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Development of an automated 7-day 96-well Caco-2 cell culture model

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Caco-2 cells are a widely accepted model to predict permeability and absorption of compounds in humans. We built up an automated 96-wellplate Caco-2 permeation model with reduced growth time. Model compounds (metoprolol, ketoprofen, verapamil, naproxen, hydrochlorthiazide), permeability markers (TEER, Lucifer Yellow, D-[1-^{14}C]-mannitol) and confocal microscopy were used to assess the utility of our method on Biomek-FX-automation. The confocal imaging data showed that Caco-2 cells formed monolayers when cultured for 7 days with initial cell density of 2.1×10^5 cells/cm^2. P-Glycoprotein was present in Caco-2 cells, localized on the plasma membrane. Permeation of model compounds was comparable to those obtained from traditional 12-wellplate experiments.

1. Introduction

About 50% of the investigational new drugs fail in trials due to their inadequate absorption, distribution, metabolism, excretion and/or toxicity (ADMET) profiles (Gombar et al. 2003). In spite of many innovations in drug delivery, oral intake is still the most desirable route for administration (Marino et al. 2005). One of the most important biophysical parameter is the absorption of a drug across intestinal epithelium (Hämäläinen and Frostell-Karlsson 2004, Pascoe et al. 2006). No matter how potent a drug is, it will be ineffective if it is not first absorbed trough the gut into the bloodstream. To save money and prevent false expectations it is invaluable to assess permeability as early as possible.

Caco-2 cell monolayers have been widely accepted by pharmaceutical companies and by regulatory authorities as a standard *in vitro*-model system to predict permeability and absorption of compounds in humans (reviewed for example in Bailey et al. 1996 and in Artursson et al. 2001). Caco-2 cells, which are derived from a human colonic adenocarcinoma, exhibit morphological and functional similarities to intestinal enterocytes. Under normal conditions of cell culture Caco-2 cells differentiate to mature cells to form monolayers. Traditionally these cells are grown on 12 and 24 wells for 21 to 28 days. This is time consuming, laborious, expensive and low throughput. To adapt the model to the needs of modern screening approaches, we aimed to develop permeation experiments more efficient by reducing the cell growth time, by automation of the permeation study and by miniaturizing the protocol and in this way put up a new and faster method which is accurate and reliable. Because complicated conditions in primary screening can be difficult and time consuming (Yamashita et al. 2000) we aimed to keep our method simplified to provide first hand information about drug permeability.

P-Glycoprotein (P-gp) is an efflux transporter expressed in various tissues (Cordon-Cardo et al. 1990) and affects the absorption, distribution and excretion of a number of clinically important drugs (reviewed in Hunter and Hirst 1997). P-gp can transport a variety of structurally unrelated compounds from the interior of plasma membranes out of the cells thereby limiting access of xenobiotics into cells (Schinkel 1997; Ambudkar et al. 1999). Due to large substrate specificity P-gp significantly affects drug absorption through intestinal mucosa after oral administration. Moreover it can indirectly enhance intestinal CYP3A4 metabolism by increasing intestinal residence time and by preventing CYP3A4 product inhibition by the removal of primary metabolites (Gan et al. 1996; Watkins 1997; Hochman et al. 2000). Thus knowledge about the substrate activity of a drug candidate for P-gp has become an integral part of drug discovery (Gao et al. 2001; Polli et al. 2001; Perloff et al. 2003). Expression of P-gp in Caco-2 cells is not steady; it varies between cell generations (Hosoya et al. 1996; Anderle et al. 1998). It is important to know the current expression level of P-gp to interpret permeation studies properly. We evaluated the expression of P-gp with confocal laser microscopic studies and functionality with bi-directional permeation studies with P-gp substrates rhodamine 123 and verapamil (Nara et al. 1994; Adachi et al. 2003; Ogihara et al. 2006).

To evaluate the functionality of our new experimental conditions we compared the permeation of compounds with known Caco-2 cell permeability across the traditionally grown monolayers (12 wellplate, 21–27 days) and the new conditions (96 wellplate, 7 days). Monolayer and tight junction formation was confirmed with confocal laser microscopy using actin antibodies.

2. Investigations, results and discussion

2.1. Optimization of experiments

Transepithelial electrical resistance measurements were used to evaluate the formation of monolayers during the cell growth. TEER measures the resistance to passive ion transport and reflects the tightness and confluence of monolayers. At first experiments were performed with cell densities of 0.93×10^5, 2.1×10^5, and 4.2×10^5 cells/cm^2 which were modifications of previously used cell densities in the literature (Alsenz and Haenel 2003) and in Multi-Screen^{TM} Caco-2 plate user guide (Millipore Corporation 2002). With initial cell density of 0.93×10^5 cells/cm^2 TEER values were low (<200 Ω) and the plateau was not reached until after 9 days of cell growth while 2.1×10^5, and 4.2×10^5 cells/cm^2 tightness of the monolayers reached the plateau after 4 days cell growth. The behavior of cell densities 2.1×10^5, and 4.2×10^5 cells/cm^2 was quite similar although TEER values of higher density were about 100–200 Ω higher (about 800 Ω). It seemed that 10 days of cell growth did not give any better results than 7 days cell growth. Actually TEER-values declined which might be an indication of increased apoptosis (Yamashita et al. 2002) We continued the experiments with densities of 1.8×10^5, 2.1×10^5, and 3.1×10^5 cells/cm^2. With the density of 2.1×10^5 cells/cm^2 the obtained TEER values were most reproducible and stayed stable at 600 ± 70 Ω (n = 16). When the obtained TEER data was combined with results from ^{14}[C]-mannitol and Lucifer Yellow permeation experiments the results indicated clearly the advantageousness of this density. ^{14}[C]-Mannitol permeation rate stayed under 0.5% per 60 min and Lucifer Yellow rejection was 99.3 ± 1.7 (n = 160). With the cell density of 1.8×10^5 cells/cm^2 the values were lower (~1% and ~98%, respectively) and with cell density of 3.1×10^5 cells/cm^2 there was a lot of variation and the TEER values started to go down after five days of cell growth (data not shown). These results are in good correlation with studies of Alsenz and Haenel (2003). Although their plate and feeding system was different they used seeding densities comparable to ours. The ratio of cell amount between 21 day growth on 12-wellplate and 7 day growth on 96-wellplate was the same (Alsenz et al. 1998; Alsenz and Haenel 2003). Balimane et al. (2004) and Marino et al. (2005) have used lower cell densities on 96-wellplates, but they grew the cells for three weeks. The optimum passage range has been suggested to be 28–65 (Briske-Anderson et al. 1997). Our studies did not reveal any difference in behavior of the cells during passages 50–72 although higher passages have been reported in some cases to affect favorable to rapid cell growth and monolayer formation (Briske-Anderson et al. 1997; Yu et al. 1997). Summary of the differences of 12-wellplate method and 96-wellplate method with selected cell density 2.1×10^5 cells/cm^2 is presented in Table 1. New method clearly shows cost effectiveness. When this method is used, 18 times less medium is needed during cultivation and growth time is 1/3 of the old 12-wellplate method. By our modifications, labor and material costs decrease significantly and more experiments can be done in less time.

Different ^{14}[C]-mannitol sample volumes and two scintillation cocktails with different combinations were investigated to obtain optimal composition for measurements. ^{14}[C]-Mannitol measurements were previously done with liquid scintillation counter using 100 µl of sample and 4000 µl scintillation cocktail (Optiphase HiSafe 2, Wallac Fisher Chemicals, Loughborough, England). We miniaturized the assay to 96-plate format and concluded that 50 µl sample volume with 150 µl scintillation cocktail (Optiphase SuperMix, Wallac Fisher Chemicals, Loughborough, England) gave the most reliable results even in these small concentrations (<0.05%). Lucifer Yellow samples were 50 µl aliquots from the donor compartments. The same sample could be used for the drug concentration assays as well as the determination of monolayer formations since Lucifer Yellow did not affect the detection of studied drugs and the studied drugs did not affect the detection of Lucifer Yellow. After optimization of cell density we continued using Lucifer Yellow assays in the further experiments due to its easiness and non-radioactivity. However, it should be borne in mind that ^{14}[C]-mannitol assay is needed if samples are colorful or in some other way disturb Lucifer Yellow measurements or vice versa.

Table 1: Summary of the differences between 96-well plate and 12-well plate methods

Assay conditions	96-well plate method	12-well plate method
Cell amount (well)	23000	75000
Cell density (cells/cm^2)	2.1×10^5	0.75×10^5
Cell growth time (d)	7	21
Assay volume AP (µl)	75	500
Assay volume BL (µl)	250	1500
Medium consumption during cultivation/well (µl)	975	18000

The efficacy of many drugs depends on their ability to cross cellular barriers. However, sometimes absorption is limited by efflux transporters present in the plasma membrane. They might play a crucial role in limiting drug absorption through drug secretion into the intestinal lumen. Multidrug resistance (MDR) ATP-binding cassette transporters are a super family of structurally related membrane proteins that mediate active translocation of a diverse range of molecules across plasma membrane (Mahadevan and Shirahatti 2005). P-gp is the protein product of the multidrug resistance (MDR1) gene which consists of two similar transmembrane domains joined by a linker region (Idriss et al. 2000). Each transmembrane domain is followed by cytoplasmic nucleotide domain which is responsible for the ATP hydrolysis associated with the drug efflux. P-gp acts as a permeability barrier present on the apical surface of the enterocytes (Balimane et al. 2006). Transportation of rhodamine 123, typical substrate of P-gp, AP-BL as well as BL-AP directions with and without verapamil, was used to study efflux activity (Fig. 1). The efflux ratio P_{app}, BL-AP/P_{app}, AP-BL has been used extensively to assess how P-gp-mediated efflux activity affects the transport of P-gp substrates (Troutman and Thakker 2003). Rhodamine 123 permeation was greater from BL-AP direction than AP-BL direction. This difference was reduced in the presence of verapamil, the P-gp inhibitor. These findings were in good correlation with studies of Troutman and Thakker (2003). Only secretory transport was affected in the presence of P-gp inhibitor and no effect was seen on the absorptive flux. These findings together with confocal laser microscopic studies (Fig. 2) confirmed that P-gp was active in our cells. It has been reported that functionality of MDR1 decreases in passages 45+ (Siissalo et al. 2007), but this was not the case with our cell line. The follow up of P-gp expression during passages 50–72 showed that expression was steady during these passages as seen also in Fig. 1. The results empha-

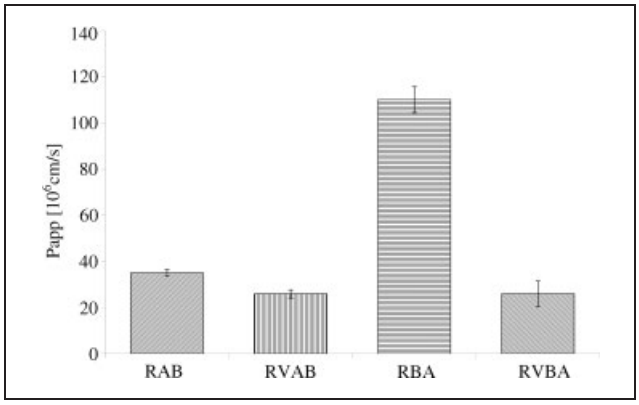

Fig. 1: Rhodamine 123 permeation apical to basolateral (AB) and and basolateral to apical (BA) directions alone (R) and in the presence of verapamil (RV). Values are presented as permeation coefficients ± SDs. (n = 7)

size the importance of defining the activity when using cells of different origin and different passages.

Confocal microscopic data showed that the cells grown in 96-wellplates for 7-, 10- and 21-days formed monolayers. Immunoreactivity of actin was easily recognizable in permeabilized Caco-2 cells (Fig. 2). In permeabilized cells subunits on the plasma membrane and in intracellular parts were labeled. In unpermeabilized cells only subunits on the plasma membrane were labeled. Stack images showed that 95% of the cells grown for 7 days existed as monolayers. Amount of multilayer structures seemed to increase in cultures grown for 21 days. Expression studies of P-gp showed labeling in Caco-2 cells grown for 7 days. Labeling seemed to be localized on the plasma membrane. Labeling was seen in permeabilized cells, but not in unpermeabilized cells. No immunostaining was observed when the primary antibodies were omitted. These results confirmed the proper monolayer formation results from TEER measurements and Lucifer Yellow and mannitol permeation experiments as well as P-gp expression from rhodamine 123 permeation experiments.

Permeability assay was put up on a Biomek FX-workstation. The suitability of the assay for automation was evaluated by several experiments. Height of tips when aspirating was studied carefully to obtain as much liquid as possible but at the same time avoiding harm to the cells. Because of the large number and the small size of wells the automated handling was a more accurate way to perform these experiments. Well-to-well variation (n = 40) of Lucifer Yellow rejection (%) on four different days is presented in Table 2. The results indicate that tips do not harm the cell monolayer and pipetting is accurate and permeation is stable between the wells. Day-to-day variation was 99.3 ± 0.55 (n = 4) indicating repeatability. Manual work is still needed for TEER measurements and maintaining the cells but for the assay precision pipetting at the same height and at the same time is crucial in the 96-well environment. Our studies indicate that the automated method is suitable for these cell experiments and gives accurate results. The method enables performance of the whole experiment with the automated system and handling of numerous samples.

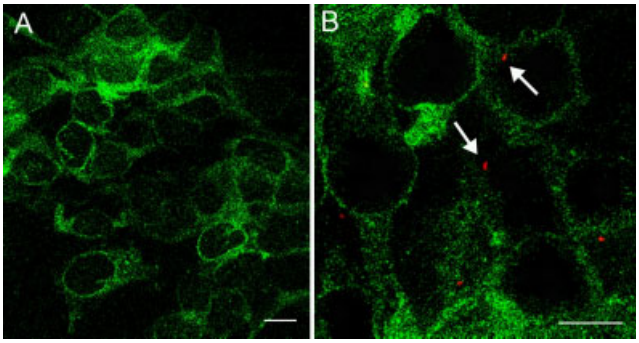

Fig. 2: Staining for actin (A) and P-glycoprotein (B) of Caco-2 cells. The cells were grown for 7 days. The cells were fixed and permeabilized with Triton X-100. Actin and P-glycoprotein were labeled with specific antibodies. Stack images were taken from the labeled cells. Arrows (B) indicate P-glycoprotein labeling. Majority of the cells (95%) were in mono-layer structures. Scale bar = 10 μm

Table 2: Well to well variation of Lucifer Yellow rejection on four different days (n = 40)

Experiment	LY rejection ± SD (%)
1	99.5 ± 1.1
2	99.7 ± 0.6
3	98.5 ± 2.9
4	99.4 ± 0.9

2.2. Permeability of model compounds

Permeability of metoprolol, ketoprofen, verapamil, naproxen, and hydrochlorthiazide across Caco-2 monolayer on 12-well plates and on 96-well plates was compared. 12-well experiments were performed manually and 96-well experiments automatically. Each test was performed several times (from 24 to 60 replicates). The pH 7.4 for both apical and basolateral sides was selected due to long term knowledge gained of Caco-2 permeation at this pH in the literature (for example Artursson et al. 1996; Markowska et al. 2001; Polli 2001) and in our lab (Laitinen et al. 2004; Tammela et al. 2004; Kreander et al. 2006; Riihimäki et al. 2007). Since we know how our cells should behave at this pH, the differences between 12- and 96-wells could be monitored more effectively. According to the pH partition theory permeability of basic drugs increases when pH increases and acidic drugs behave in the opposite way. In the lumen absorption is more complicated due to variable pH in different parts of the intestine. Therefore we cannot completely mimic the situation in the intestine using Caco-2 cell model systems with fixed pH.

The obtained P_{app} values from transport experiments of metoprolol, ketoprofen, verapamil, naproxen and hydrochlorthiazide in 12- and 96-wellplates are presented in Table 3. Metoprolol is a basic, rapidly transported drug and its permeability in Caco-2 cells correlates well with absorption in human jejenum (Lennernäs et al. 1996). Ketoprofen and naproxen are acidic compounds. Their permeability is assumed also to be mediated by monocarboxylic acid transporter MCT1 (Tsuji et al. 1994; Tamai et al. 1995, Choi et al. 2005). Carrier mediated monacarboxylic acid transport is demonstrated to be functioning in Caco-2 cells in accordance with those carriers in intestinal brush-border membrane (Tsuji et al. 1994; Takanaga et al. 1994). Verapamil is a known substrate of efflux protein P-gp (Tsuruo et al. 1981, Orlowski et al. 1996; Collett et al. 2004) and hydrochlorthiazide is a low permeable compound with two dissociation groups, and pH gradient does not affect its permeability (Yamashita et al. 2000).

As seen in Table 3 differences between 12- and 96-well assays were quite minor. Metoprolol permeation was high and hydrochlorthiazide permeation was low as expected. Verapamil permeability was high. At the concentration

Table 3: Permeation of the model compounds across Caco-membranes in 12- and 96-well plates. Values are presented as permeation coefficients ± SDs

Compound	Permeation characteristic	$P_{app\ (12\text{-well plate})}$	n	$P_{app\ (96\text{-well plate})}$	n
Metoprolol	High, transcellular	29.0 ± 3.9	60	27.3 ± 4.8	56
Verapamil	High transcellular, P-gp substrate	25.6 ± 2.9	57	27.6 ± 6.4	56
Ketoprofen***	High, transcellular, active transport	24.5 ± 3.5	58	33.5 ± 1.5	56
Naproxen*	High, transcellular, active transport	32.5 ± 0.9	24	36.9 ± 1.6	28
Hydrochlorthiazide	Low, paracellular	7.2 ± 2.7	24	7.7 ± 4.6	28

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

level 0.25 mM used, most probably the active efflux has been saturated (Garrigos et al. 1993; Balimane et al. 2006) and the effect of P-gp activation could not be seen. It has been shown that only at very low donor concentrations (≪0.05 mM) the effect of P-gp is detected. (Doppenschmitt et al. 1999; Balimane et al. 2006). Data analysis with unpaired t-test showed small difference in the permeability of naproxen and significant difference in the permeability of ketoprofen. These compounds are transported not only via transcellular but also via active transport. In Caco-2 cells carrier mediated transport could be variable and generally lower than seen *in vivo* (Hu and Borchardt 1990; Lennernäs et al. 1996). It might be that in the different growth circumstances carrier expression is different and that could explain the differences between these two formats. Deviation on the other hand was very small, especially in the case of naproxen, indicating that permeation is stable. The real magnitude of influence of carrier mediated transport could only be confirmed with characterization of all transporters involved.

Ketoprofen and naproxen possess similar physicochemical parameters with molecular weights of 254.3 and 230.3, LogP 3.12 and 3.18, pKa 4.6 and 4.2, respectively. Ketoprofen, on the other hand, is more soluble and has greater polar surface area 54.4 $Å^2$ than naproxen 46.5 $Å^2$. It seems that ketoprofen acts differently in the minor environment in 96-wellplates compared to in 12-wellplates. More detailed studies need to be conducted to find out the reason for the different behavior. Despite the small difference in naproxen permeation the magnitude of the permeation data from 96-wellplate and from 12-wellplate experiments and permeation characteristics are similar. This indicates that also acidic compounds with active transport could be studied accurate with our 96-well permeation model.

Although behavior of ketoprofen on 96-wellplates remains to be clarified, the similarity of the other results indicates that our new model is suitable for permeation experiments and gives reliable first information about the compound permeability. Based on results of these experiments the most interesting compounds could then be selected for more detailed permeation studies and further drug discovery purposes.

2.3. Conclusions

The aim of this study was to develop a rapid permeability screening method for drug discovery process. The method could be used to provide first information of permeability of pharmacologically active compounds.

The confocal imaging data showed that Caco-2 cells form monolayers, when cultured for 7 days on the Multi-Screen™ Caco-2 plates with initial cell density of 2.1×10^5 cells/cm^2. Proper TEER-values, ^{14}[C]-mannitol and Lucifer Yellow permeation data showed that intact monolayer was formed. P-gp labeling showed that P-gp was present in Caco-2 cells and seemed to be localized on the plasma membrane. Permeation of model compounds metoprolol, verapamil, naproxen, and hydrochlorthiazide in 96-well plates was comparable to those obtained from traditionally used 12-well plate experiments. Ketoprofen acted differently on 96-well plates but permeability of the very similar compound naproxen indicates that also acidic compounds with active transport could be studied accurately with our 96-well permeation model. Automation of the protocol made it more accurate and enables the handling of a large number of samples. Based on these results we managed to build up a fast miniaturized and automated protocol to make a first permeation evaluation of new compounds in the drug discovery process.

3. Experimental

3.1. Materials

Dulbecco's modified Eagle medium (DMEM), foetal bovine serum (FBS), Hank's balanced salt solution (HBSS) and HEPES solution were bought from Gibco Invitrogen Corporation (Paisley, Scotland, UK). Phosphate buffered saline (PBS), nonessential amino acids (NEAA), L-glutamine, antibiotic mixture (10 000 IU/ml Penicillin G, 10 000 µg/ml streptomycin) and trypsin were obtained from Cambrex Bio Science (Verviers, Belgium). D-[1-^{14}C]-mannitol was bought from Ammersham Pharmacia Biotech UK Ltd. (Ammersham, England) and Lucifer Yellow and rhodamine 123 from Fluka Chemie AG (Buchs, Switzerland). Acetonitrile was purchased from Rathburn (Walkerburn, Scotland) and formic acid from Riedel-de Haën (Seelze, Germany). MilliQ water was prepared with Millipore Milli-Q plus system (Molsheim, France). DMSO was obtained from Merck (Darmstadt, Germany). Metoprolol, ketoprofen and naproxen were purchased from ICN Biomedicals Inc. (Aurora, Ohio, USA) and verapamil, ranitidine, hydrochlorthiazide and Triton X-100 from Sigma Chemical Co (St.Louis, MO, USA).

3.2. Cell culture

Caco-2 cells (originating from American Type Culture Collection) were kindly donated by Professor Arto Urtti, Drug Discovery and Development Technology Center, Faculty of Pharmacy, University of Helsinki, Finland. Cells were grown in a medium comprised of Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/l glucose and supplemented with 10% (v/v) heat inactivated (56 °C/30 min) foetal bovine serum (FBS), 1% (v/v) glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), and 1% Minimum Essential Medium (MEM) nonessential amino acids. Cultures were maintained at 37 °C in an incubator (BB 16 Gas Incubator, Haeraeus Instruments Gmbh, Hanau, Germany) in an atmosphere of 95% air and 5% CO_2 at 95% humidity. Cells were harvested with trypsin-EDTA and seeded on to polycarbonate filter membranes with pore size of 0.4 µm and growth areas of 1.0 cm^2 in clusters of 12 wells (Corning Costar Corporation, Cambridge, MA, USA) at a density of 0.75×10^5 cells/cm^2 or onto polycarbonate filter membranes with pore size of 0.4 µm and growth areas of 0.11 cm^2 in clusters of 96 wells (MultiScreen™ Caco-2, Millipore, USA) at a density of $0.93–4.2 \times 10^5$ cells/cm^2. The growth medium was changed every second day. Passages from 50 to 72 were used for the permeability experiments.

3.3. Preparation of test solutions

Metoprolol, verapamil, ketoprofen, naproxen and hydrochlorthiazide were dissolved into HBSS containing 10 mM HEPES (pH 7.4) to concentrations of 250 µM. After dilution pH levels were checked and corrected to pH 7.4 if necessary. ^{14}[C]-Mannitol 0.6 µCi/ml, Lucifer Yellow 100 µg/ml and rhodamine 123 50 µM (to apical side) and 5 µM (to basolateral side) solutions

were prepared in the similar manner. The concentrations were selected based on literature and knowledge of our previous studies (User guide; Millipore Corporation 2002, Laitinen et al. 2004; Tammela et al. 2004; Kreander et al. 2006; Riihimäki et al. 2007; Siissalo et al.). Solutions were kept at −20 °C until used.

3.4. Optimization of cell growth and experiments on 96-well plates

7-, 10- and 21-day experiments were done at first with cell densities of $0.93\times$-, $2.1\times$-, and 4.2×10^5 cells/cm^2 and then based on obtained results with cell densities of $1.8\times$-, $2.1\times$-, 3.1×10^5 cells/cm^2. The monolayer formation was evaluated daily by measuring transepithelial electrical resistance (TEER) (Evom-X, World Precision Instruments Inc. USA) and permeation of paracellular markers, fluorescent Lucifer Yellow and radioactive ^{14}[C]-mannitol, were used after adequate cell growth for evaluation within experiments. The ^{14}[C]-activity was measured with Microbeta® Trilux liquid scintillation counter (PerkinElmer Life and Analytical Science/Wallac Oy, Turku, Finland) and the Lucifer Yellow permeation was determined with a Varioskan scanning spectrofluorometer and spectrophotometer (Thermo Electron Corporation, Vantaa, Finland) with exitation-wavelength 430 nm and emission-wavelength 535 nm. Functionality of P-gp was assessed with permeation studies with known P-gp substrates rhodamine 123 alone and with verapamil apical to basolateral (AP-BL) as well as basolateral to apical (BL-AP) directions. Samples were collected from AP-BL experiments after 0, 20, 40, 60 and 120 min and from BL-AP experiments after 0, 3.5, 7, 10 and 15 min to maintain sink conditions. The experiments were conducted and the results were calculated as described under "Permeability experiments". The Varioskan scanning spectrofluorometer and spectrophotometer with exitation-wavelength 485 nm and emission-wavelength 535 nm was used to obtain the results.

Automated Caco-2 permeation experiments were put up on a Biomek FX workstation equipped with a 96-channel head (Beckman Coulter Inc., CA, USA). Pipetting parameters and robotic handling during the process was optimized and the results of these experiments were compared to those obtained from manually performed experiments. The workstation was used to perform all pipetting steps as well as sampling.

3.5. Light microscope immunohistochemistry

The labeling of the Caco-2 cells was based on Maimone and Merlie (1993). The cells grown for 7-, 10- and 21-days on MultiScreen™ Caco-2 plates were washed with phosphate buffered saline (PBS) and incubated with fixative (1% paraformaldehyde, 100 mM L-lysine and 10 mM sodium metaperiodate) for 20 min at room temperature. After rinsing 3 times with PBS for 5 min cells were permeabilized with 1% Triton X-100 in PBS for 10 min and washed three times in PBS. The cells were stored overnight in 10% foetal bovine serum in PBS. For staining, the wells were incubated in 100 µl of primary antibody [actin; sc-8312, rabbit (Santa-Cruz, California, USA), P-gp; C219, mouse monoclonal (Signet Laboratories, Dedham, USA), diluted in 10% foetal calf serum in PBS] and incubated for 2 h at room temperature. Wells were rinsed three times in PBS and incubated in 100 µl of secondary antibody [actin; sc-2090, goat anti-rabbit conjugated with fluorochrome, (Santa-Cruz, California, USA), P-gp; T-2762, tetramethylrhodamine, Molecular Probes, Carlsbad, California, USA] in 10% foetal calf serum in PBS, and incubated for 1 h at room temperature. After incubation the wells were washed three times in PBS for 5 min at a time. After the washings the bottoms of the wells were removed and placed onto a drop of Vectashield (Vector Laboratories, Burlingame, USA). The excess of Vectashield was removed and the edges of the coverslips were sealed by nailpolish. Light microscope images were captured by using a confocal microscopy (Zeiss LSM 510, Germany) with oil 63x objective (plan apochromat, oil 63x/1.4 numeric aperture).

3.6. Image processing

Stack images were taken from the cells. The analyzed images were from the last third of the cell facing the coverslip. The brightness of the confocal microscopy images was adjusted to the whole image (Adobe Photoshop 6.0, Adobe Systems, San Jose, CA). The images were reduced to the desired size (300 dpi).

3.7. Permeability experiments

Model compounds for permeation tests were selected based on their different permeability according to FDA (2000). Permeation of 250 µM metoprolol, ketoprofen, verapamil, naproxen and hydrochlorthiazide was evaluated both on 96-well plates and 12-well plates AP-BL direction.

The permeation test was performed in an incubator (Stuart, orbital incubator S150, Bibby Sterilin Limited, USA), in humified atmosphere shaking 75 rpm at 37 °C. After TEER-value measurement the cell monolayers were washed twice with HBSS solution. After washing cell monolayers were allowed to equilibrate for half an hour prior to experiments. TEER-values were measured again and the apical solution was changed to HBSS containing the drugs. Samples were collected after 0, 20, 40, 60 and 120 min by moving the insert into a new receiver well containing fresh HBSS-solution After 120 min monolayers were washed again with HBSS solution and TEER values were measured to assure the monolayer integrity. Experiments were performed under sink conditions i.e. moved before >10% of the drug had been transported. By the use of sink conditions the influence of the drug diffusing back from the receiving to the donor compartment could be minimized. Samples were kept at −20 °C until analyzed. The apparent permeability coefficients P_{app} (cm/s) were calculated according to equation:

$$P_{app} = (dQ/dt)/(A \times 60 \times C_0)$$

Where dQ/dt is the amount of compound transported within a given time period, A is the surface area of the monolayer (cm^2), 60 is the conversion from minutes to seconds, C_0 is the initial drug concentration on the donor compartment (µg/ml).

3.8. Sample analysis

Drug samples from permeability studies were analyzed using a reversed-phase HPLC system (PerkinElmer Series 200 LC pump and autosampler and PE Nelson 600 series link, Norwalk, CT, USA) equipped with a diode array detector (PerkinElmer LC 235 C, Norwalk, CT, USA). A Gemini C18 column (250 mm × 4.6 mm; 5 µm, Phenomenex, USA) with a C18 guard column (SecurityGuard™, Phenomenex, USA) was used with a flow rate of 1 ml/min. For determination of ketoprofen and naproxen, the mobile phase consisted of acetonitrile (HPLC-grade) and 0.1% (v/v) aqueous formic acid (pro analysis). For determination of metoprolol, verapamil and hydrochlorthiazide mobile phase consisted of acetonitrile (HPLC-grade) and MilliQ water. The injection volume for the samples was 20 µl. The separations were performed by gradient elution. For determination of metoprolol, a 5 min isocratic section was followed by a linear gradient 5–40 (B%) in 5 min and followed by an 8 min isocratic section ($\lambda = 200$ nm). Ketoprofen and naproxen samples were analyzed with the same mobile phase where a 5 min isocratic section was followed by a linear gradient 60–95 (B%) in 2 min and followed by an 8 min isocratic section ($\lambda = 255$ nm). For determination of verapamil, an 8 min isocratic section was followed by a linear gradient 20–70 (B%) in 5 min and followed by a 7 min isocratic section ($\lambda = 200$ nm). The amount of hydrochlorthiazide was determined with a method where a 5 min isocratic section was followed by a linear gradient 20–70 (B%) in 5 min and followed by a 5 min isocratic section ($\lambda = 220$ nm).

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