Application of Caco-2 Cell Line in Herb-Drug Interaction Studies: Current Approaches and Challenges

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ABSTRACT - The Caco-2 model is employed in pre-clinical investigations to predict the likely gastrointestinal permeability of drugs because it expresses cytochrome P450 enzymes, transporters, microvilli and enterocytes of identical characteristics to the human small intestine. The FDA recommends this model as integral component of the Biopharmaceutics Classification System (BCS). Most dedicated laboratories use the Caco-2 cell line to screen new chemical entities through prediction of its solubility, bioavailability and the possibility of drug-drug or herb-drug interactions in the gut lumen. However, challenges in the inherent characteristics of Caco-2 cell and inter-laboratory protocol variations have resulted to generation of irreproducible data. These limitations affect the extrapolation of data from pre-clinical research to clinical studies involving drug-drug and herb-drug interactions. This review addresses some of these caveats and enumerates the plausible current and future approaches to reduce the anomalies associated with Caco-2 cell line investigations focusing on its application in herb-drug interactions.

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INTRODUCTION

Oral administration of drugs and xenobiotics is the most convenient route of drug administration (1,2). Oral drug absorption is complex; characterized by multiple challenges including the inherent characteristics of the formulation, the physiological conditions and the physicochemical properties of the drugs (3). Additionally, intestinal enterocytes present a well-structured defensive system to modulate entry of drugs and xenobiotics from gut lumen into the systemic circulation. Metabolism and transport of drugs across intestinal membrane are therefore multifaceted and dynamic process involving both passive and active transport mechanisms (Figure 1). Hence, transmembrane absorption is generally recognized as a basic condition to ensure efficient systemic availability of drugs via the gastro-intestinal tract.

Research scientists and pharmaceutical companies employ cell-based assays such as Caco-2 cells and Mardin-Darby canine kidney (MDCK); artificial lipid-based systems including parallel artificial membrane permeability assay (PAMPA) as preclinical high throughput screening models for evaluation of intestinal permeability of drugs and herbal supplements (4,5,6,7). The Caco-2 cell line derived from human colon adenocarcinoma is considered the most common _in vitro_ model used for investigation and prediction of intestinal drug absorption (8). It undergoes spontaneous enterocytic differentiation in appropriate culture to become polarized cells expressing apical and basolateral surfaces with well-established tight junctions. The polarized cells depict several functions of normal enterocytes including expression of brush border enzymes, some cytochrome (CYP) isoenzymes and phase II enzymes (9). The model is also employed in identification of substrates and/or inhibitors of drug transporters (10). Caco-2 cell line is used for screening of conventional drugs and new chemical entities for potential drug-drug interactions. However, its application in most dedicated laboratories and research institutions for screening herbal medications for likely herb-drug interactions is gaining popularity.

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In recent years, consumption of herbal remedies either in combination with conventional drugs or alone has become a common practice in patients suffering from chronic diseases such as HIV/AIDS and cancer (11,12). Unfortunately, most physicians are unaware of this habit which may likely cause therapeutic failure and/or toxicity due to high propensity of such individuals experiencing herb-drug interactions (13).

Currently, studies on herb-drug interactions have increased due to the awareness of the possible threats it may pose to both patients and healthcare providers in an attempt to achieve optimum therapeutic objectives. The pharmacokinetic herb-drug interactions are mostly attributed to inhibition or induction of drug metabolism enzymes and transporters or renal drug clearance. The use of Caco-2 cell line to screen herbal remedies for possible herb-drug interactions may reduce the probability of associated therapeutic failure, adverse effects, and cost of treatment and withdrawal of herbal products from the market.

Although most researchers and pharmaceutical industries prefer Caco-2 as an in vitro model for investigation of intestinal drug absorption, mixed reports have been published regarding its in vivo correlation with humans (14). The deficiencies associated with Caco-2 cell line as a preclinical model for prediction of intestinal permeability may yield inaccurate results in herb-drug interactions investigations. This review aims to appraise the utilization of Caco-2 cell lines in preclinical studies, address some of the associated limitations and the emerging approaches to resolve these problems. The emerging approaches addressed in this review may be beneficial to minimize current and future challenges likely to undermine the application of Caco-2 cell line for herb-drug interaction studies.

Search strategy and selection criteria

The review was conducted systematically by searching the databases of EBSCOHOST, MEDLINE, PUBMED, EMBASE, GOOGLE SCHOLAR, and COCHRAINE libraries for original researches that include the following combination search terms: "Caco-2 cell line", "drug permeability and/or absorption", "herb-drug interactions", "pitfalls and current approaches". The literature searches were limited to publications that are available online in English language. There was no limitation to dates of publication nevertheless in cases of recurrence of search results, the most recent was preferred.

Mechanisms of intestinal drug absorption

Absorption of herbal supplements and drugs mainly occurs at the duodenum and jejunum segments of small intestine due to high concentrations of villi and microvilli which provide a large surface area (15, 16, 17, 18). The epithelial cells in these regions are heterogeneous with enterocytes responsible for the absorption of most pharmacological agents. The enterocytes are polarized with distinct apical and basolateral membranes separated by tight junctions. This architectural composition of polarized enterocytes restricts the passage of drugs and xenobiotics through either passive diffusion or carrier-mediated processes (19). The large surface area serves as a conduit for efficient permeation of drugs through various mechanisms including passive and active pathways. Passive diffusion encompasses two routes: the paracellular pathway which facilitates permeation of hydrophilic drugs of low molecular weight and peptides via aquaporins at the tight junctions between the enterocytes; and the transcellular pathway responsible for movement of lipophilic drugs across lipid cell membrane of the enterocytes. Presence of tight junctions in the intercellular space limits the free flow of compounds traversing the intestinal epithelium via the paracellular process (20). The carrier-mediated transport processes involve both facilitated and active mechanisms engineered by transport proteins responsible for uptake or efflux of drugs in the intestine as shown in figure 1 (21,22, 23).

Uptake and efflux transporters are localized at both the apical side and basolateral membranes of the intestinal enterocytes. Efflux transporters commonly expressed in the intestine membranes include P-glycoprotein (P-gp), multidrug resistance proteins (MRP 1–6), and breast cancer resistance protein (BCRP). These are members of the ATP-binding cassette (ABC) transport proteins which utilise ATP as energy source to transport substrates against a concentration gradient (24,25,26) from the cytoplasm of intestinal cells back to the intestinal lumen or to the blood.

Primary active efflux transporters notably P-gp, MRP2, and BCRP are well expressed on brush-border apical membrane of the enterocytes and reported to form major hindrance to intestinal absorption of drugs (27,28). (29,30).
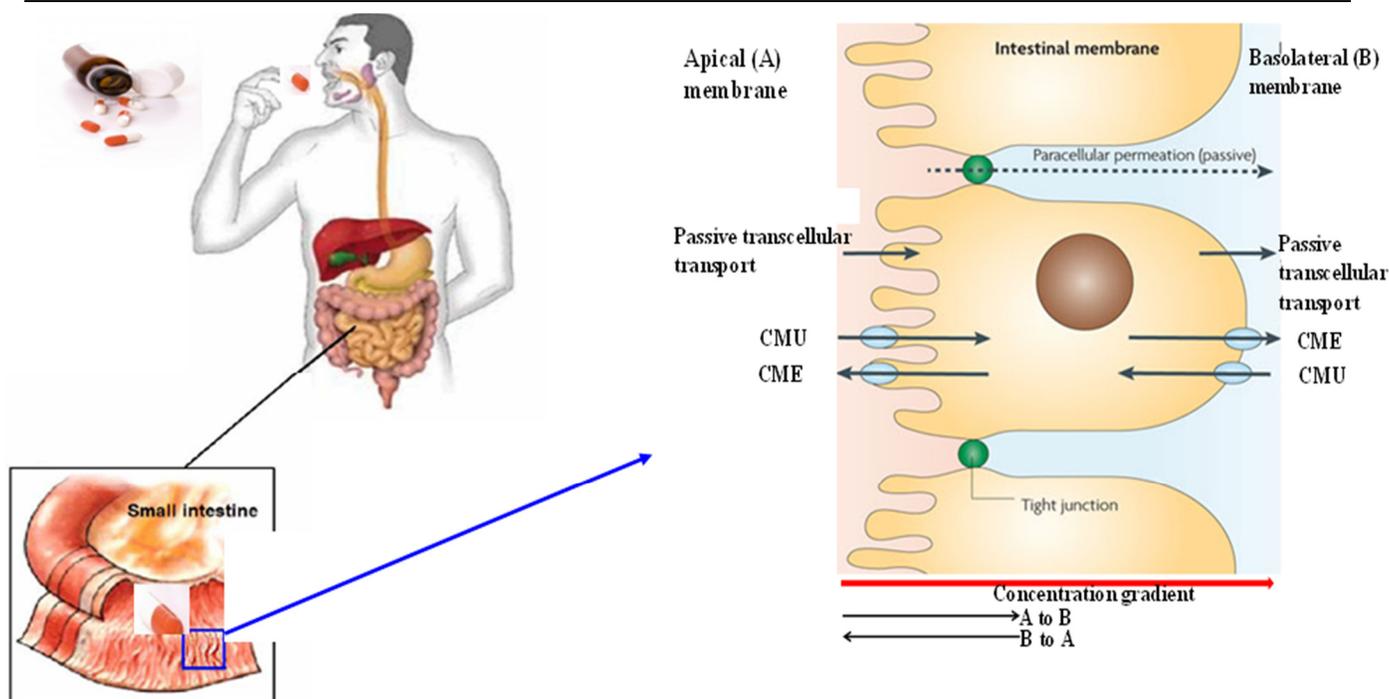
Figure 1. Pathways of drug absorption through intestinal enterocytes modified from Sugano et al. (27): carrier-mediated uptake (CMU), carrier-mediated efflux (CME), absorptive direction (A to B) and excretive direction (B to A).

These have the tendency to reduce oral bioavailability of specific drugs by expulsion from the enterocytes into intestinal lumen. P-gp is a well-recognized efflux transporter which influences absorption and excretion of several drugs due to its wider range of substrate specificity (31). BCRP is capable to extrude glucuronides and sulfate conjugates formed in the enterocytes into the lumen, although its main function relates to excretion of drugs and xenobiotics into breast milk and a defensive role at the placental barrier (32). MRP2 plays an important role in detoxification of drug and xenobiotics which have undergone phase II metabolism to facilitate excretion (33,34). Unlike efflux transporters, apical membrane uptake transporters increase absorption of drugs from the lumen into the enterocytes (35,36). Finally, receptor-mediated endocytosis could also play a role in drug absorption.

Uptake transporters e.g., peptide transporter (PEPT), organic anion transporter (OAT), organic anion transporting polypeptide (OATP), organic cation transporter (OCT) are members of solute carrier (SLC and SLCO) superfamilies and function independent of ATP but rather transport drugs according to their concentration gradient (Figure 2).

In the Caco-2 cell permeability assessment of drugs and herbal supplements, adequate expression of transport proteins can be utilized to predict the possible degree of bioavailability upon co-administration (37). Most transporters aforementioned can be expressed in Caco-2 cell under appropriate culture media. Table 1 below describes the uptake and efflux transporters that are likely to be expressed in the Caco-2 cell line. Despite the possibility of expression of most transporters in the Caco-2 cell, some transporters like hPEPT1 are poorly developed except under modified conditions such as tease out or molecularly engineered manipulations. Few of these limitations will be subsequently discussed in detail.

Caco-2 cell line
The Caco-2 cell line is the most common and extensively characterized cell-based model for the assessment of absorption of drugs via the intestinal membrane enterocytes (38, 39, 40).
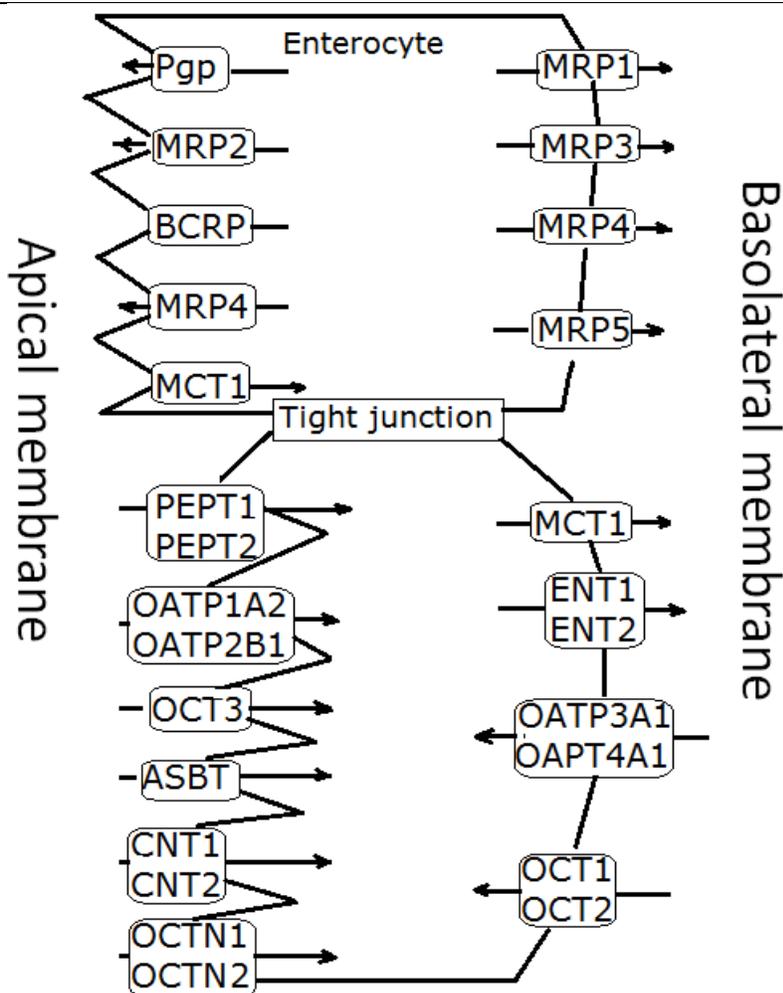
Figure 2. Localization of ABC and SLC transporters in human small intestine adapted from Smetanová et al. (38)

It is derived from heterogenous human epithelial colorectal adenocarcinoma cells, developed through research by Jorgen Fogh (1975) at Sloan-Kettering Institute for Cancer Research (41). The Caco-2 cells have the intrinsic ability to initiate spontaneous differentiation on reaching confluence in the presence of normal culture conditions. At confluence, there is progressive development of brush border. The surface occupied by each cell gradually reduces from 5 to 20 days post-confluence and intimate intercellular junctions are developed. During the same period, the length and density of microvilli increase. The Caco-2 cells form well - developed tight junctions after 21 days of culturing. At day - 30 post-confluence, the cell surface reaches minimum with typical enterocytes-like morphology. Most cells develop complete brush borders with tall and regular microvilli when the full structural polarization is achieved after 30 days (42) . The complete polarized Caco-2 cells resemble human small intestinal mucosa cells expressing brush borders, tight junctions and, efflux and uptake transporters at both apical and basolateral compartments.

The Caco-2 cell line fairly represent intestinal biochemical barrier due to expression of membrane efflux proteins (P-gp, MRP 1-3), CYP450 isoenzymes and phase II conjugating enzymes such as sulfotransferase, UDP-glucuronyltransferase, glutathione S-transferase (43,44) . Table 2 summarizes the characteristics of a parental Caco-2 cell line. Permeability of substrate via the Caco-2 cell monolayer has proven to correlate closely with oral absorption in humans compared with other parental cell-based models. Currently, Caco-2 monolayers are applied for screening the potential effects of herbs and new chemical entities (NCE) on absorption of drugs.

Table 2. Characteristics of Caco-2 cell line

Parameter	Description
Origin	Human colorectal adenocarcinoma
Growth in culture	Monolayer epithelial cells
Differentiation	14-21 days after confluence in standard culture medium
Morphology	Polarised cells, with tight junctions, apical brush border
Electrical resistance	High transepithelial electrical resistance (TEER)
Digestive enzymes	Typical membranous peptidases and disaccharidases of the small intestine
Active transport	Amino acids, sugars, vitamins, hormones
Membrane ionic transport	Na^+/K^+ ATPase, H^+/K^+ ATPase, Na^+/H^+ exchange, $Na^+/K^+/Cl^-$ co-transport, apical Cl^- channels

Comparison of Caco-2 with other cell-based models for intestinal permeability studies

The Madin-Darby canine kidney (MDCK) is considered as an alternative to Caco- 2 cell for permeability studies. The MDCK cell line is derived from the normal dog kidney. When cultured in an appropriate media, MDCK differentiate into columnar epithelial cells with clear tight junctions similar to Caco-2 formed within on a semi-permeable membrane. The differentiated MDCK cell maintains characteristics of kidney epithelial cells such as asymmetric distribution of enzymes and vectorial transport of sodium and water from the apical to the basolateral membrane (45). The MDCK has a shorter cultivation period (3 - 5 days) compared to 21 days of Caco -2 cell line. Furthermore, the transepithelial electrical resistance (TEER) of MDCK cells is lower and closer to that of small intestine compared to Caco-2 cell. This often results in a higher permeability coefficient of hydrophilic compounds in MDCK cells than Caco-2 cell. The shorter culture duration is advantageous since it reduces cost, time, and enhances high throughput. The MDCK cell line appears to be more robust and need less rigorous nutritional schedule over a short incubation period relative to Caco-2 cell. However, these cells are derived from dog kidney and therefore there is a high propensity of differences in the expression levels of some transporters and metabolic activity as compared to Caco-2 cell (46).

LLC-PK1 cells derived from pig kidney epithelial have also been employed as an alternative model to Caco-2 cells for assessing permeability of test compounds. Studies have reported the use of LLC-PK1 for characterization of passive absorption of test compounds (47,48,49).

The human adenocarcinoma HT29-MTX model is used to investigate the role of intestinal mucus on drug absorption across the intestinal barrier. The HT29-MTX is conditioned to acquire the morphological and mucin producing features of goblet cells by culturing parental HT29 in a medium containing 10^{-6}M methotrexate for six months (50,51). Unlike Caco-2, HT29-MTX develops sparse microvilli on the apical side and reaches confluence 3 days later than the former. However, expression of goblet cells in HT29-MTX increases absorption of lipophilic compounds compared to Caco-2 monolayer.

TC-7, a subclone of Caco-2 cells, is also used for permeability screening of tests compounds. The TC-7 clone displays morphological features of brush-border membrane, microvilli and tight junctions similar to Caco-2 monolayer (19). There is a good correlation of passive transcellular absorption of compounds through TC7 and the parental Caco-2 cell monolayer comparable to the extent of permeability in humans. The TC-7 model therefore offers an alternative to Caco-2 for intestinal permeability assessment of test compounds. In addition, TC-7 has an advantage over Caco-2 by expressing high levels of CYP3A4 enzymes well represented in the intestine. However, TC-7 unlike Caco-2 cell lacks transport proteins and therefore its application is skewed towards metabolism of drug employing CYP3A4.

2/4/A1 originates from fetal rat intestine and is considered to mimic intestinal passive paracellular permeability in humans better than Caco-2 monolayer (52,53). This immortalized cell is reported to differentiate monolayers with tight junctions, brush-border membrane enzymes and transporter proteins. Unlike, Caco-2, tight junctions expressed in 2/4/A1 are loose and better for studies of compounds absorbed in the human intestine via the paracellular route. The model has been proven to

be excellent for prediction of poorly absorbed compounds such as mannitol and creatinine better than Caco-2 but comparable to that of human jejunum (52,53). Table 3 summarises the comparison of Caco-2 with other cell-based models for intestinal permeability analysis.

Generalized Caco-2 cells study protocol
Caco-2 Cell Culture
Caco-2 cells are seeded in standard cell culture medium (consisting of Dulbecco's modified Eagle's medium supplemented with fetal bovine serum, nonessential amino acids, penicillin, and streptomycin) onto 24 transwell membrane inserts at specified cells concentration under a temperature of $37^{o}C$ in an atmosphere of 5% CO_2 and 90% relative humidity. The cell monolayers are employed for transport studies 15 - 21 days after seeding between different passages (54).

Cell viability
Caco-2 cells are seeded onto Petri dishes (area 9.2 cm^2) at a density of 2×10^5 cells/cm^2 and cultured for 24 h. Investigated compound or transport medium (control) is added and incubated for 2 h. The cells are then released from the dishes and incubated with 0.4% Trypan blue for 5 min and counted under a microscope (55).

Assessment of monolayer integrity
The integrity of Caco-2 cell monolayer can be determined by monitoring the paracellular permeability of ion and hydrophilic compounds using TransEpithelial Electrical Resistance (TEER) and ^{14}C-mannitol respectively after the experiment. Mannitol is water soluble, cell membrane impermeable and non-ionisable small molecule with molecular weight of 182 g/mol and radius 4.1 nm. The low permeability of mannitol (<0.5% per hour) is used as a criterium to determine integrity of the Caco-2 monolayer. It is imperative to assess the integrity of the monolayer with ^{14}C-mannitol after the actual permeability experiment or during the permeability experiment in the absence and presence of test compounds and control. TEER provides faster and reliable alternative method of assessing Caco-2 cell monolayer intensity. The TEER of monolayer is measured post equilibrating the monolayer in a transport buffer with ERS Voltmeter. The integrity of the monolayer is confirmed by measuring the TEER after each permeability experiment (56).
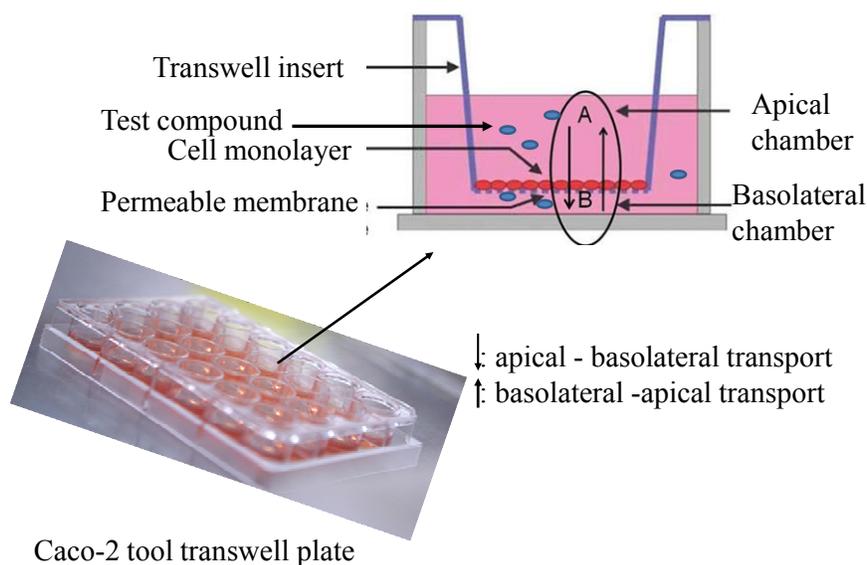

↓: apical - basolateral transport
↑: basolateral -apical transport

Figure 3. Caco-2 cell in transwell plate (57**)**

Bidirectional transport assay

Prior to all experiments, the inserts (figure 3) are washed twice and equilibrate with pre-warmed transport medium at 37^oC for 30 min. The transport experiment is initiated by addition of diluted probe substrate (positive control) in the presence or absence of the test substrate to either the apical (for apical to basolateral transport; AP - BL) or basolateral (for basolateral to apical transport; BL - AP) side of the monolayer. The monolayers are then placed in an incubator at 37°C. The samples are withdrawn from either compartment at different time intervals (e.g. 30, 60, 90 and 120 min) for H PLC analysis or liquid scintillation spectrometry (58,59,60).

Data analysis of transport

The apparent permeability coefficient, Papp (cm/s) is determined as:

$$p_{app} = \left(^{\delta Q}/_{\delta t}\right) \times (^1/_A \times \quad c_o)$$

Where $^{\delta Q}/_{\delta t}$ is the permeability rate, the amount of drug appearing in the receiver as a function of time (nmol/s), C_o is the initial concentration in the donor chamber (nmol/ml), and A is the surface area of the monolayer (cm^2) as published by Engdal and Nilsen (37).

The direction of probe substrate transport in the monolayer can be determined as passive or active using the Papp uptake ratio:

$$P_{app} \text{uptake} \quad \text{ratio} = P_{app}\left(\frac{AP\text{-}BL}{BL\text{-}AP}\right)$$

The net substrate flux can be calculated as the difference between the B–A and the A–B flux, J:

$$JNet = J(B-A) - J(A-B)(nmol\ cm^{-2}h^{-1})$$

Where: J= P_{app}*$C_o$$(nmol\ cm^{-2}h^{-1})$

Percentage inhibition is calculated according to:

$$\%\text{inhibition} = \left(100 - \frac{JNet,\ inhibition}{JNet,\ control}\right) \times 100$$

Evidence of herb-drug interactions in Caco-2 cell model at transporter level

The Caco-2 cell model is capable of expressing intestinal efflux and uptake transporters which regulate permeation of drugs, and herbs from intestinal lumen to systemic circulation. Herbal remedies contain numerous phytochemical compounds with functional groups identical to those found in conventional drugs. The availability of compounds with similar functional groups in both herbal medicines and drugs causes substrate overlap at receptor sites to generate herb-drug interactions when co-administered (61,62). Clinical studies have reported impact of herb-drug interactions on disposition of therapeutics in patients and healthy individuals. For example, the most common implicated herbal medicine, St. John's wort (SJW) is reported to reduce bioavailablity of different drugs including digoxin (63, 64,65), amitriptyline (66), oral contraceptives (ethinyl estradiol and norethindrone) (67), or cyclosporine (68,69, 70, 71, 72, 73, 74, 75, 76) . The widespread effect of SJW on drugs for management of diverse pathological conditions has been attributed to the induction of P-gp by a bioactive compound hyperforin, modulating the expulsion of these drugs from enterocytes into the intestinal lumen. The Caco-2 cell line is used as a pre-clinical model to predict the possibility of herb-drug interactions ex *vivo* subsequent to clinical trials.

The prediction of drug-drug and herb-drug interactions involving drug transporters is inferred from pre-clinical evidence from cell lines and animal models. In vitro studies have shown that, green tea, garlic and milk thistle extracts inhibit activity of P-gp (77,78,79) whilst genisten, a bioactive compound in soybean, inhibits activities of both P-gp and MRP2 (80, 81) . In vitro study conducted in human embryonic kidney (HEK) 293 cells stably expressing OATP-B (HEK/OATP-B cells) employing extracts of bilberry, enchinacea, green tea, banana, grape seed, gingkgo and soybean produced significant inhibition of OATP-B mediated uptake of estrone-3-sulfate (82).

In a recent study, 50 ml of ''untreated'' Red Yeast Rice aqueous extract (RYR) of a concentration 0.5 mg/ml were added into the apical compartment of Caco-2 tissue culture plates in the presence of verapamil as a P-gp probe substrate. The study showed a 2-fold increase in the net absorption of verapamil from apical membrane to the basolateral compartment, an indicative of a potential inhibitory

effect of RYR on intestinal P-gp. A further study was conducted in intact animal to confirm the result from caco-2 cell line. The result showed 30 minutes post-administration of verapamil in pre-treated "untreated" RYR rat models produced a 2-fold plasma concentration of P-gp probe substrate compared to the control which concurred with the outcome of the in vitro study (83).

Ginseng is one of the most recognised traditional Chinese medicines reported to exhibit anticancer effect due to presence of 20(S)-Ginsenoside Rh2 [20(S)-Rh2], a trace element in gingseng (84 ,85) . In 2010, a study conducted by Zhang and others revealed that presence of 20(S)-Rh2 significantly reduced transport of three classical P-gp probe substrates; digoxin, fexofenadine, or etoposide across Caco-2 monolayers in basolateral to apical direction but increased digoxin or etoposide transport in the apical to basolateral direction. The result reveals that 20(S)-Rh2 inhibits efflux activity of P-gp whilst uptake transporters in the apical membrane are induced which could result in potential systemic toxicity of the substrates. Further study was conducted in Sprague-Dawley rats intragastrically treated with 20(S)-Rh2 in the presence of the three P-gp probe substrates as a follow-up investigation of the in vitro result. The outcome of this in vivo study revealed an increase in AUC and C_{max} for all the substrates without any impact on their terminal elimination half-life. This suggests that the bioactive compound increase absorption of the substrates by inhibiting P-gp similar to that observed in the in vitro study (86).

Oga and co-workers (2012) investigated the impact of herbal medicines used for treatment of malaria on P-glycoprotein mediated efflux in Caco-2 cell monolayer in the presence and absence of digoxin (87). Their investigation showed a significant inhibition of digoxin from basolateral to apical membrane of Caco-2 cell monolayer when extracts from *Vernonia amydalina*, *Carica papaya* and *Tapinanthus sessilifolius* were independently applied. Thus, these herbal medicines may increase bioavailability of antimalaria drugs once concomitantly administered. The increase systemic circulation of antimalaria drugs could become toxic to the liver (88).

Thus concomitant administration of herbs and drugs which are substrates for P-gp may either cause therapeutic failure and pre-systemic toxicity due to induction of the transporter or systemic toxicity as a result of inhibition of the efflux transporter. The role of in vitro cell culture systems in preclinical studies involving HDI therefore cannot be overemphasized. The Caco-2 cell is therefore often employed to assess functions of intestinal membrane transporters especially P-gp during HDI studies since most herbs are consumed orally.

Common Pitfalls in Caco-2 Assays
Variations in passive and carrier-mediated transports
Pharmaceutically relevant uptake transport proteins (e.g. peptide transporters, organic cation transporter and OAT) are under-expressed in Caco-2 monolayer compared to the level of expression in vivo (89,90,91). Studies have demonstrated poor permeability of β-lactam antibiotics (e.g. cephalexin and amoxicillin) and ACE inhibitors across Caco-2 cell monolayer although these dipeptide transporter substrates are completely absorbed in vivo (92) . Also, it has been shown that there is significant variation of mRNA expression levels of efflux transporters in Caco-2 cell monolayer compared to small and large intestines in humans. For example, the transcription of BCRP (ABCG2) is about a 100-fold lower in Caco-2 cells compared with jejunum (99,93 ,94). The differences in transporter proteins expression in Caco-2 cell monolayer generate false negative findings that may not correlate with in vivo studies.

The source of Caco-2 cell and inter-laboratory variations in protocol design contribute to discrepancies in paracellular permeability of compounds in the monolayer. Studies have shown that, the source of Caco-2 cell could provide a 100-fold variation in the permeability of mannitol (95) and 20-fold differences in TEER due to different culture conditions and composition of cell subpopulation (30). Inter-laboratory differences in TEER values influence the estimation of passive transport of compounds since the TEER value is used to determine the expression of tight junction. For instance, the low molecular weight hydrophilic compounds (e.g. metformin, ranitidine, atenolol, furosemide or hydrochlorothiazide) are well-absorbed in humans. However, poor permeability of these compounds has been observed in Caco-2 cell monolayer similar or even less than that of mannitol. This drug transport abnormality poses a problem to properly predicting the in vivo-in vitro correlation of

data from different laboratories due to variable outcomes.

Low expression of CYP3A4

Intestinal CYP3A4 plays a vital role in first pass metabolism of drugs and herbs. An ideal cell–based model for intestinal permeability studies should mimic the human gastrointestinal enterocytes by expressing metabolic enzymes together with the lipid bilayer interspersed with transporters and tight junctions to give a fair representation of the inter-play between CYP3A4 and P-gp. The Caco-2 cell monolayer fails to simulate in vivo intestinal environment due to low expression of CYP3A4 which is considered as the main drug-metabolizing enzyme in the human epithelial cells.

Loss of compounds - Membrane retention and non-specific binding

Membrane retention also called cacophilicity refers to the tendency of a drug to reversibly bind to Caco-2 cells causing incomplete recovery and inaccurate data interpretation. Incomplete recovery is especially common with lipophilic drug candidates. Several factors including: the amount of drugs that appeared in the receiver compartment, the initial concentration in the donor compartment, and the surface area of the physical barrier (e.g, lipid bilayers and cell monolayer) affect permeability of drugs. The drug can also non-specifically bind to plastic devices (transwell plates), reducing the concentration of drug in the donor and receiver compartments during incubation period and therefore leading to underestimation of permeability estimates, and false negatives. Non-specific binding can also occur during transfer of cultured sample together with the drug into another container. Few approaches may be helpful to reduce this problem. The final donor concentration may be preferred to initial donor concentration at the termination of incubation since the non-specific binding occurs relatively faster. The second approach involves addition of bovine serum albumin to the receiver compartment to minimize non-specific binding of lipophilic drugs, thus, improve the assay recovery and overall predictability (96,97,98). Application of organic solvent such as acetonitrile into containers ready to receive transferred cultures has also been helpful to reduce loss of compounds due to non-specific binding to plastic devices.

Lack of adequate solubility

The permeability assessment of test compounds in Caco-2 is underestimated due to poor aqueous solubility. Organic cosolvent such as DMSO and propylene glycol are added to the donor compartment to avert this problem. However, the Caco-2 cell monolayer can tolerate these organic solvent at a concentration ~1 - 2% v/v. Beyond this concentration, the cell tight junction is compromised and renders the monolayer impracticable. However, some solvents such as Tween 80, cremophor and pluronic which are known inhibitors of efflux transporters must be avoided (99,100,101). Intestinal pH variations from the upper small intestine to the distal large intestine determine the nature of compounds absorbed at a specific region. For example, weak acidic drugs remain unchanged in the upper small intestine due to high pH, therefore passive transcellular transport emerge as the dominant route of absorption into the circulation. Caco-2 studies are incapable of simulating the changes of intestinal pH system since it is performed at constant pH conditions (apical pH of 6.5 and basolateral pH of 7.4). Any pH modification in design of Caco-2 cell protocol may affect its cyto-architecture and underestimate the permeability characteristics.

Other experimental variability

Different laboratories operate under diverse experimental conditions such as culturing, passage number and culture duration which account for variations in permeability values. The inter-laboratory variations affect the expression levels of efflux transporters which depend on the age of the cell cultures (19). The Caco-2 subclones expressing high levels of efflux transporters are currently preferred to establish structure-transport relationship for efflux transporters. Few challenges have been encountered during analysis of samples using various instruments such as HPLC or LC-MS. These instruments are expected to produce accurate results due to their sensitivity. However, presence of high salt component in transport buffer (e.g. HBSS) interferes with ionization and hence complicates development of steady LC-MS methods.

Current and future approaches

Quality high throughput devices are employed by dedicated laboratories for screening large quantities of new drugs. Most of these new devices are

developed through integration of advance knowledge in molecular biology and combinatorial computer into conventional in vitro models. A few of these recent developments in Caco-2 cell permeability assay which may eventually replace the conventional techniques in the coming years are discussed below.

Automation and Robotic systems

Cell culturing requires several hours of repetitive, painstaking manual procedures in order to maintain absolute sterility. Additionally, maintenance of favourable environmental conditions via the cell culture cycle; growth, harvesting, re-seeding and analysis are important. Cell growth therefore demands careful regulation of several factors in an attempt to achieve viable and consistent Caco-2 cell lines. In conditions where large quantities of permeability assay of compounds are analysed, there is a high propensity of cell contamination. This difficulty has propelled pharmaceutical companies to embrace the use of automated Caco-2 cell culture system. An automated Caco-2 system can be employed to study 200-500 compounds within a month without any increase in the cost of operation.

Robotic systems are capable of performing both the cell culture and high throughput screening assay of drug candidates. Most robotic systems perform multi-purpose tasks such as plate coating, seeding and cell feeding. The robotic system uses key components (articulating arms and plate carousel) to automate the cell culture procedure. The articulating arms hold plates at different angles allowing the user to dispense media on the wall of the plate, reducing possible cell or membrane damage (19).

Ready-to-use Caco-2 models

Modified Caco-2 cell models which are ready for permeability analysis within culturing time of 3-7 days have been produced by dedicated laboratories to curtail the long period associated with parental culture procedure. Some of these modified models are currently used for permeability analysis of new drugs. Typical example of modified models, Biocoat™ HTS Caco-2 Assay System promotes rapid differentiation of Caco-2 cells. The system is accompanied by a specialized media formulation that facilitates differentiation of the cell monolayer within three days. Major pharmaceutical industries globally now depend on Biocoat™ HTS Caco-2 Assay System for oral permeability evaluation of new drugs. CacoReady™ is another innovative cell-based and ready to use kit for intestinal permeability analysis. The kits composed of 24 and 96 insert-integrated permeable supports seeded with differentiated and polarized Caco-2 barriers on polycarbonated microporous filters. A comparative study to assess permeability efficient of parental Caco-2 and Biocoat™ HTS Caco-2 assay system using parabens; a preservative commonly used in food, cosmetics and pharmaceutical industries showed better absorption in ready-to-use Caco-2 as illustrated by figure 4 (102). Ready-to-use Caco-2 cell has several advantages over parental Caco-2: high flexibility, time saving and cost effective, easy to set up, high reproducibility and adaptable to automated procedures and high throughput screening. However, low expression of enzymes and transporters has been reported due to inadequate duration for culturing (103,104).

Transfected Caco-2 cell line

The advances made in modern molecular biology and genetic information are exploited fully to enhance functional expression of transporters and CYP using Caco-2 cell lines. The expression level of specific transporters in Caco-2 may be manipulated either transiently or stably according to the preference of the researcher (105). Construction of transfected Caco-2 cell line is relevant to transporters such as PEPT1 that cannot be expressed under normal culture conditions. In a study conducted to characterized the cellular uptake mechanism of amino acid ester prodrugs of nucleoside antiviral drugs in the transiently transfected Caco-2 cells overexpressing a human intestinal peptide transporter, hPEPT1 (Caco-2/hPEPT1 cells), the transfected cell line produced five times higher uptake than parental Caco-2 (106) . However, induction of efflux transporters is often easier and well-expressed in parental Caco-2 compared to transfected cells. Hence, transfection of efflux transporters in Caco-2 cell is not recommended.

The Caco-2 cell line can also be transfected with extra-chromosomal CYP vectors. Caco-2 cells transfected with CYP3A4 have been successfully employed to investigate the interplay of CYP enzymes and efflux pumps at the intestinal epithelium (107). Nevertheless, transfected Caco-2 harbouring extra-chromosomal CYP have been withdrawn from the market due to its lack of long-term stability.
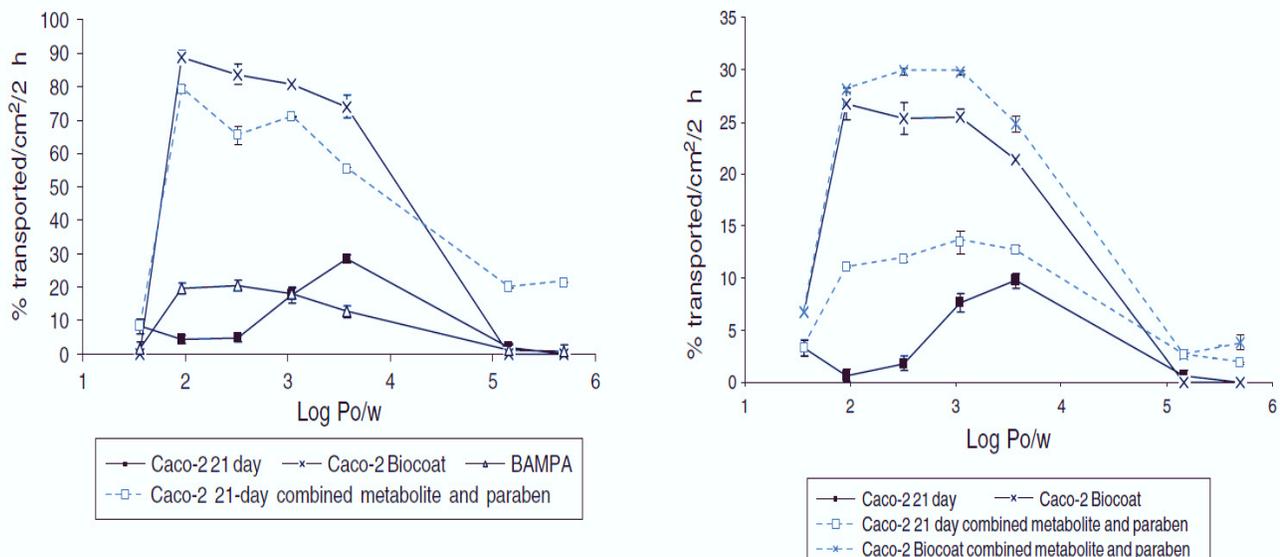
Figure 4. comparative permeability assessment between parental and ready-to-use Caco-2 models using paraben adopted from Lakeram et al. (110).

Molecular biology technology has been used to modulate the expression of nuclear receptors that are principal regulators of gene expression for CYP enzymes. Caco-2 cells transfected with human PXR and HNF4 genes showed an increased expression of both nuclear receptors under CYP3A4 promoter control (108).

The functional expressions of efflux and uptake transporters in Caco-2 monolayer depend on the passage number and other inter-laboratory conditions. These variations have resulted in inconsistent expression of drug transporter during permeability assays. In recent years, dedicated laboratories have introduced engineered cell lines which explicitly over-express a specific efflux or uptake transporter for better examination of drug-transporter interactions. The newly constructed cell line selectively express transporter of interest and enhance interaction study with a specific transporter in isolation. Bidirectional investigations conducted in engineered Caco-2 cell line are devoid of interference from other transporter since only a specific transporter is teased out. The functional expression of transporters in transfected cell line appears more stable than parental Caco-2 cell.

Knockdown Caco-2 cell line
Selectively knocking down specific endogenous genes is an alternative to construct modified Caco-2 cell line with a decreased transporter expression.

Several attempts have been made to produce Caco-2 cell lines deficient in P-glycoprotein. Hilgendorf and co-workers were first to use antisense technique to reduce transcription and activity of P-gp. Currently, modern siRNA technology is employed to reduce P-gp activity in Caco-2 cells. Although siRNA technology drastically reduced P-gp expression, conflicting results have been reported (109). Celius and others (110) reported that complete abolishment of P-gp is unachievable whilst Watanabe and co-workers (111) experiment produced complete loss of both MDR1 mRNA and P-gp expression. The application of siRNA technology in Caco-2 cell line must be done with caution to prevent misrepresentation of data.

CONCLUSION

The Caco-2 model is widely used as a tool for permeability and/or absorption screening of test compounds during decision-making process in the early stages of drug discovery. The use of Caco-2 cell line for herb-drug interactions screening is increasingly gaining grounds in the academia and dedicated laboratories due to its popularity and acceptability. The recommendation of Caco-2 cell line by the FDA as an integral component of the Biopharmaceutics Classification System (BCS) has given this model an advantage over the other cell-based models (112). Consequently, the routine

application of Caco-2 cell for screening of biopharmaceutical parameters of test compounds such as herbal medicines in dedicated laboratories requires reliable protocol to generate reproducible results. The pitfalls enumerated in this review may undermine the credibility of results generated from different laboratories due to protocol variations. However, the incorporation of current and future technologies discussed in this paper into Caco-2 cell line study protocol during herb-drug interactions investigations may reduce some of these caveats. The dedicated laboratories engaged in screening of herbs and NCE must therefore embrace the paradigm shift of the emerging technologies associated with the application of Caco-2. This will improve the reproducibility of results from different laboratories and help decision making bodies to give right recommendations for clinical trials based on the pre-clinical information

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Table 1. Selected important membrane transporters involved in intestinal drug absorption

Transporter	Location		Expression in Caco-2 cell line	Substrates	Inhibitors
	Tissue	Subcellular			
P-gp/MDR1(*ABCB1*)	Intestine, liver, kidney, brain, placenta, adrenal, testes	Apical	+	Digoxin,fexofenadine, indinavir, vincristine, colchicine, topotecan, paclitaxel	Ritonavir,cyclosporine, verapamil,erythromycin, ketocoanzole,itraconazole, quinidine
BCRP(*ABCG2*)	Intestine, liver, breast, placenta	Apical	+	Daunorubicin, doxorubicin, topotecan, rosuvastatin, sulfasalazine	Elacridar (GF120918), gefitinib
MRP1(ABCC1)	Ubiquitous	Basolateral	+	Adefovir, indinavir	?
MRP2 (*ABCC2*)	Liver, intestine and kidney	Apical	+	Indinavir, cisplatin,	Cyclosporine, delaviridine, efavirenz, emtricitabine
MRP3(ABCC3)	Liver, intestine, kidney and bile ducts	Basolateral	+	Etoposide, methotrexate, tenoposide	Delaviridine, efavirenz, emtricitabine
MRP4 (*ABCC4*)	Prostate, lung, muscle, pancreas and bladder	Apical	+	Adefovir, tenofovir, methotrexate, topotecan	Celecoxib, diclofenac
MRP5(ABCC5)	Ubiquitous	Basolateral	+	?	?
MRP6(ABCC6)	Liver and kidney	Basolateral	+	Cisplatin, daunorubicin	?
PepT1(*SLC15A1*)	Intestine, kidney	Apical	+	Ampicillin, amoxicillin, captopril, valacyclovir	Glycyl-proline
OATP 2B1(*SLCO2B1*)	Intestine, liver, kidney, brain	Apical	?	Pravastatin	Rifampicin, cyclosporine
MCT1(*SLC16A1)*	Intestine, erythrocyte	Apical	+	?	?

Keys: += presence and ? = unknown

Table 3. Comparison of Caco-2 with other cell-based models for intestinal permeability studies

Cell lines	Origin	Specific characteristics	Strengths	Weaknesses	References
Caco-2	Human colon adenocarcinoma	Most established cell model Spontaneous differentiation and expression of some relevant efflux transporters Interlaboratory variations in expression of uptake transporters	Well developed and characterized Easy maintenance Good reproducibility, robustness and functional property of human intestinal cells. Extensively used, large database available	Absence of mucous secreting goblet cells Not suitable for paracellular transport Low expression of metabolizing enzymes 21-days culture period	([i], 78)
HT29-MTX	Human colon	Contains mucus-producing goblet cells	Expression of mucin	Not suitable for carrier-mediated transport Unstable mucus layer	([ii], iii)
LLC-PK1	Pig kidney	Polarized cells with low intrinsic transporter expression Suitable for transfections	Characterization of passive transport	Absence of P-gp	(65, 67)

MDCK	Dog kidney	Polarized cells with low intrinsic expression of efflux transporters Suitable for transfections	3-5 days culture period Ideal for transfection purposes	Under-expression of major efflux transporters High expression of CYP 3A5 and less of CYP 3A4	([iv], [v])
TC7	Caco-2 subclone	Low expression of P-gp Stable expression of CYP3A4 High taurocholic acid transport	High CYP 3A4 expression	Similar to Caco-2	([vi])
2/4/A1	Rat fetal intestinal epithelial cells	Temperature-sensitive Suitable for paracellularly absorbed compounds (leakier pores)	Produces tight junction similar to human intestine Expression of both transporters and metabolizing enzymes	Low expression of protein Good for medium to low throughput screening.	(19)

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