

# Diclofenac and Its Acyl Glucuronide: Determination of In Vivo Exposure in Human Subjects and Characterization as Human Drug Transporter Substrates In Vitro<sup>S</sup>

Yueping Zhang, Yong-Hae Han,<sup>1</sup> Siva Prasad Putluru, Murali Krishna Matta, Prashant Kole, Sandhya Mandekar, Michael T. Furlong,<sup>2</sup> Tongtong Liu, Ramaswamy A. Iyer, Punit Marathe, Zheng Yang, Yurong Lai, and A. David Rodrigues<sup>3</sup>

*Pharmaceutical Candidate Optimization, Bristol-Myers Squibb Company, Princeton, New Jersey*

Received August 26, 2015; accepted December 28, 2015

## ABSTRACT

Although the metabolism and disposition of diclofenac (DF) has been studied extensively, information regarding the plasma levels of its acyl- $\beta$ -D-glucuronide (DF-AG), a major metabolite, in human subjects is limited. Therefore, DF-AG concentrations were determined in plasma (acidified blood derived) of six healthy volunteers following a single oral DF dose (50 mg). Levels of DF-AG in plasma were high, as reflected by a DF-AG/DF ratio of  $0.62 \pm 0.21$  ( $C_{max}$  mean  $\pm$  S.D.) and  $0.84 \pm 0.21$  (area under the concentration-time curve mean  $\pm$  S.D.). Both DF and DF-AG were also studied as substrates of different human drug transporters in vitro. DF was identified as a substrate of organic anion transporter (OAT) 2 only ( $K_m = 46.8 \mu\text{M}$ ). In contrast, DF-AG was identified as a substrate of numerous OATs ( $K_m = 8.6, 60.2, 103.9,$  and  $112 \mu\text{M}$  for

OAT2, OAT1, OAT4, and OAT3, respectively), two organic anion-transporting polypeptides (OATP1B1,  $K_m = 34 \mu\text{M}$ ; OATP2B1,  $K_m = 105 \mu\text{M}$ ), breast cancer resistance protein ( $K_m = 152 \mu\text{M}$ ), and two multidrug resistance proteins (MRP2,  $K_m = 145 \mu\text{M}$ ; MRP3,  $K_m = 196 \mu\text{M}$ ). It is concluded that the disposition of DF-AG, once formed, can be mediated by various candidate transporters known to be expressed in the kidney (basolateral, OAT1, OAT2, and OAT3; apical, MRP2, BCRP, and OAT4) and liver (canalicular, MRP2 and BCRP; basolateral, OATP1B1, OATP2B1, OAT2, and MRP3). DF-AG is unstable in plasma and undergoes conversion to parent DF. Therefore, caution is warranted when assessing renal and hepatic transporter-mediated drug-drug interactions with DF and DF-AG.

## Introduction

Diclofenac (DF) is a nonsteroidal anti-inflammatory drug widely used for the treatment of postoperative pain, rheumatoid arthritis, osteoarthritis, ankylosing spondylitis, and acute gouty arthritis. It is highly bound to plasma proteins and is rapidly and almost completely absorbed following an oral dose (John, 1979; Peris-Ribera et al., 1991). In addition, the pharmacokinetic profile of DF is linear within the dose range of 25–150 mg. Depending on the formulation, the absolute bioavailability of DF varies from 50 up to 90% (Willis et al., 1979). Early analyses demonstrated that DF is extensively metabolized in animals and man either by hydroxylation followed by conjugation or by direct conjugation to form an acyl- $\beta$ -D-glucuronide (DF-AG) (Riess et al., 1978; John, 1979; Stierlin et al., 1979). DF-AG found in the bile is unstable and likely undergoes hydrolysis in the gastrointestinal track to

release parent DF, which undergoes enterohepatic recirculation (Stierlin and Faigle, 1979). However, regardless of the apparent predominance of oxidative pathways in DF elimination, significant oxidative metabolism of DF-AG itself (forming 4'-hydroxy DF-AG) has been reported, suggesting that a substantial fraction of DF-AG formed in the liver could be converted to its hydroxy metabolites in vivo (Kumar et al., 2002). More importantly, direct DF glucuronidation to form DF-AG has been proposed as the major clearance pathway (~75% of total metabolic clearance) (Kumar et al., 2002). So although hydroxylation of DF is major in the presence of NADPH-fortified human liver microsomes, and CYP2C9 is dominant, the CYP2C9 genotype is not associated with clinically meaningful changes in DF pharmacokinetics (Rodrigues, 2005).

In rodents, the biliary secretion of DF-AG is governed by active transport. For instance, in bile duct-cannulated Groningen Yellow transport deficient (TR-) rats that lack the functional canalicular ATP binding cassette C family (ABCC) transporter multidrug resistance protein 2 (Mrp2), virtually no hepatobiliary excretion of DF-AG was found, suggesting Mrp2-mediated biliary secretion (Seitz and Boelsterli, 1998; Seitz et al., 1998). Similarly, the disposition of DF-AG (following an i.v. and oral DF dose) is greatly impacted in mice lacking canalicular Mrp2 and breast cancer resistance protein (BCRP), reflected in the altered DF-AG/DF ratios in bile (decreased) and plasma (increased)

This research was supported by Bristol-Myers Squibb, Co.

<sup>1</sup>Current affiliation: Enzychem Lifesciences Corp., Seoul, Korea.

<sup>2</sup>FORUM Pharmaceuticals Inc., Waltham, Massachusetts.

<sup>3</sup>Pharmacokinetics, Dynamics and Metabolism, World Wide Research and Development, Pfizer, Inc. Groton Laboratories, Groton, Connecticut  
[dx.doi.org/10.1124/dmd.115.066944](http://dx.doi.org/10.1124/dmd.115.066944)

<sup>S</sup>This article has supplemental material available at [dmd.aspetjournals.org](http://dmd.aspetjournals.org).

**ABBREVIATIONS:** AUC, area under the plasma concentration versus time curve;  $AUC_{tot}$ , total AUC; BCRP, breast cancer resistance protein; CCK-8, cholecystokinin octapeptide; CsA, cyclosporine A; DDI, drug-drug interaction; DF, diclofenac; DF-AG, diclofenac acyl- $\beta$ -D-glucuronide; ESI, electrospray ionization; FTC, fumitremorgin C; HBSS, Hanks' balanced salt solution; HEK-293, human embryonic kidney 293; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MK571, 3-[[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoyl)ethylsulfanyl]methylsulfanyl] propionic acid; MRP, multidrug resistance protein; OAT, organic anion transporter; OATP, organic anion-transporting polypeptide.

(Lagas et al., 2009). The sinusoidal efflux of DF-AG into blood, once formed in the liver, is largely dependent on Mrp3 (Lagas et al., 2009). Recently, it was reported that the plasma concentration of DF-AG is 90% lower in Mrp3 gene knockout mice (versus wild-type mice), confirming that basolateral efflux of DF-AG is mediated by Mrp3 (Scialis et al., 2015). These results support the fact that various transporters play an important role in the distribution and elimination of DF-AG. When compared with rodents, however, it is worth noting that less of the human dose ( $\leq 30\%$  vs.  $> 50\%$ ) is recovered in bile/feces (Riess et al., 1978; John, 1979; Lagas et al., 2010). In humans, a larger fraction of the radiolabeled dose (oral or i.v.) is recovered in the urine ( $\sim 60\%$ ). This implies that the urinary excretion of DF and DF-AG could be altered in subjects with severe renal impairment (Kendall et al., 1979). Because DF-AG is present in human urine and bile (John, 1979; Stierlin and Faigle, 1979), its disposition by renal transporters should also be considered.

As it is increasingly recognized that many drug-drug interactions (DDIs) occur at the level of drug transporter proteins, understanding transporter-mediated disposition of DF and DF-AG, its major metabolite (Kumar et al., 2002), is important. Therefore, one of the aims of the present study was to characterize both as substrates of liver and kidney transporters in vitro. In contrast to the rodent (Lagas et al., 2010), only limited data are available for DF-AG in human plasma (Hammond et al., 2014), with earlier studies largely focused on dose recovery and profiling of human excreta (John, 1979; Stierlin and Faigle, 1979). Therefore, a second aim of the study was to determine DF-AG exposure in normal healthy human volunteers following a single oral DF dose. For the first time, DF-AG/DF ratios in human plasma are reported, and in vitro drug transporter data are provided for DF-AG beyond MRP2, BCRP, and organic anion-transporting polypeptides (OATPs).

## Methods and Materials

**Chemicals and Reagents.** [ $^3\text{H}$ ]estradiol-17 $\beta$ -D-glucuronide (34.3 Ci/mmol), [ $^3\text{H}$ ]cholecystokinin octapeptide (CCK-8; 97.5 mCi/mmol), and [ $^3\text{H}$ ]estrone-3-sulfate salt (45.6 Ci/mmol) were purchased from PerkinElmer Life and Analytical Science (Waltham, MA). [ $^3\text{H}$ ]penciclovir (1.1 Ci/mmol) was purchased from Moravak Biochemicals (Brea, CA). Benzbromarone, estradiol-17 $\beta$ -D-glucuronide, CCK-8, estrone-3-sulfate salt, and rifampin were purchased from Sigma-Aldrich (St. Louis, MO). Fumitremorgin C (FTC) was purchased from Alexis Biochemicals (San Diego, CA). Membrane vesicles prepared from Sf9 cells expressing human MRP2, MRP3, and BCRP were purchased from Genomembrane, Inc. (Yokohama, Japan). DF and DF-AG were purchased from Toronto Research Chemicals (Toronto, Canada). [ $^3\text{H}$ ]DF-AG (purity:  $> 97\%$ ) was synthesized at Bristol-Myers Squibb (Princeton, NJ). Cell culture reagents including Dulbecco's modified Eagle's medium, fetal calf serum, trypsin, Hanks' balanced salt solution (HBSS), nonessential amino acids, and L-glutamine were purchased from Mediatech (Herndon, VA). Biocoat poly-D-lysine-coated 24-well plates were purchased from BD Biosciences (Bedford, MA). BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL). 3-[[3-[2-(7-Chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoyl)ethylsulfanyl)methylsulfanyl] propionic acid (MK571) was obtained from Cayman Chemical Company (Ann Arbor, MI). All other chemicals and reagents were purchased from Sigma-Aldrich.

**Determination of DF and DF-AG in Plasma Samples of Healthy Human Volunteers.** Exposures of DF and DF-AG were determined in an open-label study of single-dose administration of 50 mg of Voltafam (diclofenac potassium, Novartis (India) Ltd., Mumbai, India) using six normal healthy subjects (males ages 22–34). Blood was collected predose and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, 6.0, 8.0, 12.0,

and 24 hours. In each case, the sample of blood (6 ml) was acidified with 80  $\mu\text{l}$  of citric acid (3.2 M) immediately after collection to stabilize the DF-AG. Plasma was prepared from each blood sample. The study protocol and volunteer informed consents were approved by an institutional review board at Biocon Bristol-Myers Squibb Research and Development Center (Bangalore, India). The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice.

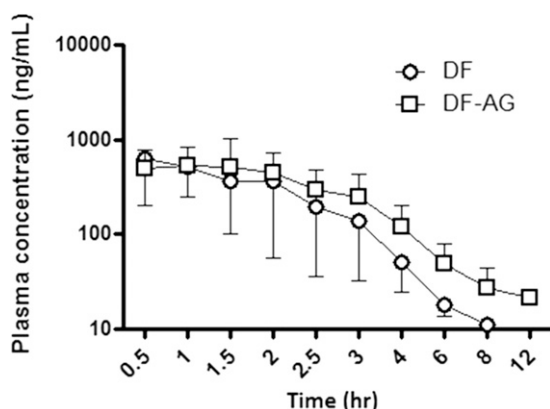
**Liquid Chromatography–Tandem Mass Spectrometry Analyt-ics of DF and DF-AG.** A 25  $\mu\text{l}$  aliquot of plasma was precipitated with acetonitrile (125  $\mu\text{l}$ ) containing deuterated DF (D4-diclofenac) as the internal standard. The samples were then centrifuged at 3000g for 5 minutes through a 96-well filter plate, and the filtrates were subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis.

Sample analysis was conducted on an API-4000 quadrupole ion trap (Applied Biosystems/MDS SCIEX, Toronto, Canada) coupled with an Acquity ultra-performance LC system (Waters Corp., Milford, MA). A BEH column (C18, 50  $\times$  2.1 mm, 1.7  $\mu\text{m}$  particles; Waters Corp.) was used for chromatographic separation. Mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). Chromatographic separation of DF and DF-AG was achieved with the following gradient at a flow rate of 0.7 ml/min: 10% B to 90% B over 1.2 minutes, 90% B maintained from 1.2 to 1.8 minutes, 90% B to 10% B from 1.8 to 2.0 minutes, return to 10% B from 2.0 to 2.2 minutes. Analytes were detected by multiple reaction monitoring using electrospray ionization (ESI) MS. ESI source was operated in the negative ion mode with multiple reaction monitoring analysis. The ESI source temperature was set at 550°C, and ion spray voltage and cell entrance and exit potentials were set at  $-4500$ ,  $-10$ , and  $-15$  V, respectively. Mass transitions were 293.9  $\rightarrow$  249.9, 297.4  $\rightarrow$  253.6, and 470.1  $\rightarrow$  193.1 for DF, d4-DF, and DF-AG, respectively. The lower limit of quantitation was 34 and 21.3 nM for DF and DF-AG, respectively.

**PK Analysis.** PK parameters were determined based on a non-compartmental approach using Kinetica (version 4.4.1; Thermo Electron Corporation, Waltham, MA). The following parameters were obtained:  $C_{\text{max}}$ , time of maximum concentration observed ( $T_{\text{max}}$ ), the area under the plasma concentration versus time curve (AUC) up to the last quantifiable time point, and total AUC ( $\text{AUC}_{\text{tot}}$ ). AUC was estimated by the mixed logarithmic-linear method in the software. Data are also reported as the DF/DF-AG  $\text{AUC}_{\text{tot}}$ , AUC up to the last quantifiable time point, and  $C_{\text{max}}$  ratio.

**Stable Expression of OATP1B1, OATP1B3, OATP2B1, Organic Anion Transporters 1, 2, 3, and 4 in Human Embryonic Kidney 293 Cells.** Human embryonic kidney 293 (HEK-293) cells are routinely cultured in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and hygromycin (100  $\mu\text{g}/\text{ml}$ ) in a humidified incubator at 37°C and 5%  $\text{CO}_2$ . The stably transfected HEK-293 cell lines expressing drug transporter proteins were established by using the Flp-In expression system (Invitrogen, Carlsbad, CA) as described previously (Han et al., 2010). In brief, the recombinant pcDNA5/FRT construct containing the open frames of OATP1B1, OATP1B3, OATP2B1, organic anion transporter 1 (OAT1), OAT2, OAT3, and OAT4 was cotransfected with pOG44, an Flp recombinase expression plasmid, into Flp-InTM HEK-293 cells. Cells stably expressing the transporters were selected in hygromycin (100  $\mu\text{g}/\text{ml}$ ) according to the manufacturer's protocol. All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5%  $\text{CO}_2$  and subcultured once a week.

**Stability of DF-AG In Vitro.** Before in vitro transport studies were conducted, the chemical stability of DF-AG was tested over a pH range (pH 5.4–7.4) using LC-MS/MS methods. DF-AG was not stable in



**Fig. 1.** Concentration-time profile in plasma of DF and DF-AG followed by administration of a single dose of 50 mg of DF. All plasma samples were prepared from blood collected under acidified conditions to ensure stabilization of DF-AG. Data are presented as the mean  $\pm$  S.D. from six healthy male human subjects.

buffer at pH 7.4 (data not shown). At 37°C, it remained stable (>87%) up to 2 hours over a pH range of 6.0–5.4. Consequently, the *in vitro* transport study was performed at pH 5.4 (cell-based assays) or pH 6.0 (membrane vesicle-based assays). Incubations were no longer than 10 minutes. Lowering the pH to stabilize DF-AG did not greatly impact transporter function (Supplemental Table 1).

**Studies with Transporter-Expressing HEK-293 Cells.** Uptake of DF and DF-AG was assessed using stably transfected HEK cells that singularly expressed human OATP1B1, OATP1B3, OATP2B1, OAT1, OAT2, OAT3, or OAT4. The stable transfected HEK-293 cells and mock control cells were seeded on BioCoat poly-D-lysine-coated 24-well plates (BD Biosciences) with cell density at  $2.5 \times 10^6$ /ml in Dulbecco's modified Eagle's medium, containing 10% fetal calf serum, nonessential amino acids (0.1 mM), and L-glutamate (2 mM), in an atmosphere of CO<sub>2</sub> (5%) and air (95%) at 37°C. After 2 days, cells were washed twice with 2 ml of prewarmed HBSS buffer. Cellular uptake was initiated at 37°C by adding 1  $\mu$ M [<sup>3</sup>H]DF-AG or DF dissolved in HBSS buffered with HEPES (pH 5.4) to cell plates, and the incubation was stopped at designated time points by removing the buffer. Time-dependent uptake was conducted prior to the assays to ensure that the transporter kinetics were determined under linear conditions. The incubation was conducted at a lower pH (5.4) to avoid the degradation of DF-AG. The cells were washed three times with ice-cold HBSS buffer. For [<sup>3</sup>H]DF-AG, the cells were lysed with 0.3 ml of 0.1% Triton X-100 (Sigma-Aldrich). The cell lysate samples were analyzed by liquid scintillation counting (LS 6500; Beckman Coulter, Inc., Fullerton, CA). DF in the cells was extracted by adding 100  $\mu$ l of water and 200  $\mu$ l of methanol with internal standard, followed by shaking for 30–45 minutes at room temperature. The plates were spun down at 4000 rpm for 15 minutes, and the clear supernatant was transferred to a clean 96-well plate. The samples were dried under nitrogen and reconstituted with a mobile phase mixture of 80% water with 0.1% formic acid and 20% acetonitrile with 0.1% formic acid for LC-MS/MS analysis. Cellular uptake was normalized to the protein content of the HEK-293 cells in each well, measured using the BCA protein assay kit. Control substrates for different transporter phenotyping assays were as follows: [<sup>3</sup>H]estradiol-17- $\beta$ -glucuronide (1  $\mu$ M, OATP1B1), [<sup>3</sup>H]estrone 3-sulfate (1  $\mu$ M, OATP2B1, OAT3, and OAT4), [<sup>3</sup>H]CCK-8 (1  $\mu$ M, OATP1B3), *p*-aminohippurate (1  $\mu$ M, OAT1), and [<sup>3</sup>H]penciclovir (0.14  $\mu$ M, OAT2). The uptake of probe substrates in transporter-expressing cells was also conducted under the same condition as DF-AG and DF uptake (pH 5.4), and known inhibitors of OATPs (sulfobromophthalein, 5  $\mu$ M; rifampin, 10  $\mu$ M) and OATs (indomethacin, 100  $\mu$ M; probenecid, 100  $\mu$ M) were

used to confirm the functional activities of each transporter in the test system.

**Transport Studies with MRP2-, MRP3-, and BCRP-Expressing Membrane Vesicles.** For efflux transporters, studies were performed using the membrane vesicles prepared from Sf9 cells expressing human BCRP, MRP2, and MRP3. Rapid filtration method was applied based on the manufacturer's protocol, with minor modification. In brief, 30  $\mu$ l of reaction medium (50 mM 2-(N-Morpholino)ethanesulfonic acid-Tris, 70 mM KCl, 7.5 mM MgCl<sub>2</sub>, pH 6.0) with 50  $\mu$ g of protein and DF-AG (50  $\mu$ M) or DF (50  $\mu$ M) was prewarmed at 37°C for 3 minutes, and the assay was started by adding 20  $\mu$ l of 4 mM ATP or AMP. The incubation was conducted at a lower pH (6.0) to avoid the degradation of DF-AG. The uptake of known substrates [<sup>3</sup>H]estradiol-17- $\beta$ -glucuronide (50  $\mu$ M, MRP2 and MRP3) and [<sup>3</sup>H]estrone 3-sulfate (1  $\mu$ M, BCRP) in the vesicles expressing each transporter was conducted under the same condition of DF-AG and DF uptake (pH 6.0), and known inhibitors of MRP2 (benzbromarone, 200  $\mu$ M), MRP3 (MK571, 200  $\mu$ M), and BCRP (FTC, 10  $\mu$ M) were used to confirm the functional activities of each transporter in the test system. Time-dependent uptake in membrane vesicles was conducted for the linearity of uptake in the vesicles. At designated time points, the reaction was stopped by the addition of 200  $\mu$ l of cold wash buffer (40 mM MES-Tris, 70 mM KCl, pH 6.0). The incubation mixture was immediately transferred to a PerkinElmer Unifilter GF/B plate, followed by a five-times wash with 250  $\mu$ l of cold wash buffer using Filter-Mate Harvester (PerkinElmer Life and Analytical Sciences). The filter plates were left to dry overnight at room temperature, and then each individual well membrane was transferred to 96-well filtration plates stacked on top of a 96-well deep receiver plate using Multi-screen Punch (EMD Millipore, Billerica, MA). DF or DF-AG in the filter membrane was extracted by adding 100  $\mu$ l of water and 200  $\mu$ l of acetonitrile with internal standard, followed by shaking for 30–45 minutes at room temperature. The plates were centrifuged at 4000 rpm for 15 minutes, dried, and reconstituted with the mobile phase mixture at 80% water with 0.1% formic acid and 20% acetonitrile with 0.1% formic acid for LC-MS/MS analysis.

**Data Analysis.** Transport kinetic parameters were estimated from eq. 1 using GraphPad Prism 5 (GraphPad Software, Inc., San Diego, CA). In the case of cooperative reaction (MRP2), the data were fitted to the Hill equation (eq. 2):

$$v = \frac{V_{\max} * S}{K_m + S} \quad (1)$$

$$v = \frac{V_{\max} * S^n}{K_m + S^n} \quad (2)$$

where  $v$  is the rate of uptake measured at the given concentration,  $V_{\max}$  is the maximal rate of uptake,  $K_m$  represents the Michaelis-Menten constant at which the uptake rate is half its maximal value,  $[S]$  is the substrate concentration, and  $[n]$  is the Hill coefficient. The comparison

TABLE 1

Pharmacokinetic parameters of DF and DF-AG following a single oral 50-mg dose of DF

Parameter	DF	DF-AG	DF-AG/DF ratio
$C_{\max}$ (nM)	3076 $\pm$ 531	1846 $\pm$ 528	0.62 $\pm$ 0.21
$T_{\max}$ (hours)	1.25 $\pm$ 0.58	1.25 $\pm$ 0.58	—
$AUC_{\text{last}}$ (nM*h)	4046 $\pm$ 635	3466 $\pm$ 1168	0.83 $\pm$ 0.20
$AUC_{\text{tot}}$ (nM*h)	4126 $\pm$ 627	3593 $\pm$ 1185	0.84 $\pm$ 0.21

$AUC_{\text{last}}$ , AUC up to the last quantifiable time point.

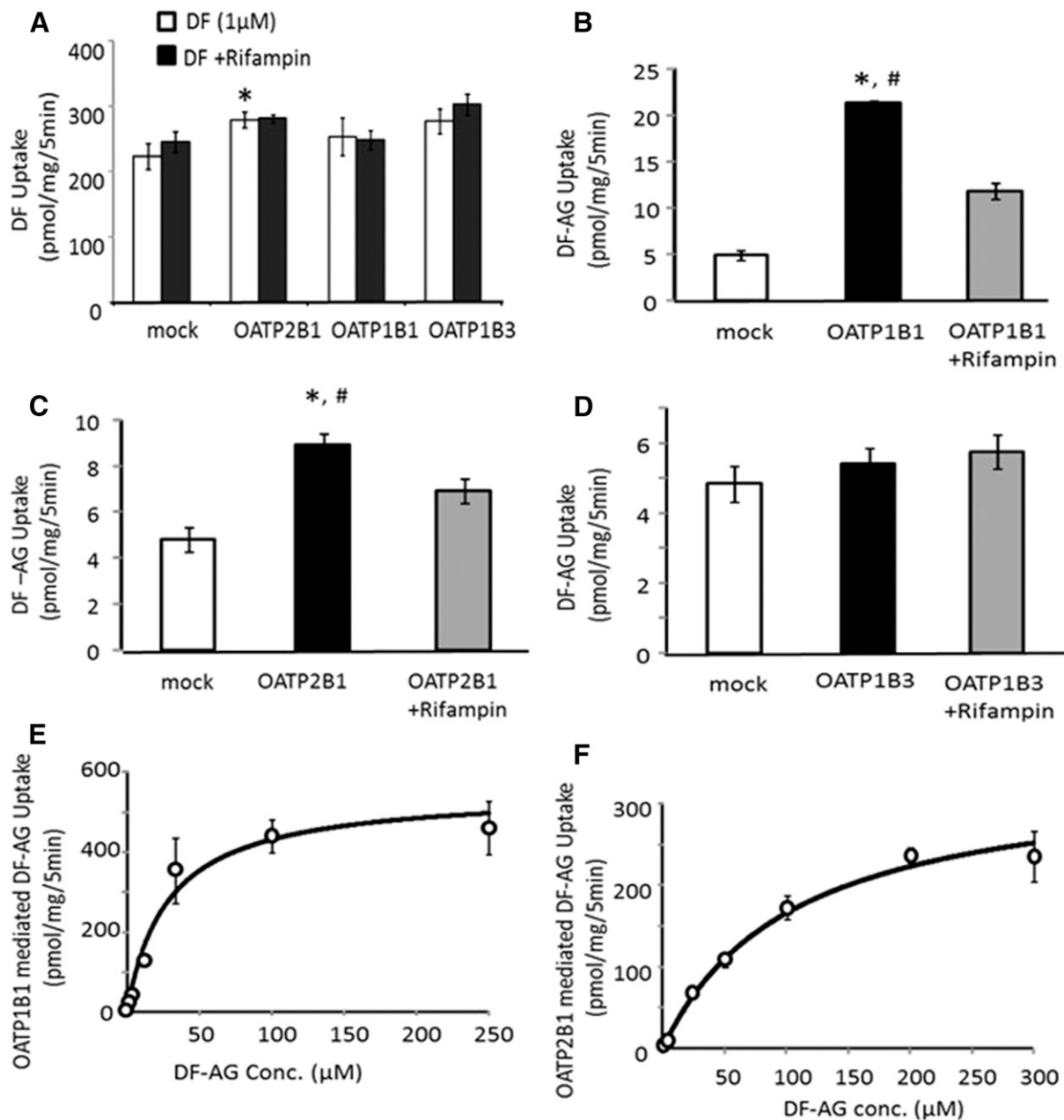
of cellular uptake and inhibition effects was made using Student's *t* test (*p* value less than 0.05 was considered statistically significant).

## Results

**Plasma Levels of DF and DF-AG.** DF and DF-AG in plasma of fasted healthy volunteers were monitored following a single 50-mg dose of DF. All plasma samples were prepared from blood collected under acidified conditions (42 mM citric acid) to ensure stabilization of DF-AG. As depicted in Fig. 1, DF and DF-AG were both detectable in plasma up to 8 and 12 hours after dosing, respectively, although sampling was carried out for 24 hours. The mean  $C_{max}$  and  $AUC_{tot}$  values for DF were 3075.9 nM and 4125.7 nM\*h, respectively

(Table 1). The level of DF-AG in plasma was comparable to that of DF ( $C_{max}$ , 1849.4 nM;  $AUC_{tot}$ , 3592.6 nM\*h) and rendered mean  $C_{max}$  and  $AUC_{tot}$  DF-AG/DF ratios of 0.62 and 0.84, respectively.

**OATP-Mediated Uptake of DF and DF-AG.** DF and DF-AG were evaluated as substrates for hepatic uptake transporters OATP1B1, 1B3, and 2B1. Although the positive control substrates behaved as expected (data not shown), uptake of DF in HEK-293 cells singly transfected with OATP1B1 or 1B3 was not significantly higher than mock cells, and the uptakes were not inhibited by rifampin, a known OATP inhibitor (Fig. 2). Although a slight but significant increase of DF uptake was observed in HEK-OATP2B1 cells as compared with the mock cells (Fig. 2), the uptake was not inhibited by rifampin. The results suggested that DF is not a substrate for hepatic OATP



**Fig. 2.** OATP-mediated uptake of DF and DF-AG. Uptake of DF and DF-AG was assessed in HEK-293 mock cells and the cells singly transfected with OATP1B1, 1B3, and 2B1, in the presence or absence of the OATP inhibitor rifampin. Concentration-dependent uptake of DF-AG was conducted in HEK cells overexpressing OATP1B1 and 2B1 cells. (A) DF uptakes. (B) OATP1B1-mediated DF-AG uptake. (C) OATP2B1-mediated DF-AG uptake. (D) OATP1B3-mediated DF-AG uptake. (E) Concentration-dependent DF-AG uptake in OATP1B1 cells. (F) Concentration-dependent DF-AG uptake in OATP2B1 cells. \**P* < 0.05 compared with mock cells; #*P* < 0.05 compared with the uptake in the presence of inhibitor. Data are presented as the mean ± S.D. (*n* = 3).

transporters. In contrast, uptake of DF-AG in the presence of HEK-OATP1B1 and OATP2B1 cells was significantly higher (>2-fold) versus mock cells. Moreover, the uptake was significantly reduced in the presence of rifampin. In contrast to OATP1B1 and 2B1, no uptake was detectable when DF-AG was incubated with HEK-OATP1B3 cells. The results suggested that DF-AG is a substrate for OATP1B1 and 2B1, but not a substrate for OATP1B3. The kinetics of OATP-mediated DF-AG transport was further assessed. Concentration-dependent and saturable uptake of DF-AG was detected with both HEK-OATP1B1 and OATP2B1 cells, characterized by a  $K_m$  of 34 and 104.6  $\mu\text{M}$ , respectively (Fig. 2; Table 2).

**OAT-Mediated Uptake of DF and DF-AG.** Similar to the OATPs, both DF and DF-AG were assessed as substrates of four different OATs (Fig. 3). A significant increase (versus mock cells) in uptake was not detected when DF was added to HEK-OAT1, -OAT3, or -OAT4 cells. Furthermore, uptake was not inhibited by 100  $\mu\text{M}$  probenecid, a known OAT inhibitor. In contrast, uptake of DF in HEK-OAT2 cells was about 2-fold higher than that in mock cells, and the uptake was inhibited by indomethacin, a known OAT2 inhibitor (Shen et al., 2015), suggesting that DF is a substrate of human OAT2. The  $K_m$  of OAT2-mediated DF uptake was 46.8  $\mu\text{M}$  (Fig. 3). On the other hand, the uptake of DF-AG in the presence of HEK-OAT1, -OAT2, -OAT3, and -OAT4 cells was about 2- to 8-fold greater versus mock cells, and was significantly inhibited by probenecid or indomethacin. The results revealed that DF-AG is a human OAT1, OAT2, OAT3, and OAT4 substrate. The kinetics of OAT-mediated transport of DF-AG was further assessed. As summarized in Table 2, the  $K_m$  of DF-AG was 60.2, 8.6, 114.1, and 103.9  $\mu\text{M}$  for OAT1, OAT2, OAT3, and OAT4, respectively (Fig. 3; Table 2).

**Transport of DF and DF-AG by Efflux Transporters.** ATP-dependent transport of DF and DF-AG in membrane vesicles expressing human MRP2, MRP3, and BCRP protein was determined. Uptake of DF in MRP2, MRP3, or BCRP membrane vesicles was not significantly increased in the presence of ATP, and the uptake was not inhibited by benzbromarone (MRP2 inhibitor), MK571 (MRP3 inhibitor), or FTC (BCRP inhibitor) (Fig. 4). The results suggested that DF is not a substrate for MRP2, MRP3, or BCRP. In contrast, uptake of DF-AG was significantly increased when vesicles overexpressing MRP2, MRP3, and BCRP were fortified with ATP. The ATP-dependent uptake was significantly inhibited by benzbromarone, MK571, and FTC, suggesting that DF-AG is transported by human MRP2, MRP3, and BCRP (Fig. 4; Table 2). DF-AG conformed to Michaelis-Menten kinetics with MRP3 and BCRP ( $K_m = 196.3$  and 152  $\mu\text{M}$ , respectively). However, ATP-dependent uptake into

MRP2-expressing vesicles was evidently sigmoidal and best described by the Hill equation ( $S_{50} = 145 \mu\text{M}$ ; Hill coefficient = 3.2).

## Discussion

In the present investigation, high exposure of DF-AG was confirmed in acidified plasma samples from human subjects after oral administration of 50 mg of DF (Fig. 1). For the first time, DF-AG/DF ratios in human plasma are reported. In addition, both DF and DF-AG were identified as relatively low- $K_m$  (<50  $\mu\text{M}$ ) OAT2 substrates (Table 2). This is important, because the transporter is expressed on the basolateral membranes of both hepatocytes and renal tubular epithelial cells (Kobayashi et al., 2005; Shen et al., 2015). In contrast to DF, DF-AG was also determined to be a substrate of a number of additional transporters located in the liver (basolateral OATP1B1 and OATP2B1; canalicular MRP2 and BCRP) and kidney (basolateral OAT1 and OAT3; apical MRP2, BCRP, and OAT4). Although uptake of DF tended to be higher with HEK cells singly expressing OATP1B3, OATP1B1, or OATP2B1 (statistically significant) than with MOCK cells, the uptake was not inhibited by rifampin, a known OATP inhibitor. It is concluded that DF is not a substrate for OATP1B1, 1B3, or 2B1, which is not consistent with the conclusion of Kindla et al. (2011) that DF is a substrate of OATP1B3. Studies by Kindla et al. (2011) demonstrate that DF uptake in HEK cells overexpressing OATP1B3 is marginally increased (~20% increase compared with control cells). However, the uptake changes appear to lack concentration dependency, and inhibitory effect with known OATP inhibitors was not provided.

DF undergoes acyl glucuronidation (forming DF-AG) and aryl hydroxylation. DF-AG is the major metabolite found in the plasma in preclinical species. It is known from in vivo rodent studies that DF-AG that is formed in the liver can be transported into bile by hepatic canalicular efflux pumps (Seitz and Boelsterli, 1998) or back-fluxed into the bloodstream by at least one sinusoidal efflux transporter, Mrp3 (Scialis et al., 2015). In addition to the aforementioned transporters, BCRP, OATs, and OATPs are found to be involved in the disposition of DF-AG. As illustrated in Fig. 5, following an oral dose, highly permeable DF passively diffuses or is actively taken up into hepatocytes via OAT2 transporter, where DF-AG is formed. DF-AG in the liver is either secreted into the bile via canalicular efflux transporters, MRP2 and BCRP, or effluxed back to the bloodstream via sinusoidal efflux transporter MRP3 (Scialis et al., 2015). DF-AG in the bile enters the intestine, undergoes hydrolysis back to DF, and can be reabsorbed into the systemic circulation from the small intestine. This is consistent with the previous report that biliary secretion of DF-AG is found to be associated with increased ulceration in the rat jejunum and ileum (Seitz and Boelsterli, 1998). In a pH 7.4 aqueous buffer, DF-AG is quickly degraded (first-order degradation half-life of 0.5 hour), resulting in the formation of parent DF and three isomeric acyl glucuronides (Ebner et al., 1999). In addition, DF-AG in the systemic circulation can be actively taken up into hepatocytes via OAT2, OATP1B1, and OATP2B1, where DF-AG can be further hydroxylated forming hydroxylated DF-AG that is subsequently secreted in the urine (Stierlin et al., 1979), resulting in greater than 60% of administered DF recovered as hydroxy conjugates in the urine and 30% in the feces in humans (Riess et al., 1978; John, 1979; Stierlin et al., 1979; Davies and Anderson, 1997) (Fig. 5). Moreover, DF-AG in the plasma can be taken up into renal proximal tubule cells via OAT1, OAT2, and OAT3, and subsequently excreted in the urine by efflux transporters MRP2 and BCRP. Interruption of the transporter functions could lead to a DDI, resulting in the increase of plasma exposure of DF-AG. It is worth noting that only less than 30% of administered DF is recovered in the

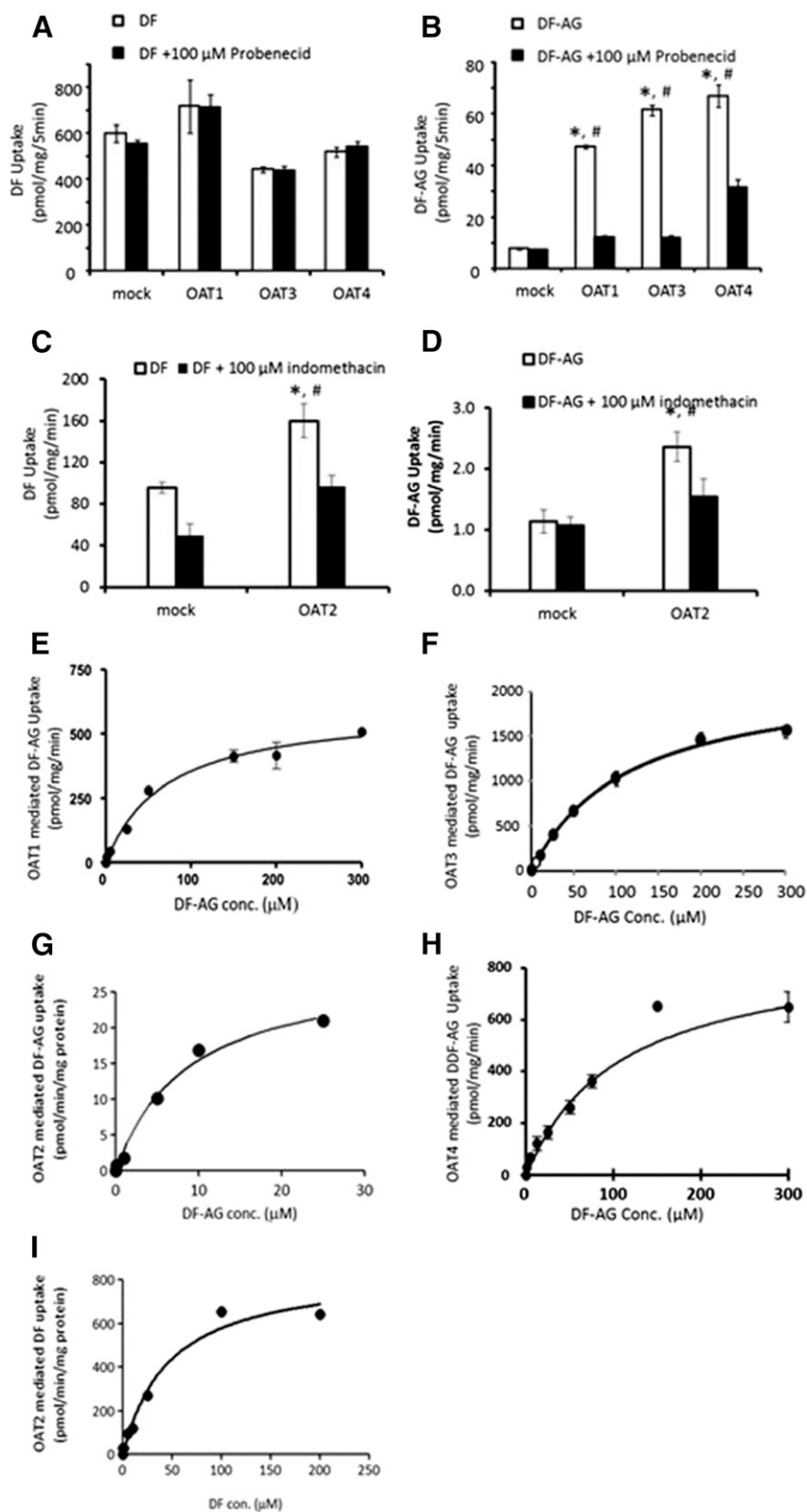
TABLE 2

Summary of kinetic data for DF and DF-AG for various human transporters in vitro

Transporter	System	$K_m \pm \text{S.E. of the Parameter Estimate}$	
		DF	DF-AG
		$\mu\text{M}$	$\mu\text{M}$
OAT2	HEK-293 Cells	46.8 $\pm$ 12.2	8.6 $\pm$ 1.9
OATP1B1	HEK-293 Cells	— <sup>a</sup>	34.0 $\pm$ 8.6
OAT1	HEK-293 Cells	— <sup>a</sup>	60.2 $\pm$ 13.5
OAT4	HEK-293 Cells	— <sup>a</sup>	103.9 $\pm$ 29.0
OATP2B1	HEK-293 Cells	— <sup>a</sup>	104.6 $\pm$ 16.7
OAT3	HEK-293 Cells	— <sup>a</sup>	114.1 $\pm$ 8.1
MRP2	Vesicles	— <sup>a</sup>	145.0 $\pm$ 7.8 <sup>b</sup>
BCRP	Vesicles	— <sup>a</sup>	152.0 $\pm$ 54.9
MRP3	Vesicles	— <sup>a</sup>	196.3 $\pm$ 58.0
OATP1B3	HEK-293 Cells	— <sup>a</sup>	—

<sup>a</sup>No detectable transport.

<sup>b</sup>Reported as  $S_{50}$ ; Hill Coefficient = 3.19  $\pm$  0.34.

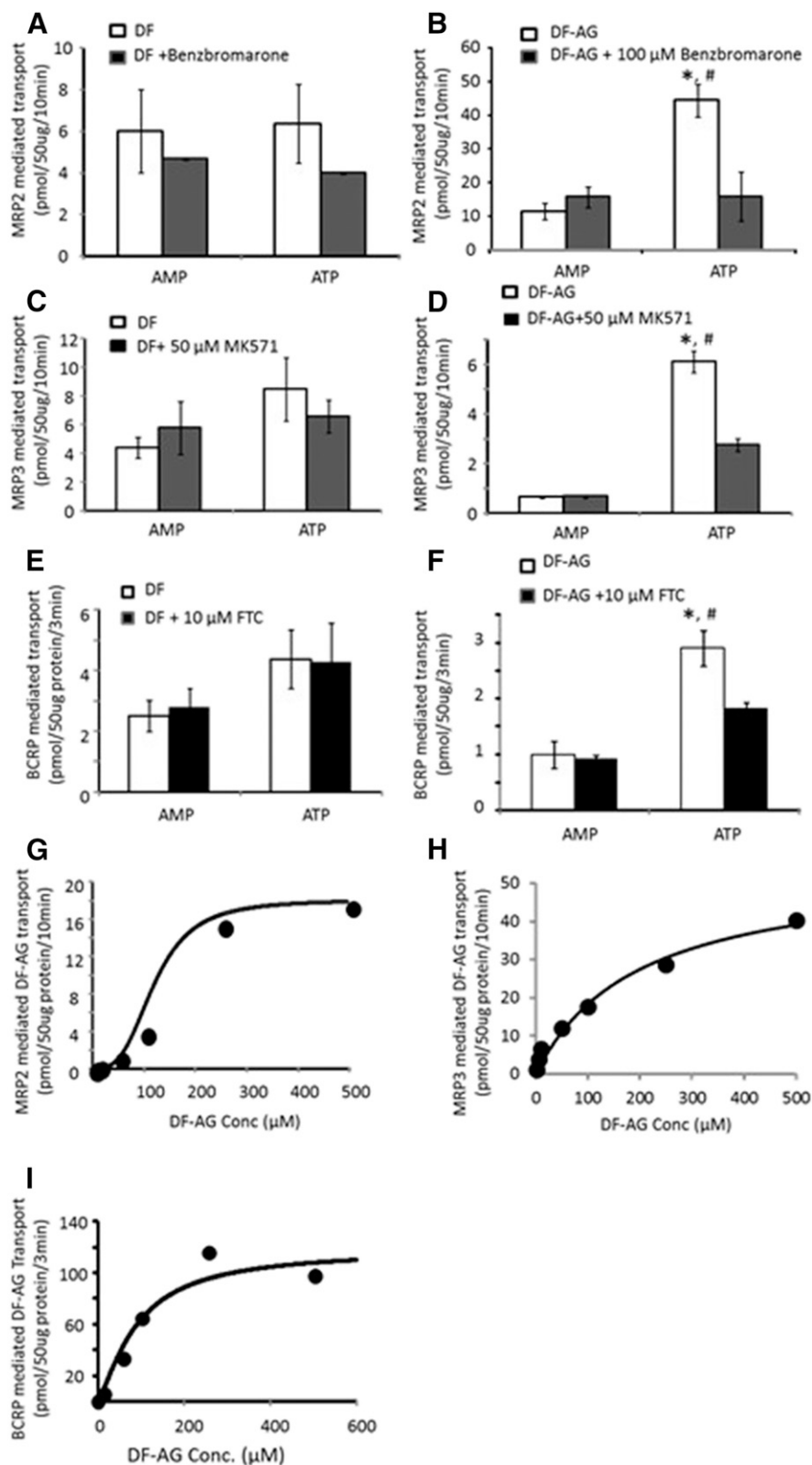


**Fig. 3.** OAT-mediated uptake of DF and DF-AG. Uptake of DF and DF-AG was assessed in HEK-293 mock cells and the cells singly transfected with OAT1, OAT2, OAT3, and OAT4, in the presence or absence of OAT inhibitors probenecid (OAT1, OAT3, and OAT4 inhibitor) and indomethacin (OAT2 inhibitor). Concentration-dependent uptake of DF and DF-AG was further assessed. (A) DF uptake in OAT1-, OAT3-, and OAT4-overexpressed HEK cells. (B) DF-AG uptake in OAT1-, OAT3-, and OAT4-overexpressed HEK cells. (C) OAT2-mediated DF uptake. (D) OAT2-mediated DF-AG uptake. (E) Concentration-dependent DF-AG uptake in OAT1 cells. (F) Concentration-dependent DF-AG uptake in OAT3 cells. (G) Concentration-dependent DF-AG uptake in OAT2 cells. (H) Concentration-dependent DF-AG uptake in OAT4 cells. (I) Concentration-dependent DF uptake in OAT2 cells. \* $P < 0.05$  compared with mock cells; # $P < 0.05$  compared with the uptake in the presence of inhibitor. Data are presented as the mean  $\pm$  S.D. ( $n = 3$ ).

urine from rats, suggesting species difference in the renal elimination for DF and its conjugates. OAT2 is expressed on both basolateral and apical membranes of human and monkey renal proximal tubules, but appears only on the apical membrane of rat proximal tubule cells (Shen et al., 2015). The basolateral expression of OAT2 in human and

monkey proximal tubules may result in an increased active uptake of DF-AG into the cells to increase the renal secretion.

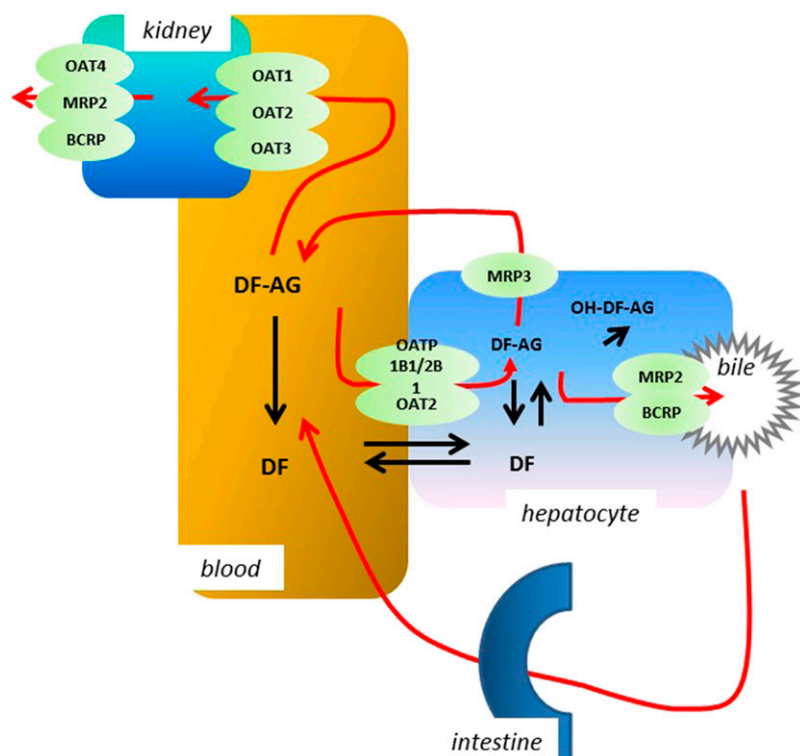
Cyclosporine A (CsA) is a potent inhibitor of a number of uptake and efflux transporters, and represents the largest proportion of reported clinical transporter-mediated DDIs. It is known to inhibit OATP1B1



**Fig. 4.** ATP-dependent transport of DF and DF-AG in membrane vesicles expressing human MRP2, MRP3, and BCRP protein. Uptake of DF and DF-AG was conducted in the vesicles in the presence or absence of inhibitors, and with or without ATP. Benzbromarone (MRP2 inhibitor), MK571 (MRP3 inhibitor), or FTC (BCRP inhibitor) was used to block transporter activities. (A) MRP2-mediated DF transport. (B) MRP2-mediated DF-AG transport. (C) MRP3-mediated DF transport. (D) MRP3-mediated DF-AG transport. (E) BCRP-mediated DF transport. (F) BCRP-mediated DF-AG transport. (G) Concentration-dependent DF-AG uptake in MRP2 vesicles. (H) Concentration-dependent DF-AG uptake in MRP3 vesicles. (I) Concentration-dependent DF-AG uptake in BCRP vesicles. \* $P < 0.05$  compared with the uptake with AMP; # $P < 0.05$  compared with the uptake in the presence of inhibitor. Data are presented as the mean  $\pm$  S.D. ( $n = 3$ ).

(Amundsen et al., 2010; Chang et al., 2013), OATP2B1 (Ho et al., 2006), BCRP (Xia et al., 2007), MRP1 (Juvale and Wiese, 2012), MRP2 (Lechner et al., 2010), and MRP3 (Kock et al., 2014) (Supplemental Table 2). CsA is the most potent inhibitor for OATPs, with  $K_i < 0.1 \mu\text{M}$  (Ho et al., 2006; Amundsen et al., 2010). It is generally accepted that inhibition of the uptake of statins into

hepatocytes causes significant DDIs (Asberg et al., 2001). It is reported that a single oral dose of CsA administered with multiple oral doses of 50 mg of DF demonstrated a significant increase in DF  $C_{\text{max}}$  and AUC with extensive variability (Mueller et al., 1993). A follow-up investigation demonstrated that the combination therapy of CsA and DF in patients with refractory rheumatoid arthritis ( $n = 20$ ) demonstrated a



**Fig. 5.** Scheme of transporter-mediated DF and DF-AG disposition. Following an oral administration, highly permeable DF passively diffuses or is actively taken up into hepatocytes via OAT2 transporter, where DF-AG is formed. DF-AG in the liver is either secreted into the bile via canalicular efflux transporters MRP2 and BCRP or effluxed back to the bloodstream via sinusoidal efflux transporter MRP3. DF-AG in the bile enters the intestine, undergoes degradation, and can be reabsorbed to the systemic circulation from the small intestine. DF-AG in the systemic circulation can be actively taken up into hepatocytes via OAT2, OATP1B1, and OATP2B1, where DF-AG can further be hydroxylated, forming hydroxylated DF-AG that is subsequently secreted in the urine.

104% increase in AUC with no change in half-life ( $T_{1/2}$ ) (Supplemental Table 3). Although the authors hypothesized that the increase in AUC was due to a reduction in first-pass metabolism (Kovarik et al., 1996), the assumption was inconsistent with human results showing that the absolute bioavailability of DF was  $90 \pm 11\%$  following oral administration of a single dose of 50 mg of  $^{14}\text{C}$ -DF (Kendall et al., 1979; Willis et al., 1979). Therefore, the interaction between CsA and DF is likely attributed to another mechanism(s) rather than a reduction of first-pass metabolism. CsA is a potent inhibitor of OATP transporters and causes clinically significant DDIs with statin drugs through the mechanism of OATP inhibition. Since DF-AG is a substrate of OATP1B1 and 2B1, CsA could decrease DF-AG uptake into hepatocytes, and therefore result in the increase of DF-AG exposure in the plasma. DF-AG is chemically unstable in the plasma and can be readily hydrolyzed to parent DF either in systemic circulation or ex vivo upon collection of plasma samples (if not acidified). Further investigation is warranted to elucidate the observed increase of DF exposure when CsA is codosed. Although both DF and DF-AG serve as OAT2 substrates, inhibition by CsA is weaker versus OATPs ( $\text{IC}_{50} > 11 \mu\text{M}$ ) (Shen et al., 2015). Likewise, inhibition of DF-AG renal clearance mediated by OAT1 and OAT3 is unlikely, because CsA is not an inhibitor of either transporter (Uwai et al., 2007). Therefore, the DDI with CsA through the inhibition of renal transporters is unlikely. However, further investigation of possible clinically relevant interactions with OAT potent inhibitors is warranted.

In conclusion, several renal and hepatic transporters may play a role in the disposition of DF-AG, whereas only OAT2 has the potential to govern DF disposition. In the kidney, OAT1, OAT2, and OAT3 can transport DF-AG into proximal tubule cells, and it is subsequently excreted in the urine through luminal efflux transporters, MRP2, BCRP, and OAT4. In the liver, OATP1B1, OATP2B1, and OAT2 are possibly involved in the hepatic uptake of DF-AG. MRP2 and BCRP actively transport DF-AG into the bile, whereas MRP3 mediates DF-AG flux back into the systemic circulation (Fig. 5). Since DF-AG is

chemically unstable, and prone to back-conversion to DF, transporter-mediated DDIs involving DF-AG could complicate the plasma exposure of parent DF. It is worth noting that acidification of plasma samples is a critical requirement to protect DF-AG from degradation and accurately determine the systemic exposure of DF-AG (Zhang et al., 2011). The findings in the current investigation have revealed the possibility for greater numbers of transporters involved in DF-AG disposition, and raise concerns regarding possible DDI with DF-AG.

#### Acknowledgments

The authors thank Dr. Hong Shen for help with the assays involving OAT2-mediated uptake of DF and DF-AG. The authors also value Drs. Donglu Zhang and William G. Humphreys for their scientific discussion. The authors also thank Jimmy Kotecha (Biocon Bristol-Myers Squibb Research and Development Center) and Jaya Patil, Priya Patil, Apurba Das, Siddangouda Patil, and Nageswara Rao (Anil of Syngene Clinical Development Team) for their help in conducting the clinical study. William Mylott and Song Zhao (PPD Laboratories, Richmond, VA) are acknowledged for providing LC-MS/MS bioanalytical method information and blood collection/stabilization conditions.

#### Authorship Contributions

*Participated in research design:* Zhang, Rodrigues, Han, Mandekar, Furlong, Iyer, Yang, Lai, Putluru, Kole, Matta.

*Conducted experiments:* Zhang, Han, Putluru, Matta, Kole, Liu.

*Contributed new reagents or analytic tools:* Zhang, Han, Rodrigues, Putluru, Matta, Kole, Mandekar, Liu.

*Performed data analysis:* Zhang, Han, Lai, Rodrigues, Putluru, Mandekar, Liu.

*Wrote or contributed to the writing of the manuscript:* Zhang, Rodrigues, Han, Putluru, Kole, Mandekar, Furlong, Iyer, Lai, Marathe, Yang.

#### References

Amundsen R, Christensen H, Zabihyan B, and Asberg A (2010) Cyclosporine A, but not tacrolimus, shows relevant inhibition of organic anion-transporting protein 1B1-mediated transport of atorvastatin. *Drug Metab Dispos* **38**:1499–1504.



- Asberg A, Hartmann A, Fjelds  E, and Holdaas H (2001) Atorvastatin improves endothelial function in renal-transplant recipients. *Nephrol Dial Transplant* **16**:1920–1924.
- Chang JH, Plise E, Cheong J, Ho Q, and Lin M (2013) Evaluating the in vitro inhibition of UGT1A1, OATP1B1, OATP1B3, MRP2, and BSEP in predicting drug-induced hyperbilirubinemia. *Mol Pharm* **10**:3067–3075.
- Davies NM and Anderson KE (1997) Clinical pharmacokinetics of diclofenac. Therapeutic insights and pitfalls. *Clin Pharmacokinet* **33**:184–213.
- Ebner T, Heinzel G, Prox A, Beschke K, and Wachsmuth H (1999) Disposition and chemical stability of telmisartan 1-O-acetylglucuronide. *Drug Metab Dispos* **27**:1143–1149.
- Hammond TG, Meng X, Jenkins RE, Maggs JL, Castelazo AS, Regan SL, Bennett SN, Earnshaw CJ, Aithal GP, and Pande I, et al. (2014) Mass spectrometric characterization of circulating covalent protein adducts derived from a drug acyl glucuronide metabolite: multiple albumin adducts in diclofenac patients. *J Pharmacol Exp Ther* **350**:387–402.
- Han YH, Busler D, Hong Y, Tian Y, Chen C, and Rodrigues AD (2010) Transporter studies with the 3-O-sulfate conjugate of 17alpha-ethinylestradiol: assessment of human kidney drug transporters. *Drug Metab Dispos* **38**:1064–1071.
- Ho RH, Tirona RG, Leake BF, Glaeser H, Lee W, Lemke CJ, Wang Y, and Kim RB (2006) Drug and bile acid transporters in rosuvastatin hepatic uptake: function, expression, and pharmacogenetics. *Gastroenterology* **130**:1793–1806.
- John VA (1979) The pharmacokinetics and metabolism of diclofenac sodium (Voltarol) in animals and man. *Rheumatol Rehabil* (Suppl 2):22–37.
- Juvale K and Wiese M (2012) 4-Substituted-2-phenylquinazolines as inhibitors of BCRP. *Bioorg Med Chem Lett* **22**:6766–6769.
- Kendall MJ, Thornhill DP, and Willis JV (1979) Factors affecting the pharmacokinetics of diclofenac sodium (Voltarol). *Rheumatol Rehabil* (Suppl 2):38–46.
- Kindla J, M ller F, Mieth M, Fromm MF, and K nig J (2011) Influence of non-steroidal anti-inflammatory drugs on organic anion transporting polypeptide (OATP) 1B1- and OATP1B3-mediated drug transport. *Drug Metab Dispos* **39**:1047–1053.
- Kobayashi Y, Ohshiro N, Sakai R, Ohbayashi M, Kohyama N, and Yamamoto T (2005) Transport mechanism and substrate specificity of human organic anion transporter 2 (hOat2 [SLC22A7]). *J Pharm Pharmacol* **57**:573–578.
- K ck K, Ferslew BC, Netterberg I, Yang K, Urban TJ, Swaan PW, Stewart PW, and Brouwer KL (2014) Risk factors for development of cholestatic drug-induced liver injury: inhibition of hepatic basolateral bile acid transporters multidrug resistance-associated proteins 3 and 4. *Drug Metab Dispos* **42**:665–674.
- Kovarik JM, Kurki P, Mueller E, Guerret M, Markert E, Alten R, Zeidler H, and Genth-Stolzenburg S (1996) Diclofenac combined with cyclosporine in treatment refractory rheumatoid arthritis: longitudinal safety assessment and evidence of a pharmacokinetic/dynamic interaction. *J Rheumatol* **23**:2033–2038.
- Kumar S, Samuel K, Subramanian R, Braun MP, Stearns RA, Chiu SH, Evans DC, and Baillie TA (2002) Extrapolation of diclofenac clearance from in vitro microsomal metabolism data: role of acyl glucuronidation and sequential oxidative metabolism of the acyl glucuronide. *J Pharmacol Exp Ther* **303**:969–978.
- Lagas JS, Sparidans RW, Wagenaar E, Beijnen JH, and Schinkel AH (2010) Hepatic clearance of reactive glucuronide metabolites of diclofenac in the mouse is dependent on multiple ATP-binding cassette efflux transporters. *Mol Pharmacol* **77**:687–694.
- Lagas JS, van der Kruijssen CM, van de Wetering K, Beijnen JH, and Schinkel AH (2009) Transport of diclofenac by breast cancer resistance protein (ABCG2) and stimulation of multidrug resistance protein 2 (ABCC2)-mediated drug transport by diclofenac and benzobromarone. *Drug Metab Dispos* **37**:129–136.
- Lechner C, Reichel V, Moenning U, Reichel A, and Fricker G (2010) Development of a fluorescence-based assay for drug interactions with human Multidrug Resistance Related Protein (MRP2; ABCC2) in MDCKII-MRP2 membrane vesicles. *Eur J Pharm Biopharm* **75**:284–290.
- Mueller EA, Kovarik JM, Koelle EU, Merdjan H, Johnston A, and Hitzzenberger G (1993) Pharmacokinetics of cyclosporine and multiple-dose diclofenac during coadministration. *J Clin Pharmacol* **33**:936–943.
- Peris-Ribera JE, Torres-Molina F, Garcia-Carbonell MC, Aristorena JC, and Pla-Delfina JM (1991) Pharmacokinetics and bioavailability of diclofenac in the rat. *J Pharmacokinetic Biopharm* **19**:647–665.
- Riess W, Stierlin H, Degen P, Faigle JW, G rardin A, Moppert J, Sallmann A, Schmid K, Schweizer A, and Sulc M, et al. (1978) Pharmacokinetics and metabolism of the anti-inflammatory agent Voltaren. *Scand J Rheumatol Suppl* **22**:17–29.
- Rodrigues AD (2005) Impact of CYP2C9 genotype on pharmacokinetics: are all cyclooxygenase inhibitors the same? *Drug Metab Dispos* **33**:1567–1575.
- Scialis RJ, Csanaky IL, Goedken MJ, and Manautou JE (2015) Multidrug Resistance-Associated Protein 3 Plays an Important Role in Protection against Acute Toxicity of Diclofenac. *Drug Metab Dispos* **43**:944–950.
- Seitz S and Boelsterli UA (1998) Diclofenac acyl glucuronide, a major biliary metabolite, is directly involved in small intestinal injury in rats. *Gastroenterology* **115**:1476–1482.
- Seitz S, Kretz-Rommel A, Oude Elferink RP, and Boelsterli UA (1998) Selective protein adduct formation of diclofenac glucuronide is critically dependent on the rat canalicular conjugate export pump (Mrp2). *Chem Res Toxicol* **11**:513–519.
- Shen H, Liu T, Morse BL, Zhao Y, Zhang Y, Qiu X, Chen C, Lewin AC, Wang XT, and Liu G, et al. (2015) Characterization of Organic Anion Transporter 2 (SLC22A7): A Highly Efficient Transporter for Creatinine and Species-Dependent Renal Tubular Expression. *Drug Metab Dispos* **43**:984–993.
- Stierlin H and Faigle JW (1979) Biotransformation of diclofenac sodium (Voltaren) in animals and in man. II. Quantitative determination of the unchanged drug and principal phenolic metabolites, in urine and bile. *Xenobiotica* **9**:611–621.
- Stierlin H, Faigle JW, Sallmann A, K ng W, Richter WJ, Kriemler HP, Alt KO, and Winkler T (1979) Biotransformation of diclofenac sodium (Voltaren) in animals and in man. I. Isolation and identification of principal metabolites. *Xenobiotica* **9**:601–610.
- Uwai Y, Motohashi H, Tsuji Y, Ueo H, Katsura T, and Inui K (2007) Interaction and transport characteristics of mycophenolic acid and its glucuronide via human organic anion transporters hOAT1 and hOAT3. *Biochem Pharmacol* **74**:161–168.
- Willis JV, Kendall MJ, Flinn RM, Thornhill DP, and Welling PG (1979) The pharmacokinetics of diclofenac sodium following intravenous and oral administration. *Eur J Clin Pharmacol* **16**:405–410.
- Xia CQ, Liu N, Miwa GT, and Gan LS (2007) Interactions of cyclosporin A with breast cancer resistance protein. *Drug Metab Dispos* **35**:576–582.
- Zhang D, Raghavan N, Wang L, Xue Y, Obermeier M, Chen S, Tao S, Zhang H, Cheng PT, and Li W, et al. (2011) Plasma stability-dependent circulation of acyl glucuronide metabolites in humans: how circulating metabolite profiles of muraglitazar and peliglitazar can lead to misleading risk assessment. *Drug Metab Dispos* **39**:123–131.

---

**Address correspondence to:** Dr. Yurong Lai, Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb Company, Route 206 and Province Line Road, Princeton, NJ 08543-4000. E-mail: yurong.lai@bms.com

---