Diclofenac and its Acyl Glucuronide: Determination of In Vivo Exposure in Human Subjects and Characterization as Human Drug Transporter Substrates In Vitro

Yueping Zhang, Yong-Hae Han, Siva Prasad Putluru, Murali Krishna Matta, Prashant Kole, Sandhya Mandlekar, Michael T. Furlong, Tongtong Liu, Ramaswamy A. Iyer, Punit Marathe, Zheng Yang, Yurong Lai and A. David Rodrigues

Pharmaceutical Candidate Optimization, Bristol-Myers Squibb Company, 3551 Lawrenceville Road, Princeton, NJ 08540
Running title: DF and DF-AG transporters in vitro and their in vivo disposition in human

Address correspondence to: Yurong Lai, PhD. Department of Metabolism and Pharmacokinetics, Bristol-Myers Squibb Company, Route 206 & Province Line Road, Princeton, NJ 08543-4000.

Telephone: 609-252-6365
Fax: 609-252-7354
E-mail: yurong.lai@bms.com

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Abbreviations: OAT, organic anion transporter; OATP, organic anion transporting polypeptide; MRP, multidrug resistance protein; BCRP, breast cancer resistance protein; DDI, drug-drug interaction; DF, diclofenac; DF-AG, diclofenac acyl-β-D-glucuronide; MK571, [3-[3-[2-(7-chloroquinolin-2-yl)vinyl]phenyl]-(2-dimethylcarbamoylethylsulfanyl)methylsulfanyl] propionic acid; FTC, fumitremorgin C
Abstract:

Although the metabolism and disposition of diclofenac (DF) has been studied extensively, information regarding the plasma levels of its acyl-β-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 ± 0.21 (Cmax mean ± SD) and 0.84 ± 0.21 (area under the concentration-time curve mean ± SD). 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 2 only (OAT2, Km = 46.8 µM). In contrast, DF-AG was identified as a substrate of numerous OATs (Km = 8.6, 60.2, 103.9, 112, µM; OAT2, OAT1, OAT4, and OAT3, respectively), two organic anion-transporting polypeptides (OATP1B1, Km = 34 µM; OATP2B1, Km = 105 µM), breast cancer resistance protein (Km = 152 µM), and two multidrug resistance proteins (MRP2, Km = 145 µM; MRP3, Km = 196 µM). 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, 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 widely used nonsteroidal anti-inflammatory drug 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 to 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-β-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 cytochrome P450 2C9 (CYP2C9) is dominant, 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 multidrug resistance protein 2 (Mrp2) deficient (TR-) rats that lack the functional canalicular ABCC transporter 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 IV and PO 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) (Lagas et al., 2009). The sinusoidal efflux of DF-AG into blood, once formed in the liver, is largely dependent on multidrug resistance protein 3 (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 to rodents, however, it is worth noting that less of the human dose (≤30% versus >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 IV) is recovered in the urine (~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 be considered also.

As it is increasingly recognized that many 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). So 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,
97.5mCi/mmol), \[^3\text{H}\]esteone-3-sulfate salt (45.6Ci/mmol) were purchased from Perking Elmer Life and
Analytical Science (Waltham, MA). \[^3\text{H}\]Penciclovir (PCV; 1.1 Ci/mmol) were purchased from Moravek
Biochemicals (Brea, CA). Benz bromarone, estradiol-17\beta-D-glucuronide, CCK-8, esteone-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 in Bristol-Myers Squibb. Cell culture reagents including Dulbecco’s
modified Eagle’s medium, fetal calf serum, trypsin, Hank’s 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). MK571 was obtained from Cayman Chemical Company
(Ann Arbor, MI). All other chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO).

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 Voltaflam® (diclofenac potassium) employing six normal healthy subjects;
males aged from 22 to 34. Blood was collected predose, 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 µL 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 (BBRC, Bangalore, India). The study was conducted in accordance with the principles of the Declaration of Helsinki and Good Clinical Practice.

**LC-MS/MS analytics of DF and DF-AG**

An aliquot of 25 µL of plasma was precipitated with acetonitrile (125 µL) containing deuterated DF (D4-diclofenac) as the internal standard (IS). The samples were then centrifuged at 3000g for 5 minutes through a 96-well filter plate and the filtrates were subjected to UPLC-MS/MS analysis.

Sample analysis was conducted on an API-4000 quadrupole ion trap (Applied Biosystems/MDS SCIEX, Toronto, Canada) coupled with an Acquity UPLC system (Waters Corp., Milford, MA). A BEH column (C18, 50 × 2.1 mm, 1.7 µm 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 1.2-1.8 minutes, 90% B to 10% B from 1.8 to 2.0 minutes, return to 10% B from 2.0-2.2 minutes. Analytes were detected by multiple reaction monitoring (MRM) using electrospray ionization mass spectrometry (ESI-MS).

Electrospray ionization (ESI) source was operated in negative ion mode with multiple reactions monitoring analysis. The ESI source temperature was set at 550°C and ion spray voltage, cell entrance (EP) and exit potentials (CXP) were set at -4500, -10 and -15 Volts respectively. Mass transitions were
293.9→249.9, 297.4→253.6 and 470.1→193.1 for DF, d4-DF and DF-AG, respectively. The lower limit of quantitation (LLOQ) was 34 nM 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\textsubscript{max}, T\textsubscript{max}, the area under the plasma concentration versus time curve up to the last quantifiable time point (AUC\textsubscript{last}) and total AUC (AUC\textsubscript{tot}). AUC was estimated by the mixed logarithmic-linear method in the software. Data are also reported as the DF/DF-AG AUC\textsubscript{tot}, AUC\textsubscript{last} and C\textsubscript{max} ratio.

Stable expression of OATP1B1, OATP1B3, OATP2B1, organic anion transporter 1 (OAT1), OAT2, OAT3, and OAT4 in HEK-293 Cells

HEK-293 cells are routinely cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and hygromycin (100 µg/mL) in a humidified incubator at 37°C and 5% CO\textsubscript{2}. The stably transfected HEK-293 cell lines expressing drug transporter proteins were established by using the Flp-In\textsuperscript{TM} expression system (Invitrogen) as described previously (Han et al., 2010). In brief, the recombinant pcDNA5/FRT construct containing the open frames of OATP1B1, OATP1B3, OATP2B1, OAT1, OAT2, OAT3 and OAT4 were co-transfected with pOG44, an Flp recombinase expression plasmid, into Flp-InTM HEK-293 cells. Cells stably expressing the transporters were selected in hygromycin (100 µg/ml) according to the manufacturer’s protocol. All cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO\textsubscript{2} and sub-cultured 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 to 7.4) using LC/MS/MS methods. DF-AG was not stable in buffer at pH7.4
(data not shown). At 37 °C, it remained stable (>87%) up to 2 hrs over a pH range of 6.0 to 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 (Supplement 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 x 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 CO2 (5%) and air (95%) at 37°C. After two days, cells were washed twice with 2 mL pre-warmed HBSS buffer. Cellular uptake was initiated at 37°C by adding 1 µM [³H]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 lower pH (5.4) to avoid the degradation of DF-AG. The cells were washed three times with ice-cold HBSS buffer. For [³H]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 µL water and 200 µL of methanol with internal standard, followed by shaking for 30 to 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 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 HEK293 cells in each well measured using the BCA protein assay kit. Control substrates for different transporter phenotyping assays were as follows: \[ ^{3} \text{H} \] estradiol-17-β-glucuronide (1 μM, OATP1B1), \[ ^{3} \text{H} \] estrone 3-Sulfate (1 μM, OATP2B1, OAT3 and OAT4), \[ ^{3} \text{H} \] CCK-8 (1 μM, OATP1B3), PAH (1 μM, OAT1), \[ ^{3} \text{H} \] PCV (0.14 μM, OAT2). The uptake of probe substrates in transporter expressing cells was also conducted under the same condition of DF-AG and DF uptake (pH 5.4) and known inhibitors of OATPs (BSP, 5 μM; rifampin 10 μM) and OATs (indomethacin, 100 μM; probenecid 100 μM), 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 μL of reaction medium (50 mM MES-Tris, 70 mM KCl, 7.5 mM MgCl2, pH 6.0) with 50 μg of protein and DF-AG (50 μM) or DF (50 μM) was pre-warmed at 37°C for 3 minutes, assay started by adding 20 μL of 4mM ATP or AMP. The incubation was conducted at lower pH (6.0) to avoid the degradation of DF-AG. The uptake of known substrates \[ ^{3} \text{H} \] estradiol-17-β-glucuronide (50 μM, MRP2 and MRP3) and \[ ^{3} \text{H} \] estrone 3-sulfate (1 μM, 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 μM), MRP3 (MK571, 200 μM), BCRP (FTC, 10 μM) 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 addition of 200 μL cold wash buffer (40mM...
MES-Tris, 70mM KCl, pH 6.0). The incubation mixture was immediately transferred to PerkinElmer Unifilter GF/B plate, followed by 5 times wash with 250 µL 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 µL water and 200 µL of acetonitrile with internal standard, followed by shaking for 30 to 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 equation 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 (equation 2)

\[
v = \frac{V_{\text{max}} \cdot S}{K_m + S}
\]

Equation 1

\[
v = \frac{V_{\text{max}} \cdot S^n}{K_m + S^n}
\]

Equation 2

where \(v\) is the rate of uptake measured at the given concentration; \(V_{\text{max}}\) is 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 of cellular uptake and inhibition
effects was made using a 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) in order to ensure stabilization of DF-AG. As depicted in Figure 1, DF and DF-AG were both detectable in plasma up to 8 and 12 hr after dosing, respectively, although sampling was carried out for 24 hr. The mean C\text{max} and AUC\text{tot} values for DF were 3075.9 nM and 4125.7 nM.hr, respectively (Table 1). The level of DF-AG in plasma was comparable to that of DF (C\text{max}, 1849.4 nM; AUC\text{tot}, 3592.6 nM*hr) and rendered mean C\text{max} and AUC\text{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. While 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 (Figure 2). Although a slight but significant increase of DF uptake was observed in HEK-OATP2B1 cells as compared to the mock cells (Figure 2), the uptake was not inhibited by rifampin. The results suggested that DF is not a substrate for hepatic OATP 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 Km 34 µM and 104.6 µM, respectively (Figure 2 and 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 (Figure 3). A significant increase (versus mock cells) in uptake was not detected when DF was added to HEK-OAT1, OAT3 and OAT4 cells. Furthermore, uptake was not inhibited by 100 µM 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 Km of OAT2 mediated DF uptake was 46.8 µM (Figure 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 Km of DF-AG was 60.2 µM, 8.6 µM, 114.1 µM and 103.9 µM for OAT1, OAT2, OAT3 and OAT4, respectively (Figure 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) (Figure 4). The results suggested that DF is not a substrate for MRP2, MRP3 and BCRP. In contrast, uptake of DF-AG was significantly increased when vesicles over-expressing 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 (Figure 4; Table 2). DF-AG conformed to Michaelis-Menten kinetics with MRP3 and BCRP (K_m 196.3 and 152 µM, respectively). However, ATP-dependent uptake into MRP2 expressing vesicles was evidently sigmoidal and best described by the Hill equation (S_{50} = 145 µM; 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 DF (Figure 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 µM) 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 cell, the uptake was not inhibited by rifampin, a known OATP inhibitor. It is concluded that DF is not a substrate for OATP1B1, 1B3 and 2B1, which is not consistent with the conclusion of Kindla et al that DF is a substrate of OATP1B3 (Kindla et al., 2011). Studies by Kindla et al.
(Kindla et al., 2011) demonstrate that DF uptake in HEK cells overexpressing OATP1B3 is marginally increased (~20% increase compared to control cells). However, the uptake changes appear to lack of 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 blood stream 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 Figure 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 blood stream 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 re-absorbed 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 hr), 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 2B1, where DF-AG can further be 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 human (Riess et al., 1978; John, 1979; Stierlin et al., 1979; Davies and
Anderson, 1997) (Figure 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 DDI resulting in the increase of plasma exposure of DF-AG. It is worth noting that there is only less than 30% of administered DF being recovered in the urine from rats, suggesting species difference of 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 (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) (Supplement Table 2). CsA is the most potent inhibitor for OATPs with Ki<0.1 µM (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 50mg DF demonstrated a significant increase in DF C_{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 104% increase in AUC with no change in T1/2 (Supplement Table 3). Although the authors hypothesized that increase in AUC was due to a reduction in first pass metabolism (Kovarik et al., 1996), the assumption was inconsistent with human ADME results that the absolute bioavailability
of DF was 90±11% following oral administration of a single dose of 50 mg ¹⁴C-DF (Kendall et al., 1979; Willis et al., 1979). Therefore, the interaction between CsA and DF is likely attributed to other mechanism(s) rather than a reduction of first pass metabolism. CsA is a potent inhibitor of OATP transporters and causes clinical 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 co-dosed. Although both DF and DF-AG serve as OAT2 substrates, inhibition by CsA is weaker versus OATPs (IC₅₀ > 11 µM) (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 in understanding the clinical 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, while 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, while MRP3 mediates DF-AG flux back into the systemic circulation (Figure 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.
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Authorship Contributions

Participated in research design: Y Zhang, D Rodrigues, YH Han, S. Mandlekar, M T. Furlong, R Iyer, Z Yang Y. Lai, S Putluru, P Kole, M Matta

Conducted experiments: Y Zhang, YH Han, S Putluru, M Matta, P Kole, T Liu

Contributed new reagents or analytic tools: Y Zhang, YH Han, D Rodrigues, S Putluru, M Matta, P Kole, S Mandlekar, T Liu

Performed data analysis: Y Zhang, YH Han, Y Lai, D Rodrigues, S Putluru, S Mandlekar, T Liu

Wrote or contributed to the writing of the manuscript: Y Zhang, D Rodrigues, YH Han, S Putluru, P Kole, S. Mandlekar, M T. Furlong, R Iyer, Y. Lai, P Marathe, Z Yang
Reference:


Rodrigues AD (2005) Impact of CYP2C9 genotype on pharmacokinetics: are all cyclooxygenase inhibitors the same? Drug Metab Dispos 33:1567-1575.


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.


Footnote:

a) Current address: Enzychem Lifesciences Corp., 15F, Trust Tower BLDG, 60 Mabang ro, Seocho gu, Seoul Korea (Y.H.); Pharmacokinetics, Dynamics & Metabolism, World Wide Research & Development, Pfizer, Inc. Groton Laboratories, CT 06340 (D.R.); FORUM Pharmaceuticals Inc., 225 Second Avenue, Waltham, MA 02451 (M.F.)

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

c) Reprint requests should be sent to:

Dr. Yurong Lai,
Route 206 & Provinceline Rd
Princeton, NJ 08540
Yurong.lai@bms.com
Figure Legend

Figure 1 Concentration-time profile in plasma of DF and DF-AG followed by administration of a single dose of 50 mg DF. All plasma samples were prepared from blood collected under acidified conditions to ensure stabilization of DF-AG. Data were presented as mean ± SD from 6 healthy male human subjects.

Figure 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 OATP1B1, 1B3 and 2B1, in the presence or absence of 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 to mock cells. #, P<0.05, compared to the uptake in the presence of inhibitor.

Data were presented as mean ± SD, n=3.

Figure 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 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 over-expressed HEK cells; B, DF-AG uptake in OAT1, OAT3 and OAT4 over-expressed 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 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 to mock cells; #, P<0.05 compared to the uptake in the presence of inhibitor. Data were presented as mean ± SD, n=3.

Figure 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) were 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 to the uptake with AMP; #, P<0.05 compared to the uptake in the presence of inhibitor. Data were presented as mean ± SD, n=3.

Figure 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 blood stream via sinusoidal efflux transporter MRP3. DF-AG in the bile enters the intestine, undergoes degradation and can be re-absorbed 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 2B1, where DF-AG can further be hydroxylated forming
hydroxylated DF-AG that is subsequently secreted in the urine.
Table 1. Pharmacokinetic parameters of DF and DF-AG following a single oral 50mg dose of DF

<table>
<thead>
<tr>
<th>Parameter</th>
<th>DF</th>
<th>DF-AG</th>
<th>DF/DF-AG ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{\text{max}}$ (nM)</td>
<td>3076±531</td>
<td>1846±528</td>
<td>0.62±0.21</td>
</tr>
<tr>
<td>$T_{\text{max}}$ (hr)</td>
<td>1.25±0.58</td>
<td>1.25±0.58</td>
<td>-</td>
</tr>
<tr>
<td>AUC$_{\text{last}}$ (nM*hr)</td>
<td>4046±635</td>
<td>3466±1168</td>
<td>0.83±0.20</td>
</tr>
<tr>
<td>AUC$_{\text{tot}}$ (nM*hr)</td>
<td>4126±627</td>
<td>3593±1185</td>
<td>0.84±0.21</td>
</tr>
</tbody>
</table>

Data are reported as the mean ± SD of six different subjects.
Table 2. Summary of kinetic data for DF and DF-AG for various human transporters in vitro

<table>
<thead>
<tr>
<th>Transporter</th>
<th>System</th>
<th>Km ± SE of the parameter estimate (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>DF</td>
</tr>
<tr>
<td>OAT2</td>
<td>HEK293 Cells</td>
<td>46.8 ± 12.2</td>
</tr>
<tr>
<td>OATP1B1</td>
<td>HEK293 Cells</td>
<td></td>
</tr>
<tr>
<td>OAT1</td>
<td>HEK293 Cells</td>
<td></td>
</tr>
<tr>
<td>OAT4</td>
<td>HEK293 Cells</td>
<td></td>
</tr>
<tr>
<td>OATP2B1</td>
<td>HEK293 Cells</td>
<td></td>
</tr>
<tr>
<td>OAT3</td>
<td>HEK293 Cells</td>
<td></td>
</tr>
<tr>
<td>MRP2</td>
<td>Vesicles</td>
<td></td>
</tr>
<tr>
<td>BCRP</td>
<td>Vesicles</td>
<td></td>
</tr>
<tr>
<td>MRP3</td>
<td>Vesicles</td>
<td></td>
</tr>
<tr>
<td>OATP1B3</td>
<td>HEK293 Cells</td>
<td></td>
</tr>
</tbody>
</table>

aReported as S_{50}; Hill Coefficient = 3.19 ± 0.34

bNo detectable transport.
Figure 1

- Plot showing the plasma concentration (ng/mL) over time (hr).
- Data points for DF and DF-AG.
- Time points: 0.5, 1, 1.5, 2, 2.5, 3, 4, 6, 8, 12 hours.
- Y-axis: Plasma concentration (ng/mL) with a log scale.
- X-axis: Time (hr).
Figure 5