11 Applications Using Caco-2 and TC7 Cells for Drug Metabolism Studies

ERIC LE FERREC[1]

[1]Université de RENNES1, IFR140, Rennes, France

OLIVIER FARDEL[1,2]

[2]Département HITC, Hopital Pontchaillou, CHU Rennes, France

11.1 SUMMARY

Gastrointestinal tract, particularly the small intestine, is the site of absorption of orally ingested xenobiotics including nutrients and drugs. The objective of this absorption is the passage of these molecules from the intestinal lumen to the blood and/or to the lymphatic circulation. Moreover, the oral route is the major route used for administration of drugs since dosing is convenient and noninvasive and many drugs are well absorbed by gastrointestinal tract. It is therefore easy to understand that knowledge and the prediction of the passage of these molecules through the intestinal barrier are essential for the development of these compounds. The complexity of this barrier justifies the existence of many predictive models and explains the numerous studies done to develop and improve these patterns. This chapter provides an overview of key issues regarding the intestinal barrier, commonly used cell models and their weaknesses. Also proposed are an example of protocol for the use of model Caco-2 cells and a set of controls for validating the model.

Encyclopedia of Drug Metabolism and Interactions, 6-Volume Set, First Edition.
Edited by Alexander V. Lyubimov.

11.2 INTRODUCTION

The intestinal barrier, in a general point of view, can be defined as the boundary between the intestinal contents and the internal medium of the human body, represented by the enteric circulation [1,2]. To be more precise, the intestinal barrier can be defined histologically, chemically, or metabolically.

11.2.1 Histological Point of View

The intestinal mucosa consists of a simple columnar epithelium, a lamina propria (collagen matrix containing blood and lymphatic vessels), and a muscularis mucosa [3]. The intestinal mucosa possesses villi, whose function is to increase the surface of absorption. The epithelium constitutes the essential element of the intestinal barrier and is composed of four major cell types: enterocytes (possessing microvilli, and implicated in the absorption), goblet cells (producing mucus), enteroendocrine cells, and Paneth cells [4]. Intestinal epithelium is a dynamic system continuously renewed from stem cells located in invaginations called *crypts of Lieberkuhn* [5]. The epithelial cells are connected by junctional complexes including adherens junctions, desmosome and especially tight junctions at the origin of the intestinal barrier impermeability.

11.2.2 Chemical Point of View

To reach the bloodstream, a molecule present in the intestinal lumen must cross several layers possessing specific physicochemical characteristics. Thus, the molecule will pass through the mucus layer (aqueous solution of mucin), the unstirred water layer (aqueous interface between the intestinal lumen and the plasma membrane), the lipid bilayer of the enterocytes plasma membrane, and the basement membrane associated with intestinal epithelium and reach the capillaries, then cross the basement membrane associated with the endothelium and the endothelium itself. All of these steps can limit the passage of a molecule.

11.2.3 Metabolical Point of View

If the liver is the organ of reference concerning the metabolism, the intestinal metabolism should not be underestimated. Indeed, the metabolic activity of the intestinal mucosa is the second largest of the human body after the liver. The metabolizing enzymes, such as aminopeptidases, dipeptidyl peptidase IV, esterase, sulfotransferase (SULT), UDP-glucuronyltranferase (UGT), glutathione-*S*-transferase (GST), cytochrome P450 (CYP) superfamily, and membrane transport systems, including P-glycoprotein (ABCB1), expressed by enterocytes, serve as a biochemical barrier [6]. Some works even seem to demonstrate that after normalization for the relative abundance of P450 enzymes in the organ, the metabolic activities of the intestine and liver are comparable. This ability to transform and/or to efflux molecules into the lumen of intestine is also a crucial element of the barrier intestinal.

The intestinal barrier is extremely complex and difficult to reproduce *in vitro*. There are many systems that attempt to mimic: organotypic models (everted gut sac, Ussing chamber), cellular models (cell lines: Caco-2, TC7, etc.), which all have advantages and limitations. The choice of one of them will depend mainly on the problematic of the molecules studied and resources available.

11.3 INTESTINAL METABOLISM

11.3.1 Longitudinal and Transverse Heterogeneities

The intestinal metabolism, in animals or humans, has been the subject of a very extensive work. Many difficulties have been encountered, leading sometimes to discordant results. Thus, one can be cited: (i) the low expression levels of intestinal metabolic enzymes relatively the liver; (ii) intra- or interspecies variability of expression of biotransformation enzymes; (iii) ethical and technical limitation for studies in humans; (iv) variability of sample preparation techniques; and (v) structural and functional heterogeneity of the intestine.

The human intestine is divided into two parts: the small intestine, subdivided into duodenum, jejunum, and ileum, and the colon. They differ in their histological structure and by their metabolism. Thus, when considering axially mouth rectum (longitudinal axis) of the gastrointestinal tract, the metabolic activity of the intestine is particularly highest in the upper part of the small intestine, with a maximum observed at the proximal jejunum [7]. It has been shown that the total P450 content increased slightly between the duodenum and the jejunum, then decreased markedly at the ileum. If this heterogeneous distribution concern phase I enzymes (i.e., CYP3A4, 2 C9, or 2 C19), phase II enzyme (i.e., GST, UDPGT) distribution is relatively homogeneous in small intestine but with a lower level of expression in the colon. These observations were consistent with the fact that absorption of most of the xenobiotics occurs in duodenum and proximal jejunum and with the fact that relative abundance of goblets cells increases from small intestine to colon. In the same way, the peptide transporter PepT1 (SLC15A1) appears to have higher expression in the duodenum than in the ileum [8]. However, the decrease of expression and/or activity, from duodenum to the colon, seems not to be associated with all membrane transport systems. Indeed, some reports has been demonstrated, by analyzing the rate of mRNA levels along the gastrointestinal tract, that expression of the drug efflux pump P-glycoprotein increases from the stomach to the colon [9], even if other studies have shown that the expression of P-glycoprotein is correlated with CYP3A4 distribution [10,11].

Another level of heterogeneity corresponds to crypt-villus axis (transverse axis) [7]. Three cellular phenomena are observed along this axis: proliferation, differentiation, and apoptosis. Proliferative cells (stem cells) reside in the cryps, while apopototic cell are observed in the top of villi. Approximately three days after the terminal differentiation, the cells reach the villus apex, undergo apoptosis and are exfoliated in the intestinal lumen. In the crypts, stem cells generate four major differentiated cell types: enterocytes, goblet cells, and enteroendocrinecells, which migrate to the top of the villus, and Paneth cells that remain at the crypt [12,13]. The junction between the crypts and the villi is the limit from which the cells develop their specific characteristics. This organization is observed not only in the small intestine but also in the colon, except for a colon that has no villi or Paneth cells [14]. The differentiation gradient of enterocytes along the crypt-villus axis results in the existence of a gradient of expression and/or activity of metabolic enzymes. It was thus shown that microsomes prepared from crypts cells contained much less CYPs that microsomes prepared from the mature villus cells. The maximum activity was observed at the middle third of the villus. A similar gradient exists for phase II enzymes, especially for GST and UDPGT

[7,15]; P-glycoprotein is also highly expressed in villus cells but is barely detectable in crypt epithelium [16].

11.3.2 First-Pass Intestinal

The first-pass effect is defined as *partial* or *complete extraction* of the dose of a xenobiotic that takes place between administration site and the systemic circulation. Metabolism is a key element of this processus. To reach the systemic circulation, a molecule, administered orally, must pass three potential sites of metabolism: the intestine, liver, and lung. To cross the intestinal barrier, a molecule can use the transcellular or the paracellular route [16]. Only molecules using the transcellular mode may be submitted to first-pass effect. During the cellular transfer, these compounds can undergo the action of phase I and phase II metabolic enzymes and can be expelled out of cells by membrane transport systems [17,18]. The first-pass effect is also facilitated by amplification systems of the intestinal luminal surface (i.e., valvulae conniventes, villi, microvilli) that allow a surface of absorption, and thus of potential metabolization, considerable. Despite the great variability of expression in humans, CYP3A enzymes, especially CYP3A4, are a key element of the intestinal first-pass effect [18–20]. These enzymes represent 80% of CYPs in the small intestine and about 50% of drugs are substrates for these enzymes. P-glycoprotein is another key element of the intestinal first-pass effect. This transporter belongs to the family of ATP-binding cassette (ABC) transporters and is present at the brush border of enterocytes. It is interesting to note that CYP3A4 and P-glycoprotein have many substrates in common and that their intestinal distributions may be correlated [10,11]. All these observations led to the hypothesis of coordinated actions between these two detoxifying proteins [21].

11.4 COMPARISON BETWEEN INTESTIN/Caco-2/TC7

11.4.1 Cellular Intestinal Model

Much attention is paid to the use of epithelial cell cultures for studies of drug transport mechanisms, especially during drug development in pharmaceutical companies. As the use of primary intestinal epithelial cells is limited by the rapid loss of their differentiated characteristics during culture, the attention has turned to the use of human established lines of tumor origin, which display a number of properties characteristic of differentiated intestinal cells with variable degrees of differentiation. Some of them, such as Caco-2, HT-29, and T84 cells have been shown to exhibit most of morphological and functional characteristics of enterocytes. Other cell lines, such as HCT8, HRT18, or SW1116, express only a partially intestinal differentiated phenotype [22]. The limitations of cell models must not be overlooked, but they offer the advantage of relative simplicity and are suitable for automated procedures. Caco-2 cells are the most used cellular model in studies on passage and transport. They were derived from a human colorectal adenocarcinoma. In culture, Caco-2 cells, despite their colonic origin, express the majority of the morphological and functional characteristics of small intestinal absorptive cells, including phase I and phase II enzymes and drug transporters; they differentiate spontaneously into polarized intestinal cells possessing an apical brush border and tight junctions between adjacent cells, and they express hydrolases and typical microvillar transporters.

Moreover, these cells, particularly Caco-2 cells, can be cultivated as monolayers of differentiated cells on a semipermeable membrane. Thus, separating the apical compartment from the basolateral compartment, which correspond to the intestinal lumen side and the serosal side, respectively, is possible. These cell lines, originated from tumors, are, however, out of the *in vivo* physiological environment; therefore, extrapolation of the data to the *in vivo* situation may be difficult; a similar conclusion can be otherwise drawn for most of *in vitro* culture systems.

11.4.2 Phase I Enzymes

Among the enzymes of phase I, CYPs have been the object of the largest number of studies [23–25]. There may be differences between these works, especially for those using human intestine as a model. These differences are related to techniques used (sample preparation, analytical techniques) and may also reflect human variability. Using enterocytes isolated from human small intestine, Zhang *et al*. have analyzed the expression of CYPs. The used technique allows the analysis of enterocytes from the villi without contamination by crypts cells. Several CYPs were detected at mRNA level: CYP1A1, 1B1, 2C, 2D6, 2E1, 3A4, and 3A5. In contrast, at the protein level, only CYP1A1, 2C, and 3A4 were detected. Other studies have demonstrated that CYP2C isoforms present in the intestine were 2C9 and 2C19 [23]. Regarding CYP3A5, it has been highlighted, at the protein level, in a small proportion of the caucasian population [26].

Despite the differences that may exist between the works, they agree that the main CYPs are those of the CYP3A family, especially the isoform 3A4. However, these CYPs are not or slightly (according to studies) expressed in Caco-2 cells [26–28]. Thus, Sun *et al*. [29], comparing the expression profile of many genes, including CYP3A4, have shown that Caco-2 cells expressed about 150 times less CYP3A4 the human duodenum. Treatment with 1α,25-dihydroxyvitamin D_3, an inducer of CYP3A4 at the mRNA level, and transfection of CYP3A4 cDNA are two ways for increasing CYP3A4 expression levels in Caco-2 cells [30,31]. However, expression levels do not reach the levels observed *in vivo*. Other CYPs have been highlighted in this cell line, at least at mRNA level, such as CYP1A1, 2E1, and 2B6. Caco-2 cells are widely used to study the passage of the intestinal barrier and is described in the literature as the reference model to study intestinal drug absorption. However, on the intestinal first-pass effect, due to the low expression of CYP3A4, it seems that these cells do not constitute a model of choice.

To overcome absence expression of CYP3A, the Caco-2 clone TC7 has been developed. This cell line, generated by passing Caco-2 cells 198 times, expresses CYP3A4 and 3A5 (at least at mRNA level) at a higher level than their parental counterparts. In addition, CYP3A4 is inducible by 1α,25-dihydroxyvitamin D_3 in this cell line. Moreover, several studies have demonstrated that TC7 cells are a good alternative to the Caco-2 parental line for drug transport studies [27,28,32].

11.4.3 Phase II Enzymes

The main phases II enzymes are present in the intestine, including UGT, SULT, acetyltransferase (ACT), and GST [29,33–35] (Table 11.1). These enzymes are characterized by their large interindividual variations and their most important expressions in the

TABLE 11.1 Example of Phase II Metabolizing Enzyme Expressed in Human Small Intestine, Caco-2 Cells and TC7 Cells[a]

	Small Intestine	Caco-2	TC7
GST	Alpha, mu, pi	A1, A2, A3, A4, O1, P1, T2, Z1	
UGT	1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A10, 2B4 2B7, 2B15, 2B28, 2B17	1A1, 1A6, 1A9, 2B9	1A1, 1A3, 1A6, 2B7
SULT	1A1, 1A2, 1A3, 1B1, 1E1, 2A1	1A1, 1A2, 1A3, 1B1, 1C1, 1C2, 2A1	1A1, 1A2, 1A3, 1B1, 1C1, 1C2, 2A1, 1E1

[a]Refs 29, 33–35, and 37–43.

small intestine compared to colon [36]. In the Caco-2 cells, at least at mRNA level, the GST class μ, π, and especially α (A1, A2, A3, and A4) has been highlighted [34,37]; UGT (especially after induction) such as the UGT 1A1, 1A6, 1A9, and 2B9; and SULT such as SULT 1A1, 1A2, 1A3, 1B1, 1 C1, 1 C2, or 2A1. From a general point of view, the activity of phase II enzymes is lower in the Caco-2 cell line than in the small intestine. However, some enzymes have higher expression in this cell line than in intestine. This is the case, for example, of the SULT 1A2, 1A3, 1 C1, or 2A1 [29,37–40].

Several studies have shown that phase II metabolism is more important in TC7 cell line than in Caco-2 cells, especially with respect to glucuronidation and sulfoconjugation levels [41] (Table 11.1). In these two cell lines, as in the enterocytes, the expression of phase II enzymes is in fact strongly linked to the differentiation state.

11.4.4 Drug Transporters

Drug transporters, belonging either to the solute carrier (SLC) family or to the ABC family, play a major role in drug absorption or secretion in the intestine. Indeed, the ABC pumps P-glycoprotein, multidrug resistance protein (MRP) 2 (ABCC2), and breast cancer resistance protein (BCRP, also known as ABCG2), located to the apical pole of enterocytes, are important in preventing the absorption of some chemicals into the systemic circulation, through expelling them in the lumen of the intestine. Other transporters located at the brush border pole of intestinal cells, such as the peptide transporter, PEPT1, or the organic anion transporters, OATP-A (SLCO1A2) and OATP-B (SLCO2B1), are by contrast involved in drug uptake into enterocytes. At the basolateral pole, organic solute transporters (OSTα/OSTβ) and MRP3 (ABCC3) may facilitate the removal of the intracellular solutes into the basolateral side, thereby enhancing the absorption of drugs.

Most of the transporters found in human intestine are also detected in Caco-2 cells, including P-glycoprotein, BCRP, MRP2, PEPT1, OATP-B, and the monocarboxylate transporter (MCT)-1 (SLC16A1) [42]. The expression of transporters in Caco-2 cells such as OATP-B, BCRP, and MRP2 differ, however, more than fivefold than that found in the ileum, indicating that Caco-2 cells do not strictly reflect the pattern of transporter expression displayed by small intestine. In addition, it is noteworthy that the source of the Caco-2 cells, the culture conditions, and the culturing time are likely to affect

transport proteins; P-glycoprotein, MRP2, and PEPT1 mRNA levels have thus been shown to differ by at least a twofold factor in two independent Caco-2 cell clones [43]. Such differences may directly contribute to heterogeneity in transport activity between Caco-2 cells cultured in different laboratories [44].

With respect to transporter expression in TC7 cells, slightly higher mRNA levels of SLC transporters were found in these cells when compared to Caco-2 cells; these differences between the two cell lines were, however, considered as marginal [45].

Functional assays with Caco-2 cells, that is, permeability studies (see the following sections), may be performed with referent substrates for transporters: digoxin, vinblastine or placlitaxel (for P-glycoprotein), sulfobromophthalein (for MRPs and OATPs), methotrexate, estrone-3-sulfate, and pheophorbide A (for BCRP) and glycylsarcosine (for PEPT1). In addition, referent inhibitors of transporters may be used: verapamil or cyclosporin A (inhibiting P-glycoprotein), probenecid (inhibiting MRPs and OATPs), and fumitremorgin A (inhibiting BCRP). A recent paper from the International Transporter Consortium has proposed guidelines for investigating whether a compound may be a substrate for P-glycoprotein or BCRP using Caco-2 cells; an efflux ratio, basal to apical versus apical to basal, >2, is in favor of a potential transport by P-glycoprotein or BCRP, which has to be confirmed by the use of transporter inhibitors [46]. Moreover, decision trees for P-glycoprotein and BCRP inhibitor interaction studies using bidirectional transport assays with Caco-2 cells are also provided in this chapter.

11.5 USING CELL MODELS

As Caco-2 cells are the most popular cellular model in studies on passage and transport, this and the following sections will only concern these cells.

11.5.1 Conditions of Culture

The complete morphological and functional differentiation of Caco-2 cells requires three weeks in culture under conditions described in Table 11.2. Many modifications of these conditions have been reported. Thus, the support of culture can be coated with, for example, type 1 collagen or matrigel, and the inactivated serum concentration decreased.

TABLE 11.2 Culture Conditions of Caco-2 Cells

	Caco-2 Cells
Culture medium	Dulbecco's modified Eagle's medium (classically)
Inactivated fetal calf serum (heat-inactivated at 56°C for 30 min)	Usually 20% (range, 5–20%)
glucose	25 mM (final concentration)
Nonessential amino acids	1%
Glutamine	2 mM (final concentration, range: 2–4 mM)
Cell density at seeding	$1 \times 10^5 - 4 \times 10^5$ cells/cm^2
pH	Usually 7.4
Medium change	Every 24–48 h

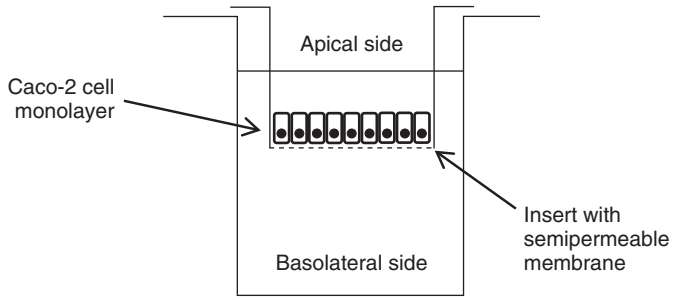

Figure 11.1 Schematic description of cells culture in nacelle system.

The enterocyte-like phenotype of Caco-2 cells is enhanced when cells are grown on semipermeable membrane (Fig. 11.1). In this case, Caco-2 cells can proliferate and differentiate in monolayer, reproducing the intestinal epithelium organization, where the apical compartment corresponds to the intestinal lumen and the basolateral compartment to the serous site. This method of culture allows the creation of an accessible apical and basolateral pole, with a possible asymmetry in the composition of culture medium. This asymmetry may allow, for example, studies of the passage of molecules from apical to basolateral side or from basolateral to apical side.

11.5.2 Essential Elements

The variation between laboratories can be associated to the heterogeneity of the Caco-2 cell line as well as to many factors influencing the morphological and functional characteristics of these cells. Among these factors, we can cite: the origin of cells, the culture conditions, the number of passage, the culture time, and the study conditions (concentration of drugs, materials used, temperature, methods of analysis, etc.) [6,47]. Thus, the transepithelial electrical resistance (TEER, a marker of the integrity of the cell monolayer) [48], or the expression of P-glycoprotein, [49–51] are influenced by cell differentiation level or by passage numbers of cells. Moreover, many studies show the influence of fetal calf concentration in the culture medium. Indeed, the serum provides hormones, growth factors, attachment factors, and other compounds necessary for growth and differentiation of Caco-2 cells. However, the exact composition of serum is often not fully characterized and varies from batch. This variation, for some authors, may favor the selection of a predominant clone over the course of passages, contributing to interlaboratory variability [52,53]. It is obvious that the removal of this variability is illusory. However, some rules can be observed to reduce it, such as to establish a master bank and a working bank to be sure to be in ranges of moderate numbers of passage, to try to always use the same serum; to strictly follow the protocols used for changing the culture medium or for reseeding cells, and to establish control of differentiation before the use of Caco-2 cells such as the analysis of the tight junction formation. These controls allow to reduce the variability of results and also to value the model.

11.5.3 Validation of Models

The use of Caco-2 model requires different controls [6,54] (Table 11.3). The first controls concerns the identification of the differentiated state of Caco-2 by measuring

TABLE 11.3 Example of Control and Methods of Control Used during Validation of the Caco-2 Cell Model[a]

	Method Proposed for the Control	
Cell monolayer integrity	Measuring the TEER	TEER measurement can reveal toxicity or the tight junctions opening. TEER range of Caco-2 cells: 150–1600 ohm cm^2
	Measuring permeability of test compounds: • mannitol • PEG 400 • Lucifer yellow	These molecules do not usually spend the intestinal barrier
Cell differentiation	By measuring expression of differentiation markers: • sucrase isomaltase • dipeptidyl peptidase IV • alkaline phosphatase aminopeptidase	The expression of these markers is correlated with cell differentiation that depends on confluence of cells
Model validation (for passage studies)	By measuring the permeability of reference molecules	Proposed molecules with their apparent permeability (Papp): • Testosterone: 73×10^{-6} cm/s • Propranolol hydrochloride: 28×10^{-6} cm/s • Methothrexate: 1.2×10^{-6} cm/s The United States pharmacopeial (USP) convention proposes these molecules. If the Papp values obtained with Caco-2 cells are the same as the values given by USP (±20%), the model can be considered to be valid

Abbreviation: TEER, transepithelial electrical resistance.
[a]Refs 6, 56, and 57.

the expression of differentiation markers such as sucrase-isomaltase (SI), dipeptidyl peptidase IV, alkaline phosphatase, or aminopeptidase. These enzymes are present at the brush border of enterocytes and some of them, such as SI, correspond to specific differentiation markers of the intestine. These checks can be performed on Caco-2 cells grown on plastic supports or on semipermeable membrane.

The second concerns the formation of cell layer and tight junctions (if cells are cultivated on semipermeable membrane). The integrity of the cell layer can be monitored by measuring TEER, which may be associated with the study of the passage of molecules such as lucifer yellow, an hydrophilic dye that does not pass the intestinal barrier.

Finally, the third control concerns the passage of the intestinal epithelium, represented by the monolayer of differentiated Caco-2 cells, by the study of passage of

references molecules. The choice of reference molecules is difficult. The following compounds have been proposed by the US Pharmacopeia: testosterone, propranolol hydrochloride, and methotrexate; permeability values obtained should not be different ±20% of the proposed values [55].

If this part is related to the use of Caco-2 cells, it should be noted that most of the concepts described are also to be associated with the use of TC7 cells. However, keep in mind that TC7 cells possess some different characteristics, compared to Caco-2 cells, that is, a lower expression of P-glycoprotein (but remains higher than in the human small intestine); a doubling time shorter; a lower TEER than Caco-2cells, and expression of CYP3A4. Thus, if controls and validation methods are the same for these two cell models, the experimental results obtained are not necessarily directly comparable.

11.6 EXAMPLE OF PROTOCOL: USING Caco-2 CELLS FOR PASSAGE STUDIES

This section provides a sample protocol for using Caco-2 cells in order to study the passage of various molecules through the cell monolayer formed by Caco-2 cells.

11.6.1 Cell Culture

The Caco-2 cells are used between passages 25 and 35. The culture medium (DMEM, 4.5 g glucose/L) is supplemented with 20% decomplemented fetal calf serum, 1% nonessential amino acids, 1% L-glutamine and antibiotics (100 μg/mL penicillin and 100 mU/mL streptomycin). It is renewed every 48 h. The cells are seeded at 100,000 cells/cm^2 on a semipermeable membrane (Transwell, 0.4 μm, COSTAR) and maintained in culture for 21 days after confluence. With these culture conditions, the cell confluence is usually obtained in four days.

11.6.2 Expression of Sucrase-Isomaltase (SI)

To determine whether our culture conditions lead to differentiated Caco-2 at the time of their use, we have studied expression of SI. The study of mRNA level of SI and β-actin was assessed by RT-PCR. The primers used for the study of mRNA of SI and β-actin of Caco-2 are: SI forward, 5′-gcccgtcttccaagtgatta-3′; SI reverse, 5′-aaattgcagggtccaga-3′; β-actin forward, 5′-ggccatctcttgctc-3′; β-actin reverse, 5′-gcccagagcaagagag-3′. The results show that, in our conditions of cell culture, enterocytic differentiation appears between the 5th and the 10th day of culture (Fig. 11.2).

11.6.3 Measurement of the TEER and Passage of Lucifer Yellow

Measurement of TEER of cell monolayer and the low passage of lucifer yellow, a dye not passing the epithelial barrier, are markers for the development of tight junctions. The TEER measure can be achieved through a volt-ohm meter (Evoma, World Precision Instruments Sarasota, FL). It is realized from the day after seeding (day 0) and after 3, 7, 14, and 22 days of culture. The intrinsic resistance of the semipermeable membrane

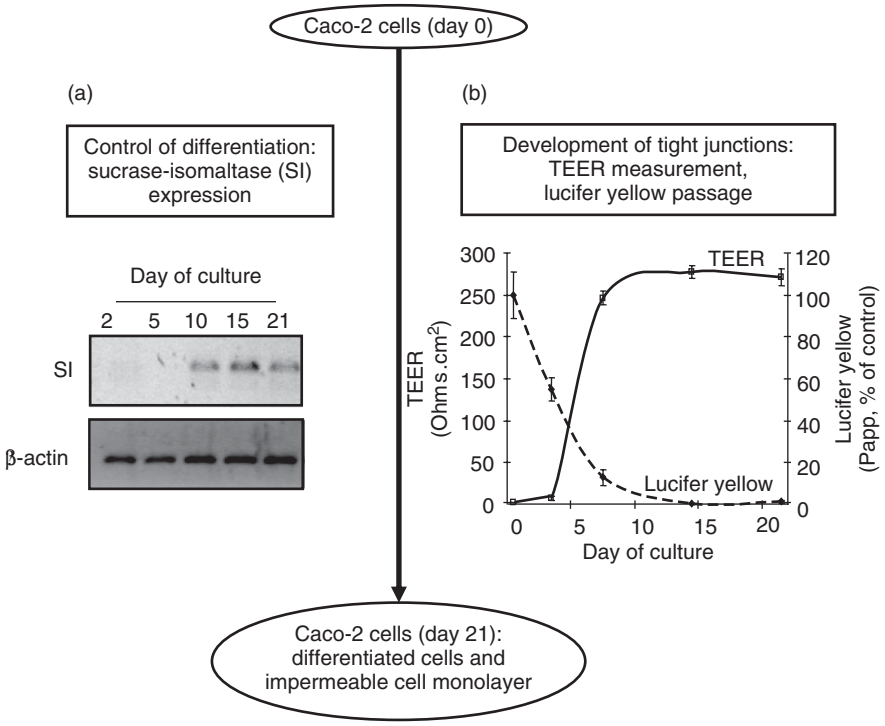

Figure 11.2 (a) Kinetic expression analysis, by RT-PCR, of sucrase-isomaltase (SI) and of β-actin in Caco-2 cells. (b) Measurement of TEER and the passage of lucifer yellow. These measurements were made from the day of seeding on semipermeable support (day 0) and after 3, 7, 14, and 22 day of culture. The results are expressed in Ohm·cm^2 for TEER and percentage of control (amount of cell-free passage) for lucifer yellow.

is subtracted from the total resistance (cell + membrane). After correcting the value obtained by the culture surface, the results are expressed in Ohm cm^2 (Ω cm^2). The permeation rate of lucifer yellow is determined by Papp calculation (see below). The sharp increase of TEER associated with the decrease in Papp of Lucifer yellow (Fig. 11.2) shows that our experimental conditions allow the formation of an impermeable cell monolayer.

11.6.4 Calculation of Apparent Permeability (Papp)

The current mode of implementation of the experience of passage is described in Fig. 11.3 [31]. There is a passage from the cell support (Fig. 11.1), which corresponds to the donor compartment or apical compartment in different acceptor compartments, or basolateral compartment, at regular time intervals. Using calculations as described in the Table 11.4, the curve representing the variations, in the acceptor compartment, of the quantity (Q) of interest molecule in function of time (T), can be built. The Papp were determined by linear regression of changes in concentrations in the acceptor compartment in function of time and by measuring the slope obtained (represented by dQ/dT, Table 11.4). The coefficient of apparent permeability (Papp) is expressed

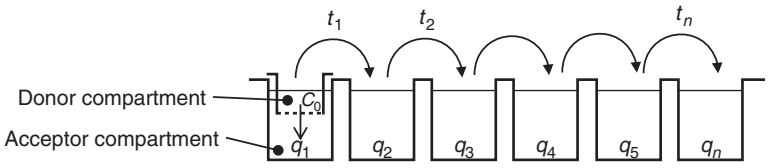

Figure 11.3 A schematic representation of the method used for measurement of passage (donor compartment to acceptor compartment) of drug across monolayer of Caco-2 cells. C_0, initial concentration; q, quantity of drug passing in acceptor compartment; t, interval time.

TABLE 11.4 Steps for Apparent Permeability (Papp) Determination

(1) Using disposition described in Figure 11.3, construction of the curve corresponding to the variations, in the acceptor compartment, of the quantity (Q) of interest molecule, in function of time (T).	Where $T_0 = 0$ and $Q_0 = 0$; $T_1 = t_1$ and $Q_1 = q_1$; $T_2 = t_1 + t_2$ and $Q_2 = q_1 + q_2$; $T_n = t_1 + t_2 + \cdots + t_n$ and $Q_n = q_1 + q_2 + \cdots + q_n$ (generally t_1, t_2, ... and t_n are identical)
(2) Determination of the slope $\mathrm{d}Q/\mathrm{d}T$ by linear regression of the curve obtained in (1).	
(3) Calculate the coefficient of apparent permeability (Papp) by: Papp (cm/s) = $(\mathrm{d}Q/\mathrm{d}T) \times [1/(A \times C_0)]$	Where C_0 = initial concentration in donor compartment; A = surface-exposed (cm^2); $\mathrm{d}Q/\mathrm{d}T$ = slope determined in (2).
(4) Construction of the curve corresponding to the slope ($\mathrm{d}Q/\mathrm{d}T$) in function of the initial concentration (C_0) in the donor compartment.	If the curve obtained is saturable, this indicates that the mode of passage probably involves a transport system. If the relationship between the slope ($\mathrm{d}Q/\mathrm{d}T$) and C_0 is linear, this indicates that the mode of passage is probably passive.

in centimeter per second. Measuring the slope ($\mathrm{d}Q/\mathrm{d}T$) as a function of the initial concentration (C_0) may be also used to determine if the passage uses a passive or active mode (Table 11.4).

The permeation rate of testosterone, propranolol hydrochloride, methotrexate, and the lucifer yellow are used as reference compounds. The US Pharmacopeia specifies the concentration tested ($C_0 = 0.3$ mM) and the Papp values to obtained (1.2×10^{-6} cm/s for methotrexate, 28×10^{-6} cm/s for propranolol, 73×10^{-6} cm/s for testosterone) [55].

Figure 11.4 shows the variations in quantity of different compounds in the acceptor compartment (basolateral) as a function of time. The results are expressed as a percentage of the initial amount at time zero in the donor compartment (apical). The slopes of linear regression curves obtained allow the calculation of various Papp (Fig. 11.4a). No significant difference was observed by comparing our values and those proposed by the US Pharmacopeia (Fig. 11.4b).

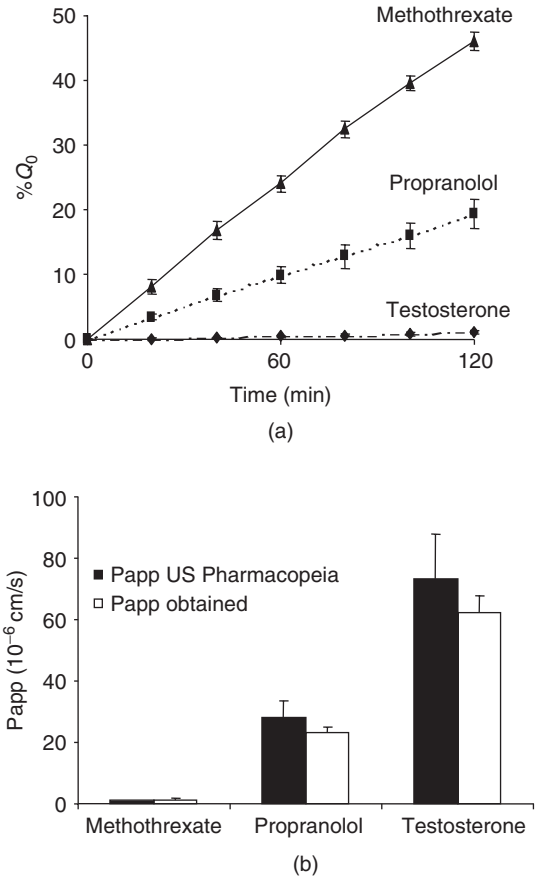

Figure 11.4 Passage study, across Caco-2 cell monolayer, of the reference molecules. (a) Evolution of methothrexate, propranolol hydrochloride, and testosterone concentrations in the acceptor compartment in function of time. Results are expressed as a percentage of the initial quantity (Q_0) in the donor compartment. (b) Comparison of apparent permeability (Papp) obtained with those proposed by the US Pharmacopeia. The results are expressed in 10^{-6} cm/s.

In summary, these results show that our culture conditions allow obtaining a fully differentiated cell monolayer.

11.7 CONCLUSION

Among the different models used to study the passage of the intestinal barrier and/or intestinal metabolism, the intestinal cell lines are the most used. Despite their faults, they are popular because of their ease of use, maneuverability, and ability to conduct studies that can last more than a few hours. However, the major problem that remains unresolved is the variability inter- and/or intralaboratory. In this section, some solutions are proposed, but they cannot solve the problem. Thus, the reference drugs proposed by the US Pharmacopeia are considered by some users as too rigid limits

and not accepted by all users. Another approach is the use of a collection of laboratory values, with internal specifications and criteria of acceptance for each assay system. The development of methods for isolation of enterocytes and goblet cells from human biopsies and culture techniques for maintaining these cells at an acceptable level of differentiation over several days is a goal that has likely to deserve further studies.

REFERENCES

1. Baumgart DC, Dignass AU. Intestinal barrier function. Curr Opin Clin Nutr Metab Care 2002;5:685–694.
2. Turner JR. Intestinal mucosal barrier function in health and disease. Nat Rev Immunol 2009;9:799–809.
3. DeSesso JM, Jacobson CF. Anatomical and physiological parameters affecting gastrointestinal absorption in humans and rats. Food Chem Toxicol 2001;39:209–228.
4. Garrison AP, Helmrath MA, Dekaney CM. Intestinal stem cells. J Pediatr Gastroenterol Nutr 2009;49:2–7.
5. Barker N, Van de Wetering M, Clevers H. The intestinal stem cell. Genes Dev 2008;22:1856–1864.
6. Shah P, Jogani V, Bagchi T, *et al*. Role of Caco-2 cell monolayers in prediction of intestinal drug absorption. Biotechnol Prog 2006;22:186–198.
7. Schwenk M. Mucosal biotransformation. Toxicol Pathol 1988;16:138–146.
8. Meier Y, Eloranta JJ, Darimont J, *et al*. Regional distribution of solute carrier mRNA expression along the human intestinal tract. Drug Metab Dispos 2007;35:590–594.
9. Fricker G, Drewe J, Huwyler J, *et al*. Relevance of p-glycoprotein for the enteral absorption of cyclosporin A: in vitro–in vivo correlation. Br J Pharmacol 1996;118:1841–1847.
10. Berggren S, Gall C, Wollnitz N, *et al*. Gene and protein expression of P-glycoprotein, MRP1, MRP2, and CYP3A4 in the small and large human intestine. Mol Pharm 2007;4:252–257.
11. Nakamura T, Sakaeda T, Ohmoto N, *et al*. Real-time quantitative polymerase chain reaction for MDR1, MRP1, MRP2, and CYP3A-mRNA levels in Caco-2 cell lines, human duodenal enterocytes, normal colorectal tissues, and colorectal adenocarcinomas. Drug Metab Dispos 2002;30:4–6.
12. Clatworthy JP, Subramanian V. Stem cells and the regulation of proliferation, differentiation and patterning in the intestinal epithelium: emerging insights from gene expression patterns, transgenic and gene ablation studies. Mech Dev 2001;101:3–9.
13. Van der Flier LG, Clevers H. Stem cells, self-renewal, and differentiation in the intestinal epithelium. Annu Rev Physiol 2009;71:241–260.
14. Peifer M. Developmental biology: colon construction. Nature 2002;420:274–277.
15. Fan MZ, Stoll B, Jiang R, *et al*. Enterocyte digestive enzyme activity along the crypt-villus and longitudinal axes in the neonatal pig small intestine. J Anim Sci 2001;79:371–381.
16. Chan LM, Lowes S, Hirst BH. The ABCs of drug transport in intestine and liver: efflux proteins limiting drug absorption and bioavailability. Eur J Pharm Sci 2004;21:25–51.
17. Galetin A, Gertz M, Houston JB. Potential role of intestinal first-pass metabolism in the prediction of drug-drug interactions. Expert Opin Drug Metab Toxicol 2008;4:909–922.
18. Lin JH, Chiba M, Baillie TA. Is the role of the small intestine in first-pass metabolism overemphasized. Pharmacol Rev 1999;51:135–158.
19. Kato M. Intestinal first-pass metabolism of CYP3A4 substrates. Drug Metab Pharmacokinet 2008;23:87–94.
20. Van Herwaarden AE, Van Waterschoot RA, Schinkel AH. How important is intestinal cytochrome P450 3A metabolism. Trends Pharmacol Sci 2009;30:223–227.

21. Suzuki H, Sugiyama Y. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. Eur J Pharm Sci 2000;12:3–12.
22. Sambruy Y, Ferruzza S, Ranaldi G, *et al*. Intestinal cell culture models: applications in toxicology and pharmacology. Cell Biol Toxicol 2001;17:301–317.
23. Galetin A, Houston JB. Intestinal and hepatic metabolic activity of five cytochrome P450 enzymes: impact on prediction of first-pass metabolism. J Pharmacol Exp Ther 2006;318:1220–1229.
24. Paine MF, Hart HL, Ludington SS, *et al*. The human intestinal cytochrome P450 "pie". Drug Metab Dispos 2006;34:880–886.
25. Zhang QY, Dunbar D, Ostrowska A, *et al*. Characterization of human small intestinal cytochromes P-450. Drug Metab Dispos 1999;27:804–809.
26. Engman HA, Lennernas H, Taipalensuu J, *et al*. CYP3A4, CYP3A5, and MDR1 in human small and large intestinal cell lines suitable for drug transport studies. J Pharm Sci 2001;90:1736–1751.
27. Raeissi SD, Guo Z, Dobson GL, *et al*. Comparison of CYP3A activities in a subclone of Caco-2 cells (TC7) and human intestine. Pharm Res 1997;14:1019–1025.
28. Raeissi SD, Hidalgo IJ, Segura-Aguilar J, *et al*. Interplay between CYP3A-mediated metabolism and polarized efflux of terfenadine and its metabolites in intestinal epithelial Caco-2 (TC7) cell monolayers. Pharm Res 1999;16:625–632.
29. Sun D, Lennernas H, Welage LS, *et al*. Comparison of human duodenum and Caco-2 gene expression profiles for 12,000 gene sequences tags and correlation with permeability of 26 drugs. Pharm Res 2002;19:1400–1416.
30. Fleet JC, Eksir F, Hance KW, *et al*. Vitamin D-inducible calcium transport and gene expression in three Caco-2 cell lines. Am J Physiol Gastrointest Liver Physiol 2002;283:G618–G625.
31. Van Breemen RB, Li Y. Caco-2 cell permeability assays to measure drug absorption. Expert Opin Drug Metab Toxicol 2005;1:175–185.
32. Carriere V, Lesuffleur T, Barbat A, *et al*. Expression of cytochrome P-450 3A in HT29-MTX cells and Caco-2 clone TC7. FEBS Lett 1994;355:247–250.
33. Gregory PA, Lewinsky RH, Gardner-Stephen DA, *et al*. Regulation of UDP glucuronosyltransferases in the gastrointestinal tract. Toxicol Appl Pharmacol 2004;199:354–363.
34. Peters WH, Roelofs HM. Time-dependent activity and expression of glutathione S-transferases in the human colon adenocarcinoma cell line Caco-2. Biochem J 1989;264:613–616.
35. Peters WH, Roelofs HM, Nagengast FM, *et al*. Human intestinal glutathione S-transferases. Biochem J 1989;257:471–476.
36. Peters WH, Kock L, Nagengast FM, *et al*. Biotransformation enzymes in human intestine: critical low levels in the colon. Gut 1991;32:408–412.
37. Scharmach E, Hessel S, Niemann B, *et al*. Glutathione S-transferase expression and isoenzyme composition during cell differentiation of Caco-2 cells. Toxicology 2009;265:122–126.
38. Galijatovic A, Walle UK, Walle T. Induction of UDP-glucuronosyltransferase by the flavonoids chrysin and quercetin in Caco-2 cells. Pharm Res 2000;17:21–26.
39. Meinl W, Ebert B, Glatt H, *et al*. Sulfotransferase forms expressed in human intestinal Caco-2 and TC7 cells at varying stages of differentiation and role in benzo[a]pyrene metabolism. Drug Metab Dispos 2008;36:276–283.
40. Munzel PA, Schmohl S, Heel H, *et al*. Induction of human UDP glucuronosyltransferases (UGT1A6, UGT1A9, and UGT2B7) by t-butylhydroquinone and 2,3,7,8-tetrachlorodibenzo-p-dioxin in Caco-2 cells. Drug Metab Dispos 1999;27:569–573.
41. Liu X, Tam VH, Hu M. Disposition of flavonoids via enteric recycling: determination of the UDP-glucuronosyltransferase isoforms responsible for the metabolism of flavonoids in intact Caco-2 TC7 cells using siRNA. Mol Pharm 2007;4:873–882.

42. Englund G, Rorsman F, Ronnblom A, *et al*. Regional levels of drug transporters along the human intestinal tract: co-expression of ABC and SLC transporters and comparison with Caco-2 cells. Eur J Pharm Sci 2006;29:269–277.
43. Maubon N, Le Vee M, Fossati L, *et al*. Analysis of drug transporter expression in human intestinal Caco-2 cells by real-time PCR. Fundam Clin Pharmacol 2007;21:659–663.
44. Hayeshi R, Hilgendorf C, Artursson P, *et al*. Comparison of drug transporter gene expression and functionality in Caco-2 cells from 10 different laboratories. Eur J Pharm Sci 2008;35:383–396.
45. Ahlin G, Hilgendorf C, Karlsson J, *et al*. Endogenous gene and protein expression of drug-transporting proteins in cell lines routinely used in drug discovery programs. Drug Metab Dispos 2009;37:2275–2283.
46. Giacomini KM, Huang SM, Tweedie DJ, *et al*. Membrane transporters in drug development. Nat Rev Drug Discov 2010;9:215–236.
47. Vachon PH, Beaulieu JF. Transient mosaic patterns of morphological and functional differentiation in the Caco-2 cell line. Gastroenterology 1992;103:414–423.
48. Markowska M, Oberle R, Juzwin S, *et al*. Optimizing Caco-2 cell monolayers to increase throughput in drug intestinal absorption analysis. J Pharmacol Toxicol Methods 2001;46:51–55.
49. Anderle P, Niederer E, Rubas W, *et al*. P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels. J Pharm Sci 1998;87:757–762.
50. Goto M, Masuda S, Saito H, *et al*. Decreased expression of P-glycoprotein during differentiation in the human intestinal cell line Caco-2. Biochem Pharmacol 2003;66:163–170.
51. Shirasaka Y, Kawasaki M, Sakane T, *et al*. Induction of human P-glycoprotein in Caco-2 cells: development of a highly sensitive assay system for P-glycoprotein-mediated drug transport. Drug Metab Pharmacokinet 2006;21:414–423.
52. Briske-Anderson MJ, Finley JW, Newman SM. The influence of culture time and passage number on the morphological and physiological development of Caco-2 cells. Proc Soc Exp Biol Med 1997;217:248–257.
53. Lentz KA, Hayashi J, Lucisano LJ, *et al*. Development of a more rapid, reduced serum culture system for Caco-2 monolayers and application to the biopharmaceutics classification system. Int J Pharm 2000;200:41–51.
54. Le Ferrec E, Chesne C, Artusson P, *et al*. In vitro models of the intestinal barrier. The report and recommendations of ECVAM Workshop 46. European centre for the validation of alternative methods. Altern Lab Anim 2001;29:649–668.
55. USP. Pharmacopeial forum. US Pharmacopeia 1998;24:6015–6023.
56. Shin HC, Kim HR, Cho HJ, *et al*. Comparative gene expression of intestinal metabolizing enzymes. Biopharm Drug Dispos 2009;30:411–421.
57. Tamura HO, Taniguchi K, Hayashi E, *et al*. Expression profiling of sulfotransferases in human cell lines derived from extra-hepatic tissues. Biol Pharm Bull 2001;24:1258–1262.