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

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Running title: DF and DF-AG transporters in vitro and their in vivo disposition in human

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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 (C_{max} 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

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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

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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

[³H]Estradiol-17β-D-glucuronide (34.3 Ci/mmol), [³H] cholecystokinin octapeptide (CCK-8,

97.5mCi/mmol), [³H]esteone-3-sulfate salt (45.6Ci/mmol) were purchased from Perking Elmer Life and

Analytical Science (Waltham, MA). [³H]Penciclovir (PCV; 1.1 Ci/mmol) were purchased from Moravek

Biochemicals (Brea, CA). Benzbromarone, estradiol-17β-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). [³H]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

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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 550ºC and ion spray voltage, cell entrance

(EP) and exit potentials (CXP) were set at -4500, -10 and -15 Volts respectively. Mass transitions were

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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_{max}, T_{max}, the area under the plasma concentration versus time curve up to the last quantifiable time

point (AUC_{last}) and total AUC (AUC_{tot}). AUC was estimated by the mixed logarithmic-linear method in

the software. Data are also reported as the DF/DF-AG AUC_{tot}, AUC_{last} and C_{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 hygromycine (100 µg/mL) in a humidified incubator at 37°C and 5% CO_2. The stably

transfected HEK-293 cell lines expressing drug transporter proteins were established by using the Flp-

In™ 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% CO2 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 2.5×10^6/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 [^{3}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 lower

pH (5.4) to avoid the degradation of DF-AG. The cells were washed three times with ice-cold HBSS

buffer. For [^{3}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 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:[³H]

estradiol-17-β-glucuronide (1 μM, OATP1B1), [³H]estrone 3-Sulfate (1 μM, OATP2B1, OAT3 and OAT4),

[³H]CCK-8 (1 μM, OATP1B3), PAH (1 μM, OAT1), [³H]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 [³H]

estradiol-17-β-glucuronide (50 μM, MRP2 and MRP3) and [³H]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

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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)

$$v = \frac{V_{max} * S}{K_m + S} \qquad\qquad \text{Equation 1}$$

$$v = \frac{V_{max} * S^n}{K_m + S^n} \qquad\qquad \text{Equation 2}$$

where v is the rate of uptake measured at the given concentration; V_{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_{max} and AUC_{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_{max}, 1849.4 nM; AUC_{tot}, 3592.6 nM*hr)

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. 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 uM 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 (Km 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.

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(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-live 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

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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 ^{14}C-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 (IC$_{50}$ > 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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Acknowledgements

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Reference:

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, Fjeldsa 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-acylglucuronide. *Drug Metab Dispos* **27:**1143-1149.

Hammond TG, Meng X, Jenkins RE, Maggs JL, Castelazo AS, Regan SL, Bennett SN, Earnshaw CJ, Aithal GP, Pande I, Kenna JG, Stachulski AV, Park BK, and Williams DP (2014) Mass spectrometric characterization of circulating covalent protein adducts derived from a drug acyl glucuronide metabolite: multiple albumin adductions 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, Muller F, Mieth M, Fromm MF, and Konig 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.

Kock 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.

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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 benzbromarone. *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 Hitzenberger 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 Pharmacokinet Biopharm* **19:**647-665.

Riess W, Stierlin H, Degen P, Faigle JW, Gerardin A, Moppert J, Sallmann A, Schmid K, Schweizer A, Sulc M, Theobald W, and Wagner J (1978) Pharmacokinetics and metabolism of the anti-inflammatory agent Voltaren. *Scand J Rheumatol Suppl*: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, Elferink RPJO, and Boelsterli UA (1998) Selective Protein Adduct Formation of Diclofenac Glucuronide Is Critically Dependent on the Rat Canalicular Conjugate Export Pump (Mrp2). *Chemical Research in Toxicology* **11:**513-519.

Shen H, Liu T, Morse BL, Zhao Y, Zhang Y, Qiu X, Chen C, Lewin AC, Wang XT, Liu G, Christopher LJ, Marathe P, and Lai Y (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, Kung 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, Li W, Ramanathan R, Yang Z, and Humphreys WG (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.

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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

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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

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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.

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Table 1. Pharmacokinetic parameters of DF and DF-AG following a single oral 50mg dose of DF

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

Data are reported as the mean ± SD of six different subjects

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Table 2. Summary of kinetic data for DF and DF-AG for various human transporters in vitro

Transporter	System	Km ± SE of the parameter estimate (µM)	
		DF	DF-AG
OAT2	HEK293 Cells	46.8 ± 12.2	8.6 ± 1.9
OATP1B1	HEK293 Cells	–[b]	34.0 ± 8.6
OAT1	HEK293 Cells	-	60.2 ± 13.47
OAT4	HEK293 Cells	-	103.9 ± 29.0
OATP2B1	HEK293 Cells	-	104.6 ± 16.7
OAT3	HEK293 Cells	-	114.1 ± 8.1
MRP2	Vesicles	-	145.0 ± 7.8[a]
BCRP	Vesicles	-	152.0 ± 54.9
MRP3	Vesicles	-	196.3 ± 58.0
OATP1B3	HEK293 Cells	-	-

[a]Reported as S_{50}; Hill Coefficient = 3.19 ± 0.34
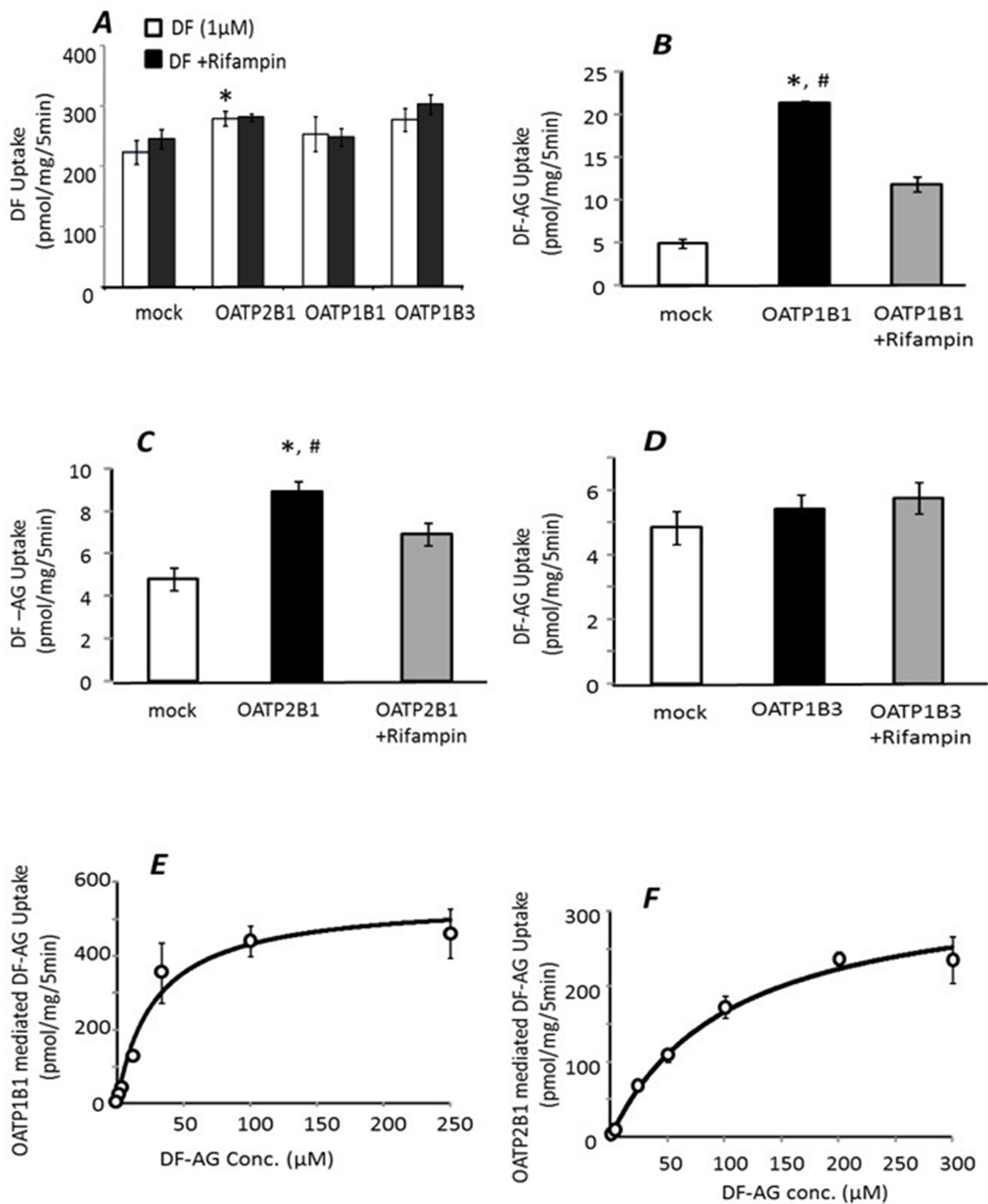
[b]No detectable transport.
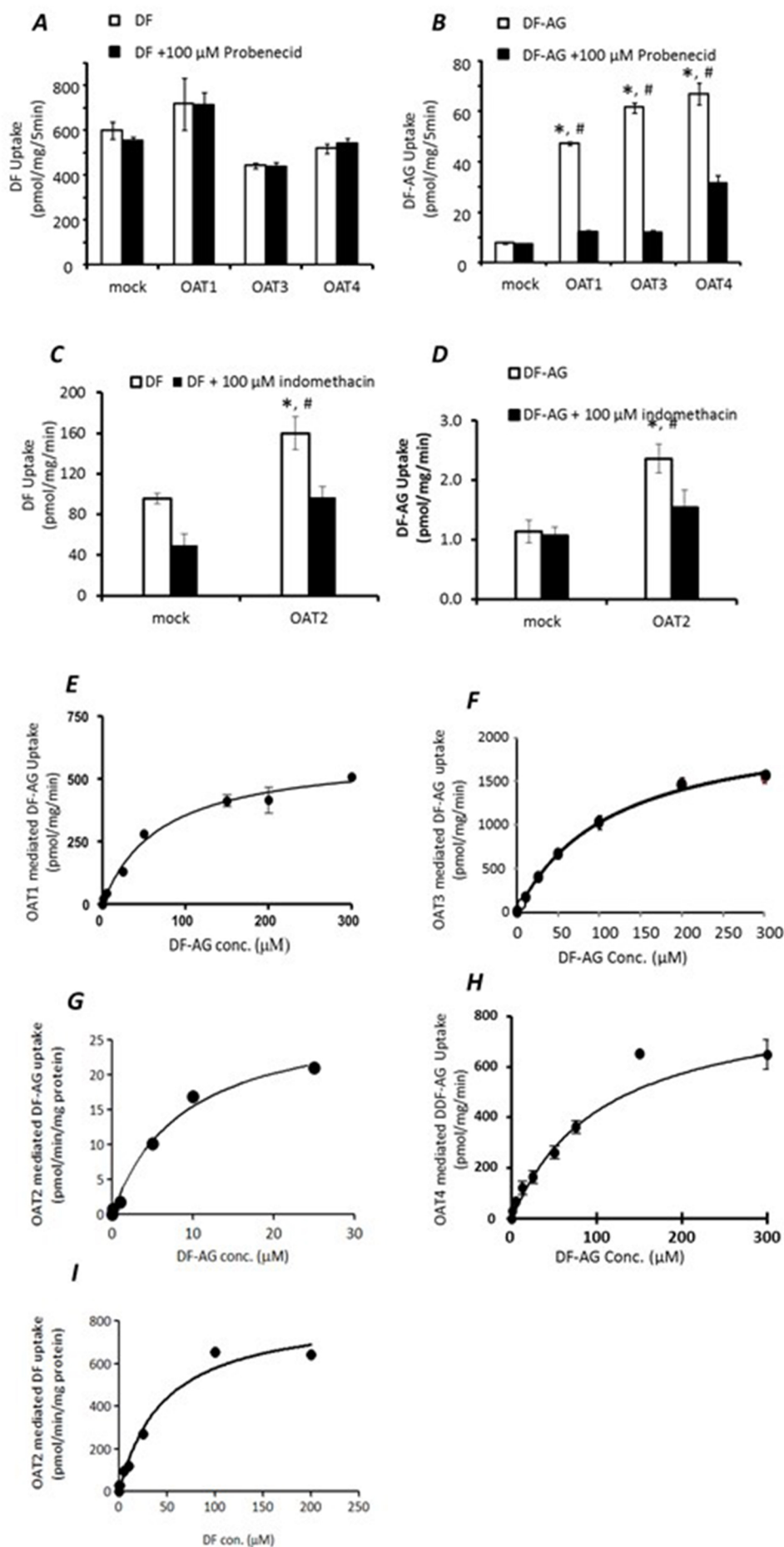
Figure 1
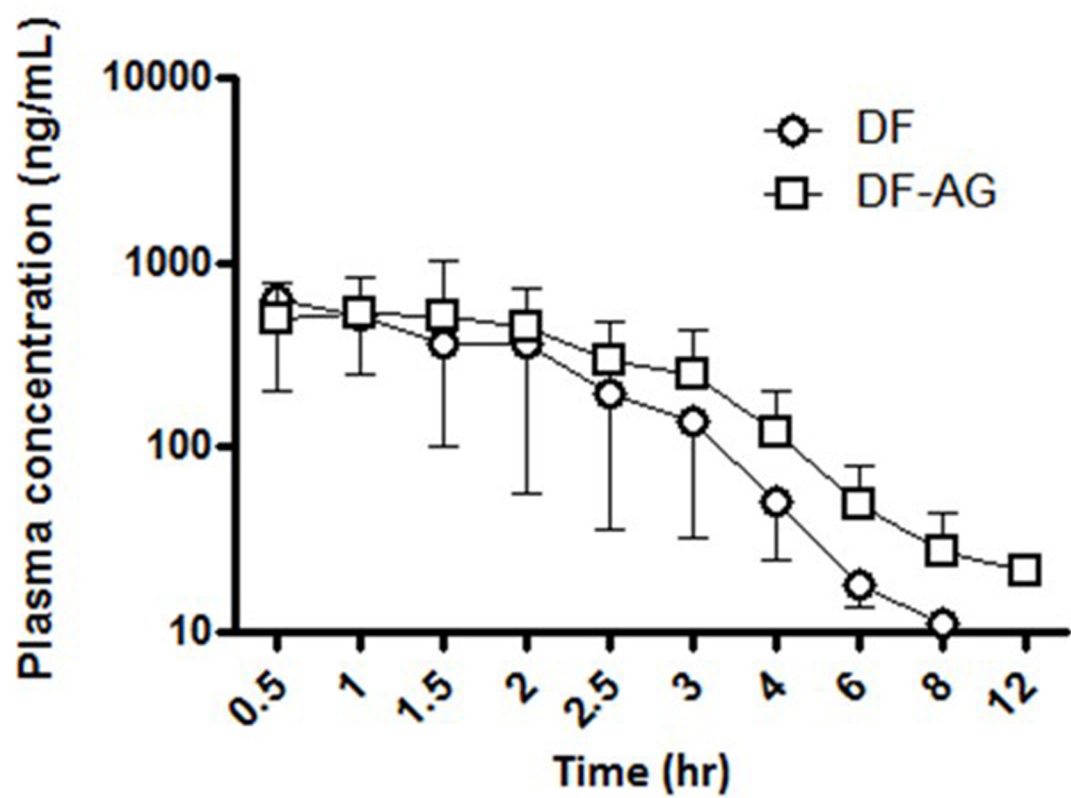
Figure 2

Figure 3

Figure 4
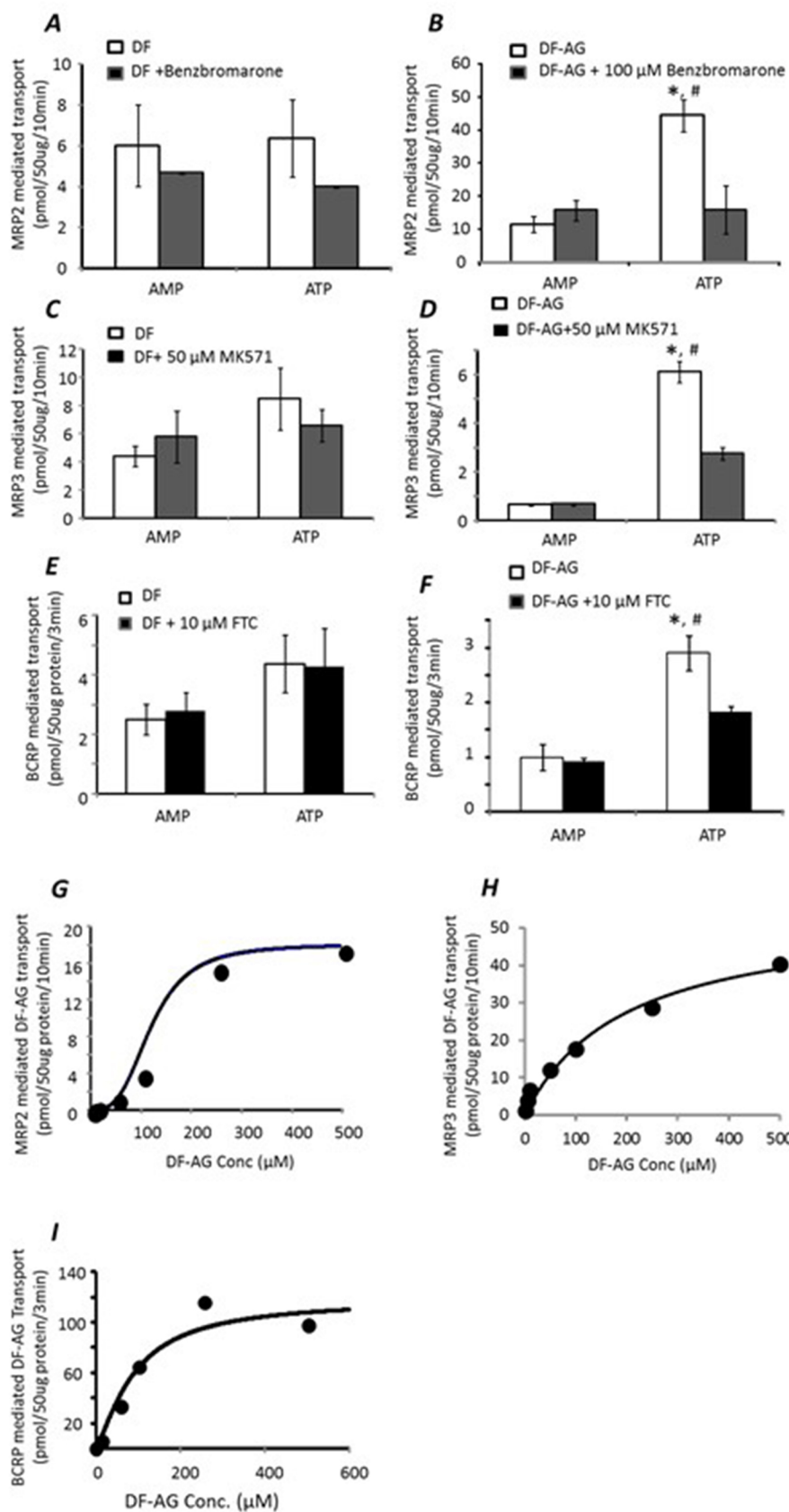
Figure 5
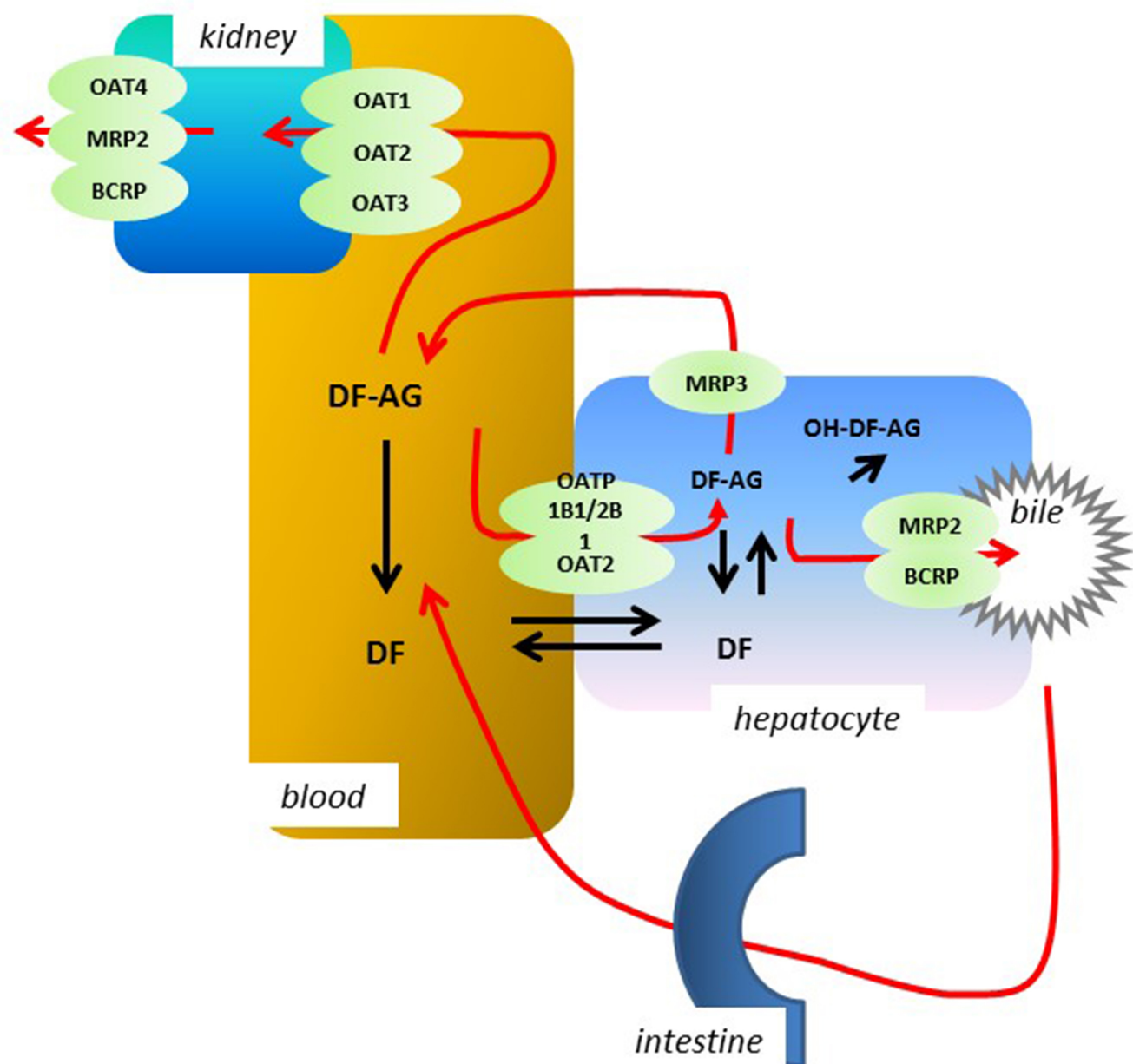
Diclofenac and its Acyl Glucuronide: Determination of *In Vivo* Exposure in Human Subjects and Characterization as Human Drug Transporter Substrates *In Vitro*

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Table 1. Comparison of transporter activity between pH 5.4/6.0 and pH 7.0.

Uptake was assessed using stably transfected HEK cells that singularly expressed human transporter.

Transporter	Substrate	Transporter activity at pH 5.4 (cell uptake) or 6.0 (vesicle uptake)	Transporter activity at pH7.4
		Mean ± SD (ul/mg/min)	Mean ± SD (ul/mg/min)
OAT1	PAH	85.02±4.38	104.85±11.29
OAT3	E3S	151.03±4.23	240.2±6.4
OAT4	E3S	167.71±20.6	199.79
OATP1B1	E-GLU	28.22±0.89	30.29±3.15
OATP1B3	CCK-8	32.53±1.04	36.7±1.6
OATP2B1	E3S 5.4	50.88±2.86	53.61±5.88
MRP2	E-Glu	186.7±26.7	150.2±20.6
MRP3	E-Glu	533.0±14.1	659.8±23.44
BCRP	MTX	171.7±24.5	60±2.39

Table 2. IC50 or Ki values of CsA against uptake and efflux transporters (resource: www.druginteractioninfo.org)

Transporter	Probe Substrate	Cell System	Ki (μM)	IC50 (μM)	Pubmed Accession #
BCRP (ABCG2)	estrone-3-sulfate	Membrane Vesicles	6.7		17220244
	methotrexate		7.8		17220244
BSEP (ABCB11)	taurocholic acid	Membrane Vesicles		0.5	21965623
				10	22961681
				1.54	205123
				4.6	24014644
				2	19520776
			9.5		12404239
				0.8	24062352
MRP1 (ABCC1)	calcein AM	Other cells		2.81	23851114
MRP2 (ABCC2)	5(6)- carboxy-2,7-dichlorofluorescein (CDCF)	Membrane Vesicles		5.55	20307659
	cholecystokinin octapeptide (CCK-8)	Membrane Vesicles	24	45.3	15665139
	vinblastine	Caco-2 cells		2.69	12134946
	vinblastine	MDCK-transfected cells		8.11	12134946
MRP3 (ABCC3)	estradiol-17-beta-glucuronide	Membrane Vesicles			24154606
MRP4 (ABCC4)	dehydroepiandrosterone sulfate (DHEAS)	Membrane Vesicles			24154606

	Substrate	Cell type			PMID
NTCP (SLC10A1)	atorvastatin	HEK293-transfected	4.8	4.8	24799396
	rosuvastatin	HeLa-transfected		0.37	16697742
	taurocholic acid	HEK293-transfected		0.8	24062352
		Hepatocytes		2.4	23516635
		Other cells		1.2	23516635
		Other cells		2.1	25740896
OATP1B1 (SLCO1B1)	8-fluorescein-cAMP	CHO-transfected		0.25	20540932
		HEK293-transfected		2.8	23219525
	atorvastatin	HEK293-transfected	0.16		25414411
			0.66	1.5	24799396
			0.82		21861202
			0.014	0.021	20519340
			0.31	0.47	20519340
	bosentan	CHO-transfected		0.3	17496208
	bosentan	HEK293-transfected	0.206		25414411
	bromosulfophthalein (BSP)	HEK293-transfected		0.694	23920221
	bromosulfophthalein (BSP)	MDCK-transfected cells		3.5	16495352
	cerivastatin	Hepatocytes (cryopreserved)	0.28		12538813
	cerivastatin	MDCK-transfected cells	0.238		12538813
	estradiol-17-beta-glucuronide	HEK293-transfected		0.05	17901929
				0.198	23179780
				0.019	23179780
				0.2	16316932
				0.118	23920221
				0.9	22587986
				0.87	23297161
				1.64	23248200
				1.25	23248200
			0.2		15535988
				0.13	23886114
		HeLa-transfected		0.37	12490595
	estrone-3-sulfate	HEK293-transfected		0.732	23920221
					22240838
				1.25	22587986
	fexofenadine (terfenadine carboxylate)	HEK293-transfected	0.0771		25414411
	fluvastatin	HEK293-transfected	0.157		25414411
	glyburide (glibenclamide)	HEK293-transfected	0.102		25414411

	mesalamine (5-ASA)	HEK293-transfected	1		21430235
	nateglinide	HEK293-transfected	0.244		25414411
	phalloidine	HEK293-transfected	0.051	0.5	14530907
	pitavastatin	HEK293-transfected		0.23	23750830
			0.228		25414411
				0.7	22587986
			0.242		16595711
		X. laevis oocytes-injected		2.91	15344842
	pravastatin	HEK293-transfected	0.184		25414411
	repaglinide	HEK293-transfected	0.0857		25414411
	rosuvastatin	HEK293-transfected		0.21	25740896
				0.89	23248200
			0.301		25414411
		HeLa-transfected		0.31	16697742
		X. laevis oocytes-injected		2.2	15289793
	torsemide	HEK293-transfected	0.486		25414411
	valsartan	HEK293-transfected	0.138		25414411
OATP1B3 (SLCO1B3)	8-fluorescein-cAMP	CHO-transfected		0.2	20540932
		HEK293-transfected		2.2	23219525
	amanitin	MDCK-transfected cells		0.3	16495352
	atorvastatin	HEK293-transfected	1.3	3.1	24799396
	bosentan	CHO-transfected		0.8	17496208
	bosentan hydroxy metabolite (Ro 48-5033)	CHO-transfected		0.5	17496208
	bromosulfophthalein (BSP)	MDCK-transfected cells		0.3	16495352
	cholecystokinin octapeptide (CCK-8)	HEK293-transfected		0.8	23297161
		MDCK-transfected cells	1.2	1.8	15665139
	dioscin	HEK293-transfected	1.43		23396419
	estradiol-17-beta-glucuronide	HEK293-transfected		0.162	23179780
				0.032	23179780
				0.057	23886114
	fexofenadine (terfenadine carboxylate)	HEK293-transfected		0.573	18180276
	pitavastatin	HEK293-transfected		0.42	23750830
	rosuvastatin	HEK293-transfected		0.13	25740896
		HeLa-transfected		0.06	16697742
OATP2B1 (SLCO2B1)	rosuvastatin	HeLa-transfected		0.07	16697742

OAT2	Creatinine	HEK293-transfected		11	25904762

Table 3. Clinical relevant drug-drug interaction with diclofenac as a victim (resource: www.druginteractioninfo.org)

Precipitant	Mechanism	Pubmed Accession #	AUC fold changes	Cmax fold changes
cyclosporine	Not provided	8970037	2.05	
diosmin	CYP2C9	17708066	1.60	1.55
fluvastatin	CYP2C9	7586933	1.25	1.14
voriconazole	CYP2C9	18034666	1.76	2.13