Transport and Metabolic Characterization of Caco-2 Cells Expressing CYP3A4 and CYP3A4 Plus Oxidoreductase

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Purpose. To further characterize CYP3A4-transfected Caco-2 cells with regard to morphological, transport, and metabolic properties, and to evaluate a different Caco-2 cell strain transfected with both CYP3A4 and oxidoreductase (OR).

Methods. Transfected Caco-2 cells, Caco-2 TC7 cells, and wild-type Caco-2 cells grown onto Millicell™ were used. We determined the morphological characteristics of transfected cell monolayers using light and transmission electron microscope. We determined the transport and metabolic capabilities of the transfected cells, TC7 cells, and wild-type cells with a variety of drugs, nutrients, and marker compounds.

Results. The transfected Caco-2 cells formed a tight monolayer with TEER values and mannitol transport similar to the untransfected parent cell strain (wild type). However, the transfected cells (grown onto Millicell™) reached maturity approximately 33% faster than the wild-type cells. Permeabilities of propranolol, nifedipine, testosterone, linopirdine, mannitol, and cephalexin were similar in transfected and wild-type Caco-2 cells. On the other hand, the transfected cells of early passages were much more metabolically active, and metabolized standard CYP3A4 substrates (e.g., testosterone and nifedipine) as much as 100 times faster than untransfected cells. In addition, metabolism of standard substrates was inhibitable by ketoconazole and TAO. Using comparable data, the transfected cells metabolized testosterone the fastest, followed by linopirdine and nifedipine (approximate ratio: 10:6:2). The metabolites of standard substrates were generally preferably excreted to the apical membrane.

Conclusion. The monolayers of newly transfected cells (CYP3A4 + OR) have a significantly increased level of CYP3A4 activities compared to untransfected cells. These cell monolayers also have desirable morphological and transport characteristics that are similar to untransfected cells.

KEY WORDS: Caco-2 cells; transfection; CYP3A4; Oxidoreductase; transport characteristics.

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INTRODUCTION

The Caco-2 cell culture model is routinely used to determine a drug candidate's absorption potential. The main reason for this model's popularity in the pharmaceutical industries is the permeabilities obtained from this model are correlated with percent absorption in humans (1–2). This screening method also uses a smaller amount of drug candidates, and produces cleaner samples for analysis. The Caco-2 cell culture model is a fast, reliable, and convenient model system for supporting drug discovery research.

A main disadvantage of the current Caco-2 model system is the lack of or underexpression of common and important oxidative metabolic enzymes of the intestinal tract (3,4). One of these enzymes is the cytochrome P-4503A4 (or CYP3A4), an enzyme that contributes significantly to the metabolism of many drugs and xenobiotics (5–7).

In an earlier study, we reported our first effort to transfect Caco-2 cells to derive cells that are more metabolically robust than the wild-type cell (3). We found that the derived cells (CYP3A4 clone 4) expressed high level of CYP3A4, and formed a tight cell monolayer. Permeabilities of mannitol, cephalexin, and propranolol were similar in transfected cells and wild-type Caco-2 cells, whereas permeability of phenylalanine was higher in the cloned transfected cells but not in bulk transfected cells. However, we did not examine the morphology of transfected cells. We also did not report whether the cell monolayers were capable of distinguishing substrates with different transport and metabolic properties. Finally, transfected cells also lost activity during cell passage with a half-life of 3–4 weeks.

In the present study, we will report our further characterization of transfected Caco-2 cells, with an emphasis on morphological characteristics and transport properties as well as metabolic capabilities for the transfected cells expressing CYP3A4 only. We will also present data on newly derived Caco-2 cells that express both CYP3A4 and oxidoreductase (OR). OR was included in the transfection cassette to boost the turnover number of CYP3A4, which was relatively low in the earlier cell variant. This strategy (of using OR to boost turnover) has been successfully used in a different cell line (8). Finally, we will present the results of our experiments that were designed to restore the expression of CYP3A4 in transfected cells.

MATERIALS AND METHODS

Materials

Untransfected Caco-2 cells (wild type) were maintained at Washington State University. These cells originated from Dr. Ronald T. Borchardt's laboratory at the University of Kansas (Lawrence, KS), which was obtained from ATCC at passage 18. Transfected Caco-2 cells as described previously (3) were obtained from Gentest Corp (Woburn, MA). Cloned Caco-2 cells, coded TC7 (passage 42), were a kind gift from Dr. Moniqué Rousset of INSERM U178 (Villejuit, France).

[^{3}H]-Mannitol and [^{3}H]-propranolol were obtained from NEN (Boston, MA). Linopirdine and its metabolites were obtained from DuPont-Merck Pharmaceuticals (Wilmington, DE). Testosterone's metabolites were obtained from Sterealoids Inc. (Wilton, NH). Oxidized nifedipine was obtained from Salford Ultrafine Chemicals and Research Ltd. (Manchester, UK).

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All other chemicals and/or drugs used were obtained from Sigma Chemical Company (St Louis, MO).

Cell Culture

The culture conditions for growing Caco-2 cells have been described previously (3,9). The seeding density, growth media, and quality control criteria were all implemented in the present study as they were described previously (3,9). The main differences were: (1) transfected cells were seeded at a density of 500,000 cells per well whereas the untransfected cells were seeded at a density of 400,000 cells per well; (2) TC7 cells were maintained in 20% fetal bovine serum (FBS), whereas other strains of cells were maintained in 10% FBS.

Transfection of Caco-2 Cells with p220CMV3A4OR Vectors

We produced p220CMV3A4 derivatives which also contained the human OR cDNA under the control of the glucocorticoid inducible MMTV-LTR (mouse mammary tumor virus long terminal repeat). The OR expression unit was inserted between the CYP3A4 expression unit and hygromycin gene (Fig. 1). Two constructs with the two possible orientations of the OR expression unit (relative to CYP3A4) were prepared and were designated p2203A4OR#7 (head to tail w.r.t. CYP3A4) and p2203A4OR#8 (tail to tail w.r.t. CYP3A4). Caco-2 cells were transfected according to a published procedure (3), and bulk hygromycin-resistant cells were selected for functional studies.

Light and Transmission Electron Microscopy

Cell monolayers were prepared for examination according to the procedure of Grushkin-Lerner (16). In brief, live cells on the intact Millicell™ inserts were rinsed with D-PBS and fixed with 3% glutaraldehyde in 0.1 M cacodylate buffer for 1hr, washed with sucrose containing cacodylate buffer, and postfixed with 2% osmium tetroxide buffered with 0.1M cacodylate buffer. Samples were dehydrated in a graded ethanol series and embedded in Spurr's resin. Semi-thin (1 mm) and thin sections (100 μm) of embedded samples were cut using a Reichert Ultracut R, and stained with either Stevenel's blue or uranyl acetate lead citrate. Sections were viewed on an Olympus BH-2 or a JEOL 1200 EX microscope.

Transport Experiments

Experiments in triplicates were performed in pH 6.0 or pH 7.4 Hank's balanced salt solution (8). Phenylalanine and cephalexin transport experiments were performed at pH 6.0, whereas transport studies of propranolol, linopirdine, and other passively absorbed drugs were performed in pH 7.4 Hank's balanced salt solution. The protocols for performing cell culture experiments were similar to those described previously (10,11). Briefly, the cell monolayers were washed three times with 37°C pH 7.4 Hank's balanced salt solution. The transepithelial electrical resistance (TEER) values were then measured and cell monolayers with TEER values of less than 400 ohms × cm^2 were discarded. This criteria of excluding cell monolayers has been found to be reliable indicator of mannitol transport in this laboratory in the last 5 years (9–11). (However, this value tends to change between labs.) The cell monolayers were loaded into a diffusion chamber and amounts of drug transported from the apical side to the basolateral side were followed as a function of time. Four samples were taken at different times, and amounts transported were determined following analysis of the samples by HPLC or liquid scintillation counter.

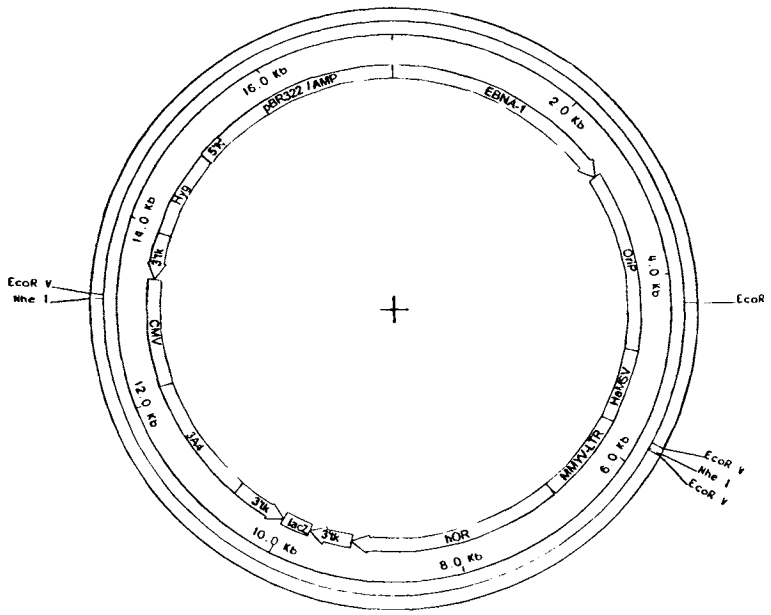

Fig. 1. Expression Cassette of p220CMV3A4OR#8 under the control of MMTV-LTR (mouse mammary tumor virus long terminal repeat). p220.2 CMV3A4 was digested with Xbal partial/CIP to yield a single cut vector. HaMSV/MMTV-LTR/Human OR/lac Z was isolated from Blue HyHo with AvrII partial, yielding a 5.2 kb fragment. This was ligated to the single cut vector, and two promising clones were identified by digestion with EcoRV and Ecl 136 II. Orientation was determined by digestion with Sac I. Confirming digests were done with AvrII, NheI, and EcoRI.

Metabolic Experiments in Intact Monolayers

Experiments in triplicate were performed in a pH 7.4 buffer. For experiments with phorbol 12-myristate 13-acetate (TPA) treatment, cell monolayers were treated overnight with 100 nM TPA prior to the start of an experiment. For experiments with 2′-deoxy-5-azacytidine (AzDC) treatment, the cell monolayers were treated with 1 μM of AzDC for different period of times. Concentrations of testosterone, nifedipine were 200 μM, whereas the concentrations of linopirdine and its metabolites and warfarin were 50 μg/ml. The experiments were performed for 3 hr in a 37°C environment chamber (Labline 3520) with an orbital shaking rate of 50 rpm.

Metabolic Experiments in Intestinal Microsomes

Microsomes of Caco-2 cells expressing CYP3A4 were prepared according to a modified method of Penman *et al.* (12). Briefly, cells harvested from monolayers grown onto plastic surface of tissue culture flasks were washed and pelleted. The pelleted cells were then resuspended in 0.1 M pH 7.4 KH_2PO_4 buffer and homogenized in a 14 ml dounce homogenizer. The cells were then further broken up with a sonicator (200 W for 20 second). After large debris was removed by centrifuging the suspension at 8,600 rpm (or 10,000 g) at 4°C, the microsomes were pelleted by centrifuging the solution at 28,000 rpm (100,000 g) for 10 min. The formed microsomes were rapidly resuspended in 0.1 M pH 7.4 KH_2PO_4 and stored in −80°C freezer until use. Activity assay was performed according to a previously published procedure (3).

Sample Analysis

The HPLC conditions for cephalexin, phenylalanine, nifedipine, and its metabolites, and 6-OH-testosterone have been described previously (3,10,11). The HPLC conditions for linopirdine and its metabolites were: column, analytical C-18 (Beckman ultrasphere 5 μm, 4 × 250 mm); mobile phase A, 15% CH_3CN + 85% 10 mM Na_2HPO_4, mobile phase B, 100% CH_3CN; gradient, 0–5 min, 0–15% B, 5–10 min, 15–40% B; wavelength, 210 nm; injection volume, 100 μl; detection limit: 0.025 μg/ml; flow rate, 1 ml/min. The mono-oxidized linopirdine had a retention time of 8.5 min, whereas the bis-N-oxidized linopirdine had a retention time of 6.5 min. The HPLC conditions for testosterone only were: column, analytical C-18 (Beckman ultrasphere 5 μm, 4 × 250 mm); mobile phase, 50% 10 mM Na_2HPO_4 + 50% CH_3CN; wavelength, 254 nm; injection volume, 25 μl; detection limit: 0.025 μg/ml; flow rate, 1 ml/min.

Data Analysis

Permeabilities were calculated using the following equation:

$$P = \text{rate of transport} / (\text{surface area} \times \text{donor concentration})$$

One-way ANOVA or unpaired Student's t-test was used to analyze the data. The prior level of significance was set at 5%, or $p < 0.05$.

RESULTS

Transfection of Caco-2 Cells with p220CMV3A4OR Vectors

Cells bearing p2203A4OR#8 were found to grow better that cells bearing p2203A4OR#7 (and also better than cells with p220CMV3A4 clone 4). CYP3A4 activities of Caco-2 cells also bearing the OR gene was approximately 2 times higher following 20 μM dexamethasone (Dex) treatment. Cells were pretreated with 5 mM dexamethasone for 2 days and microsomes were prepared from cells bearing p2203A4OR#8. The testosterone 6β-hydroxylase activity was found to be 1045 pmol/(mg min) and the cytochrome P450 content 72 pmol/mg. Both values were significantly higher than obtained with p220CMV3A4#4 (testosterone 6β-hydroxylase activity was 983 pmol/(mg min) and the cytochrome P450 content 45 pmol/mg). However, the turnover number was unchanged (15 min−1) indicating no improvement in turnover per unit enzyme. Indeed, OR levels, as measured by cytochrome C reductase activity were essentially unchanged [47 nmol/(mg min) versus 39 nmol/(mg min)]. These results indicate that while it is possible to increase CYP3A4 levels relative to those obtained in CYP3A4-clone 4, expression of OR in this system did not result in significantly higher enzyme activity.

Cell Growth Effects

Monolayer protein contents, rates of 6-OH-testosterone formation, and TEER values were monitored as a function of post-seeding time in CYP3A4-expressing cells and Caco-2 TC7 cells (Fig. 2). The results indicated that these CYP3A4-expressing cells were metabolizing testosterone to 6-OH-testosterone the fastest at days 12–15 post seeding. In contrast, we did not detect significant amount of 6-OH-testosterone when Caco-2 TC7 cells or wild-type Caco-2 cells were used. In addition, the metabolite formed by CYP3A4-expressing cell monolayers was preferably found in the apical (AP) side. The preference ratio (defined as AP formation rate over basolateral (BL) formation rate) was initially at 2.3 (at day 5), rapidly decreased to 1.44 (at day 8), and then stabilized at approximately 1.33 afterwards (Fig. 2). This ratio or directional flux of metabolites was also observed using the other tested compounds except for midazolam at a concentration of 50 μM (Fig. 3). It was also observed for CYP3A4+OR cells.

The results also showed that CYP3A4-expressing cells formed tight layers with TEER peaked at 12–15 days post-seeding. The cells continued to grow past fifteen days (as indicated by a higher protein content), but the monolayers were less tight (lower TEER value). In contrast, Caco-2 TC7 cells had higher TEER values and less protein as the cell further matured. The latter pattern was also observed with wild type cells (10). It is possible that this difference in growth is due to the introduction of transfection vector system, since CYP2A6-expressing Caco-2 cells also had similar pattern of growth (3). The underlying mechanism for this vector effect is currently unknown.

Based on these results, the best windows for doing transport experiments were between 12–15 days post-seeding for the CYP3A4-expressing cells, and 19–22 days for Caco-2 TC7 cells. It should be pointed out that the expression of CYP3A4 gradually decreased as the cells underwent several passages.

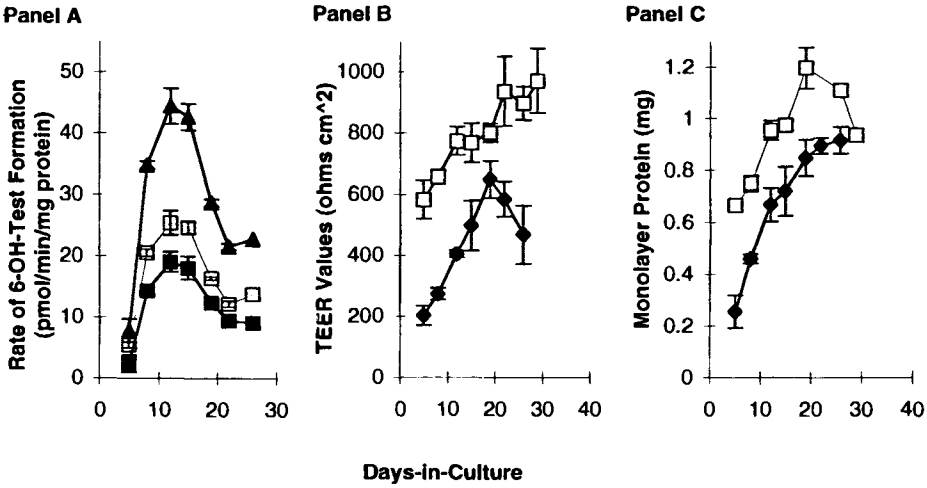

Fig. 2. Growth Effects on Various Cellular Functions of Caco-2 CYP3A4#4 cells and Caco-2 TC7 cells. Panel A, total rates (solid triangles) of metabolism of testosterone by Caco-2 CYP3A4 (clone 4) cells were obtained by measuring the rate of accumulation of 6β-hydroxy-testosterone at both the apical (AP, open squares) and the basolateral (BL, solid squares) sides. The metabolite was preferentially excreted to the AP media. Panel B, TEER values of TC7 cell monolayers (open squares) and Caco-2 CYP3A4 cell monolayers (solid diamonds). Panel C, amount of protein associated with monolayers of TC7 cells (open squares) and Caco-2 CYP3A4 cells (solid diamonds).

At a rate of 1 passage per week, the half-life was approximately 4 weeks (3).

Light and Electron Microscopy

Microscopic studies were carried out to compare if the transfected cells share morphological characteristics of the wild type Caco-2 cells. Light microscopy at a high magnification indicated that CYP3A4-expressing cells formed a tight monolayer (Fig. 4). These cells were relatively uniform in size (Fig. 4), and the cells were dividing, even after they have formed a monolayer (not shown). The monolayers have characteristic features associated with normal epithelial membrane including microvilli, tight junctions, and other junctional complexes. Compared to untransfected cells grown for the same length of time (not shown), these cells grew much faster (as seen in more rapid percent increase in the protein content as a function of time-post seeding). They also formed tight and differentiated monolayer in a shorter period of time (two weeks), comparing with a normal maturing time of approximately three weeks for untransfected cells.

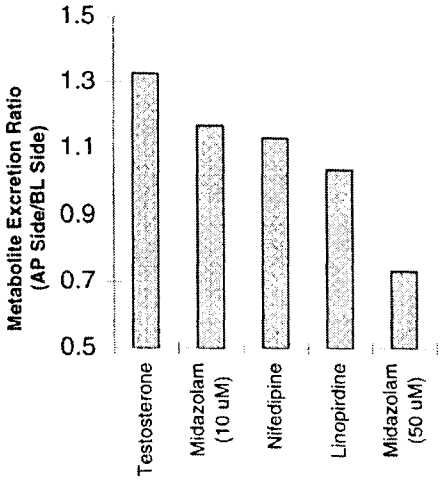

Fig. 3. Ratio of metabolite excretion using intact cell monolayers of CYP3A4-expressing Caco-2 cells (clone 4). The test compounds were used at concentrations of 200 μM for testosterone and nifedipine, and of 50 μg/ml for linopirdine, and 10 and 50 μM for midazolam. The experiments were performed for 3–4 hours, with sampling from both the receiver side and the donor (loading) side.

Transport of Drugs

Transport of ^{3}H-propranolol (a passively absorbed drug) and ^{3}H-mannitol (a leakage marker) was measured in four different variants of Caco-2 cells (Table 1). The results indicated these cells have high permeabilities to propranolol, indicating membrane lipid components are probably not significantly different. These cells also have low permeabilities to mannitol, suggesting the monolayers were tight. It is quite interesting that TC7 cells were more permeable to propranolol and testosterone but less permeable to mannitol.

Transport of linopirdine and nifedipine was similar in TC7 and CYP3A4-expressing cells. On the other hand, transport of testosterone was 1.3 fold higher ($p < 0.05$) but transport of warfarin was 35% lower ($p < 0.05$) in TC7 cells than in CYP3A4-expressing cells.

To determine if the Caco-2 cell monolayers can be used to distinguish compounds of increasing polarity, permeabilities of linopirdine, and two of its metabolites (Fig. 5), mono-N-oxidized-linopirdine (lino-M1) and bis-N-oxidized-linopirdine (lino-M2) were determined in TC7 cells. The results indicated permeability decreased as the polarity of the compound increased, indicating the usefulness of this model system to select drug candidates with only a subtle change in structures.

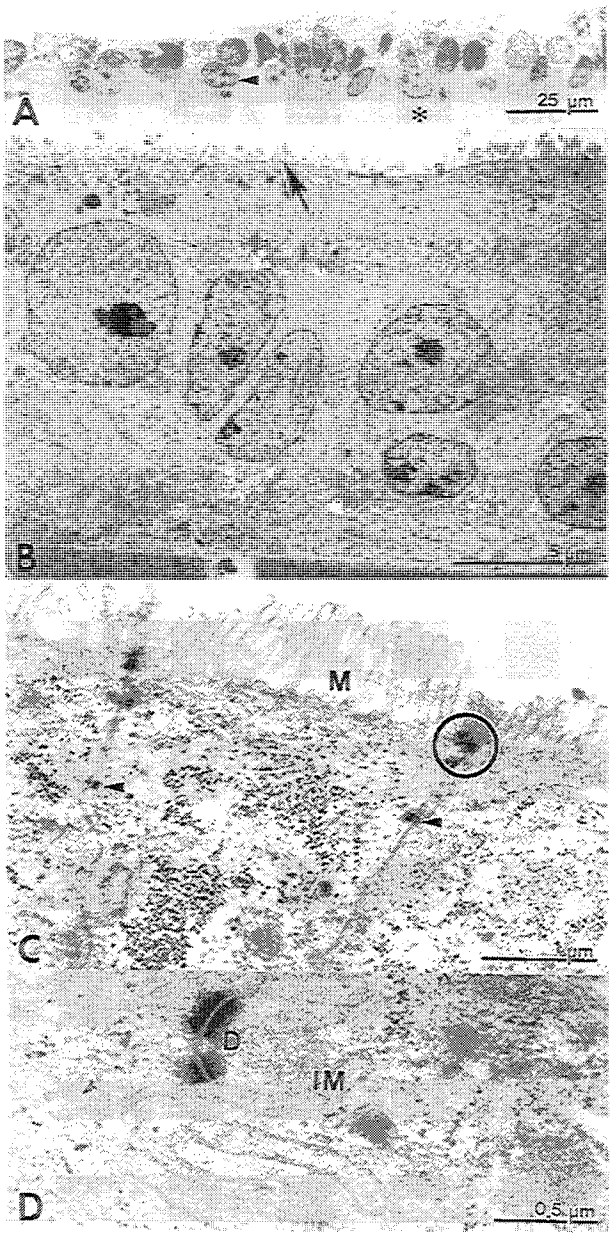

Fig. 4A–D. Light microscopy of CYP3A4-expressing cell monolayers grown for 14 days. A. Light micrograph showing a monolayer of transfected cells grown on Millicell (*). Ovoid nuclei (arrowhead) can be observed throughout the monolayer. B. Transmission electron micrograph of transfected cells. Cell membranes are closely associated and exhibit numerous microvilli (arrowhead) at the apical surface. C. Higher magnification view of the apical portion of transfected Caco-2 cells displaying brush border-like surface (BB) and well defined junctional complexes (circle). Lateral cell surfaces are juxtaposed and little intercellular space is observed. D. Interdigitating plasma membranes (IM) are visible near the apical portion of two adjacent cells. Demosomes (D) are also visible in these regions.

Metabolism of Drugs in Intact Cell Monolayers

Metabolism of testosterone, nifedipine, midazolam, linopirdine, and warfarin were measured in the presence or the absence of inhibitors and a phorbol ester (phorbol 12-myristate 13-acetate, TPA, except for midazolam) (Table 2). The results indicated known CYP3A4 substrates testosterone, nifedipine, midazolam, and linopirdine were metabolized by the transfected cells. On the other hand, TC7 cells did not metabolize these substrates. with the exception of nifedipine, which was poorly metabolized by these cells. Metabolism of these drugs in CYP3A4-expressing cells was generally enhanced by overnight treatment of cells with 100 nM TPA, and inhibited by the presence of ketoconazole. Metabolism of testosterone in CYP3A4-expressing cells was also inhibited significantly by troleandomycin (TAO), a characteristic inhibitor of CYP3A4 (14). These results suggest the transfected cells expressed CYP3A4 since testosterone, nifedipine, midazolam, and linopirdine (15) were typical substrates of CYP3A4. Previous studies have shown these enzymes were recognized, using a western plot, by antibodies that cross-react with human CYP3A4 (3).

Stabilization of Expressed CYP3A4 Activities

Previous studies indicated that the expressed CYP3A4 lost activities as the cells underwent normal passage. We hypothesized that the loss may be due to: (1) oxidative stress caused by the expression of CYP3A4; or (2) the methylation of inserted gene segments. We first supplemented media with 50 μM glutathione, 20 μM vitamin E, and 1 μM ketonconazole. For both CYP3A4 and CYP3A4+OR cells, the supplementation did not result in any significant change in CYP3A4 activities over time. There was, however, a slight increase (10–20%) in the half-life of the activities. We then tested the effect of 1 μM 2′-deoxy-5-azacytidine (AzDC), a DNA-methylation inhibitor (17), on the CYP3A4 activities expressed by cells of two different passages.

We first tested the importance of treatment schedule on the cellular CYP3A4 activities. The results indicated the cellular CYP3A4 activities increased when cells were exposed to 1 μMAzDC, regardless of the exposure pattern (Fig. 6). On the other hand, the highest increase (about 300%) were found to be associated with longest exposure (Fig. 6). Additional overnight treatment with TPA further increased CYP3A4 activities in all treatment groups. We also measured the protein content of each cell monolayers after AzDC treatment. We found a treatment scheme that did not affect protein content. This treatment scheme used 1 μM AzDC 4 days after cells are seeded to the polycarbonate membrane. Taken together, this result suggests that we can use AzDC to treat the cell monolayers without affecting normal cell growth.

We then tested the effect of AzDC treatment on the restoration of enzyme activity. The results indicated that AzDC was able to restore the specific activity of the cell monolayers to a specific level that appeared to be independent of the passage number (Fig. 7).

Metabolism of Drugs By Caco-2 Cell Microsomes

In microsomes prepared from transfected Caco-2 cells, the metabolism of testosterone was inhibited by 50 μM triacetyloleandomycin (TAO) (76%, $p < 0.05$) and 100 μM SKF 525A (30%, $p < 0.05$). However, metabolism increased 90% in the presence of 50 μM β-naphthoflavone (BNF) ($p < 0.05$). β-Naphthoflavone has been shown to increase CYP3A4 activities in microsomal preparations. Nifedipine metabolism was also significantly inhibited by 50 μM TAO and 100 μM SKF525A

Table I. Transport Properties of Model Compounds in Caco-2 Cell Variants

Parameters or drugs	Permeabilities ($\times 10^{-6}$ cm/sec)			
	Wild type	TC7 clone	CYP3A4#4	CYP3A4 + OR#8
Day-in-Culture	20	18–22	13	13–16
TEER	473–568	800–1000	551	466–477
Passive Diffusion				
propranolol	91.3 ± 3.4	132 ± 6	92.7 ± 1.7	114 ± 3
mannitol	4.4 ± 0.3	1.7 ± 0.3	5.5 ± 0.15	5.5 ± 0.9
linopirdine	ND	52.7 ± 4.2	44.8 ± 2.0	ND
lino-M1	ND	1.77 ± 0.15	ND	ND
lino-M2	ND	0.39 ± 0.05	ND	ND
testosterone	ND	81.8 ± 3	34.8 ± 0.6	ND
nifedipine	ND	29.3 ± 0.1	28.4 ± 1.2	ND
warfarin	ND	24.2 ± 5.2	37.6 ± 3.7	ND
Carrier-Mediated				
phenylalanine	2.5 ± 0.3	6.55 ± 0.30	4.3 ± 0.45	6.7 ± 1.2
cephalexin	3.0 ± 0.15	8.72 ± 0.15	3.0 ± 0.27	3.1 ± 0.2

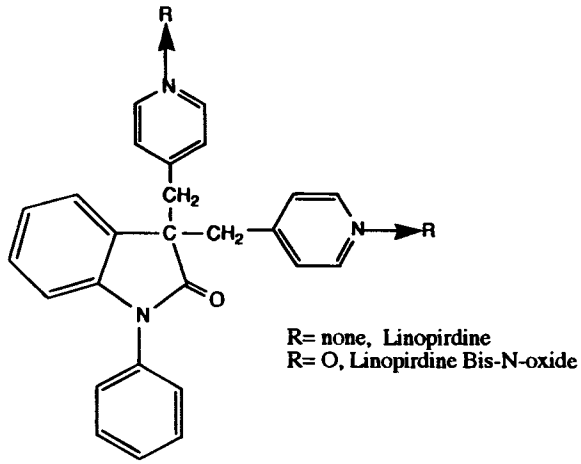

Fig. 5. Structures of linopirdine and its metabolites.

(approximately 50% for both inhibitors). In contrast, microsomes prepared from TC7 cells formed detectable amount of metabolites within the 30-min reaction time, but the amount was too small to quantify.

DISCUSSION

The results of transport studies indicated that permeabilities of passively absorbed drugs are not necessary the same in different "clones" of cells (Table I). Previously published literature data from different laboratories have shown that the permeability of drugs may be different in different laboratory (16). Our results further support the notion that different "clones" of Caco-2 cells have different permeabilities to different drugs.

For drugs (nutrients) transported by a carrier-mediated mechanism, the difference between drugs transported via carrier-mediated pathways were more obvious (Table I). For example, growth selected cloned cells appeared to have a

Table 2. Metabolic Properties of Model Compounds in Caco-2 Cell Variants

Parameters/drugs	Metabolic rates (pmol/min/mg protein)		
	TC7 clone	CYP3A4#4	CYP3A4 + OR#8
Drugs			
testosterone (Tes)	BDL[a]	7–32	5.20 ± 0.48
nifedipine (Nif)	1	2–10	1.36 ± 0.08
linopirdine (Lin)	BDL	12.3 ± 1.4	ND
midazolam	ND	1.09 ± 0.12	ND
warfarin	BDL	BDL	ND
Drug + Modulator (% Control)			
Tes + ketoconazole	BDL	7.0 ± 0.0	ND
Nif + ketoconazole	12 ± .16	5 ± 0.2	ND
Nif + TPA	ND[b]	145 ± 5	ND
Lin + ketoconazole	ND	57.6 ± 3.8	ND
Lin + TPA	ND	ND	4.71 ± 0.07[c]
Tes + TPA	ND	183 ± 21	7.57 ± 0.44[c]

[a] "BDL" stands for "below detection limit" of 0.25 pmol/min/mg protein.
[b] "ND" stands for "not determined."
[c] Actual rates of metabolism with a unit of pmol/min/mg protein.

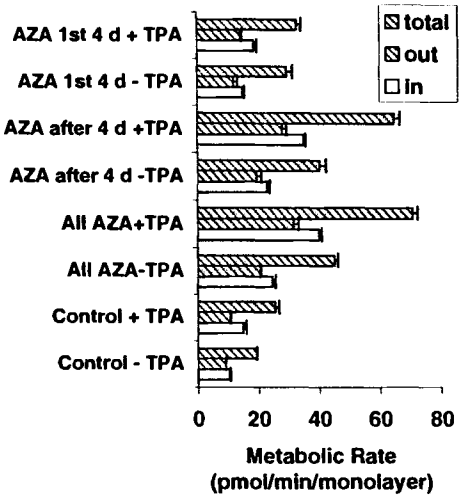

Fig. 6. Effects of length and treatment pattern on CYP3A4 activities of CYP3A4-expressing Caco-2 cells (clone 4) in the presence (+TPA) or absence (−TPA) of 24-hour treatment with TPA. The activities were expressed as metabolite formation rate in the apical media (in) and basolateral media (out). Testosterone was used as a CYP3A4 probe because it is metabolized to 6β-OH-testosterone by this enzyme. The treatment groups were as follows: no treatment with 2′-deoxy-5-azacytidine or AzDC (Control), presence of AzDC at all time (All AzDC), presence of AzDC from after 4 days in culture to the end (AzDC after 4 d), and presence of AzDC for the first 4 day (AzDC 1st 4 d). Experiments were performed with 200 μM testosterone for 3 hours at 37°C, and cells were grown for 14 days.

significant higher expression of large neutral amino acid carrier that transport phenylalanine than the wild type Caco-2 cells, suggesting the importance of expression of this transporter in the cell selection process. On the contrary, only TC7 cells had a higher peptide transport capability. The latter is interesting since other three "strains" of cells were from the same original source (i.e., the wild-type Caco-2 cells). Previously, we have shown that this wild-type Caco-2 cell had a lower expression of peptide transport transporter function than another wild-type Caco-2 cell (10). Together, these results suggest that amino acid carriers and peptide carriers may be under different regulatory control, which is similar to our previous observation (10).

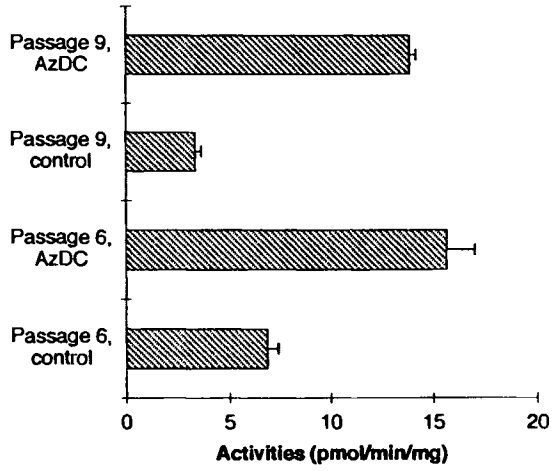

Fig. 7. Stabilization of CYP3A4 enzyme activities with 2′-deoxy-5-azacytidine (AzDC). The cells (14 days) were treated with 1 μM AzDC 4 days after the cells were seeded and the treatment lasted until the date of experiment. Cells (Caco-2 3A4#4) of consecutive passages were used. Experiments were performed with 200 μM testosterone for 3 hours at 37°C. Testosterone was used as a CYP3A4 probe because it is metabolized to 6β-OH-testosterone by this enzyme.

A main rationale for incorporating CYP3A4 into the Caco-2 cells is to improve our understanding of the dynamics of the transport process. Compared to microsomal studies, where drugs are exposed to the maximal exposure to the enzymes, metabolism studies using intact cells more directly reflect the dynamics of the transport and metabolism processes, where drug molecules can only gain contact with the cellular enzyme for a brief period of time. Protein binding may also limit the exposure of the drug molecules to metabolic enzymes. On the other hand, cellular metabolism study may be carried out for a longer period of time (up to 6 hours), whereas microsomal studies are generally carried out for 30 min for fear of losing enzyme activity over time (14).

Lastly, the results of our studies indicated that instability of the expressed CYP3A4 were not due to insufficient expression of OR, or oxidative stress. Rather, it is primarily due to DNA-methylation since AzDC was able to restore about 90% of the activity. However, DNA-methylation is probably not the only factor, since treatment did not restore the activity to the level of 20–25 pmol/min/mg protein exhibited by early passages of cells. Further work may be necessary to achieve optimal expression of CYP3A4 in transfected cells.

In conclusion, transfected Caco-2 cells formed good quality cell monolayers that are tight and transport drugs and nutrients at rates comparable to untransfected cells. In addition, transfected Caco-2 cell monolayers as well as microsomes prepared from transfected Caco-2 cells metabolized standard substrates of CYP3A4, and the metabolism was inhibited by typical CYP3A4 inhibitors such as ketoconazole and TAO. Finally, AzDC may be used in the cell culture media to improve the expression level of CYP3A4. Taken together, the transfected cell monolayers have robust metabolic capabilities and are similar to untransfected cell monolayers with respect to their permeabilities to selected model drugs.

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