1 $\pm$ 0.1	0.91 $\pm$ 0.01
Levetiracetam	1.1 $\pm$ 0.1	1.0 $\pm$ 0.1	1.03 $\pm$ 0.06

^a At the end of preincubation, cells were incubated with midazolam for the measurement of CYP3A4 activity, and then the cell pellets were collected for mRNA extraction.

^b Cells were washed with PBS to remove the pretreatment drugs and then treated with 0.5 nM 1 α ,25(OH) $_2$ D $_3$ for 24 hrs. qRT-PCR analysis was performed for measurement of TRPV6 mRNA content in the cell lysate. Data are normalized to the level of GAPDH mRNA and presented as the ratio of the indicated drug treatment to vehicle-pretreated control (0.1% DMSO/1 α ,25(OH) $_2$ D $_3$). Mean $\pm$ SD are shown;

*, p < 0.05.

Table 2
Enhancement of $1\alpha,25(\text{OH})_2\text{D}_3$ elimination by rifampin pretreatment and inhibition by DHB in LS180 cells

LS180 cells were pretreated with 50 μM rifampin or 0.1% DMSO (vehicle) for 48 hrs. At the end of preincubation period, cells were washed and treated with the CYP3A4 inhibitor DHB (20 μM) or culture media alone for 4 hrs, washed again and then treated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ + DHB (20 μM) for the indicated time, ranging from 0 to 24 hrs. At that point, cell media was collected for the measurement of $1\alpha,25(\text{OH})_2\text{D}_3$ concentration, as described in Methods. Data are presented as a percentage of the starting concentration. Mean $\pm$ SD of results from 3 experiments is shown.

Pretreatment	0.1% DMSO		50 μM Rifampin	
	(-) DHB	(+) DHB	(-) DHB	(+) DHB
	<i>(% $1\alpha,25(\text{OH})_2\text{D}_3$ Dose Remaining in Media)</i>			
0 hr	100 $\pm$ 6.9	100 $\pm$ 6.6	100 $\pm$ 6.9	100 $\pm$ 4.4
12 hr	78.6 $\pm$ 2.6	82.1 $\pm$ 3.8	76.3 $\pm$ 2.0	80.1 $\pm$ 1.5
16 hr	75.9 $\pm$ 3.2	83.0 $\pm$ 4.0	63.5 $\pm$ 2.2 *	76.3 $\pm$ 5.2 #
24 hr	68.9 $\pm$ 7.1	77.4 $\pm$ 13.61	56.0 $\pm$ 6.7 *	68.4 $\pm$ 6.2 #
Half-life (hr)	44	66 #	28	44

*, $p < 0.05$ for effect of rifampin, compared to corresponding DMSO pretreated cells;

$p < 0.05$ for effect of DHB in corresponding DMSO or rifampin pretreated cells. In addition, there was no significant difference between $1\alpha,25(\text{OH})_2\text{D}_3$ concentration in cells pretreated with DMSO and no DHB compared to cells pretreated with rifampin and DHB, at all of the time-points examined. An apparent (recognizing the limited sampling time) elimination half-life was calculated using mean concentrations and assuming monoexponential elimination kinetics. The regression coefficient (r) was > 0.96 for all four of the datasets.

Table 3
Effect of rifampin pretreatment on $1\alpha,25(\text{OH})_2\text{D}_3$ elimination in MOCK-LS180 and hPXR.1-LS180 cells

LS180 cells were pretreated with 50 μM rifampin or 0.1% DMSO (vehicle) for 48 hrs. At the end of preincubation period, cells were washed and then treated with 0.5 nM $1\alpha,25(\text{OH})_2\text{D}_3$ for the indicated time, ranging from 0 to 24 hr. At that point, cell media was collected for the measurement of $1\alpha,25(\text{OH})_2\text{D}_3$ concentration, as described in Methods. Data are presented as a percentage of the starting concentration. Mean $\pm$ SD of results from 3 experiments is shown.

Cell Types	MOCK-LS180		hPXR.1-LS180	
Pretreatment	DMSO	Rifampin	DMSO	Rifampin
<i>(% $1\alpha,25(\text{OH})_2\text{D}_3$ Dose Remaining in Media)</i>				
0 hr	100 $\pm$ 3.6	100 $\pm$ 4.5	100 $\pm$ 4.2	100 $\pm$ 3.8
4 hr	93.5 $\pm$ 4.3	94.4 $\pm$ 4.7	94.4 $\pm$ 6.2	77.7 $\pm$ 3.1 *
8 hr	86.0 $\pm$ 3.3	88.0 $\pm$ 0.7	84.5 $\pm$ 2.2	64.4 $\pm$ 7.4 *
12 hr	82.9 $\pm$ 6.9	80.5 $\pm$ 3.2	86.3 $\pm$ 5.3	51.3 $\pm$ 9.6 *
24 hr	75.1 $\pm$ 2.7	60.9 $\pm$ 1.5 *	69.2 $\pm$ 6.7	46.0 $\pm$ 5.7 *
Half-life (hr)	67	33	46	22 *

* $p < 0.05$ for effect of rifampin, compared to corresponding DMSO pretreated cells. An apparent (recognizing the limited sampling time) elimination half-life was calculated using mean concentrations and assuming monoexponential elimination kinetics. The regression coefficient (r) was > 0.92 for all four of the datasets.