Influence of cyclooxygenase inhibitors on the function of the prostaglandin transporter OATP2A1 expressed in human gastroduodenal mucosa

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Running Title
Impact of COX inhibitors on the OATP2A1 function

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Abbreviations
a.u. arbitrary units
ASA acetylsalicylic acid
COX cyclooxygenase
K_m Michaelis-Menten constant
NSAIDs non-steroidal anti-inflammatory drugs
OATP organic anion transporting polypeptide
PGE_2 prostaglandin E_2
SLCO solute carrier organic anion transporter
VC vector control
V_max maximal uptake rate

Section
1. Gastrointestinal, Hepatic, Pulmonary, and Renal
2. Metabolism, Transport, and Pharmacogenomics
Abstract

The human organic anion transporting polypeptide 2A1 (OATP2A1) is a prostaglandin transporter expressed in several tissues and plays an important role for local distribution of prostaglandins, which contribute to the integrity of gastric mucosa. Blockade of prostaglandin pathways by cyclooxygenase (COX) inhibitors has been associated with serious side effects such as gastrointestinal ulceration and bleeding. However, little is known regarding the OATP2A1 expression in the upper gastrointestinal tract and the potential impact of cyclooxygenase inhibitors on the OATP2A1 function. We first investigated the expression of OATP2A1 mRNA and protein in human gastroduodenal mucosa using human biopsy specimens obtained from antrum, corpus, and duodenum. The results indicate that OATP2A1 is expressed in the neck region and deep pyloric glands of antrum and in parietal cells of gastric corpus. Secondly, we examined various COX inhibitors for their effects on the OATP2A1 transporter activity. Using HEK293 cells expressing OATP2A1, we found that diclofenac and lumiracoxib are potent inhibitors of the OATP2A1-mediated transport of PGE$_2$ with IC$_{50}$ values of 6.2 ± 1.2 µM and 3.1 ± 1.2 µM. In contrast, indomethacin, ketoprofen, and naproxen led to a significant stimulation of OATP2A1-mediated PGE$_2$ transport by 162.7 ± 13.9 %, 77.2 ± 3.6 %, and 32.3 ± 4.9 %, respectively. Taken together, our results suggest that various clinically used COX inhibitors have differential impact on the function of the prostaglandin transporter OATP2A1 in human stomach and these effects may contribute to differences in gastrointestinal side effects of COX inhibitors.
Introduction

The SLCO (solute carrier organic anion transporter) gene family encodes for the organic anion transporting polypeptides (OATPs) mediating uptake of various drugs and endogenous compounds (Hagenbuch and Meier, 2003; Hagenbuch and Meier, 2004; König et al., 2006). The rodent prostaglandin transporter Oatp2a1 was first identified in rats in 1995 (Kanai et al., 1995). The human prostaglandin transporter OATP2A1 was cloned from an adult kidney cDNA library and found to be expressed in several tissues such as lung, kidney, and intestine (Lu et al., 1996). Both human OATP2A1 and rat Oatp2a1 were found to transport various prostaglandins including PGE1, PGE2, PGD2, and PGF2α (Kanai et al., 1995; Lu et al., 1996). It was previously suggested that OATP2A1 might be involved in the termination of PGE2 effects by mediating the cellular uptake and the subsequent oxidation of prostaglandins in cytoplasm (Pitt et al., 1983; Schuster, 2002). A study by Nomura and colleagues (Nomura et al., 2005) further demonstrated that the cellular reuptake of prostaglandins is an essential component in the metabolic clearance of prostaglandins. The authors further proposed a model where the synthesis, release, reuptake, and oxidation of prostaglandins occur in the same cell in the kidney, but in a compartmentalized manner (Nomura et al., 2005). However, the nature of compartmentalization is not clearly understood and it remains to be investigated whether OATP2A1 would have similar functions in other organs.

The non-steroidal anti-inflammatory drugs (NSAIDs) are inhibitors of COX-1 and COX-2. The decrease in prostaglandin and thromboxane production leads to the desired analgesic, antipyretic, and anti-inflammatory therapeutic effects. On the other hand, these drugs cause frequently mucosal damage such as gastrointestinal erosions, ulceration, and severe bleedings. Given that the integrity of gastroduodenal mucosa is highly dependent on the effects of locally synthesized prostaglandins (Gudis and Sakamoto, 2005), we raised the questions whether and where the prostaglandin transporter OATP2A1 is expressed in human gastroduodenal mucosa.
Furthermore, we studied whether clinically used COX inhibitors influence the transport activity of OATP2A1, potentially contributing to gastrointestinal side effects.

In the present study, we report the differential expression of the human prostaglandin transporter OATP2A1 in human gastroduodenal mucosa and that different COX inhibitors are capable of inhibiting or stimulating the OATP2A1-mediated transport of PGE₂.
Methods

Chemicals

[^3]H]Prostaglandin E2 ([^3]H]PGE2; 185.6 Ci / mmol) was obtained from PerkinElmer Life Sciences
GmbH (Rodgau-Jügesheim, Germany). Unlabeled PGE2 was purchased from Cayman
Chemicals (Tallinn, Estonia) and poly-D-lysine hydrobromide from Sigma-Aldrich Chemie GmbH
(Taufkirchen, Germany). Sodium butyrate was purchased from Merck KGaA (Darmstadt,
Germany). Acetylsalicylic acid, salicylic acid, diclofenac, ibuprofen, indomethacin, ketoprofen,
naproxen were purchased form Sigma-Aldrich. Novartis (Nuremberg, Germany) kindly provided
the COX-2 selective inhibitor lumiracoxib. Acetylsalicylic acid, diclofenac, ibuprofen,
indomethacin, ketoprofen, and naproxen were dissolved in dimethyl sulfoxide. Lumiracoxib and
salicylic acid were dissolved in ethanol. All other chemicals and reagents, unless stated
otherwise, were obtained from Carl Roth GmbH + Co. KG (Karlsruhe, Germany) and were of the
highest grade available.

Cell culture and generation of a HEK293 cell line stably expressing OATP2A1

Human embryonic kidney (HEK293) cells were cultured in minimum essential medium containing
10 % heat-inactivated fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin at
37 °C and 5 % CO2. The cells were routinely subcultured by trypsinization using trypsin
(0.05 %)-EDTA (0.02 %) solution. All cell culture media supplements were obtained from
Invitrogen GmbH (Karlsruhe, Germany).

The OATP2A1 expression plasmid was prepared by subcloning the SLCO2A1 reference
sequence (NM_0005630) from the original pCMV-SPORT6-OATP2A1 plasmid [purchased from
Invitrogen (Karlsruhe, Germany)] into the pcDNA3.1 (+)-vector (Invitrogen GmbH). HEK293 cells
were transfected with the plasmid pcDNA3.1 (+)-OATP2A1 using Effectene transfection reagent
kit (Qiagen GmbH, Hilden, Germany). After geneticin (G-418; 800 µg/ml) treatment, single
colonies were selected and characterized for OATP2A1 mRNA and protein expression using real-time PCR, immunofluorescence, and immunoblot analyses. HEK293-VC (VC: vector control) cells were established by the same method using the plasmid lacking the insert for transfection.

**Real-Time PCR**

OATP2A1 mRNA expression was measured by quantitative real-time PCR using the LightCycler 2 System (ROCHE) and normalized to the housekeeping gene β-actin (expressed as arbitrary units a.u.). PCR was performed using Light Cycler FastStart DNA MasterPLUS SYBR Green I reagents (ROCHE) and the following primers: OATP2A1 (fw 5'-GTGGTGAACCAGGAGGAAAAG-3'; rev 5'-GTATAGGCAGGTGGGAAGAG-3', amplicon size of 369 bp) and β-actin (fw 5'-TGACGGGGTCACCCACACTGTGCCCATCTA-3', rev 5'-CTAGAAGCATTTGGTGAGGACGAT-GGAGGG-3', amplicon size of 661 bp). PCR fragments were amplified with an initial denaturation step of 10 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s, annealing for 10 s at 64 °C and extending for 30 s at 72 °C. After DNA amplification a melting curve analysis was performed.

**Immunoblot analysis**

HEK293-OATP2A1 and HEK293-VC cells were seeded in poly-D-lysine coated cell culture plates (diameter: 10 cm) at an initial density of 4.5x10^6 cells per plate. After 24 h, cells were treated with 10 mM sodium butyrate for additional 24 hours before immunoblot analysis. Pelleted HEK293 cells were resuspended in protein storage buffer (100 mM Tris-HCl and 1 mM EDTA, pH 7.4) containing protease inhibitors (mini-complete protease inhibitor cocktail tablets, Roche Diagnostics-Applied Science, Mannheim, Germany). Protein concentrations were determined by bicinchoninic acid assay (BCA Protein Assay Kit; Thermo Scientific, Rockford, IL). Five
micrograms of total protein were diluted with Laemmli buffer (62 mM Tris-HCl, 2 % SDS, 10 % glycerol, 0.01 % bromophenol blue, 0.4 mM DTT) and incubated at 95 °C for 5 min before separation on 10 % SDS-polyacrylamide gels. BenchMark Pre-Stained Protein Ladder (Invitrogen GmbH, Karlsruhe, Germany) was used to visualize the protein molecular weight ranges. The proteins were transferred onto a nitrocellulose membrane (PROTRAN WHATMAN® Schleicher and Schuell, A. Hartenstein GmbH, Würzburg) using a tank blotting system from Bio-Rad (Munich, Germany). The membrane was incubated with a polyclonal mouse anti-human OATP2A1 antibody (Abnova; Taipei City, Taiwan) or a mouse monoclonal anti human β-actin antibody (Sigma-Aldrich, Taufkirchen, Germany). As secondary antibody, horseradish peroxidase-labeled goat anti mouse Fab-fragments (Dianova, Hamburg, Germany) were used. HEK293 cells and HEK293-VC cells served as negative controls. Protein was visualized on autoradiography films (GE Healthcare UK Ltd, Buckinghamshire, UK) using ECL™ Western blotting detection reagents (GE Healthcare) and a film developer (Kodak, Germany). Films were scanned in order to perform a semiquantitative expression analysis using the Gel-Pro® Analyzer software version 4.5.00.0 (Media Cybernetics Europe, Buckinghamshire, UK). The cross-reactivity of the OATP2A1 antibody against other human OATPs was tested by performing an immunoblot analysis using homogenates of cells stably expressing OATP1A2, OATP1B1, OATP1B3, OATP2B1, OATP4A1, and OATP4C1. No cross-reactivity against the tested OATPs was observed.

**Immunofluorescence microscopy**

HEK293-OATP2A1 and HEK293-VC cells were seeded on poly-D-lysine coated coverslips placed in 12-well plates at an initial density of 5.5x10^5 cells / well. After 24 h, cells were treated with 10 mM sodium butyrate for 24 h. The immunofluorescence staining was performed according to a previously published protocol (Lee et al., 2005). The cells were washed with TBS (Tris-buffered saline) and subsequently fixed in ice-cold 70% methanol for 10 min. Thereafter,
the cells were permeabilized for 10 min using TBS / Triton (0.4%). The cells were blocked with 2% bovine serum albumin followed by incubation with a polyclonal mouse anti-human OATP2A1 antibody (Abnova; Taipei City, Taiwan). A Cy2-conjugated AffiniPure goat anti-mouse IgG (Dianova, Hamburg, Germany) was used as secondary antibody. The nuclei were counterstained with the SYTOX® Orange dye (Invitrogen GmbH, Karlsruhe, Germany). The coverslips were inversely placed with an aqueous mounting medium (Thermo Scientific, Pittsburgh, PA) on a microscope slide. The fluorescence was visualized using a confocal laser scanning microscope Axiovert 100M (Carl Zeiss GmbH, Jena, Germany). The images were further processed using the Zeiss LSM Image Browser version 4.2.0.121 and ADOBE® PHOTOSHOP® CS2 version 9.0.2 (Adobe Systems Inc.).

**Transport assays**

The uptake experiments were performed as previously described (Seithel et al., 2007). Briefly, HEK293-OATP2A1 and HEK293-VC cells were seeded in poly-D-lysine coated 12-well plates at an initial density of 7.5x10^5 cells / well. After 24 h, cells were treated with sodium butyrate for 24 h prior to the uptake experiments. First, cells were washed with prewarmed (37 °C) uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM K_2HPO_4, 1.2 mM MgSO_4, 1.5 mM CaCl_2, 5 mM glucose, and 12.5 mM HEPES, pH 7.3). Subsequently, cells were incubated with a mix of radiolabeled and nonradiolabeled PGE_2 at 37 °C for 5 min. For analyzing the inhibition of OATP2A1-mediated PGE_2 uptake, 0.5 µM PGE_2 was co-incubated with different concentrations of the unselective COX inhibitors acetylsalicylic acid, salicylic acid, diclofenac, ibuprofen, indomethacin, ketoprofen, naproxen, and the selective COX-2 inhibitor lumiracoxib at 37 °C for 5 min. Afterwards the cells were washed three times with ice-cold uptake buffer and lysed with 0.2% sodium dodecyl sulfate (SDS). An aliquot was mixed with 4 ml of scintillation cocktail (Ultima Gold XR, PerkinElmer Life Sciences GmbH; Rodgau-Jügesheim, Germany) and the intracellular accumulation of radioactivity was determined by liquid scintillation counting (TriCarb 2800,
PerkinElmer Life Sciences GmbH). The appropriate protein concentration of each well was determined by bicinchonic acid assay (BCA Protein Assay Kit; Thermo Scientific, Rockford, IL). All experiments were performed at least in two separate experiments with a total of at least six wells per concentration studied.

Immunohistochemical analysis

Paraffin-embedded biopsy samples from healthy subjects as well as macroscopically and microscopically judged healthy stomach sections of corpus, antrum, and duodenum were taken from the tissue archives of the Institute of Pathology (Friedrich-Alexander-University Erlangen-Nuremberg) following local ethical guidelines. Ten different samples from each region (antrum, corpus, and duodenum) were investigated. The immunohistochemical analysis was performed as previously described (Glaeser et al., 2007). Briefly, paraffin sections were deparaffinized with xylene (Merck KGaA; Darmstadt, Germany). After rinsing with ethanol, an antigen retrieval procedure was performed using citrate buffer, pH 6.0 (DCS GmbH & Co. KG; Hamburg, Germany). Afterwards the sections were incubated with 10% H₂O₂ in Tris (pH 7.4, 100 mM) for 20 min at room temperature to quench endogenous peroxidase activity. After incubation of the sections with the blocking buffer Powerblock Universal Blocking Reagent (Biogenex, San Ramon, CA) for 60 min, tissue sections were incubated overnight with a polyclonal mouse anti-human OATP2A1 antibody (Abnova; Taipei City, Taiwan). Slides were subsequently incubated with biotinylated goat anti-mouse immunoglobulin and peroxidase-conjugated streptavidin (BioGenex, San Ramon, California). The proteins were visualized through a color reaction using 3-amino-9-ethylcarbazole or 3,3’-diaminobenzidine (AEC One Step Solution; Liquid DAB Substrate Pack; BioGenex, San Ramon, California) as substrates. Tissue sections were counterstained with Mayer’s hematoxylin (BioGenex, San Ramon, California). The expression of OATP2A1 in antrum, corpus, and duodenum was graded using an immunohistochemical rating score (IRS) according to Remmele et al. (Remmele and Stegner, 1987). Briefly, the intensity was
rated as followed: 0= no staining, 1= weak staining, 2= moderate staining, 3= strong staining. The amount of positive stained cells was rated as followed: 0= no cells, 1= <10 %, 2= 10-50 %, 3= 51-80 %, 4= >80 %. For the calculation of IRS, the scores of intensity and amount of positive cells were multiplied. Sporadic occurrence of positively stained edges of the tissue section was judged as an artificial effect and was therefore excluded from the IRS analysis. The specificity of immunoreactive signals for OATP2A1 was verified by negative controls, which were incubated with blocking buffer or polyclonal antiserum that had been neutralized by preincubation with the antigenic peptide (Abnova; Taipei City, Taiwan) at 37 °C for 1 h.

Analysis of OATP2A1 expression in human gastroduodenal biopsy samples

Samples for the investigation of mRNA and protein expression were obtained from a previously published study (Venerito et al., 2006; Wex et al., 2008). Briefly, healthy Helicobacter pylori-negative volunteers (n=9) underwent a gastroduodenoscopy without any drug treatment. The biopsies from antrum, corpus, and duodenum were used to determine mRNA and protein expression of OATP2A1 as described above. All subjects gave written informed consent. The local ethics committees of the Otto-von-Guericke-University (Magdeburg, Germany) and Friedrich-Alexander-University (Erlangen-Nuremberg, Germany) approved the study.

Data and statistical analysis

The percentage of uptake inhibition or stimulation was calculated from control experiments in the absence of inhibitors or stimulators (100 % uptake). The corresponding IC$_{50}$ or EC$_{50}$ values for inhibition or stimulation were calculated by fitting the data to a sigmoid dose-response regression curve (Prism 4.01 2004; GraphPad Software, San Diego, CA). The IC$_{50}$ or EC$_{50}$ value is the concentration at which 50 % inhibition or stimulation of substrate uptake is obtained. All data are presented as mean ± standard error mean (SEM) unless stated otherwise. The expression of
OATP2A1 mRNA and protein in antrum, corpus, and duodenum was analyzed using one-way analysis of variance (one-way ANOVA) with a Bonferroni's multiple comparison test. The influence of COX inhibitors at a concentration of 100 µM on the OATP2A1 transport was analyzed using one-way ANOVA with a Dunnett's multiple comparison test. A p-value ≤ 0.05 was considered statistically significant.
Results

Expression of OATP2A1 in human stomach

First, we investigated the mRNA and protein expression of OATP2A1 in antrum, corpus, and duodenum of nine healthy subjects without any medications. The OATP2A1 expression was normalized to that of β-actin. The OATP2A1 mRNA expression was highest in antrum and the difference between the antrum and the duodenum was found to be statistically significant (3.3-fold; p<0.001, one-way ANOVA, Bonferroni’s multiple comparison test; figure 1A). Interindividual variability of OATP2A1 mRNA expression among the nine volunteers was highest in antrum (8.5-fold) compared to corpus (6.4-fold) and duodenum (3.9-fold), respectively. The OATP2A1 protein expression however did not show statistical significance among antrum, corpus, and duodenum (p>0.05, one-way ANOVA, Bonferroni’s multiple comparison test; figure 1B). Figure 1C shows a representative immunoblot of OATP2A1 in biopsy samples obtained from human antrum, corpus, and duodenum.

Localization of OATP2A1 in human stomach

Following our immunoblotting analysis indicating OATP2A1 expression in human gastroduodenal mucosa, we performed immunohistochemical staining in order to investigate the localization of OATP2A1. The results showed the most intense staining of OATP2A1 in the antrum mucosa (n= 10). Positive cytoplasmic and granular staining was found in the neck region and deep pyloric glands of antrum (figure 2A). Human corpus mucosa (n= 10) showed a cytoplasmic granular staining also within the neck region predominantly in parietal cells. In antrum and corpus, a possible luminal staining along the deep glands could be observed (figure 2A, B). In duodenum (n= 10) several cells from the surface to the crypts in the mucosa and Brunner’s glands showed positive staining for OATP2A1 (figure 2C). The immunohistochemical rating score (IRS) revealed values (mean ± SD) of 6.7 ± 3.2 for antrum, 4.3 ± 2.6 for corpus, and 1.9 ± 1.8 for duodenum. The positive staining in the parietal cells was substantially attenuated by
preincubation of the OATP2A1 antibody with the antigenic peptide, indicating a specific binding of the antibody (figure 2D and E).

Characterization of the HEK293-OATP2A1 cell line stably expressing OATP2A1

In order to investigate the influence of non-selective and selective cyclooxygenase inhibitors on the OATP2A1 function, we established a cell line stably expressing OATP2A1. Using real-time PCR and immunoblotting analysis, we confirmed that the selected clone of the HEK293-OATP2A1 cell line has elevated levels of OATP2A1 mRNA and protein expression compared to the HEK293-VC cell line (figure 3A and 3B). The HEK293-OATP2A1 cells showed specific signals around 60 kDa and 80 kDa, which could not be detected in the HEK293-VC cells and HEK293 parental cells. We further investigated the cellular localization of OATP2A1 using confocal microscopy. OATP2A1 was localized in cytoplasm and on the plasma membrane of cells (figure 3C). OATP2A1 expressed in HEK293 cells was functionally active as an uptake transporter of PGE₂. HEK293-OATP2A1 cells were able to mediate the uptake of PGE₂ (0.5 µM) with an uptake ratio of 17.4 compared to HEK293-VC cells (p<0.0001). Figure 3D shows the uptake of 0.5 µM PGE₂ at 5 minutes into the HEK293-OATP2A1 and HEK293-VC cells.

Screening of COX inhibitors for the influence on the OATP2A1 function

In order to investigate a potential influence of COX inhibitors on the function of OATP2A1, we screened commonly used NSAIDs such as ASA (acetylsalicylic acid), salicylic acid, indomethacin, diclofenac, ibuprofen, ketoprofen, naproxen, and the selective COX-2 inhibitor lumiracoxib. ASA and salicylic acid (100 µM) showed significant inhibition of the PGE₂ transport by 18 % and 27 % (p<0.01). Diclofenac and lumiracoxib were found to be potent inhibitors reducing the PGE₂ transport by more than 95% (p<0.01). In contrast, indomethacin, ibuprofen, ketoprofen, and naproxen showed a statistically significant stimulation of PGE₂ transport at the tested concentration of 100 µM (p<0.01; figure 4).
Inhibition of OATP2A1-mediated PGE\(_2\) transport by diclofenac and lumiracoxib

Following the initial screening shown in figure 4, we investigated the impact of varying diclofenac and lumiracoxib concentrations on the PGE\(_2\) transport (figure 5). The IC\(_{50}\) values of diclofenac and lumiracoxib for inhibition of OATP2A1-mediated PGE\(_2\) uptake were 6.2 ± 1.2 µM and 3.1 ± 1.2 µM, respectively.

Stimulation of OATP2A1-mediated PGE\(_2\) transport by indomethacin, ketoprofen, and naproxen

Based on the results from the initial screening (figure 4), we further investigated the influence of varying concentrations of indomethacin, ibuprofen, ketoprofen, and naproxen on the OATP2A1-mediated PGE\(_2\) transport. As shown in figure 6, the sigmoidal curve fitting revealed that indomethacin, ketoprofen, and naproxen showed a concentration-dependent stimulation of PGE\(_2\) transport by 162.7 ± 13.9 %, 77.2 ± 3.6 %, and 32.3 ± 4.9 %, respectively. The EC\(_{50}\) value for indomethacin was 1.1 ± 1.4 µM. Ibuprofen did not show a dose-dependent stimulation or inhibition of OATP2A1-mediated PGE\(_2\) transport (data not shown).
Discussion

Here, we report that the prostaglandin transporter OATP2A1 is expressed in human gastroduodenal mucosa and that its transporter function can be inhibited or stimulated by commonly used COX inhibitors. Our study is the first detailed investigation of the OATP2A1 expression in various regions of human stomach and duodenum. Our findings regarding interindividual variability in OATP2A1 expression and differential impact of commonly used COX inhibitors on the OATP2A1 function may be important in obtaining a better understanding of the mechanisms leading to the gastrointestinal side effects associated with various COX inhibitors.

In the present study, we investigated the OATP2A1 expression at mRNA and protein levels in human biopsy samples. The real-time PCR analysis indicated that the OATP2A1 mRNA expression was highest in the antrum, followed by corpus and duodenal mucosa. The immunohistochemical staining and semiquantitative IRS analysis revealed similar findings in terms of the OATP2A1 protein expression. The immunoblotting analysis also indicated the highest expression in samples obtained from the antrum compared to corpus and duodenum. While most of these data were consistent, the OATP2A1 expression levels in the duodenal biopsies based on the immunoblotting analysis appeared to be higher than expected compared to the immunohistochemical staining. These observed differences may be due to variability of OATP2A1 expression in the duodenal mucosa among individuals and may also reflect regional expression differences within the duodenum. However, it could also be the result of possible differences during the biopsy collection. Taken together, our results support the expression of OATP2A1 in the human gastric and duodenal mucosa and the potential importance of OATP2A1 in prostaglandin signaling.

Of note, our immunohistochemical analysis showed the expression of OATP2A1 in parietal cells and gastric glands with a granular cytoplasmic staining pattern, suggesting a possible expression of OATP2A1 in cytoplasmic vesicles. It has been reported that COX-1 and COX-2 are localized in endoplasmic reticulum and canalicular membranes of parietal cells and gastric...
glands of human stomach under normal and certain pathological conditions (Jackson et al., 2000; Bhandari et al., 2005). These results are in line with the previous studies reporting that OATP2A1/Oatp2a1 is often expressed in the same cells where prostaglandin synthesis and oxidation take place (Bao et al., 2002; Nomura et al., 2005). The authors further proposed a model of prostaglandin metabolism where the prostaglandin synthesis, transport, and degradation take place in a compartmentalized manner. Supporting this model, Oatp2a1 expression in rat kidney was localized in cytoplasmic vesicles along different regions of the collecting duct and epithelial and interstitial cells in the inner and outer medulla (Bao et al., 2002).

Given the OATP2A1 expression in the gastroduodenal mucosa, we further investigated whether COX inhibitors influence the function of OATP2A1. We generated a HEK293 cell line stably expressing OATP2A1, which showed a significantly higher transport of PGE₂ compared to the control cells. For the initial screening, various commonly used COX inhibitors were examined for their impact on the OATP2A1 function using the concentration of 100 µM. This concentration was pharmacologically relevant corresponding to the therapeutic plasma concentrations for most compounds except for indomethacin (1-3 µM), diclofenac (2-10 µM), and lumiracoxib (4-20 µM).

First, our results indicated that diclofenac and lumiracoxib are potent inhibitors of OATP2A1 activity with comparable IC₅₀ values. These results are not surprising because the chemical structures of the two compounds are closely related. It should be noted that diclofenac and lumiracoxib have a relatively low risk of gastrointestinal complications (Henry et al., 1996). This reduced risk of diclofenac and lumiracoxib was associated with the preferential or complete inhibition of COX-2 at therapeutic doses, respectively (Tegeder et al., 1999; Brune, 2007). It remains to be further investigated whether the low risk of gastrointestinal complications of these drugs may also be related to their inhibitory effects on the OATP2A1 function. On the other hand, indomethacin, ketoprofen, and naproxen stimulated the OATP2A1-mediated PGE₂ uptake in a concentration-dependent manner. While the molecular mechanism underlying the
stimulatory effect of these compounds on the OATP2A1 function is unknown, similar findings showing the stimulation of uptake transporter activities by drugs and endogenous substrates have been previously reported of transporters other than OATP2A1 (Grube et al., 2006; Bachmakov et al., 2008). Regarding OATP2A1, Lu et al. also observed a slight increase of 14 % in the OATP2A1-mediated PGE$_2$ transport at a concentration of 10 µM indomethacin, whereas at 100 µM indomethacin the PGE$_2$ uptake was decreased to 82.5 % of control (Lu et al., 1996). The discrepancy to our study may be explained by differences in the total substrate concentration of PGE$_2$. Currently, it is not known whether the differential impact of COX inhibitors on the OATP2A1 function may contribute to the gastrointestinal side effects of COX inhibitors. NSAID-induced gastroduodenal complications are most frequent in the antrum, followed by the corpus (Kamada et al., 2006) and our results indicate that OATP2A1 expression levels follow the same order. It is known that the blockade of cyclooxygenases by COX inhibitors is a major cause for NSAID-induced gastrointestinal ulcerations. Our study indicates that some COX inhibitors may also modulate the activity of OATP2A1 in addition to the well-known inhibition of cyclooxygenases, thus influencing the concentrations of prostaglandins in gastric mucosa. The role of OATP2A1 in NSAID-induced ulcerations and bleedings remains to be further elucidated.

Furthermore, Brune et al. demonstrated that parietal cells of stomach are capable of trapping salicylates (Brune et al., 1977). This seems to be of special interest considering the localization of OATP2A1 in parietal cells in human stomach. Whether transport proteins such as OATPs mediate this trapping needs to be investigated.

Taken together, we characterized the expression of the prostaglandin transporter OATP2A1 in the human gastroduodenal mucosa, where it is predominantly expressed in antrum and corpus mucosa. Furthermore, we demonstrate that the activity of OATP2A1 is distinctly modulated by unselective and selective COX inhibitors, suggesting a potential pathophysiological role of OATP2A1 in the gastrointestinal side effects of COX inhibitors.
References


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Footnotes

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Legends for figures

Figure 1: Expression of OATP2A1 mRNA and protein in human stomach and duodenum (n=9).
A) OATP2A1 mRNA expression was determined in human biopsy samples from different regions of the stomach and duodenum obtained from healthy subjects. The mRNA expression was quantified using Real-Time PCR, normalized to the expression of the housekeeping gene β-actin and expressed as arbitrary units (a.u.). Significant differences were observed between antrum and duodenum (**p<0.001, one-way ANOVA, Bonferroni’s multiple comparison test). B) Protein expression of OATP2A1 in human stomach and duodenum. The expression was determined by immunoblot analysis and expressed as arbitrary units (a.u.). No significant differences were observed between antrum, corpus, and duodenum. C) Representative immunoblot of OATP2A1 in human antrum, corpus, and duodenum. All data are displayed as single values and mean (horizontal lines).

Figure 2: Localization of OATP2A1 in human gastroduodenal tissues. Intense staining of antrum mucosa (A) in the neck region and pyloric glands was observed. Staining occurred in parietal cells of corpus mucosa (B) within the neck region and possible luminal expression in deep glands. Staining of duodenum (C) with positive cells throughout surface and crypts was observed. Preincubation without (D) or with (E) the antigenic peptide. The signal was almost abolished after preincubation with the peptide (E). A), B), and C): 100x-fold magnification and stained with AEC, D) and E): 200x-fold magnification and stained with DAB.

Figure 3: Characterization of the HEK293-OATP2A1 cell line stably expressing OATP2A1. A) The generated HEK293-OATP2A1 cell line showed an elevated expression of OATP2A1 mRNA compared to the HEK293 cells transfected with the empty vector (VC=vector control). B) Immunoblot of OATP2A1 in HEK293-OATP2A1 cells, HEK293-VC, and HEK293 parental cells. At the molecular weight of around 60 and 80 kDa, a specific signal was detected in HEK293-
OATP2A1 cells, which was not detectable in HEK293-VC and HEK293 parental cells. C) Immunofluorescence analysis of HEK293-OATP2A1 cells (left) and HEK293-VC cells (right) using confocal microscopy. OATP2A1 was visualized using a Cy2-conjugated secondary antibody (green fluorescence). The localization of OATP2A1 is shown in x-y layer, x-z-layer, and y-z layer. OATP2A1 was localized in cytoplasm and on the plasma membrane of the HEK293-OATP2A1, whereas no staining is detectable in the HEK293-VC cells. The nuclei were stained red (400x-fold magnification with 4-fold zoom). D) Uptake of 0.5 µM PGE2 into HEK293-OATP2A1 and HEK293-VC cells. The HEK293-OATP2A1 cells showed a significantly higher uptake (17.4-fold) of PGE2 compared to HEK293-VC cells (***p<0.0001, paired t-test with Welch's correction). All data are displayed as mean ± SEM.

Figure 4: Influence of different COX inhibitors on the OATP2A1-mediated PGE2 (0.5 µM) transport (5 min). 100 µM of COX inhibitors were used. The data are expressed as percent of control (white column, PGE2 uptake without COX inhibitors). Acetylsalicylic acid (ASA) and salicylic acid showed a slight inhibition whereas diclofenac and lumiracoxib displayed a strong inhibition of the PGE2 uptake. Indomethacin, ibuprofen, ketoprofen, and naproxen showed a significant stimulation of the PGE2 uptake. All data are presented as mean ± SEM. **p<0.01, one-way ANOVA with a Dunnett's multiple comparison test.

Figure 5: Concentration-dependent effects of diclofenac and lumiracoxib on the OATP2A1 mediated PGE2 (0.5 µM) transport (5 min). All data are presented as mean ± SEM.

Figure 6: Concentration-dependent effects of indomethacin, ketoprofen, and naproxen on the OATP2A1-mediated PGE2 (0.5 µM) transport (5min). The data are expressed as percent of control (uptake without COX inhibitors). All data are presented as mean ± SEM. *p<0.05, **p<0.01, one-way ANOVA with a Dunnett's multiple comparison test.
Figure 1

A

OATP2A1 mRNA expression [a.u.]

Antrum  Corpus  Duodenum

B

OATP2A1 protein expression [a.u.]

Antrum  Corpus  Duodenum

C

HEK293-VC  HEK293-OATP2A1  Antrum  Corpus  Duodenum

85 kDa  60 kDa  40 kDa

β-actin

OATP2A1
**Figure 3**

**A**

- Bar graph showing OATP2A1 mRNA expression [a.u.] with bars for VC and HEK-OATP2A1.

**B**

- Western blotting image with protein bands at 85 kDa, 60 kDa, and 40 kDa labeled OATP2A1 and β-actin.

**C**

- Confocal microscopy images of HEK293-OATP2A1 and HEK293-VC with x-y, y-z, and x-z planes.

**D**

- Bar graph showing PGE$_2$ uptake (pmol/mg protein)$^{-1}$min$^{-1}$ with bars for VC and HEK-OATP2A1.
The bar chart illustrates the effects of various non-steroidal anti-inflammatory drugs (NSAIDs) on PGE2 uptake, as compared to a control group. The x-axis represents different NSAIDs: control, ASA, salicylic acid, indomethacin, diclofenac, lumiracoxib, ibuprofen, ketoprofen, and naproxen. The y-axis indicates the percentage of PGE2 uptake. Each bar is accompanied by a significance level (indicated by **), suggesting a statistically significant difference from the control group.
A

\[
\text{IC}_{50} = 6.2 \, \mu M
\]

B

\[
\text{IC}_{50} = 3.1 \, \mu M
\]

figure 5
A

EC$_{50}$ = 1.1 μM

PGE$_2$ uptake [%]

indomethacin [μM]

B

PGE$_2$ uptake [%]

ketoprofen [μM]

C

PGE$_2$ uptake [%]

naproxen [μM]

figure 6