Oral availability of cefadroxil depends on ABCC3 and ABCC4.

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Transport of cefadroxil by ABCC3 and ABCC4.

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List of abbreviations

AUC, area under the curve; E217ßG, estradiol-17ß-glucuronide; H⁺-coupled peptide transporter 1, PepT1;
Abstract

Some cephalosporins, like cefadroxil, are orally available. PepT1 mediates the transport of cephalosporins across the apical membrane of enterocytes. It is not known which mechanism(s) is (are) responsible for the subsequent transport of cephalosporins across the basolateral membrane towards the circulation. We have tested whether ABCC3 and/or ABCC4 are involved in the latter process. Transport experiments with plasma membrane vesicles expressing these transporters were used to determine whether ABCC3 and ABCC4 can transport cephalosporins in vitro. The involvement of Abcc3 and Abcc4 in the transport of cefadroxil from enterocytes was subsequently studied using intestinal explants from wild-type, Abcc3+/-, Abcc4+/-, and Abcc3+/-/Abcc4+/- mice in an Ussing chamber setup. Finally, appearance of cefadroxil in portal blood was investigated in vivo after intra jejunal administration of cefadroxil in wild-type, Abcc3+/-, Abcc4+/-, and Abcc3+/-/Abcc4+/- mice. ABCC3- and ABCC4-mediated transport of estradiol-17ß-glucuronide (E217ßG) was dose dependently inhibited by cephalosporins in vesicular transport experiments. Furthermore, transport of cefadroxil by ABCC3 and ABCC4 was saturable with K_m values of 2.5±0.7 and 0.25±0.07 mM, respectively. Transport of cefadroxil from the apical to the basolateral side of jejunal tissue explants was unchanged in Abcc3+/- but significantly reduced (approximately two-fold) in Abcc4+/- and Abcc3+/-/Abcc4+/- when compared to wild type tissue. Upon instillation of cefadroxil in the jejunum, portal- and peripheral blood concentrations were similar in Abcc3+/- and Abcc4+/- but approximately two-fold reduced in Abcc3+/-/Abcc4+/- compared to wild type mice. Our data demonstrate that intestinal absorption of cefadroxil depends partly on ABCC3 and ABCC4.


**Introduction**

Infectious diseases are often treated with cephalosporins (Livermore, 2009) which belong to the class of β-lactam antibiotics. Due to antibiotic resistance there is a continuous need for the development of new cephalosporins (Livermore, 2009). Knowledge of their pharmacokinetics is therefore of eminent interest. One way of classifying cephalosporins is the route of administration: topical, oral or parenteral. Oral administration is the most uncomplicated form of administration but requires passage through the enterocytes. Since it is known that cephalosporins are ionized at physiological pH and have very low lipid solubility, luminal enterocytic uptake was considered to be transporter mediated (Tsuji, et al., 1981). The H⁺-coupled peptide transporter 1 (PepT1) was identified as the transporter involved (Nakashima, et al., 1984). Similarly, cephalosporins can not passively cross the basolateral membrane, implicating that transport over the basolateral membrane must also be transporter mediated. A transporter known to mediate transport of cephalosporins (like ceftriaxone, cefaperazone and cephalexin) is the efflux pump multidrug resistance associated protein 2 (Mrp2/Abcc2) (Oude Elferink and Jansen, 1994; Kato, et al., 2008). ABCC2 is a broad-spectrum transporter and can transport many different substrates including unconjugated anions, glucuronate-, glutathione- and sulphate conjugates and furthermore, among others, leukotriene C4 and methotrexate (Oude Elferink and de, 2007). ABCC2 has overlapping substrate specificity with its close homologs ABCC3 and ABCC4 (Borst, et al., 2007; Rius, et al., 2008). While ABCC2 resides in the apical membrane of enterocytes (Mottino, et al., 2000), ABCC3 localizes to the basolateral membrane of epithelial cells including enterocytes (Scheffer, et al., 2002). The subcellular localization of ABCC4 is cell type dependent: In prostate tubuloacinar cells,
hepatocytes and choroid plexus epithelium ABCC4 is localized on the basolateral membrane, but in renal proximal tubule cells ABCC4 resides at the apical membrane (Russel, et al., 2008). It is not exactly clear where ABCC4 resides in the intestine: in the colonic cell line HT29-CL19A the protein was localised to both the apical and basolateral membrane, with a higher expression apically (Li, et al., 2007). However, data of Caco2 cells show preferential basolateral expression (Ming and Thakker, 2010). In the present study we explored whether ABCC3 and/or ABCC4 are able to transport cefadroxil in plasma membrane vesicles from cells overexpressing the corresponding genes. In addition, we used Ussing chamber experiments and intestinal uptake experiments with wild-type, Abcc3+/−, Abcc4+/− and Abcc3+/−/Abcc4+/− mice to test whether Abcc3 and/or Abcc4 are involved in the basolateral transport of cefadroxil in vivo.
Materials and methods

Materials

Acetonitrile (HPLC grade) was from Baker (Mallinckrodt Baker, Deventer, The Netherlands). [\(^3\)H]estradiol-17β-glucuronide ([\(^3\)H]E\(_2\)17βG) was from Amersham (Amersham, Roosendaal, The Netherlands). All other chemicals and reagents were from Sigma (Sigma-Aldrich, Zwijndrecht, The Netherlands).

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee (IUCAC) of the Academic Medical Center. Wild type and Abcc3\(^+/\) (Zelcer, et al., 2006) mice were bred at the Animal Institute of the Academic Medical Center. Abcc4\(^+/\) (Assem, et al., 2004) and Abcc3\(^+/\)/Abcc4\(^+/\) mice, generated by crossing Abcc3\(^+/\) and Abcc4\(^+/\) mice, were bred at the Netherlands Cancer Institute (Amsterdam, The Netherlands). The animals used were 9 to 16 weeks old. All animals were on a 99% FVB/N background and kept in a temperature-controlled environment with a 12-h light/dark cycle. Standard diet (CRM; SDS diets, Germany) and water were available ad libitum.

Methods

Preparation of membrane vesicles

The cDNA of the ABCC2 gene (Paulusma, et al., 1997) was cloned into the pFastBac1 (Life Technologies, Breda, The Netherlands) donor plasmid. This was followed by transformation of the donor plasmid into DH10Bac (Life Technologies, Breda, The Netherlands) cells, which allowed for transposition of the ABCC2 gene into bacmid DNA. ABCG2, ABCC1, ABCC3 and ABCC4-recombinant baculovirus were a kind gift.
from Professor P. Borst (Bakos, et al., 2000; Breedveld, et al., 2004; van Aubel, et al., 2002; Zelcer, et al., 2001). Sf21 cells grown at 27°C were infected with ABCC1, ABCC2, ABCC3, ABCC4 or ABCG2-recombinant baculovirus. Isolation of membrane vesicles was as described before (de Waart, et al., 2006). In brief, cells were harvested 2 days (ABCC3), 3 days (ABCC1, ABCC2 and ABCG2) or 4 days (ABCC4), respectively, postinfection and resuspended in ice-cold hypotonic buffer (250 mM Mannitol/ 20 mM HEPES/Tris-buffered (pH 7.4)/2 mM EDTA) supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin), and incubated for 30-60 min on ice. Cells were homogenized using a tight-fitting Dounce homogenizer. The cell homogenate was layered on top of a discontinuous sucrose gradient (i.e. 56%, 38% and 19% sucrose) and centrifuged for 3.2 h at 141000g (SW28 rotor and a Beckman Optima L-90K centrifuge (Beckman, Mijdrecht, The Netherlands)). The 19%-38% interface was collected, washed and resuspended in 250 mM sucrose/ 20 mM HEPES/Tris-buffered pH 7.4. Membrane vesicles were obtained after revesiculation of the preparation by passing it 30 times through a 27-gauge needle. Vesicles were aliquoted and stored at –80°C until use. Protein content was determined using the BCA method.

**Western blotting and protein analysis**

Membrane vesicles were fractionated by 6% SDS-PAGE, blotted on nitrocellulose membranes (Schleicher&Schuell, Dassel, Germany) which were blocked in phosphate-buffered saline (PBS)/5% milk powder/0.05% Tween-20. The following antibodies were used: anti-ABCG2; BXP-21 (Maliepaard, et al., 2001), anti-ABCC1; MRPr1 (Scheffèr, et al., 2000), anti-ABCC2; M2III6 (Scheffèr, et al., 2000), anti-ABCC3; M3III21 (Scheffèr,
et al., 2000) and anti-ABCC4; M4I10 (Leggas, et al., 2004) (all antibodies were kind gifts of Dr. G.L. Scheffer (Free University Hospital, Amsterdam, The Netherlands)). Immune complexes were visualized with horseradish-peroxidase-conjugated immunoglobulins (Biorad, Veenendaal, The Netherlands) and detected using the ECL Western blot detection kit (Amersham, Roosendaal, The Netherlands).

**Vesicular transport assays**

Vesicular transport studies were performed using the rapid filtration technique as described (Heijn, et al., 1992). Briefly, 5-50 µg membrane vesicles were added to pre-warmed (37°C) reaction-mixture containing [³H]estradiol-17β-glucuronide or [³H]cefadroxil (0.05 and 0.2 µCi respectively, in 250 mM sucrose, 20 mM HEPES/Tris (pH 7.4)/ 20 mM MgCl₂/ 30 mM creatine phosphate/ 1.2 mg creatine kinase per ml/ with or without ATP (8 mM). Uptake was terminated by the addition of 20 vol of ice-cold 250 mM sucrose, 20 mM HEPES/Tris pH 7.4. The suspension was immediately filtered through 0.2 µm nitrocellulose filters under vacuum. The filters were washed three times and counted in a liquid scintillation counter.

**Calculation of half-maximal inhibition**

Transport of [³H]estradiol-17β-glucuronide was plotted against cephalosporin concentration. The program GraphPad using non-linear regression was used to calculate the concentration of cephalosporin, at which halfmaximal inhibition was achieved.

**Ussing chamber experiments and quantification of cefadroxil**

Jejunal tissues from wild-type, Abcc3⁺/⁺, Abcc4⁺/⁺ and Abcc3⁺/⁺/Abcc4⁺/⁺ animals were mounted in an Ussing chamber. The basolateral compartment contained 1.5 ml basolateral buffer which consisted of Krebs/ bicarbonate, glucose (10 mM) and HEPES.
(10 mM, pH 7.4). Furthermore, the apical compartment contained 1.5 ml apical buffer which consisted of Krebs/ bicarbonate, mannitol (10 mM) and MES (10 mM, pH 6.0). Both buffers were continuously gassed with carbogene. Temperature was set at 37 ± 1°C. After 5-10 min equilibration the experiment was started by the addition of 100 µl cefadroxil containing 8 or 80 nmol (in apical buffer) at the apical side. Cefadroxil was quantified by reversed phase High-performance liquid chromatography (HPLC) with UV detection (Dionex, Amsterdam, The Netherlands). In brief, 100 µl sample was applied to a BDS Hypersil C18, 3 µm, 15 cm HPLC column (Thermo scientific, Waltham, USA). Starting eluent consisted of 10% acetonitrile/ 90% ammonium formate (20 mM), pH 3.5, followed by a linear gradient to 30% acetonitrile in the same buffer in 20 min. Cefadroxil had a retention time of 14 min. Detection of cefadroxil was done at 260 nm. Quantification of cefadroxil was done by using a calibration curve of cefadroxil.

**In vivo experiment: Jejunal administration of cefadroxil and portal blood collection**

Mice were anesthetized with a combination of Hypnorm (VetaPharma, Leeds, UK; 11.8 mg/kg fluanisone and 0.37 mg/kg fentanyl citrate) and Valium (Centrafarm, Etten-Leur, The Netherlands; 5.9 mg/kg Diazepam). Body temperature was maintained between 35°C and 37°C by keeping the mice on thermostatted heating pads. After induction of anaesthesia, the vena porta was cannulated, followed by ligation of 10 cm of the middle part of the jejunum. 500 µl cefadroxil (5 µM; 2µCi) was subsequently injected into the ligated jejunum and portal blood samples were collected after indicated time points. At the end of the experiment a peripheral blood sample was taken by cardiac puncture. Mice were subsequently anaesthetized and liver, the ligated jejunum, kidneys and gallbladder
were collected. After the addition of H_2O_2 (30%) to blood, jejunum, kidney, liver and jejunum radioactivity was measured by liquid scintillation counting.

**Statistical analyses**

Statistical differences were determined by an unpaired Student’s t-test. All data were expressed as means ± standard deviation.
Results

To study the contribution of ABCC3 and ABCC4 in the transport of cephalosporins, we expressed the human proteins in Spodoptera Frugiperda 21 (Sf21) insect cells. ABCC2 protein was expressed as a positive control for cephalosporin transport. Transport activity was determined by measuring uptake of substrates into isolated membrane vesicles. First, ABCC2-, ABCC3- and ABCC4-dependent uptake of the model substrate E2ßG was studied in the absence and presence of cefadroxil. A concentration-dependent inhibition of E2ßG uptake by cefadroxil was observed in membranes expressing ABCC2 (Fig. 1A), ABCC3 (Fig. 1B) and ABCC4 (Fig. 1C), albeit with very different IC50’s. The same inhibition studies were performed for nine other cephalosporins (table 1). For all cephalosporins the concentration at which E2ßG uptake was inhibited by 50% (IC50) was determined and plotted as a function of the molecular weight of the cephalosporins (Fig. 1D and Supplemental Figure 1). For all three ABC transporters a near linear, inverse correlation between IC50-values and molecular weight of the cephalosporins was observed. The orally-available cephalosporins (see Table 1) have the lowest molecular weight, indicating that molecular weight/bulkiness of the substrate is an important determinant for oral availability. Moreover, the orally available cephalosporins show the largest difference with regard to IC50-values of cephalosporins towards transport of E2ßG mediated by ABCC4 versus ABCC2 and ABCC3, respectively. Competitive inhibition is an indication that the inhibitor may be a transported substrate. Cefadroxil is an orally prescribed cephalosporin that is commercially available in a radiolabelled form allowing its use in direct transport experiments. We, therefore, used cefadroxil as a model substrate for orally prescribed cephalosporins, in vesicular transport experiments.
ABCG2 did not transport cefadroxil. However, membrane vesicles containing ABCC1, ABCC2, ABCC3 and ABCC4 all showed ATP-dependent uptake of cefadroxil (Fig. 2). In line with the inhibition experiments, transport of cefadroxil mediated by ABCC4 was three- to six-fold higher compared to transport by ABCC2 and ABCC3 (Fig. 2). Furthermore, cefadroxil transport was time dependent (Fig. 3A-C) and saturable (Fig. 3D-E) for ABCC1, ABCC3 and ABCC4 with \( K_m \) values of 3.9±0.6, 2.5±0.7 and 0.25±0.07 mM, respectively (Supplemental Table 1 and Supplemental Figure 2).

We subsequently tested whether there could be a role for Abcc3 and/or Abcc4 in vivo in the transport of cefadroxil across the basolateral membrane of the enterocyte in Ussing chamber experiments. No difference was found in transport of cefadroxil from the apical to the basolateral compartment using jejunal tissue from wild type and Abcc3⁻/⁻ mice. Transport of cefadroxil, (5 µM), was reduced to about 50% of normal levels, when jejunal tissue of Abcc4⁻/⁻ and Abcc3⁻/⁻/Abcc4⁻/⁻ mice were used (Fig. 4). Similar results were found with 50 µM cefadroxil (data not shown). Finally, in intestinal loop experiments we found that injection of cefadroxil in the ligated jejunum resulted in the time-dependent appearance of cefadroxil in portal blood of wild type mice (Fig. 5). The appearance of cefadroxil in portal blood of Abcc3⁻/⁻ and Abcc4⁻/⁻ mice was however not different from wild type mice, the latter result being in contrast to the Ussing chamber data. Interestingly, the appearance of cefadroxil in portal blood of Abcc3⁻/⁻/Abcc4⁻/⁻ mice was reduced. Similar results were found for peripheral blood: A significantly lower concentration of cefadroxil was found in Abcc3⁻/⁻/Abcc4⁻/⁻ mice in comparison with wild type mice. There was no difference in the cefadroxil tissue content of intestine, kidney and liver nor in its urinary excretion between the mice strains tested. However, an
increased amount of cefadroxil was noticed in the very low (less than 0.4% of dose) biliary content in *Abcc3*⁻/⁻/Abcc4⁻/⁻ versus wild type mice (Fig. 5D).
Discussion

Orally prescribed cephalosporins are efficiently taken up in the gut. At the luminal side of the small intestine the dipeptide transporter PepT1 is involved in the import of cephalosporin. At present the transporter(s) involved in the translocation of cephalosporins from the enterocytes into blood is (are) still unknown. ABCC2 transports several cephalosporins like ceftriaxone, cefaperazone and cephalixin (Kato, et al., 2008; Oude Elferink and Jansen, 1994). Because of its apical expression, ABCC2 can not be responsible for the transport of oral cephalosporins into blood. As ABCC3 and ABCC4 are related transporters we tested their ability to mediate transport of one of the orally prescribed cephalosporins, namely cefadroxil. In this article we show that both ABCC3 and ABCC4 mediate transport of cefadroxil albeit with different kinetics: of the ABC transporters under study ABCC4 mediates transport with the highest affinity (Fig. 2 and 3). This is in line with an earlier report showing that the non-oral available cephalosporins ceftizoxime, cefazolin, cefotaxime and cefmetazole are substrates of ABCC4 (Ci, et al., 2007). In this paper the $K_m$ values of 18 and 81 µM were stated for MRP4-mediated transport of respectively ceftizoxime and cefazolin (Ci, et al., 2007).

Since cefadroxil is a smaller compound than both these cephalosporins the $K_m$ value of 246 µM is higher as expected. This is based on our findings that an inverse correlation exists between molecular weights of cephalosporins and IC$_{50}$ values (Fig. 1D) and a relation between $K_m$ and IC$_{50}$ values. Our in vivo experiments with wild type, $Abcc3^{-/-}$ and $Abcc4^{-/-}$ single knockout mice showed no difference in transport of cefadroxil from luminal to serosal side (Fig 5). We have two explanations for this result: one explanation is that murine Abcc3 and Abcc4, unlike their human orthologues used in the vesicular
transport experiments, are unable to mediate transport of cefadroxil (Fig. 2 and 3). This explanation is unlikely as we observed in Ussing chamber experiments that transport was reduced in intestinal explants from Abcc4−/− mice compared to wild type mice. Another explanation is that these transporters can compensate for the loss of each other. Therefore, we extended the experiments with Abcc3−/−/Abcc4−/− mice. The Abcc3−/−/Abcc4−/− mice had reduced levels of cefadroxil in their portal blood (Fig. 5). As similar results were found in Ussing chamber experiments using jejunal explants from Abcc3−/−/Abcc4−/− and wild type mice, conclude that both Abcc3 and Abcc4 transport cefadroxil and when one transporter is lacking the other can fully compensate. Human ABCC4 transports cefadroxil at higher rates than ABCC3 in vesicular transport experiments. Expression of murine Abcc3 is higher than murine Abcc4 in the gut. This might be the reason that although Abcc3 transports cefadroxil only at moderate rates it can still compensate for the loss of Abcc4. As the difference in expression level between Abcc3/ABCC3 and Abcc4/ABCC4 in jejunum is similar in mouse and man we speculate that also in the human situation ABCC3 and ABCC4 are involved in transport of cefadroxil from enterocyte to blood (Taipalensuu, et al., 2001; Maher, et al., 2005).

The potential involvement of Abcc4 in the basolateral transport of cefadroxil is important since literature data are not consistent about the cellular localization of Abcc4. In the human colonic cell line HT29-CL19A, Li et al. found Abcc4 in both the apical and basolateral membrane, with a higher expression apically (Li, et al., 2007). In Caco2 cells Ming and Thakker detected Abcc4 mainly in basolateral membrane (Ming and Thakker, 2010). Our data that intestinal Abcc4 influences cefadroxil uptake supports the notion that Abcc4 is present at the basolateral membrane of the enterocytes. Our functional data
suggest that Abcc4 is present at the basolateral membrane of enterocytes and confirm the data obtained in Caco2 cells by Ming and Thakker (Ming and Thakker, 2010).

Abcc3⁻/⁻/Abcc4⁻/⁻ mice still show a considerable amount of transport of cefadroxil over the basolateral membrane of enterocytes. Theoretically, Abcc1 could be responsible for the residual transport of cefadroxil as we found that human ABCC1 is able to mediate transport of cefadroxil (Fig 2 and 3). However, Abcc1 is expressed in the small intestine, mainly in the crypts (Peng, et al., 1999; Kato, et al., 2009) which does not colocalize with PepT1, which is abundantly present in the villus tip with decreasing levels towards the villus base. The localization of Abcc1 therefore argues against a role of Abcc1 in the basolateral efflux of cefadroxil in enterocytes. We can, however, not fully exclude that there is a role of Abcc1 in the efflux of cefadroxil as this transporter protects against the intestinal toxicity evoked by methotrexate (Kato, et al., 2009). This means that Abcc1 is pumping methotrexate out of the cell at a physiological relevant speed. The same might be the case for Abcc1 mediated transport of cefadroxil into blood from the enterocyte, especially in the absence of Abcc3 and Abcc4 in the used murine model: Abcc3⁻/⁻/Abcc4⁻/⁻ mice.

Oral administration is a preferable route of administration of drugs. In general, the oral available cephalosporins are low molecular weight molecules. Kato et al. showed a correlation between size and biliary excretion for several cephalosporins (Kato, et al., 2008). This can be partially caused by a size-dependent affinity for the transporter mainly responsible for the biliary excretion, Abcc2/ABCC2 (Kato, et al., 2008). In line with data of Kato et al., we found a size dependent inhibition by cephalosporins of ABCC2-mediated transport of E₂₁₇βG (Fig 1D). Also, a size-dependent inhibition by
cephalosporins was found by us for ABCC3- and ABCC4-mediated transport of E₂₁₇βG (Fig 1D), but the slope for ABCC2 is steeper than for ABCC4 and similar for ABCC3. This could indicate that the influence of size is relatively big for ABCC2 (which prevents net uptake) and relatively small for ABCC4 (which stimulates net uptake). Interestingly, an inverse selectivity of PepT1 was shown to be dependent on molecular weight as well, with small cephalosporins being preferentially transported. Hence, the combined selectivity of the uptake transporter Pept1 and the efflux transporter ABCC2 may determine the typical pharmacokinetic behaviour of cephalosporins.

In conclusion, the data presented in this paper demonstrate that murine intestinal uptake depends partly on Abcc3 and Abcc4. We therefore speculate that in the human situation oral availability involves, at least partly, uptake via ABCC3 and ABCC4.
Authorship contributions:

Participated in research design: de Waart and Oude Elferink.

Conducted experiments: de Waart, Kunne and Duijst.

Contributed new reagents or analytic tools: van de Wetering and Paulusma.

Performed data analysis: de Waart.

Wrote or contributed to the writing of the manuscript: de Waart, van de Wetering, Paulusma and Oude Elferink.
Reference List


Legends

Figure 1:

Inhibition of estradiol-17β-glucuronide transport by cephalosporins.

Cephalosporin inhibition of E₂₁⁷ßG (0.05 μCi; 1.0 µM) transport into, ABCC2, ABCC3
and ABCC4 containing Sf21 membrane vesicles.

Shown are the measurements of the IC₅₀ values for E₂₁⁷ßG (0.05 μCi; 1.0 µM) transport
mediated by (A) ABCC2 (solid line), (B) ABCC3 (dashed line) and (C) ABCC4 (dotted
line), inhibited by different concentrations of cefadroxil. (D) IC₅₀ values for inhibition of
E₂₁⁷ßG transport by cephalosporins. Transport mediated by ABCC2, ABCC3 and
ABCC4 versus molecular weight of these cephalosporins.

Figure 2:

Vesicular transport assay.

Transport of cefadroxil (0.2 μCi; 10µM) with (black bars) or without (white bars) ATP
into mock-transduced, ABCC1, ABCC2, ABCC3, ABCG2 and ABCC4 expressing
membrane vesicles. Incubation time is 6 min. Amount total protein used is 10 µg. Data
represent the average ± SD. Shown is the result of one out of two independent
experiments. Significance was tested using two-sided Student’s t-test: *P<0.05 for
transport of ABCC1, ABCC2, ABCC3 and ABCC4 expressing membrane vesicles versus
membrane vesicles from mock transduced cells.

Figure 3:

Time- and concentration-dependent transport.
Time-dependent transport of cefadroxil (0.2 µCi; 10 µM) into plasma membrane vesicles from Sf21 cells expressing ABCC1 (A), ABCC3 (B) and ABCC4 (C). Open squares (with dotted line) and open diamonds represent incubations with or without ATP, respectively. Filled triangles (with solid line) represent ATP dependent transport. Concentration-dependent transport of cefadroxil (0.2 µCi; 10 µM) into plasma membrane vesicles (open diamonds with solid line) from Sf21 cells expressing ABCC1 (D), ABCC3 (E) and ABCC4 (F). Shown are the average ± SD of ATP-dependent transport of a representative experiment with triplicate incubations performed 3-4 times.

**Figure 4:**
Transport of cephadroxil across jejunal explants in Ussing chambers.
Appearance of cefadroxil on the basolateral side of jejunal explants from Abcc3−/−, Abcc4−/− and Abcc3−/−/Abcc4−/− and wild type mice. Cefadroxil was applied to the apical compartment of Ussing chambers at a final concentration of 5 µM. At indicated time points samples were taken from the basolateral side and analyzed by HPLC. Shown is the average ± standard deviation of 3-4 experiments with triplicate incubations. ‡, p < 0.05 comparing appearance of cefadroxil using tissue from Abcc4−/− versus wild type mice for indicated time points. #, p < 0.05 comparing appearance of cefadroxil using tissue from Abcc3−/−/Abcc4−/− versus wild type mice for indicated time points.

**Figure 5:**
In vivo uptake of cefadroxil by the jejunum.
Appearance of cefadroxil in portal (closed symbols) and peripheral blood (open symbols) of Abcc3<sup>-/-</sup> versus wild type mice (A), Abcc4<sup>-/-</sup> versus wild type mice (B) and Abcc3<sup>-/-</sup>/Abcc4<sup>-/-</sup> versus wild type mice (C) after injection of 500 µl cefadroxil (5 µM; 2µCi) into ligated jejunum. Portal blood was collected after indicated time points, peripheral blood after the last portal blood sample was drawn. Appearance of cefadroxil in bile of Abcc3<sup>-/-</sup>/Abcc4<sup>-/-</sup> versus wild type mice (D) after injection of 500 µl cefadroxil (5 µM; 2µCi) into ligated jejunum. Data represent average ± SD of at least 5 mice. Significance was tested using two-sided Student’s t-test: *P<0.05 for Abcc3<sup>-/-</sup>/Abcc4<sup>-/-</sup> versus wild type mice.
Table 1:
Cephalosporins used in the inhibition studies.

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