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Caco-2 cell monolayers as a tool to study simultaneous phase II metabolism and metabolite efflux of indomethacin, paracetamol and 1-naphthol

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ABSTRACT

The human intestinal cell line, Caco-2, was used to study compounds – indomethacin, paracetamol and 1-naphthol – that undergo intestinal phase II metabolism followed by apical and/or basolateral efflux of the metabolites and/or parent compounds. The interplay was studied during permeability experiments across fully differentiated Caco-2 cell monolayers. The parent compounds and their glucuronide and/or sulfate metabolites were detected by LC–MS/MS. Conjugation of the model compounds and effluxes of their metabolites were observed. The efflux of indomethacin glucuronide was apical, but complementary basolateral efflux was observed at the highest indomethacin concentration (500 μM), probably due to apical saturation. Paracetamol glucuronide was not formed in these experiments, but apical and basolateral effluxes of paracetamol sulfate were observed. A typical bell-shaped inhibition curve was observed for the formation of 1-naphthol glucuronides, indicating substrate or product inhibition of the UGT enzyme(s) at higher 1-naphthol concentrations (200 μM and 500 μM). Based on these results, the fully differentiated Caco-2 cell monolayers can be applied as a platform for qualitative *in vitro* studies, where phase II metabolism and efflux activities are ongoing simultaneously.

1. Introduction

One of the pending challenges in drug development is the prediction of the intestinal drug absorption. During the absorption from the gastrointestinal tract, a drug is exposed to several active barrier mechanisms, where the kinetics of the processes involved could limit the absorption or have a role in drug interactions. Several metabolic enzymes and efflux transporters are known to be functional in the enterocytes (Pang et al., 2007). In recent years, the interplay of these active processes has attracted a lot of research interest (Jeong et al., 2005).

Unlike the classic example of the co-regulated MDR1 (Multidrug resistance protein 1, P-glycoprotein) and CYP3A4 (Cytochrome P450 enzyme 3A4) working in parallel and sharing many substrates and inhibitors (Wacher et al., 1995; Christians, 2004; Cerveny et al., 2007), the interplay of phase II drug metabolism and efflux is concatenated, i.e. the enzymes and efflux proteins work in a serial manner. The conjugated metabolites produced by enzymes such as UDP-glucuronosyltransferases (UGTs) or sulfotransferases (SULTs) are substrates for the apical MRP2 (Multidrug resistance associated protein 2) (or BCRP [Breast cancer resistance protein]) or basolaterally localized MRPs like MRP3 (Haimeur et al., 2004). Thus, in this co-operation the role of the efflux proteins is to prevent the intracellular accumulation of phase II metabolites. This interplay has so far been mainly studied for flavonoids and toxins, but it may also take place during the absorption of perorally administered drugs susceptible to glucuronidation or sulfation (Zhang et al., 2007; Sergent et al., 2008; Sun et al., 2008).

There is a recognized need to develop new *in vitro* methods for intestinal absorption studies and further improve the existing methods (EMEA [European Medicines Agency], 1997). The currently used methodologies range from isolated cell membranes and other subcellular fractions to *in vivo* knockout animals (Xia et al., 2007; Van de Kerkhof et al., 2007). In the more simplified methods, only a limited number of active processes can be observed. For example, cell membranes transfected with a specific efflux transporter are deficient of the possible compensatory role of other transporters sharing the same substrates or inhibitors. On the other hand, most of the more complex systems are not fully characterized, which complicates the interpretation of the results with regards to the contributions of each active process.

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Table 1
Physicochemical properties of the model compounds used in this study.

Compound	MW	pK_a	Log P_{oct}	PSA
Indomethacin	357.8	4.1	3.1	68.5
Paracetamol	151.2	9.5	0.3	49.3
1-Naphthol	144.2	9.3	2.8	20.2

MW = molecular weight [g/mol], pK_a = acid dissociation constant, log P_{oct} = octanol/water partition coefficient, PSA = polar surface area [Å^2].

Interspecies differences in the expression and functionality of efflux transporters and metabolic enzymes are known limitations of preclinical *in vivo* studies and non-human based cell lines (Xia et al., 2006; Komura and Iwaki, 2007).

The Caco-2 cell line, originating from human colorectal carcinoma, has been utilized as a model for intestinal absorption for over 20 years (Hilgers et al., 1990; Artursson and Karlsson, 1991). Grown as monolayers on filters, fully differentiated Caco-2 cells deserve the status as the "golden standard" among the *in vitro* models for the intestinal epithelium (Hubatsch et al., 2007). There is some variation in the characteristics of Caco-2 cells grown in different laboratories, which emphasizes the need for intralaboratory standardization (Hayeshi et al., 2008). Our Caco-2 cell line has been thoroughly characterized with regards to the expression and functionality of efflux proteins and UGT enzymes (Siissalo et al., 2007, 2008, 2009), while other groups have reported SULT expression and functionality in differentiated Caco-2 cells (Meinl et al., 2008). Sulfation and glucuronidation of a drug compound in Caco-2 monolayers, followed by efflux of the metabolites, has been reported only for raloxifene and methyldopa (Chikhale and Borchardt, 1994; Jeong et al., 2004). Recently, theoretical pharmacokinetic models of Caco-2 cells incorporating metabolism and efflux have been constructed (Sun and Pang, 2008; Sun et al., 2008). With verified extensive phase II metabolism and efflux functions, Caco-2 could also be used as a platform for routine qualitative *in vitro* studies of the interplay between intestinal drug conjugation and efflux.

In this study, we have examined the phase II metabolism of three model compounds in differentiated Caco-2 monolayers, followed by the efflux of their metabolites into the apical and/or basolateral sides of the cell monolayer (Table 1). Indomethacin was chosen as an example of a drug compound susceptible to intestinal glucuronidation (Mano et al., 2006). Paracetamol (acetaminophen) was used as a model drug known for its detoxification both via sulfation and glucuronidation (Prescott, 1980). In addition, 1-naphthol was included as a common model compound for high glucuronidation activity (Siissalo et al., 2008). The contribution of CYP (cytochrome P450) metabolism in Caco-2 cells was not considered significant.

2. Materials and methods

2.1. Materials

Indomethacin was purchased from Fluka (Buchs, Switzerland), paracetamol (acetaminophen) from Hawkins Inc. (Minneapolis, MN, USA) and 1-naphthol from Sigma–Aldrich (St. Louis, MO, USA). The cell culturing reagents were purchased from Euroclone (Pero, Italy) except for foetal bovine serum and HBSS (Hank's buffered salt solution) 10× concentrate from Gibco Invitrogen Corporation (Carlsbad, CA, USA) and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) from Sigma (St. Louis, MO, USA). All the plasticware were obtained from Corning B.V. Life Sciences (Schiphol-Rijk, Netherlands).

HPLC (high performance liquid chromatography) grade acetonitrile was obtained from Merck (Darmstadt, Germany) and ammonium acetate from BDH Laboratory Supplies (Poole, UK). Water was in-house freshly prepared with Direct-Q (Millipore Oy, Espoo, Finland) purification system and UP grade (ultra pure, 18.2 MW). Other chemicals were obtained mainly from Sigma Chemical Company (St. Louis, Missouri, USA) and VWR International (Espoo, Finland) and were of the highest purity available.

2.2. Cell cultures

Human colon adenocarcinoma (Caco-2) cells were obtained from American Type Culture Collection (Rockville, MD, USA) at passage 18. The cells were maintained at +37 °C in an atmosphere containing 5% CO_2 at 95% relative humidity, in a medium consisting of DMEM (Dulbecco's Modified Eagle Medium, high glucose 4.5 g/l), 10% HIFBS (Heat Inactivated Foetal Bovine Serum, inactivation at +56 °C for 30 min), 1% NEAA (Non Essential Amino Acids), 1% L-glutamine, penicillin (100 IU/ml) and streptomycin (100 μg/ml).

The cells were grown in 75 cm^2 plastic flasks and harvested weekly with 0.25% trypsin. The cells (passages 34–41) were seeded at 6.8×10^4 cells/cm^2 onto polycarbonate filter membranes (pore size 0.4 μm, filter area 1.1 cm^2) in 12-well Transwell® insert plates (Corning Cat. No. #3401), and grown for 21–28 days for the transport experiments. The medium was changed three times a week.

2.3. Permeability experiments

Transport and metabolism of the model compounds in Caco-2 monolayers was studied both in apical to basolateral (A → B) and basolateral to apical (B → A) transport directions at pH 7.4. The cell monolayers were washed twice with preheated HBSS–HEPES (pH 7.4; 10 mM HEPES) and equilibrated for 30 min at 37 °C. The integrity of the cell monolayers was verified by measuring transepithelial electrical resistance (TEER above 250 Ω cm^2) across each monolayer before and after the experiment using the Millipore Millicell®-ERS device (Bedford, MA, USA) at 37 °C.

Indomethacin (5 μM, 20 μM, 50 μM, 200 μM or 500 μM), paracetamol (20 μM, 50 μM, 200 μM, 500 μM or 1 mM) or 1-naphthol (5 μM, 20 μM, 50 μM, 200 μM or 500 μM) solutions were placed in the apical (700 μl) or basolateral (1700 μl) chamber and a donor sample of 200 μl was drawn immediately. During the experiments, samples were taken from both apical (400 μl) and basolateral (1 ml) compartments at 30 min, 60 min and 120 min and replaced with the parent solution (donor chamber) or study buffer (receiver chamber). At the end of the experiments, the filters were washed briefly with cold buffer, cut off with a scalpel and transferred into separate microcentrifuge tubes with 250 μl cold buffer. The cells were disrupted by freeze-thawing the filters and the tubes were centrifuged at 13,000 rpm for 5 min (Eppendorf Centrifuge 5415D, Eppendorf AG, Hamburg, Germany), after which 200 μl samples were drawn from each supernatant for the determination of residual model compounds and metabolites in the cells. All the samples were stored at −20 °C until they were sent for LC–MS/MS analysis.

2.4. Liquid chromatography–mass spectrometry

The samples were thawed at room temperature (RT), shaken and centrifuged for 10 min at 16,100 × *g* (Eppendorf 5415D, Eppendorf AG, Hamburg, Germany) and pipetted to Total Recovery vials (Waters Corporation, Milford, MA, USA) until the LC–MS/MS analysis.

A Waters Alliance 2695 chromatographic system (Waters Corp., Milford, MA, USA) with autosampler, vacuum degasser and column oven was used. The analytical column used was a Waters XBridge Shield RP18 (2.1 mm × 50 mm, 3.5 μm), together with Phenomenex Luna-C18 precolumn, 4.0 mm × 2.0 mm, 3.0 μm (Phenomenex, Torrance, CA, USA). The eluents were 2 mM ammonium acetate (A, pH 7.0) and acetonitrile (B). For 1-naphthol, indomethacin and

their conjugates a linear gradient elution from 10% B to 90% B in 1 min was employed, followed by 2 min isocratic elution with 90% B and column equilibration with flow rate of 0.4 ml/min. For paracetamol and its conjugates a linear gradient elution from 5% B to 90% B in 2.5 min was employed, followed by column equilibration with flow rate of 0.3 ml/min. In all analyses the column temperature was 35 °C. The flow was split post-column to Waters 2996 photo-diode-array (PDA) detector and a mass spectrometer ion source by using an Acurate Post-Column Stream Splitter (LC Packings, Amsterdam, The Netherlands) with ratio of 3:1, respectively. LC–MS/MS data were acquired with a Micromass Quattro Micro triple quadrupole mass spectrometer (Micromass Ltd., Manchester, England) equipped with a Z-Spray electrospray ion source, using a negative ionisation and a multiple reaction monitoring (MRM) mode. The monitored MRM transitions were m/z 319 > 143 for 1-naphthol glucuronide (collision energy 20 eV, cone voltage 30 V), m/z 356 > 312 for indomethacin (10 eV, 32 V), m/z 532 > 356/193 for indomethacin glucuronide (14/30 eV, 22 V), m/z 436 > 356/97 for indomethacin sulfate (14/30 eV, 22 V), m/z 150 > 107 for paracetamol (20 eV, 32 V), m/z 326 > 150/193 for paracetamol glucuronide (22 eV, 26 V) and m/z 230 > 150 for paracetamol sulfate (18 eV, 32 V). In addition, 1-naphthol was monitored by UV detection at wavelength of 295 nm. Nitrogen was used as both desolvation and nebulising gases, while argon was used as collision gas at 3.8×10^{-3} mbar pressure. The mass spectrometer and HPLC system were operated under Micromass MassLynx 4.0 software.

2.5. Data analysis

The amounts of parent compounds and metabolites removed in the samples and added during donor solution replacements were accounted for in the calculations. Apparent permeability coefficients (P_{app}, cm/s) were calculated based on Eq. (1).

$$P_{app} = \frac{dQ/dt}{A \times C_0 \times 60} \quad (1)$$

where dQ/dt = cumulative transport rate (nmol/min), A = surface area of the cell monolayer (1.1 cm^2) and C_0 = initial concentration in donor compartment (nmol/ml).

Efflux ratios were calculated from the apparent permeability coefficients (Eq. (2)).

$$\text{Efflux ratio} = \frac{P_{app,B\rightarrow A}}{P_{app,A\rightarrow B}} \quad (2)$$

3. Results

3.1. Indomethacin

The permeability of indomethacin across Caco-2 monolayers was quite consistent in both directions over the studied concentration range, except for 5 μM (Fig. 1). Indomethacin glucuronides were formed in all the studies, but at the lowest parent compound concentrations the metabolite concentrations were under the limit of quantification (Fig. 2). The efflux of indomethacin glucuronide was apical. Nonetheless, complementary role of basolateral efflux was observed at the highest indomethacin concentration (500 μM) in the A → B direction, as the apical efflux was probably saturated. Residual intracellular amounts of indomethacin were small and no residual glucuronides were detected.

3.2. Paracetamol

The permeability of paracetamol was passive and consistent in both directions over the studied concentration range except for

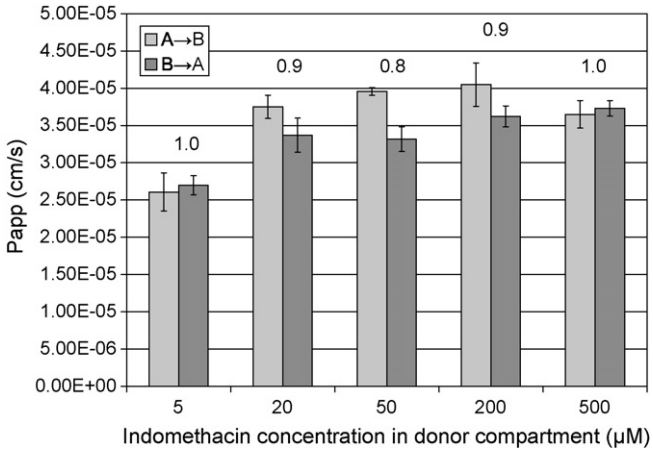

Fig. 1. Transport of parent indomethacin across the Caco-2 monolayers during the metabolism/permeability studies ($P_{app} \pm$ SD, $n = 3$). Efflux ratios (B → A vs. A → B) for each pair of experiments are presented above the respective bars.

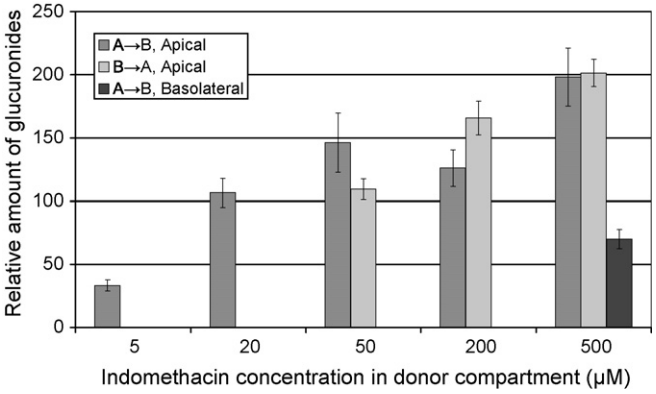

Fig. 2. The relative amounts of indomethacin glucuronide observed in the apical and basolateral Transwell® compartments at 120 min during the Caco-2 permeability studies of indomethacin at different initial parent donor concentrations ($n = 3$).

1000 μM (Fig. 3). Paracetamol glucuronidation was not detected in our studies, but paracetamol sulfate was observed in both chambers at all the parent compound concentrations and in both directions (Fig. 4). Based on these results, there was no obvious saturation or inhibition of the sulfation or the apical and basolateral efflux of paracetamol sulfate. No intracellular residuals were found, except for small amounts of sulfate in the cells used for A → B experiments.

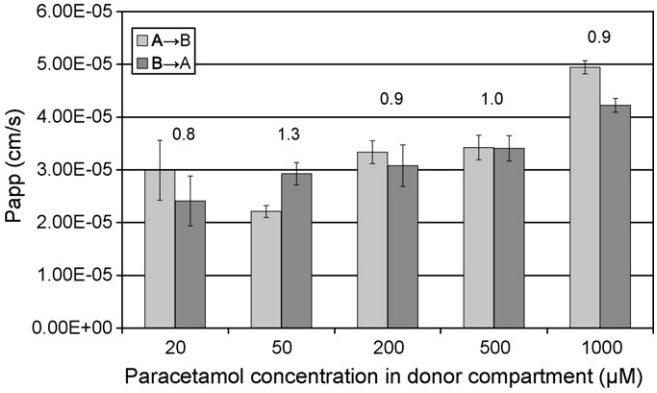

Fig. 3. Transport of parent paracetamol across the Caco-2 monolayers during the metabolism/permeability studies ($P_{app} \pm$ SD, $n = 3$). Efflux ratios (B → A vs. A → B) for each pair of experiments are presented above the respective bars.

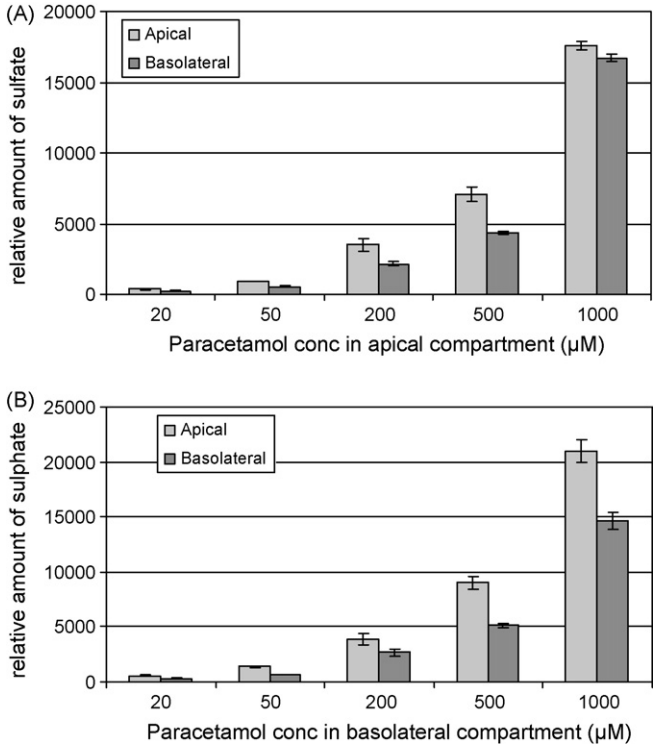

Fig. 4. The relative amounts of paracetamol sulfate observed in the apical and basolateral Transwell® compartments at 120 min during the Caco-2 permeability studies of paracetamol at different initial parent donor (A, apical and B, basolateral) concentrations ($n = 3$).

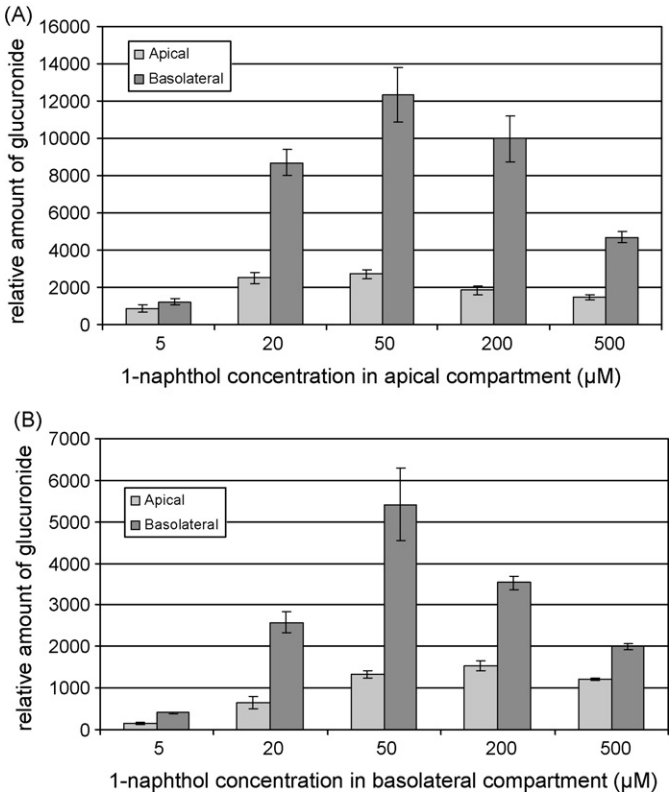

Fig. 6. The relative amounts of 1-naphthol glucuronide observed in the apical and basolateral Transwell® compartments at 120 min during the Caco-2 permeability studies of 1-naphthol at different initial parent donor (A, apical and B, basolateral) concentrations ($n = 3$).

3.3. 1-Naphthol

The apparent permeability of 1-naphthol was higher at higher donor concentrations (Fig. 5). Formation of 1-naphthol glucuronide was substantial in the permeation studies: at the lowest concentration studied (5 μM) the concentrations of parent compound were below the limit of quantification in the receiver compartment, whereas glucuronides were detected in both compartments at all time points. Both apical and basolateral efflux of 1-naphthol glucuronide were observed at all the studied concentrations, but the efflux was higher to the basolateral than to the apical side of Caco-2 monolayers (Fig. 6). Apical efflux of naphthol glucuronide was saturated at 1-naphthol concentrations above 20 μM. A typical bell-shaped inhibition curve was observed for the basolateral naphthol glucuronides, indicating substrate or product inhibition of the UGT enzyme(s) at higher 1-naphthol concentrations (200 μM and 500 μM). Negligible residual amounts of both parent compound and glucuronide were found in the cells, except for the lowest 1-naphthol donor concentrations.

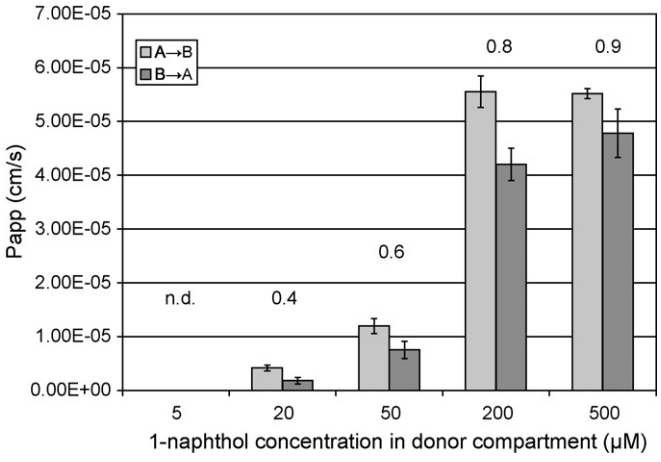

Fig. 5. Transport of parent 1-naphthol across the Caco-2 monolayers during the metabolism/permeability studies ($P_{app} \pm SD$, $n = 3$). Efflux ratios (B → A vs. A → B) for each pair of experiments are presented above the respective bars.

4. Discussion

Of the three model compounds used in this study, passive permeability of indomethacin and paracetamol across Caco-2 cell monolayers has been previously reported (Laitinen et al., 2004; Neuhoff et al., 2005). Glucuronidation of paracetamol and 1-naphthol has been observed in Caco-2 cell microsomes and disrupted cells (Abid et al., 1995; Siissalo et al., 2008). Inability of Caco-2 cells to produce paracetamol sulfate has been reported, but in that case the cells had been grown in flasks instead of on filters and, thus, the cell differentiation may have been incomplete (Prueksaritanont et al., 1996). Paracetamol is a known substrate for UGT1A1, 1A6, 1A9 and 2B15, as well as at least SULT1A1 (Falany, 1997; Blanchard et al., 2004; Mutlib et al., 2006). Adequate cell differentiation has been shown to be critical for the expression of all of these enzymes in Caco-2 cells, with the notable exception of UGT1A6 (Meinl et al., 2008; Siissalo et al., 2008). Sulfotransferases often have much higher affinity for the substrates they share with the UGTs (Van den Hurk et al., 2002; Gamage et al., 2006). Hence, the UGTs mainly contribute at high substrate concentrations. During the permeability experiments, the intracellular paracetamol concentration in Caco-2 cells may not be high enough for glucuronidation.

Indomethacin glucuronidation did not exhibit inhibition pattern as observed for 1-naphthol in this study. On the other hand, the

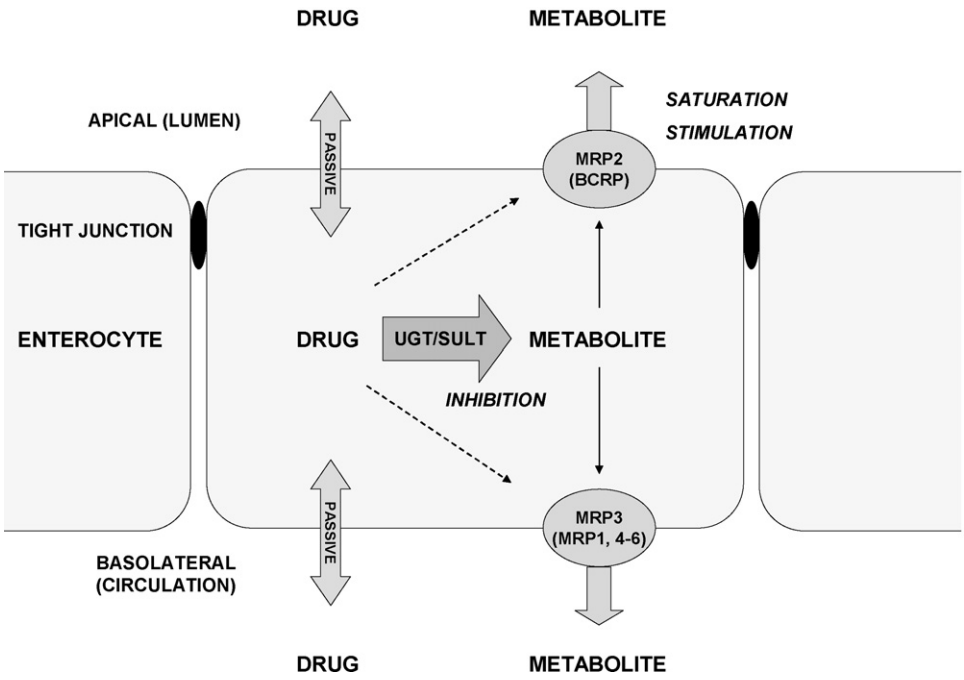

Fig. 7. Summary of the active processes observed in Caco-2 cells during the permeability experiments. Passive permeability of the studied compounds is presented as passive transport across the apical and basolateral lipid bilayers. UGT/SULT metabolism of indomethacin, paracetamol and 1-naphthol is coupled with apical and/or basolateral efflux of the metabolites and/or parent compound. Saturation of the apical efflux of 1-naphthol glucuronide, saturation and possible stimulation of the apical efflux of indomethacin glucuronide and inhibition of 1-naphthol glucuronidation are indicated. UGT = UDP-glucuronosyltransferase, SULT = sulfotransferase, MRP = multidrug resistance associated protein, BCRP = breast cancer resistance protein.

increase in the formation of indomethacin glucuronide was not linear with the increase in the parent compound concentration (Fig. 2). Since the linearity in the analytics of the glucuronide was not fully established, definitive quantitative conclusions for indomethacin glucuronidation in Caco-2 monolayers cannot yet be drawn. It can be estimated qualitatively that the UGTs involved in the glucuronidation of indomethacin in the differentiated Caco-2 cells may include UGT1A1, UGT1A3, UGT1A9 and UGT2B7 (Sacolovic et al., 2000; Mano et al., 2007; Siissalo et al., 2008). It has, however, been reported that at higher indomethacin concentrations the glucuronidation process and, thus, the formation of the glucuronides is decreased (Kuehl et al., 2005). Indomethacin itself is not a substrate for MRP2, so the reported concentration-dependent competitive inhibition of MRP2 may well be due to the efflux of glucuronides (Kouzuki et al., 2000; El-Sheikh et al., 2007). Indomethacin may also stimulate the MRP2-mediated efflux in a substrate and concentration-dependent manner, perhaps accounting for the profoundly apical efflux of indomethacin glucuronide at the lower concentrations (Zelcer et al., 2003). At the presumably highest intracellular glucuronide concentrations, the observed basolateral efflux provides an example of the complementary role of the basolateral efflux proteins in the case of saturation of the apical efflux (Fig. 2).

The glucuronidation of 1-napthtol has previously been studied in Caco-2 cells at different stages of differentiation (Mizuma et al., 2004). However, these observations have not been fully linked to the efflux of the metabolites or the data from enzyme expression studies. The observations in the current study are mainly due to glucuronidation of 1-naphthol by UGT1A6 (Siissalo et al., 2008), followed by the apical efflux by MRP2 and/or BCRP and the basolateral efflux by MRP3 and/or the other basolateral MRPs (Haimeur et al., 2004). The higher efflux of 1-naphthol glucuronide to the basolateral than to the apical side of Caco-2 monolayers has also previously been observed by others (Mizuma et al., 2004). This preference for the basolateral side could be due to several factors: higher affinity of 1-naphthol glucuronide to basolateral efflux transporters, higher capacity of basolateral efflux systems, or the inhibition of apical efflux by the glucuronide conjugates.

In conclusion, it was clearly demonstrated that fully differentiated Caco-2 cell monolayers can be regarded a promising *in vitro* method for phase II metabolism/efflux studies. Active processes may take place in the cells even if the permeability of the parent compound appears passive. Knowledge on the expression and kinetics of all the involved active components is crucial for the prediction of *in vivo* absorption of compounds susceptible for phase II metabolism. As both parent compounds and conjugates may, in addition to being substrates, act as stimulating an/or inhibitory modulators of different efflux proteins, complete unravelling of all the interactive processes requires the use of a number of experimental settings. Here we have shown that many complex processes can be observed during regular Caco-2 permeability experiments and the kinetics were entirely explained with the actions of metabolic enzymes and efflux proteins known to be expressed and functional in the fully differentiated Caco-2 cells (Fig. 7).

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