Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers

Ina Hubatsch, Eva G E Ragnarsson & Per Artursson

Department of Pharmacy, Uppsala University, Box 580, SE-751 23 Uppsala, Sweden. Correspondence should be addressed to P.A. (per.artursson@farmaci.uu.se).

Published online 23 August 2007; doi:10.1038/nprot.2007.303

Permeability coefficients across monolayers of the human colon carcinoma cell line Caco-2, cultured on permeable supports, are commonly used to predict the absorption of orally administered drugs and other xenobiotics. This protocol describes our method for the cultivation, characterization and determination of permeability coefficients of xenobiotics (which are, typically, drug-like compounds) in the Caco-2 model. A few modifications that have been introduced over the years are incorporated in the protocol. The method can be used to trace the permeability of a test compound in two directions, from the apical to the basolateral side or vice versa, and both passive and active transport processes can be studied. The permeability assay can be completed within one working day, provided that the Caco-2 monolayers have been cultured and differentiated on the permeable supports 3 weeks in advance.

INTRODUCTION

In the intestine, a single layer of epithelial cells covers the inner intestinal wall and forms the rate-limiting barrier to the absorption of dissolved drugs. As a consequence, the proper reconstitution of a human differentiated epithelial cell monolayer *in vitro* allows the prediction of oral drug absorption in humans[1]. The human colon carcinoma cell line Caco-2 has been found to serve this purpose well. In culture, this cell line slowly differentiates into monolayers with a differentiated phenotype with many functions of the small intestinal villus epithelium. Caco-2 cells grown on permeable filters (**Fig. 1**) have, therefore, become the golden standard for *in vitro* prediction of intestinal drug permeability and absorption[2]. The most common application of this cell line can be seen in **Figure 2a**. In this figure, the permeability of Caco-2 monolayers to a series of orally administered, passively transported drugs is plotted as a function of the absorbed fraction of these drugs after oral administration in humans. Once this curve has been established in a research laboratory, the *in vitro* permeability of a new compound in Caco-2 cells can be used to predict the absorption in humans, provided that confounding factors such as the drug solubility and metabolism can be accounted for with the help of other assays[3]. The Food and Drug Administration provides a list of reference drugs that can be used to establish a curve corresponding to that presented in **Figure 2a**[4].

Caco-2 cell monolayers are also used to elucidate drug transport mechanisms, as many of the brush border enzymes and transport proteins that mediate the active uptake or efflux of drugs in the intestine are functional in Caco-2 cells. An example of such a study is given in **Figure 2b**, where the absorption mechanism of a representative of a new class of anti-HIV compounds was determined. In this example, it was shown that the compound was degraded by the brush border peptidase CD26 to one active and one inactive compound and that the two resulting compounds were transported across Caco-2 cells by different mechanisms[5]. In this context, it should be noted that Caco-2 cells do not express all important intestinal drug-metabolizing enzymes, such as CYP3A4, to an appreciable extent, although this enzyme can be induced by dihydroxy-vitamin D3[6,7].

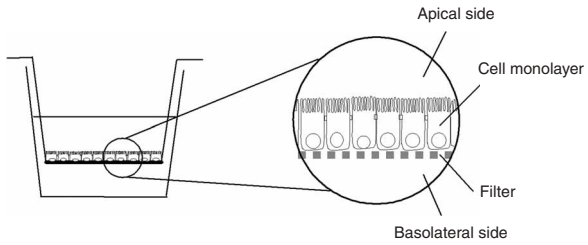

Figure 1 | Diagram of a Caco-2 monolayer grown on a permeable filter support.

If carefully controlled, the Caco-2 cell monolayers can perform consistently within a single laboratory for decades. However, the polyclonal Caco-2 cell line is rather unstable, and, without good quality control, it may alter its performance over time and with the passage number. Below, we present our protocol for the cultivation and evaluation of Caco-2 cell monolayers grown on permeable supports, followed by our procedures for the assessment of drug toxicity, drug permeability and active drug transport across Caco-2 monolayers. It should be noted that, depending on the compounds to be studied, variants of the protocols may be necessary. For instance, if compounds of low solubility are to be studied, the inclusion of solubility-enhancing agents, such as serum albumin, may be necessary[8]. The protocols are based on 20 years of experience and have, without any dramatic alterations, resulted in the consistent performance of Caco-2 cell monolayers in drug transport studies in our laboratory over this period of time.

Overview of the procedure

The experimental procedure is comprised of two parts, the cultivation of the Caco-2 cells on filter supports (Steps 1–11) and the drug transport experiment (Steps 12–20). Procedures for appropriate controls of the monolayer integrity are given in **Box 1** and in the REAGENT SETUP section. **Box 2** describes procedure for determining paracellular permeability to [^{14}C]mannitol. **Box 3** contains notes concerning the sampling options. **Box 4** describes considerations for studying active transport.

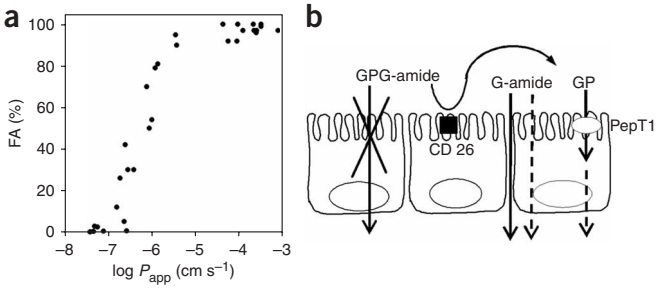

Figure 2 | Application of the technique in absorption and drug metabolism studies. (**a**) Correlation between the fraction absorbed (FA) in humans and the permeability across Caco-2 monolayers (P_{app}) (redrawn with permission from ref. 19). (**b**) Example of the outcome of a mechanistic study in the Caco-2 cell monolayers. In this case, the active tripeptide GPG-amide was shown to be a prodrug that is degraded by the brush border hydrolase dipeptidyl peptidase IV (CD26) to form G-amide and the dipeptide GP. The inactive metabolite GP is transported across the apical barrier by the peptide transporter PEPT1, whereas the small hydrophilic active metabolite G-amide permeated the monolayer via the paracellular route (modified from ref. 5).

MATERIALS

REAGENTS

- Caco-2 cells (ATCC) ▲ **CRITICAL** Use only about ten passages (i.e., cells within a defined interval of passages). We use Caco-2 cells of a high passage number (95–105), but it is possible to use cells of lower passage numbers (e.g., 40–50). The phenotypes of Caco-2 cells taken from high and low passage intervals will differ. ▲ **CRITICAL** After thawing a vial of Caco-2 cells from the stock, cultivate the cells for two passages before seeding cells on the filter supports to stabilize the cell phenotype.
- Culture medium (see REAGENT SETUP)
- Trypsin/EDTA solution (see REAGENT SETUP)
- Hank's balanced salt solution without phenol red (HBSS; see REAGENT SETUP)
- DMSO (99.5% purity) as co-solvent ▲ **CRITICAL** Compounds obtained from drug discovery settings and sparingly water-soluble compounds are often dissolved in DMSO. In this case, it is important to ensure that the compounds do not precipitate after dilution in HBSS. DMSO may affect the monolayer integrity and the active transport processes. We therefore recommend that the maximum final concentration of DMSO should not exceed 1% for passive permeability studies and should be less for studies of active transport processes, but this is transporter dependent and should be investigated in each case. We include DMSO in both the apical and basolateral compartments to avoid precipitation of the transported compound. ▲ **CRITICAL** It is advisable to ensure that the Caco-2 monolayers tolerate the co-solvent at the concentration applied. This is done by conducting a transport experiment with a paracellular marker such as [^{14}C]mannitol in the absence and in the presence of the co-solvent. ▲ **CRITICAL** DMSO might interact with transport proteins. The influence on the transporter activity will not be revealed by the flux of a paracellular marker such as mannitol. To ensure that the transport protein to be studied is not impaired by the applied DMSO concentration, a transport experiment with a transport protein-specific substrate in the absence and presence of the co-solvent can be performed. Note that the sensitivity to DMSO may vary for different active transport proteins. The efflux transporter MRP2 (ABCC2) tolerates a maximum of 0.5% DMSO with maintained activity, whereas in our hands the peptide transporter PepT1 (SLC15A1) maintains its activity at 1% (vol./vol.) DMSO.
- Paracellular marker ([^{14}C]mannitol) (e.g., supplied by NEN Life Science Products) ! **CAUTION** Radioactive.
- Solvents for analytical procedures (scintillation fluid or HPLC solvents)
- Compound of interest for transport experiments (see REAGENT SETUP)
- Bovine serum albumin (Fraction V, Sigma), required if assessing highly lipophilic compounds

EQUIPMENT

- Incubator with CO_2 connection (using either 10% or 5% CO_2 in air) and water-saturated atmosphere for cell culture
- LAF-bench
- Cell counter (NucleoCounter; ChemoMetec A/S, inclusive of counting cassettes)
- Incubator (37 °C) with humid atmosphere and a calibrated orbital shaker (3 mm orbital diameter; IKA-Schüttler MTS4)
- Heating plate for use during sampling (conducted at 37 °C), for example, Kitazato stage warmer (Shizuoka-ken)
- Transepithelial resistance (TER) measurement device, Endohm tissue resistance measurement chamber (WPI) with Millicell-ERS-voltmeter (Millipore) or with Evohm epithelial voltmeter (WPI), or comparable device ▲ **CRITICAL** The TER equipment should be equilibrated before the experiments and cleaned after the experiments as advised by the manufacturer.
- Filters, such as Corning Costar Transwell permeable supports, polycarbonate membrane pore size 0.4 μm, diameter 12 mm (12-well plates, cat. no. 3401) or the corresponding product from Millipore. ▲ **CRITICAL** Uncoated filters with a pore diameter of 3 μm are not recommended, as they allow the cells to crawl through the pores to the opposite side of the filter, resulting in a double monolayer.
- 12-well cell culture clusters with lids (Corning Costar, cat. no. 3512) or corresponding product
- Analytical equipment (scintillation counter, UV-plate reader, fluorescence plate reader or HPLC(-MS))

BOX 1 | EVALUATION OF CACO-2 CELL MONOLAYERS GROWN ON PERMEABLE SUPPORTS: QUALITY CONTROL STEPS RECOMMENDED

When establishing the technique for growing Caco-2 monolayers on filter supports, it is necessary to control the morphology of the cell monolayer. In addition, controls should be made when larger changes in the culture conditions, for example, a new batch of serum, are introduced. The morphology is preferably evaluated by confocal microscopy. Usually, a single stain with rhodamine-phalloidin (to visualize actin)[17] is sufficient to ensure that a single layer of cells is present. Alternatively, immunohistochemical staining of the tight junction proteins ZO-1 or fluorescent nuclear stains can be used[17] (see **Fig. 3b,c**). ? **TROUBLESHOOTING**

For each batch of seeded cells, a quantitative integrity control of the monolayers by performing a transport experiment with a hydrophilic paracellular marker is also recommended. We routinely use [^{14}C]mannitol for this purpose. The control can be done on separate monolayers (see **Box 2**) or in parallel with the compound to be tested in the transport experiment. In the latter case, note that all samples will be radioactive because of the paracellular marker [^{14}C]mannitol. The fluorescent compound lucifer yellow is regarded as a non-radioactive alternative to evaluate monolayer integrity, but higher donor concentrations are required for detection to be possible[20,21].

As a quality control of each filter, the TER is routinely measured before the start of transport experiments (after replacing the cell culture medium with HBSS at the end of the washing step) using the Endohm tissue resistance measurement chamber (at 37 °C). In addition, the TER is measured at the end of the transport experiment. When establishing the technique of culturing Caco-2 cells on filter supports, TER might be controlled at different days during the maintenance period to follow the monolayer development.

BOX 2 | DETERMINATION OF THE PARACELLULAR PERMEABILITY TO [^{14}C]MANNITOL IN THE APICAL-TO-BASOLATERAL DIRECTION BY PERFORMING A REPLACEMENT EXPERIMENT

Time required: up to 2 h without scintillation counting

1. Pre-warm the HBSS (pH 7.4) to 37 °C.
2. Prepare the donor solution to obtain a final concentration of approximately 2.5–5 × 10^5 dpm ml^{-1} (if the specific radioactivity of the stock is 50 mCi $mmol^{-1}$ and the concentration is 0.1 mCi ml^{-1}, then 1 µl mannitol stock per ml HBSS corresponds to 2 µM mannitol); incubate at 37 °C.
3. Prepare vials for sampling.
4. Decant medium from 3 to 4 filter inserts with Caco-2 monolayers, place them in a fresh 12-well cluster and wash the monolayers with HBSS for 15 min (1.5 ml on the basolateral side, 0.5 ml on the apical side) at 37 °C.
5. Measure the TER values.
6. Add 1.2 ml HBSS per well in 3 to 4 wells of a fresh 12-well plate.
7. Decant the HBSS from the monolayers and place the filter inserts in the wells with the fresh HBSS prepared in Step 6.
8. Add 0.45 ml of the donor solution to each filter directly and slowly (apically) and immediately withdraw 0.05 ml samples (c0) into scintillation vials.
9. Incubate the filters with the cell monolayers on an orbital shaker at 37 °C.
10. After 30 min, withdraw samples of 0.6 ml from the basolateral sides of the filters and replace them with 0.6 ml of the pre-warmed HBSS.
11. Incubate the filters again under shaking and then repeat point 10 (at 30 min intervals) up to 2 h. Note that the duration of the mannitol experiment is dependent on the predicted duration of the subsequent transport experiment. If a 30 min experiment is predicted, then it is sufficient to run the mannitol experiment for 30 min.
12. At the end of the experiment, withdraw a 0.05 ml sample from the apical side of each filter (required for calculation of the mass balance).
13. Voluntary step: Measure the TER values. **! CAUTION** The TER equipment will be contaminated by radioactivity.
14. Determine radioactivity and calculate the permeability coefficient.

REAGENT SETUP

Culture medium Dulbecco's modified Eagle's medium (DMEM) high glucose (4,500 mg $liter^{-1}$ glucose) with L-glutamine (without pyruvate) (Gibco/Invitrogen, cat. no. 41965-039). Two versions of DMEM are required: (a) complete DMEM for the maintenance of cells in culture flasks: 500 ml DMEM + 50 ml fetal calf serum (FCS) + 5 ml 100× nonessential amino acids (Gibco/Invitrogen, cat. no. 11140-035) and (b) DMEM-PEST for maintenance of the cells on filter supports: 500 ml DMEM + 50 ml FCS + 5 ml 100× nonessential amino acids + 5 ml PEST (penicillin 10,000 U ml^{-1}–streptomycin 10,000 µg ml^{-1} solution (100×); Gibco/Invitrogen, cat. no. 15140-122). ▲ **CRITICAL** Before buying a new batch of FCS, it is advisable to test different sera to evaluate which serum batch performs well with the Caco-2 cultures. To do this, cultivate the cells for 1 week in flasks with the new FCS batch. Then evaluate the growth pattern and the quantity of cells harvested from the next two passages in culture flasks. Also, seed cells on filters and maintain for 3 weeks; then control the monolayer formation (see **Box 1**), measure the TER (see Step 14) and perform transport experiments with mannitol (see **Box 2**) plus bidirectional transport experiments with a selected influx (e.g., glycyl-sarcosine) and efflux substrate (e.g., digoxin).

Trypsin/EDTA solution 40 ml PBS without Ca/Mg + 5 ml EDTA (2% sodium salt) + 5 ml trypsin (10× stock solution 2.5% (wt/vol); Gibco/Invitrogen, cat. no. 15090046, or Sigma, cat. no. T4549). ▲ **CRITICAL** A more concentrated trypsin solution than is normally used is necessary to detach the cells from the flasks. Therefore, the time window between detachment and cell death is narrower than for most other cell lines. Trypsinization at a lower cell density (70% confluence) is recommended if it proves to be difficult to detach the cells.

Hank's balanced salt solution without phenol red A powder, with ten packages, each making 1 liter, can be obtained from Sigma (cat. no. H1387). This HBSS should be buffered by adding HEPES (end concentration 25 mM, if a pH of 7.4 is desired) and $NaHCO_3$ (end concentration 0.35 g $liter^{-1}$) to the HBSS solution. Adjust the pH to 7.4 and sterile-filter the solution. If a pH of 6.5 is desired, use 10 mM methanesulfonic acid instead of HEPES. ▲ **CRITICAL** Whether a pH of 6.5 or 7.4 is used on the apical side depends on the type of transport study. In the standard setting, we recommend that a pH of 7.4 is applied on both sides of the monolayer, as this allows direct comparison of the permeabilities in both directions without a need to consider pH-dependent effects on the charge of the ionizable compounds generally studied[9,10]. However, to mimic the acid microclimate of the small intestine[11,12] or to study proton-dependent transport mechanisms, an apical pH of 6.5 should be used.

Compound of interest for transport experiments Dissolve the compound in buffered HBSS at the desired concentration (to obtain the donor solution) just before the experiment. The pH of the donor solution should be checked and, if necessary, adjusted. The initial donor concentration will be dependent on the solubility of the compound, the toxicity of the compound and the method of analysis (because of its detection limit). ▲ **CRITICAL** It is critical to ensure that

BOX 3 | NOTES CONCERNING THE SAMPLING OPTIONS

Replacement experiments are experiments where a sample is withdrawn from the receiver chamber at a specific point in time and the withdrawn volume is replaced by buffered HBSS. As only a part of the transported amount of the substance is sampled, the concentration in the receiver compartment will rise during each sampling interval (even if the time intervals chosen are the same). Care must be taken that the receiver concentration does not exceed 10% of the donor concentration per time interval to maintain the 'sink' of the receiver.

To avoid impaired 'sink conditions' for very rapidly transported substances ($P_{app} > 10^{-4}$ cm s^{-1}), 'transfer experiments' are required (possible for experiments in the ab direction). In this case, a receiver plate is prepared, which contains HBSS (measured volume, here 1.2 ml) for each filter in as many wells as there are sampling time points plus one. At the start of the experiment, the filter is inserted into the first well, the donor solution is added to the apical side and then the filter is moved at each point in time from one HBSS-containing well to the next. Care must be taken that there is no solution transferred from one well to the next. In principle, the transfer experiments are experiments where all the receiver solution is exchanged at each sampling.

BOX 4 | STUDYING ACTIVE TRANSPORT

Many researchers, including us, prefer to perform transport experiments in both directions across the cell monolayers. The ratio between the two permeability coefficients obtained, which is usually named the efflux ratio or the uptake ratio, can then be used as a first indication of the involvement of an active transport process. We recommend the following:

1. A low concentration, typically in the low micromolar range, is used to avoid saturation of the transporter.
2. Perform a transport experiment as in **Box 2**.
3. Perform a transport experiment as in **Box 2**, but with the compound being added to the basolateral compartment instead of the apical side and with the samples being taken from the apical side.
4. Calculate the permeability coefficients.
5. If there is a significant difference between the permeability coefficients in the two directions, as shown by, for example, an efflux ratio of 1.5–2.0, then a transport experiment with increasing donor concentrations should be performed to investigate if the flux rate is concentration-dependent and if the transport is saturable. Traditionally, kinetic parameters such as K_M and V_{max} have been derived from such experiments. Such kinetic parameters should be considered as apparent parameters, as in Caco-2 cells several transport proteins may contribute to the net flux of the compound. Moreover, in the case of ABC transporters, which pick up substrates from the cell membrane, the contribution of membrane partitioning may be significant[22–25].
6. If there is no clear difference between the permeability coefficients in the two directions, the transport might be passive. Alternatively, the transport protein could depend on a proton gradient, or the transport protein is not expressed in the Caco-2 monolayers. Examples of intestinal transport proteins that are dependent on a proton gradient are the peptide transporter PEPT1 (SLC15A1)[26] and the organic anion transporter OATP-B (SLCO2B1)[27]. For these transporters, a more acidic pH in the apical chamber is recommended to create the proton gradient. When performing transport experiments in the presence of a pH gradient, the proportions of unionized and ionized species of a charged compound will differ with pH. For passively permeating compounds, this will lead to different permeabilities in the two transport directions, as only the uncharged fraction will permeate the cell membrane[9,10]. Therefore, the contribution of a proton-dependent transporter on the transport might be examined by inhibition experiments.
7. As an alternative, inhibitors of the transport proteins can be used. The inhibitor concentration should be kept constant during the entire experiment. It is also advisable to preincubate the Caco-2 monolayers with the inhibitor during the wash period. Ideally, specific inhibitors should be used to inhibit the transport process. However, there are only a few specific inhibitors. In the screening situation, a rather nonspecific inhibitor is sometimes preferred to obtain broad simultaneous inhibition of several efflux transporters. This all-in-one procedure is useful for qualitative assessment of the contribution of active transport to permeability.

the compound does not affect the integrity of the cell monolayer. A toxic effect is revealed as an increased permeability to the paracellular marker [^{14}C]mannitol in the presence of the compound. A change in the [^{14}C]mannitol permeability is a more sensitive marker of integrity than measurement of TER values, which are more variable in the simple commercial setup for routine use. Note that mannitol has a very low permeability across Caco-2 monolayers, so, if a highly permeable compound is studied, a small change in mannitol permeability may be acceptable, although this must be judged from case to case. The procedure for measuring the (paracellular) [^{14}C]mannitol permeability across the Caco-2 monolayer is laid out in **Box 2**. In our laboratory, an intact cell monolayer of Caco-2 cells typically has a [^{14}C]mannitol permeability of $1.2 \pm 0.5 \times 10^{-7}$ cm s^{-1}, and we consider permeability coefficients higher than 5×10^{-7} cm s^{-1} to be indicative of compromised cell monolayers and values above 1×10^{-6} cm s^{-1} to demonstrate destroyed integrity of the cell monolayers to drug like compounds. Note that the measurement of the paracellular permeability will give an evaluation of the integrity of the monolayer but will not show whether the compound affects a specific active transport process (see also REAGENTS).

For studies of active transport, a low donor concentration of 10 μM or less should be used initially to avoid saturating the transport proteins if the analytical procedure is sensitive at this level. Care must be taken to avoid nonspecific adsorption to plastic surfaces when using very low donor concentrations. For determination of passive transport, which is the dominating transport mechanism during the absorption of orally administered drugs, a high donor concentration (in the millimolar range) can be applied. By this approach, active transport proteins (if any) will be saturated, resulting in a flux that is dominated by the passive permeability.

PROCEDURE

Cultivation of Caco-2 cell monolayers grown on permeable supports ● TIMING Start 21–29 d in advance of actual experiment

1| Trypsinize Caco-2 cells from maximally (90%) confluent Caco-2 cultures: remove the medium by aseptical decantation, rinse with PBS (15 ml per 75 cm^2 flask), decant PBS and add the trypsin/EDTA solution (5 ml per 75 cm^2 flask); rinse the cells and pour off the majority of the trypsin/EDTA so that only about 500 μl to 1 ml remains in the flask; the remaining trypsin must wet the entire cell layer. Incubate the flask (closed lid) at 37 °C for 6–15 min (use the shortest time possible) and check the detachment of the cells from the plastic surface by mildly knocking the sidewall of the flask with your palm. As soon as the cells are detached, immediately stop trypsinization by resuspending the cells in complete DMEM.

▲ **CRITICAL STEP** The cells should be seeded on filter supports 21–29 d before the experiment.

? TROUBLESHOOTING

2| Transfer the cells to a test tube and allow the debris and large cell aggregates (if any) to sediment. Transfer the supernatant to a new test tube, take an aliquot and count the cells, for example, by counting the nuclei of permeabilized cells after DNA staining with propidium iodide using a NucleoCounter.

▲ **CRITICAL STEP** The percentage of dead cells must not exceed 5%. This is assessed by counting the total number of cells as well as the number of non-viable cells. When using the NucleoCounter, non-viable cells are counted by omitting the cell permeabilization step according to the manufacturer's procedure.

3| Spin down the cells (5 min at 1,500 r.p.m., 16 cm swing-bucket rotor, i.e., ca. 400*g*) and remove the supernatant.

4| Resuspend the cells in DMEM-PEST (at a concentration of 0.6×10^6 cells ml^{-1}).

5| Place the desired number of filters in 12-well cell culture clusters; pre-wet the filters (12 mm diameter) with about 0.1 ml of medium (for at least 2 min) before seeding the cells. Seed by dispensing 0.5 ml of the resuspended cell solution on each filter. The seeding density used in our laboratory is 2.6×10^5 cells cm^{-2} (300,000 cells for each 12 mm diameter filter).
▲ **CRITICAL STEP** We recommend that quadruplicate filters are used initially, but once experience has been gained, triplicates are sufficient.
? TROUBLESHOOTING

6| Fill the basolateral chamber with 1.5 ml DMEM-PEST.

7| Incubate the plate with the filter supports at 37 °C and 10% CO_2 in a humid atmosphere for 6 h (if seeding is done in the morning) or overnight (16 h; if seeding is done at the end of the day).
▲ **CRITICAL STEP** The length of this incubation should not exceed 16 h.

8| Remove the apical medium and replace with 0.5 ml of DMEM-PEST. This step is done to remove non-adherent cells and to reduce the risk of multilayer formation.

9| Maintain cells, every second day, as follows: aspirate the medium from the basolateral side of all wells and then carefully and slowly from the apical side (of all filters in a plate). Replace the aspirated medium with fresh DMEM-PEST, first in the apical compartments and then in the basolateral compartments; the volumes we use for 12-well plates are 1.5 ml for the basolateral side and 0.5 ml for the apical side.
▲ **CRITICAL STEP** Do not touch the filter surface with the pipettes! Slow pipetting and avoiding physical contact between the pipette tip and the monolayer are essential for maintenance of monolayer integrity.

10| Repeat Step 9 every second day for a total of 21–29 d.
▲ **CRITICAL STEP** As a rule, we perform transport experiments with Caco-2 monolayers that have been grown on filter inserts for 21–29 d. By 21 d, the monolayers have become differentiated with regard to the expression of many transport proteins and brush border hydrolases. Note that procedures relevant to the use of short-term cultures are available, but, in general, such cultures express less well-differentiated features, although, recently, clear improvements have been made[13]. However, further characterization of the short-term cultures is needed. It is essential for the outcome of the transport experiments that the cells form monolayers, not multilayers. Therefore, when establishing this method, it is important to investigate the morphology of the cell monolayer. **Box 1** gives further information about appropriate 'quality control' experiments that should be performed.

Final preparations for the transport experiment

11| Change the culture medium 12–24 h before the experiment.
▲ **CRITICAL STEP** Longer periods without feeding before starting the experiment should be avoided because the cells may have consumed the essential nutrients and adapted to a more starved phenotype.

Experimental procedure ● TIMING Up to 2 h, depending on the sampling intervals (excluding the time required to analyze the samples)

12| Prepare donor solutions (containing the compound of interest; see REAGENT SETUP); pre-warm all solutions used in subsequent steps to 37 °C; pre-warm (to 37 °C) an incubator with humidified atmosphere (but without CO_2, as the solutions are buffered) equipped with an orbital shaker. Place a heating plate to be used during the sampling on the working bench.

13| Wash the filter supports with the cell monolayers to remove residual medium by transferring the cell monolayers (medium decanted, not aspirated) into new 12-well clusters containing buffered HBSS (1.5 ml per well) and carefully adding buffered HBSS (0.5 ml) to the apical side. Incubate the filter inserts under gentle shaking (IKA-Schuettler MTS4 orbital shaker 100 r.p.m.) for 15–20 min at 37 °C.

14| Measure the TER values using an Endohm tissue resistance measurement chamber (at 37 °C) according to the manufacturer's instructions. The TER values should also be measured after the experiment.
▲ **CRITICAL STEP** The TER is highly dependent on the temperature[14]. If measured at room temperature (e.g., 25 °C) rather than at 37 °C, an erroneously high TER will be obtained.
▲ **CRITICAL STEP** If chopstick electrodes are used, the background resistance will be higher. Correct the background resistance by subtracting the resistance value obtained with cell-free filters from the TER of filters with Caco-2 monolayer. If an Endohm tissue resistance measurement chamber is used, the background is typically much lower.
? TROUBLESHOOTING

15| Perform transport experiments in either the apical-to-basolateral (absorptive) direction (option A) or the basolateral-to-apical (secretory) direction (option B). Generally, we use final volumes (i.e., after withdrawal of the c0-sample) of 0.4 ml apically and 1.2 ml basolaterally for 12-mm filter supports during the transport experiment. These volumes have been chosen as they give the same surface levels of the solutions in the two chambers so that no effect of hydrostatic pressure difference is obtained.

(A) Apical-to-basolateral (ab) transport experiments

(i) Remove the washing solutions by decanting and transfer the filter inserts into new clusters containing the basolateral HBSS.
▲ CRITICAL STEP Decanting instead of aspirating the washing solution reduces the risk of compromising the monolayers. Care must be taken to leave as little residual liquid as possible on the monolayers; by placing the edge of the filter support against the edge of the 12-well plate, the residual volume is typically reduced to 35–45 µl.

(ii) Add donor solution to the filter inserts ($t = 0$) and immediately take a sample (c0) from the donor solution in the apical compartment.
▲ CRITICAL STEP The c0 sample has to be taken directly to prevent a part of the compound from being transported before the sampling is done.

(B) Basolateral-to-apical (ba) transport experiments

(i) Remove the washing solutions by decanting and transfer the filter inserts into new clusters containing the basolateral donor solution.
▲ CRITICAL STEP Decanting instead of aspirating the washing solution reduces the risk of compromising the monolayers. Care must be taken to leave as little residual liquid as possible on the monolayers; by placing the edge of the filter support against the edge of the 12-well plate, the residual volume is typically reduced to 35–45 µl.

(ii) Add HBSS to the apical side of the filter inserts ($t = 0$) and immediately take a sample (c0) from the donor solution in the basolateral compartment.
▲ CRITICAL STEP The c0 sample has to be taken directly to prevent a part of the compound from being transported before the sampling is done.
? TROUBLESHOOTING

16| Incubate the (lid-covered) plate with the filter supports in the incubator at 37 °C on a calibrated orbital shaker at 500 r.p.m. to minimize the impact of the unstirred water layer.
▲ CRITICAL STEP It has been shown that the permeability of highly permeable compounds is dependent on the thickness of the unstirred water layer[2,15]. Inadequate stirring will result in underestimation of the permeability coefficients for highly permeable compounds (i.e., those with a permeability coefficient larger than 2×10^{-5} cm s^{-1})[15,16]. Note, however, that too vigorous shaking will affect the cell monolayer integrity.

17| Sample from the receiving compartment at appropriate time points (for the ab experiment, the basolateral compartment; for the ba experiment, the apical compartment). Typically, when performing a replacement experiment, half of the volume in the receiver chamber is sampled and replaced by the same volume of pre-warmed buffered HBSS.

The sampling is generally performed at 4–5 different time points, but in permeability screening, a single time point is often used for qualitative assessment. If the simplest and most commonly used expression is applied to calculate the permeability, sampling intervals should be chosen so that the transport experiment is completed before the receiver concentration exceeds 10% of the donor concentration per time interval to maintain sink conditions. Sampling times will vary from 5 min for very rapidly transported compounds, that is, usually more lipophilic drugs, to 1 h for compounds that are transported more slowly, such as hydrophilic compounds that are dependent on the paracellular route. A detailed step-by-step example for performing a transport experiment is given in **Box 2**.

18| Take a final sample from the donor chamber for the calculation of the mass balance.

19| Measure the TER values.
■ PAUSE POINT Depending on the stability of the test compound, the samples may or may not be stored before quantification.
? TROUBLESHOOTING

Analytical procedures

20| Time required is dependent on the analytical method used and analysis is normally performed by an automated process. The quantification of the samples will be dependent on the test compound used. Appropriate methods are scintillation counting for radioactive-labeled compounds and, for other compounds, analysis by HPLC with either UV, fluorescence or MS detection or analysis using a UV or fluorescence-plate reader/spectrophotometer.

? TROUBLESHOOTING

Modification of the experimental procedure (Steps 15–20) to avoid loss of highly lipophilic compounds

If a significant fraction of the compound disappears during the transport experiment, a poor mass balance will result and the permeability coefficient obtained becomes unreliable. By introducing BSA in the transport buffer in the receiving chambers, this problem can sometimes be avoided[8]. Note that the inclusion of BSA, which binds drugs and other xenobiotics to a variable

extent, will create an additional sink in the receiver chamber. Therefore, the permeability coefficient obtained in the presence of BSA will be higher than that in its absence. We include BSA at a 4% final concentration in the basolateral chamber, which closely mimics the physiological situation.

If the analysis of the samples is to be done by HPLC(-MS), we use the following sample pretreatment to clean BSA from the samples: the BSA is precipitated by adding two volumes of acetonitrile to one volume of sample, vortexing for 5 s and centrifuging for 10 min at 12,000 r.p.m. (=15,000*g*) (Eppendorf centrifuge 5403 with a fixed-angle rotor 16F24-11); the supernatant containing the compound is immediately withdrawn.

See **Table 1** for further troubleshooting advice.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1	Cells do not detach during trypsinization	Cell layer in the culture flask is too confluent	Trypsinize at lower confluence (70–90%)
1 and **Box 1**	Multilayer formation	There are too many cells and/or cell aggregates	Remove cell aggregates before seeding Reduce the number of cells added to the filters
		The FCS has not been properly tested	Use another source of FCS
5	Cells are observed on both sides of the filter	The pore diameter is too large	Use filters with 0.4 μm pore diameter
14	Variable TER values are detected before the transport experiment	Poor cell culture technique, for example, owing to poorly resuspended cells or perforation of some monolayers by physical contact from the pipette	Improve cell culture technique
	Low TER values are observed in all filters before the experiment	Damaged or non-confluent monolayer	Improve the cell culture technique
		The FCS has not been properly tested	Use another source of FCS that passes the recommended tests
15	The donor concentration is lower than anticipated	The compound has precipitated or adsorbed to the test tube	Use freshly prepared solutions
			Include DMSO as a co-solvent
			Use glass vials
			Consider addition of BSA
15–19	Poor mass balance at the end of the experiment	The compound has adsorbed in significant amounts to the experimental device (the plastic of the 12-well plate, the filter device or the filter itself)	Add BSA to the basolateral transport buffer as an additional sink (attention: if BSA is added in the donor compartment, the free concentration of the compound might be changed)
		The compound is retained inside the cells or in the cell membranes	Lyse the cells and determine the cellular concentration
		The compound is metabolized	Use LC-MS/MS to detect metabolites
19	Low or no TER values after the experiment	Toxic compound or co-solvent	Perform the recommended integrity test with mannitol (see **Box 1**) to check the test compound/co-solvent toxicity and find nontoxic conditions
		Too vigorous shaking or pipetting	Calibrate shaker to allow reproducible shaking at a rate that does not compromise the monolayers
			Improve the pipetting technique

ANTICIPATED RESULTS

Calculations

The apparent permeability coefficient (P_{app}, unit: cm s^{-1}) is determined from the amount of compound transported per time; P_{app} is usually calculated according to the following equation:

$$P_{app} = (dQ/dt)(1/(AC_0))$$

where dQ/dt is the steady-state flux (dpm s^{-1} or µmol s^{-1}), A is the surface area of the filter (cm^2) and C_0 is the initial concentration in the donor chamber (in dpm $liter^{-1}$ or µM).

This calculation requires that the sink conditions are fulfilled, that is, the receiver concentration should not exceed 10% of the donor concentration.

The reduction in donor concentration should preferably also be taken into account after every sampling (i.e., the donor concentration should be recalculated by subtracting the cumulative amount transported to the receiver chamber for each time interval). The 'cumulative fraction transported' FA_{cum} is defined as

$$FA_{cum} = \frac{1}{A}\sum_{k=1}^{i}\frac{[C_R(t_k) - fC_R(t_k-1)]V_R}{[C_D(t_{k-1})+C_D(t_k)]/2}$$

where the factor $f = (1 - V_s/V_R)$ is a sample replacement factor. The equation gives a 'weighted normalized cumulative amount of the transported drug', where the amount of transported drug in each time interval is weighted by the inverse average driving force (donor concentration for the interval). The 'cumulative fraction transported' is not a dimension-free ratio, but has the unit of a length (for derivation of the equation, see ref. 17). The variables C and V denote the concentration and volume in the donor (index D) or receiver (index R), respectively.

A general equation that does not require sink conditions can also be applied[17]. In this 'non-sink'- analysis, P_{app} is determined by nonlinear curve fitting of

$$C_R(t) = [M/(V_D+V_R)]+(C_{R,0} - [M/(V_D+V_R)])e^{-P_{app}A(1/V_D+1/V_R)t}$$

where V_D is the volume of the donor compartment, V_R is the volume of the receiver compartment, A is the area of the filter, M is the total amount of substance in the system, $C_{R,0}$ is the concentration of the substance in the receiver compartment at the start of the time interval and $C_R(t)$ is the concentration of the substance at time t measured from the start of the time interval.

The mass balance (recovery) is defined as the sum of the drug recovered from the acceptor chamber and the drug remaining in the donor chamber at the end of the experiment, divided by the initial donor amount. It is calculated according to

$$\text{recovery}\,(\%) = (C_{D(fin)}V_D+\Sigma(C_{S(t)}V_{S(t)})+C_{R(fin)}V_{R(fin)})100/(C_{D(0)}V_{D(0)})$$

where C_D and C_R are the concentrations on the donor (D) and receiver (R) sides of the monolayer at the start (0) or end (fin) of the experiment, $C_{S(t)}$ denotes the concentrations of the samples withdrawn at different time points, t, and V is used for each of the respective volumes.

The mass balance should be as high as possible. A mass balance of 90% will generally give a 10% error in the calculated P_{app} value. According to our experience, a mass balance of $>80\%$ gives an acceptable approximation of the P_{app} value. A lower mass balance will result in underestimation of the P_{app} values but can be acceptable for qualitative studies, aiming at identification of highly permeable compounds. It is not advisable to use general correction terms for a low recovery, as the processes involved in the loss of the compound are compound-specific[18] (see **? TROUBLESHOOTING** section).

The efflux ratio is defined as the quotient of the secretory permeability and the absorptive permeability ($P_{app,ba}/P_{app,ab}$). The uptake ratio is the inverse of the efflux ratio, that is, $P_{app,ab}/P_{app,ba}$.

Typical results

Confocal micrographs of Caco-2 cell monolayers grown on permeable supports should reveal cell monolayers, and not multilayer formation (see **Fig. 3**), and tight junctions between the cells.

TER values of the Caco-2 monolayers might depend on the Caco-2 clone used, on the number of days after seeding and on the passage number and culture conditions (including the material the filter support is made of). In our laboratory, standard TER values at 37 °C are 260 ± 65 Ω cm^2. Cell monolayers with TER values below 165 Ω cm^2 are discarded.

The apparent permeability coefficient for the paracellular marker mannitol in our laboratory is 1.2 ± 0.5 × 10^{-7} cm s^{-1}. The value for the apparent permeability coefficient obtained in our laboratory for a rapidly transported compound that is completely absorbed from the human intestine is larger than 2–5 × 10^{-6} cm s^{-1} (see **Fig. 2a**).

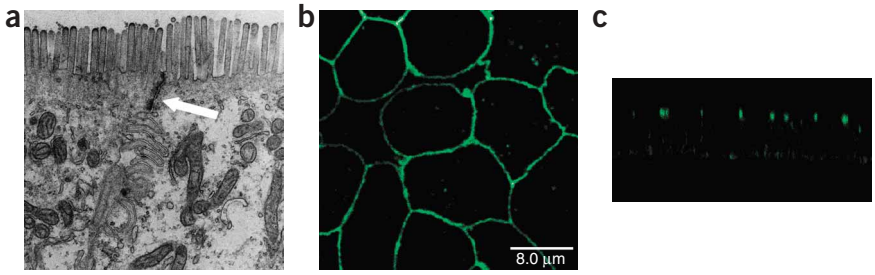

Figure 3 | Morphology of Caco-2 cell monolayers. (**a**) Transmission electron micrograph of the apical parts of two Caco-2 cells grown for 21 d on a polycarbonate filter. The transmission electron micrograph shows well-differentiated cells with microvilli and a tight junction between the cells (arrow). (**b**) Confocal micrograph showing a top view of a Caco-2 monolayer where the cell borders can be distinguished by immunocytochemical staining of the tight junction protein ZO-1 (green). (**c**) The optical cross-sections (x–z direction) with ZO-1 staining.

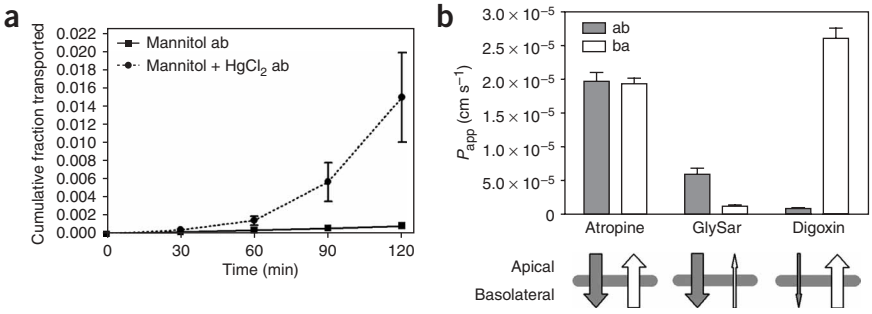

Figure 4 | Anticipated results. (**a**) The 'cumulative fraction transported' of the paracellular marker [^{14}C]mannitol across intact (solid line) and compromised (dotted line) cell monolayers. In this case, the integrity was compromised because a toxic compound was present. Mean values ± s.d.; $n = 3$. (**b**) Examples of results from transport experiments of passively and actively transported compounds; the P_{app} values are shown for the transport experiments in directions ab and ba for the passively and actively transported compounds, respectively. Atropine: only passive transport occurs; note that the monolayer permeability to the compound is equal in both directions. Glycyl-sarcosine (GlySar): an actively transported PEPT1 substrate; the uptake ratio ($P_{app,ab}/P_{app,ba}$) is about 5. Digoxin: an actively transported P-glycoprotein substrate; the efflux ratio ($P_{app,ba}/P_{app,ab}$) is 26; the transport experiments were conducted with HBSS pH 7.4 with the exception of glycyl-sarcosine, where the apical pH was 6.5 (because PEPT1 is a proton-dependent transport protein). The arrows below the figure illustrate the fluxes of the substance.

Figure 4a shows a typical result for the 'cumulative fraction transported' for a transport experiment of the kind described in **Box 2**. Note that deviations from linearity can be observed, for example, as a result of a lag phase or a burst phase in the first time interval. The latter might be obtained if radiolabeled (and especially ^{3}H-labeled) compounds are used, as these might contain impurities. In other cases, if the transport rate increases with time, the monolayer might become impaired during the experiment. In **Figure 4b**, results from passive and active transport experiments are compared.

ACKNOWLEDGMENTS This work has been supported by the Swedish Research Council, the Swedish Animal Welfare Agency and EU-grant no. LSHB-CT-2004-512051.

COMPETING INTERESTS STATEMENT The authors declare no competing financial interests.

Published online at http://www.natureprotocols.com
Reprints and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Artursson, P., Palm, K. & Luthman, K. Caco-2 monolayers in experimental and theoretical predictions of drug transport. *Adv. Drug Deliv. Rev.* **46**, 27–43 (2001).
2. Stenberg, P., Norinder, U., Luthman, K. & Artursson, P. Experimental and computational screening models for the prediction of intestinal drug absorption. *J. Med. Chem.* **44**, 1927–1937 (2001).
3. Artursson, P. & Karlsson, J. Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells. *Biochem. Biophys. Res. Commun.* **175**, 880–885 (1991).
4. Food and Drug Administration. FDA Guidance for industry, waiver of *in vivo* bioavailability and bioequivalence studies for immediate-release solid oral dosage forms based on a biopharmaceutics classification system. (Food and Drug Administration, Baltimore, MD, 2000). http://www.fda.gov/cder/guidance/3618fnl.htm (2007).
5. Hubatsch, I., Lazorova, L., Vahlne, A. & Artursson, P. Orally active antiviral tripeptide glycyl-prolyl-glycinamide is activated by CD26 (dipeptidyl peptidase IV) before transport across the intestinal epithelium. *Antimicrob. Agents Chemother.* **49**, 1087–1092 (2005).
6. Schmiedlin-Ren, P. *et al.* Expression of enzymatically active CYP3A4 by Caco-2 cells grown on extracellular matrix-coated permeable supports in the presence of 1alpha, 25-dihydroxyvitamin D3. *Mol. Pharmacol.* **51**, 741–754 (1997).
7. Engman, H.A. *et al.* CYP3A4, CYP3A5, and MDR1 in human small and large intestinal cell lines suitable for drug transport studies. *J. Pharm. Sci.* **90**, 1736–1751 (2001).
8. Neuhoff, S., Artursson, P., Zamora, I. & Ungell, A.L. Impact of extracellular protein binding on passive and active drug transport across Caco-2 cells. *Pharm. Res.* **23**, 350–359 (2006).
9. Neuhoff, S., Ungell, A.L., Zamora, I. & Artursson, P. pH-dependent bidirectional transport of weakly basic drugs across Caco-2 monolayers: implications for drug–drug interactions. *Pharm. Res.* **20**, 1141–1148 (2003).
10. Neuhoff, S., Ungell, A.L., Zamora, I. & Artursson, P. pH-dependent passive and active transport of acidic drugs across Caco-2 cell monolayers. *Eur. J. Pharm. Sci.* **25**, 211–220 (2005).
11. Fallingborg, J. *et al.* pH-profile and regional transit times of the normal gut measured by a radiotelemetry device. *Aliment. Pharmacol. Ther.* **3**, 605–613 (1989).
12. Yamashita, S. *et al.* Optimized conditions for prediction of intestinal drug permeability using Caco-2 cells. *Eur. J. Pharm. Sci.* **10**, 195–204 (2000).
13. Yamashita, S. *et al.* New and better protocols for a short-term Caco-2 cell culture system. *J. Pharm. Sci.* **91**, 669–679 (2002).
14. Delie, F. & Rubas, W. A human colonic cell line sharing similarities with enterocytes as a model to examine oral absorption: advantages and limitations of the Caco-2 model. *Crit. Rev. Ther. Drug Carrier Syst.* **14**, 221–286 (1997).
15. Karlsson, J. & Artursson, P. A method for the determination of cellular permeability coefficients and aqueous boundary layer thickness in monolayers of intestinal epithelial (Caco-2) cells grown in permeable filter chambers. *Int. J. Pharm.* **71**, 55–64 (1991).
16. Avdeef, A. *et al.* Caco-2 permeability of weakly basic drugs predicted with the double-sink PAMPA pKa(flux) method. *Eur. J. Pharm. Sci.* **24**, 333–349 (2005).
17. Tavelin, S., Grasjo, J., Taipalensuu, J., Ocklind, G. & Artursson, P. Applications of epithelial cell culture in studies of drug transport. *Methods Mol. Biol.* Vol. 188 (ed. Wise, C.) 233–272 (Humana Press, Totowa, New Jersey, 2002).
18. Osth, K., Grasjo, J. & Bjork, E. A new method for drug transport studies on pig nasal mucosa using a horizontal Ussing chamber. *J. Pharm. Sci.* **91**, 1259–1273 (2002).
19. Bergstrom, C.A. *et al.* Absorption classification of oral drugs based on molecular surface properties. *J. Med. Chem.* **46**, 558–570 (2003).
20. Hidalgo, I.J., Raub, T.J. & Borchardt, R.T. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* **96**, 736–749 (1989).
21. Konsoula, R. & Barile, F.A. Correlation of *in vitro* cytotoxicity with paracellular permeability in Caco-2 cells. *Toxicol. In Vitro* **19**, 675–684 (2005).
22. Bentz, J., Tran, T.T., Polli, J.W., Ayrton, A. & Ellens, H. The steady-state Michaelis–Menten analysis of P-glycoprotein mediated transport through a confluent cell monolayer cannot predict the correct Michaelis constant Km. *Pharm. Res.* **22**, 1667–1677 (2005).
23. Gatlik-Landwojtowicz, E., Aanismaa, P. & Seelig, A. Quantification and characterization of P-glycoprotein–substrate interactions. *Biochemistry* **45**, 3020–3032 (2006).
24. Korjamo, T., Kemilainen, H., Heikkinen, A.T. & Monkkonen, J. Decrease in intracellular concentration causes the shift in Km value of efflux pump substrates. *Drug Metab. Dispos.* doi: 10.1124/dmd.107.016014.
25. Matsson, P. *et al.* A global drug inhibition pattern for the human ABC transporter BCRP (ABCG2). *J. Pharmacol. Exp. Ther.* doi: 10.1124/jpet.107.124768.
26. Adibi, S.A. The oligopeptide transporter (Pept-1) in human intestine: biology and function. *Gastroenterology* **113**, 332–340 (1997).
27. Sai, Y. *et al.* Predominant contribution of organic anion transporting polypeptide OATP-B (OATP2B1) to apical uptake of estrone-3-sulfate by human intestinal Caco-2 cells. *Drug Metab. Dispos.* **34**, 1423–1431 (2006).