Role of Organic Anion-Transporting Polypeptides for Cellular Mesalazine (5-Aminosalicylic Acid) Uptake

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ABSTRACT:
The therapeutic effects and metabolism of mesalazine (5-aminosalicylic acid) in patients with inflammatory bowel disease require intracellular accumulation of the drug in intestinal epithelial cells and hepatocytes. The molecular mechanisms of mesalazine uptake into cells have not been characterized so far. Using human embryonic kidney cells stably expressing uptake transporters of the organic anion-transporting polypeptide (OATP) family, which are expressed in human intestine and/or liver, we found that mesalazine uptake is mediated by OATP1B1, OATP1B3, and OATP2B1 but not by OATP1A2 and OATP4A1. Moreover, genetic variations (∗1b, ∗5, ∗15) in the SLCO1B1 gene encoding OATP1B1 reduced the $K_m$ value for mesalazine uptake from 55.1 to 16.3, 24.3, and 32.4 μM, respectively, and the respective $V_{max}$ values. Finally, budesonide, cyclosporine, and rifampin were identified as inhibitors of OATP1B1, OATP1B3, and OATP2B1-mediated mesalazine uptake. These in vitro data indicate that OATP-mediated uptake and its modification by genetic factors and comedications may play a role for mesalazine effects.

Introduction

Mesalazine (5-aminosalicylic acid, mesalamine) represents a drug of first choice for the treatment of mild to moderate ulcerative colitis. Moreover, long-term use of mesalazine in patients with chronic inflammatory bowel disease reduces the risk of development of colorectal cancer in patients with ulcerative colitis (Velayos et al., 2005). Recent in vitro data indicate that an important underlying mechanism of the chemopreventive action of mesalazine is that mesalazine causes cells to reversibly accumulate in the S phase and activate an ataxia telangiectasia-mutated and Rad3-related kinase-dependent checkpoint with subsequent increases in the maintenance of genomic stability and countering carcinogenesis (Luciani et al., 2007; Koelink et al., 2010).

Mesalazine is believed to act intracellularly in epithelial cells of the gut with topical delivery from the luminal side of the intestine (Schwab and Klotz, 2001). Sufficient delivery of the active drug to the inflamed area is an important determinant of therapeutic efficacy, whereas the extent of drug absorption into the systemic circulation might influence tolerability (Schwab and Klotz, 2001; Klotz and Schwab, 2005). In patients with ulcerative colitis there was indeed a negative correlation between mucosal concentrations of mesalazine and the severity of colonic inflammation (Frieler et al., 2000). Absorbed mesalazine is metabolized in the intestinal epithelium and in liver to pharmacologically inactive N-acetyl-mesalazine (Schwab and Klotz, 2001).

Uptake transporters such as members of the organic anion-transporting polypeptide family (OATPs) (gene symbol SLCO), which are located in the luminal membrane of enterocytes and in the basolateral membrane of hepatocytes, are well recognized determinants of drug absorption, distribution, and effects (Nies et al., 2009; Fahrmayr et al., 2010). OATPs transport a broad spectrum of endogenous compounds and drugs (Fahrmayr et al., 2010). OATP1B1, OATP1B3, and OATP2B1 are the three major hepatic OATPs mediating uptake of their substrates from the portal venous blood into the hepatocytes. Genetically determined variations in the SLCO1B1 gene affecting OATP1B1 function (e.g., SLCO1B1∗1b, ∗5, and ∗15) have a major impact on the plasma concentrations, therapeutic effects, and side effects of multiple drugs including HMG-CoA reductase inhibitors (Marzolini et al., 2004; Link et al., 2008; Fahrmayr et al., 2010; Niemi, 2010). Moreover, inhibition of specific uptake transporters by concomitantly administered drugs is an important mechanism of drug-drug interactions (for review see Kindla et al., 2009; Fahrmayr et al., 2010).

OATP1A2 and OATP2B1 expression has also been reported for enterocytes in the small intestine, thereby affecting drug absorption (Kobayashi et al., 2003; Glaeser et al., 2007). For example, coadministration of grapefruit juice with the antihistaminic drug fexofenadine leads to significantly reduced plasma concentrations of the OATP
substrate fexofenadine (Glaeser et al., 2007). At the cellular level, inhibition of OATP1A2-mediated fexofenadine uptake by naringin, a major constituent of grapefruit juice, was identified as the underlying mechanism (Bailey et al., 2007). OATP1A2, OATP1B1, OATP1B3, and OATP2B1 expression has also been reported for human colon (Hilgendorf et al., 2007; Meier et al., 2007) and OATP1B3 expression for colorectal cancer (Ballester et al., 2006; Lee et al., 2008). Of interest, Wojtal et al. (2009) recently reported up-regulation of OATP2B1 and OATP4A1 in inflamed compared with noninflamed tissue samples from terminal ileum and colon from patients with Crohn’s disease and significant up-regulation of OATP2B1 and OATP4A1 in paired colonic specimens from patients with ulcerative colitis.

At the present time, there are no published data on molecular mechanisms of mesalazine uptake into cells. In this study, we aimed to answer the following questions: 1) Is mesalazine uptake into cells mediated by members of the OATP family?; 2) Do genetic variations in the SLCO1B1 gene affect mesalazine uptake?; and 3) Could concomitantly administered drugs affect intracellular mesalazine concentrations via inhibition of OATP transporters? These questions were addressed by using human embryonic kidney (HEK) cell lines stably expressing human OATP1A2, OATP1B1, OATP1B3, OATP2B1, and OATP4A1 and HEK cells expressing the polymorphic OATP1B1 variants OATP1B1*1b (SLCO1B1c.388A>G, OATP1B1p.N130D), *5 (SLCO1B1c.521T>C, OATP1B1p.V174A), and *15 (SLCO1B1c.388A>G+521T>C, OATP1B1p.N130D+V174A).

Materials and Methods

Chemicals. [3H]Sulfobromophthalein ([3H]BSP) (7585 GBq/mmol) was obtained from Hartmann Analytic (Braunschweig, Germany). Unlabeled sulfobromophthalein and poly-d-lysine hydrobromide were purchased from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany). [3H]Mesalazine (26.2 Ci/mmol) was purchased from ViTrax (Placentia, CA), unlabeled mesalazine was obtained from Sigma-Aldrich (Taufkirchen, Germany). G418 (Genetin) disulfate and hygromycin were from Invitrogen (Groningen, The Netherlands).

Cloning of the SLCO1A2 cDNA Encoding Human OATP1A2. The SLCO1A2 coding sequence (NM_134431.3) was cloned by reverse transcription reaction and subsequent polymerase chain reaction from human brain cDNA (Human Total RNA Master Panel II; Takara Bio Europe/Clontech, Saint-Germain-en-Laye, France) into the pcDNA3.1(+) vector (Invitrogen GmbH, Karlsruhe, Germany) and subsequently subcloned into the retroviral vector pQCXIN (Takara Bio Europe/Clontech). HEK293 cells were transfected with the plasmid pQCXIN-OATP1A2 using a retroviral gene transfer vector pQCXIN (Takara Bio Europe/Clontech). HEK293 cells stably expressing human OATP1A2 and OATP4A1 were established as described previously (Seithel et al., 2007). Cell clones were screened by quantitative reverse transcription-PCR for mRNA expression, and the protein synthesis was verified by immunoblot analysis (Seithel et al., 2007). Finally, the cell clones with the highest mRNA and corresponding protein expression were used for subsequent transport experiments.

Uptake and Uptake Inhibition Studies. Uptake assays were performed as described previously (Seithel et al., 2007). In brief, 700,000 HEK-OATP cells (stably expressing an OATP protein) or the respective HEK-Co cells [HEK-Co/418 or HEK-Co/Hy; control cell lines transfected with the empty expression vectors pcDNA3.1(+) and pcDNA3.1(Invitrogen, respectively) were seeded in 12-well plates coated with 0.1 mg/ml poly-d-lysine (Greiner Bio-One, Frickenhausen, Germany) and cultured for 2 days. Twenty-four hours before uptake or uptake inhibition experiments, cells were induced with 10 mM sodium butyrate (Merck KGaA, Darmstadt, Germany) to obtain higher levels of recombinant protein expression (Cui et al., 1999). Before uptake experiments, cells were washed with prewarmed uptake buffer (142 mM NaCl, 5 mM KCl, 1 mM KH2PO4, 1.2 mM MgSO4, 1.5 mM CaCl2, 5 mM glucose, and 12.5 mM HEPES, pH 7.3). [3H]BSP or [3H]mesalazine was dissolved in uptake buffer, and unlabeled BSP or mesalazine was added to reach the final concentrations. For uptake inhibition experiments, mesalazine (as an inhibitor of OATP-mediated BSP uptake) or several drugs (as inhibitors of OATP-mediated mesalazine uptake) were added in the respective concentrations into the uptake solution. The cells were incubated with the uptake solution for 10 min and subsequently washed three times with ice-cold uptake buffer to remove radioactivity bound to the cell membrane. An incubation time of 10 min was used for mesalazine, because experiments have demonstrated linearity of uptake up to 10 min using mesalazine in a concentration of 20 μM. After the cells were lysed with 0.2% SDS, the intracellular accumulation of radioactivity was determined by liquid scintillation counting (TriCarbo 2800; PerkinElmer Life and Analytical Sciences GmbH, Rodgau, Germany), and the appropriate protein concentrations was determined by a bichinchoninic acid assay (BCA Protein Assay Kit; Thermo Fisher Scientific, Bonn, Germany). K i values for inhibition of OATP1B1-mediated mesalazine uptake were determined using different mesalazine (7.5 or 10, 50, and 100 μM) and inhibitor concentrations. For pravastatin and budesonide as inhibitors of OATP1B1-mediated mesalazine uptake, 1, 10, and 25 μM concentrations of the respective drug and for rifampicin and cyclosporine 0.1, 1, and 10 μM concentrations were used. These data were analyzed by the method of Dixon, in which the reciprocal velocity (1/V) is plotted against the inhibitor concentration.

Statistical Analysis. The uptake experiments were repeated at least two times on different days with n = 3, i.e., for each concentration and time point 6 (and for control experiments up to 12) separate wells were investigated. All data are presented as the mean ± S.D. Transport kinetics were calculated using Prism 3.01 (GraphPad Software Inc., San Diego, CA). The calculated parameters were maximum transport rate (V max) and Michaelis-Menten constant (K m). K m values were calculated using the nonlinear regression (one-site binding) curve fit from the same software package. Multiple comparisons were analyzed by analysis of variance with a subsequent Dunnett’s multiple comparison test by using Prism 3.01. p < 0.05 was required for statistical significance.

Results

Inhibition of OATP1A2-, OATP1B1-, OATP1B3- and OATP2B1-Mediated BSP Uptake by Mesalazine. The influence of increasing concentrations of mesalazine on OATP1A2-, OATP1B1-, OATP1B3- and OATP2B1-mediated BSP uptake is shown in Fig. 1. Mesalazine...
had no significant effect on OATP1A2- and OATP2B1-mediated BSP uptake. Significant, concentration-dependent inhibition of BSP uptake by mesalazine was found for OATP1B1- and OATP1B3-mediated BSP uptake (Fig. 1).

**FIG. 1. Inhibition of OATP1A2-, OATP1B1-, OATP1B3- and OATP2B1-mediated BSP uptake by mesalazine. Inhibitory effects of mesalazine on OATP-mediated BSP (0.05 µM for OATP1B1 and 1 µM for OATP1A2, OATP1B3, and OATP2B1) uptake were investigated after a 10-min incubation. Data are shown as the percentage of the BSP uptake in the absence of mesalazine. Each value is the mean value ± S.D. ***, p < 0.01 versus control.**

**FIG. 2. OATP1B1-, OATP1B3-, and OATP2B1-mediated uptake of mesalazine (20 µM) in HEK-OATP1B1, HEK-OATP1B3, and HEK-OATP2B1 cells and the respective vector control cells. ***, p < 0.01 versus control.**
observed lower $K_m$ and lower $V_{\text{max}}$ values ($p < 0.01$) for mesalazine uptake mediated by the polymorphic variants $SLCO1B1^*1b$, $^*5$, and $^*15$ compared with wild type $SLCO1B1^*1a$ (Table 1).

### Influence of Drugs on OATP1B1-, OATP1B3-, and OATP2B1-Mediated Mesalazine Uptake

Finally, the impact of known OATP inhibitors or of drugs used for treatment of inflammatory bowel disease on OATP1B1-, OATP1B3-, and OATP2B1-mediated mesalazine uptake was investigated. Pravastatin (100 µM) significantly inhibited mesalazine uptake mediated by all hepatic OATPs ($p < 0.01$) (Fig. 5). At the lower concentration of 10 µM, pravastatin significantly inhibited OATP1B1-mediated mesalazine uptake ($p < 0.05$) (Fig. 5). Azathioprine had only a modest effect on OATP1B1-, OATP1B3-, and OATP2B1-mediated mesalazine uptake (Fig. 5). Cyclosporine and rifampin were particularly strong inhibitors of OATP1B1- and OATP1B3-mediated mesalazine uptake but also significantly inhibited OATP2B1-mediated mesalazine uptake. Of interest, budesonide at 10 and 100 µM inhibited OATP1B1-mediated ($p < 0.05$), OATP1B3-mediated ($p < 0.01$) and OATP2B1-mediated ($p < 0.01$) mesalazine uptake (Fig. 5). We also analyzed the effect of these drugs (in a concentration of 100 µM) on OATP1B1-, OATP1B3-, and OATP2B1-mediated BSP uptake and could confirm these results (data not shown). As shown for OATP1B1-, OATP1B3-, and OATP2B1-mediated mesalazine uptake, pravastatin, budesonide, cyclosporine, and rifampin significantly inhibited BSP uptake, whereas clarithromycin inhibited only OATP1B1- and OATP1B3-mediated BSP uptake and azathioprine had no significant effect on OATP-mediated BSP uptake. Additional Dixon plot analyses were indicative of competitive inhibition of OATP1B1-mediated mesalazine uptake by pravastatin, budesonide, cyclosporine, and rifampin with $K_i$ values of 7.2, 2.1, 0.7, and 1.0 µM, respectively (data not shown).

### Discussion

This is the first study showing that intracellular accumulation of mesalazine is mediated by members of the OATP uptake transporter family, which are expressed in small and large intestine and in the liver. The desired therapeutic effects of mesalazine in the intestine require a sufficient intracellular accumulation of the drug (Schwab and Klotz, 2001; Luciani et al., 2007; Koelink et al., 2010). On the basis of our data, it can be concluded that mesalazine is a substrate for the OATP family members OATP1B1, OATP1B3, and OATP2B1. These transporters are not only the most important OATP uptake transporters in the basolateral membrane of hepatocytes, thus being an essential step for the hepatic elimination of drugs (Kindl et al., 2009), but they are also expressed in human healthy colon and colorectal cancer (Ballestero et al., 2006; Hilgendorf et al., 2007; Meier et al., 2007; Lee et al., 2008). Wojtal et al. (2009) recently reported up-regulation of OATP2B1 and OATP4A1 in inflamed compared with noninflamed tissue originating from terminal ileum and colon from patients with Crohn’s disease and significant up-regulation of OATP2B1 and OATP4A1 in paired colonic specimens from patients with ulcerative colitis, indicating that the up-regulation of OATP2B1 could lead to enhanced accumulation of the OATP2B1 substrate mesalazine in the inflamed tissues.

Previous clinical studies indicate that a fraction of mesalazine and its metabolite N-acetyl-mesalazine are secreted back from the enterocytes into the gut lumen (Goebell et al., 1993; Layer et al., 1995). For other drugs, efflux transporters expressed in the luminal membrane of enterocytes such as P-glycoprotein, multidrug resistance-associated protein 2, and breast cancer resistance protein are well recognized determinants of intracellular accumulation in the enterocytes and efflux into the gut lumen. The molecular mechanism of secretion of mesalazine back into the gut lumen is still unclear, because recent in vitro data by Xin et al. (2006) showed that mesalazine is not a

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**Fig. 3.** Kinetics of OATP1B1-, OATP1B3-, and OATP2B1-mediated net uptake of mesalazine. Net uptake was calculated as the difference between total uptake into HEK-OATP1B1, HEK-OATP1B3, and HEK-OATP2B1 cells and the respective vector control cells.

**Fig. 4.** Impact of the $SLCO1B1$ genetic variants $^*1b$, $^*5$, and $^*15$ compared with wild-type ($^*1a$) OATP1B1-mediated mesalazine net uptake. Net uptake was calculated as the difference between total uptake in the different HEK-OATP1B1 cells and the respective control cells. **, $p < 0.01$, wild-type ($^*1a$) versus $^*1b$, $^*5$, and $^*15$. 

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**Table 1.** Kinetic data of mesalazine uptake by OATP family members.

<table>
<thead>
<tr>
<th>OATP Family Member</th>
<th>$K_m$ (µM)</th>
<th>$V_{\text{max}}$ (pmol * mg Protein * min$^{-1}$)</th>
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<tr>
<td>OATP1B1</td>
<td>$K_m = 55.1 \pm 13.4$ µM</td>
<td>$V_{\text{max}} = 28.3 \pm 2.0$ pmol * mg Protein * min$^{-1}$</td>
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<tr>
<td>OATP1B3</td>
<td>$K_m = 77.4 \pm 8.1$ µM</td>
<td>$V_{\text{max}} = 85.1 \pm 3.1$ pmol * mg Protein * min$^{-1}$</td>
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<tr>
<td>OATP2B1</td>
<td>$K_m = 188.9 \pm 40.5$ µM</td>
<td>$V_{\text{max}} = 28.3 \pm 2.0$ pmol * mg Protein * min$^{-1}$</td>
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substrate of the efflux pumps P-glycoprotein and multidrug resistance-associated protein 2.

Mesalazine shows the desired therapeutic effects in inflammatory bowel disease via topical mechanisms in the intestine. Nevertheless, after oral administration or administration in the form of enemas or suppositories, mesalazine and its metabolite reach significant concentrations in the systemic circulation (approximately 20 μM for mesalazine) (Lück et al., 2009), which have been linked to the tolerability of drugs, including statins and certain oral antidiabetic drugs (Fahrmayr et al., 2010). For example, recent studies reported a strong dependence of simvastatin acid plasma concentrations on the SLCO1B1 genotype (Pasanen et al., 2006) and a clear relationship between SLCO1B1 variants and the risk for statin-induced myopathy (Link et al., 2008). Our in vitro data clearly show impaired mesalazine uptake by cells stably expressing the variants SLCO1B1*1b, *5, and *15 compared with those expressing wild-type SLCO1B1. The clinical consequences of our observation need to be clarified in further studies, but we postulate that mesalazine plasma concentrations and possibly some of its side effects will depend on the SLCO1B1 genotype. To the best of our knowledge, the impact of SLCO1B1 genotypes on mRNA or protein expression in the colon has not been investigated.

In addition to genetic factors, local drug concentrations at the site of action, drug disposition in the systemic circulation, and drug effects can considerably be altered by concomitantly administered drugs. An important mechanism of drug-drug interactions is inhibition of drug uptake by a second, concomitantly administered drug (for review, see Fahrmayr et al., 2010). Because we were able to show that mesalazine is a substrate of OATP1B1, OATP1B3, and OATP2B1, we determined the impact of drugs known to inhibit OATPs and of other drugs used for treatment of inflammatory bowel disease on OATP1B1-, OATP1B3-, and OATP2B1-mediated mesalazine uptake. It should be noted that transporters located in the luminal membrane of enterocytes and in the basolateral membrane of hepatocytes might be exposed to relatively high concentrations of a potential inhibitor dissolved in the intestinal lumen and the portal venous circulation.

<table>
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<th>Table 1: Kinetic parameters of mesalazine uptake by SLCO1B1<em>1a and its variants SLCO1B1</em>1b, SLCO1B1<em>5, and SLCO1B1</em>15</th>
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<tr>
<td><strong>K_m</strong> (μM)</td>
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<tr>
<td>SLCO1B1*1a</td>
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<tr>
<td>SLCO1B1*1b</td>
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<tr>
<td>SLCO1B1*5</td>
</tr>
<tr>
<td>SLCO1B1*15</td>
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</table>

* The clinical consequences of our observation need to be clarified in further studies, but we postulate that mesalazine plasma concentrations and possibly some of its side effects will depend on the SLCO1B1 genotype. To the best of our knowledge, the impact of SLCO1B1 genotypes on mRNA or protein expression in the colon has not been investigated.

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**Fig. 5.** Inhibition of OATP1B1-, OATP1B3-, and OATP2B1-mediated mesalazine (20 μM) uptake by different drugs. The inhibitory effect on OATP1B1-, OATP1B3-, and OATP2B1-mediated mesalazine uptake was investigated using 10 and 100 μM concentrations of the potential inhibitors. Data are shown as the percentage of the transporter-mediated mesalazine uptake in the absence of potential inhibitors (Co). Each value is the mean value ± S.D. Prava, pravastatin; Aza, azathioprine; Clari, clarithromycin; Bude, budesonide; CsA, cyclosporine; Rifa, rifampin. *p < 0.05; **p < 0.01 versus control.
blood, respectively, compared with the concentrations in the systemic circulation. The OATP substrate pravastatin preferentially inhibited OATP1B1-mediated mesalazine uptake but also had a significant effect on OATP1B3 and OATP2B1 transport function at the higher 100 μM concentration. The macrolide antibiotic clarithromycin significantly inhibited OATP1B1- and OATP1B3-mediated mesalazine uptake at the higher concentration. The OATP inhibitors cyclosporine and rifampin had a more pronounced effect on mesalazine transport by OATP1B1 and OATP1B3 compared with that of OATP2B1. Azathioprine had no relevant effect on OATP function, whereas budesonide significantly affected mesalazine transport by all OATPs. These in vitro data indicate that local mesalazine concentrations and thus its effects could be reduced by some concomitantly administered drugs in vivo. However, it should be noted that we do not have reliable measured values of inhibitor concentrations in the gut lumen or the portal vein, making it very difficult to reliably predict the clinical consequences of these in vitro data.

Taken together, we identify here the molecular mechanisms of mesalazine uptake into intestinal cells and hepatocytes. Moreover, our data indicate that some comedication and genetic factors may modify intracellular mesalazine accumulation. Variability in mesalazine cellular uptake is likely to explain a fraction of the large interindividual variability in systemic plasma and local tissue concentrations of mesalazine. Because Wojtal et al. (2009) recently showed significant up-regulation of SLC23A1 mRNA in inflamed versus noninflamed colon in patients with ulcerative colitis, we postulate that increased intracellular accumulation of mesalazine contributes to the beneficial effects of this drug in the treatment of ulcerative colitis. Further studies are required to assess in greater detail the expression and effects of this drug in the treatment of ulcerative bowel disease.

**References**


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**Authorship Contributions**

**Participated in research design:** König, Klotz, and Fromm.

**Conducted experiments:** König, Glaser, and Mandery.

**Contributed new reagents or analytic tools:** Keiser.

**Performed data analysis:** König, Glaser, Mandery, and Fromm.

**Wrote or contributed to the writing of the manuscript:** König, Glaser, Keiser, Mandery, Klotz, and Fromm.