

# Organic Anion Transporter 3 (Oat3/Slc22a8) Interacts with Carboxyfluoroquinolones, and Deletion Increases Systemic Exposure to Ciprofloxacin

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## ABSTRACT

Carboxyfluoroquinolones, such as ciprofloxacin, are used for the treatment of numerous infectious diseases. Renal secretion is a major determinant of their systemic and urinary concentration, but the specific transporters involved are virtually unknown. In vivo studies implicate the organic anion transporter (OAT) family as a pivotal component of carboxyfluoroquinolone renal secretion. Therefore, this study identified the specific renal basolateral OAT(s) involved, thereby highlighting potential sources of carboxyfluoroquinolone-drug interactions and variable efficacy. Two heterologous expression systems, *Xenopus laevis* oocytes and cell monolayers, were used to determine the roles of murine and human renal basolateral mOat1/hOAT1 and mOat3/hOAT3. Ciprofloxacin was transported by mOat3 in both systems ( $K_m$  value,  $70 \pm 6 \mu\text{M}$ ) and demonstrated no interaction with mOat1 or hOAT1. Furthermore, ciprofloxacin, norfloxacin, ofloxacin, and gatifloxacin exhibited concentration-dependent inhibition of transport on mOat3 in cells with

inhibition constants of  $198 \pm 39$ ,  $558 \pm 75$ ,  $745 \pm 165$ , and  $941 \pm 232 \mu\text{M}$ , respectively. Ciprofloxacin and gatifloxacin also inhibited hOAT3. Thereafter, in vivo elimination of ciprofloxacin was assessed in wild-type and Oat3 null mice [Oat3(−/−)]. Oat3(−/−) mice exhibited significantly elevated plasma levels of ciprofloxacin at clinically relevant concentrations ( $P < 0.05$ , male mice;  $P < 0.01$ , female mice). Oat3(−/−) mice also demonstrated a reduced volume of distribution (27%,  $P < 0.01$ , male mice; 14%,  $P < 0.01$ , female mice) and increased area under the concentration-time curve (25%,  $P < 0.05$ , male mice; 33%,  $P < 0.01$ , female mice). Female Oat3(−/−) mice had a 35% ( $P < 0.01$ ) reduction in total clearance of ciprofloxacin relative to wild type. In addition, putative ciprofloxacin metabolites were significantly elevated in Oat3(−/−) mice. The present findings indicate that polymorphisms of and drug interactions on hOAT3 may influence carboxyfluoroquinolone efficacy, especially in urinary tract infections.

Ciprofloxacin is a broad-spectrum antimicrobial that is used in the treatment of numerous infectious diseases, including those afflicting the skin (Lipsky et al., 1999), bone (Gasem et al., 2003), gastrointestinal tract (Hsieh et al.,

1998), genitourinary system (Wagenlehner et al., 2006), lungs (Jones, 2002), and meninges (Gogos et al., 1991). It is also a preferred agent for the prevention and treatment of anthrax (Meyerhoff et al., 2004). Its mechanism of action is through the effective inhibition of DNA gyrase, thus preventing DNA replication in susceptible bacteria (Gellert et al., 1977; Sugino et al., 1977). Ciprofloxacin is a carboxylic acid-containing fluoroquinolone (carboxyfluoroquinolone) that undergoes renal and hepatic elimination, with ~50% (oral) or ~80% (intravenous) appearing in the urine as parent compound and metabolites 24 h after administration in humans (Höffken et al., 1985). Approximately 20 to 40% of circulating

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**ABBREVIATIONS:** LLC-PK<sub>1</sub>, porcine kidney cells; AUC, area under the concentration-time curve; CHO, Chinese hamster ovary cells; CHO-FRT, empty vector-transfected Chinese hamster ovary; CHO-mOat1, mOat1-transfected CHO; CHO-mOat3, mOat3-transfected CHO; hOAT2, human organic cation transporter 2; HPLC, high-performance liquid chromatography; mOat1, murine organic anion transporter 1; mOat3, murine organic anion transporter 3; OAT/Oat, organic anion transporter; OAT1/hOAT1, human organic anion transporter 1; OAT3/hOAT3, human organic anion transporter 3; Oat3(−/−), Oat3 null; V<sub>dss</sub>, apparent volume of distribution at steady state; V<sub>max</sub>, maximum transport rate; ANOVA, analysis of variance; HEK, human embryonic kidney; MUSC, Medical University of South Carolina; M1, desethyleneciprofloxacin; M2, sulfociprofloxacin; M3, oxociprofloxacin; M4, formylciprofloxacin.

This information is important for identifying potential renal etiologies of variable carboxyfluoroquinolone efficacy and concomitantly administered drug toxicity (e.g., methotrexate), such as xenobiotic competition for tubular transport

(Micromedex Healthcare Series, 2007; VanWert and Sweet, 2008) and transporter polymorphisms (Bleasby et al., 2005; Erdman et al., 2006), especially for the treatment of infectious diseases of the urinary tract. Although a detailed mechanistic understanding of carboxyfluoroquinolone renal transport has not been achieved, some advances have been made in understanding the general flux of carboxyfluoroquinolones across tubular cells (Table 1). Basolateral uptake of carboxyfluoroquinolones seems to involve an organic anion transport mechanism, because levofloxacin inhibited basolateral *p*-aminohippurate uptake into opossum kidney cells (Matsuo et al., 2001), and basal uptake of levofloxacin was not inhibited by tetraethylammonium or cimetidine in LLC-PK<sub>1</sub> cells derived from porcine kidney (Ohtomo et al., 1996). These observations are consistent with a role for the basolateral organic anion exchangers Oat1 and Oat3 in the renal uptake of carboxyfluoroquinolones (Sweet et al., 1997, 1999, 2002, 2003; Cihlar et al., 1999). However, a recent study demonstrated that levofloxacin can inhibit creatinine transport by human renal basolateral organic cation transporter 2 (hOCT2)

Carboxyfluoroquinolone	System	Major Finding	Suggested Possible Role for Transporter Family				Specific Transporter	Reference		
			Anionic		Cationic					
			Apical	Basolateral	Apical	Basolateral				
Ciprofloxacin	In vivo in humans (male and female)	Renal clearance was reduced to ~1/3 of normal by probenecid	✓	✓	—	—	No	Jaehde et al., 1995		
Norfloxacin	In vivo in humans, rabbits, and dogs (all male)	Renal clearance was reduced to ~1/2 of normal by probenecid, no effect was apparent in dogs	✓	✓	—	—	No	Shimada et al., 1983		
Ofloxacin	In vivo in humans	Reduced the plasma clearance of procainamide	—	—	✓	✓	No	Martin et al., 1996		
	Rat renal cortical apical membrane vesicles	Inhibited uptake of TEA, cephalixin, and cephadrine with no effect on PAH transport	✓	—	✓	—	No	Okano et al., 1990		
Ciprofloxacin, enoxacin, enrofloxacin, fleroxacin, norfloxacin, ofloxacin, and pefloxacin	In vivo: stop-flow renal peritubular capillary perfusion in rats	All inhibited renal basolateral uptake of PAH and NMeN (weakly to moderately)	—	✓	—	✓	No	Ullrich et al., 1993		
Ofloxacin	In vivo: i.v. administration in rats	Cimetidine and probenecid reduced total and renal clearance	✓	✓	✓	✓	No	Foote and Halstenson, 1998		
Levofloxacin	LLC-PK <sub>1</sub> cells	Inhibited TEA apical transport; basolateral transport was not inhibited by TEA or cimetidine	—	—	✓	No role	No	Ohtomo et al., 1996		
	OK cells	Inhibited PAH basolateral uptake and apical efflux; transport was unaffected by PAH	✓	✓	—	—	No	Matsuo et al., 2001		
	hOCT2 expressing HEK293 cells	Inhibited creatinine uptake	—	—	—	✓	hOCT2	Okuda et al., 2006		
	Isolated perfused rat kidney	Cimetidine and quinolones prolonged the transepithelial transit time	✓	—	✓	—	No	Ito et al., 2000		

✓, suggested role in transport; —, undetermined role in transport; no role, study specifically suggested no role for the transporter family and membrane; NMeN, *N*<sup>1</sup>-methyl-nicotinamide; OK, opossum renal proximal tubular cells; PAH, *p*-aminohippurate; TEA, tetraethylammonium.

(Okuda et al., 2006). Investigations using LLC-PK<sub>1</sub> cells and rat renal cortical apical membrane vesicles have demonstrated that ofloxacin and levofloxacin are capable of inhibiting tetraethylammonium transport across the apical membrane (Okano et al., 1990; Ohtomo et al., 1996). This finding suggests that zwitterionic fluoroquinolones interact with organic cation transporters at the brush border membrane as well. However, there are conflicting studies on the involvement of apical OATs in that one investigation reported no effect of ofloxacin on renal apical *p*-aminohippurate transport in rat (Okano et al., 1990), whereas another study reported that levofloxacin (an ofloxacin enantiomer) inhibits apical efflux of *p*-aminohippurate in opossum kidney cells (Matsuo et al., 2001). Although this discrepancy may be the result of species differences in apical transport, clearly, the contribution of individual renal transporters in carboxyfluoroquinolone elimination remains ambiguous.

Despite more than 2 decades of investigation into carboxyfluoroquinolone transport, the specific interaction of carboxyfluoroquinolones with organic anion transporters has not been examined using heterologous expression systems. Therefore, we investigated the interaction of the two major renal basolateral organic anion transporters, Oat1 and Oat3, with ciprofloxacin and other carboxyfluoroquinolones by conducting a series of experiments in which we used *Xenopus laevis* oocytes and Chinese hamster ovary (CHO) cells expressing the murine and human transporters. Evidence was gathered indicating a significant involvement of murine Oat3 (mOat3) and human OAT3 (hOAT3). Further data corroborating this observation were obtained in vivo using intact wild-type and Oat3 knockout (Oat3<sup>-/-</sup>) mice.

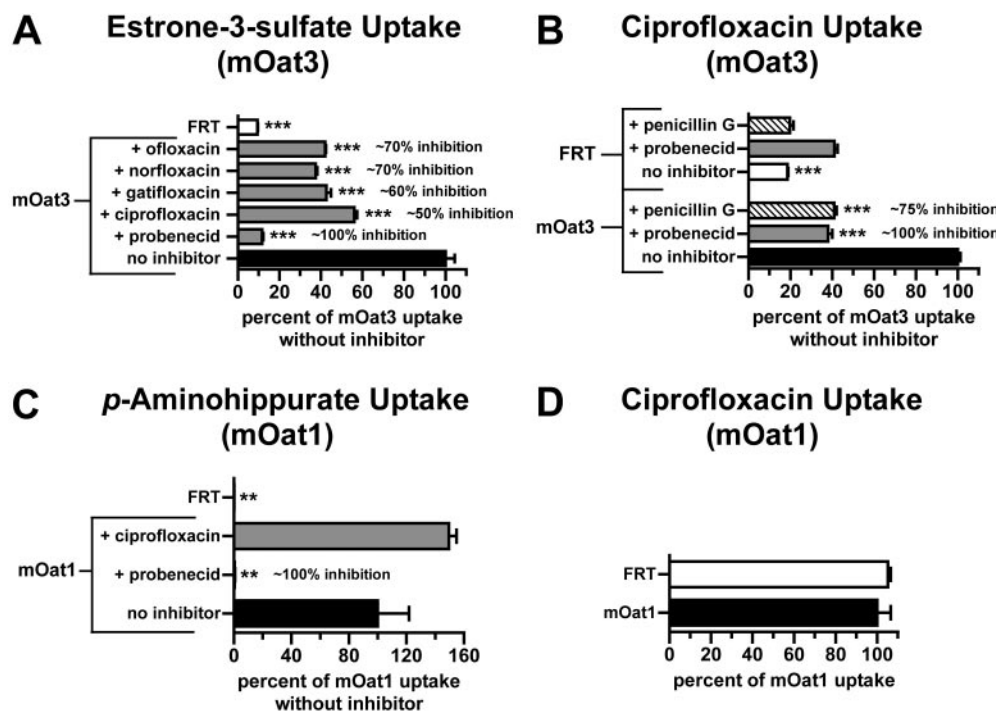
## Materials and Methods

**Chemicals and Materials.** [<sup>3</sup>H]*p*-Aminohippurate and [<sup>3</sup>H]estrone-3-sulfate were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA) and American Radiolabeled Chemicals (St. Louis, MO), respectively. Ciprofloxacin and gatifloxacin were

purchased from Fisher Scientific (Suwanee, GA) and LKT Laboratories, Inc. (St. Paul, MN), respectively. Norfloxacin and ofloxacin were purchased from MP Biomedicals (Solon, OH). The high-performance liquid chromatography (HPLC) column and precolumn filter were acquired from Phenomenex (Torrance, CA).

**Animals.** Female *X. laevis* were housed in 15-gallon aquariums and maintained on a 12-h light/dark cycle. Frogs were fed and moved to clean, aged water every other day. Male and female Oat3<sup>-/-</sup> (backcrossed onto C57BL/6 eight times) and wild-type age-matched C57BL/6 mice (10–12 weeks old, ~24 g) were also used in the present study (VanWert et al., 2007; VanWert and Sweet, 2008). Mice were allowed food and water ad libitum and were housed in animal facilities maintained by the Medical University of South Carolina (MUSC) Division of Laboratory Animal Resources. The MUSC program for laboratory animal care has an assurance statement on file with the National Institutes of Health Office for the Protection from Research Risks/Department of Health and Human Services and has maintained full accreditation with the Association for Assessment and Accreditation of Laboratory Animal Care since 1987. All animal procedures were approved by the MUSC Institutional Animal Care and Use Committee [AR no. 2080 (*X. laevis*) and AR no. 2082 (*M. musculus*)] and carried out in accordance with the Guide for the Care and Use of Laboratory Animals.

**Isolation of Stably Transfected Cell Lines.** Mouse organic anion transporter 1-expressing CHO cells (CHO-mOat1) were generated using a method adapted from that described previously for the generation of mouse organic anion transporter 3-expressing (CHO-mOat3) and empty vector-transfected (CHO-FRT) CHO cell lines (VanWert and Sweet, 2008). In brief, a full-length mOat1 restriction fragment was ligated into the pcDNA5/FRT vector, and this construct was used to transfect CHO-FlpIn cells (Invitrogen, Carlsbad, CA) plated into a 12-well tissue culture plate (3 × 10<sup>5</sup> cells/well). Two days after plating, cells were transfected with 0.3 μg of pcDNA5/FRT-mOat1 plasmid DNA and 2.7 μg of pOG44 plasmid DNA for 24 h using 2 μl of Transfectin Lipid Reagent (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's directions. After 24 h of transfection at 37°C/5% CO<sub>2</sub>, the medium was changed to fresh Ham's F-12 with serum, and the cells were grown for an additional 24 h. The cells were then thoroughly detached with trypsin, and all cells were plated into a 25-cm<sup>2</sup> tissue culture flask containing 700



**Fig. 1.** Carboxyfluoroquinolone inhibition and transport in mouse organic anion transporter-expressing Chinese hamster ovary cells. [<sup>3</sup>H]Estrone-3-sulfate, [<sup>3</sup>H]*p*-aminohippurate, or unlabeled ciprofloxacin uptake was measured at room temperature for 15 min in cells expressing mOat1 or mOat3. Empty vector-transfected Chinese hamster ovary cells (FRT) serve as a reference for nonspecific accumulation. A, [<sup>3</sup>H]estrone-3-sulfate (5 μM) uptake in CHO-mOat3 cells in the absence (no inhibitor) or presence of carboxyfluoroquinolones or probenecid (1 mM). B, ciprofloxacin (100 μM) uptake in CHO-mOat3 cells in the absence (no inhibitor) or presence of Oat3 inhibitors (1 mM). C, [<sup>3</sup>H]*p*-aminohippurate (145 nM) uptake in CHO-mOat1 cells in the absence (no inhibitor) or presence of ciprofloxacin or probenecid (1 mM). D, ciprofloxacin (100 μM) uptake in CHO-mOat1 cells. Values shown are means ± S.E. and significant differences were assessed between mOat3 or mOat1 without inhibition and the indicated bars (\*\*, *P* < 0.01; \*\*\*, *P* < 0.001). The percentage of inhibition was calculated after correcting for nonspecific accumulation (i.e., in FRT cells).



$\mu\text{g/ml}$  Hygromycin B (Invitrogen) in Ham's F-12 with serum. Untransfected cells were treated in parallel to detect positive resistance of transfected cells to Hygromycin B. Transfected and untransfected cells were treated with Hygromycin B for approximately 4 weeks. After testing positive for transport of [ $^3\text{H}$ ]p-aminohippurate, cells were maintained in 125  $\mu\text{g/ml}$  Hygromycin B.

The stable cell line expressing hOAT1 was generated previously in MDCKII cells (Aslamkhan et al., 2003). The HEK293 cell line stably expressing hOAT3 was also established using the Flp-In expression system. In brief, full-length hOAT3 cDNA was amplified from pcDNA3.1V5/His TOPO-hOAT3 and ligated into the pEF5/FRT/V5-DEST vector, resulting in the pEF5/FRT/V5-DEST-hOAT3 vector. Sequencing confirmed this construct to be 100% identical with that of GenBank accession number AB042505 (Aslamkhan et al., 2006). HEK293-FlpIn cells were plated into a 60-mm tissue culture dish ( $1 \times 10^6$  cells) using Dulbecco's modified Eagle's medium (high glucose) (Invitrogen) with 10% fetal bovine serum. One day after plating, cells were transfected with 1  $\mu\text{g}$  of pEF5/FRT/V5-DEST-hOAT3 plasmid DNA and 9  $\mu\text{g}$  of pOG44 plasmid DNA using 20  $\mu\text{l}$  of Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Twenty-four hours after transfection, the media were changed, and the cells were grown for an additional 24 h, followed by selection via plating into a 25-cm<sup>2</sup> tissue culture flask containing Dulbecco's modified Eagle's media with 10% fetal bovine serum and 50  $\mu\text{g/ml}$  Hygromycin B (Invitrogen) for 4 weeks. An empty vector (pEF5/FRT/V5-DEST) HEK293 stable cell line was also generated. Stable cell lines were maintained in 10  $\mu\text{g/ml}$  Hygromycin B.

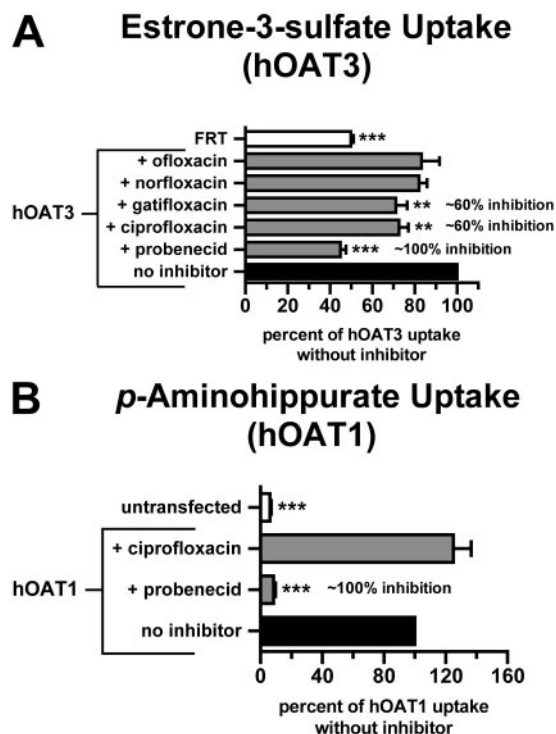
**X. laevis Oocyte Transport Assays.** *X. laevis* oocytes were obtained from dissected ovaries by collagenase A treatment, and substrate uptake assays were performed 3 days after injection with 20 ng of capped cRNA as described previously (Sweet et al., 2000; Youngblood and Sweet, 2004). Oocytes were randomly divided into experimental groups of 5 for estrone sulfate transport and 25 for ciprofloxacin transport, and they were incubated for 60 min at room temperature in oocyte Ringer 2 medium (82.5 mM NaCl, 2.5 mM KCl, 1 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaOH, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, 1 mM pyruvic acid, and 5 mM HEPES, pH 7.6) containing 5  $\mu\text{M}$  [ $^3\text{H}$ ]estrone-3-sulfate (0.5  $\mu\text{Ci/ml}$ ) or 40  $\mu\text{M}$  unlabeled ciprofloxacin. Unlabeled ciprofloxacin (500  $\mu\text{M}$ ) was present or absent in the [ $^3\text{H}$ ]estrone-3-sulfate uptake solution as indicated. Individual oocyte radioactivity was measured by liquid scintillation spectroscopy with external quench correction for measuring [ $^3\text{H}$ ]estrone-3-sulfate uptake. To determine unlabeled ciprofloxacin accumulation, HPLC was used on five pooled oocytes per sample (see below).

**Cell Line Transport Assays.** Procedures for cell uptake assays were adapted from protocols reported previously (Schnabolk et al., 2006; VanWert and Sweet, 2008). Cells were seeded in 24-well ( $2 \times 10^5$  cells/well for [ $^3\text{H}$ ]estrone-3-sulfate and [ $^3\text{H}$ ]p-aminohippurate uptake) or 12-well ( $4 \times 10^5$  cells/well for ciprofloxacin uptake) tissue culture plates and grown for 2 days (37°C/5% CO<sub>2</sub>) in the absence of antibiotics. Before the initiation of transport experiments, the culture medium was removed, and the cells were equilibrated for 10 min with transport buffer (Hanks' balanced salt solution containing 10 mM HEPES, pH 7.4; Sigma-Aldrich, St. Louis, MO). The equilibration medium was removed before a final application of 500  $\mu\text{l}$  (24-well plate) or 1000  $\mu\text{l}$  (12-well plate) of transport buffer containing 0.1 to 100  $\mu\text{M}$  [ $^3\text{H}$ ]estrone-3-sulfate (0.5  $\mu\text{Ci/ml}$ ), 0.144 to 10  $\mu\text{M}$  [ $^3\text{H}$ ]p-aminohippurate (0.5  $\mu\text{Ci/ml}$ ), or 20 to 1000  $\mu\text{M}$  unlabeled ciprofloxacin in the absence or presence of inhibitors at the indicated concentrations. After incubation at room temperature or 37°C for the specified times, the uptake solutions were removed, and the cells were rapidly rinsed three times with 1 ml of ice-cold transport buffer. In [ $^3\text{H}$ ]estrone-3-sulfate and [ $^3\text{H}$ ]p-aminohippurate transport experiments, the cells were dissolved in 500  $\mu\text{l}$  of 1 M NaOH, neutralized with 50  $\mu\text{l}$  of 10 M HCl, and assayed for radioactivity by liquid scintillation counting. In ciprofloxacin transport experiments, the cells were processed for HPLC (see below). For all cell transport experiments, aliquots were removed and analyzed for protein con-

tent using (mouse cell lines) a BCA protein assay kit (Pierce, Rockford, IL) or (human cell lines) Bradford assay kit (Bio-Rad Laboratories). Uptake was then normalized to protein content in lysates. Transformations for kinetic calculations were performed using GraphPad Prism software (GraphPad Software Inc., San Diego, CA), and the  $K_m$  and  $V_{max}$  values were calculated from the x and y intercepts of the Lineweaver-Burk plot, respectively.  $K_i$  values were calculated assuming competitive inhibition. All experiments were performed at least three times in triplicate.

**Ciprofloxacin Elimination.** Ciprofloxacin (5 mg/kg body weight) was administered in normal saline (5  $\mu\text{l/g}$  body weight) by bolus tail vein injection to unanesthetized animals. Serial blood samples ( $\sim 35 \mu\text{l}$ ) were obtained at 1, 5, 10, 15, 20, and 30 min in heparinized capillary tubes and centrifuged to isolate plasma as described previously (VanWert et al., 2007; VanWert and Sweet, 2008). Ten microliters of plasma was then processed for HPLC as described in the following section.

**High-Performance Liquid Chromatography.** Ciprofloxacin was extracted from *X. laevis* oocytes as follows: five oocytes were placed in a microcentrifuge tube, dried with a gentle stream of nitrogen gas, and homogenized in 40  $\mu\text{l}$  of mobile phase A (50 mM formic acid, pH 3.5 via ammonia) with an ultrasonic probe. The homogenate was centrifuged at 21,000g for 5 min, and 25  $\mu\text{l}$  of supernatant was directly injected into the HPLC system via an autosampler. The HPLC system consisted of a Waters 2695 separations module (Waters, Milford, MA), a Phenomenex Luna C18(2) 250 mm  $\times$  4.6 mm, 5- $\mu\text{m}$  reversed-phase column preceded by a Krud-



**Fig. 2.** Carboxyfluoroquinolone inhibition in human organic anion transporter-expressing cells. [ $^3\text{H}$ ]Estrone-3-sulfate or [ $^3\text{H}$ ]p-aminohippurate uptake was measured for 15 min in cells expressing hOAT3 (HEK293 cells) or hOAT1 (MDCKII cells), respectively. Empty vector-transfected HEK293 cells (FRT) or untransfected MDCKII cells served as a reference for nonspecific accumulation. A, [ $^3\text{H}$ ]estrone-3-sulfate (100 nM) uptake in hOAT3 cells in the absence (no inhibitor) or presence of carboxyfluoroquinolones or probenecid (1 mM). B, [ $^3\text{H}$ ]p-aminohippurate (10  $\mu\text{M}$ ) uptake in hOAT1 cells in the absence (no inhibitor) or the presence of ciprofloxacin or probenecid (1 mM). Values shown are means  $\pm$  S.E., and significant differences were assessed between hOAT3 or hOAT1 without inhibition and the indicated bars (\*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ). Percentage of inhibition was calculated after correcting for nonspecific accumulation (i.e., in FRT or untransfected cells).

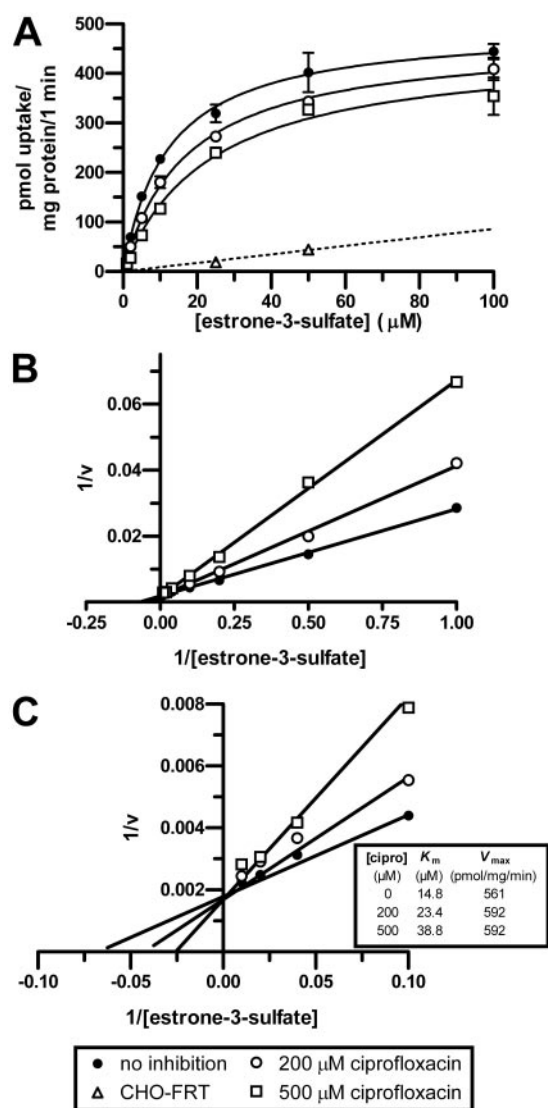
Katcher guard/filter (Phenomenex), and a Waters 474 scanning fluorescence detector. To resolve ciprofloxacin, an isocratic elution was used [60% A/40% B (HPLC-grade methanol), 800  $\mu$ l/min; retention time,  $\sim$ 8 min]. Quantification was performed via a published procedure (Joos et al., 1985) using peak areas with fluorescence detection (excitation and emission  $\lambda$  of 278 and 456 nm, respectively). The external standard curve was consistently linear ( $R^2 > 0.998$ ) and was repeated for each day of analysis.

Ciprofloxacin was extracted from cell monolayers in the 12-well plate by applying 250  $\mu$ l of 70°C distilled and deionized water and pipetting aggressively to lift the cells. Cellular detachment was verified microscopically, and cells were placed in a microcentrifuge tube before disrupting with an ultrasonic probe. Membranous debris was pelleted by centrifugation at 21,000g for 20 min, and 230  $\mu$ l of supernatant were then placed in a fresh tube. Twenty microliters of the supernatant was taken and diluted to 60  $\mu$ l with water for

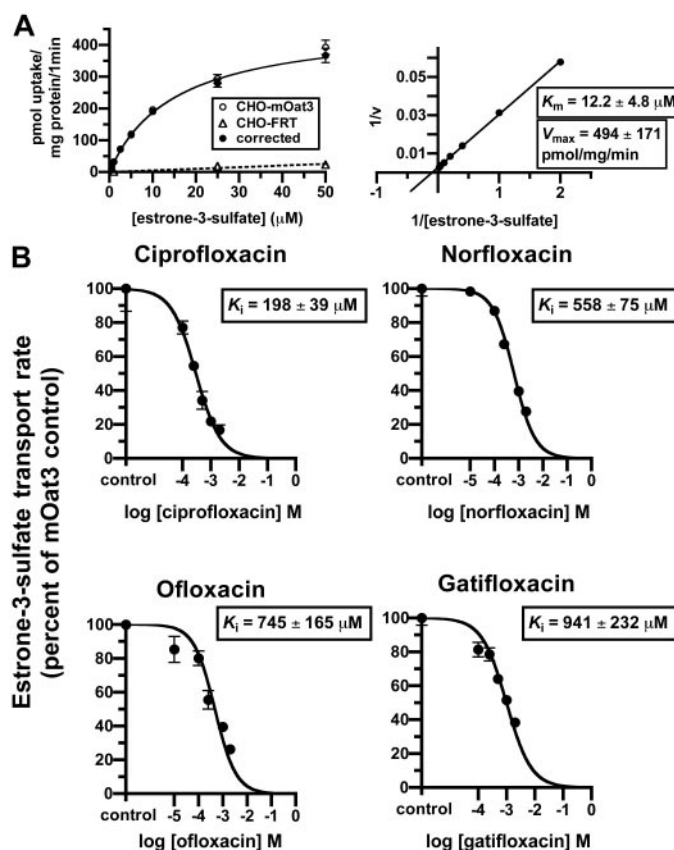
duplicate 25- $\mu$ l samples in the BCA protein assay. To precipitate protein that could potentially block the column, 10  $\mu$ l of 4-hydroxyphenylacetic acid was added to the remaining 210  $\mu$ l of supernatant to achieve a final concentration of 500 ng/ $\mu$ l. This turbid solution was vortexed vigorously, and protein was pelleted at 21,000g for 10 min. Thirty microliters of this supernatant was injected for HPLC under conditions identical with those for the oocyte assay.

Ciprofloxacin was quantified in mouse plasma using a reported method with minor adaptations (Joos et al., 1985). Standards made in plasma and 10- $\mu$ l plasma samples from injected animals were vortexed after adding four volumes of 6% (w/v) trichloroacetic acid in HPLC-grade water. They were then centrifuged at 21,000g for 5 min to pellet the protein. Thirty microliters of supernatant was used directly for HPLC separation and detection as described above for oocytes.

**Kinetic and Statistical Analyses.** Transport and inhibition curves and kinetics were determined using Prism software (GraphPad Software).  $K_i$  values were determined assuming competitive inhibition. Pharmacokinetic determinations were performed using WinNonlin software (Pharsight Corporation, Mountain View, CA).



**Fig. 3.** Competitive-type inhibition of mOat3 by ciprofloxacin. The  $V_{max}$  and observed  $K_m$  values for [ $^3$ H]estrone-3-sulfate uptake were determined in CHO-mOat3 cells at room temperature in the absence or presence of ciprofloxacin at 200 or 500  $\mu$ M. A, the [ $^3$ H]estrone-3-sulfate transport rate was assessed at 1, 2, 5, 10, 25, 50, and 100  $\mu$ M for 1 min. The saturation curves shown were generated after correction for nonspecific accumulation in empty vector-transfected cells (CHO-FRT). B, Lineweaver-Burk transformation of data shown in A. C, the graph from B was magnified to illustrate the x and y intercepts. Data points are shown as means  $\pm$  S.E.

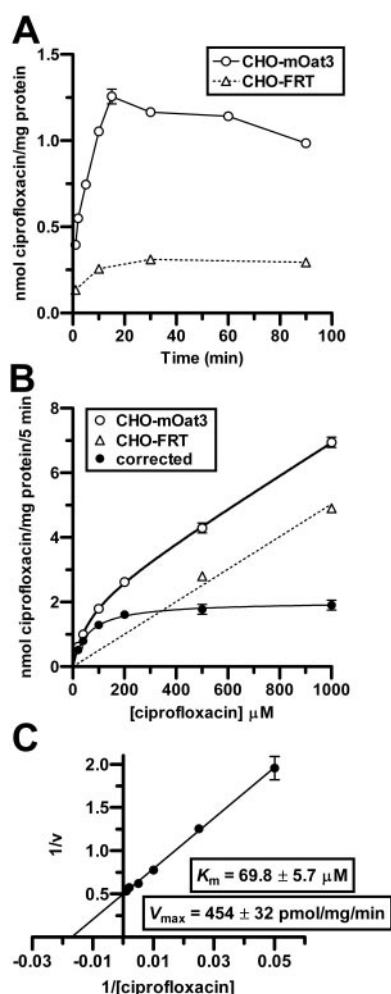


**Fig. 4.** mOat3 inhibition constants ( $K_i$  values) of carboxyfluoroquinolones. The  $K_m$  value for [ $^3$ H]estrone-3-sulfate uptake was determined in CHO-mOat3 cells and was subsequently used to calculate  $K_i$  values for carboxyfluoroquinolones. mOat3-mediated [ $^3$ H]estrone-3-sulfate uptake was determined by subtracting accumulation in empty vector-transfected cells (CHO-FRT). A, left, [ $^3$ H]estrone-3-sulfate uptake was measured for 1 min at 0.5, 1, 2, 5, 10, 25, and 50  $\mu$ M to determine saturable transport (corrected). Right, Lineweaver-Burk transformation using corrected data to calculate the  $K_m$  value for [ $^3$ H]estrone-3-sulfate. B, [ $^3$ H]estrone-3-sulfate (1  $\mu$ M) uptake was measured for 1 min in the absence or presence of carboxyfluoroquinolones (ciprofloxacin and gatifloxacin at 100, 250, 500, 1000, and 2000  $\mu$ M; norfloxacin and ofloxacin at 10, 100, 250, 1000, and 2000  $\mu$ M) to determine  $K_i$  values. Inhibition curves, and thus  $K_i$  values, were determined using the "one-site competition" model in GraphPad Prism (i.e., assuming competitive-type inhibition). Kinetic constants shown are means  $\pm$  S.E. of three to five experiments performed in triplicate, and graphs are single representative experiments.

Noncompartmental intravenous bolus modeling was used and yielded lower coefficients of variation compared with other models. Statistical significance of elimination curves was determined using two-way ANOVA. One-way ANOVA with Tukey's multiple comparison test was used for Figs. 1 and 2. A two-tailed Student's *t* test was used for all other comparisons. The  $\alpha$  for significance was set at 0.05.

## Results

**Interaction of Ciprofloxacin with mOat3 in *X. laevis* Oocytes.** In mOat3-expressing *X. laevis* oocytes, ciprofloxacin (500  $\mu$ M) significantly inhibited mOat3-mediated estrone-3-sulfate (5  $\mu$ M) uptake ( $\sim 40\%$  in 60 min at room temperature,  $P < 0.05$ ; data not shown). When examined directly as a substrate, ciprofloxacin (40  $\mu$ M) accumulated in mOat3-expressing oocytes to a level significantly higher than in water-injected/control oocytes ( $\sim 2$ -fold greater in 60 min at room temperature,  $P < 0.01$ ; data not shown).

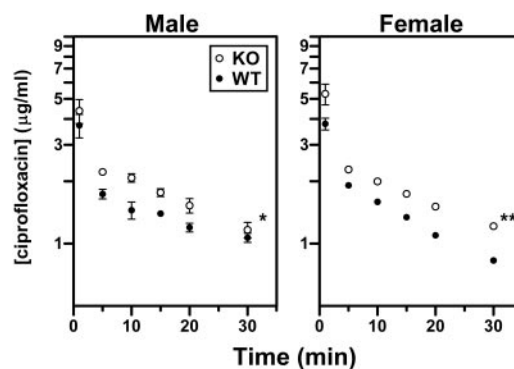


**Fig. 5.** Ciprofloxacin transport kinetics in CHO-mOat3 cells. Ciprofloxacin uptake was measured in CHO-mOat3 or empty vector-transfected (CHO-FRT) cells at room temperature. A, ciprofloxacin (100  $\mu$ M) accumulation was determined by HPLC at 1, 2, 5, 10, 15, 30, 60, and 90 min to determine the linear accumulation phase. B, ciprofloxacin uptake at 5 min was determined over a range of concentrations (20, 40, 100, 200, 500, and 1000  $\mu$ M). "Corrected" curve represents the accumulation mediated by mOat3 (i.e., the difference between uptake in CHO-mOat3 and CHO-FRT cells). C, Lineweaver-Burk transformation using corrected data from B to calculate  $K_m$  and  $V_{max}$  values for ciprofloxacin from the x and y intercepts. Values shown are means  $\pm$  S.E. from three determinations performed in triplicate.

**Interaction of Carboxyfluoroquinolones with mOat3/hOAT3 and mOat1/hOAT1 in Cell Monolayers.** The stably transfected mOat3-expressing (CHO-mOat3) and mOat1-expressing (CHO-mOat1) cell lines demonstrated marked accumulation of standard substrates relative to empty vector-transfected (CHO-FRT) cells: more than 10-fold greater for estrone-3-sulfate on mOat3 (Fig. 1A), and more than 150-fold greater for *p*-aminohippurate on mOat1 (Fig. 1C). In CHO-mOat3 cells, mOat3-mediated estrone-3-sulfate uptake was significantly inhibited (50–70%) by carboxyfluoroquinolones, whereas probenecid inhibited virtually all transport (Fig. 1A). Ciprofloxacin accumulated in CHO-mOat3 cells to more than five times its concentration in CHO-FRT cells (Fig. 1B). Penicillin G and probenecid inhibited mOat3-mediated ciprofloxacin uptake by 75 and 100%, respectively, after correction for nonspecific accumulation (i.e., in CHO-FRT cells; Fig. 1B). In CHO-mOat1 cells, mOat1-mediated *p*-aminohippurate uptake was not inhibited by a 7000-fold molar excess of ciprofloxacin but was abolished by the same molar excess of probenecid (Fig. 1C). Ciprofloxacin accumulation in CHO-mOat1 and CHO-FRT cells was virtually identical (Fig. 1D).

The stably transfected hOAT3- and hOAT1-expressing cell lines also demonstrated significantly greater accumulation of standard substrates relative to control cells (Fig. 2). Similar to mOat3, hOAT3-mediated estrone-3-sulfate uptake was significantly inhibited by ciprofloxacin and gatifloxacin ( $\sim 60\%$  inhibition after correction for nonspecific accumulation; Fig. 2A). In addition, there was a nonsignificant trend for inhibition ( $\sim 20\%$ ) by both norfloxacin and ofloxacin. Probenecid again inhibited all mediated transport. As with mOat1, hOAT1-mediated *p*-aminohippurate uptake was unaffected by ciprofloxacin but was abolished by probenecid (Fig. 2B).

After demonstrating that carboxyfluoroquinolones interact with mOat3 and hOAT3, experiments were conducted to characterize their inhibition type and potency. It was determined that ciprofloxacin is a competitive-type inhibitor of estrone-3-sulfate transport on mOat3 (Fig. 3). This was reflected in the positive correlation between the ciprofloxacin concentration and the observed  $K_m$  value, together with an unaltered  $V_{max}$  value (Fig. 3C, embedded table). Thereafter,



**Fig. 6.** Ciprofloxacin plasma elimination after intravenous bolus administration. Ciprofloxacin was administered to male and female wild-type (WT) and Oat3(−/−) (KO) mice at a dose of 5 mg/kg to achieve clinically observed plasma levels. Serial plasma samples were acquired at 1, 5, 10, 15, 20, and 30 min and assayed by HPLC to determine total (free and bound) ciprofloxacin levels. Values shown are means  $\pm$  S.E. ( $n = 4$  WT and 6 KO male mice; 3 WT and 5 KO female mice), and significant differences are between entire curves using two-way ANOVA (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ).



$K_i$  values were determined for four carboxyfluoroquinolones assuming competitive inhibition: ciprofloxacin, norfloxacin, ofloxacin, and gatifloxacin (Fig. 4B). To calculate the  $K_i$  values, estrone-3-sulfate's  $K_m$  value for transport was first determined in CHO-mOat3 cells ( $12.2 \pm 4.8 \mu\text{M}$ ; Fig. 4A). All four carboxyfluoroquinolones demonstrated a concentration-dependent inhibition of estrone-3-sulfate transport on mOat3, and ciprofloxacin exhibited the greatest potency.

Kinetics of ciprofloxacin transport in CHO-mOat3 cells were then determined to demonstrate saturability and provide an additional means of assessing its affinity for mOat3. A time course study indicated that ciprofloxacin accumulation in CHO-mOat3 cells was very rapid over the first 1 to 2 min and then was approximately constant for the next 14 min (Fig. 5A). Saturation kinetics were then determined for ciprofloxacin transport in CHO-mOat3 cells using a 5-min uptake duration to approximate the initial rate (Fig. 5B). Background accumulation in control CHO-FRT cells was also determined. mOat3-mediated ciprofloxacin accumulation was clearly saturable with a  $K_m$  value of  $69.8 \pm 5.7 \mu\text{M}$  and a  $V_{\text{max}}$  value of  $454 \pm 32 \text{ pmol/mg protein/min}$  (Fig. 5, B and C).

**Ciprofloxacin Plasma Elimination in Wild-Type and Oat3(−/−) Mice.** The administered ciprofloxacin dose (5 mg/kg) was chosen to yield plasma concentrations that are clinically observed in humans (i.e., 3.6–5.0  $\mu\text{g/ml}$ ; Fig. 6) (Micromedex Healthcare Series, 2007). It is noteworthy that ciprofloxacin plasma levels were significantly higher in male and female Oat3(−/−) mice compared with wild type (Fig. 6). Female knockout mice demonstrated an apparent reduction in the terminal elimination rate (i.e., slope) compared with wild type, although this trend was not obvious in male mice. Noncompartmental pharmacokinetic analysis also revealed abnormalities in Oat3(−/−) mice (Table 2). Both genders of Oat3(−/−) mice exhibited a significantly lower apparent volume of distribution ( $V_{\text{dss}}$ ) and higher area under the concentration-time curve (AUC) compared with wild-type ( $V_{\text{dss}}$  reduction of 27% for male mice, 14% for female mice; AUC increased by 25% for male mice, 33% for female mice). For female Oat3(−/−) mice exclusively, these abnormalities resulted in a significant (35%) reduction in total plasma clearance of ciprofloxacin (Table 2). Furthermore, sex differences in pharmacokinetics of ciprofloxacin were observed exclusively in wild-type mice and indicated that female mice have a significantly lower volume of distribution (~18%), shorter half-life (~40%), and greater plasma clearance (~39%). Oat3 deletion abolished these sex differences.

TABLE 2

Pharmacokinetic analysis of intravenous bolus ciprofloxacin in wild-type and Oat3(−/−) mice

Ciprofloxacin was administered by intravenous bolus injection at a 5 mg/kg dose, and serial plasma samples were analyzed (see Fig. 6). Noncompartmental pharmacokinetic analysis was performed. Data are shown as mean  $\pm$  S.E. Plasma clearance was calculated using the AUC from zero to infinity.

Sex	Genotype	Pharmacokinetic Parameter			
		$V_{\text{dss}}$	Half-Life	AUC	Plasma Clearance
		$\text{ml/kg}$	$\text{min}$	$\text{min} \cdot \text{ng}/\mu\text{l}$	$\text{ml/min/kg}$
Male	Wild type ( $n = 4$ )	$2391 \pm 141$	$35.3 \pm 2.6$	$47.9 \pm 2.5$	$49.5 \pm 3.3$
	Oat3(−/−) ( $n = 6$ )	$1755 \pm 107^{**}$	$30.8 \pm 6.1$	$60.0 \pm 2.7^*$	$47.9 \pm 7.0$
Female	Wild type ( $n = 3$ )	$1949 \pm 66^{\dagger}$	$21.2 \pm 1.0^{**}$	$47.3 \pm 1.4$	$69.0 \pm 2.8^{**}$
	Oat3(−/−) ( $n = 5$ )	$1673 \pm 13^{**}$	$28.7 \pm 2.9$	$62.8 \pm 2.2^{**}$	$44.9 \pm 3.0^{**}$

\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ , significant differences for Oat3(−/−) compared with wild type for the same sex.

$^{\dagger}$ ,  $P < 0.05$ ;  $^{\ddagger}$ ,  $P < 0.01$ , significant differences for female compared with male for the same genotype.

Two putative ciprofloxacin metabolite peaks were observed in serum samples from all mice (Figs. 7 and 8). The peaks were absent at 1 min after administration and became overt at later time points. An attempt was made to identify the putative metabolites by administering 50 mg/kg ciprofloxacin (intravenous bolus) to a mouse, acquiring total blood at 30 min, and isolating the “metabolite” fractions during subsequent HPLC for analysis by mass spectrometry. The early eluting putative metabolite (Figs. 7 and 8; retention time of ~7.3 min) yielded an abundant peak at a mass-to-charge ratio of 346.3 when assessed with electrospray ionization in the positive ion mode. The late-eluting “metabolite” (Figs. 7 and 8; retention time of ~8.5 min) showed an abundant peak at a mass-to-charge ratio of 316.1. Male Oat3(−/−) mice exhibited significantly heightened accumulation of the early eluting “metabolite” compared with wild type (Fig. 8A), whereas female Oat3(−/−) mice exhibited significantly heightened accumulation of both “metabolites” (Fig. 8, A and B). Furthermore, although the female-to-male ciprofloxacin plasma ratio was not far from unity within each genotype at 30 min (i.e., ratio of 1.04 for Oat3(−/−) and 0.78 for wild-type), female mice showed far greater levels of the “metabolites”, particularly the late-eluting one, compared with male mice (Fig. 8).

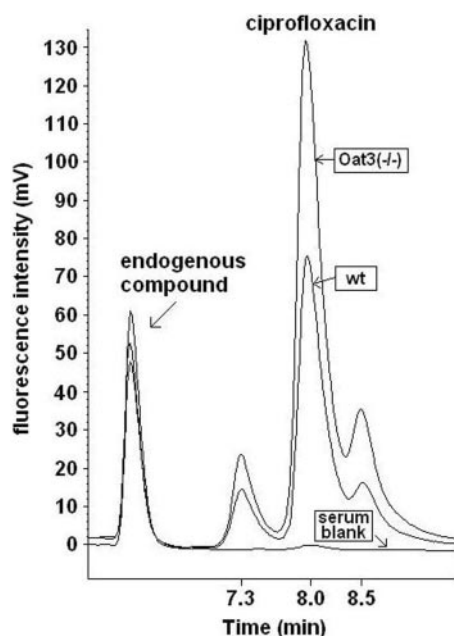
## Discussion

Fluoroquinolones exhibit an impressive spectrum of antimicrobial activity and remain one of the most commonly prescribed classes of pharmacotherapeutics. Some carboxylic acid-containing fluoroquinolones, or carboxyfluoroquinolones, such as ciprofloxacin and levofloxacin, are among the most useful in treating afflicted patients. In general, carboxyfluoroquinolones can undergo both renal and hepatic elimination (Micromedex Healthcare Series, 2007), with the renal component demonstrating a marked bias for secretory transport (Shimada et al., 1983; Jaehde et al., 1995). Therefore, perturbations in tubular secretory transport function can significantly increase systemic, and decrease urinary, levels of carboxyfluoroquinolones (Jaehde et al., 1995), potentially resulting in enhanced systemic efficacy but failure to eradicate renal and postrenal pathogens. This is of pivotal importance to patients with ascending or complicated urinary tract infections, because carboxyfluoroquinolones are used as first-line treatment (Warren et al., 1999).

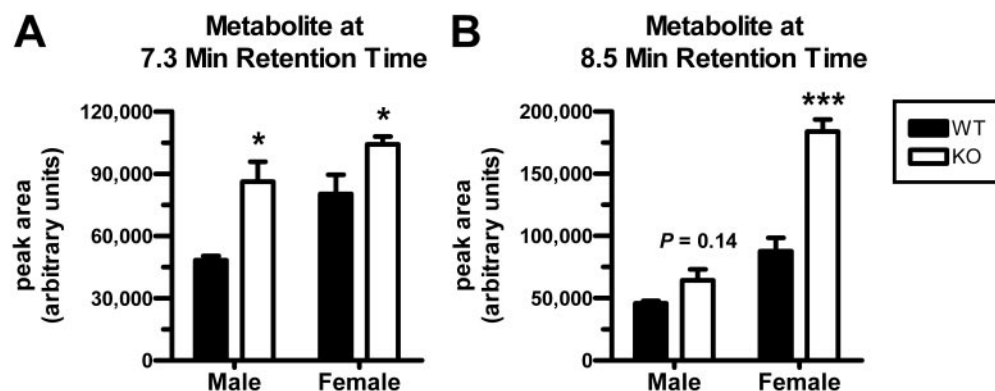
Although renal secretion of carboxyfluoroquinolones is well accepted as a major route of elimination, the specific transport mechanism for this pathway remains ambiguous. Thus

far, investigators have suggested possible roles for organic anion, organic cation, and undetermined transporter families expressed on both basolateral and apical plasma membranes (Table 1). A recent *in vitro* study suggested a role for basolateral human organic cation transporter 2 (hOCT2) specifically, based on the ability of levofloxacin and a premarket fluoroquinolone to inhibit creatinine transport (Okuda et al., 2006). The promiscuous interaction of carboxyfluoroquinolones with various transport systems stems from their zwitterionic nature, bearing both amine and carboxylate moieties on opposite ends.

Although a role for an individual organic anion transporter in carboxyfluoroquinolone renal secretion has not been reported, three *in vivo* studies in particular provide substantial evidence that the OAT family plays a pivotal role (Shimada et al., 1983; Jaehde et al., 1995; Foote and Halstenson, 1998).



**Fig. 7.** Representative HPLC chromatograms of ciprofloxacin extracted from plasma acquired 30 min after administration. Isocratic elution and fluorescence detection was used for quantifying ciprofloxacin in wild-type and Oat3(-/-) mice. Overlaid ciprofloxacin peaks are shown from wild-type (wt) and Oat3(-/-) mouse plasma, in addition to a serum blank. Peaks to the immediate left and right of ciprofloxacin (i.e., putative metabolites) were not found in spiked plasma or samples acquired 1 min after administration. The unidentified endogenous compound was observed in blank serum (highest endogenous peak) and both genotypes (two lower endogenous peaks), providing an indication of HPLC sample injection consistency.



**Fig. 8.** Comparison of putative ciprofloxacin metabolites between wild-type and Oat3(-/-) mice 30 min after intravenous bolus administration. Metabolites were apparent in chromatograms at later time points after ciprofloxacin administration as shown in Fig. 7. A, metabolite eluting at 7.3 min. B, metabolite eluting at 8.5 min. Values shown are means  $\pm$  S.E. ( $n$  = 4 WT and 6 KO male mice; 3 WT and 5 KO female mice), and significance was assessed between genotypes within the same sex (\*,  $P$  < 0.05; \*\*\*,  $P$  < 0.001).

Two of the investigations demonstrated that probenecid can reduce the renal clearance of ciprofloxacin and norfloxacin to approximately one third or one half, respectively, of that in the uninhibited state in humans (Shimada et al., 1983; Jaehde et al., 1995). In a subsequent investigation, it was shown that probenecid can produce a similar effect on ofloxacin in rats, reducing renal clearance to approximately one half of normal (Foote and Halstenson, 1998). In the latter investigation, cimetidine also markedly reduced renal clearance of ofloxacin in rats, maintaining the putative role for organic cation transporters. However, it is now established that cimetidine also interacts substantially with organic anion transporters 1 and 3 (Burckhardt et al., 2003; Tahara et al., 2005; Erdman et al., 2006). Because it is primarily uncharged at physiological pH (Burckhardt et al., 2003), its effects on ofloxacin secretion may be the result of inhibition of either organic cation or anion transporter families. Given the convincing *in vivo* evidence substantiating the role of the OAT family, we probed further into the mechanism of this interaction by assessing the ability of carboxyfluoroquinolones to interact with murine and human renal basolateral Oat1/OAT1 and Oat3/OAT3 *in vitro* and, thereafter, Oat3 *in vivo* using wild-type and Oat3(-/-) mice.

The present results indicate that carboxyfluoroquinolones, especially ciprofloxacin, exhibit a significant interaction with murine and human Oat3/OAT3 (Figs. 1 and 2). Furthermore, ciprofloxacin interacts with mOat3/hOAT3 selectively, demonstrating no inhibition of mOat1/hOAT1 and no transport by mOat1, the other major renal basolateral transporter (Figs. 1 and 2). Ciprofloxacin's concentrative accumulation by and affinity for mOat3 suggested that this transporter may play a significant role *in vivo* in the elimination of ciprofloxacin (Figs. 4 and 5). The *in vivo* role of mOat3 was thus confirmed by comparing elimination in wild-type and Oat3(-/-) mice (Fig. 6 and Table 2). Pharmacokinetic differences between genotypes in this investigation are expected to be caused by differences in transport and not differences in hemodynamics or glomerular function, because these potential confounding factors have been ruled out in two previous studies by measuring inulin plasma elimination in the same mouse colony (VanWert et al., 2007; VanWert and Sweet, 2008). The consequence of Oat3 deletion in the present study, at clinically relevant ciprofloxacin levels, is similar to the effect of probenecid in previous studies in humans and rats (Shimada et al., 1983; Jaehde et al., 1995; Foote and Halstenson, 1998). Therefore, it is likely that OAT3 plays a significant role in humans in the renal secretion of carboxyfluoroquinolones. Thus, the clear role of mOat3 in carboxyfluoro-



quinolone elimination and the lack of interaction with mOat1/hOAT1, and previous reports of hOAT3 polymorphisms yielding nonfunctional hOAT3 protein suggest that there is a human population that may respond poorly to carboxyfluoroquinolone therapy when prescribed for the eradication of renal and post-renal pathogens.

The present findings also suggest that mOat3 contributes significantly to the distribution of ciprofloxacin in both genders, but its role in total clearance is more overt in female mice (Fig. 6 and Table 2). Furthermore, deletion of Oat3 abolished all pharmacokinetic differences between male and female mice, indicating that differential Oat3 function exists between genders, and female mice may depend more on this transporter than male mice. Similar observations have been reported for two other pharmacotherapeutic Oat3 substrates, namely penicillin G and methotrexate, in that Oat3 deletion had a greater impact on their total clearance in female compared with male mice (VanWert et al., 2007; VanWert and Sweet, 2008). Moreover, the observation that female mice may depend more on Oat3 function than male mice is also consistent with known sex differences in the expression of Oat3 mRNA in the currently used C57BL/6 mouse colony (i.e., female kidney expressed twice the level of Oat3 mRNA relative to male) (VanWert et al., 2007). Given our current knowledge, it is unclear whether this sex difference in mOat3 dependence can be extrapolated to other species. For example, female rats express lower levels of Oat3 protein in the proximal tubule than male rats (Ljubojevic et al., 2004), and rabbits failed to exhibit any sex differences in Oat3 mRNA or protein levels (Groves et al., 2006). Gender differences in humans have not been reported; therefore, clinical predictions regarding sex differences in dependence on hOAT3 are not warranted at this time.

Finally, two putative ciprofloxacin metabolites were observed during HPLC analysis, both of which were completely absent in spiked plasma standards and early time points but were highly evident in later time points (Fig. 7). Each of the suspected metabolites exhibited heightened accumulation in Oat3(−/−) mice (Figs. 7 and 8), suggesting that ciprofloxacin levels are not elevated as a result of impaired metabolism in Oat3(−/−) mice, but rather as a consequence of perturbed secretory transport. For both genotypes, the metabolites accumulated to a greater extent in female mice. In addition, the late-eluting metabolite was markedly heightened in female Oat3(−/−) mice compared with wild-type or male mice of either genotype (Fig. 8B).

Four ciprofloxacin metabolites have been identified in humans: desethyleneciprofloxacin (M1, or the 2-aminoethyl-amino metabolite), sulfociprofloxacin (M2), oxociprofloxacin (M3), and formylciprofloxacin (M4) (Zeiler et al., 1987). The M1 and M3 metabolites have also been observed in animals (rats and monkeys) (Siefert et al., 1986). All of these metabolites retain the carboxylate moiety, suggesting that they may also be substrates for Oat3. In support of this, probenecid has been shown to prolong the half-life of the 2-aminoethyl-amino metabolite (M1) and impact its plasma level to a greater extent than the parent compound in humans (Jaehde et al., 1995). The early eluting (7.3 min) HPLC peak in the present study yielded an abundant mass spectrometry peak with a mass-to-charge ratio of 346.3 and is consistent with the metabolite oxociprofloxacin (M3), which has a molecular weight of 345.3 before protonation and has been identified

previously in animals (Siefert et al., 1986). The late eluting (8.5 min) HPLC peak does not seem to correspond to any of the metabolites of ciprofloxacin reported previously (M1–M4). Further studies are required to positively identify the compound represented by this peak.

In conclusion, the results of this investigation show that renal basolateral mOat3/hOAT3 interacts with carboxyfluoroquinolones in vitro, and the murine ortholog plays a significant role in ciprofloxacin elimination at clinically observed concentrations in vivo. In contrast, mOat1/hOAT1 does not interact with ciprofloxacin, indicating that this other major renal basolateral OAT is not involved in the elimination of ciprofloxacin. Therefore, hOAT3 polymorphisms, some of which have already been demonstrated as highly dysfunctional (Erdman et al., 2006), should be considered a potential source of variable carboxyfluoroquinolone efficacy in tissues and especially throughout the urinary tract. Furthermore, numerous drug interactions involving carboxyfluoroquinolones, whether affecting the carboxyfluoroquinolone level or concomitant drug level, probably occur via competition for transport on hOAT3. Such carboxyfluoroquinolone-drug interactions have been documented in humans (Micromedex Healthcare Series, 2007) and are known to involve substrates/inhibitors, which have been demonstrated to interact with mOat3 in vivo [e.g., methotrexate (VanWert and Sweet, 2008) and probenecid]. Pharmacotherapeutic OAT3 substrates (e.g., penicillin G and nonsteroidal anti-inflammatory drugs) should therefore be administered with caution in patients who are receiving a carboxyfluoroquinolone for a urinary tract infection, because blood and tissue levels of the quinolone may be therapeutic or super-therapeutic, whereas urinary levels may fail to reach the minimum effective concentration.

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