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Up-Regulation of Transporters and Enzymes by the Vitamin D Receptor Ligands, 1α,25-Dihydroxyvitamin D_3 and Vitamin D Analogs, in the Caco-2 Cell Monolayer

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ABSTRACT

The effects of 1α,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] on gene expression and function were studied in Caco-2 cells. Microarray analyses, real-time quantitative polymerase chain reactions, and Western blotting were used to determine the mRNA and protein expression of transporters and enzymes after 1,25$(OH)_2D_3$ or vehicle (0.1% ethanol) treatment for 1, 3, 6, and 10 days. The mRNA and protein expressions of the apical sodium-dependent bile acid transporter, oligopeptide transporter 1, multidrug resistance-associated protein (MRP) 3, and sulfotransferase 1E1 remained unchanged with 1,25$(OH)_2D_3$ treatment, whereas those for CYP3A4, multidrug resistance protein 1, and MRP2 were significantly increased ($P < 0.05$). 1,25$(OH)_2D_3$ treatment significantly enhanced MRP4 protein expression by increasing protein stability without affecting mRNA expression, as confirmed in cycloheximide experiments. Marked increase in 6β-hydroxylation of testosterone by CYP3A4 was also observed in the 6-day 1,25$(OH)_2D_3$-treated (100 nM) cell lysate. The transport of [^{3}H]digoxin, the P-glycoprotein (P-gp) substrate, after treatment with 100 nM 1,25$(OH)_2D_3$ for 3 days revealed a higher apparent permeability (P_{app}) value in the basal (B)-to-apical (A) direction over that of vehicle treatment ($15.1 \pm 0.53 \times 10^{-6}$ versus $11.8 \pm 0.58 \times 10^{-6}$ cm/s; $P < 0.05$), whereas the P_{app} in the A-to-B direction was unchanged; the efflux ratio was increased (from 5.8 to 8.0). Reduced cellular retention of 5-(and-6)-carboxy-2′,7′-dichlorofluorescein, suggestive of higher MRP2 activity, was observed in the 3-day 100 nM 1,25$(OH)_2D_3$-treated cells over controls. Higher protein expression of CYP3A4, MRP2, P-gp, and MRP4 was also observed after a 6-day treatment with other vitamin D analogs (100 nM 1α-hydroxyvitamin D_3, 1α-hydroxyvitamin D_2 or Hectorol, and 25-hydroxyvitamin D_3) in Caco-2 cells, suggesting a role of 1,25$(OH)_2D_3$ and analogs in the activation of enzymes and transporters via the vitamin D receptor.

The intestine plays an important role in the absorption of orally administered drugs. The expression and proximity of metabolic enzymes and efflux transporters in the enterocyte contribute to intestinal first-pass removal and delimit the tissue accumulation of endo- and xenobiotics. In the small intestine, cytochrome P450 3A4 (CYP3A4) accounts for approximately 70% of total cytochrome P450 content and is responsible for the metabolism of approximately 50% of drugs currently in use (Pelkonen et al., 2008). The ATP-dependent drug efflux P-glycoprotein (P-gp), encoded by the multidrug resistance 1 gene (*MDR1*) located in the apical membrane of the enterocyte, is involved in the active excretion of a variety of lipophilic, cationic drugs from the intestine (Artursson et al., 2001). The multidrug resistance-associated proteins (MRPs), another important subfamily of

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ABBREVIATIONS: P-gp, P-glycoprotein; MDR1, multidrug resistance protein 1; MRP, multidrug resistance-associated protein; PXR, pregnane X receptor; CAR, constitutive androstane receptor; VDR, vitamin D receptor; VDRE, vitamin D response element; SULT, sulfotransferase; ASBT, apical sodium dependent bile acid transporter; PEPT1, oligopeptide transporter 1; 1,25$(OH)_2D_3$, 1α,25-dihydroxyvitamin D_3; 25$(OH)D_3$, 25-hydroxyvitamin D_3; l$\alpha$$(OH)D_3$, 1$\alpha$-hydroxyvitamin D_3; l$\alpha$$(OH)D_2$, 1$\alpha$-hydroxyvitamin D_2 or Hectorol; CDF, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein; CDF-DA, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate; DMEM, Dulbecco's modified Eagle's medium; HPLC, high-performance liquid chromatography; RT-qPCR, real-time quantitative polymerase chain reaction; aRNA, aberrant RNA; FBS, fetal bovine serum; EfR, efflux ratio; ITS, insulin-transferrin-selenium; SSC, standard saline citrate; TEER, transepithelial electrical resistance; A, apical; B, basolateral; HBSS, Hanks' balanced salt solution; P_{app}, apparent permeability; PBS, phosphate-buffered saline; DBP, vitamin D binding protein; PMEA, 9-(2-phosphonylmethoxyethyl)adenine; POM, pivaloxymethyl.

ATP-binding cassette transporters, are involved in the transport of unconjugated amphiphilic anions and glutathione, glucuronide, and sulfate conjugates. MRP2 is an efflux transporter localized in the apical membrane, whereas MRP1, MRP3, and MRP5 are basolateral efflux transporters. The localization of MRP4 in enterocytes has been inferred to exist at the basolateral membrane for efflux (Prime-Chapman et al., 2004).

The expression and function of drug metabolic enzymes and transporters are under regulation of nuclear receptors. In addition to the pregnane X receptor (PXR), the constitutive androstane receptor (CAR), farnesoid X receptor, and glucocorticoid receptor (for review, see Urquhart et al., 2007), the vitamin D receptor (VDR) is known to induce enzyme and transporter genes in the intestine. These include the human CYP3A4 (Thummel et al., 2001), SULT2A1 (Song et al., 2006), murine Mrp3 (McCarthy et al., 2005), and the rat apical sodium-dependent bile acid transporter (Asbt) (Chen et al., 2006).

1α,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the biologically active form of vitamin D, is the natural ligand of the VDR. Vitamin D is synthesized in the skin from its precursor, 7-dehydrocholesterol, in response to ultraviolet light, and it is converted to 25-hydroxyvitamin D_3 [25$(OH)D_3$] in the liver and then to 1,25$(OH)_2D_3$ by 1α-hydroxylase in the kidney (Darwish and DeLuca, 1996). Upon activation, the ligand-receptor complex recruits coactivators and heterodimerizes with the retinoid X receptor and then binds to the vitamin D response element (VDRE) and regulates target gene expression. During the past three decades, evidence has accumulated that 1,25$(OH)_2D_3$ mediates its biological activities through specific binding to the nuclear VDR. The therapeutic potential of 1,25$(OH)_2D_3$ is limited by its tendency to induce hypercalcemia (Prudencio et al., 2001). Vitamin D analogs that have lower hypercalcemic effects are potential therapeutic agents that can be used for treatment of human diseases and disorders (Stein and Wark, 2003). 1α-Hydroxyvitamin D_3 [1α$(OH)D_3$] is a prodrug of the active form of vitamin D_3 and undergoes metabolic conversion by 25-hydroxylase in the liver or intestine to 1,25$(OH)_2D_3$ before exerting its effect (Brickman et al., 1976). 1α-Hydroxyvitamin D_2 [1α$(OH)D_2$] or Hectorol is activated by 25-hydroxylase to produce 1,25$(OH)_2D_2$, a biologically active form of vitamin D_2, and it is reported to display significantly reduced hypercalcemia at therapeutic dosages for the treatment of secondary hyperparathyroidism (Brown, 2001).

The Caco-2 cell monolayer is a well established human carcinoma cell line that differentiates spontaneously in culture upon reaching confluence to become enterocyte-like cells that contain transporters and enzymes (Artursson et al., 2001). Effects of 1,25$(OH)_2D_3$ and the vitamin D analogs on the expression and activity of enzymes and transporters in the human intestine may thus be conveniently ascertained in Caco-2 cells due to high expression level of the VDR. Most transporters, with the exception of breast cancer resistance protein, are present at reasonably high levels (Taipalensuu et al., 2001). It had been demonstrated that CYP3A4 expression was up-regulated by 1,25$(OH)_2D_3$ via binding to the VDRE (Schmiedlin-Ren et al., 1997). More recently, a functional VDRE has been identified in the human MDR1 gene (Saeki et al., 2008). However, the role of the VDR on other target genes is unknown. In this study, we investigated the effects of 1,25$(OH)_2D_3$ and the vitamin D analogs on the expression and function of major metabolic enzymes and transporters in Caco-2 cells. To confirm that CYP3A4 function was catalytically induced by 1,25$(OH)_2D_3$ in Caco-2 cells, the activity of testosterone 6β-hydroxylase, a marker of induction of CYP3A4 (Chan et al., 2004), was measured. Digoxin, a prototypic substrate of P-gp, was used to evaluate P-gp function in Caco-2 cells (Cavet et al., 1996), whereas the fluorescent MRP2 substrate, 5-(and-6)-carboxy-2′,7′-dichlorofluorescein (CDF), whose diacetate prodrug 5-(and-6)-carboxy-2′,7′-dichlorofluorescein diacetate (CDF-DA) readily diffuses across the cell membrane for hydrolysis by intracellular esterases to form CDF, was used to evaluate MRP2 function (Tian et al., 2004).

Materials and Methods

Materials

1α,25$(OH)_2D_3$, lα$(OH)D_3$, 25$(OH)D_3$, cycloheximide, and glucose were purchased from Sigma-Aldrich Canada (Mississauga, ON, Canada). lα$(OH)D_2$ was kindly provided by Dr. Peter Bonate (Genzyme, Cambridge, MA). Testosterone and 6β-hydroxytestosterone were obtained from Sigma-Aldrich (St. Louis, MO). CDF-DA, Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum, 0.05% trypsin-EDTA, penicillin-streptomycin, and nonessential amino acids were all obtained from Invitrogen (Carlsbad, CA). A 12-well Transwell plate was purchased from Corning Life Sciences (Lowell, MA). [^{3}H]Digoxin (specific activity, 40 mCi/μmol), whose purity exceeded 98% as verified by HPLC, and [^{14}C]mannitol (specific activity, 51 mCi/mmol) were procured from PerkinElmer Life and Analytical Sciences (Boston, MA). The materials and reagents for real-time quantitative polymerase chain reaction (RT-qPCR) were purchased from Applied Biosystems (Foster City, CA). Reagents for mRNA sample preparation were from Sigma-Aldrich and Ambion (Austin, TX). Anti-CYP3A4, MRP2, and P-gp antibodies were purchased from Abcam (Cambridge, MA). Anti-villin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-VDR and anti-Cyp3A4 antibody were from Thermo Fisher Scientific (Waltham, MA) and BD Biosciences (Mississauga, ON, Canada), respectively. Donkey anti-rabbit IgG, sheep anti-mouse IgG, and the enhanced chemiluminescence were purchased from GE Healthcare (Baie d'Urfe, QC, Canada). Other antibodies were kindly provided by the following investigators: anti-PEPT1 (Dr. Wolfgang Sadee, The Ohio State University, Columbus, OH), anti-ASBT (Dr. Paul A. Dawson, Wake Forest University School of Medicine, Winston-Salem, NC), anti-MRP3 (Dr. Yuichi Sugiyama, University of Tokyo, Tokyo, Japan), and anti-MRP4 (Dr. John D. Schuetz, St. Jude Children's Research Hospital, Memphis, TN).

Cell Culture

Caco-2 cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were allowed to undergo two passages before use. Cells then were cultured in DMEM supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin under an atmosphere of 5% CO_2 and 95% relative humidity at 37°C. Cells used in the studies were between passages 30 and 50. Caco-2 cells were seeded in a density of 2.5×10^4 cells/cm^2 in 60-mm dishes; the medium was changed every other day, with the exception of treatment days. For the treatment with 1,25$(OH)_2D_3$ and vitamin D analogs (10 or 100 nM), cells were treated with control medium containing 0.1% ethanol or 1,25$(OH)_2D_3$ and vitamin D analogs in 0.1% ethanol daily on day 15 for six consecutive days, on day 18 for three consecutive days, or on day 20 for 1 day; cells were then harvested on day 21. For studies investigating the effect of 1,25$(OH)_2D_3$ on MRP4 protein stability, Caco-2 cells were first treated with 100 nM 1,25$(OH)_2D_3$ or ethanol

(0.1%) for 6 days and then with cycloheximide (10 μg/ml) to arrest protein synthesis, followed by protein isolation for MRP4 determination at 0, 8, 24, and 48 h after the cycloheximide treatment. For studies determining the effect of $25(OH)D_3$ on gene expression in the presence or absence of FBS, Caco-2 cells were treated with 100 nM $1,25(OH)_2D_3$, $25(OH)D_3$, or ethanol (0.1%) for 6 days in the presence of 10% FBS or in the serum-free condition, wherein a 1% mixture of insulin-transferrin-selenium (ITS; Invitrogen) was used instead.

DTEx Microarray Analysis

Total RNA (~2 μg) was converted to aRNA using the MessageAmp II aRNA amplification kit (ABI, Chicago IL; and Ambion) according to the manufacturers' instructions. Labeled cDNA was prepared from purified aRNA using Cy5-labeled random nonamers and SuperScript II reverse transcriptase (Invitrogen, Burlington, ON, Canada) according to the manufacturer's instructions. Cy5-labeled cDNA was purified on QIAquik PCR purification columns (QIAGEN, Mississauga ON, Canada). Purified Cy5-labeled cDNA was quantified using a spectrophotometer (NanoDrop Inc., Wilmington, DE). Cy5-labeled cDNA (~3 μg) was denatured at 95°C for 10 min and hybridized to a DTEx microarray slide [145 absorption, distribution, metabolism, and excretion-associated genes printed on Nexterion E slides (Schott, Louisville, KY)] in Nexterion 1× Hyb solution for 18 h at 45°C. Before hybridization, each DTEx microarray slide was denatured in boiled water for 60 s and blocked in Nexterion Block E for 15 min at 45°C. After hybridization, the DTEx microarray slides were washed once for 15 min at 45°C in each of the following solutions: 2× SSC/0.2% SDS, 2× SSC, and 0.2× SSC. The DTEx microarray slides were "spin-dried" at 1200 rpm for 2 min at room temperature and then scanned in a ProScanArray HT (PerkinElmer, Woodbridge, ON, Canada). After image acquisition, fluorescent signals from the DTEx microarray were assessed using QuantArray. Results were exported to GeneLinker Gold (Improved Outcomes Software, Kingston, ON, Canada) for normalization, hierarchical cluster analysis, and matrix plot images. Four independent DTEx microarray experiments were performed for each RNA sample in this study. Each gene on the DTEx microarray was printed in quadruplicate, resulting in $n = 16$ for each gene interrogated in the DTEx microarray analysis.

Real-Time Quantitative Polymerase Chain Reactions

RNA levels of the drug transporters and enzymes were analyzed by RT-qPCR. Total RNA was isolated from cells using TRI Reagent (Sigma Chemical Co., Oakville, ON, Canada). The first-strand cDNA was synthesized by using the First Strand cDNA synthesis kit (Fermentas, Burlington, ON, Canada). cDNA samples then were subjected to qPCR assays with the fluorescent dye SYBR Green methodology and an ABI 7500 detector (Applied Biosystems, Foster City, CA). Gene-specific primers, designed with the Primer3 software (Whitehead Institute for Biomedical Research, Cambridge, MA), are summarized in Table 1. Primers were optimized and validated with the comparative C_T method, as described in the manufacturer's manual. Reactions were performed using the following protocol: 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. A dissociation curve protocol was linked to every PCR assay to ensure the specificity of the PCR product. The expression of specific genes was normalized to the value of villin mRNA of the same sample. Relative quantification was obtained by the comparative threshold cycle ($\Delta\Delta C_T$) method (RQ software, version 1.2.3; Applied Biosystems).

Western Blot Analysis

Cellular proteins were extracted by dissolving cells in the CelLytic cell lysis reagent (Sigma-Aldrich). Protein concentration was measured by the method of Lowry et al. (1951). Sixty micrograms of whole cell protein was mixed with 4× gel loading buffer (250 mM Tris-HCl, pH 6.8, 8% SDS, 20% glycerol, 0.2% bromphenol blue, and 0.4 M dithiothreitol), separated on a 7.5% SDS-polyacrylamide gel under reducing conditions, and transferred to nitrocellulose membranes (Hybond ECL; GE Healthcare). Nonspecific antibody binding was blocked by preincubation of the membranes with 5% skim milk in 1× Tris-buffered saline for 1 h at room temperature. Membranes were then incubated overnight at 4°C with the appropriate primary antibodies at different dilutions with 2% skim milk in 1× Tris-buffered saline containing 0.1% Tween 20. After washing, they were incubated with donkey anti-rabbit IgG or sheep anti-mouse IgG at 1:1000 dilutions for 1 h at room temperature. Bands were visualized according to the enhanced chemiluminescence kit per the manufacturer's instructions. Villin served as loading control. Band intensity was analyzed with the ImageJ 1.32 software (National Institutes of Health, Bethesda, MD), and protein expression was presented as the ratio of target proteins band intensity to villin band intensity in the same blot.

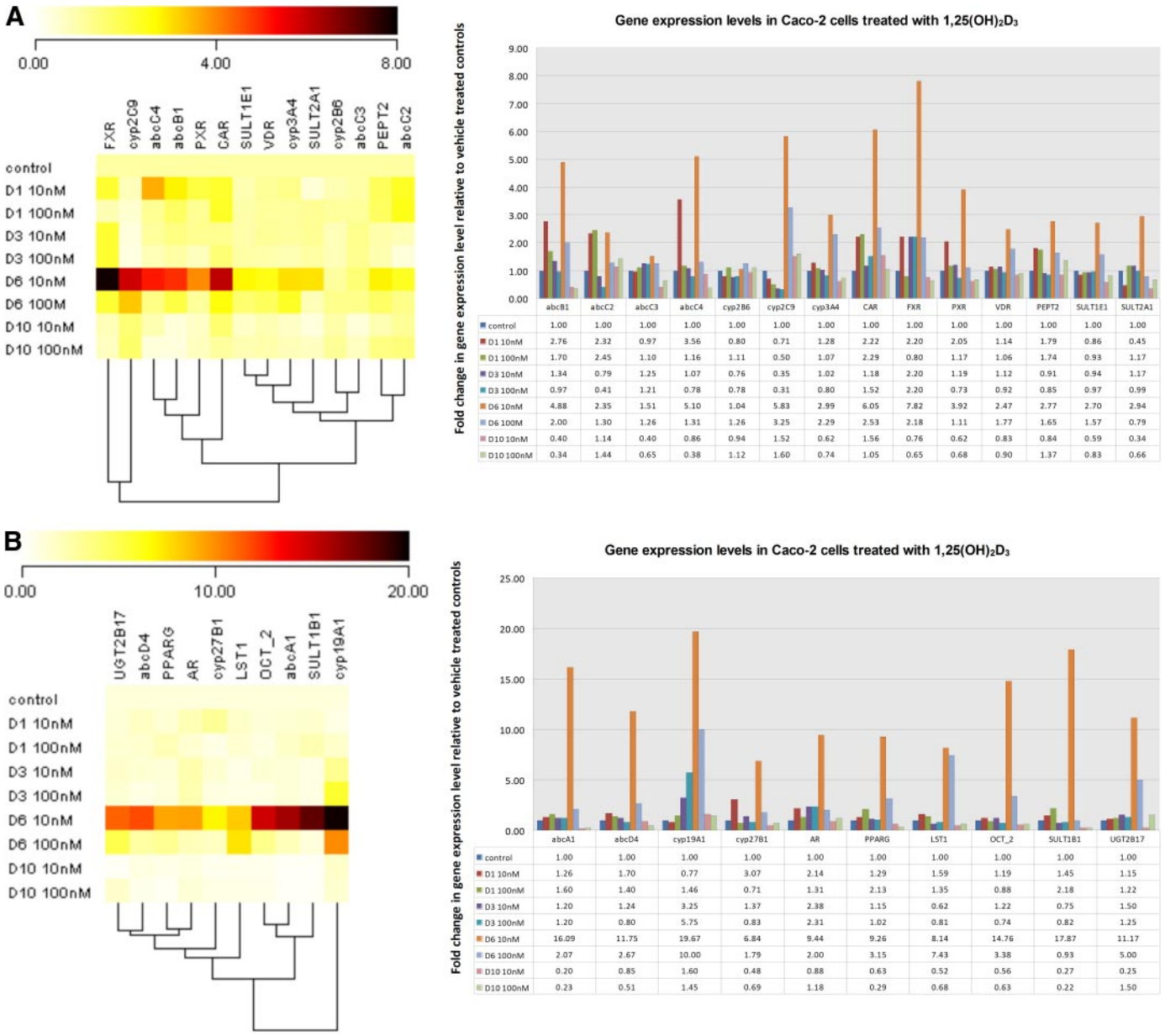
Functional Studies in Caco-2 Cells

CYP3A4-Mediated Testosterone 6β-Hydroxylation after 6 Days of Treatment with 100 nM $1,25(OH)_2D_3$. Cell lysate (4 mg), testosterone (200 μM), and Tris-HCl buffer (50 mM; pH 7.4) in a volume of 470 μl were preincubated for 5 min at 37°C. Thereafter, an NADPH-regenerating system composed of 25 μl of solution A and 5 μl of solution B (BD Biosciences) was added to initiate the reaction. The reaction was terminated at 60 min upon addition of 1 ml of ice-cold acetonitrile that contained 500 ng/ml desmethyldiazepam (internal standard for HPLC). The solution was added to 25 μl of 1 N NaOH and extracted against 5 ml of ethyl acetate after mixing and centrifugation at 5000*g* (for 5 min). The supernatant was removed and dried under nitrogen gas and then reconstituted in 100 μl of methanol. The 6β-hydroxytestosterone and testosterone in the reconstituted sample were assayed by a validated HPLC method with the following conditions (Brimer et al., 2000): the HPLC system (Shimadzu, Kyoto, Japan) consisted of an SCL-10A system controller, LC-10AT pump, and SIL-10A XL autoinjector; and an Altima C18 reverse-phase column (4.6 × 250 mm; particle size, 10 μm; Alltech Associates, Deerfield, IL) and precolumn [2.2 × 0.34 cm i.d.

TABLE 1
Primer sets for quantitative real-time PCR

Gene	GenBank No.	Forward (5′→3′ Sequence)	Reverse (5′→ 3′ Sequence)
GAPDH[a]	NM_002046	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC
VDR	NM_001017535	GACATCGGCATGATGAAGGAG	GCGTCCAGCAGTATGGCAA
CYP3A4	NG_008421	CATTCCTCATCCCAATTCTTGAAG T	CCACTCGGTGCTTTTGTGTATCT
CYP1A2	NM_000761	CAAGGGACACAACGCTGA	CCAGGACTTCCCCGATACA
SULT1E1	NM_005420	AAACAATTAGATGAGATGAAT	ATTTGGATGACCAGCCAC
ASBT	NM_000452	GCCCCAAAAAGCAAAGATCA	GCTATGAGCACAATGAGGATGG
PEPT1	NM_005073	TCCACCGCCATCTACCATAC	GGACAAACACAATCAGGGCT
MDR1	NM_000927	TGCTCAGACAGGATGTGAGTTG	AATTACAGCAAGCCTGGAACC
MRP2	NM_000392	CAAACTCTATCTTGCTAAGCAGG	TGAGTACAAGGGCCAGCTCTA
MRP3	NM_003786	CTTAAGACTTCCCCTCAACATGC	GGTCAAGTTCCTCTTGGCTC
MRP4	NM_005845	AATGTGACCGTCCATCCTCC	AGGTTTGGCCTTCTTGGGA

GAPDH, glyceraldehyde-3-phosphate dehydrogenase.



	abcB1	abcC2	abcC3	abcC4	cyp2B6	cyp2C9	cyp3A4	CAR	FXR	PXR	VDR	PEPT2	SULT1E1	SULT2A1
control	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D1 10nM	2.76	2.32	0.97	3.56	0.80	0.71	1.28	2.22	2.20	2.05	1.14	1.79	0.86	0.45
D1 100nM	1.70	2.45	1.10	1.16	1.11	0.50	1.07	2.29	0.80	1.17	1.06	1.74	0.93	1.17
D3 10nM	1.34	0.79	1.25	1.07	0.76	0.35	1.02	1.18	2.20	1.19	1.12	0.91	0.94	1.17
D3 100nM	0.97	0.41	1.21	0.78	0.78	0.31	0.80	1.52	2.20	0.73	0.92	0.85	0.97	0.99
D6 10nM	4.88	2.35	1.51	5.10	1.04	5.83	2.99	6.05	7.82	3.92	2.47	2.77	2.70	2.94
D6 100M	2.00	1.30	1.26	1.31	1.26	3.25	2.29	2.53	2.18	1.11	1.77	1.65	1.57	0.79
D10 10nM	0.40	1.14	0.40	0.86	0.94	1.52	0.62	1.56	0.76	0.62	0.83	0.84	0.59	0.34
D10 100nM	0.34	1.44	0.65	0.38	1.12	1.60	0.74	1.05	0.65	0.68	0.90	1.37	0.83	0.66

	abcA1	abcD4	cyp19A1	cyp27B1	AR	PPARG	LST1	OCT_2	SULT1B1	UGT2B17
control	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
D1 10nM	1.26	1.70	0.77	3.07	2.14	1.29	1.59	1.19	1.45	1.15
D1 100nM	1.60	1.40	1.46	0.71	1.31	2.13	1.35	0.88	2.18	1.22
D3 10nM	1.20	1.24	3.25	1.37	2.38	1.15	0.62	1.22	0.75	1.50
D3 100nM	1.20	0.80	5.75	0.83	2.31	1.02	0.81	0.74	0.82	1.25
D6 10nM	16.09	11.75	19.67	6.84	9.44	9.26	8.14	14.76	17.87	11.17
D6 100nM	2.07	2.67	10.00	1.79	2.00	3.15	7.43	3.38	0.93	5.00
D10 10nM	0.20	0.85	1.60	0.48	0.88	0.63	0.52	0.56	0.27	0.25
D10 100nM	0.23	0.51	1.45	0.69	1.18	0.29	0.68	0.63	0.22	1.50

Fig. 1. DTEx microarray analysis of enzyme and transporter gene expression after ethanol (control; 0.1% as vehicle) and 1,25$(OH)_2D_3$ (0–100 nM) treatment for 0, 1, 3, 6, or 10 days (D1, D3, D6, or D10). Expression levels of genes that were regulated by 1,25$(OH)_2D_3$ (A) and genes displaying the highest differential levels of expression in response to 1,25$(OH)_2D_3$ treatment (10 and 100 nM, B) in Caco-2 cells for 1, 3, 6, or 10 consecutive days; ethanol (0.1%) was used for treatment in the control group. Cells were then harvested on day 21, and the expression levels of 1,25$(OH)_2D_3$-regulated genes were determined by DTEx microarray analysis and normalized to that of the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase.

filled with μBondapak C18/Corasil 37–55 μm (Waters, Milford, MA)]. The mobile phase, consisting of methanol and water (58:42, v/v), was used at the flow rate of 1.0 ml/min. The effluent was monitored at 254 nm with an SPD-10A UV detector (Shimadzu). Data acquisition and integration were performed with the Star-Chrom LITE HPLC data system software (D-Star Instruments, Manassas, VA). The retention times of 6β-hydroxytestosterone and testosterone were 9.2 and 22 min, respectively. The enzymatic activity of CYP3A4 was expressed as picomoles of 6β-hydroxytestosterone formed per gram of protein per minute.

Transport Studies for [^{3}H]Digoxin (5 μM) in Transwell Plates after 3 Days of Treatment with 10 and 100 nM 1,25$(OH)_2D_3$. For transport studies, Caco-2 cells were used at passages between 39 and 50 and seeded in a density of 8×10^4 cells/cm^2 in Transwell plates with a pore size of 0.4 μm and a growth area of 1.13 cm^2 (Corning Inc., Corning, NY). The medium was changed every other day up to day 10 and then daily thereafter. Before and after the transport experiment was performed, the integrity of the monolayer was checked by measurement of the transepithelial electrical resistance (TEER) values with a millicell-ERS volt/ohm meter (Millipore, Billerica, MA). Monolayers that demonstrated a resistance value between 600 and 1000 Ω · cm^2 were used for the transport experiments. Routine analysis of TEER values showed normal development of the tight junctions (>600 Ω · cm^2). [^{3}H]Digoxin ($5960 \pm 3.69 \times 10^3$ dpm/ml with 5 μM unlabeled digoxin) or CDF-DA (10 μM) was dissolved in dimethyl sulfoxide (0.1%). [^{14}C]Mannitol ($1.90 \pm 0.52 \times 10^3$ dpm/ml), a marker of paracellular transport, was used for each set of studies to assess membrane integrity.

The cell monolayer at 21 to 24 days of culture was used for transport studies. The procedure was similar to that described previously (Artursson et al., 2001). Before the experiment, the incubation medium was gently suctioned off and replaced by prewarmed

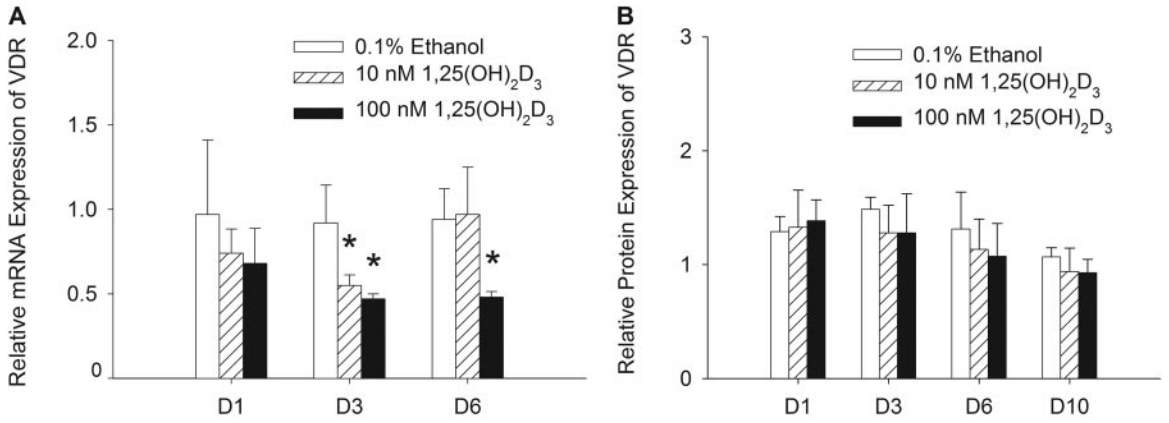


Fig. 2. Effects of $1,25(OH)_2D_3$ on VDR mRNA and protein expression in Caco-2 cells. Caco-2 cells were treated with $1,25(OH)_2D_3$ (10 or 100 nM) on day 20, 18, 15, or 11 for 1, 3, 6, or 10 consecutive days, whereas the control group was treated with ethanol (0.1%). Cells were harvested on day 21, and the expression of VDR mRNA and protein was determined by using qPCR and Western blotting and normalized to that of the house-keeping gene villin. A, data are the mean ± S.D. of qPCR quantifications from three independent cultures. B, data are the mean ± S.D. of the density histograms from three separate Western blot analyses. ∗, $P < 0.05$ between the $1,25(OH)_2D_3$-treated and ethanol-treated groups.

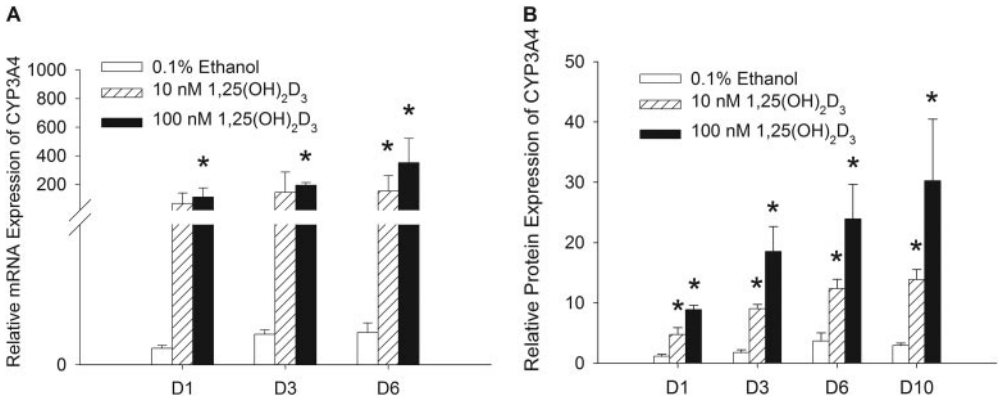


Fig. 3. Effects of $1,25(OH)_2D_3$ on CYP3A4 expression in Caco-2 cells. Caco-2 cells were treated with $1,25(OH)_2D_3$ (10 or 100 nM) on day 20, 18, 15, or 11 for 1, 3, 6, or 10 consecutive days, whereas the control group was treated with ethanol (0.1%). Cells were harvested on day 21 and the expression of mRNA and protein was determined by using qPCR and Western blotting and normalized to that of the house-keeping gene villin. A, data are the mean ± S.D. of qPCR quantifications from three independent cultures. B, data are the mean ± S.D. of the density histograms from three separate Western blot analyses. ∗, $P < 0.05$ between the $1,25(OH)_2D_3$-treated and ethanol-treated groups.

transport HBSS buffer and incubated for 60 min at 37°C thereafter. The transport experiment for the apical-to-basolateral (A→B) or basolateral-to-apical (B→A) direction was initiated upon gentle removal of the transport buffer in the donor compartment and placement of 0.55 or 1.55 ml of [^{3}H]digoxin into the donor compartment; the receiver compartment was filled with 1.5 or 0.5 ml of blank transport buffer. An aliquot of 0.05 ml of sample was quickly withdrawn from the donor compartment to define the loading concentration, C_0, to result in starting volumes of 0.5 ml in the apical compartment and 1.5 ml in the basolateral compartment. TEER values were measured at various stages to ensure that the integrity of the monolayer was retained with the buffer changes. At 20, 40, 60, and 90 min, 750 and 250 μl were removed from the receiver compartment for the A→B and B→A transport studies, respectively. The volume removed was replenished by prewarmed blank transport buffer to ensure the sink conditions. At the end of the experiment, 250 and 750 μl of sample were collected from the apical side and the basolateral side, respectively. Cells collected at the end of the transport study were washed three times with 2 ml of ice-cold HBSS; 750 μl of NaOH (0.2 N) was added to digest the cells, and the sample was back-neutralized by the addition of 150 μl of HCl (1 N).

The radioactivities of samples were measured by liquid scintillation spectrometry (model 5801 beta counter; Beckman Coulter Canada, Inc., Mississauga, ON, Canada) upon addition of 9.5 ml of scintillation fluor (Ready Safe; Beckman Coulter Canada, Inc.). The cellular contents and amounts in the donor and receiver compartments were used for the mass balance considerations or the estimation of recovery. The apparent permeability coefficient (P_{app}, centimeter per second) of [^{3}H]digoxin across Caco-2 monolayers was expressed in centimeter per second and calculated as shown in eq. 1:

$$P_{app} = \frac{\Delta A_R/\Delta t}{\text{Area} \times 60 \times C_0} \quad (1)$$

where ΔA_R is the amount accumulated in the receiver side during the interval time (Δt), area is the surface area of the filter/membrane of the Transwell (square centimeters), and C_0 is the initial drug concentration in the donor chamber. The efflux ratio, EfR is given by the ratio of P_{app} in the (B→A) to that in the (A→B) direction:

$$\text{EfR} = \frac{P_{app(B\rightarrow A)}}{P_{app(A\rightarrow B)}} \quad (2)$$

CDF Transport Studies in Transwell Plates after 3 Days of Treatment with 10 and 100 nM $1,25(OH)_2D_3$ in Caco-2 Cells. For the definition of MRP2 function, 10 μM CDF-DA, the nonfluorescent prodrug that was dissolved in transport buffer, was added into either the apical or basolateral compartment of the Transwell plates. At 10, 20, and 40 min, 750 and 250 μl were removed from the receiver compartment for the A→B and B→A transport studies, respectively. The volume removed was replenished by prewarmed, blank transport buffer to ensure the sink conditions. At the end of

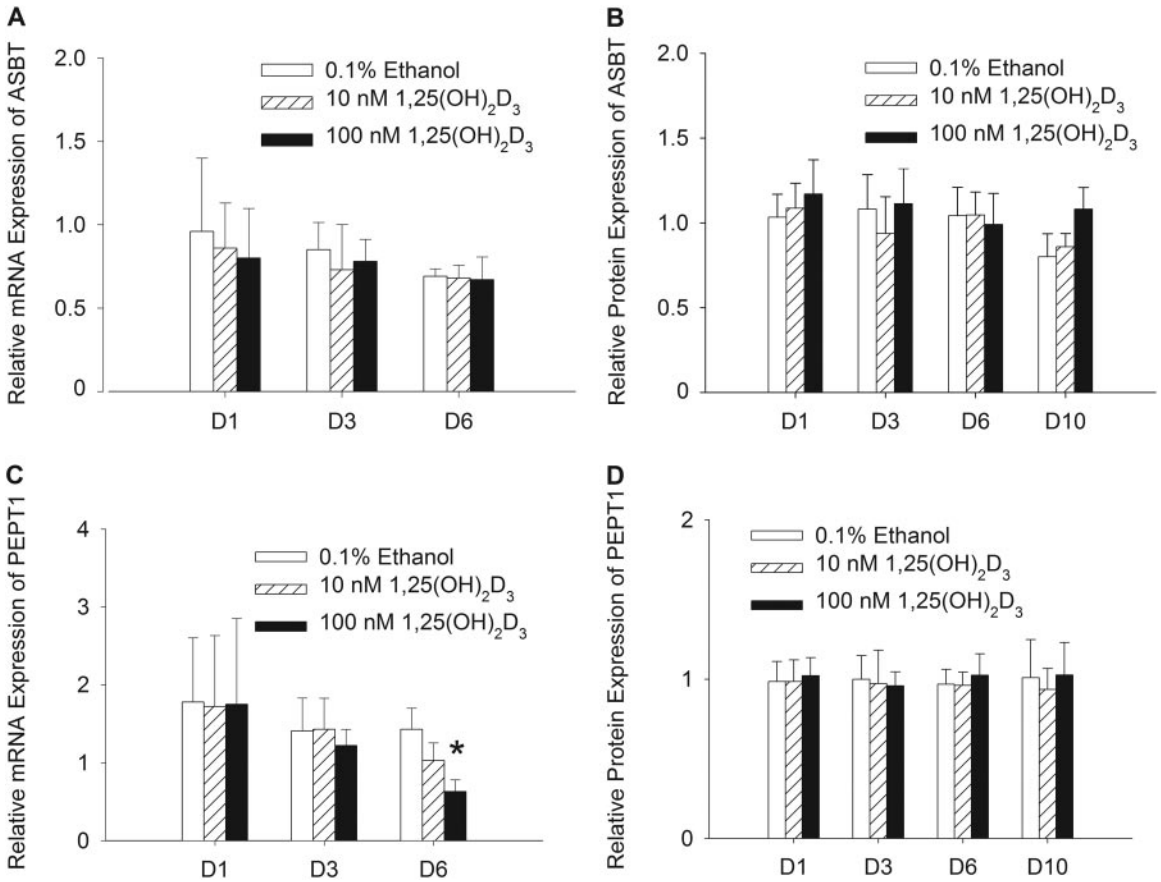


Fig. 4. Effects of $1,25(OH)_2D_3$ on ASBT and PEPT1 expression in Caco-2 cells. Caco-2 cells were treated with $1,25(OH)_2D_3$ (10 or 100 nM) on day 20, 18, 15, or 11 for 1, 3, 6, or 10 consecutive days, whereas the control group was treated with ethanol (0.1%). Cells were harvested on day 21, and the expression of mRNA and protein was determined by using qPCR and Western blotting and normalized to that of the house-keeping gene villin. A and C, data are the mean ± S.D. of qPCR quantifications from three independent cultures. B and D, data are the mean ± S.D. of the density histograms from three separate Western blot analyses. *, $P < 0.05$ between the $1,25(OH)_2D_3$-treated and ethanol-treated groups.

the experiment, cells were lysed with 500 μl of 1% Triton X-100 in PBS for 20 min at room temperature. All retrieved samples were stored at −20°C until analyses. The extent of transport (transport efficiency) of the fluorescent CDF metabolite in the A-to-B or B-to-A direction was assessed as shown in eq. 3:

$$\text{Transport Efficiency} = \frac{\text{CDF amount in receiver side}}{\text{CDF amount in receiver side} + \text{CDF amount in cell}} \quad (3)$$

CDF fluorescence in the receiver compartment and in cell lysate were analyzed by a microplate fluorescence reader (λ_{ex} = 485 nm; λ_{em} = 590 nm) (SpectraMax Gemini XS; Molecular Devices, Sunnyvale CA).

CDF Accumulation in Caco-2 Cells in Culture Dish. The extent of accumulation of CDF, an index of MRP2 function, in Caco-2 cells (Tian et al., 2004) was also assessed in Caco-2 cells grown on a culture dish. Caco-2 cells were seeded with a density of 2.5×10^4 cells/cm^2 in a six-well plate and treated with control medium containing 0.1% ethanol or $1,25(OH)_2D_3$ (100 nM) daily on day 18 for three consecutive days. On day 21, triplicate plates were washed three times with HBSS and incubated with 10 μM CDF-DA in transport buffer for 30 min at 37°C. At the end of incubation, CDF fluorescence in the lysed cells was assayed.

Data Analysis

Student's *t* test and analysis of variance were used to determine statistical difference between control and treatment groups. Results were expressed as mean ± S.D. A *P* value of <0.05 was considered as statistically significant.

Results

DTEx Microarray Analysis of Enzymes and Transporters in $1,25(OH)_2D_3$-Treated Caco-2 Cells

DTEx microarray analysis of Caco-2 cells treated with $1,25(OH)_2D_3$ (0, 10, and 100 nM for 1, 3, 6, and 10 days) showed that maximal differential expression occurred after 6 days of treatment with 10 nM $1,25(OH)_2D_3$. At this point, 99 genes were up-regulated at least 2-fold (range, 2–20-fold) compared with vehicle-treated controls. This group of differentially expressed, up-regulated genes included 37 ABC transporters (range, 2–16-fold), 13 cytochromes P450 (range, 2–20-fold), 15 nuclear xenobiotic receptors (range, 2–10-fold), 23 solute ligand carrier transporters (range, 2–15-fold), 5 sulfotransferases (range, 2–15-fold), and 6 UDP-glucuronosyltransferases (range, 2–11-fold). Genes that were regulated by $1,25(OH)_2D_3$ treatment are shown in Fig. 1A. The 10 most up-regulated genes are shown in Fig. 1B. A close alignment between $1,25(OH)_2D_3$ concentration and days of induction with increased DTEx signal was not achieved, an observation that probably can be attributed to the toxic effects of $1,25(OH)_2D_3$ in the system.

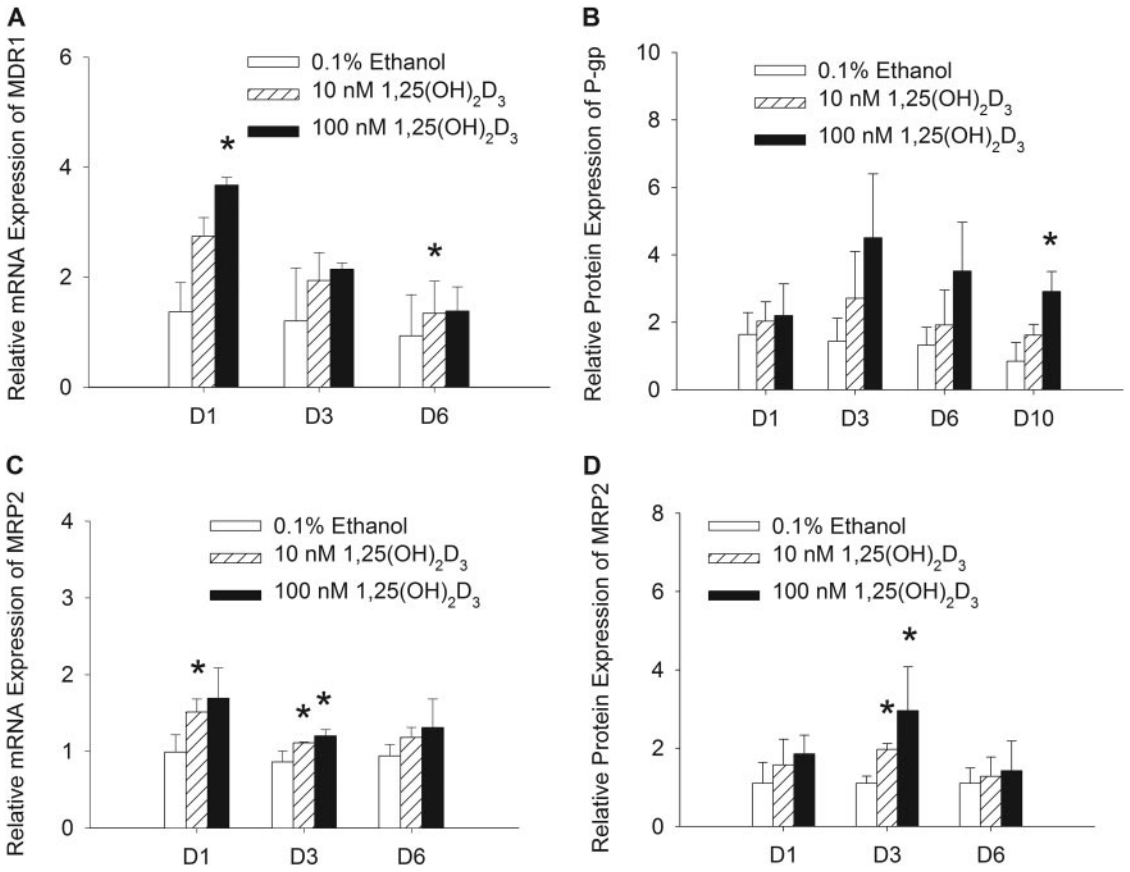


Fig. 5. Effect of $1,25(OH)_2D_3$ on P-gp and MRP2 expression in Caco-2 cells. Caco-2 cells were treated with $1,25(OH)_2D_3$ (10 or 100 nM) on day 20, 18, 15, or 11 for 1, 3, 6, or 10 consecutive days, and the control group was treated with ethanol (0.1%). Cells were harvested on day 21, and the expression of mRNA and protein were determined by using qPCR and Western blotting and normalized to that of the house-keeping gene villin. A and C, data are the mean ± S.D. of qPCR quantifications from three independent cultures. B and D, data are the mean ± S.D. of the density histograms from three separate Western blot analyses. *, $P < 0.05$ between the $1,25(OH)_2D_3$-treated and ethanol-treated groups.

Effects of $1,25(OH)_2D_3$ on Gene Expression in Caco-2 Cells

The mRNA and protein levels of the house-keeping gene villin were unchanged and independent of the duration and concentration of $1,25(OH)_2D_3$ used in the treatments. There was a decrease in VDR mRNA level in response to $1,25(OH)_2D_3$; a decreasing trend of VDR protein expression, although statistically insignificant, was observed (Fig. 2, A and B). $1,25(OH)_2D_3$ (10 and 100 nM) resulted in a dramatic increase in CYP3A4 mRNA and protein expression in a dose-dependent manner in Caco-2 cells, with the maximal response being achieved at the concentration of 100 nM after 6 or 10 days of treatment (Fig. 3, A and B). The changes for CYP3A4 mRNA and protein levels were consistent with findings of Schmiedlin-Ren et al. (1997). No significant change was observed for the mRNA expression of other phase I and phase II enzymes, such as CYP1A2, SULT1E1, SULT2A1, and SULT1A1, or the protein level of CYP1A2 (data not shown).

No change was observed for the absorptive transporters PEPT1 and ASBT (Fig. 4), with the exception of a small decrease in PEPT1 mRNA at 100 nM $1,25(OH)_2D_3$ after the 6-day treatment (Fig. 4C). For apical efflux transporters, MDR1 mRNA levels in the treated groups were significantly up-regulated upon exposure to $1,25(OH)_2D_3$ for 1 or 6 days at the designated concentrations, and P-gp protein levels were increased significantly for the 10-day treatment group compared with controls (Fig. 5, A and B). Increased MRP2 mRNA and protein levels were observed after treatment for 1 or 3 days, being higher after the 3-day treatment for the protein level (Fig. 5, C and D).

$1,25(OH)_2D_3$ failed to elicit any change in MRP3 mRNA and protein levels (Fig. 6, A and B), but it exhibited a significant inductive effect on MRP4 protein expression of 1.8-, 1.6-, or 1.9-fold after 3-, 6-, or 10-day treatment with 100 nM $1,25(OH)_2D_3$, respectively; however, mRNA levels of MRP4 remained unaltered (Fig. 6, C and D), suggesting a post-transcriptional regulation of $1,25(OH)_2D_3$ on MRP4 in the Caco-2 cells.

Post-Transcriptional Modulation Instead of de Novo Protein Synthesis for $1,25(OH)_2D_3$-Mediated Increase in MRP4 Protein

To determine the underlying mechanism for the up-regulation of MRP4 protein by $1,25(OH)_2D_3$, Caco-2 cells were incubated with the protein synthesis inhibitor cycloheximide (10 μg/ml) after induction with $1,25(OH)_2D_3$ (100 nM for 6 days). At 0, 8, 24, and 48 h after the addition of cycloheximide, samples were retrieved and subjected to Western blot analysis. As shown in Fig. 7, levels of MRP4 fell to less than 62% of the initial value by 8 h after cycloheximide treatment and 44% by 48 h in the vehicle-treated group. In the $1,25(OH)_2D_3$-treated group, however, degradation of MRP4 was arrested, and MRP4 levels showed a 1.3-fold increase for

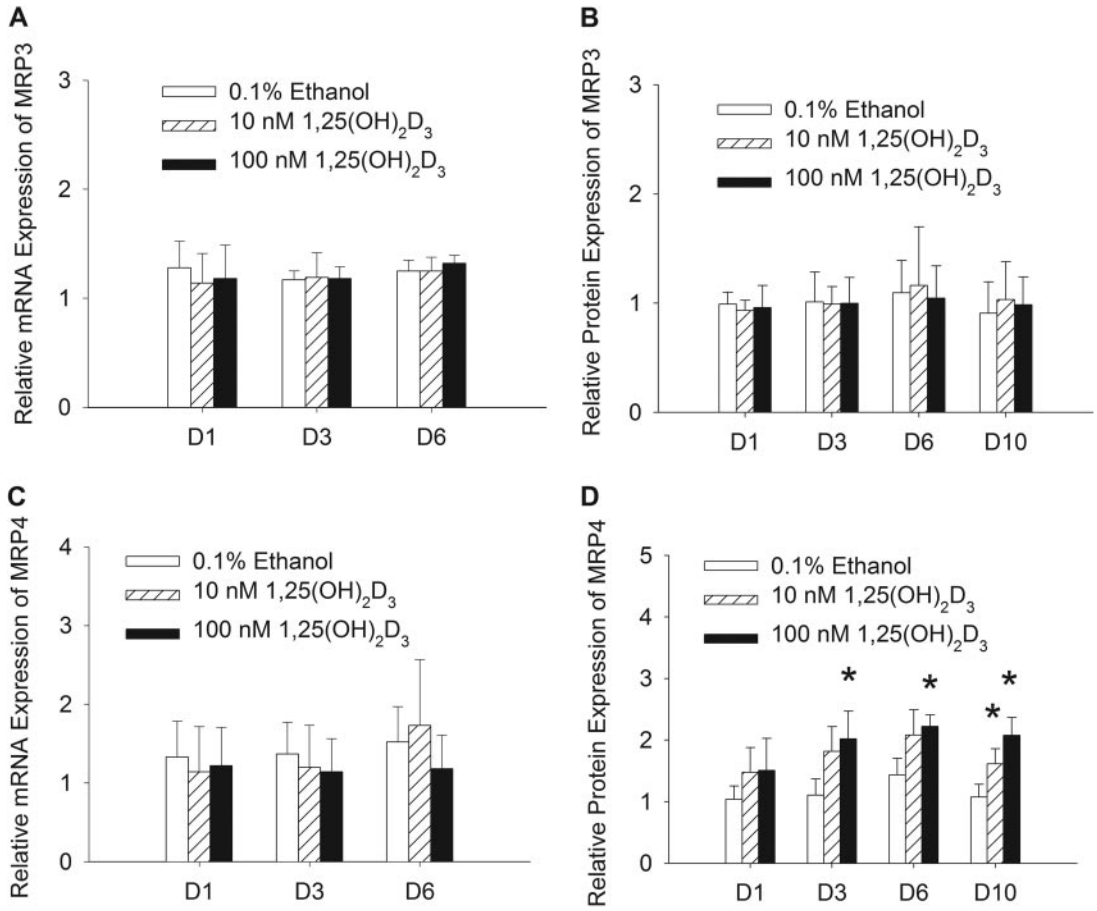


Fig. 6. Effects of $1,25(OH)_2D_3$ on MRP3 and MRP4 expression in Caco-2 cells. Caco-2 cells were treated with $1,25(OH)_2D_3$ (10 or 100 nM) on day 20, 18, 15, or 11 for 1, 3, 6, or 10 consecutive days, whereas the control group was treated with ethanol (0.1%). Cells were harvested on day 21, and the expression of mRNA and protein was determined by using qPCR and Western blotting and normalized to that of the house-keeping gene villin. A and C, data are the mean ± S.D. of qPCR quantifications from three independent cultures. B and D, data are the mean ± S.D. of the density histograms from three separate Western blot analyses. *, $P < 0.05$ between the $1,25(OH)_2D_3$-treated and ethanol-treated groups.

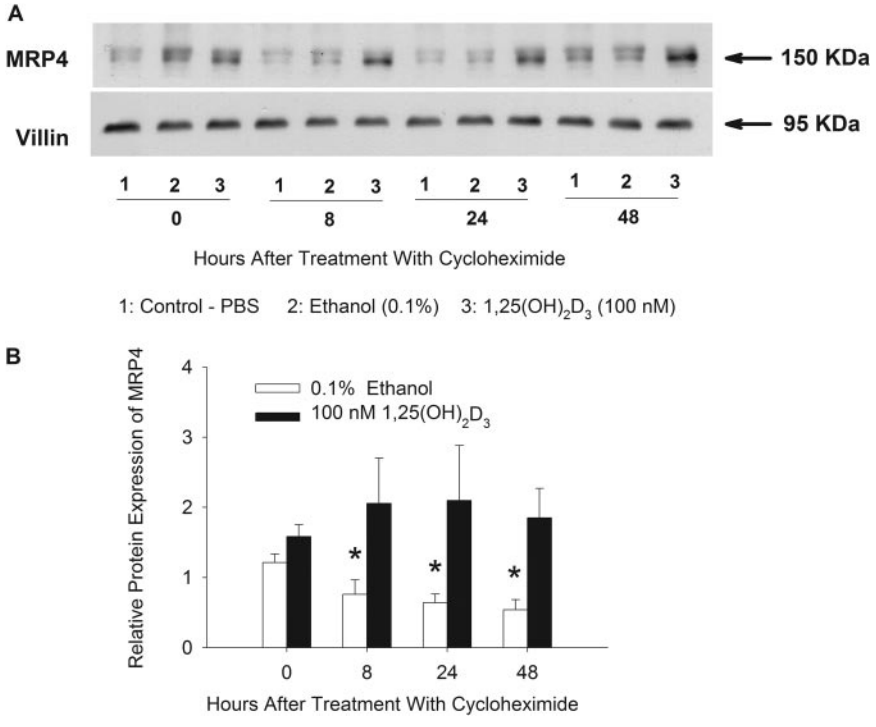


Fig. 7. $1,25(OH)_2D_3$ improved MRP4 protein stability in Caco-2 cells. Caco-2 cells were treated with 100 nM $1,25(OH)_2D_3$ on day 15 for six consecutive days, whereas the control group was treated with ethanol (0.1%). The protein synthesis inhibitor cycloheximide (10 μg/ml) was added and incubated with the cells on day 21. A, cell extracts were collected at the indicated times for determination of protein expression using Western blotting and normalized to the expression of the house-keeping gene villin. B, density histograms from three separate Western blot analyses (mean ± S.D.). *, $P < 0.05$ between the $1,25(OH)_2D_3$-treated and ethanol-treated groups.

TABLE 2

Apparent permeability coefficients (P_{app} $\times 10^{-6}$ cm/s) and net efflux ratio of bidirectional [^{3}H]digoxin and [^{14}C]mannitol transport for 60 min across the Caco-2 cell monolayer

Caco-2 cells were treated with 1,25(OH)$_2$D$_3$ (10 and 100 nM) on day 18 for three consecutive days. The transport experiment was performed on day 21 for 60 min. Results are mean ± S.D. in triplicates.

Treatment	P_{app} for [^{3}H]Digoxin[a]			P_{app} for [^{14}C]Mannitol[b]		
	A→B	B→A	EfR	A→B	B→A	EfR
Control PBS	1.7 ± 0.4	11.5 ± 1.8	6.8	0.7 ± 0.2	1.5 ± 0.4	2.2
Ethanol (0.1%)	2.1 ± 0.4	11.8 ± 0.6	5.8	0.8 ± 0.3	1.1 ± 0.1	1.3
1,25(OH)$_2$D$_3$ (10 nM)	2.1 ± 0.3	12.7 ± 1.4	6.1	1.2 ± 0.2	1.7 ± 0.2	1.4
1,25(OH)$_2$D$_3$ (100 nM)	1.9 ± 0.3	15.1 ± 0.5*	8.0	1.3 ± 0.3	1.9 ± 0.7	1.5

* $P < 0.05$ compared with ethanol-treated group.
[a] Slope of cumulative amount of [^{3}H]digoxin or [^{14}C]mannitol vs. time plot.

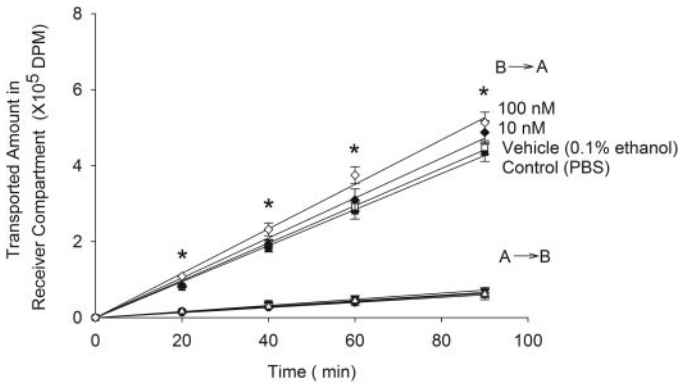


Fig. 8. Cumulative amounts of [^{3}H]digoxin transported for the A→B and B→A direction at various times in the receiver compartment of the Caco-2 cell monolayer. Caco-2 cells were treated with ethanol (0.1%) and 1,25(OH)$_2$D$_3$ (10 and 100 nM) on day 18 for three consecutive days. The transport study was performed on day 21. Data are presented as mean ± S.D. ($n = 3$). *, $P < 0.05$ between the 1,25(OH)$_2$D$_3$-treated and ethanol-treated groups.

the 8-h sample relative to that at time 0, followed by a gradual decrease of MRP4 protein expression for the next 16 h. These data suggest that 1,25(OH)$_2$D$_3$ had probably increased the stability of MRP4 protein. However, whether 1,25(OH)$_2$D$_3$ directly affects MRP4 protein or other related proteinase activities needs to be further investigated.

Functional Studies of 1,25(OH)$_2$D$_3$ on CYP3A4 and Transporters

Effect of 1,25(OH)$_2$D$_3$ on CYP3A4 Catalytic Activity. To determine the correlation between the higher CYP3A4 protein levels observed with 1,25(OH)$_2$D$_3$ treatment in Caco-2 cells lysate and catalytic activity, the rate of formation of 6β-hydroxytestosterone from 200 μM testosterone by CYP3A4 was assessed. With a 6-day treatment of 100 nM 1,25(OH)$_2$D$_3$ in Caco-2 cells, the rate of 6β-hydroxytestosterone formation was dramatically increased from undetectable levels in vehicle-treated and PBS control samples to 202 ± 41.2 pmol/min/g cellular protein.

Effects of 1,25(OH)$_2$D$_3$ on P-gp Function: [^{3}H]Digoxin Transport across the Caco-2 Monolayer. The effects of the 3-day treatment of 1,25(OH)$_2$D$_3$ on P-gp function were evaluated by measuring the transport of [^{3}H]digoxin across the Caco-2 cell monolayer. The TEER values for different treatment groups at the end of the 3-day treatment, measured before and after transport studies, remained high (>600 Ω · cm^2) (data not shown). [^{3}H]Digoxin transport across the PBS control Caco-2 monolayer showed marked asymmetry, with the P_{app} value for B→A ($11.5 \pm 1.8 \times 10^{-6}$ cm/s, with the rate or $\Delta A_R/\Delta t$ in eq. 1 being estimated as the slope of cumulative amount in receiver side versus time plot) exceeding that for the A→B direction ($1.7 \pm 0.4 \times 10^{-6}$ cm/s); the efflux ratio was 6.8 (Table 2). Values of the vehicle (0.1% ethanol)-treated cells were similar to those of the PBS controls. 1,25(OH)$_2$D$_3$ (100 nM in 0.1% ethanol) treatment had no significant effect on the A-to-B permeability (Table 2; Fig. 8) but increased the P_{app} value of [^{3}H]digoxin significantly in the B-to-A direction from 11.8 to 15.1×10^{-6} cm/s ($P < 0.05$), resulting in an increased efflux ratio of 8.0. In instances where 1,25(OH)$_2$D$_3$ was used for treatment, the apparent permeability of mannitol had remained unchanged. These data suggest the presence of functionally active P-gp protein in the 100 nM 1,25(OH)$_2$D$_3$-treated Caco-2 cells.

Effect of 1,25(OH)$_2$D$_3$ after 3 Days of Treatment on MRP2 Function. The effective transport of the fluorescent CDF after application of CDF-DA into either the apical or basolateral, donor side, is shown. There was asymmetry in the accumulation of CDF in the receiver side and retention in the cell from the B-to-A (Fig. 9A) and A-to-B direction (Fig. 9B); CDF preferably accumulated in the apical side and not the basolateral side. With basolateral administration, the retention of CDF in the cell was significantly lower with 100 nM 1,25(OH)$_2$D$_3$ treatment, and there was no change in CDF appearance in the receiver, apical side (Fig. 9A). With apical administration, the appearance of CDF in the basolateral, receiver side was lower with 1,25(OH)$_2$D$_3$ treatment, but there was no change in CDF fluorescence in the cellular compartment (Fig. 9B). The plot of the transport efficiency (eq. 3) revealed the trend of transmembrane transport, showing a significantly higher transport at the apical membrane compared with the basolateral membrane, a decreasing trend of net basolateral efflux, and an increasing net apical efflux with 1,25(OH)$_2$D$_3$ treatment (Fig. 9C). These trends were, however, not statistically significant, suggesting that changes in MRP2 function were minor.

Results on CDF accumulation (administered as CDF-DA) in control and 1,25(OH)$_2$D$_3$-treated Caco-2 cells cultured on culture dishes are summarized in Fig. 9D. Consistent with increased MRP2 protein expression in 1,25(OH)$_2$D$_3$-treated Caco-2 cells, the fluorescence associated with the MRP2 substrate CDF inside the cells was similar between the control and ethanol (0.1%)-treated cells, but the fluorescence was significantly decreased after 1,25(OH)$_2$D$_3$ (100 nM) treatment for 3 days (44.0 ± 2.5 versus 29.2 ± 1.5 fluorescence/mg protein/min), suggesting appreciable excretion of CDF with 1,25(OH)$_2$D$_3$ treatment.

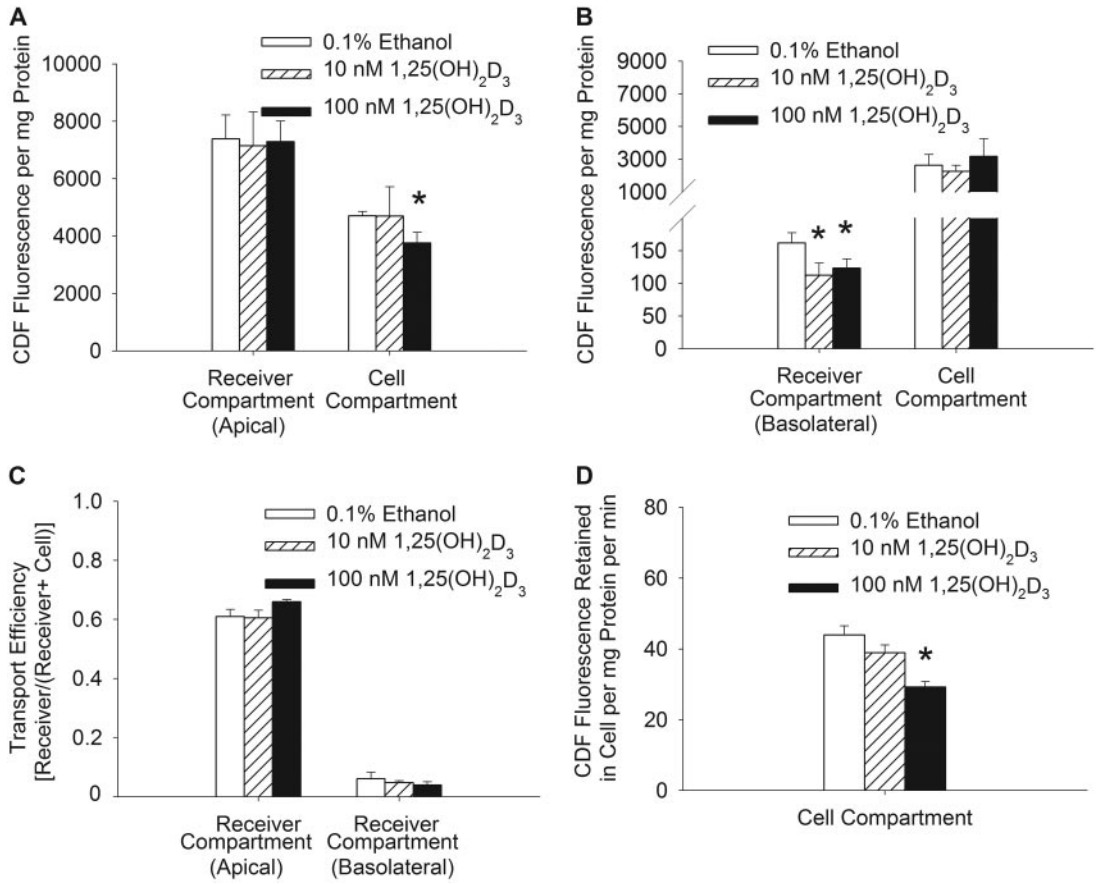


Fig. 9. $1,25(OH)_2D_3$ on MRP2 function in Caco-2 cells. CDF accumulation, measured as fluorescence, in the receiver side and in cells for ethanol (0.1%) and $1,25(OH)_2D_3$ (10 and 100 nM) treated Caco-2 cells after 40 min of incubation after basolateral (A) and apical (B) dosing of the precursor 10 μM CDF-DA, and transport efficiency (C) (amount of CDF in receiver side/amounts of CDF in receiver and cell) at the apical or basolateral membrane over 40 min in the Caco-2 cell monolayer in Transwell plates. Data are presented as mean ± S.D. ($n = 3$). *, $P < 0.05$ between the $1,25(OH)_2D_3$-treated and ethanol-treated groups. D, CDF retention/accumulation in cultured Caco-2 cells grown on culture dishes, measured as fluorescence, for the ethanol- (0.1%) and $1,25(OH)_2D_3$-treated Caco-2 cells after 30 min of incubation.

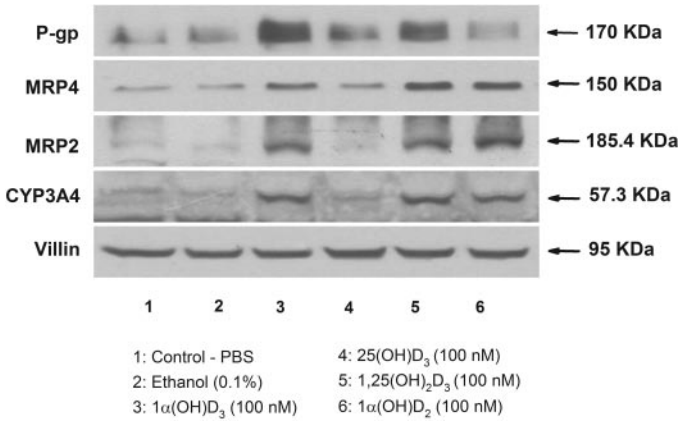


Fig. 10. Effects of vitamin D analogs on genes expression in Caco-2 cells. Caco-2 cells were treated with 100 nM $1\alpha,25(OH)_2D_3$, $1\alpha(OH)D_3$, $1\alpha(OH)D_2$, and $25(OH)D_3$ on day 15 for six consecutive days, whereas the control group was treated with ethanol (0.1%) in the course of culture. Cells were harvested on day 21, and protein expression was determined using Western blot and normalized to that of the house-keeping gene villin.

Effects of Vitamin D Analogs on Gene Expression in Caco-2 Cells Cultured in FBS

Effects of $1\alpha(OH)D_3$ and $1\alpha(OH)D_2$ on P-gp, MRP2, MRP4, and CYP3A4 protein expression were similar to those of $1,25(OH)_2D_3$ (Fig. 10; Table 3). Because all of the vitamin D analogs and $1,25(OH)_2D_3$ were tested at the 100 nM concentration, the relative potencies could not be directly compared because the analogs require activation to furnish the active forms. $1\alpha(OH)D_3$, $1,25(OH)_2D_3$, and $1\alpha(OH)D_2$ were all capable of significantly inducing P-gp, MRP2, MRP4, and CYP3A4 protein expression in Caco-2 cells after 3 or 6 days of treatment. MRP2 and P-gp protein expressions were highest after 3 days of treatment, and a higher induction on MRP4 and CYP3A4 protein expression was achieved after six consecutive days of treatment with the vitamin D analogs. However, $25(OH)D_3$ failed to exert any effect.

Effects of the Vitamin D Analog $25(OH)D_3$ on Protein Expression in Caco-2 Cells Cultured in Absence of FBS

The role of the vitamin D binding protein (DBP) in FBS on the availability of free $25(OH)D_3$ was further investigated by culturing the cells during the six consecutive treatment days in absence of 10% FBS; for this, an ITS-containing medium was used. $1,25(OH)_2D_3$ was again used as a standard for comparison with $25(OH)D_3$ (Fig. 11). In the absence of FBS (replaced by ITS containing medium), significantly higher inductive activities of $25(OH)D_3$ toward P-gp and MRP2 protein were observed compared with those with FBS in the culture medium (Fig. 11, C and D). Approximately 1.4- and 2-fold increases in P-gp and MRP2 protein expression over

TABLE 3
Relative protein expression in Caco-2 cells after treatment with vitamin D analogs for 3 and 6 days
Results are means ± S.D. from triplicate experiments; the mean value for the ethanol (0.1%) group was expressed as 1.0.

Treatment	Relative Protein Expression after 3 Days of Treatment				Relative Protein Expression after 6 Days of Treatment			
	MRP2	MRP4	P-gp	CYP3A4	MRP2	MRP4	P-gp	CYP3A4
Ethanol (0.1%)	1.0 ± 0.4	1.0 ± 0.2	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.2	1.0 ± 0.4	1.0 ± 0.6	1.0 ± 0.2
1α(OH)D_3 (100 nM)	3.0 ± 0.5*	2.1 ± 0.5*	2.6 ± 0.8*	4.0 ± 0.6*	2.1 ± 0.9	3.6 ± 0.5*	1.3 ± 0.6	5.6 ± 0.7*
25(OH)D_3 (100 nM)	1.5 ± 0.7	0.7 ± 0.4	1.3 ± 0.4	0.8 ± 0.1	1.7 ± 0.9	1.1 ± 0.2	1.1 ± 0.3	1.1 ± 0.1
1,25$(OH)_2D_3$ (100 nM)	3.4 ± 0.5*	2.5 ± 0.8*	2.9 ± 1.0*	4.4 ± 0.6*	2.7 ± 1.1	4.7 ± 1.1*	1.8 ± 0.5	5.8 ± 0.9*
1α(OH)D_2 (100 nM)	3.9 ± 1.0*	2.3 ± 0.7*	2.5 ± 0.9*	3.4 ± 1.4*	3.1 ± 0.8*	2.7 ± 0.9*	1.5 ± 0.3	4.9 ± 0.6*

* $P < 0.05$ compared with ethanol-treated group.

the vehicle-treated groups were achieved in response to 25(OH)D_3 in the absence of FBS, and the activities were less than that for 1,25$(OH)_2D_3$. Unexpectedly, protein expressions of CYP3A4 and MRP4 in Caco-2 cells cultured with 25(OH)D_3 in absence of FBS were similar to those cultured in FBS and were slightly but insignificantly higher than those of controls (Fig. 11, B and E). An amelioration of inductive effects for CYP3A4 and MRP4 in the absence of FBS was further observed for 1,25$(OH)_2D_3$ (Fig. 11, B and E), results that were consistent with observations of Schmiedlin-Ren et al. (1997), who speculated that FBS was essential to prevent the toxicity of 1,25$(OH)_2D_3$ by limiting its free concentration. The same reasoning was adopted here for the observations on CYP3A4 and MRP4, genes that may be sensitive to toxic effects of 1,25$(OH)_2D_3$ (see lane 4 of Fig. 11A) and 25(OH)D_3 (Fig. 11A, lane 3).

Discussion

Vitamin D is used for increasing calcium absorption and regulating bone mineral metabolism, and it is increasingly being used for the prevention and treatment of cancer and cardiovascular and autoimmune diseases (Holick, 2004; Schwartz and Skinner, 2007). Normally, 1,25$(OH)_2D_3$ exists at low levels in blood after vitamin D intake (DeLuca, 1988). However, upon administration of the commercially available 1,25$(OH)_2D_3$ via injection (calcitriol injection, Calcijex) or capsule (calcitriol, Rocaltrol) for the treatment of hypocalcemia, osteoporosis, hypoparathyroidism, hypoparathyroidism, osteomalacia, rickets, renal osteodystrophy (Bailie and Johnson, 2002), or cancer (Beer and Myrthue, 2006), high systemic levels of 1,25$(OH)_2D_3$ are attained. Consequently, due to the marked toxicity associated with 1,25$(OH)_2D_3$, vitamin D analogs that possess less hypercalcemic effects and toxicity are being developed as new drug entities (Masuda and Jones, 2006). The contribution of this class of 1,25$(OH)_2D_3$ products/vitamin D analogs to the regulation of genes that modulate drug disposition via the VDR has not been systematically examined.

Inductive effects of 1,25$(OH)_2D_3$ on transporters and enzymes have been observed in Caco-2 cells because of high levels of VDR and transporter and enzyme genes, rendering the system well suited for investigation (Taipalensuu et al., 2001). Our results demonstrated that there was a decrease in VDR mRNA level in 1,25$(OH)_2D_3$-treated cells compared with vehicle-treated cells (Fig. 2). Different responses of the VDR to 1,25$(OH)_2D_3$ with respect to VDR expression or VDR binding activity have been noted (Halline et al., 1994). 1,25$(OH)_2D_3$ could result in the up-regulation of VDR in undifferentiated Caco-2 cells, or would lose the inducing capability, or even down-regulate VDR expression and VDR binding activity in fully differentiated cells (Halline et al., 1994), suggesting factors other than VDR levels are important in the VDR response to 1,25$(OH)_2D_3$.

Our results showed that the 1,25$(OH)_2D_3$ treatment of Caco-2 cells significantly induced the mRNA and protein expression of various genes in a dose- and time-dependent manner. Results on the overall induction of CYP3A4 expression from microarray analyses (Fig. 1), RT-qPCR, Western blotting (Fig. 3, A and B), and the rate of 6β-hydroxylation of testosterone in 1,25$(OH)_2D_3$-treated Caco-2 cells in the present study were consistent with those of previous reports (Schmiedlin-Ren et al., 1997; Chan et al., 2004). Our results on the up-regulation of MDR1 mRNA and increased P-gp protein (Fig. 5, A and B) were validated functionally by the higher apical efflux of [^{3}H]digoxin across the 1,25$(OH)_2D_3$-treated Caco-2 cell monolayer (Fig. 8; Table 2). The parallel changes in MDR1 mRNA and P-gp protein expression, similar to those for CYP3A4, suggest coordinate, transcriptional modulation of CYP3A4 and MDR1 by the VDR. The activation of both CYP3A4 and MDR1 via the PXR is unlikely because low levels of PXR exist in Caco-2 cells, and the VDR/vitamin D pathway had previously failed to stimulate PXR expression (Schmiedlin-Ren et al., 2001). Due to the inability of 1,25$(OH)_2D_3$ to activate PXR even in the mucosal villus of the small intestine (Thummel et al., 2001), it was concluded that it was VDR not PXR that mediated the 1,25$(OH)_2D_3$-induced gene expression of CYP3A4 and MDR1 in Caco-2 cells. In vivo, both PXR and VDR could mediate the regulation of MDR1 and CYP3A4 (Lemaire et al., 2004; Satsu et al., 2008), rendering it difficult to resolve the VDR versus the PXR effects when vitamin D analogs and PXR-activating ligands such as rifampin are administered concomitantly.

Song et al. (2006) reported that SULT2A1 is a target for transcriptional activation by vitamin D mediated through VDR and CCAAT/enhancer-binding protein α in Caco-2 cells. However, we did not detect a significant change in mRNA of SULT2A1, SULT1A1, or SULT1E1 from qPCR assay, a discrepancy that could be explained by the different Caco-2 cells among laboratories and the differing cell culture conditions. We used Caco-2 cells that were fully differentiated and grown in DMEM with 10% FBS for 21 days, whereas cells used by Song et al. (2006) were grown in DMEM supplemented with 5% charcoal-dextran-stripped FBS and cultured only for 3 days. We were not able to detect SULT2A1 protein because these may surface only when cells are kept in a confluent stage for an extended period beyond 21 days, as suggested by Meinl et al. (2008).

No change in protein and mRNA levels of ASBT, PEPT1, and MRP3 was detected using Western blot and qPCR after

$1,25(OH)_2D_3$ treatment (Figs. 4 and 6, A and B). By contrast, changes in mRNA levels of the apical efflux transporter MRP2 were commensurate with increased protein (Fig. 5, C and D). The increased MRP2 expression correlated with the lower cellular retention of CDF, which was formed through the rapid hydrolysis after entry of the precursor CDF-DA (Fig. 9D). Due to the lack of involvement of MRP4 (Pratt et al., 2006) and lack of change in mRNA or protein expression of MRP3 (Fig. 6, A and B), the alternate route of efflux of CDF (Tian et al., 2004), namely, the reduction in cellular accumulation of CDF in Caco-2 cells, was found to correlate well with increased MRP2 function after $1,25(OH)_2D_3$ treatment. A similar reduction was noted in the cells in Transwell transport studies in the B-to-A direction (Fig. 9A).

For MRP4, an increase in protein and not mRNA, probably the result of post-transcriptional regulation, was observed (Fig. 6, C and D). This post-transcriptional regulation of MRP4 expression was also observed in primary biliary cirrhosis (Zollner et al., 2007). The regulation mechanism, confirmed by cycloheximide treatment of Caco-2 cells, is probably due to $1,25(OH)_2D_3$ being able to enhance MRP4 protein stability (Fig. 7). We attempted to assess the transport of [^{3}H]PMEA ([^{3}H]adefovir) as the substrate of MRP4 (Reid et al., 2003) after the addition of [^{3}H]bis(POM)-PMEA (or

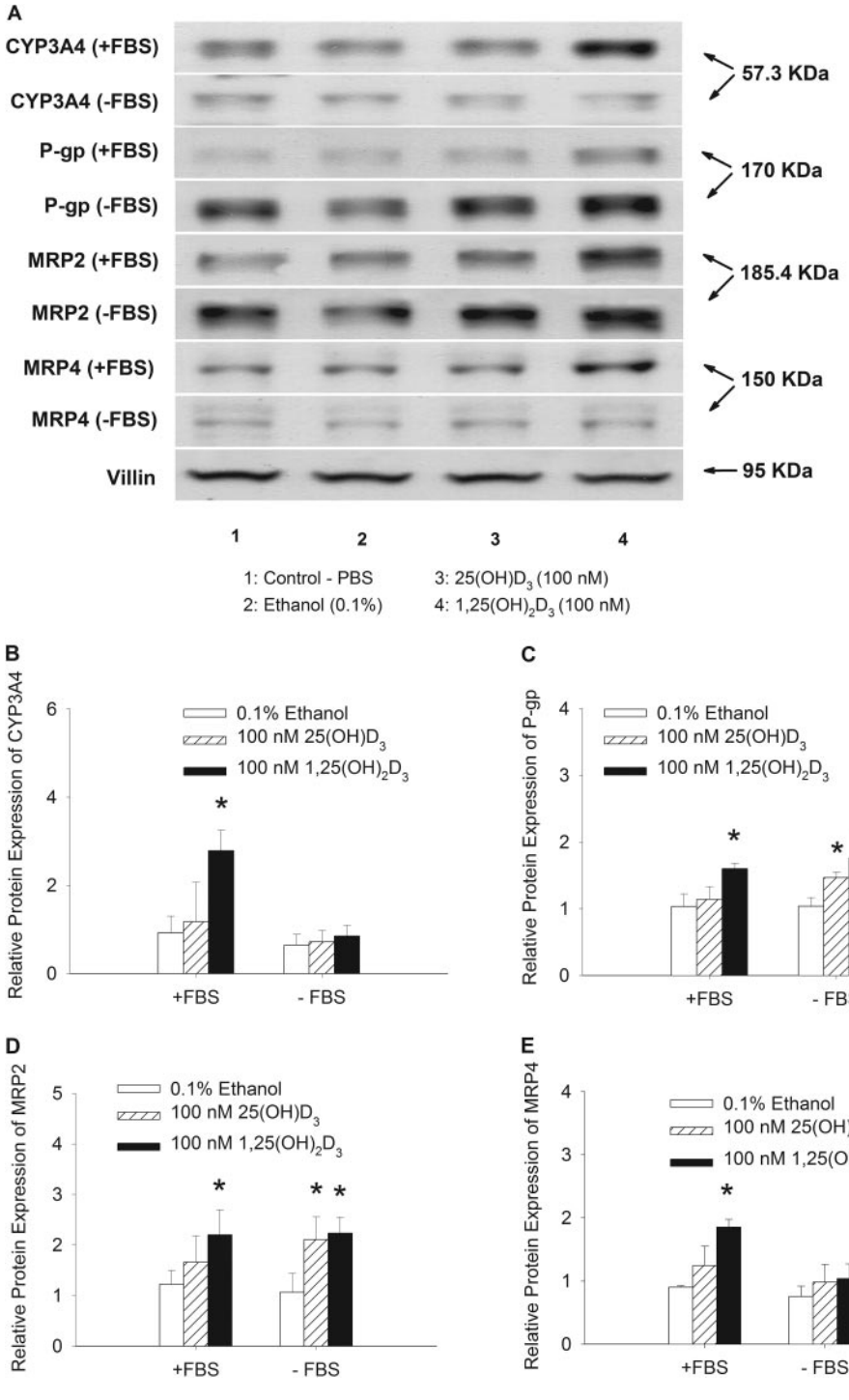


Fig. 11. Effects of $25(OH)_2D_3$ on P-gp, MRP2, MRP4, and CYP3A4 genes expression in Caco-2 cells. Cells were treated with 100 nM $1\alpha,25(OH)_2D_3$ and $25(OH)D_3$ on day 15 for six consecutive days in the absence (−FBS) or presence (+FBS) of 10% FBS in cell culture medium. Control group was treated with ethanol (0.1%). Cells were harvested on day 21. Protein expression was determined using Western blot and normalized to that of the housekeeping gene villin (A). The relative intensity of protein expression from Western blot analyses, and the summarized data (density histograms) were shown. B to E, mean ± S.D. ($n = 3$) for CYP3A4 (B), P-gp (C), MRP2 (D), and MRP4 (E). *, $P < 0.05$ between the ethanol- and $1,25(OH)_2D_3$-treated groups.

[^{3}H]adefovir dipivoxil) into donor (apical or basolateral) compartments after 1,25(OH)$_2$D$_3$ pretreatment in Transwell plates. The complexity of the multiple transporters: P-gp for the handling of [^{3}H]bis(POM)-PMEA; involvement of MRP4 and other MRP proteins for active transport of [^{3}H]bis(POM)-PMEA metabolites, mono(POM)-PMEA and PMEA, at both the apical and basolateral membranes (Annaert et al., 1998); and the varying extents of induction of P-gp, MRP2, and MRP4 by 1,25(OH)$_2$D$_3$ drastically compromised data interpretation. A thorough investigation is underway to examine the involvement of MRP4 with use of specific inhibitors.

The inert vitamin D analogs that require activation to the respective active species on induction of expression of major drug metabolic enzymes and transporters were also investigated. Our studies demonstrated, for the first time, the inductive effects of vitamin D analogs lα(OH)D$_3$, 25(OH)D$_3$, and 1α(OH)D$_2$ on CYP3A4, MDR1, MRP2, and MRP4 protein expression in Caco-2 cells (Fig. 10). The potencies of these vitamin D analogs to induce target genes expression depend on the binding affinities of the analogs toward the DBP and the binding affinity of the activated ligand(s) for the VDR. It is known that some nonrenal cells, including those of the prostate (Lou et al., 2004), colon, and pancreas, also express 1α-hydroxylase and 25-hydroxylase, which may lead to the local conversion of 25(OH)D$_3$, 1α(OH)D$_3$, and 1α(OH)D$_2$ to the active forms 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$, establishing the VDR signaling pathway among these cells. It has been noted that 25(OH)D$_3$ binds more tightly to DBP than 1,25(OH)$_2$D$_3$, whereas 1,25(OH)$_2$D$_3$ binds more tightly to the VDR than 25(OH)D$_3$ (Norman et al., 2001). Due to its high binding affinity for the DBP, the effect of 25(OH)D$_3$ on gene expression becomes limited due to reduced cellular availability of free 25(OH)D$_3$. Our observation in this study showed that the effectiveness of 25(OH)D$_3$ on gene expression in Caco-2 cells was lowered in the presence of FBS, a source of DBP, whereas much higher activity was re-established in the absence of FBS (Fig. 11). However, 25(OH)D$_3$, at a high concentration (1 μM), was reported to stimulate gene expression in the presence of FBS in Caco-2 cells (Taparia et al., 2006), implying that the interactions related to 25(OH)D$_3$ might be possible because the circulating concentration of 25(OH)D$_3$ is 1000-fold higher than that of 1,25(OH)$_2$D$_3$ in vivo (Taparia et al., 2006). All of the analogs—lα(OH)D$_3$, 25(OH)D$_3$, and 1α(OH)D$_2$—activated CYP3A4, MDR1, MRP2, mRNA, and protein expression and increased MRP4 protein, although absent for MRP3 (Fig. 10; Table 3). The activity of 25(OH)D$_3$ was masked when FBS was present in the culture medium (Fig. 11). Harada et al. (1993) had demonstrated previously that 1,25(OH)$_2$D$_3$ was more potent than lα(OH)D$_3$ and 25(OH)D$_3$ in bone calcium mobilization.

This study on Caco-2 cells suggests statistically significant changes in CYP3A4, MDR1, MRP2, and MRP4 protein with 1,25(OH)$_2$D$_3$. Several other genes were stimulated by 1,25(OH)$_2$D$_3$, as revealed from the positive and significant changes with microarray analyses (Fig. 1) that directed us to areas for exploration. Similar changes were observed with the inert vitamin D analogs, albeit with slightly lower inducing activities, suggesting that the Caco-2 cells possess enzymes to form the active 1,25(OH)$_2$D$_3$ and 1,25(OH)$_2$D$_2$. These changes on transporters and enzymes in the Caco-2 cells after 1,25(OH)$_2$D$_3$ treatment attest to the importance of VDR in the regulation of transporters and enzymes. In addition to PXR and CAR, the VDR is another important nuclear receptor that influences drug disposition.

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