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TRANSFECTED MDCK CELL LINE WITH ENHANCED EXPRESSION OF CYP3A4 AND P-GLYCOPROTEIN AS A MODEL TO STUDY THEIR ROLE IN DRUG TRANSPORT AND METABOLISM

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Abstract

The aim of this study was to characterize and utilize MDCK cell line expressing CYP3A4 and P-glycoprotein as an in vitro model for evaluating drug-herb and drug-drugs of abuse interactions. MDCK cell line simultaneously expressing P-gp and CYP3A4 (MMC) was developed and characterized by using expression and activity studies. Cellular transport study of 200 μ M cortisol was performed to determine their combined activity. The study was carried across MDCK-WT, MDCK-MDR1 and MMC cell lines. Similar studies were also carried out in the presence of 50 μ M naringin and 3 μ M morphine. Samples were analyzed by HPLC for drug and its CYP3A4 metabolite. PCR, qPCR and western blot studies confirmed the enhanced expression of the proteins in the transfected cells. The vivid CYP3A4 assay and ketoconazole inhibition studies further confirmed the presence of active protein. Apical to basal transport of cortisol was found to be ten and three fold lower in MMC as compared to WT and MDCKMDR1 respectively. Higher amount of metabolite was formed in MMC than in MDCK-WT indicating enhanced expression of CYP3A4. Highest cortisol metabolite formation was observed in MMC cell line due to the combined metabolic activities of CYP3A4 and P-gp. Transport of cortisol increased fivefold in presence of naringin in MMC and doubled in MDCKMDR1. Cortisol transport in MMC was significantly lower than that in WT in presence of naringin. The permeability increased three fold in presence of morphine which is a weaker inhibitor of CYP3A4. Formation of 6 β -hydroxy cortisol was found to decrease in presence of morphine and naringin. This new model cell line with its enhanced CYP3A4 and P-gp levels in addition to short culture time can serve as an invaluable model to study drug-drug interactions. This cell line can also be used to study the combined contribution of efflux transporter and metabolizing enzymes towards drug-drug interactions.

Keywords

Efflux; Metabolism; Drug Interaction; in vitro models; Dual transfection

Introduction

Identification of drug candidates that can permeate across the intestinal membrane and resists or bypass first-pass metabolism is an important step in oral drug development. Historically, empirical screens (for instance, permeability through cell monolayers and

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metabolic stability with liver sub cellular fractions) were routinely performed to select the best possible candidates for further drug development ¹.

A number of *in vitro* systems were employed as models to investigate drug transport across intestine. Among the intestinal cells, a human colon carcinoma cell line (Caco-2), having similar characteristics of normal intestinal absorptive cells, has become the “work-horse” for scientists in the field of oral absorption. However, these cells require 21 days of growth. Also, low expressions of endogenous efflux transporters and metabolizing enzymes have become an impediment to select it as a model for the assessment of drug-drug interactions. Madin-Darby Canine Kidney (MDCK) cells, a renal epithelial cell line, when grown onto Transwells®, differentiate into columnar epithelium and can form tight junctions in 4–6 days ². However, MDCK cells, derived from dog kidney lack certain biochemical properties of intestinal cells. The importance of this limitation depends on the application. A good correlation has been established with regard to permeability across MDCK and Caco-2 cell monolayers and with human *in vivo* bioavailability data ³.

Cytochrome P450 (CYP) is the largest family of metabolizing enzymes out of which cytochrome P450 3A4 (CYP3A4) is the major contributor to drug metabolism. Watkins et al has reported that about 50 to 70% of currently administered drugs are metabolized by CYP3A4⁴. The expression of CYP3A4, like all other CYPs varies from location to location in the gastro intestinal tract. CYP3A4 is highly expressed in the liver and intestine which accounts for approximately 30% of hepatic CYP and more than 70% of intestinal CYP.

P-glycoprotein (P-gp), a product of the multidrug resistance (MDR1) gene, was first characterized in the 1970s as the ATP dependent transporter responsible for emergence of drug resistance due to efflux from cancer cells. P-gp is present at high levels in kidney and adrenal gland, at intermediate levels in liver, small intestine, colon and lung, and at low levels in prostate, skin, spleen, heart, skeletal muscle, stomach and ovary ^{5–6}. P-gp is also expressed in brain ^{7–10}, choroid plexus ¹¹, cornea ¹² and placenta ¹³. This efflux protein displays a broad range of substrate specificity such as cyclosporin-A, taxol, dexamethasone, lidocaine, erythromycin, ketoconazole, rifampicin, gatifloxacin, protease inhibitors and many anti-cancer agents ^{14–21}.

When multiple drug therapies are indicated, drug–drug interactions (DDIs) become an important consideration for physicians and patients undergoing treatment. It has been estimated that adverse drug reactions are the fourth to sixth leading causes of death in US hospitals, exceeding deaths by pneumonia and diabetes ²². Major causes of pharmacokinetic drug-drug interactions are either due to inhibition or induction of a metabolizing enzyme and efflux transporters by the respective interacting agents ^{23–24}. It is hypothesized that the metabolizing enzymes and the efflux proteins may together play a synergistic role in limiting the overall bioavailability of therapeutic agents. This is primarily because most agents, which are substrates for P-gp, are also substrates for CYP3A4. Due to similarity in substrate specificity of both CYP3A4 and P-gp, these proteins appear to act synergistically ²⁵. This synergism during the first pass effect has been recognized as a major barrier to oral absorption of many clinically effective drugs ^{26–29}. Close chromosomal location of P-gp and CYP3A4 genes, their expression in mature enterocytes, and their similar substrate specificity suggest that the function of these two groups of proteins may be complementary in nature and may form a coordinated barrier to drug absorption ³⁰. Studies by Benet et al. have also pointed towards a functional correlation linking *in-vivo* activity of P-gp and CYP3A4 in the small intestine ^{31–32}.

There are several factors that point towards a possible interplay between P-gp and CYP3A4 in drug disposition. Cellular localization of P-gp and CYP3A4 are in close proximity (P-gp

at apical brush border membranes of villous enterocytes³³ and CYP3A4 at the endoplasmic reticulum below it^{34–35}. Wachter et al. first pointed out the high overlap in substrates of both these proteins²⁹. Hence, bioavailability of those co-substrates will be restricted by both P-gp mediated efflux and CYP3A4-mediated metabolism^{29, 35–36}. Lan et al suggested that P-gp can contribute to the extent of metabolism by CYP3A4 by affecting its availability to the enzyme³⁷.

Several *in vitro* models such as Caco-2, MDCK have been extensively used to delineate drug-drug interactions mediated by P-gp and CYP3A4. But limited or lack of constitutive expression of these proteins has been recognized as intrinsic weakness of these models. A rapid and stable *in vitro* cell culture model, MDCK-MDR1, with enhanced expression of P-gp has been developed to study the absorption barrier, particularly the efflux barrier (P-gp) at small intestine². But MDCK-MDR1 alone cannot simulate exact environment present in the small intestine as it expresses only P-gp. Since small intestine is shown to express significant amounts of CYP3A4 in addition to efflux transporters, MDCK-MDR1 can be regarded as an incomplete model of small intestine.

Therefore, in our current study, we developed and characterized a modified MDCK cell line expressing both CYP3A4 and P-gp simultaneously as an *in vitro* model for evaluating oral absorption. Further, we attempted to test the hypothesis that drug-drug interactions involving both Pgp and CYP3A4 are synergistic. To substantiate this, the effect of morphine and naringin (as interacting agents) was examined on the transport of 6 β hydroxycortisone (cortisol), a well known P-gp and CYP3A4 substrate^{38–41}. A thorough understanding of such interplay between efflux proteins and metabolizing enzymes may facilitate the measurement of individual contribution of efflux and metabolic barriers.

Materials and Methods

Morphine, naringin, cortisol and 6- β -Hydroxycortisol were obtained from Sigma Chemical Company (St. Louis, MO). All culture media and fetal bovine serum were obtained from Invitrogen (Carlsbad, CA). Lopinavir was provided as a generous gift by Abbott Laboratories. Human intestinal microsomes were obtained from Xenotech LLC (Kansas City, MO). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). Culture flasks (75cm² growth area), polyester transwells (pore size of 0.4 μ m and 12mm diameter) and 96-well plates (0.32cm² growth area per well) were purchased from Corning Costar Corp. (Cambridge, MA). G418, Lipofectamine™ 2000 transfection reagent, Trizol®-LS reagent and other molecular biology reagents were purchased from Invitrogen (Carlsbad, CA). All other chemicals were products of commercial biological grade and were obtained from Sigma Chemicals (St. Louis, MO) and Fisher Scientific.

Cell Culture

All the unmodified cell lines (MDCK-WT, HepG2 and Caco-2) were obtained from American Type Culture Collection (Manassas, VA). MDCK-MDR1 cells were a generous gift from Dr. Peter Borst, Netherland's Cancer Institute. MDCK-WT and Caco-2 cells as well as MDCK-MDR1 (passages 4–12) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 4.5 g/L glucose, supplemented with 10% (v/v) fetal bovine serum, penicillin-streptomycin (100 Units/L and 100 μ g/mL, respectively), and 1% (v/v) nonessential amino acids. Cells were maintained at 37°C in a humidified atmosphere of 5% CO₂ in air and subcultured once 80% confluent. Semi-confluent cell monolayers were subcultured every 4 days using 0.25% trypsin containing 0.537 mM EDTA. HepG2 cells were grown in Minimum Essential Medium (MEM) containing Earl's salts and 10% (v/v) heat inactivated fetal bovine serum under similar conditions.

Plasmid Preparation and Cell Transfection

Plasmid expression vectors were constructed according to standard protocols. We obtained human CYP3A4 cDNA (NM_017460.3) insert as 10 µg transfection-ready plasmid (pCMV6-Neo- CYP3A4) from Origene Technologies. The custom synthesized plasmid already contained neomycin resistance gene for stable transfection. The reconstituted plasmid was transfected with Lipofectamine™ 2000 Transfection Reagent (Carlsbad, CA). CYP3A4-NEO was introduced into MDCK-WT and MDCK-MDR1 cells according to manufacturer instructions. Briefly, cells were plated 1 day before the transfection in T-75 flasks or 35-mm tissue culture plates at an appropriate cell density. Once 70–80 % confluent, plasmid was transfected in complete growth medium without a selection agent. Seventy-two hrs post transfection, cells were sub cultured (1:10 ratio) and grown in the medium containing selection agent, G418 at a concentration of 400 µg/mL. Cells were maintained in the selection medium for about 2–3 weeks with fresh medium added every 24 hrs. Once single cell stable colonies resistant to G418 were observed, cells were sub cultured again and distinct colonies were isolated using cloning cylinders. The cells were grown in the presence of G418 to maintain the expression of the transfected genes.

Cell cytotoxicity Assay

MTT assay is a very sensitive cell viability assay and was performed as reported earlier^{42–43}. Reagents were prepared as per manufacturer's protocol. Viability assay was carried out in 96-well plates. Transfections were performed in scaled down manner exactly like in the T-75 flasks described earlier. Following transfections, cells were washed once with warm PBS and then replaced with 100 µL of fresh warm PBS in each well. Cells were incubated in a humidified 5% CO₂ atmosphere for a period of 48 h to evaluate the cytotoxic effects. The commercial assay method (CellTiter® 96 AQueous Non-radioactive Cell Proliferation Assay Kit) involves a colorimetric procedure for determining the number of proliferating cells in culture. Cell viability was measured as the rate of fluorescent metabolite production (color formation) over the course of the reaction which is proportional to number of viable cells present. Color determination was measured at 485nm (reference at 590nm) using a 96-well microtiter plate reader (SpectraFluor Plus; Tecan, Maennedorf, Switzerland).

RT-PCR Analysis

Total RNA was isolated from cells using Trizol-LS® reagent according to manufacturer's instructions. RNA was measured by UV absorbance at 260 nm. Briefly, 1 µg of total RNA was mixed with 2 µl of oligo dT₁₅ primer and denatured at 70 °C for 10 minutes. After denaturation, it was reverse transcribed with 1 µl (10 units) per reaction mixture of MMLV Reverse Transcriptase (Promega, Madison, WI). PCR was carried out in 20-µL volumes containing 20 ng of each tissue cDNA as a template and 0.5 µM of each primer with Taq polymerase (Promega). Primers used for the amplification are included in Table 1. PCR conditions are as follows. All samples were preheated at 94 °C for 2 min and then subjected to denaturing conditions at 94 °C for 10 s. Subsequent to annealing at 60°C for 45 s, genes were amplified at 72 °C for 1.5 min (35 cycles).

Quantitative Real Time PCR

Real-time PCR was conducted with an ABI 5700 GeneAmp Sequence Detection System (Perkin – Elmer, Applied Biosystems, Foster City, CA) capable of detecting fluorescence between 500 to 660 nm and Roche - LightCycler® 480 System. Primers were designed with OligoPerfect™ Designer (Invitrogen Corp. Carlsbad, CA). All the primers were designed such that the amplicons generated will be 100–150 bp long to increase the efficiency of simultaneous amplification of target and reference genes (Table 2). PCR mixture has a final

volume of 40 μ L and contains the relevant template cDNA, 250 nM each of forward and reverse primers for gene of interest, 1X Mg free PCR buffer (Promega), 3.75 mM MgCl_2 , 0.025U Taq Polymerase (Promega), 200 μ M dNTPs, 1X ROX reference dye and 1X SYBR Green (Invitrogen). The specificity of the target amplicon was examined by melting curve analysis⁴⁴⁻⁴⁵. Quantitation was carried out using comparative Ct method.

Samples for real-time PCR were prepared in triplicate. Quantitative values were obtained above the threshold PCR cycle number (Ct) at which an increase in signal associated with an exponential growth for PCR products is detected. The relative mRNA levels in each sample were normalized according to the expression levels of GAPDH/ β -actin. For these studies, initial experiments were carried out to ensure that the target and GAPDH/ β -actin genes were amplified with equal efficiencies. An induction ratio (treated/untreated) was determined from the relative expression levels of the target gene using $2^{-\Delta\text{Ct}}$ ($\Delta\text{Ct} = \text{Ct}_{\text{target gene}} - \text{Ct}_{\text{GAPDH}}$)⁴⁶.

Immunoblotting

Confluent cells grown on T-75 flask (75 cm^2 growth area) were washed twice with PBS and harvested with a cell scraper in 5 mL PBS. Cell suspension was centrifuged at 1500 rpm for 10 minutes and the pellet was resuspended in freshly prepared lysis buffer (PBS containing 0.1% NP40 and protease inhibitor cocktail) for 15 minutes on ice. Cells were first ultrasonicated for 30 s and then centrifuged at 12000 rpm for 10 minutes at 4°C. Total protein lysate was collected and stored at -80°C , until further use. Protein content was determined with Bradford method (Bio-Rad Protein Estimation Kit, Hercules, CA). The proteins (20 μ g/lane) were then separated on ready-made Novex[®] 8–16% Tris-bis Gels (Invitrogen, Carlsband, CA) for 2 hrs at 120 V. Proteins were then transferred onto PVDF membranes for 2.5 hrs at 30V on ice by standard techniques. The blot was incubated in freshly prepared blocking buffer (3% w/v BSA and 5% non fat dry milk in phosphate buffered saline) overnight at room temperature. It was then probed with antibody specific to CYP3A4 (Xenotech LLC, Kansas City, KS) at 37°C for 2–3 hrs. Following five 10-minute washes in PBST (Phosphate buffered saline + 0.1% Tween 20), the blots were probed with secondary antibody in PBST (1:2000 anti-mouse IgG-HRP). The blots were finally washed three times with PBST and then subjected to enhanced chemiluminescence using SuperSignal West Dura Kit (Pierce Biotechnology, Rockford, IL) and visualized by ChemiImager 8900 digital imaging system (Alpha Innotech, San Leandro, CA). The images of immunoreactive staining were measured and analyzed by AlphaEase software (Alpha Innotech).

VIVID Assay

CYP3A4 activity in MDCK-MDR1-CYP3A4 cells was assayed with VIVID[™] CYP3A4 Red Substrate (Invitrogen), which was prepared as a 20mM stock solution in acetonitrile. Following transfection, the assay medium was removed and cells were washed once with warm PBS. Reagents were added according to the manufacturer's protocol. Fluorescent readings were monitored kinetically at 37°C over a period of 30 minutes with a 96 well plate reader at an excitation wavelength of 530 nm and an emission wavelength of 585 nm. Activity was measured as fluorescent metabolite production from the metabolism of a Vivid[®] CYP450 substrate over the course of the reaction.

Cellular Transport Studies

Transwell diffusion system was used for these studies. Prior to an experiment, cell monolayers grown on the Transwell[™] inserts were rinsed with Dulbecco's Phosphate Buffered Saline (DPBS) and incubated at 37°C. Drug solutions [cortisol alone and in combination with morphine (3 μ M) or naringin (50 μ M)] in DPBS (pH 7.4) were placed in

the donor chamber. The receiver chamber was filled with DPBS. Sampling from the receiver chamber was done at predetermined time intervals and fresh DPBS solution was added to maintain sink conditions in receiver chamber. The samples were then analyzed by HPLC ⁴⁷. All experiments are performed at 37°C. The effective permeability (cm/sec) was determined using the following Equation:

$$P = \frac{dC/dt}{C * AV * 60}$$

P is the permeability in cm/sec, dC/dt represents the slope of plot of concentration (nmoles) versus time (min), C denoted the concentration of drug (nmoles/cm³), A is the surface area of the chamber (cm²), and V is the volume of each half-chamber (cm³).

Data Analysis

All experiments were conducted at least in triplicate and results are expressed as mean ± SD. For comparing the treatment group (i.e., in the presence of inhibitors) with control, one-way analysis of variance followed by Dunnett's test was applied to determine significance of data. For comparisons between two groups, *t* test was used. The prior level of significance was set at *p* < 0.05. Statistical comparisons were performed by student T test.

Results

EXPRESSION OF CYP3A4 mRNA IN MDCK-MDR1

RT-PCR analysis was carried out to confirm the expression of CYP3A4 mRNA after transfection. MDCK-MDR1 (host cells) and Caco-2 cells were used for the comparison purposes. Results suggested that transfected MDCK-MDR1 cells were able to express high level CYP3A4 mRNA (Figure 1)

IMMUNOBLOTTING

Since purpose of developing this cell line is to have functionally active CYP3A4 protein, we carried out immunoblot analysis to establish the presence of CYP3A4 protein using CYP3A4 specific monoclonal antibody (Xenotech LLC.). Immunoblot analysis showed that MDCK-WT and MDCK-MDR1 transfected with CYP3A4 expressed significant amounts of CYP3A4 protein (Lanes 3 and 5 in Figure 2) as compared to the host cell line. Human intestinal microsomes were considered as positive controls (Lanes 7 through 9 in Figure 2).

CELL VIABILITY AFTER CELL TRANSFECTIONS

Since transfection reagents and foreign DNA can affect the cell survival, post transfection cell viability assays were conducted. Neither components of the transfection mixture alone nor transfection reagent and plasmid mixture produced any significant toxicity at the concentrations utilized (Figure 3).

COMPARATIVE CYP3A4 mRNA ANALYSIS IN TRANSFECTED VS NON-TRANSFECTED CELLS

Quantitative Real Time PCR was employed to compare the CYP3A4 mRNA in transfected cell lines relative to non-transfected cell lines. MDCK-WT cells were used as the control cell line which was observed to express moderate level of CYP3A4 mRNA. Both MDCK-WT-CYP3A4 and MDCK-MDR1-CYP3A4 cells appeared to express CYP3A4 mRNA more than 6–7 times compared to non transfected cells. Transfected cells were also compared

against model cell lines such as Caco-2 (intestine) and HepG2 (liver) both of which showed just over 2-fold expression compared to MDCK-WT cells (Figure 4).

VIVID™ METABOLISM ASSAY FOR CYP3A4 FUNCTIONAL ACTIVITY

Functional activity of CYP3A4 enzyme was then measured under similar treatment conditions by VIVID CYP3A4 assay kit containing Red fluorescent substrate. Figure 2 clearly indicates transfection resulted in higher CYP3A4 protein expression. Fluorescent metabolite formation in both MDCK-WT-CYP3A4 and MDCK-MDR1- CYP3A4 cell lines were almost 5–6 fold higher than host cells suggesting enhanced expression and activity of CYP3A4 (Figure 5). Non-transfected cells did cause basal metabolism, albeit at a very low level. Baculosomes which were used as the positive control for these studies showed similar activity to the two transfected cell lines.

In another study, we evaluated CYP3A4 transfected MDCK-MDR1 cell line for its ability to express functionally active CYP3A4 protein by comparing with recombinant CYP3A4 expressed in Baculosomes. Ketoconazole, a prototypic CYP3A4 inhibitor, was used as a positive control for CYP3A4 inhibition (Figure 6).

TRANSPORT OF CORTISOL ACROSS MDCK-WT, MDCK-CYP3A4, MDCK-MDR1 AND MDCK-MDR1-CYP3A4

Transport of cortisol was carried out in all of the 4 cell lines and there was a significant decrease in active cortisol transport in the dual transfected verses the mono transfected verses the non-transfected cell lines (Figure 7a).

On similar lines a significantly higher amount of cortisol metabolite was formed in MDCK-WT transfected with CYP3A4 than MDCK-WT (Figure 7b). Maximum metabolite of cortisol (6 β -hydroxy cortisol) was formed in MDCK-MDR1-CYP3A4 cells as compared to others (Figure 7b).

TRANSPORT OF CORTISOL IN PRESENCE OF MORPHINE AND NARINGIN ACROSS TRANSFECTED CELLS

Transport of cortisol was conducted in the presence of known interacting agents, morphine (drug of abuse) and naringin (herbal drug). No significant difference in the cortisol permeability was observed in MDCK-WT cell line in presence of either of the interacting agents (Figure 8a & figure 9). A significant increase in cumulative transport of cortisol was observed in presence of both interacting agents in both the mono transfected cell lines (figure 8b and 8c). Naringin was found to be a stronger interacting agent when the transport was carried out across MDCK-MDR1-CYP3A4 cell line (Figure 8d).

Discussion

Efflux transporters and metabolizing enzymes are known to play an essential role in the disposition of drugs in various organs^{48–49}. In the gastrointestinal system these proteins play a key role in reducing the absorption of orally administered substrates. It has been proposed that both P-gp and CYP3A4 act synergistically to lower the absorption of their common substrates. During *in vitro* screening, contribution of both P-gp and CYP3A4 towards drug disposition is studied separately. Due to the lack of existence of an *in vitro* system to study synergistic activity of these proteins, there overall contribution may be under estimated. Similarly, it is essential to screen potential drug interactions occurring upon co-administration of common substrates in a system which can accommodate this synergism so that more accurate projections can be made⁵⁰. To develop such a system, MDCK cells were transfected with MDR1 and CYP3A4 genes in order to over-express both proteins.

Thus, both the proteins can be studied for their synergism in an isolated system, as MDCK cells do not express a significant amount of other efflux proteins.

MDCK cells already transfected with MDR-1 gene were then transfected with CYP3A4 containing plasmid. Figures 1, 2 and 4 clearly indicate transfection generating higher expression of CYP3A4 at both mRNA and protein levels. MDCK-MDR1-CYP3A4 cells of passage number 1–4 were utilized for all the studies. The expression levels of CYP3A4 and P-gp were found to be consistent across all the passages utilized. To confirm whether these protein expression levels also translated into increased activity of the protein, VIVID CYP activity assay was performed. This proprietary fluorescent substrate based assay from Invitrogen has been extensively used to measure the activity of CYP3A4 in *in vitro* systems. The assay results further confirmed that the protein being expressed in the transfected cell line was active (Figure 5).

Inhibitory assays are also often performed to confirm the presence of an active protein in an *in vitro* system. Inhibitory potency of ketoconazole in well-established *in vitro* assay employing recombinant CYP3A4 expressed in Baculosomes. The inhibitory activity of ketoconazole in this system was already reported with an IC₅₀ value of 0.04 μ M⁵¹. Apparent IC₅₀ values obtained for ketoconazole in our cell line assay was 0.21 μ M (Figure 6). One possible explanation for this lower inhibitory effect of ketoconazole observed in the cell based assays is the complex membrane structure of the cell line. Reduced cell membrane permeability and transport may result in lower intracellular concentrations of the inhibitor compared with Baculosomes hence resulting in elevated IC₅₀. These results clearly suggest the importance of this model cell line expressing simultaneously efflux proteins and metabolizing enzymes in assessing the contribution of each protein.

Similar to what was observed in Figure 5, a significantly higher amount of cortisol metabolite was formed in MDCK-WT transfected with CYP3A4 than MDCK-WT (Figure 7b). However, metabolite formation in MDCK-WT which does not express CYP3A4 was an unexpected result. A quick review of the literature suggested that CYP3A5, a close homolog of CYP3A4 also metabolizes cortisol to 6 β -hydroxy cortisol⁵². In addition, it was already reported that kidney expresses CYP3A5 in significant amounts but not CYP3A4⁵³. Since MDCK was derived from kidney, it is no surprise that it exhibited basal metabolism of CYP3A5. Therefore, we conclude that increased metabolite formation in MDCK-WT-CYP3A4 cells is due to combined metabolism of transfected CYP3A4 as well as endogenous CYP3A5.

Even more surprising was the increased metabolite formation in MDCK-MDR1. These studies also indicated that MDCK-MDR1 not only expresses CYP3A5 natively but the efflux activity is also synergistic with the natively expressed metabolizing enzyme (Figure 7b). MDCK-MDR1 which itself was derived from MDCK-WT is expected to express the same endogenous levels of CYP3A5 as MDCK-WT. In the case of common substrates for both CYP3A4/5 and P-gp, the latter effluxes the substrates out of the cell and in doing so it keeps the concentrations of substrates exposed to CYP3A4/5 metabolism well below the saturation leading to enhanced metabolism. The major metabolite of cortisol (6 β -hydroxy cortisol) was formed at the highest amount in MDCK-MDR1-CYP3A4 cells as compared to others (Figure 7b). This increased cortisol metabolite formation was demonstrated in the MDCK-MDR1-CYP3A4 cells due to the combined metabolic activity of CYP3A5, CYP3A4 and MDR1. Due to this very high metabolism and efflux observed in MDCK-MDR1-CYP3A4 no measurable amount of active drug was observed in the first hour of transport (Figure 7a).

The primary objective behind developing these transfected cell lines is to generate a model for studying drug-drug interactions. To validate this model, transport of cortisol was carried out along with known interacting agents, morphine (drug of abuse) and naringin (herbal drug). These two interacting agents did not generate any significant difference in the permeability when the transport was carried out in MDCK-WT cell line. Even though both MDCK-MDR1 and MDCK-CYP3A4 cell lines did show increase in cumulative transport of cortisol in presence of either interacting agent, neither of these cells were able to differentiate much between the interacting capabilities of the two interacting agents. There was more significant difference in the transport levels achieved in the absence and presence of the interacting agents when the transport of cortisol was carried out in the dual transfected cell line. Therefore, better differentiation could be made between the intractability of the two agents in this cell line (Figure 8a–d and 9). Also, permeability of a paracellular marker such as mannitol was studied to check if transfection could alter the membrane integrity of cells and passive permeability of substrates. No significant difference in mannitol transport was evident in MDCK-WT, MDCK-CYP3A4 and MDCK-MDR1-CYP3A4 cells suggesting the transfection process did not alter the membrane integrity (data not shown). Based on all the above observations, MDCK-MDR1-CYP3A4 cell line may be able to better predict more physiologically relevant interactions. Hence this cell line may prove to be a better model to study drug-drug interaction as it would incorporate the synergy between efflux and metabolism.

Conclusion

We developed a new cell line expressing both P-gp and CYP3A4 simultaneously. CYP3A4 cDNA was transfected into MDCK-WT and MDCK-MDR1 cells. Both gene expression and functional activity studies suggested that MDCK is a workable model and could express the CYP3A4 protein after transfection. This result also corroborates the previous report that MDCK was a better model for CYP3A4 transfection than other cell lines⁵⁴. For the very first time, it was also shown that MDCK cells constitutively express CYP3A5 owing to their kidney source. We also showed that the wild type and mono-transfected cell line can cause over estimations of permeability. These models are not adequate to differentiate between interacting agents. The dual transfected cell line due to higher interplay between efflux and metabolism may yield better estimates of drug transport and also is a good cell line for studying drug-drug interactions.

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Figure 1.
Expression of CYP3A4. 1. Molecular weight markers 2. MDCK-MDR1 cells 3. Caco-2 cells
4. MDCK-MDR1 cells transfected with CYP3A4.

Figure 2.

Immunoblot showing 56.5 kD band for CYP3A4 protein: lane 1) molecular marker, lane 2) MDCK-WT, lane 3) MDCK-WT-CYP3A4, lane 4) MDCK-MDR1, lane 5) MDCKMDR1-CYP3A4, lane 6) blank, lanes 7 through 9) human intestinal microsomes. Lanes 2–5 were loaded with 20 μ g microsomal protein where as lanes 7 to 9 were loaded with 10, 5 and 2.5 μ g microsomal protein respectively.

Figure 3. Cell viability after the transfection procedure. Data was presented as mean $\pm$ SD. 1- Medium; 2- Plasmid only 3; Transfection reagent only and 4- Transfection reagent and Plasmid

Figure 4. Comparative CYP3A4 mRNA levels in MDCK-WT, MDCK-MDR1, MDCK-WT-CYP3A4, MDCK-MDR1-CYP3A4, Caco-2 and HepG2 cells after CYP3A4-NEO transfection. All data were presented as mean $\pm$ SD. * indicates statistical significance at $p < 0.05$ with $n = 6$

Figure 5.

Flourescent metabolism assay for determining CYP3A4 functional activity in the transfected cells. All data were presented as mean $\pm$ SD. * indicates statistical significance at $p < 0.05$ with $n = 6$

Figure 6.

Determination IC₅₀ value for inhibition of CYP3A4-mediated metabolism of VIVID by ketoconazole in MDCK-MDR1-CYP3A4 cells in 96-well plates. Various concentrations of ketoconazole were incubated in the presence of 20 μM Vivid for 40 minutes. Values are expressed as the % of control rates (absence of inhibitor).

Figure 7a

Figure 7b

Figure 7.

A) Transport of 200 μ M cortisol in MDCK-WT and MDCK-MDR1 cells alone and transfected with CYP3A4. B) Time dependent formation of CYP3A4-mediated metabolite of cortisol (6 β -hydroxy cortisol). $n = 6 \pm$ S.D.

Figure 8a

Figure 8b

Figure 8c

Figure 8d

Figure 8.

Transport of 200 μ M cortisol alone or in presence of Morphine (3 μ M) or Naringin (50 μ M) across (A) MDCK-WT, (B) MDCK-MDR1, (C) MDCK-CYP3A4, (D) MDCK-MDR1-CYP3A4 cell lines. $n = 4 \pm$ S.D.

Figure 9.

Permeability values for transport of 200 μ M cortisol alone or in presence of Morphine (3 μ M) or Naringin (50 μ M) across MDCK-WT and MDCK-MDR1 cells alone and transfected with CYP3A4. * indicates statistical significance at $p < 0.05$ with $n = 4$ when compared to transport in wt cell line for that combination. ¥ indicates statistical significance at $p < 0.05$ with $n = 4$ when compared to transport of cortisol in that respective cell line.

Table 1

Reverse Transcription-Polymerase Chain Reaction Primers for CYP3A4

	Sequence (5'→3')	Template	Length	Tm	GC%
Forward	AATCACTGTTGGCGTGGGG	2155-2173	19	54.80	57.89
Reverse	GCATCGAGACAGTTGGGTGT	2735-2716	20	54.54	55.00

Product length = 581

Table 2

Quantitative Real-Time Polymerase Chain Reaction Primers for CYP3A4

	Sequence (5'→3')	Template	Length	T _m	GC%
Forward	GCACCAATTAACTCCTCCTGAGCT	1890-1912	23	56.27	52.17
Reverse	AGTTCTGACAAAAGGCCCCACG	2187-2167	21	56.96	57.14