

RESEARCH PAPER

Ibuprofen is a non-competitive inhibitor of the peptide transporter hPEPT1 (SLC15A1): possible interactions between hPEPT1 substrates and ibuprofen

Diana Højmark Omkvist, Birger Brodin and Carsten Uhd Nielsen

Department of Pharmaceutics and Analytical Chemistry, The Faculty of Pharmaceutical Sciences, University of Copenhagen, Copenhagen, Denmark

Correspondence

Dr Carsten Uhd Nielsen,
Department of Pharmaceutics
and Analytical Chemistry, Faculty
of Pharmaceutical Sciences,
University of Copenhagen,
Universitetsparken 2, DK-2100
Copenhagen, Denmark. E-mail:
cun@farma.ku.dk

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BACKGROUND AND PURPOSE

Recently, we identified etodolac as a possible ligand for the human intestinal proton-coupled peptide transporter (hPEPT1). This raised the possibility that other non-steroidal anti-inflammatory drugs, and especially ibuprofen, could also interact with hPEPT1. Here, we have assessed the interactions of ibuprofen with hPEPT1.

EXPERIMENTAL APPROACH

The uptake of [¹⁴C]Gly-Sar, [³H]ibuprofen and other radio-labelled compounds were investigated in Madin–Darby canine kidney cells (MDCK)/hPEPT1, MDCK/Mock, LLC-PK₁ or Caco-2 cells. The transepithelial transport of ibuprofen and hPEPT1 substrates was investigated in Caco-2 cell monolayers.

KEY RESULTS

Ibuprofen concentration dependently inhibited hPEPT1-mediated uptake of Gly-Sar in MDCK/hPEPT1 cells ($K_i^{app} = 0.4$ mM) but uptake of ibuprofen in Caco-2 cells and MDCK/hPEPT1 cells was not inhibited by hPEPT1 substrates. The maximum uptake rate for Gly-Sar uptake was reduced from 522 pmol·min⁻¹·cm⁻² to 181 pmol·min⁻¹·cm⁻² and 78 pmol·min⁻¹·cm⁻² in the presence of 0.5 mM and 1 mM ibuprofen, respectively. The interaction between ibuprofen and hPEPT1 was thus non-competitive. In LLC-PK₁ cells, ibuprofen (1 mM) did not influence the transporter-mediated uptake of glycine or α -methyl-D-glycopyranoside. In Caco-2 cell monolayers the absorptive transport of δ -aminolevulinic acid was reduced by 23% and 48% by ibuprofen (1 and 10 mM), respectively. Likewise the transport of Gly-Sar was reduced by 23% in the presence of ibuprofen (1 mM).

CONCLUSIONS AND IMPLICATIONS

Ibuprofen is a non-competitive inhibitor of hPEPT1. As ibuprofen reduced the transepithelial transport of δ -aminolevulinic acid, drug–drug interactions between ibuprofen and hPEPT1 drug substrates at their site of absorption are possible if administered together.

Abbreviations

A–B, apical to basolateral; ABBA, 2-(2-aminobenzoyl)benzoic acid; ALA, δ -aminolevulinic acid; B–A, basolateral to apical; BSA, bovine serum albumin; Caco-2, Human colon adenocarcinoma cells; E3S, oestrone-3-sulfate; HBSS, Hanks' balanced salt solution; MDCK, Madin–Darby canine kidney cells; MES, 2-[N-morpholino]ethanesulfonic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NSAID, Non-steroidal anti-inflammatory drug; PAH, *p*-aminohippuric acid

Introduction

Ibuprofen is a widely used drug belonging to the structurally diverse, non-steroidal anti-inflammatory drug (NSAID) class, which also includes drugs such as naproxen, acetylsalicylic acid and etodolac. Recently, we identified etodolac as a possible ligand for the human proton-coupled peptide transporter (hPEPT1) (Larsen *et al.*, 2009), and this led us to speculate that ibuprofen might also interact with this transporter. hPEPT1 is expressed in the apical membrane of the small intestinal enterocytes (Liang *et al.*, 1995; Ogihara *et al.*, 1996), where it facilitates the absorption of a number of drug substances such as β -lactam antibiotics, anti-viral pro-drugs and δ -aminolevulinic acid (ALA), a drug used in photodynamic cancer therapy (Addison *et al.*, 1975; Nakashima *et al.*, 1984; Balimane *et al.*, 1998; Doring *et al.*, 1998; de Vruet *et al.*, 1998; Sugawara *et al.*, 2000). The recommended oral dose of ibuprofen is 400 mg, three times daily, resulting in an estimated initial intestinal luminal concentration of up to 8 mM (400 mg dose dissolved in 250 mL). Ibuprofen is absorbed rapidly and almost completely from the intestine (Martin *et al.*, 1990; Cheng *et al.*, 1994). In excised rat jejunum, mounted in an Ussing chamber, a net lumen-to-serosal transepithelial flux of ibuprofen was found. This net flux was caused by the intestinal acidic microclimate and was thus due to differential partitioning, caused by the pH gradient (Legen *et al.*, 2003). Removing the luminal mucus abolished polarized transport in rat jejunum and, using artificial membranes, it was concluded that ibuprofen is transported passively (Legen *et al.*, 2003). Ibuprofen is a substrate for the transporter OAT3 (Khamdang *et al.*, 2002; Takeda *et al.*, 2002b) and an inhibitor of OAT2, OAT4 and SMCT1 (Khamdang *et al.*, 2002; Takeda *et al.*, 2002b; Itagaki *et al.*, 2006), and there are conflicting reports as to whether it is a substrate for OAT1 (Mulato *et al.*, 2000; Khamdang *et al.*, 2002). Ibuprofen has also been reported to inhibit the transport of substrates for a number of other transporters such as MRP2, MRP4, monocarboxylate transporter 1 (MCT1), oatp1a1, oatp1a3 and oatp1a4 (Tamai *et al.*, 1995; Masuda *et al.*, 1997; Mulato *et al.*, 2000; Cha *et al.*, 2001; Khamdang *et al.*, 2002; Shitara *et al.*, 2002; El-Sheikh *et al.*, 2007; Cui and Morris, 2009). However, of these transporters only MRP2, MRP4, SMCT1 and MCT1 are expressed in the intestine.

The aim of the present study was to investigate whether ibuprofen interacted with hPEPT1 and if this could have implications for the transport of ibuprofen or hPEPT1 substrates across the intestinal epithelium. We found that ibuprofen interacted with hPEPT1 as a non-competitive inhibitor of

hPEPT1-mediated transport. Ibuprofen was transported passively across Caco-2 cells, but significantly inhibited the intestinal transepithelial transport of hPEPT1 substrates such as Gly-Sar and the drug substance ALA, indicating that drug–drug interactions between hPEPT1 substrates and ibuprofen could occur in the small intestine.

Methods

Cell culture

Madin–Darby canine kidney cells (MDCK) stably transfected with the *hPEPT1* cDNA (MDCK/hPEPT1) or the empty vector pcDNA3.1/V5&His (MDCK/Mock) were kindly donated by Bristol-Meyers Squibb Company (New Brunswick, NJ, USA) (Herrera-Ruiz *et al.*, 2004). MDCK and Caco-2 cells were seeded in culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U·mL⁻¹), streptomycin (100 μ g·mL⁻¹), 1% L-glutamine and 1% non-essential amino acids and passaged once (Caco-2) or twice (MDCK) a week. Caco-2 cells (passage 26–36) were seeded onto either Transwell™ tissue culture (Corning Inc., Life Sciences, Lowell, MA, USA) treated polycarbonate membranes (pore size: 0.4 μ m, growth area: 1.13 cm²) at a density of 10⁵ cells per filter or in 24-well plates (1.9 cm²) at a density of 1.7 \times 10⁵ cells per well. Cells seeded onto polycarbonate membranes were used between day 25 and 28 and cells seeded into 24-well plates were used 6 days after seeding. MDCK/hPEPT1 cells (passage 17–31) and MDCK/Mock cells (passage 17–18) were seeded at a density of 1 \times 10⁵ cells per well in 24-well plates or 4 \times 10⁴ cells per well in 96-well plates and used 3 days post-seeding. LLC-PK₁ cells were seeded in culture flasks and passaged in 1:1 DMEM : Nutrient mixture F-12 supplemented with 10% fetal bovine serum, penicillin (100 U·mL⁻¹) and streptomycin (100 μ g·mL⁻¹). The LLC-PK₁ cells were used in passage 110–112 and cells were seeded on to Transwell™ tissue culture treated polycarbonate filters at a density of 10⁵ cells per filter and used 10 days post-seeding.

All cell lines were grown in an atmosphere of 5% CO₂, 95% O₂ and 37°C and the culture medium was changed every second or third day.

In vitro uptake studies in MDCK/hPEPT1 and MDCK/Mock cells

MDCK/hPEPT1 and MDCK/Mock cells were pre-incubated with Hanks' balanced salt solution (HBSS), pH 7.4 [HBSS supplemented with 10 mM HEPES and 0.05% bovine serum albumin (BSA)] for 15 min. After aspirating the HBSS, the experiment

was initiated by addition of 400 μL HBSS, pH 6.0 [HBSS supplemented with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and 0.05% BSA] containing 18 μM [^{14}C]Gly-Sar (1 $\mu\text{Ci}\cdot\text{mL}^{-1}$) or 0.1 μM [^3H]ibuprofen (0.5 $\mu\text{Ci}\cdot\text{mL}^{-1}$) and other compounds as indicated. During incubation the cells were continuously rotated at 37°C on an orbital shaker (Heidolph Unimax 2010, Kelheim, Germany). After 5 min, the solutions were removed and the cells were washed three times with ice-cold HBSS buffer. The cells were detached by adding 400 μL 0.1% Triton-X in H_2O and incubating at 37°C for at least 30 min. The cell homogenate was transferred to a scintillation vial and 2 mL scintillation fluid was added. The radioactivity was counted by liquid scintillation spectrometry in a Packard TriCarb 2100TR liquid scintillation counter (Meriden, CT, USA).

In vitro uptake studies of ibuprofen in Caco-2 cells

Uptake experiments in Caco-2 cells were essentially performed as described in the previous section. The Caco-2 cells were pre-incubated in HBSS buffer pH 6.0 on the apical side and HBSS buffer pH 7.4 on the basolateral side. After the experiment the Caco-2 cells including polycarbonate filters were detached from the filter support and transferred to scintillation vials.

In vitro uptake studies of α -methyl-D-glycopyranoside and glycine in LLC-PK₁ cells

Uptake studies in LLC-PK₁ cells were performed essentially as described for Caco-2 cells. Exceptions were that uptake experiments with α -methyl-D-glycopyranoside were performed in HBSS buffer pH 7.4 without glucose [CaCl₂ (1.26 mM), MgCl₂ (0.49 mM), MgSO₄ (0.41 mM), KCl (5.33 mM), KH₂PO₄ (0.44 mM), NaHCO₃ (4.17 mM), NaCl (137.9 mM), Na₂HPO₄ (0.34 mM), HEPES (10 mM), BSA (0.05%)] and the uptake experiments were performed with HBSS buffer pH 7.4 on both apical and basolateral side.

Transepithelial transport studies in Caco-2 cell monolayers

The integrity of the Caco-2 cell monolayer was assessed by transepithelial electric resistance measurements at room temperature before initiating the experiments. The Caco-2 cells were pre-incubated in HBSS buffer pH 6.0 on the apical side and HBSS buffer pH 7.4 on the basolateral side. In the transport studies with [^3H]ibuprofen, the cells were pre-incubated with experimental buffers 15 min before the start of the experiment. Apparent permeabilities [P_{app} ($\text{cm}\cdot\text{s}^{-1}$)] of the radiolabelled compounds across

the Caco-2 cells were studied in both the absorptive [apical to basolateral (A–B)] and exsorptive (basolateral to apical) directions. Experiments were initiated by adding medium with radiolabelled compound with or without ibuprofen. When present, ibuprofen was added in both receiver and donor compartment. Samples from the receiver compartment were taken at various time points with or without 1 mM ibuprofen: 10, 20, 30, 40 and 60 min; and with or without 10 mM ibuprofen: 15, 30, 60, 90 and 120 min. After each sampling, the receiver compartment was replenished with the same volume of fresh medium. The radioactivity in samples was analysed by liquid scintillation spectrometry after adding 2 mL of scintillation fluid.

Fluorescence-based studies of hPEPT1 translocation

Translocation via hPEPT1 is electrogenic and changes in membrane potential may thus be used as a surrogate marker for substrate transport. The recently characterized and validated MDCK/hPEPT1 FLIPR[®] membrane potential assay (Molecular Devices, Berkshire, UK) (Faria *et al.*, 2004; Frolund *et al.*, 2010; Omkvist *et al.*, 2010) was used to measure substrate transport via hPEPT1. Gly-Sar and ibuprofen were applied to the MDCK/hPEPT1 FLIPR[®] membrane potential assay in absence or presence of the recently identified high affinity hPEPT1 inhibitor, Met-Pro-Pro (Omkvist *et al.*, 2010). Growth media were aspirated and replaced with 50 μL of FLIPR[®] membrane potential assay kit dye stock solution reconstituted in HBSS buffer pH 6.0. The cells were incubated for 30 min at 37°C to ensure dye equilibrium across the cell membrane. The assays were initiated by addition of 50 μL test solution in HBSS buffer pH 6.0 with FLIPR[®] membrane potential assay kit dye. Sample-induced changes in fluorescence (indicative of changes in membrane potential) were measured on a NOVostar platereader (BMG LabTech, Offenburg, Germany) at 37°C for 72 s. The probe was excited at 544 nm and emission light was collected at 590 nm.

Evaluation of ibuprofen toxicity

Cellular toxicity of the investigated compounds was examined using a 3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyl tetrazolium bromide (MTT) assay (Mosmann, 1983). MDCK/hPEPT1 cells were grown for 3 days on the bottom of 96-well plates. The growth medium was aspirated, 100 μL test compound in HBSS (0.05–20 mM) or sodium lauryl sulphate in HBSS (0.05–10 mM) (positive control) or HBSS (negative control) were added and the cells were incubated at 37°C on an orbital shaker. After 5 min the solutions were removed and 100 μL

2.4 mM MTT was added and the cells were incubated in the dark at 37°C for 90 min. To dissolve the formed formazan salt 100 µL of a solution of 10 mM HCl, 50% isobutanol and 0.4 mM sodium lauryl sulfate was added and the cells were incubated for at least 3 h. Absorbance was measured at 590 nm on an ELISA plate reader (Bie & Berntsen, Herlev, Denmark).

Data treatment

Affinity determinations. Cellular uptake of Gly-Sar in the presence of increasing concentrations of ibuprofen was fitted to a four-parameter logistic equation (Equation 1) to obtain an IC₅₀ value:

$$U = U_{\min} + \frac{U_{\max} - U_{\min}}{1 + 10^{(\log I - \log IC_{50}^{app})}} \quad (1)$$

U is % inhibition of the Gly-Sar flux at inhibitor concentration I, U_{max} is the initial flux value of Gly-Sar ([I] = 0 mM) ~100%, U_{min} is the lowest measured flux value of Gly-Sar at maximum inhibitor concentration and I is the concentration of inhibitor (mM).

According to Cheng and Prusoff (1973), IC₅₀ equals K_i for non-competitive inhibitors. Results are given as the fitted K_i^{app} value ± standard error based on results from seven concentrations in three individual cell monolayers.

Fluorescence-based studies of hPEPT1 translocation. The fluorescence signal was normalized according to the initial fluorescence signal, to account for unequal loading of the monolayers or seeding of cells. The area under curve (AUC) of the normalized fluorescence signal was subtracted from the AUC caused by addition of a sample with HBSS, pH 6.0. The AUC values were then expressed as a percentage of the AUC caused by the addition of 20 mM Gly-Sar (later referred to as response), as described previously (Omkvist *et al.*, 2010).

Determination of interaction type between ibuprofen and hPEPT1. The uptake rate of Gly-Sar in the presence or absence of 0.5 and 1 mM ibuprofen was fitted to the Eadie–Hofstee equation (Equation 2):

$$V = -K_m \frac{V}{S} + V_{\max} \quad (2)$$

V is the uptake rate of Gly-Sar (pmol·min⁻¹·cm⁻²), V_{max} is the maximum uptake rate (pmol·min⁻¹·cm⁻²), K_m is the Michaelis constant (mM) and S is the Gly-Sar concentration (mM).

Transepithelial transport across Caco-2 cell monolayers. The transepithelial transport of compounds across Caco-2 cell monolayers is expressed as the

apparent permeability (P_{app}, cm·s⁻¹) and was calculated using Equation 3:

$$P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \quad (3)$$

dQ/dt is the mass of compound (Q, pmol) accumulated in the receiver compartment over time (t, minute), as a function of the area (A, cm²) and the initial concentration of compound in the donor compartment at steady-state conditions. Steady state was assumed when no more than 10% of the initial amount of compound was transported to the receiver compartment. When more than 10% was transported, non-steady-state calculations were used (Equation 4).

$$P_{app}^{nss} = \frac{K \times V_d \times V_r}{V_t \times A} \quad K = -\ln\left(\frac{C_{\infty} - C_{r(t)}}{C_{\infty}}\right) \times \frac{1}{t} \quad (4)$$

P_{app}^{nss} is the apparent permeability estimated at non-steady state (cm·s⁻¹), V_d is the volume of the donor compartment (mL), V_r is the volume of the receiver compartment (mL), V_t is total volume in both compartments (mL), A is the area of the cell monolayers (cm²), C_∞ is concentration in compartments at equilibrium (M), C_{r(t)} is the time-dependent concentration in receiver compartment (M) and t is the time (minute).

Statistical analysis

Statistical analysis was performed using either an unpaired Student's *t*-test or a one-way analysis of variance (ANOVA) followed by Dunnett's post-test.

Statistical analysis and data fitting were performed using GraphPad Prism (version. 4.01, GraphPad Software Inc., San Diego, CA, USA).

Materials

DMEM, penicillin, streptomycin, L-glutamine, non-essential amino acids, HBSS and geneticin were from Invitrogen (Taastrup, Denmark). Fetal bovine serum was purchased from Biotech line (Slangerup, Denmark). The human colon adenocarcinoma cell line (Caco-2) and the proximal tubule porcine kidney cell line LLC-PK₁ were obtained from American Type Culture Collection (Manassas, VA, USA). Gly-Pro, Gly-Sar, Triton X-100, HEPES, MES, BSA 2-(2-aminobenzoyl)benzoic acid, *p*-aminohippuric acid (PAH), oestrone-3-sulfate (E3S), MgSO₄, MgCl₂, CaCl₂, 1:1 DMEM : Nutrient mixture F-12 and MTT were from Sigma-Aldrich (Brøndby, Denmark). [³H]mannitol (11.7 Ci·mmol⁻¹), [¹⁴C]α-methyl-D-glycopyranoside (310 mCi·mmol⁻¹), [¹⁴C]glycine (87 mCi·mmol⁻¹) and Ultima Gold scintillation fluid were from PerkinElmer (Boston, MA, USA). [³H]ibuprofen (5 Ci·mmol⁻¹), [³H]etoposide

(20 Ci·mmol⁻¹) and [¹⁴C]δ-aminolevulinic acid (ALA) (55 mCi·mmol⁻¹) were from American Radio-labelled Chemicals Inc. (St. Louis, MO, USA). [³H]L-carnosine (10 Ci·mmol⁻¹) was purchased from Moravak Biochemicals, Inc. (Brea, CA, USA). [¹⁴C]mannitol (56.5 mCi·mmol⁻¹) was from Larodan (Malmö, Sweden). [³H]metoprolol (29.7 Ci·mmol⁻¹) was purchased from Vitrox (Placentia, CA, USA). [¹⁴C]Gly-Sar (56 mCi·mmol⁻¹) was from GE-Healthcare (Freiburg, Germany). Sodium lauryl sulphate, KCl, KH₂PO₄, NaHCO₃, Na₂HPO₄ and isobutanol were from Merck (Darmstadt, Germany). MP probe included in the FLIPR[®] membrane potential assay kit was purchased from Molecular Devices (Berkshire, UK). NaCl was from VWR (Herlev, Denmark). Paracetamol, naproxen and piroxicam were obtained from Chr. Olesen Pharmaceuticals A/S (Gentofte, Denmark), whereas acetylsalicylic acid was from Fagron (Copenhagen, Denmark). Drug and molecular target nomenclature follows Alexander *et al.* (2009).

Results

Ibuprofen inhibits Gly-Sar uptake in MDCK/hPEPT1 cells

The ability of paracetamol and five structurally diverse NSAIDs (acetylsalicylic acid, etodolac, ibuprofen, naproxen and piroxicam) to inhibit hPEPT1-mediated [¹⁴C]Gly-Sar uptake was investigated in MDCK/hPEPT1 cells (Figure 1). Ibuprofen, piroxicam, etodolac and Gly-Pro, a high-affinity natural substrate for hPEPT1, inhibited the uptake of Gly-Sar significantly, whereas acetylsalicylic acid, paracetamol and naproxen did not interfere with Gly-Sar uptake. None of the tested compounds were toxic in a MTT assay at the concentration examined (0.5 mM) (data not shown). Ibuprofen was selected for further investigation and in MDCK/hPEPT1 cells [¹⁴C]Gly-Sar uptake was significantly inhibited to approximately 6% of the control uptake in the presence of 10 mM ibuprofen and 20 mM Gly-Pro (Figure 2A). The uptake of Gly-Sar in Caco-2 cells grown in 24-well plates was also investigated in absence or presence of 1 mM ibuprofen and 20 mM Gly-Pro (Figure 2B). Ibuprofen (1 mM) and Gly-Pro (20 mM) inhibited the uptake of Gly-Sar to 46% and 12% of the control uptake, respectively. The ability of ibuprofen to inhibit the uptake of [¹⁴C]Gly-Sar in MDCK/hPEPT1 cells was further investigated. As illustrated in Figure 2C, ibuprofen inhibits Gly-Sar uptake in a concentration-dependent manner with an estimated K_i^{app} of 0.4 mM ($pK_i^{app} = 0.4 \pm 0.1$ mM) (Figure 2C). The two stereoisomers of ibuprofen were investigated separately for their ability to

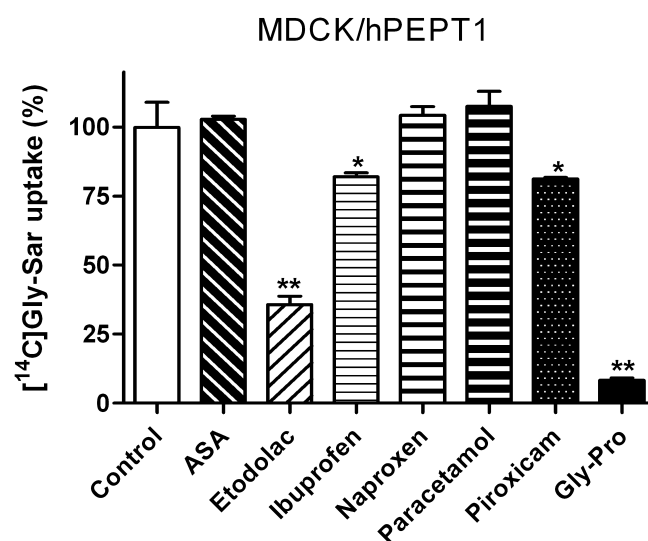


Figure 1

Uptake of 18 μM [¹⁴C]Gly-Sar in Madin–Darby canine kidney cells (MDCK)/human proton-coupled peptide transporter (hPEPT1) cell monolayers in presence or absence of 0.5 mM acetylsalicylic acid (ASA), etodolac, ibuprofen, naproxen, paracetamol, piroxicam or 20 mM Gly-Pro. The uptake was measured for 5 min with an extracellular pH of 6.0. Data represent mean ± SEM of six cell monolayers. * $P < 0.05$ and ** $P < 0.01$, analysed by one-way ANOVA followed by Dunnett's post-test (the control column used as control).

inhibit Gly-Sar uptake, and they were found to be equally potent (data not shown). Ibuprofen is thus a high affinity ligand of hPEPT1-mediated transport. To verify that the effect of ibuprofen is not due to unspecific cellular toxicity, a MTT toxicity assay was performed. Ibuprofen concentrations below 10 mM did not affect MDCK/hPEPT1 cell viability, whereas cell viability was significantly affected by the presence of ibuprofen in concentrations above 10 mM (Figure 2D).

Ibuprofen is not a substrate for hPEPT1

We then investigated if ibuprofen was a substrate for hPEPT1 using two different approaches; one using [³H]ibuprofen to measure direct cellular uptake and another using a fluorescence-based hPEPT1 translocation assay. Apical uptake of [³H]ibuprofen into MDCK/hPEPT1 cells was not inhibited by 20 mM Gly-Pro, whereas the uptake was significantly reduced in the presence of 20 mM ampicillin, 2-(2-aminobenzoyl)benzoic acid (ABBA) or ALA (Figure 3A). A similar pattern was observed in MDCK/Mock cells, where 20 mM of Gly-Pro did not inhibit the uptake of [³H]ibuprofen, whereas 20 mM of ABBA significantly reduced the uptake of ibuprofen (Figure 3B). The magnitude of cellular [³H]ibuprofen uptake was furthermore similar in MDCK/hPEPT1 and MDCK/Mock cells (Figure 3A,B).

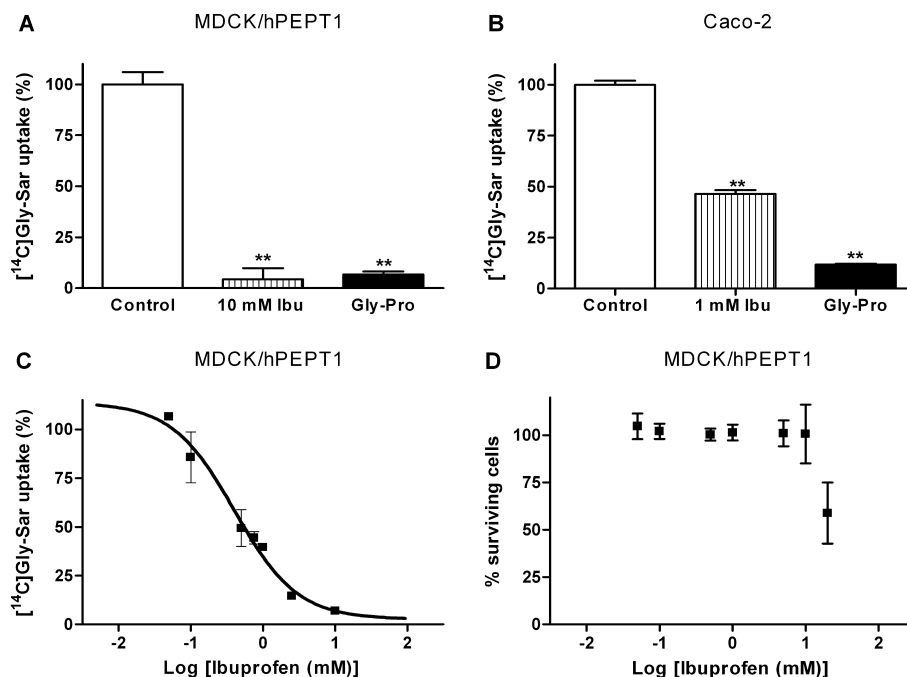


Figure 2

(A) Uptake of 18 μM [^{14}C]Gly-Sar in the presence or absence of 20 mM Gly-Pro or 10 mM racemic ibuprofen (Ibu) in Madin–Darby canine kidney cells (MDCK)/human proton-coupled peptide transporter (hPEPT1) cells. (B) Uptake of 18 μM [^{14}C]Gly-Sar in the presence or absence of 20 mM Gly-Pro or 1 mM racemic Ibu in Caco-2 cells grown in 24-well plates. (C) Uptake (%) of 18 μM [^{14}C]Gly-Sar uptake as a function of increasing ibuprofen concentrations (0–10 mM). The solid line represents the fit of the experimental data to Equation 1 and K_i^{app} was determined to be 0.4 mM ($\text{p}K_i^{\text{app}} = 0.4 \pm 0.1$ mM). (D) The viability of MDCK/hPEPT1 cells in the presence of ibuprofen (0.05–20 mM) assessed via a 3-(4, 5-dimethylthiazol-2-yl)2, 5-diphenyl tetrazolium bromide test. Uptake was measured for 5 min using a 2-[N-morpholino]ethanesulfonic acid buffer with a pH of 6.0. Results in A + C are mean \pm SD and in B + D are mean \pm SEM of three individual cell monolayers in two to three passages. $**P < 0.01$, analysed by one-way ANOVA followed by Dunnett's post-test.

The [^3H]ibuprofen uptake was investigated in MDCK/hPEPT1 cells using three concentrations: 0.0001, 0.1 and 0.5 mM ibuprofen. Here 0.1 mM reduced the uptake of [^3H]ibuprofen by 39%, whereas 0.5 mM reduced it by 50% (Figure 3C). The uptake of [^3H]ibuprofen into MDCK/hPEPT1 cells was further measured in the absence or presence of 1 mM PAH or 30 μM E3S (Figure 3D). The ibuprofen uptake was not affected by PAH, whereas the uptake was reduced approximately 50% by E3S. Collectively, these results point to the fact that ibuprofen is not a substrate for hPEPT1, but at the same time indicates that ibuprofen is a substrate for another transporter in MDCK cells.

The apical uptake of [^3H]ibuprofen in the intestinal Caco-2 cell line was investigated in the presence or absence of Gly-Pro and ampicillin. The apical uptake rate of [^3H]ibuprofen was 10 ± 1 $\text{fmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$, and the uptake was not inhibited by neither 20 mM Gly-Pro (8 ± 3 $\text{fmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$) nor 20 mM ampicillin (9 ± 2 $\text{fmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$) (data not shown).

To provide further evidence that ibuprofen was an inhibitor of hPEPT1-mediated substrate trans-

port, a membrane potential sensitive fluorescence based translocation assay was used. Met-Pro-Pro completely blocked the fluorescence response elicited by 5 mM Gly-Sar (Figure 4). Addition of 5 mM ibuprofen to the assay caused an increase in the fluorescence signal but no suppression of the signal was observed in presence of the hPEPT1 inhibitor Met-Pro-Pro (Figure 4). Ibuprofen caused a depolarization of the membrane potential by a mechanism that appeared to be unrelated to hPEPT1-mediated substrate translocation. Thus, ibuprofen inhibited translocation via hPEPT1 without being transported. Ibuprofen was therefore identified as a novel inhibitor of hPEPT1.

Ibuprofen is a non-competitive inhibitor of hPEPT1

The mechanism of ibuprofen inhibition of hPEPT1 substrate transport was investigated. The concentration-dependent uptake of Gly-Sar was measured in the absence or presence of 0.5 mM or 1 mM ibuprofen in MDCK/hPEPT1 cells. In Figure 5 the results are displayed in an Eadie–Hofstee plot. The apparent V_{max} values were 522 $\text{pmol}\cdot\text{min}^{-1}\cdot\text{cm}^{-2}$

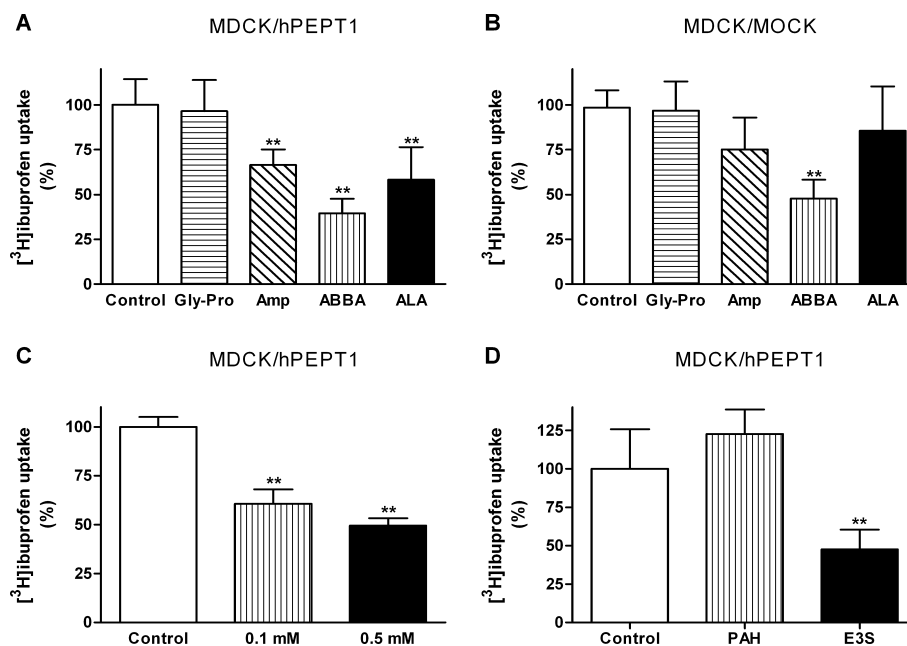


Figure 3

Uptake of ibuprofen in Madin–Darby canine kidney cells (MDCK)/human proton-coupled peptide transporter (hPEPT1) or MDCK/Mock cells. Uptake was measured for 5 min with a buffer pH of 6.0. (A,B) Uptake of [³H]ibuprofen (0.25 μ Ci-well⁻¹, 0.1 μ M) in MDCK/hPEPT1 (A) or MDCK/Mock (B) cell monolayers in the absence or presence of 20 mM Gly-Pro, 20 mM ampicillin (Amp), 20 mM 2-(2-aminobenzoyl)benzoic acid (ABBA) or 20 mM δ -aminolevulinic acid (ALA). The results are mean \pm SD of four to five individual cell monolayers. (C) Uptake of [³H]ibuprofen (0.25 μ Ci-well⁻¹, 0.1 μ M) in MDCK/hPEPT1 cells in the absence or presence of 0.1 mM or 0.5 mM ibuprofen. Results are mean \pm SEM of 9–10 individual cell monolayers from three different cell passages. (D) Uptake of [³H]ibuprofen (0.25 μ Ci-well⁻¹, 0.1 μ M) in MDCK/hPEPT1 cells in the absence or presence of 1 mM *p*-aminohippuric acid (PAH) or 30 μ M oestrone-3-sulfate (E3S). The results are mean \pm SD of four individual cell monolayers. ***P* < 0.01, analysed by one-way ANOVA followed by Dunnett's post-test.

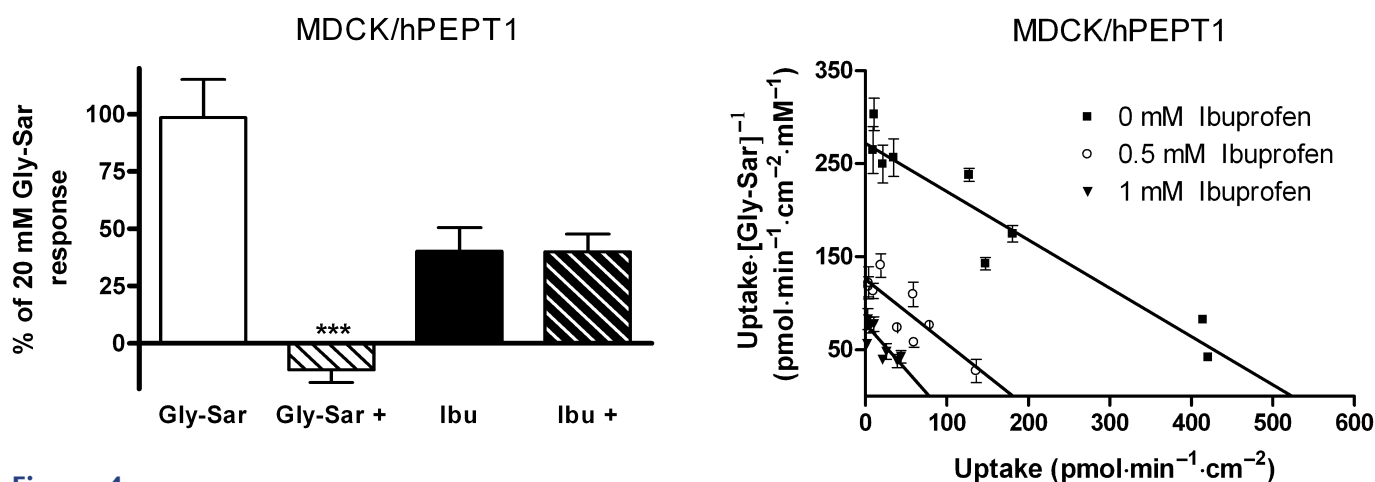


Figure 4

The percentage change in fluorescence, relative to the response elicited by 20 mM Gly-Sar, measured in the Madin–Darby canine kidney cells (MDCK)/human proton-coupled peptide transporter (hPEPT1) FLIPR® membrane potential translocation assay after addition of ibuprofen (Ibu) (5 mM) and Gly-Sar (5 mM) in the absence or presence of 0.25 mM Met-Pro-Pro (+). The cells were cultured for 3 days and change in fluorescence was measured for 72 s with an apical pH of 6.0. The results are mean \pm SEM, of six cell monolayers. Statistical analysis was performed using an unpaired Student's *t*-test comparing the response in presence or absence of Met-Pro-Pro, ****P* < 0.001.

Figure 5

Concentration-dependent uptake of Gly-Sar (36 μ M [¹⁴C]Gly-Sar) in the absence or presence of 0.5 mM or 1 mM ibuprofen. The uptake was measured for 5 min with a buffer pH of 6.0. Results are mean \pm SD of three individual cell monolayers. Data are displayed in an Eadie–Hofstee plot. The solid lines represent the linear regression of the results. MDCK, Madin–Darby canine kidney cells; hPEPT1, human proton-coupled peptide transporter.

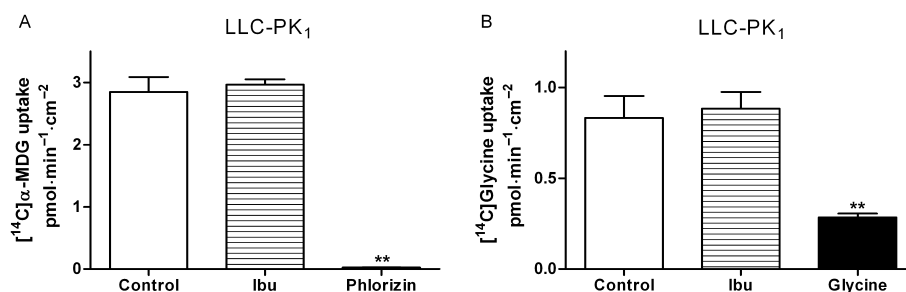


Figure 6

(A) Apical uptake of [^{14}C]α-methyl-D-glycopyranoside (α-MDG) (3 μM) in LLC-PK₁ cell monolayers in the absence or presence of 1 mM ibuprofen (Ibu) or 1 mM phlorizin. (B) Apical uptake of [^{14}C]glycine (11 μM) in LLC-PK₁ cell monolayers in the absence or presence of 1 mM Ibu or 20 mM unlabelled glycine. Uptake was measured for 5 min with an apical and basolateral pH of 7.4. Data represent mean ± SEM of two individual cell monolayers in three passages. ** $P < 0.01$, analysed by one-way ANOVA followed by Dunnett's post-test.

Table 1

Apparent permeability coefficients of the transepithelial transport of ibuprofen across Caco-2 cell monolayers

pH		Apparent permeability ($\cdot 10^{-5} \text{ cm} \cdot \text{s}^{-1}$)		Ratio
Apical	Basolateral	Absorptive Apical → basolateral	Exsorptive Basolateral → apical	$P_{\text{abs}}/P_{\text{exs}}$
6.0	6.0	3.0 ± 0.1	2.4 ± 0.2	1.2
6.0	7.4	9.3 ± 0.7	0.3 ± 0.1	28
7.4	6.0	0.5 ± 0.1	9.8 ± 0.9	0.05
7.4	7.4	2.5 ± 0.1	2.3 ± 0.3	1.1

Values represent mean ± SEM of four to five individual cell monolayers in three individual Caco-2 cell passages.

[95% confidence interval (CI) 453–618, $\text{pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$], 181 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ (95% CI 142–253 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) and 78 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$ (95% CI 60–118 $\text{pmol} \cdot \text{min}^{-1} \cdot \text{cm}^{-2}$) for uptake of Gly-Sar, Gly-Sar with 0.5 mM ibuprofen and Gly-Sar with 1 mM ibuprofen, respectively. The K_m values were 1.9 mM (95% CI 1.6–2.3 mM), 1.4 mM (95% CI 1.1–2.2 mM) and 1.0 mM (95% CI 0.7–1.6 mM) for uptake of Gly-Sar, Gly-Sar with 0.5 mM ibuprofen and Gly-Sar with 1 mM ibuprofen, respectively. The slopes of the Eadie–Hofstee plot were not significantly different from each other, whereas the intercepts were. The V_{max} values for Gly-Sar uptake are thus significantly different for the concentrations of ibuprofen tested here, which suggested that ibuprofen inhibited substrate transport via hPEPT1 in a non-competitive manner.

Ibuprofen does not inhibit α-methyl-D-glycopyranoside and glycine uptake in LLC-PK₁ cells

To investigate if the ibuprofen inhibition of substrate transported via hPEPT1 was specific, the uptake of the SGLT1 substrate α-methyl-D-

glycopyranoside into LLC-PK₁ cells was investigated in the presence or absence of 1 mM ibuprofen and 1 mM of the SGLT1 inhibitor phlorizin (Figure 6A). Ibuprofen did not reduce the uptake of α-methyl-D-glycopyranoside at the concentration investigated, whereas 1 mM phlorizin completely abolished the uptake (Figure 6A). In addition, the uptake of [^{14}C]glycine into LLC-PK₁ cells was measured in presence or absence of 1 mM ibuprofen and 20 mM of unlabelled glycine (Figure 6B). Unlabelled glycine reduced the uptake to approximately 34% of the control while ibuprofen did not appear to inhibit the uptake (Figure 6B).

Uptake and transepithelial transport of ibuprofen across Caco-2 cell monolayers

The transepithelial transport of 0.1 μM ibuprofen was investigated in Caco-2 cell monolayers, grown for 27 days, in buffers of varying pH (Table 1). Without a pH gradient across the monolayer the absorptive and exsorptive permeability of ibuprofen was similar. When a pH gradient was present, the permeability of ibuprofen was approximately 30 times larger in the direction of increasing pH. The

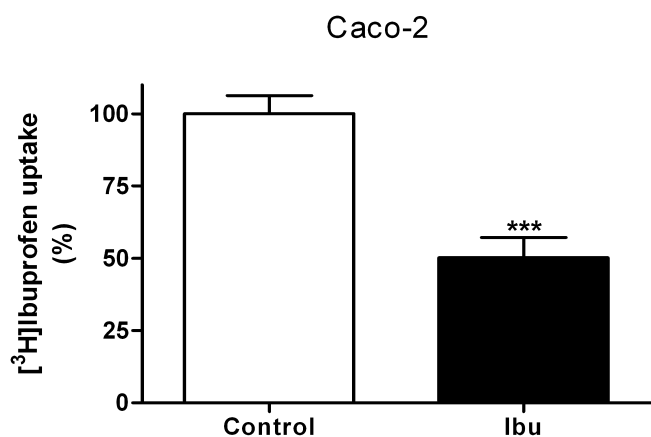


Figure 7

Uptake of 0.1 μM [^3H]ibuprofen in Caco-2 cells in 24-well plates in the presence or absence of 1 mM racemic ibuprofen. Uptake was measured for 5 min using a 2-[N-morpholino]ethanesulfonic acid buffer with a pH of 6.0. Results mean \pm SEM of 3 individual cell monolayers in three passages. Statistical analysis was performed using an unpaired Student's *t*-test, *** $P < 0.001$.

cellular concentration of ibuprofen was estimated to be 40 nM (cellular compartment 2 μL), whereas the initial concentration in donor compartment was 100 nM, hence no cellular accumulation of ibuprofen was observed. However, the uptake of [^3H]ibuprofen into Caco-2 cells grown for 6 days in 24-well plates was decreased to 50% of the control value in the presence of 1 mM unlabelled ibuprofen (Figure 7).

Trans epithelial transport of hPEPT1 substrates across Caco-2 cell monolayers in the presence of Ibuprofen

The possibility that ibuprofen could affect the trans epithelial transport of hPEPT1 substrates was then investigated. The trans epithelial transport of hPEPT1 substrates ALA, Gly-Sar and L-carnosine was measured in the presence or absence of ibuprofen. The A–B permeability of ALA and Gly-Sar was significantly larger than the basolateral to apical transport (Figure 8). The A–B transport of Gly-Sar and ALA was significantly inhibited by 1 mM ibuprofen ($P < 0.05$), whereas no change in A–B permeability was observed for L-carnosine (Figure 8). In the presence of 10 mM ibuprofen an increase in the A–B flux of all tested compounds were observed after approximately 40 min (data not shown). A time-dependent effect of ibuprofen on the barrier properties of the Caco-2 cell monolayers was observed and therefore the A–B P_{app} in the presence of 10 mM ibuprofen was calculated from data obtained within the first 30 min of the transport study. The presence of 10 mM ibuprofen inhibited the A–B transport of

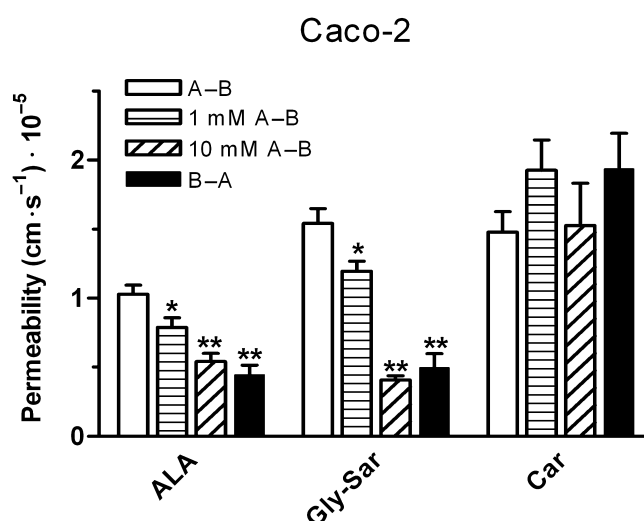


Figure 8

Apical to basolateral transport (A–B) of [^{14}C] δ -aminolevulinic acid (ALA) (0.5 $\mu\text{Ci}\cdot\text{mL}^{-1}$, 9 μM), [^{14}C]Gly-Sar (0.5 $\mu\text{Ci}\cdot\text{mL}^{-1}$, 9 μM) and [^3H]L-carnosine (Car) (0.5 $\mu\text{Ci}\cdot\text{mL}^{-1}$, 50 nM) in the absence or presence of 1 and 10 mM ibuprofen and basolateral to apical transport (B–A) of ALA, Gly-Sar and Car in Caco-2 cells. The apical pH was 6.0 and basolateral pH was 7.4. Apparent permeability coefficients (P_{app}) were calculated from flux data. To avoid possible toxic effects of ibuprofen at high concentrations, only data points from 15 and 30 min was used to calculate flux values in the presence of 10 mM ibuprofen. Data represent mean \pm SEM of five to nine cell monolayers from three individual passages. * $P < 0.05$ and ** $P < 0.01$, analysed by one-way ANOVA followed by Dunnett's post-test (the A–B P_{app} used as control).

ALA and Gly-Sar to the same level as the B–A transport in absence of ibuprofen (Figure 8), thus abolishing the hPEPT1 mediated polarized transport. 10 mM ibuprofen did not affect A–B permeability of L-carnosine. Ibuprofen thus inhibits trans epithelial transport of some hPEPT1 substrates. To verify that ibuprofen did not have additional effects on the barrier properties of Caco-2 cells the A–B transport of metoprolol (transcellular marker), mannitol (paracellular marker) and etoposide (marker for efflux transport) in the presence or absence of 1 and 10 mM ibuprofen were examined. In addition, the basolateral to apical (B–A) transport of etoposide was also investigated in the absence or presence of 1 mM ibuprofen. The A–B permeability of mannitol was $3.2 \times 10^{-6} \pm 0.4 \times 10^{-6} \text{ cm}\cdot\text{s}^{-1}$ and the permeability was not significantly different in the presence of 1 or 10 mM ibuprofen, respectively. Metoprolol had an A–B permeability of $4.6 \times 10^{-6} \pm 1.3 \times 10^{-6} \text{ cm}\cdot\text{s}^{-1}$ and the permeability was not influenced by the presence of 1 mM ibuprofen. However, a significant ($P < 0.01$) increase in the absorptive permeability of metoprolol was seen in the presence of 10 mM ibuprofen. The A–B and B–A permeability of etoposide was $2.86 \times 10^{-5} \pm 0.3 \times 10^{-5} \text{ cm}\cdot\text{s}^{-1}$ and $5.0 \times 10^{-5} \pm$

0.4×10^{-5} , respectively, and these permeabilities were not affected by the presence of ibuprofen.

Discussion

Ibuprofen is administered orally in doses of 200–400 mg every 6 h when used as an over-the-counter medication (Davies, 1998). When the indication is rheumatoid arthritis the maximal dose is up to 3200 mg a day, that is 800 mg doses four times daily (Davies, 1998). Assuming that the ibuprofen dose is taken with 250 mL fluid, initial intestinal concentrations would be from 3.9 mM (200 mg dose) to 15.5 mM (800 mg dose) using a molecular weight of ibuprofen of $206.3 \text{ g}\cdot\text{mol}^{-1}$. We have determined the K_i -value for the non-competitive interaction of ibuprofen with hPEPT1, to be 0.4 mM *in vitro*. The intestinal concentrations of ibuprofen are thus likely to be much higher than the K_i -value of ibuprofen for hPEPT1. This indicates that, even at the lowest doses of ibuprofen, hPEPT1 will be fully saturated at the ibuprofen concentrations likely to be present in the intestine. Accordingly, ibuprofen was shown to reduce the absorptive transport of δ -aminolevulinic acid and Gly-Sar in Caco-2 cell monolayers. Therefore our *in vitro* data suggest that drug–drug interactions may occur, but obviously *in vivo* data are necessary to establish whether such drug–drug interactions are present in clinical settings. We therefore hypothesize that ibuprofen could reduce the absorption of hPEPT1 substrates when dosed together, and drug–drug interaction may occur in the small intestine at the level of intestinal absorption.

Ibuprofen is a non-competitive inhibitor of substrate transport via hPEPT1

The uptake of Gly-Sar was measured in the presence or absence of five NSAIDs and paracetamol. Ibuprofen inhibited Gly-Sar uptake via hPEPT1, and also piroxicam, which is a NSAID structurally unrelated to ibuprofen, likewise inhibited Gly-Sar uptake in MDCK/hPEPT1 cells. Etodolac, which we had previously identified as a possible hPEPT1 ligand (Larsen *et al.*, 2009), was confirmed to interact with hPEPT1. Naproxen, which is structurally related to ibuprofen, did not inhibit Gly-Sar uptake nor did acetylsalicylic acid or paracetamol. This indicates that NSAIDs do not, in general, inhibit hPEPT1-mediated transport but specific structural features of individual NSAID drugs might interfere with hPEPT1-mediated transport. Ibuprofen was shown to have affinity for hPEPT1 with a K_i^{app} value of 0.4 mM, which is comparable to that of naturally occurring dipeptides and drug substances such as ceftibuten, L-cephalexin,

L-valaciclovir and ALA (Nielsen *et al.*, 2002a). We subsequently employed different strategies to investigate whether ibuprofen was a substrate or an inhibitor of hPEPT1. The uptake of [^3H]ibuprofen was not inhibited by Gly-Pro in neither MDCK/hPEPT1 cells nor Caco-2 cells. In the MDCK/hPEPT1 FLIPR[®] membrane potential assay the hPEPT1 inhibitor Met-Pro-Pro did not affect the response elicited by ibuprofen. Collectively, the data all point to the conclusion that ibuprofen inhibits hPEPT1-mediated transport, without being transported itself. The type of inhibition of hPEPT1-mediated transport by ibuprofen was investigated, and based on Figure 5 we suggest that the interaction is of a non-competitive nature. To our knowledge the only other reported non-competitive inhibitors of hPEPT1 transport are quinapril, spirapril, nateglinide and glibenclamide (Sawada *et al.*, 1999; Terada *et al.*, 2000; Zhu *et al.*, 2000; Knütter *et al.*, 2008). The non-competitive inhibition of quinapril and glibenclamide may be caused by a direct interaction with hPEPT1 (Sawada *et al.*, 1999; Zhu *et al.*, 2000).

To assess whether the observed interaction between ibuprofen and hPEPT1 was a general effect of ibuprofen on membrane transporters, the uptake of α -methyl-D-glycopyranoside and glycine was measured in the presence or absence of ibuprofen using LLC-PK₁ cell monolayers. Ibuprofen did not seem to interfere with either α -methyl-D-glycopyranoside or glycine uptake. Thus, ibuprofen does not appear to have a general effect on membrane transporters.

Renal MDCK cells stably transfected with hPEPT1 were used as an *in vitro* model to investigate ibuprofen interactions with hPEPT1. In MDCK/hPEPT1 cells the uptake of ibuprofen was concentration-dependent and inhibited by ALA, ampicillin, ABBA and E3S but not by PAH and Gly-Pro. This indicates that a non-hPEPT1 carrier-mediated mechanism is involved in the cellular uptake of ibuprofen in renal MDCK cells. Ibuprofen is reported to be a substrate for OAT3 and possibly OAT1 (Khamdang *et al.*, 2002; Takeda *et al.*, 2002a). However, PAH is also a substrate for both carriers excluding them as the carrier involved in ibuprofen transport in the MDCK/hPEPT1 cells (Cha *et al.*, 2001; Groves *et al.*, 2003). E3S is a substrate for both *oatp1a1*, *oatp1a3* and *oatp1a4* and PAH is not a ligand for *oatp1a1* and *oatp1a4* (the OATP/SLCO nomenclature defined by Hagenbuch and Meier, 2004, is used; Noe *et al.*, 1997; Hagenbuch *et al.*, 2000; van Montfoort *et al.*, 2002; Hagenbuch and Meier, 2004). *Oatp1a1*, *oatp1a4* and *oatp1a5* have been identified at the mRNA level in rat kidney, whereas *oatp1a1* and *oatp1a4* have also been identified at the protein level (Hagenbuch and Meier, 2003).

Ampicillin significantly inhibited the uptake of ibuprofen in MDCK/hPEPT1 cells. The structures of penicillin G and ampicillin are very similar and only differ by the presence of an extra amine group in ampicillin. Penicillin G has been reported to inhibit the substrate uptake via *oatp1a1* (Shitara *et al.*, 2002). Whether any of the above-mentioned transporters are expressed in the MDCK cells is presently unknown, thus it remains purely speculative whether these carriers are mediating ibuprofen transport in the MDCK/hPEPT1 cells.

Uptake and transepithelial transport of ibuprofen in intestinal Caco-2 cell monolayers

The transepithelial transport of ibuprofen was strongly dependent on the pH values of the buffers used in the donor and receiver solution. We did not find any polarized transport of ibuprofen with similar pH values of 6.0/6.0 or 7.4/7.4 on the donor and receiver side. Even though the initial apical uptake of radiolabelled ibuprofen into Caco-2 cells was inhibited by unlabelled ibuprofen, no accumulation of ibuprofen in the Caco-2 cells could be observed at the end of the transport study. The predominant transcellular absorption mechanism for ibuprofen in Caco-2 cells thus appear to be passive diffusion driven by the pH gradient (pH partitioning) (Shore *et al.*, 1957; Neuhoff *et al.*, 2005). Comparable results were obtained by Legen *et al.* (2003) in rat jejunum and artificial membranes. In intestinal cells ibuprofen has been suggested to inhibit substrate transport via the proton-coupled MCT1 (Tamai *et al.*, 1995). Therefore MCT1 might mediate the apical uptake of ibuprofen, but as no polarized transepithelial transport of ibuprofen was observed in the Caco-2 cell model, MCT1 does not seem to be a major determinant for transepithelial ibuprofen transport.

Interactions between ibuprofen and hPEPT1 substrates

The initial luminal concentration of ibuprofen was estimated to be between 3.9 mM and 15.5 mM. This corresponds to approximately 10–40 times the K_i^{app} of ibuprofen determined for hPEPT1. As ibuprofen is a non-competitive inhibitor of hPEPT1, the theoretical maximum transport rate would be reduced by at least 90% at the luminal ibuprofen concentrations likely to occur *in vivo*. Therefore, we investigated the effect on transepithelial transport of hPEPT1 substrates across Caco-2 cell monolayers using 1 or 10 mM ibuprofen. The relatively high permeability coefficients obtained in our Caco-2 cell transport experiments suggest a relatively high paracellular transport. We observed absorptive polarized tran-

sepithelial transport for ALA and Gly-Sar, whereas no polarized transport was observed for L-carnosine. For L-carnosine this pattern was expected, since even though L-carnosine is a substrate for hPEPT1, the transepithelial absorption mechanism for L-carnosine is not hPEPT1 mediated (Nielsen *et al.*, 2002b). ALA was recently also identified as a substrate for the proton-coupled amino acid transporter, hPAT1 (Anderson *et al.*, 2010; Frolund *et al.*, 2010). In Caco-2 cell monolayers, Frolund *et al.* (2010) found that only the proton-coupled carriers hPEPT1 and hPAT1 contributed to intestinal absorption of ALA. However, at the concentration used here, 9 μ M ALA and 9 μ M Gly-Sar, only hPEPT1-mediated transport accounts for the total apical transport. In Caco-2 cell monolayers 1 mM of ibuprofen significantly inhibited the absorptive transport of ALA and Gly-Sar. This confirms that the transport of hPEPT1 substrates is affected by the presence of ibuprofen in concentrations likely to be present in the lumen, following oral administration of ibuprofen. At 1 mM, ibuprofen did not affect the A–B transport of the passively transported metoprolol and mannitol, which confirmed that ibuprofen did not cause a non-specific effect on Caco-2 cells. Etoposide is a substrate for at least three efflux transporters, that is P-gp (ABCB1), BCRP (ABCG2) and MRP2 (ABCC2), which are expressed in the apical membrane of intestinal cells (Cui *et al.*, 1999; Guo *et al.*, 2002; Yuan *et al.*, 2009). Ibuprofen (1 mM) did not alter the A–B or B–A transport of etoposide and therefore did not appear to interact with the efflux transporters responsible for etoposide transport. In dose regimens including ibuprofen and a hPEPT1 substrate, inhibition of hPEPT1-mediated transport may be expected. The pharmacokinetic consequence of this may be an increased time to reach maximal plasma concentration and/or a reduced absorption fraction. *In vivo* studies are needed to clarify if the proposed interaction between ibuprofen and hPEPT1-substrates has clinical relevance in humans.

In conclusion, we have identified ibuprofen as a non-competitive inhibitor of hPEPT1 and drug–drug interactions at the level of intestinal drug absorption via hPEPT1 are anticipated in dose regimens consisting of ibuprofen and hPEPT1 substrates.

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Conflicts of interest

The authors state no conflict of interest.

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