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Review Article

Caco-2 models for xenobiotic metabolism and transportation

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Abstract

The intestine plays an important role in the absorption, metabolism, and transportation of xenobiotics including drugs and foods. Therefore, an *in vitro* intestinal model for predicting bioavailability of xenobiotics is important for the drug and food industries. Caco-2 is a human colon carcinoma cell line widely employed as a model to represent xenobiotic absorption, transportation, and metabolism in the human intestinal tract. However, Caco-2 has limitations as an intestinal model due to the low expression of metabolizing enzymes and the long time period required for cell differentiation. Hence, several modified Caco-2 models have been developed to overcome these limitations and to establish Caco-2 models that correlate well with human intestine. These include xenobiotic-induced cytochrome P450 and transporter expression models, nuclear receptor gene-modified Caco-2 cells, and a novel Caco-2 culturing method that mimics the physiological characteristics of the human intestine and accelerates the growth rate of Caco-2.

Keywords: colon carcinoma cell, Caco-2, cytochrome P450, transporter, intestinal model

1. Introduction

Drugs and nutrients mainly enter into the body by oral administration (Ferrec & Fardel, 2012). The intestine is considered a large surface area organ for food and drug absorption according to its very long length and plentiful supply of microvillus on the surface of enterocytes (Kaminsky & Zhang, 2003). Likewise, the intestine plays a role in xenobiotic metabolism and transportation, which affect xenobiotics entering the blood circulation system (Kaminsky & Zhang, 2003; Vaessen *et al*., 2017). Therefore, predicting absorption, permeation, transportation, and metabolism of xenobiotics using intestinal models is important (Vaessen *et al*., 2017).

Caco-2 is a human colon carcinoma cell line (Rousset, 1986) that is widely used as a model of the intestinal barrier for examination of drug and xenobiotic permeation, metabolism, and transportation due to its good correspondence with human intestinal morphology (Boulenc *et al*., 1992; Meunier, Bourrié, Berger, & Fabre, 1995; Natoli *et al*., 2011; Vaessen *et al*., 2017). Despite being a human colorectal carcinoma cell line, Caco-2 has a spontaneous differentiation property that provides a morphology more like a small intestinal enterocyte than other colorectal carcinoma cell lines (Borlak & Zwadlo, 2003; Maubon *et al*., 2007). However, Caco-2 has limitations as an intestinal model. For instance, Caco-2 expresses only intestinal enterocyte morphology, while the human intestine is composed of several cell types, e.g. Paneth cells, enteroendocrine cells, and goblet cells (Ferrec & Fardel, 2012; Lea, 2015). Caco-2 also exhibits low expression of cytochrome P450 (CYP) oxidative metabolizing enzymes and does not display other physiological factors that influence xenobiotic uptake and transportation, such as mucus on the small intestinal surface and movement of fluid in the lumen (Lea, 2015). Therefore, several culturing methods have been developed to modify the Caco-2 models to suit specific study purposes (Küblbeck *et al*., 2016; Lea, 2015; Li, Sai, Kato, Tamai, & Tsuji, 2003; Sun, Chow, Liu, Du, & Pang, 2008).

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2. Xenobiotic-Induced Monolayer Caco-2 Models

CYPs and transporters are regulated by transcriptional processes through ligand-activated nuclear receptors including the aryl hydrocarbon receptor (AhR), the constitutive androstane receptor (CAR), and the pregnane X receptor (PXR) (Guengerich, 2013; Zanger & Schwab, 2013). Generally, an inducer will bind to its specific nuclear receptor to form an inducer-receptor complex before it dimerizes with a specific nuclear translocase such as the AhR nuclear translocator (ARNT), the retinoid X receptor (RXR), and the glucocorticoid receptor (GR). The activated dimeric complex subsequently binds to a specific response element on the CYP or transporter gene, resulting in an increase in transcriptional expression (Guengerich, 2013). There are many clinical drugs and substances, such as Aroclor 1254, rifampicin, dexamethasone, phenobarbital, carbamazepine, tenofovir, and efavirenz, that can be used as inducers to examine expression and activity of CYPs and transporters (Borlak & Zwadlo, 2003; Boulenc *et al*., 1992; Brück, Strohmeier, Busch, Drozdzik, & Oswald, 2017; Mukhopadhya *et al*., 2016). These clinical drugs and substances have been employed in Caco-2 monolayers to predict the impact of related substances on CYPs and transporters in human intestine (Ferrec & Fardel, 2012; Lea, 2015; Li *et al*., 2003).

Aroclor 1254, a typical CYP inducer, is a polychlorinated biphenyl compound. Aroclor 1254 induces CYP1A via the AhR-ARNT signaling pathway (Borlak & Zwadlo, 2003; Guengerich, 2013), while CYP2 and CYP3 are induced via the retinoic acid receptor (RAR)-RXR pathway. It remains unclear which pathway Aroclor 1254 uses to mediate its interactions with ATP-binding cassette (ABC) transporters (Borlak & Zwadlo, 2003). Aroclor 1254 was used to induce CYPs and transporters in Caco-2 from Deutsche Sammlung von Mikroorganisme und Zellkulturen, Germany (Caco-2/DSMZ). The cells were subcultured every 5 to 6 days until the fifth passage, and then they were divided into 3 groups, including control and cells treated with Aroclor 1254 (10 μM) for 24 and 72 hours (Borlak & Zwadlo, 2003). Aroclor 1254 did not change Caco-2 morphology while it induced expression of *CYP1A1*, *CYP1A2*, *CYP2C*, *CYP2E1*, and *CYP3A5* by 2.4 to 80 fold. *CYP1A2* showed the highest expression after the 72 hour-treatment while *CYP2E1* exhibited the lowest expression with the 24 hour-treatment. Expression of *CYP2D6* was decreased by Aroclor 1254, while *CYP3A4* and *CYP2B6*/*7* were not detected. Regarding the transporters, Aroclor 1254 elevated expression of ABC subfamily B member 1 (*ABCB1*), *ABCC1*, *ABCC3*, *ABCC4*, and *ABCC5* with the highest induction at 24 hours (Table 1) (Borlak & Zwadlo, 2003).

Caco-2 (INSERM U-178, Villejuif, France) at passages no. 72 to 98 were cultured for 35 days before treatment for 3 days with typical CYP inducers acting via AhR (50 μM β-naphthoflavone, 50 μM 3-methylcholanthrene, 100 μM isosafrole, and 1 nM dioxin), PXR (50 μM rifampicin), CAR (2 mM phenobarbital), and GR (50 μM dexamethasone) (Boulenc *et al*., 1992; Zanger & Schwab, 2013). Isosafrole and phenobarbital did not induce *CYP1A1* and *CYP1A2* expression while 3-methylcholanthrene, dioxin, and β-naphthoflavone extensively induced expression of *CYP1A1* (Table 1) (Boulenc *et al*., 1992). Caco-2 (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures) at passages no. 16 to 19 were cultured for 21 days before incubation for 48 hours with carbamazepine (500 μM), efavirenz (10 μM), hypericin (0.5 μM) plus hyperforin (1 μM), or rifampicin (100 μM), which induce expression of ABC, solute carrier organic anion (SLCO) or solute carrier (SLC) transporters through the PXR-RXR signaling pathway (Brück *et al*., 2017). Expression of ABCB1, ABCC2, ABCG2, SLCO family member 2B1 (SLCO2B1), and SLC family member 15A1 (SLC15A1) mRNAs and proteins were not modified by the treatments (Table 1).

To further determine activity of a major efflux transporter ABCB1, Caco-2 was seeded in a transwell-plate with polyethylene terephthalate (PET) filter membrane insert for 21 days before treatment for 48 hours with carbamazepine (500 μM), efavirenz (10 μM), hypericin (0.5 μM) plus hyperforin (1 μM), or rifampicin (100 μM). The efflux ratio of digoxin and talinolol (substrates of ABCB1) was not changed, but a significant decrease was found after co-treatment with an ABCB1 inhibitor, valspodar (PSC833; 10 μM) (Table 2) (Brück *et al*., 2017). Seeding of Caco-2/ATCC at passage no. 30 to 60 in a collagen coated plate was suitable for determining expression of *CYP3A4* and transporters (*ABCB1*, *ABCC2*, *ABCC3*, and *SLC15A1*) while seeding in a transwell-plate with polycarbonate (PC) filter membrane insert was fit for examining ABCB1 activity. The Caco-2/ATCC cells were cultured for 14 days, then treated for 72 hours with the following tested compounds (10 μM); all-*trans* retinoic acid (ATRA, which acts via an RAR-RXR ligand to induce *CYP3A* and *ABCB1* expression), rifampicin and paclitaxel (typical inducers of *CYP3A* and *ABCB1* expression that act through the steroid xenobiotic receptor (SXR) or PXR pathways), progesterone and quercetin (SLC and ABC transporter-stimulators that act via the progesterone and estrogen receptors) (Notas *et al*., 2012; Li *et al*., 20; Zanger & Schwab, 2013), or ascorbic acid (10 μM), or *L*-Lysine (20 mM) (Li et al, 2003). Quercetin significantly increased expression of *SLC15A1* and *ABCC3* while ATRA significantly elevated expression of ABCB1 and *CYP3A4*. Ascorbic acid and *L*-lysine increased CYP3A4 expression (Table 1). Ascorbic acid and *L*-lysine were not involved in the nuclear receptor regulatory mechanism, but might be crucial co-factors for CYP expression (Li et al, 2003; Belin, Kaya, Burtey & Fontes, 2010). For ABCB1 activity, after seeding Caco-2 in a transwell-plate for 24 days, the differentiated Caco-2 cells were treated for 72 hours with ATRA (10 μM). Then the cells were incubated with [^{3}H]-digoxin (a ABCB1 substrate) and [^{14}C]-mannitol (a paracellular transporter reference marker) and the digoxin permeability ratio was calculated. ATRA significantly elevated the digoxin permeability ratio, which represented an increase in ABCB1 activity (Table 2) (Li *et al*., 2003).

Anti-retroviral drugs can cause drug interactions and clinical problems by interfering with drug transportation, especially in the main absorption site (the intestine), and affecting drug bioavailability (Foy, Sperati, Lucas, & Estrella, 2014; Mukhopadhya *et al*., 2016). Intestinal transporters, particularly ABCB1, are normally regulated via the PXR signaling pathway, therefore exposure to protease inhibitor antiretroviral drugs can induce expression of ABCB1 transporters (Gupta *et al*., 2008). Caco-2 (Health Protection

Table 1. Xenobiotic-induced Caco-2 monolayer models for the conventional culturing method

Caco-2 origins		Treatment substances (concentration/incubation time)	Results	References
Caco-2/ DSMZ	25 cm^2 flask	Aroclor 1254 (10 μM/24, 72 h)	↑ *CYP1A1*, *CYP1A2*, *CYP2C*, *CYP2E1*, *CYP3A5*, *ABCB1*, *ABCC1*, *ABCC3*, *ABCC4*, *ABCC5* ↓ *CY2D6*	Borlak & Zwadlo, 2003
Caco-2/ Pr. A. Zweibaum	180 cm^2 flask	Dioxin (1 nM/72 h) β-naphthoflavone (50 μM/72 h) 3-methylcholanthene (50 μM/72 h)	↑ *CYP1A1*	Boulenc *et al.*, 1992
		Phenobarbital (2 mM/72 h) Isosafrole (100 μM/72 h)	– *CYP1A1*, *CYP1A2*	
		Rifampicin (50 μM/72 h) Dexamethasone (50 μM/72 h)	↑ *CYP1A1*, *CYP1A2*	
Caco-2/ ATCC	6-well-plate	Quercitin (10 μM/72 h)	↑ *SLC15A1*, *ABCC3*	Li *et al*., 2003
		ATRA (10 μM/72 h)	↑ *ABCB1*, *CYP3A4*	
		Ascorbic acid (10 μM/72 h) *L*-Lysine (20 mM/72 h)	↑ *CYP3A4*	
		Rifampin (10 μM/72h) Paclitaxel (10 μM/72h) Progesterone (10 μM/72 h)	– *CYP3A4*, *SLC15A1*, *ABCB1*, *ABCC2*, *ABCC3*	
Caco-2/ DSMZ	6-well-plate	Carbamazepine (500 μM/48 h) Efavirenz (10 μM/48 h) Hypericin (0.5 μM) + hyperforin (1 μM)/48 h Rifampicin (100 μM/48 h)	– *ABCB1*, *ABCC2*, *ABCG2*, *SLCO2B1*, *SLC15A1*	Brück *et al.*, 2017

Note: ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganisme und Zellkulturen; ATRA, all-trans retinoic acid; (↑) increase, (↓) decrease, and (–) no change of expression/activity

Table 2. Xenobiotic-induced Caco-2 monolayer models for the inserted plate-Caco-2 culturing system

Caco-2 origins		Treatment substances (concentration/incubation time)		Results	References
Caco-2/ ATCC	24-transwell-plate with PC filter membrane insert	ATRA (10 μM/72 h)		↑ ABCB1 activity	Li *et al*., 2003
Caco-2/ DSMZ	24-transwell-plat with PET filter membrane insert	Carbamazepine (500 μM/48 h) Efavirenz (10 μM/48 h) Hypericin (0.5 μM) + hyperforin (1 μM)/48 h Rifampicin (100 μM/48 h)		– ABCB1 activity	Brück *et al.*, 2017
		Carbamazepine (500 μM/48 h) Efavirenz (10 μM/48 h) Hypericin (0.5 μM) + hyperforin (1 μM)/48 h Rifampicin (100 μM/48 h)	+ Valspodar (PSC833, 10 μM)	↓ ABCB1 activity	
Caco-2/ HPA	24-transwell-plate with PC filter membrane insert	Tenofovir (5 mM/72 h)		↑ *SLC7A5*, *SLC2A1*, *MVP*, *ABCC5* ↓ *SLC15A2*, *SLC7A9*, *ABCA1*, *AQP1*	Mukhopadhya *et al.*, 2016
		Darunavir (250 μM/72 h)		↓ *ABCC1*, *SLC15A2*	
		Dapivirin (10 μM/72 h)		↓ *ABCA1* ↑ *SLC3A2*	
Caco-2/ ATCC	12-transwell-plate with Matrigel coated Teflon membrane insert	1α,25-Dihydroxyvitamin D3(0.05 - 1 μM/2 wk)		↑ *CYP3A4*, *CYP3A5* [dose dependent]	Schmiedlin-Ren *et al.*, 1997
		25-(OH)-D3 (0.05 - 10 μM/2wk)		↑ *CYP3A* [dose dependent]	
		25-(OH)-D3 (15 - 20 μM/2 wk)		↓ *CYP3A*	
		Vitamin D3 (0.05 - 1 μM/2 wk)		– *CYP3A*	

Note: ATCC, American Type Culture Collection; DSMZ, Deutsche Sammlung von Mikroorganisme und Zellkulturen; HPA, Health Protection Agency culture collections; ATRA, all-trans retinoic acid; (↑) increase, (↓) decrease, and (–) no change of expression/activity

Agency culture collections, Salisbury, UK) was cultured in a transwell-plate with PC filter membrane insert for 21 days before treatment for 72 hours with 3 anti-retroviral drugs, i.e. tenofovir (5 mM), darunavir (250 μM), and dapivirin (10 μM). Tenofovir elevated expression of *SLC7A5*, *SLC2A1*, major vault protein (*MVP*), and *ABCC5*, while *SLC15A2*, *SLC7A9*, *ABCA1*, and aquaporin 1 (*AQP1*) expressions were decreased. Darunavir decreased *ABCC1* and *SLC15A2* expression while dapivirin reduced *ABCA1* expression with an increase in *SLC3A2* expression (Table 2) (Mukhopadhya *et al*., 2016).

A vitamin D derivative, 1α,25-dihydroxyvitamin D3, increased CYP3A4 activity in Caco-2 (Sambuy *et al*., 2005) resulting in an increase in 1'-hydroxymidazolam, a midazolam metabolite, which is a specific marker for CYP3A4 activity (Sambuy *et al*., 2005; Schmiedlin-Ren *et al*., 1997). A vitamin D derivative can increase expression of CYP3A through dimerization of the vitamin D receptor and RXR, followed by binding at the vitamin D response element of the *CYP3A* promoter, resulting in elevation of CYP3A transcription (Schmiedlin-Ren *et al*., 1997). Caco-2 (Caco-2/ATCC, HTB-37, passage no. 24 to 27) was cultured in a transwell-plate with matrigel coated Teflon membrane insert. The cells were incubated for 2 weeks with different concentrations of 1α,25-dihydroxyvitamin D3, vitamin D3 (0.05 to 1 μM), and 25-hydroxyvitamin D3 (25-(OH)-D3; 0.05 to 20 μM). All concentrations of 1α,25-dihydroxyvitamin D3 induced expression of CYP3A4 mRNA while CYP3A5 expression was slightly induced in a concentration-dependent pattern. Expression of CYP3A protein and CYP3A catalytic activity were examined (Schmiedlin-Ren *et al*., 1997). Incubation of Caco-2 with 1α,25-dihydroxyvitamin D3 (0.05 μM) for 2 weeks induced expression of CYP3A protein. On the other hand, 25-(OH)-D3 induced expression of CYP3A protein in a concentration-dependent pattern but those increased expressions were reduced by higher concentrations (15 and 20 μM). In addition, CYP3A catalytic activity was evaluated by incubation of midazolam (4 μM) in an apical chamber for 6 hours, followed by determination of 1'-hydroxymidazolam metabolite in the apical and basolateral chambers. Incubation of Caco-2 with 25-(OH)-D3 and 1α,25-dihydroxyvitamin D3 for 2 weeks increased CYP3A catalytic activity in a dose-dependent pattern, except at the two higher concentrations (15 and 20 μM), where they were reduced, which was seen in a decrease in the 1'-hydroxymidazolam level in the apical chamber. Hence, 1α,25-dihydroxyvitamin D3 possessed the highest induction potential due to the highest amount of 1'-hydroxymidazolam found in the apical chamber. In contrast, vitamin D3 did not alter CYP3A (Table 2) (Schmiedlin-Ren *et al*., 1997).

3. Genetically Modified Caco-2 Models

Levels of CYP mRNA in Caco-2 cells are usually lower than in intact intestinal cells. Expression of *CYP1A*, *CYP2C*, *CYP2D*, *CYP2E*, *CYP2J*, and *CYP3A* was approximately 5-fold lower in Caco-2 cells than in intact human jejunum cells (Vaessen *et al*., 2017), while *CYP2A*, *CYP2C*, *CYP2D*, *CYP2J2*, *CYP3A4*, and *CYP4F2* expression was 3-fold lower in Caco-2 cells than in intact human duodenal cells (Sun *et al*. 2002). Therefore, the major limitation of Caco-2 is low CYP expression (Korjamo *et al*., 2006; Küblbeck *et al*., 2016; Sun *et al*., 2008). Modulation of CYP expression is mediated by specific ligands activating nuclear receptors (Honkakoski & Negishi, 2000). Therefore, genetic modification of Caco-2 with nuclear receptor-transfected genes has been employed to improve CYP expression for studies on drug metabolism and drug absorption (Korjamo *et al*., 2006; Küblbeck *et al*., 2016).

Caco-2 (passages no. 33 to 43) including wide type (Caco-2/ATCC, HTB-37), human constitutive androstane receptor-transfected Caco-2 (Caco-2/h*CAR*), and human pregnane X receptor-transfected Caco-2 (Caco-2/h*PXR*) were examined for expression of CYPs and transporters. All cell-types were subcultured 2 times per week. The cells were cultured in 6-well-plates for one day for an undifferentiated cell model, and in 12-transwell-plates with uncoated PC filter membrane inserts for 21 days for a differentiated cell model. Expression of *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, *CYP3A7*, *ABCG2*, *ABCB1*, *ABCC2*, *SLC15A1*, *SLC16A1*, and *SLCO2B1* mRNAs and CYP activity were determined in both undifferentiated and differentiated Caco-2/ATCC, Caco-2/h*CAR*, and Caco-2/h*PXR*. Melatonin (10 μM), coumarin (5 μM), bupropion (50 μM), diclofenac (25 μM), omeprazole (10 μM), and midazolam (5 μM) were used as substrates for CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2C19, and CYP3A4, respectively. All substrates were incubated with the cells for 120 minutes, except midazolam for 60 min. For transporter activity, mannitol and antipyrine were used as reference compounds, paclitaxel was used as an ABCB1 substrate, vinblastine as an ABCB1 and ABCC2 substrate, and glycyl-sarcosine as an SLC15A1 substrate (Küblbeck *et al*., 2016). Both Caco-2/h*CAR* and Caco-2/h*PXR* exhibited greater expression of *CYP1A2*, *CYP2A6*, *CYP2B6*, *CYP2C9*, *CYP2C19*, *CYP3A4*, and *CYP3A7* than wild type Caco-2/ATCC. Particularly, *CYP2C9* showed the highest expression in Caco-2/h*CAR* while Caco-2/h*PXR* exhibited the highest *CYP3A4* expression. *PXR* and *CAR* are major CYP-nuclear receptors that are normally present at very low levels or even absent in wild-type Caco-2 cells. Thus, transfection of *PXR* or *CAR* into Caco-2 cells can increase the level and stability of these nuclear receptors, resulting in enhanced CYP expression and capacity (Küblbeck *et al*., 2016). CYP expression was higher in differentiated Caco-2 cells than in undifferentiated Caco-2, except for *CYP1A2*, which showed no difference in expression between differentiated and undifferentiated cells. CYP3A7 was similar between differentiated and undifferentiated in all types of Caco-2. All transporters exhibited higher expression in Caco-2/h*PXR* and Caco-2/h*CAR* than the wild type, except for ABCG2 that showed lower expression in Caco-2/h*PXR* compared to the wild type. *SLCO2B1* and *SLC15A1* were expressed in differentiated Caco-2 higher than in undifferentiated Caco-2 of all types, while ABCC2 was expressed significantly more in differentiated than in undifferentiated Caco-2/h*CAR*. In contrast, expression of ABCB1 and SLC16A1 in undifferentiated Caco-2 was higher than in differentiated Caco-2. Activity of transporters, in terms of paclitaxel (ABCB1 activity) and vinblastine (ABCC2 activity) efflux ratio and glycyl-sarcosine (SLC15A1 activity) uptake ratio from basal to apical sides, were not different in Caco-2/h*PXR* and Caco-2/h*CAR*. Conversely, ABCB1 and SLC15A1 activities from apical to basal sides in Caco-2/ATCC were significantly higher than in Caco-2/h*PXR* and Caco-2/h*CAR*. CYP1A2 and CYP2B6 activities were very low in all types of

Caco-2 while CYP2C activity was 2-fold higher in Caco-2/h*PXR* than Caco-2/ATCC (Table 3) (Küblbeck *et al*., 2016).

Three different Caco-2, i.e. Caco-2/ATCC HTB 37 at passages no. 35 to 55, Caco-2/h*PXR* at passages no. 18 to 32, and Caco2-transfected murine constitutive androstane receptor (Caco-2/m*CAR*) at passages no. 12 to 26, were cultured in 12-transwell-plates with uncoated PC filter membrane inserts for 21 days as differentiated models. The cells were incubated with rifampicin (10 μM; an h*PXR* inducer), 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPO BOP, 1 μM; a m*CAR* inducer), androstenol (10 μM; a m*CAR* inhibitor), and 1α,25-dihydroxyvitamin D3 (250 nM; a vitamin D3 receptor activator) for 3, 7, and 14 days (Korjamo *et al*., 2006). TCPOBOP induced expression of *CYP2B6* and *CYP2C9* in differentiated Caco-2/m*CAR* for all treatment periods. Treatment with rifampicin for 14 days significantly increased *CYP3A4*, *CYP2C9*, *ABCB1*, and *ABCC2* expression in differentiated Caco-2/h*PXR*. On the other hand, treatment with 1α,25-dihydroxyvitamin D3 for 14 days dramatically elevated *CYP3A4* expression by 900-fold in differentiated Caco-2/ATCC (Table 3) (Korjamo *et al*., 2006).

It is also important to consider the impact that silencing genes in Caco-2 cells can have in pharmaceutical research, particularly drug development. A lentivirus transfected knockout Caco-2/ATCC cell line containing *ABCB1*, *ABCG2*, and *ABCC2* shRNA (small hairpin RNA) and showing an 80% reduction in expression compared to wild-type was used to examine the transport of statin drugs. Simvastatin, lovastatin, atorvastatin, fluvastatin, and rosuvastatin have been reported to be substrates of these transporters, but the major transporter(s) for each statin remain controversial. Statins (each 10 μM) were added to Caco-2 and Caco-2 knockout cells cultured in 12-transwell-plates with coated PC filter membrane inserts and the level of each statin was determined from the basolateral to apical chambers. There was no difference in the level of lovastatin and simvastatin between the *ABCB1*, *ABCG2*, or *ABCC2*-knockout and the control cells, while levels of atorvastatin, fluvastatin, and rosuvastatin were significantly lower in the *ABCB1*, *ABCG2*, or *ABCC2*-knockout cells than in the control. These findings revealed that atorvastatin, fluvastatin, and rosuvastatin were substrates of ABCB1, ABCG2, and ABCC2 transporters, while lovastatin and simvastatin were not (Li *et al*., 2011). Hence, a gene-knockout Caco-2 model can be a useful tool to clarify intestinal drug transportation and potential drug interactions.

Table 3. Genetically modified Caco-2 cell models

Caco-2 origins	Treatment substances (concentration/ incubation time)	Results (compared group or Caco-2 cell types)		References
		Undifferentiated Caco-2 in 6-well-plate	Differentiated Caco-2 in 12-transwell-plate with PC filter membrane insert	
Caco-2/ ATCC (Wild type)	NT	↑ *ABCB1*[b,c,*], *SLCO15A1*[b,c,*], *SLC16A1*[*]	↑ *CYP2A6*[#], *CYP2B6*[#], *CYP2C9*[#], *CYP2C19*[#], *CYP3A4*[#] ↑ *ABCC2*, *SLCO2B1*, *SLC15A1*[#] ↑ ABCB1[b,c], SLCO15A1[b,c] activities	Küblbeck *et al.*, 2016
	1α,25-Dihydroxyvitamin D3 (250 nM/14 days)	N/A	↑ *CYP3A4*[d]	Korjamo *et al.*, 2006;
Caco-2/ h*PXR*	NT	↑ *CYP1A2*[a], *CYP2A6*[a], *CYP2B6*[a], *CYP2C9*[a], *CYP2C19*[a], *CYP3A4*[a], *CYP3A7*[a] ↑ *ABCB1*[a,*], *ABCC2*[a], *SLCO2B1*[a], *SLC15A1*[a], *SLC16A1*[a,*] ↓ *ABCG2*[a]	↑ *CYP1A2*[a], *CYP2A6*[a,#], *CYP2B6*[a,#], *CYP2C9*[a,#], *CYP2C19*[a,#], *CYP3A4*[a,#], *CYP3A7*[a] ↑ CYP2C9[a], CYP2C19[a] activities ↑ *ABCB1*[a], *ABCC2*[a,#], *SLCO2B1*[a,#], *SLC15A1*[a,#], *SLC16A1*[a] ↓ *ABCG2*[a] – ABCB1[c], ABCC2[c], SLC15A1[c] activities	Küblbeck *et al.*, 2016
	Rifampicin (10 μM/14 days)	N/A	↑*CYP3A4*[d], CYP2C9[d], ABCB1[d], ABCC2[d]	Korjamo *et al.*, 2006;
Caco-2/ h*CAR*	NT	↑ *CYP1A2*[a], *CYP2A6*[a], *CYP2B6*[a], *CYP2C9*[a], *CYP2C19*[a], *CYP3A4*[a], *CYP3A7*[a] ↑ *ABCB1*[a,*], *ABCC2*[a,*], *ABCG2*[a], *SLCO2B1*[a,#], *SLC15A1*[a,#], *SLC16A1*[a,*]	↑ *CYP1A2*[a], *CYP2A6*[a,#], *CYP2B6*[a,#], *CYP2C9*[a,#], *CYP2C19*[a,#], *CYP3A4*[a,#], *CYP3A7*[a] ↑ *ABCB1*[a], *ABCC2*[a], *ABCG2*[a,] *SLCO2B1*[a,#], *SLC15A1*[a,#], *SLC16A1*[a] – ABCB1[b], ABCC2[b], SLC15A1[b] activities	Küblbeck *et al.*, 2016
Caco-2/ m*CAR*	TCPOBOP (1 μM/3, 14, 21 days)	N/A	↑ *CYP2B6*[d], *CYP2C9*[d]	Korjamo *et al.*, 2006

Note: ATCC, American Type Culture Collection; h*CAR*, human constitutive androstane receptor; h*PXR*, human pregnane X receptor; m*CAR*, murine constitutive androstane receptor; TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene; NT, non-treated; N/A, data not available; (↑) increase, (↓) decrease, and (–) no change of expression/activity. [a] Result compared to Caco-2/ATCC (wild type), [b] Result compared to Caco-2/h*PXR*, [c] Result compared to Caco-2/h*CAR*, [d] Result compared to non-treated group, [*] Result compared to differentiated Caco-2 model, [#] Result compared to undifferentiated Caco-2 model.

4. Novel Culturing Methods in Caco-2 Models

A Caco-2 printed 3D-culturing model was established to mimic physiological conditions of the human intestine (Shen, Meng, & Zhang, 2015). Caco-2/ATCC at passages no. 35 to 45 (2×10^5 cells/cm^2) were cultured upside down for 21 days on a hanging 3D printed PC membrane insert in a 24-transwell-plate and in a normal 24-transwell-plate with PC filter membrane insert. Marker enzymes on the brush border of intestinal cells, histomorphology, cell viability, and alkaline phosphatase levels were monitored for cell differentiation (Ferrec & Fardel, 2012; Shen *et al*., 2015). ABCB1 and ABCC2 transporter activities were examined via the efflux ratio of rhodamine 123 (an ABCB1 substrate) and 5-carboxyfluorescein diacetate (5-CFDA; an ABCC2 substrate) after incubation for 0.5, 1, 1.5, and 2 hours (Figure 1) (Shen *et al*., 2015). Caco-2 on the hanging 3D-printed insert had higher cell viability than those in the normal 24-transwell-plate with no difference in histomorphology. The hanging 3D-printed insert significantly increased levels of alkaline phosphatase and γ-glutamyl transferase by 30 to 100% after incubation for 10 to 21 days. Moreover, the hanging 3D-printed insert culture showed increased ABCB1 and ABCC2 activities as 2- to 7-fold increases in the efflux ratios of rhodamine 123 and 5-CFDA compared to normal 24-transwell-plate cultures (Table 4) (Shen *et al*., 2015).

A multi-chamber microfluidic intestinal barrier model was developed to mimic the physiological conditions of human intestine by generating fluid flow in a microfluidic chamber (Tan *et al*., 2018). Caco-2/ATCC at passages no. 40 to 50 were seeded in a multi-chamber microfluidic platform with thiolene coated Teflon membrane and a 12-transwell-plate with PC filter membrane insert. Histomorphology and cell viability were determined on day 11. ABCB1 activity was determined by incubation of rhodamine 123 with Caco-2 in the microfluidic system for 80 min (Figure 2). Caco-2 cells in the microfluidic culturing system were more than 95% viable after seeding for 11 days. Within three days, Caco-2 cells in the microfluidic culturing system had differentiated into columnar cells close in appearance to healthy human intestinal cells with a height of 40 to 50 μm. This height was 2-fold that of Caco-2 cultured in 12-transwell-plates for 21 days. ABCB1 is expressed in Caco-2 as an efflux pump, pumping substrates from the basolateral to the apical side. ABCB1 activity was observed in the multi-chamber microfluidic intestinal barrier system after incubation with rhodamine 123 for 80 min (Table 4) (Tan *et al*., 2018).

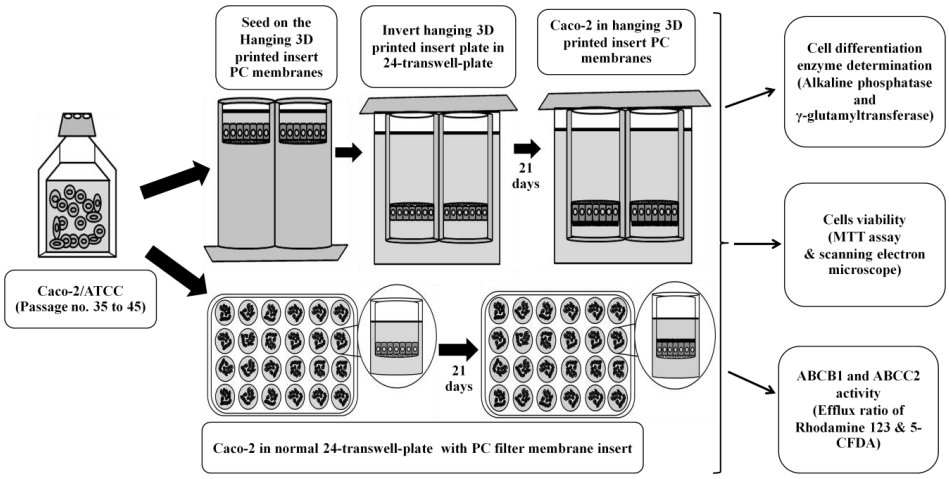

Figure 1. Caco-2 culturing on hanging 3D-printed insert model

Table 4. Novel culturing methods for Caco-2 models

Caco-2 origins		Results* (compared culturing methods)	References
Caco-2/ATCC	Hanging 3D printed with PC filter membrane insert	↑ Physiological condition, Cell viability ↑ Alkaline phosphatase, γ-Glutamyl transferase ↑ ABCB1, ABCC2 activities	Shen *et al.*, 2015
Caco-2/ATCC	Multi-chamber microfluidic with thiol-ene coated Teflon filter membrane insert	↑ Physiological fluid flow ↑ ABCB1 activities ↑ Rate of cell growth with intestinal cell appearance in 3 days	Tan *et al.*, 2018
Caco-2/ HDDC	Gut-on-chip microdevice fabrication (Microchem SU-8 100 mold)	↑ Physiological condition similar to intact intestinal environment ↑ CYP3A4 activity ↑ Rate of cell differentiation into 4 cell types (i.e. basal crypts with extended villi epithelium cell, mucus-secretory cell, enteroendocrine cell, and Paneth cell) within 4 days	Kim & Ingber, 2013

Note: ATCC, American Type Culture Collection; HDDC, Harvard Digestive Disease Center; NT, Non-treated; 5-CFDA, 5-carboxyfluorescein diacetate; (↑) increase. *Result compared to normal 12/24-transwell-plate.

A novel *in-vitro* model of Caco-2 cells cultured under micro-physiological conditions, or a "gut-on-chip", has been reported to mimic the shear stress, fluid flow, and circulation physiological conditions of intact intestinal cells. The gut-on-chip system accelerates the differentiation of Caco-2 cells to polarized microvilli-columnar cells, which are similar to intact intestinal cells, faster than in a conventional transwell-plate. Moreover, the fluid flow and shear stress system increases the paracellular permeability of Caco-2 cells, a feature that also appears in *in-vivo* models (Franco, Silva, & Cristofoletti, 2021). Gut-on-chip is a microfabrication of clear silicone rubber forming two layers of microchannels containing a porous polydimethylsiloxane membrane coated with collagen and Matrigel that mimics the environment of the human intestine (Kim & Ingber, 2013; Franco, Silva, & Cristofoletti, 2021). Caco-2 cells (obtained from the Harvard Digestive Disease Center) were seeded into the upper layer of the microdevice and after the cells attached, the device was perfused with fluid flow at 30 mL per hour and fluid shear stress around 0.02 dyne per cm^2 to both the upper and lower layers, and results were compared to a conventional transwell-plate. The Caco-2 cells in the gut-on-chip model presented four differentiated cell types; basal crypts with extended villi epithelial cells, mucus-secretory cells, enteroendocrine cells, and Paneth cells, within 4 days. In addition, CYP3A4 activity was higher in the gut-on-chip model than in the conventional transwell-plate after 150 hours, as determined by P450-Glo CYP3A4 assay kit (Kim & Ingber, 2013). Interestingly, A 3D perfused gut-on-chip or OrganoPlate three-lane (Mimetas BV) model has been used for co-culture of Caco-2 (intestinal enterocyte), HT29-MTX-E12 (goblet cell), and THP-1 or MUTZ-3 (immune cell) to examine the anti-inflammatory activity of a compound relative to each cell in the human intestinal inflammatory mechanism (Gijzen *et al*., 2020). Therefore, the gut-on-chip is a novel human intestine model that has been shown to be useful for studies of drug development, and is a good model to explore human intestinal diseases.

5. Conclusions

Caco-2 shows limited expression of CYPs, particularly for the major CYP3A4 isoform, and requires a long culturing period for differentiation. Hence, several improved Caco-2 models have been developed for examination of drug metabolism and transportation. There are three approaches that have been used, consisting of xenobiotic induced Caco-2 monolayer models, genetically modified Caco-2 models, and novel Caco-2 culturing methods. All three approaches have been established to enhance CYP and transporter expression and shorten the time for cell differentiation. Xenobiotic-induced Caco-2 monolayer models are simple models using typical CYP- or transporter-inducers to enhance expression. CYP3A4, which is normally of very low abundance in Caco-2, can be highly induced by 1α,25-dihydroxyvitamin D3 via vitamin D receptor binding, while other CYPs and transporters, e.g. CYP1A1, CYP3A5 and ABCB1, are inducible by typical substrates/inducers such as β-naphthoflavone, Aroclor 1254, and ATRA. These xenobiotic-induced monolayer models show pros and cons.

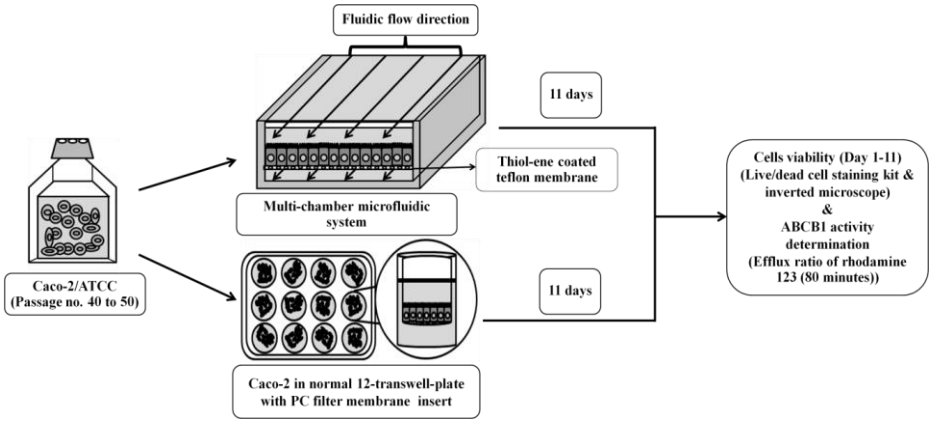

Figure 2. Caco-2 culturing on multi-chamber microfluidic intestinal barrier model

Table 5. Advantages and disadvantages of Caco-2 models

Model type	Benefits	Limitations
Xenobiotic induced Caco-2 monolayer models	- Easy for culturing and treatment - Less steps of experiment - Economic design	- Low expression of catalytic enzymes - Require long time (2 to 3 weeks) for cell differentiation
Genetic modified Caco-2 models	- Increase expression of CYPs and transporters	- Transfection is complicate. - Require long time (2 to 3 weeks) for cell differentiation
Novel Caco-2 culturing models	- Increase growth rate - Shorten time for cell differentiation - Mimic human intestinal physiology - Increase activity of transporters	- High investment - Few reports on drugs metabolism and transporters

They are simple to conduct in both culturing and treatment phases with economical experimental design, but they require long culturing periods (from 2 to 3 weeks) for cell differentiation. Genetically modified Caco-2 models created by transfection with *PXR* and *CAR* nuclear transporter genes show improved CYP and transporter expression via the up-regulation of their related expression mechanisms. However, these models still require long culturing periods for cell differentiation; and transfection is a complicated, time-consuming process. Novel Caco-2 culturing methods have been developed to mimic human intestinal physiology, increase growth rate, and shorten the period of cell differentiation. Hanging 3D-printed multi-chamber micro-fluidic and micro-physiological (gut-on-chip) platforms have shown improved cell alignment, and the fluid flow increases growth rate and the activity of efflux transporters and CYP3A4 beyond those in conventional or inserted plate-Caco-2 culturing systems. However, these novel methods require expensive, specialized technological materials, and to date, there are a very few reports using this technology in the context of drug metabolism and transportation (Table 5). In conclusion, an optimal Caco-2 model for drug metabolism and transportation is dependent on the specific aim, which needs to be incorporated into the experimental design of each individual study.

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