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Research Paper

Metabolic and Efflux Properties of Caco-2 Cells Stably Transfected with Nuclear Receptors

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Purpose. To characterise in detail the patterns of expression and functional activities of CYP and efflux pump genes in Caco-2 cells stably transfected with human Pregnane X Receptor or murine Constitutive Androstane Receptor.
Materials and Methods. Cell lines transfected with nuclear receptors were treated with established ligands, and gene expression of CYP and efflux pump genes were quantified by qRT-PCR and Western blot. P-glycoprotein activity was assessed by measuring calcein-AM accumulation and bidirectional permeability coefficients of digoxin and quinidine. CYP activities were measured with both fluorescent and non-fluorescent substrates.
Results. hPXR and mCAR upregulated some CYP and efflux pump genes ligand dependently. P-glycoprotein level was increased, but CYP3A4 protein remained below the limit of detection. P-glycoprotein activity was markedly elevated in Caco/mCAR cells and more modestly in Caco/hPXR cells. CYP3A4 activity remained lower than that in vitamin D-treated Caco-2 cells.
Conclusions. Nuclear receptors can modulate the expression of metabolic genes in Caco-2 cells, but the overall level of metabolism could not be efficiently controlled. P-glycoprotein activity increased, but CYP activities remained very low.

KEY WORDS: Caco-2; CAR; drug absorption; metabolism; PXR.

INTRODUCTION

In vitro absorption experiments with cultured cell lines are widely used to predict the absorption of drug molecules across the intestinal epithelium. The epithelium possesses several active disposition mechanisms, such as active efflux pumps [P-glycoprotein, MRP2, BCRP (1,2)] and the oxidative cytochrome P450 enzyme system. The most abundant isoenzyme is CYP3A4, but several other isoenzymes have also been detected (3–5). Well known examples of drugs, whose pharmacokinetics are affected by these intestinal components, are digoxin (6) and midazolam (7). Drug–drug interactions are possible if these proteins are either induced (8) or inhibited (9). Transporters and CYPs often work cooperatively, and it has been shown that intact cells possessing both mechanisms can give a better estimate of the *in vivo* situation than simple isolated systems like microsomes (10,11). Two studies that use computational methods also suggest that metabolism and efflux pumps may work synergistically at the epithelium and prevent the absorption of molecules that are substrates to both CYPs and efflux pumps (12,13).

The Caco-2 cell line is the most widely used model of intestinal epithelium (14). Two major problems with this cell line, however, are the wide interlaboratory variability (14) and defective oxidative metabolism (15). To activate disposition mechanisms, several modifications of the parent cell line have been introduced. These modifications usually alter only one activity, such as transfection with cDNAs encoding MDR1, CYP3A4 or CYP3A4 and CYP oxidoreductase (16–18). Chemical activation of Caco-2 cells with 1α,25-dihydroxyvitamin D_3 can increase CYP3A4 activity by activation of the vitamin D receptor (19), and P-glycoprotein activity can be increased with cytotoxic vincristine (20). Such CYP3A4 transfected and vitamin D-treated cells are able to mimic intestinal CYP3A4 mediated first-pass metabolism during absorption (10,21). We recently introduced two novel

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ABBREVIATIONS: A-B, apical-to-basolateral; AQ, absorptive quotient; B-A, basolateral-to-apical; BCRP, breast cancer resistance protein; BFC, 7-benzyloxy-4-(trifluoromethyl)-coumarin; CYP, cytochrome P450; HFC, 4-(trifluoromethyl)-coumarin; HNF4, hepatic nuclear factor 4; hPXR, human pregnane X receptor; mCAR, murine constitutive androstane receptor; MDR1, multidrug resistance 1 gene (P-glycoprotein); MRP2, multidrug reistance associated Protein 2; SQ, secretory quotient; VD3, 1a,25-dihydroxyvitamin D_3.

Caco-2 cell lines that are transfected with orphan nuclear receptors, namely the human pregnane X receptor (Caco/hPXR) and the murine constitutive androstane receptor (Caco/mCAR) (22). Since these two orphan nuclear receptors regulate several genes involved in xenobiotic metabolism (23), our approach can, in principle, control the general level of xenobiotic metabolism. Such a model could be used both in mechanical studies on the role of active and passive processes at the intestinal level and for screening potential new molecules.

The purpose of the present study was to characterise the inducibility of metabolic genes in Caco-2 cell lines stably transfected with nuclear receptors and the resulting changes in efflux pump and CYP activities. The effect of established nuclear receptor ligands on the gene expression was studied on the mRNA and protein level, and the functional activity of P-glycoprotein and CYP3A4 was assessed.

MATERIALS AND METHODS

Chemicals

Commercial chemicals used for activating the cell lines were rifampicin (Sigma, St. Louis, MO), 5α-androsten-3α-ol (androstenol; Stearaloids, Newport, Rhode Island) and 1α,25-dihydroxyvitamin D_3 (VD3; Leo Pharma, Denmark). 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) was synthesized and purified according to Honkakoski *et al.* (24). Tritium-labelled digoxin and mannitol were bought from Perkin-Elmer (Boston, MA), tritium-labelled quinidine from American Radiolabelled Chemicals (St. Louis, MO) and unlabelled quinidine from Sigma (St.Louis, MO). Calcein-acetoxymethylester (Calcein-AM) was from Calbiochem (La Jolla, CA) and 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC) from BD Gentest (Franklin Lakes, NJ). The commercial efflux pump and CYP inhibitors were cyclosporin A, ketoconazole, progesterone (Sigma, St. Louis, MO) and verapamil (MP Biomedicals, Irvine, CA). GF120918 was a gift from GlaxoSmithKline (Research Triangle Park, NC).

Cell Lines and Culture Conditions

Caco-2 cells (ATCC HTB-37) were obtained from the American type culture collection (Manassas, VA) and used between passages 35–55. These wild type cells (Caco/WT) were used as control cell lines. The generation of stable transfectants Caco/hPXR and Caco/mCAR has recently been described (22). These cell lines were used between 18–32 and 12–26 passages after transfection, respectively. Cells treated with vitamin D (Caco/VD3) were obtained from Caco/WT cells by adding 250 nM 1α,25-dihydroxyvitamin D_3 to the growth medium. The culture conditions were similar to those described in our previous report (22).

Different activation schemes for nuclear receptors were used in this study. The applied concentrations were: rifampicin 10 μM (hPXR activator) (25), TCPOBOP 1 μM (mCAR activator) (26), androstenol 10 μM (mCAR inhibitor) (27) and vitamin D 250 nM (28). Cells grown on filters were induced with chemicals for 4, 7 or 14 days at the end of the 21-day growth period. The cells used for calcein-AM experiments were activated for the full 4-day growth period.

Quantitative RT-PCR

The mRNA levels of MDR1 (multidrug resistance protein 1), MRP2 (multidrug resistance associated protein 2), BCRP (breast cancer resistance protein) and cytochrome P450 isoforms CYP2B6, CYP2C9 and CYP3A4 were measured by quantitative RT-PCR as described previously (22). Briefly, the total RNA of appropriately grown cells was isolated (TRI reagent, Sigma, St. Louis, MO), treated with DNase (DNA free, Ambion, Austin, TX), quantified (RiboGreen, MolecularProbes, Netherlands) and reverse transcribed (Fermentas, Hanover, MD). The cDNA was amplified and detected using TaqMan chemistry (Applied Biosystems, UK). The primers and probes were custom-made (22). The gene expression levels were determined according to the comparative C_t-method and normalised to β-actin.

Western Blotting

CYP3A4 and MDR1 protein expression were quantified using antibodies. The cells were suspended into the lysis buffer (29) and allowed to stand on ice for 30 min. The supernatant was collected, and the protein concentration was measured with Bradford reagent according to the manufacturer's instructions (Sigma, St. Louis. MO). Samples were mixed with sample buffer and boiled for 1 min. Samples (50 μg for CYP3A4, 20 μg for MDR1) were electrophoresed into 12% SDS-PAGE gel and blotted onto a nitrocellulose membrane (Biorad, Hercules, CA).

For CYP3A4 immunoblot the membrane was rinsed with Tris buffered saline (TBS) and blocked with 5% fat-free milk powder for 1 h at room temperature. The anti-CYP3A4 antibody was provided by Dr. Robert J.Edwards (Imperial College School of Medicine, London, UK) (30). Blocked membrane was incubated at RT for 1 h in a 1:5,000 dilution of primary antibody in TBS containing 0.5% fat-free milk powder. The membrane was washed three times for 5 min with TBS containing 0.1% Tween20, incubated in a 1:12,500 dilution of Goat anti-rabbit HRP conjugated secondary antibody (Promega, Madison, WI) for 1 h and washed again three times for 5 min with TBS. Proteins were detected using SuperSignal west chemiluminescent substrate (Pierce, Rockford, IL). The exposure time was 30 s. Human CYP3A4 Supersomes (BD Gentest, Franklin Lakes, NJ) were used as standards.

For MDR1 immunoblot the membrane was blocked with 3% fat-free milk powder in phosphate-buffered saline (PBS) containing 0.3% Tween20 for 2 h at room temperature. The primary antibody (P-glycoprotein antibody [C494], AbCam, UK) was diluted 1:500 in PBS containing 0.5% BSA and 0.3% Tween20 and incubated with the membrane overnight at +4°C. The membranes were washed three times for 10 min with PBS-0.3% Tween 20. The anti-mouse secondary antibody (Promega, Madison, WI) was diluted 1:2,000 in PBS containing 3% fat-free milk powder and 0.3% Tween20 and incubated with the membrane overnight at +4°C. The membranes were washed three times for 10 min and the proteins were detected by chemiluminescence. The total cellular protein of MDCK-MDR1 cells that were grown on filters for 4 days was used as a positive control.

Calcein-AM Experiments

P-glycoprotein activity was assayed by incubating the cells with calcein-AM and measuring cellular retention of fluorescent calcein. The method was adapted from Eneroth *et al.* (20) and Korjamo *et al.* (22) with minor modifications. Briefly, the cells were grown on 96-well plates for 4 days. Activation chemicals were present for the whole growth period. The cells were washed and preincubated at +37°C for 15 min with Hanks' balanced salt solution (HBSS) buffered with 25 mM Hepes that contained inhibitors at the desired concentrations. Calcein-AM was added and the plates were incubated for a further 20 min. Test solutions were replaced with ice cold buffer, and fluorescence was measured with VICTOR^2 (Wallac, Finland) using 480 nm for excitation and 535 nm for emission. The reported relative fluorescence values were obtained using formula (sample fluorescence—background fluorescence)/10^5.

Permeability Experiments

Apparent permeability coefficients of P-glycoprotein substrates [^{3}H]-digoxin (1 μl/ml) and [^{3}H]-quinidine (1 μM cold, 1 μl/ml labelled) along with a paracellular marker molecule [^{3}H]-mannitol (1 μl/ml) were determined using 21-day-old cell monolayers grown on Transwell polycarbonate filters (membrane diameter 12 mm, pore size 0.4 μm; Costar, Corning, NY). Both apical-to-basolateral (A–B) and basolateral-to-apical (B–A) permeabilities were determined. In the indicated cases the activation chemicals were present in the culture medium for the last 14 days. The method was adapted from Korjamo *et al.* (22) with minor modifications. Briefly, monolayers were washed with HBSS-Hepes (pH 7.4) and allowed to equilibrate for 20–30 min with plain buffer or buffer containing GF120918 (2 μM) (31). Transepithelial electrical resistance was measured before the experiment to check the monolayer integrity. Due to the differences in monolayer tightness, the cut-off limits for the cell lines were Caco/WT: 330 Ωcm^2, Caco/hPXR: 200 Ωcm^2 and Caco/mCAR: 900 Ωcm^2. The experiment was initiated by adding the donor solution. Samples were taken from the receiver side at regular intervals up to 90 min. To check the mass balance, a sample was taken from the final donor. Apparent permeability coefficients were calculated under sink conditions according to the equation $P_{app} = J/AC_0$ (cm/s), where J = flux across the cell monolayer (nmol/s), A = area of the filter (cm^2) and C_0 = initial donor concentration. We checked the influence of the unstirred water layer by conducting the experiments at various stirring rates (Titramax 1000, Heidolph, Germany). The apparent permeability of digoxin and mannitol remained unchanged, and a stirring rate of 250 rpm was used thereafter. More efficient stirring increased the apparent permeability of quinidine, and therefore the practical maximum of 450 rpm was used.

Both passive and active transport contribute to the apparent permeability. It has been pointed out, e.g., by Troutman and Thakker (32) and Collet *et al.* (33), that P-glycoprotein activity should be evaluated by comparing the total apparent permeability (Papp) to the passive permeability (P_{pass}) determined in the presence of an inhibitor. Therefore, we also present absorptive (AQ) and secretory (SQ) quotients described by Troutman and Thakker (32). The absorptive quotient describes the percentage by which P-glycoprotein is able to reduce absorptive transport. Similarly, the secretory quotient reflects the relative increase in secretory transport. The formulas are $AQ = (P_{pass} - P_{app})/P_{pass}$ and $SQ = (P_{app} - P_{pass})/P_{pass}$.

CYP Metabolic Experiments

Initially, we measured the activity of nine different CYP isoforms in modified cell lines using the LC/MS cocktail approach described by Turpeinen *et al.* (34) with minor modifications. Substrates were administered in HBSS-Hepes at 2–60 μM concentrations, and the incubation time was up to 24 h. Only Caco/VD3 cells produced detectable amounts of metabolites of midazolam and omeprazole indicating CYP3A4 activity. Therefore, we switched to a more sensitive fluorescent probe.CYP3A4 activity was measured with a coumarin derivative (7-benzyloxy-4-(trifluoromethyl)-coumarin) that is dealkylated into fluorescent 4-(trifluoromethyl)-coumarin (HFC) by CYP3A4, CYP1A2 and to a lesser extent by CYP2B6 (35). Cells were grown and activated as in the permeability experiments. Filters were washed with HBSS-Hepes that contained 1% DMSO. BFC (50 μM) was added in both apical and basolateral compartments with or without a potent CYP3A4 inhibitor ketoconazole (5 μM). Cells were incubated at +37°C for 6 h, and 80 μl samples were collected from both chambers into B&W Isoplates (Wallac, Finland). A standard curve (5–500 nM) was constructed from HFC (Sigma, St. Louis, MO). Fluorescence was recorded with VICTOR^2 (Wallac, Finland). In initial experiments the cells were also lysed and the cellular fluorescence measured, but accumulation of HFC was low (<40% of the amount in the apical chamber) and correlated well with the extracellular HFC. Therefore samples were later taken only from the buffer. To hydrolyse possible conjugates the samples were also initially treated with 5 U of glucuronidase/sulfatase enzymes (Sigma, St. Louis, MO) at +37°C for 1 h; but the amounts of HFC were not elevated. As Matrigel coating has previously been shown to increase CYP3A4 activity (28), we also tested its effect on metabolic activity in our cell lines. Filters were coated with 30 μg of Matrigel (BD Gentest, Franklin Lakes, NJ) according to the manufacturer's instructions. Metabolic activities were, however, not markedly elevated.

Statistical Analysis

All cell lines and treatments were compared to the Caco/WT cells with the programs in the SPSS package (SPSS inc. Chicago, IL) using one-way ANOVA and Dunnett's pairwise test. The effects of CAR and PXR ligands on the Caco/mCAR and Caco/hPXR cells were tested with ANOVA or *t*-test depending on the number of groups.

RESULTS

Quantitative RT-PCR

The mRNA expression of several metabolic genes was changed in fully differentiated cells (Table I). Caco/hPXR cells showed elevated levels of CYP3A4, MDR1 and

Table I. Quantitative PCR Results of Filter-grown (21 days) Caco-2 Cell Lines

Cell line	Treatment	Days	CYP3A4	CYP2B6	CYP2C9	MDR1	MRP2
Caco/WT	DMSO	14	1.0 ± 0.3	1.0 ± 0.3	1.0 ± 0.2	1.0 ± 0.5	1.0 ± 0.3
	Rifampicin	14	1.3 ± 0.4	0.8 ± 0.3	0.9 ± 0.2	1.3 ± 0.4	0.9 ± 0.2
	TCPOBOP	14	1.0 ± 0.5	0.9 ± 0.3	0.8 ± 0.2	1.0 ± 0.3	1.2 ± 0.4
	Androstenol	14	1.2 ± 0.5	0.9 ± 0.3	1.0 ± 0.2	1.0 ± 0.4	1.2 ± 0.4
Caco/hPXR	DMSO	14	21.2 ± 4.7[b]	1.4 ± 0.5	2.4 ± 0.6	6.5 ± 1.5	1.4 ± 0.3
	Rifampicin	14	39.7 ± 11.2[b]	1.5 ± 0.4	2.9 ± 0.7[b]	11.7 ± 1.3[b]	2.3 ± 0.5[b]
	Rifampicin	7	42.0 ± 12.5[b]	1.4 ± 0.3	2.5 ± 0.5	9.8 ± 1.3[b]	2.1 ± 0.5[b]
	Rifampicin	3	29.5 ± 6.7[b]	1.1 ± 0.3	1.9 ± 0.4	6.0 ± 1.4	1.3 ± 0.3
Caco/mCAR	DMSO	14	1.3 ± 0.4	0.4 ± 0.1	2.4 ± 0.5	3.7 ± 1.9	1.1 ± 0.2
	TCPOBOP	14	4.2 ± 1.1	2.5 ± 0.5[b,c]	4.2 ± 0.8[b,c]	7.0 ± 2.7[b]	1.9 ± 0.3
	TCPOBOP	7	3.7 ± 1.0	2.0 ± 0.4[b,c]	3.9 ± 0.7[b,c]	6.6 ± 2.4[b]	2.0 ± 0.4[b]
	TCPOBOP	3	3.5 ± 0.8	1.8 ± 0.5[c]	3.6 ± 0.7[b]	4.7 ± 2.6	1.3 ± 0.2
	Androstenol	14	3.9 ± 1.4	0.2 ± 0.1	2.5 ± 0.5	5.3 ± 2.0	1.3 ± 0.3
	Androstenol	7	3.5 ± 1.0	0.3 ± 0.1	3.7 ± 0.7[b]	6.9 ± 3.8	1.6 ± 0.2
	Androstenol	3	3.3 ± 0.9	0.3 ± 0.1	3.2 ± 0.7[b]	6.2 ± 2.5	1.6 ± 0.3
Caco/VD3[a]	Vitamin D3	14	900 ± 140[b]	1.4 ± 0.1	1.9 ± 0.1	3.3 ± 1.2	1.7 ± 0.3

Total RNA was isolated, treated with DNase and reverse transcribed using random priming. Samples were amplified using TaqMan-chemistry. mRNA levels were calculated using comparative C_t-method. Individual expression levels were normalised to β-actin and compared to Caco/WT expression levels. Results are expressed as mean ± S.E.M. $n = 5$.

[a] Data taken from (22).

[b] Significantly different from Caco/WT DMSO according to ANOVA and Dunnett's *post hoc* test, $p < 0.05$.

[c] Significantly different from the corresponding cell line treated with DMSO according to ANOVA and Dunnett's *post hoc* test, $p < 0.05$.

CYP2C9 mRNA. In the uninduced Caco/mCAR cells, the expression of CYP2B6, CYP2C9 and MDR1 was slightly altered. BCRP did not respond to any modifications or treatments (0.7 to 1.1 times Caco/WT), and these data were omitted from Table I. For comparison, data from Caco/VD3 cells were taken from our previous study (22).

Nuclear receptor ligands were able to modulate gene expression in Caco/hPXR and Caco/mCAR cells, but these ligands had no effect on the gene expression of Caco/WT cells. CYP3A4, MDR1 and MRP2 genes showed the best and time-dependent responses to rifampicin treatment in Caco/hPXR cells (Table I), but the changes did not reach statistical significance. A prototypical mCAR activator, TCPOBOP, induced the most pronounced activation on the CYP2B6 gene. In addition, the CYP2C9 gene was significantly activated by TCPOBOP, but the other genes responded to a lesser extent. The mCAR inhibitor androstenol was able to decrease only CYP2B6 transcription.

The influence of nuclear receptors on the gene expression of relatively undifferentiated Caco-2 cells was also measured (Table II). The mRNA levels of all genes were higher in Caco/hPXR than in Caco/WT cells, but rifampicin treatment did not increase the expression any further. mCAR transfection was most effective on CYP2B6, CYP2C9 and MDR1, which also responded to TCPOBOP. The inhibitory action of androstenol is clear only with the CYP2B6 gene, as was also the case with filter grown cells. Furthermore, Table II shows that the expression of all the studied genes is increased during the differentiation of Caco-2 cells.

Western Blot

CYP3A4 and MDR1 gene expression was quantified at the protein level (Fig. 1). CYP3A4 was only detectable in Caco/VD3 cells, which had approximately 0.4 nmol of CYP3A4 per milligram of protein. The CYP3A4 protein from the cells migrated at slightly faster than CYP3A4 from commercial microsomal preparation. MDR1 gene product P-glycoprotein was barely detected in the Caco/WT cells. The stable transfectants showed constitutive expression of P-glycoprotein, that was yet increased by nuclear receptor ligands.

Calcein-AM

P-glycoprotein function was evaluated in calcein-AM experiments (Fig. 2). The basal calcein retention was lower in

Table II. Quantitative PCR Results of Caco-2 Cell Lines Grown on Plastic (4 days)

	Treatment	CYP3A4	CYP2B6	CYP2C9	MDR1	MRP2
Caco/WT	–	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0	1.0 ± 0.0
Caco/hPXR	–	11.3 ± 1.7	4.2 ± 0.2	4.1 ± 0.9	2.3 ± 0.0	2.1 ± 0.5
	Rifampicin	11.0 ± 2.8	3.0 ± 0.8	3.2 ± 0.5	1.9 ± 1.2	1.8 ± 1.2
Caco/mCAR	–	0.9 ± 0.7	4.8 ± 2.7	6.2 ± 0.3	4.9 ± 1.9	1.8 ± 0.7
	TCPOBOP	2.9 ± 0.5	15.9 ± 5.3	11.6 ± 3.0	9.6 ± 1.4	2.7 ± 0.4
	Androstenol	0.9 ± 0.4	2.9 ± 0.9	10.3 ± 5.0	5.4 ± 0.7	2.4 ± 0.4
Caco/WT 21 days		11.2 ± 9.2	6.4 ± 0.5	21.6 ± 14.8	8.9 ± 3.6	4.1 ± 2.9

See footnote of table 1 for experimental details. Results are presented as a mean ± S.E.M. of two independent experiments.

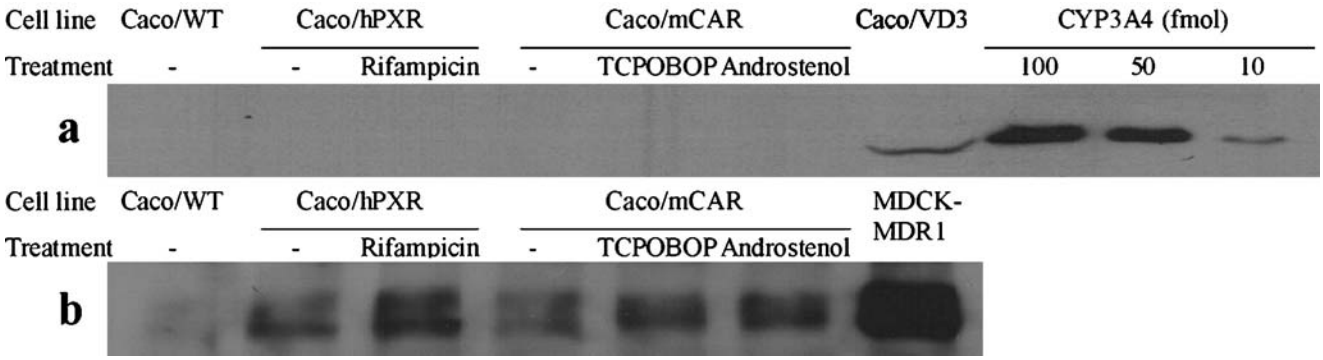

Fig. 1. Western blot data. Cells were frown on filters for 21 days and activated with indicated chemicals for the last 14 days. (a) 50 μg of total cellular protein was blotted onto nitrocellulose membrane and probed with CYP3A4 specific antibody. Commercially available microsomes were used as standards. No other specific bands were detected even after long exposure times. (b) 20 μg of total cellular protein was blotted into nitrocellulose membrane and probed with MDR1 specific antibody. Total protein form MDCK-MDR1 cells was used as a positive control.

both stable transfectants than in the Caco/WT cells. Inhibitors elevated the fluorescence values in Caco/mCAR cells to about the same level as in Caco/WT cells. Therefore, retention factors (inhibitor/vehicle ratios) were also significantly higher in Caco/mCAR cells. In Caco/hPXR cells, however, the fluorescence levels remained lower than in the wild type cells and the retention factors were only non-significantly elevated. Nuclear receptor ligands were also tested as inhibitors, but they did not increase the cellular retention of calcein (data not shown).

Permeability Experiments

Mannitol permeability was used to check monolayer integrity. Permeabilities were Caco/WT: 1.85 (±0.17) $\times 10^{-6}$ cm/s, Caco/hPXR: 1.97 (±0.11) $\times 10^{-6}$ cm/s and Caco/mCAR: 1.03 (±0.09) $\times 10^{-6}$ cm/s. We recovered virtually 100% of the applied digoxin and mannitol, but the mass balance for quinidine was about 80–90%, probably due to the cellular retention.

Digoxin was our P-glycoprotein model substrate with low passive permeability. We checked with Caco-WT cells whether the nuclear receptor ligands directly affected digoxin permeability e.g., by inhibiting some transporters. The transport of digoxin was highly polarised in the absence and essentially unpolarised in the presence of the P-glycoprotein inhibitor GF120918 (Fig. 3). Rifampicin moderately decreased the B–A permeability digoxin (from 16.9 $\times 10^{-6}$ cm/s to 13.0 $\times 10^{-6}$ cm/s) in the Caco/WT cells when present in the transport buffer, but none of the ligands affected the permeability when they were used only as activators during the growth period. The passive component was similar in Caco/WT and Caco/hPXR cells, but Caco/mCAR cells formed a tighter permeability barrier. Caco/hPXR had both decreased absorptive and increased secretory transport, indicating elevated P-glycoprotein activity. Rifampicin treatment tended to further increase P-glycoprotein-mediated digoxin transport. Caco/mCAR cells showed decreased A–B permeability, but the secretory transport was similar to that in the Caco/WT cells. Surprisingly, mCAR ligands affected digoxin permeability in ways that were opposite to what was anticipated. TCPOBOP reduced and androstenol increased the difference between inhibited and uninhibited digoxin permeability.

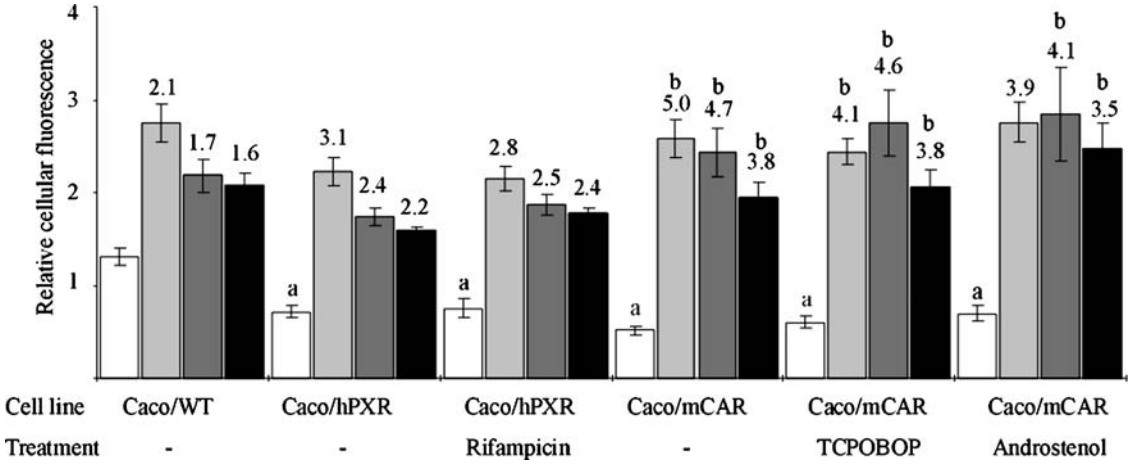

Fig. 2. Cellular retention of calcein after incubating calcein-AM with or without P-glycoprotein inhibitors. *Bars* represent background corrected relative cellular fluorescence (mean ± S.E.M.). Treatments were 1% DMSO (white), 15 μM cyclosporin A (*light grey*), 170 μM progesterone (*dark grey*) and 600 μM verapamil (*black*). *Numbers above bars* are inhibitor-to-vehicle fluorescence ratios. n = 4–7. [a]Fluorescence significantly different from corresponding Caco/WT fluorescence according to ANOVA and Dunnett's *post hoc* test, $p < 0.05$ [b]Inhibitor-to-vehicle ratio significantly different from corresponding Caco/WT value according to ANOVA and Dunnett's *post hoc* test, $p < 0.05$.

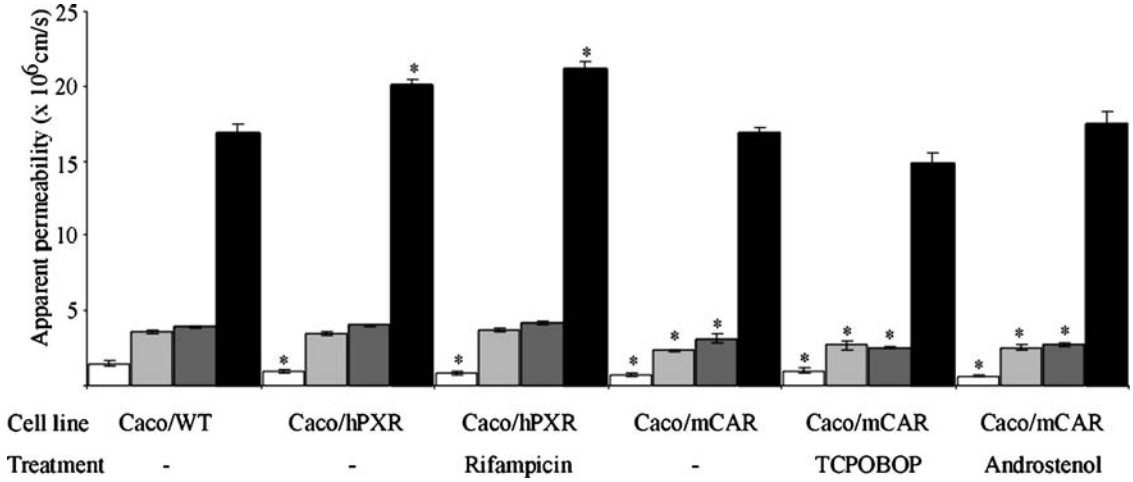

Fig. 3. Apparent permeability coefficients of digoxin. The transport buffer was spiked with radiolabelled digoxin and bidirectional apparent permeability coefficients were measured. *Bars* represent mean ± S.E.M. Permeability coefficients were measured in the apical-to-basolateral direction without (*white*) or with (*light grey*) the inhibitor (GF120918) and in the basolateral-to-apical direction with (*dark grey*) or without (*black*) the inhibitor. $n = 5–8$. *Significantly different from Caco/WT according to ANOVA and Dunnett's *post hoc* test, $p < 0.05$.

Quinidine is a good P-glycoprotein substrate with high passive permeability. The results of the permeability experiments (Fig. 4) show that passive permeability was similar in Caco/WT and Caco/hPXR cells but moderately lower in Caco/mCAR cells. Increased P-glycoprotein activity in the stable transfectants was evident from the decreased absorptive permeability. B–A permeability was, however, elevated only in Caco/mCAR cells. Statistical testing was omitted because the small number of replicates ($n = 2$) does not allow reliable testing.

The relative role of P-glycoprotein in permeability can be evaluated with absorptive and secretory quotients (32), which are presented along with the classical apical-to-basolateral/basolateral-to-apical (A–B/B–A) ratio in Table III.

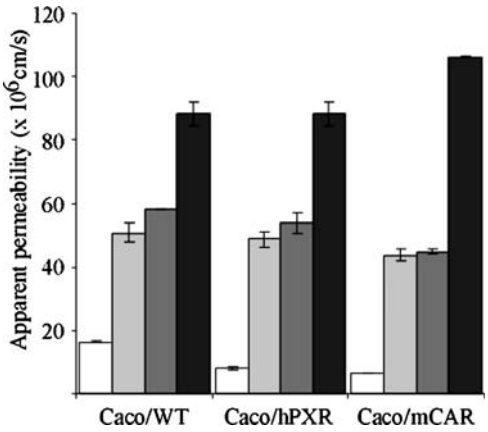

Fig. 4. Apparent permeability coefficients of quinidine. Transport buffer containing 1 μM unlabelled quinidine was spiked with radiolabelled quinidine and bidirectional apparent permeability coefficients were measured. *Bars* represent mean ± S.E.M. Permeability coefficients were measured in the apical-to-basolateral direction without (*white*) or with (*light grey*) the inhibitor (GF120918) and in the basolateral-to-apical direction with (*dark grey*) or without (*black*) the inhibitor. $n = 2$. Statistical testing was omitted due to small number of replicates.

Both modified cell lines were able to attenuate the absorption of digoxin (AQ values) more than the Caco/WT cells were. Also based on the secretory quotient, the P-glycoprotein activity seems to be elevated in the modified cell lines. Rifampicin induced a minor elevation in both quotients in the Caco/hPXR cells. As evident also from Fig. 3, androstenol treatment increased both AQ and SQ in the Caco/mCAR cells for digoxin, while TCPOBOP had smaller effect on these parameters. The A–B/B–A-ratios were significantly lower in both modified cell lines than in Caco/WT cells. The AQ of quinidine was elevated similarly in both modified cell lines in comparison to the Caco/WT cells. SQ, however, was considerably increased only in the Caco/mCAR cells (Table III). Moreover, the reduction of A–B/B–A-ratio of quinidine in stable transfectants clearly indicates increased P-glycoprotein activity.

CYP3A4 Activity

Metabolic activity of CYP3A4 was measured with a coumarin derivative BFC with and without ketoconazole (Fig. 5). The ability of CYP3A4 to metabolise BFC was confirmed using commercial CYP3A4 preparation (data not shown). In general, the metabolic activities in Caco-2 cell lines were very low since CYP3A4-mediated product formation after 6 h was less than 0.1% of the initial substrate concentration. Caco/WT had the lowest and Caco/VD3 the highest CYP3A4-activity. The modified cell lines fell between these two extremes. Uninhibited Caco/WT and Caco/VD3 cells produced an apical concentration of 21.7 and 42.1 nM, respectively. Ketoconazole reduced these values to 18.4 and 24.6 nM.

DISCUSSION

First-pass metabolism at the intestinal epithelium is an established fact for certain compounds (7) but whether it is generally significant or just a peculiarity affecting only a marginal number of compounds is still being debated (36,37).

Table III. The Relative Effect of P-glycoprotein Activity on Digoxin and Quinidine Permeability

		Digoxin			Quinidine		
	Treatment	AQ[a]	SQ[b]	A–B/B–A[c]	AQ[a]	SQ[b]	A–B/B–A[c]
Caco/WT	–	0.58	3.3	0.089	0.68	0.52	0.184
Caco/hPXR	–	0.73	4.0	0.045[d]	0.84	0.64	0.092
	Rifampicin	0.77[d]	4.1	0.040[d]	N.D.	N.D.	N.D.
Caco/mCAR	–	0.69	4.4	0.043[d]	0.85	1.37	0.063
	TCPOBOP	0.64	4.8[d]	0.067	N.D.	N.D.	N.D.
	Androstenol	0.75[d]	5.5[d]	0.036[d]	N.D.	N.D.	N.D.
Caco-2[e]		0.85	2.0	0.049	0.63	0.95	0.191
MDCK-MDR1[e]		0.75	3.8	0.053	0.67	3.90	0.067

Apparent permeability coefficients are represented as Papp $\times 10^6$ cm/s. *ND* Experiment not done.
[a] Absorptive quotient = $(P_{pass}-P_{app})/P_{pass}$ (32).
[b] Secretory quotient = $(P_{app}-P_{pass})/P_{pass}$ (32).
[c] P_{app} apical-to-basolateral/P_{app} basolateral-to-apical.
[d] Significantly different from Caco/WT according to ANOVA and Dunnett's *post hoc* test, $p < 0.05$.
[e] Data taken for comparison from Troutman and Thakker (32).

Therefore, an *in vitro* model that could express considerable and preferably regulatable amounts of CYP enzymes and efflux pumps would be useful in mechanistic studies. Our aim was to study how two nuclear receptors (hPXR and mCAR) affect CYP and efflux pump activities in the Caco-2 cell line and whether we could use known ligands to regulate the general level of metabolism in these cells.

Gene Expression

Genes involved in xenobiotic metabolism need to be regulated efficiently in response to exposure to various chemicals. Indeed, orphan nuclear receptors, which are thought to be key regulators of xenobiotic metabolism, have a wide ligand specificity and several target genes that are partially overlapping (38,39). Many important CYP-genes (e.g. 2B6, 2C9 and 3A4) and efflux pumps (e.g. MDR1 and MRP2) are target genes for PXR or CAR or both (23). BCRP expression has been correlated to PXR expression in small intestinal samples (2), but direct evidence of its regulation is missing.

Both PXR (25) and CAR (40) are expressed in the small intestine. Caco-2 cells do not express PXR (41), but during differentiation CAR transcription is elevated (40). Nuclear receptors may show some basal activity, which may be due to the presence of endogenous ligands or some chemicals in the cell culture medium. PXR has broader ligand specificity than CAR, and most of the PXR ligands activate the receptor and increase the expression of target genes (42). It should be noted, however, that not all the PXR target genes respond similarly to all activators, which is probably due to differences in receptor–coactivator interactions (43). In contrast to PXR, CAR usually shows considerable basal activity, and its ligands can either increase (44,45) or decrease (27,46) the expression of the target genes.

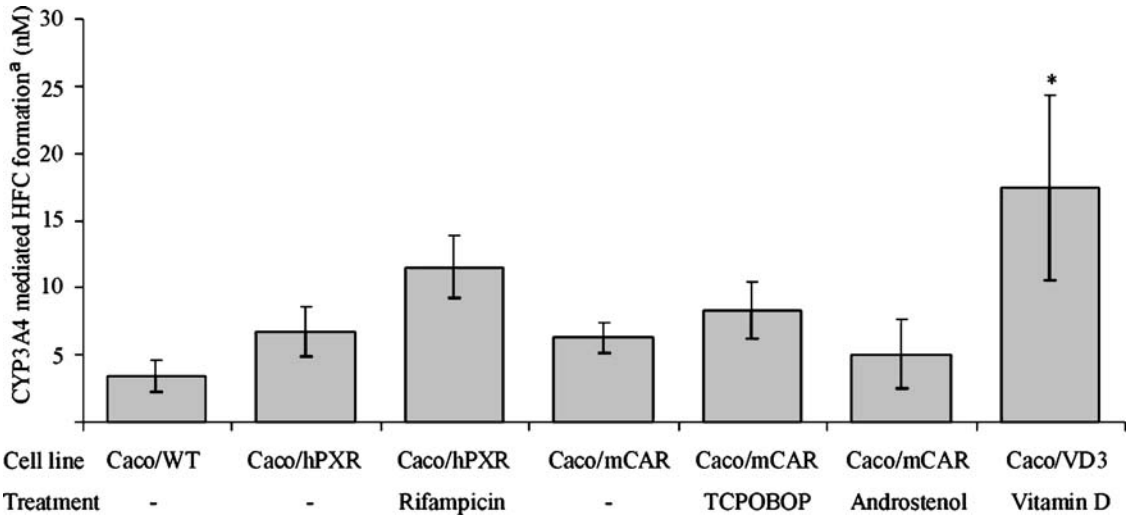

Fig. 5. CYP3A4 mediated metabolism in modified cell lines. Cells were incubated in HBSS-Hepes containing 50 μM BFC for 6 h with or without 5 μM ketoconazole. The concentration of fluorescent metabolite HFC was analysed from the apical chamber. CYP3A4 mediated metabolism was calculated by subtracting the HFC concentration in inhibited wells from that of the uninhibited wells. Bars represent mean ± S.E.M. $n = 3–6$. [a]CYP3A4 mediated HFC formation = C_{apical} (without ketoconazole) − C_{apical} (with ketoconazole) *Significantly different from Caco/WT according to ANOVA and Dunnett's *post hoc* test, $p < 0.05$.

mRNA Level

Modified Caco-2 cell lines showed changes in the patterns of gene expression. Caco/hPXR cells had higher uninduced levels of expression of CYP3A4 and MDR1 than Caco/mCAR cells (Table I). A previous study showed that transient hPXR transfection alone does not affect CYP3A4-reporter gene activity in Caco-2 cells, but rifampicin treatment causes nearly 20-fold activation (47). Compared to this, our Caco/hPXR cells showed considerable basal activation of CYP3A4 gene (>20-fold), but further increase with rifampicin was modest (1.9-fold). RT-PCR results (Table I) show, that hPXR can activate CYP3A4 gene in Caco-2 cells only modestly compared to vitamin D-treatment. Vitamin D acts through the vitamin D receptor, but this response has been shown to depend on the cell line (19). The activation of CYP2B6 in our Caco/mCAR cells caused by TCPOBOP was strong and in line with the results of a previous study with mouse hepatocytes in which 6.5-fold induction of the CYP2B10 gene was reported (46). A recent study reported that stably transfected human CAR could upregulate MDR1, CYP3A4 and CYP2B6 in an intestinal cell line to a greater extent than in our study, but there were differences in activation profiles between different clones, possibly due to the random integration of the vector (48). Androstenol could decrease the expression of CYP2B6, but other genes were unaffected or even activated. The mechanism of this activation is not clear but it may include differences in the cellular environment (48) or co-regulator requirements of promoters (43) or insufficient HNF4 expression (47,49). It can be concluded that hPXR and mCAR can alter gene expression in Caco-2 cells, but complete control of the expression of metabolic genes was not achieved, probably partly due to improper expression of the necessary auxiliary proteins in the Caco-2 cells.

We also studied gene expression and induction in less differentiated cells grown on plastic multi-well plates for 4 days that are used for calcein-AM experiments (Table II). In this case, TCPOBOP was able to activate Caco/mCAR cells, but rifampicin had virtually no effect on Caco/hPXR cells. Again, one explanation for these patterns of regulation might be the expression of nuclear receptor coregulators. The original data of Sun *et al.* (50) reveal that during the Caco-2 cell differentiation the expression of at least three nuclear receptor coregulators is elevated. Therefore, the lack of coactivators may hamper the nuclear receptor function in these relatively undifferentiated cells.

Protein Level

Changes in transcription should also alter protein levels. The findings of Taipalensuu *et al.* (51) suggest that the transcription and protein synthesis of the MDR1 gene correlated well in several adherent cell lines used for permeability studies. Our Caco-2 cells had clearly less P-glycoprotein than the positive control cell line MDCK-MDR1, which differs considerably from the results of Troutman and Thakker (32) who reported only a 1.5 fold difference. Nuclear receptor transfections produced only small additional increases in P-glycoprotein levels.

Previous studies from other laboratories have shown that Caco-2 cells express very low levels of CYP3A4 and CYP1A1 but not CYP1A2, CYP2C9, CYP2D6 or CYP2E1 proteins (52,53). Our CYP3A4 Western blot method could detect protein only from Caco/VD3 cells (Fig. 1a). The amount of CYP3A4 in whole-cell lysate is 0.4 pmol/mg, which is 40 times less than in reported the original publication (28). It should be noted, however, that the Matrigel coating used in the original study was omitted in our study since we did not observe a significant effect on CYP3A4 activity. Nuclear receptor transfections, especially hPXR, were able to increase CYP3A4 transcription but not to an extent similar to vitamin D treatment, and the protein levels remained below the limit of detection. This result points out the difficulty of upregulating CYP gene expression in Caco-2 cells.

Functional Activities

Small changes in transcription or even in protein level do not necessarily change the functional properties of the cell. This is especially true if the outcome of an assay depends on several mechanisms, including passive diffusion or the activity of other proteins. Therefore, we evaluated P-glycoprotein activity with two experimental settings that were cellular retention of calcein after calcein-AM exposure and bidirectional permeability experiments with digoxin and quinidine. CYP enzyme activities were initially tested with a cocktail approach (34); but since only traces of midazolam and omeprazole metabolites were produced in the positive control cell line (Caco/VD3), we used later a fluorescent probe for CYP3A4.

CALCEIN-AM

P-glycoprotein activity in the calcein-AM experiments (Fig. 2) and the mRNA level of MDR1 (Table II) showed a rank order correlation Caco/mCAR > Caco/hPXR > Caco/WT. In contrast to MDR1 transcription, nuclear receptor ligands did not change the results of calcein-AM experiments. The total fluorescence in the presence of inhibitors was moderately but consistently lower in Caco/hPXR than in other cell lines. A similar finding was previously made with vincristine-treated Caco-2 cells (20). As fluorescent calcein is effluxed by MRP1 and MRP2 (54), differences in their expression could change the basal retention of calcein. However, this is probably not the explanation, since the cellular fluorescence in Caco/hPXR cells remains lower than in the Caco/WT cells even in the presence of MDR1/MRP1/MRP2 inhibitor cyclosporin A. One possible reason is the moderately slower growth rate of Caco/hPXR cells, which can be seen during routine subculturing. Caco/hPXR cells reach confluence on 96-well plates in 24 h like Caco/WT cells, but may form less cell mass during the total 4-day growth period. Of course, the intracellular esterase activity also affects the formed fluorescence, and it is possible that this activity is lowered in Caco/hPXR cells.

Permeability

Bidirectional permeability experiments can reveal P-glycoprotein activities in cell lines. Previously, we have tested

the permeability properties of our transfected cell lines with several compounds including P-glycoprotein substrates verapamil and rhodamine-123 (22). Those two compounds could not make differences between our cell lines, because their permeability is only partly dependent on the P-glycoprotein activity as discussed earlier (22). For this study we chose digoxin and quinidine as model substrates, since they both are good P-glycoprotein substrates (8,55) and their passive permeabilities differ considerably. However, the difficulty of finding marker molecules that would be sole substrates of a single transporter is illustrated by a generally accepted P-glycoprotein marker molecule digoxin. In rat and human kidney, in addition to P-glycoprotein, digoxin permeability involves basolateral OATP transporter (56). An OATP substrate rifampicin has been shown to modulate digoxin transport in perfused rat liver (11). We also observed changes in digoxin permeability if rifampicin was added to the transport buffer. Thus, the apparent permeability of digoxin does not depend solely on the P-glycoprotein activity and the passive diffusion properties of the monolayer.

Changes in the P-glycoprotein activity are not necessarily obvious from individual permeability values. We used digoxin and quinidine at concentrations that were below K_m-values (57) and differences in apparent permeability should reflect altered P-glycoprotein levels. In all cell lines the passive permeability of both molecules was essentially unpolarised, but Caco/mCAR cells had slightly lower absolute permeability coefficients (Figs. 3 and 4), which is, at least for digoxin, caused by differences in paracellular permeability. Consistent with mRNA and protein data, both P-glycoprotein substrates permeated the Caco/mCAR and Caco/hPXR cells slower than the wild type cells, and thus the relative attenuation of absorption (Absorptive quotient) was increased (Table III). The increase in P-glycoprotein activity in the B–A direction was less evident because the absolute permeability values were not consistently higher in the modified cell lines. Still the relative contribution of P-glycoprotein to the secretory permeability (Secretory quotient) was at least modestly elevated in each case. In line with the mRNA and protein data, rifampicin could increase P-glycoprotein activity but the magnitude of the effect was small. Paradoxically, mCAR inhibitor androstenol caused apparent increase and activator TCPOBOP caused slight decrease in P-glycoprotein activity.

Digoxin has been classified as a high responder and quinidine as a low responder to P-glycoprotein activity according to their sensitivity to changes in P-glycoprotein expression (32). Our results show that generally digoxin SQ was more sensitive to P-glycoprotein level than quinidine SQ. However, changes in AQ values were very similar for both substrates. Comparison to Caco-2 and MDCK-MDR1 literature data (Table III) shows, that P-glycoprotein expression is not the sole factor determining the magnitude AQ and SQ, but the expression of other transporters, the paracellular permeability and probably also the details of experimental settings modulate the outcome.

CYP Activity

The lack of functional cytochrome P450 enzymes in Caco-2 cells is a major obstacle in studying intestinal first-pass metabolism. Previous studies have approached this problem with different methods. Crespi *et al.* (17) transfected Caco-2 cells with CYP cDNAs and later also with CYP oxidoreductase (18), but this method was reported to be unstable. Schmiedlin-Ren *et al.* increased CYP3A4 activity with vitamin D treatment (28). Our modifications can upregulate endogenous CYP gene transcription (Table I). The CYP activities of the stable transfectants were tested with a cocktail that has substrates for CYP isoforms 1A2, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1 and 3A4 (34). Despite increased CYP transcription, no detectable metabolites were formed in Caco/WT, Caco/hPXR and Caco/mCAR cells. Traces of midazolam and omeprazole metabolites were produced in Caco/VD3 cells, indicating CYP3A4 activity. Next we tested CYP3A4 metabolism with 7-benzyloxy-4-(trifluoromethyl)-coumarin (BFC), which is metabolised into a fluorescent compound by CYP3A4 and to some degree by CYP1A2 and CYP2B6 (35). We used ketoconazole as CYP inhibitor at 5 μM concentration. This concentration is sufficient to inhibit CYP3A4 but not CYP1A2 and CYP2B6 (34). Therefore, CYP3A4-mediated metabolism could be deduced. The activity levels were very low, but they agreed moderately with the RT-PCR results (Fig. 5, Table I) and also with the Western blot experiment (Fig. 1a). In addition to CYP proteins, the observed metabolism also depends on CYP oxidoreductase activity. It is possible that the activity of this auxiliary protein also limited the metabolic capacity in our cell lines. Yet another reason for low metabolic activity may be the insufficient incorporation of heme into CYP apoenzyme.

Previous studies with vitamin D-induced cells have used extracellular matrix coating, which has been reported to increase CYP activity (28,58). Therefore, we tested the effect of Matrigel coating on CYP3A4 activity. The level of metabolism increased moderately, but the strong induction described by Schmiedlin-Ren *et al.* (28) was not observed. A possible reason for this could be the clonal differences in vitamin D response described by Engman *et al.* (58).

CONCLUSIONS

The present study shows that gene expression in Caco-2 cells can be modulated by nuclear receptors and corresponding ligand treatments. These changes may also be seen at the functional level, but the amplitude is usually much lower than that of mRNA. Increase in efflux pump activity is much more evident than in CYP activities, which is probably inherent to this cell line since the wild type Caco-2 cells express several efflux pumps but CYP activities are virtually absent. These experiments point out the general difficulty of modifying the Caco-2 cells and finding an assay that would clearly indicate changes in a single pump activity since the result of an experiment may depend on several factors including passive diffusion, transport proteins and enzyme activities.

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