Targeted Metabolomics Identifies Glucuronides of Dietary Phytoestrogens as a Major Class of MRP3 Substrates In Vivo

KOEN VAN DE WETERING,* WOUTER FEDDEMA,* J. BERND HELMS,‡ JOS F. BROUWERS,‡ and PIET BORST*

*Division of Molecular Biology, The Netherlands Cancer Institute, Amsterdam; and ‡Department of Biochemistry and Cell Biology, Faculty of Veterinary Science, Utrecht University, The Netherlands

BACKGROUND & AIMS: The physiologic function of the efflux transporter Multidrug Resistance Protein 3 (MRP3) remains poorly defined. In vitro, MRP3 transports several glucuronidated compounds, but the compounds transported under physiologic conditions are unknown. Knowledge of the compounds transported by MRP3 in vivo would greatly contribute to the elucidation of the physiologic function of this transport protein. **METHODS:** We used targeted metabolomics to identify substrates of MRP3 in vivo. Liquid chromatography coupled to mass spectrometry was used to specifically screen in plasma and urine of mice for compounds containing a glucuronic acid moiety. **RESULTS:** We found that several highly abundant compounds containing a glucuronic acid moiety have a much lower abundance in plasma and urine of *Mrp3*$^{(-/-)}$ than of wild-type mice. We identified these as phytoestrogen-glucuronides, and we show that MRP3 transports these compounds at high rates and with high affinity in vitro. **CONCLUSIONS: We have identified the efflux transporter MRP3 as a major factor in the disposition of phytoestrogens, a class of compounds to which mammals are exposed via food of plant origin. Our targeted metabolomics approach is not restricted to MRP3 but applicable to many other transport proteins for which knockout mouse models are available. Similar screens could be developed for sulpho- and glutathione-conjugates, further increasing the potential of identifying new physiologic transporter substrates.**

Multidrug Resistance Protein 3 (MRP3; ABCC3) belongs to the adenosine triphosphate (ATP)-binding cassette (ABC) family of membrane transporters[1] and couples the energy released by the hydrolysis of ATP to transport of a substrate over the plasma membrane out of the cell.[2,3] In vitro experiments have shown that MRP3 is a typical organic anion transporter, like other MRPs. Its substrates include (some) anionic drugs, bile acids, and compounds conjugated to a glutathione-, sulphate-, or glucuronic acid moiety (ie, phase II metabolites).[2,3] Initial studies focused on the function of MRP3 in the liver because it was hypothesized to protect the hepatocytes against the toxic accumulation of bile acids during cholestasis. After *Mrp3*$^{(-/-)}$ mice became available, this hypothesis was disproved,[4,5] and the physiologic function of MRP3 remains largely unknown.[5]

MRP3 is highly expressed in epithelial cells of liver, gut, and kidney,[6] organs also known for their high glucuronidating capacity.[7] Glucuronidation is an important detoxification mechanism, and a major function of MRP3 may therefore lie in the disposal of glucuronic acid conjugates of xenobiotics to which organisms, like humans, are constantly exposed via their diet. Indeed, several glucuronosyl conjugates of drugs have been identified as MRP3 substrates in vivo[8,9] as well as bilirubin-glucuronide and glucuronidated bile salts.[4,5]

MRP3 is localized in the basolateral membrane of epithelial cells[6] and transports its substrates toward the circulation for urinary excretion. The abundance of MRP3 substrates in plasma and urine should therefore be reduced in knockout mice lacking MRP3. We have focused on a screen for glucuronosyl conjugates that are lower in plasma and urine of *Mrp3*$^{(-/-)}$ than of wild-type (WT) mice. By this approach, we identified several new substrates of MRP3, all glucuronidated phytoestrogens. Our results show that a major physiologic function of MRP3 is to transport the glucuronic acid conjugates of phytoestrogens from the gut epithelium into the circulation, for subsequent urinary excretion.

Materials and Methods

Materials

Oasis HLB solid phase extraction (SPE) cartridges (30 mg) and the preparative Atlantis C18 reversed phase column (10 μm, 250 × 19 mm) were from Waters (Milford, MA). Analytical Luna C18(2) reversed phase columns (3 μm, 150 × 2.0 mm and 3 μm, 150 × 4.6 mm) were from Phenomenex (Torrance, CA). Creatine phos-

Abbreviations used in this paper: ABC, ATP-binding cassette; ATP, adenosine triphosphate; BSA, bovine serum albumin; cDNA, complementary DNA; CID, collision-induced dissociation; HEK, Human Embryonic Kidney cells; LC, liquid chromatography; MRM, multiple reaction monitoring; MRP3, Multidrug Resistance Protein 3; MS, mass spectrometry; m/z ratio, mass-to-charge ratio; PXR, pregnane-X-receptor; SPE, solid phase extraction; WT, wild-type.

0016-5085/09/$36.00
doi:10.1053/j.gastro.2009.06.052

phate and creatine kinase were from Roche (Almere, The Netherlands). [^{3}H]genistein, [^{3}H]equol, and [^{3}H]uridine-diphosphate-glucuronic acid were from American Radiolabeled Chemicals (St. Louis, MO). Standard rodent diet (AM-II) and modified standard AIM-II diet containing 20% soy meal were from Arie Blok (Woerden, The Netherlands; for composition of the diets see the online Supplementary Materials and Methods). Acetonitrile and methanol were from Biosolve (Valkenswaard, The Netherlands). All other chemicals and reagents were from Sigma Chemical Co. (St. Louis, MO).

Animals

Mrp3$^{(-/-)}$ mice have been described.[5] All mice used were on a 99% FVB genetic background, received food and water ad libitum, and were housed in constant temperature rooms with a 12-hour light/12-hour dark cycle. Mouse handling and experimental procedures were conducted in accordance with institutional guidelines for animal care and use.

Collection and Processing of Blood and Urine Samples of WT and Mrp3$^{(-/-)}$ *Mice*

Twenty-four-hour urine samples of 10- to 12-week-old mice fed a standard rodent diet (AM-II) or a diet containing 20% soy meal were collected in metabolism cages. Afterwards, mice were placed in their normal cages and kept there for 7 days before blood was collected by cardiac puncture using heparinized syringes. Plasma was obtained after centrifugation (1500*g*, 10 minutes, 4°C). Five hundred microliters plasma was mixed with 500 μL H_2O and 100 μL acetic acid, centrifuged at 10,000*g* for 5 minutes, and applied to a preconditioned (1 mL methanol, 1 mL H_2O) Oasis HLB SPE cartridge (1 cc, 30 mg). The cartridge was washed 2× with 1 mL 2% acetic acid in 5% methanol before elution with 2% NH_4OH in 92% methanol. For urine, 50% of the 24-hour urine samples were extracted using the above-described protocol for plasma, using an acetic acid concentration of 10%. Samples were evaporated until dry under vacuum and stored at −80°C until analysis. Prior to analysis by liquid chromatography (LC)/mass spectrometry (MS), plasma and urine samples were dissolved in 35 μL and 120 μL, respectively, of buffer A (see below under High-Pressure Liquid Chromatography heading).

Deglucuronidation of Plasma and Urine Samples of WT Mice

Two milliliters pooled WT mouse plasma was subjected to SPE on a 6-mL, 200-mg OASIS HLB cartridge using the conditions described above. The pellet was dissolved in 1800 μL phosphate buffer (75 mmol/L, pH 6.8), divided in 2 fractions of 900 μL to which 100 μL phosphate buffer (control) or 100 μL β-glucuronidase (25 U/μL) was added, incubated at 37°C, and processed by SPE as described above. WT mouse urine (an equivalent of 50% of the urine produced during 24 hours by 1 animal) was directly diluted in phosphate buffer to a final volume of 1800 μL and subsequently processed as described for plasma.

High-Pressure Liquid Chromatography

For the LC/MS experiments, the analytical high-pressure liquid chromatography system consisted of 2 Series 200 micropumps (Perkin Elmer, Waltham, MA). Separations were performed on a reversed-phase Luna C18(2) (Phenomenex) 3 μm, column (150 mm × 2 mm) at a flow rate of 200 μL/min, with a total run time of 35 minutes and detection by MS (see below). The mobile phases consisted of 10 mmol/L ammonium formate, pH 8.2 (buffer A), and 10 mmol/L ammonium formate, pH 8.2, in 95% acetonitrile (buffer B). Samples were separated using the following gradient A/B (vol/vol): 0–20 minutes, 10/90 to 35/65; 20–22 minutes, 35/65 to 0/100; 22–26 minutes, 0/100; 26–28 minutes, 0/100 to 10/90; 28–35 minutes, 10/90. The injection volume was 20 μL, equivalent to 285 μL plasma or 8.3% of the amount of urine excreted by a mouse during 24 hours. The equivalent of 2.75% of the urine produced during 24 hours was injected from mice on a diet containing 20% soy meal.

MS

An API4000 Qtrap quadrupole iontrap hybrid mass spectrometer (MDS Sciex, Concord, ON, Canada) was used throughout this study and operated in the negative electrospray ionization mode. Compounds containing a glucuronic acid moiety have a specific fragmentation pattern following collision-induced dissociation (CID) because they specifically lose 176 daltons.[10] The mass spectrometer was programmed to scan for deprotonated molecules that showed a neutral loss of 176 daltons after CID, and MS parameters were optimized using commercially available glucuronides (not shown). The following parameters were used throughout the study: curtain gas: 10 psi; collision-activated dissociation gas: high; ion spray voltage: −4500 V; cone temperature: 500°C; gas flow 1: 30 psi; gas flow 2: 20 psi: decluster potential: −65 V; entrance potential: 10 V; collision energy: −39 V.

The same MS parameters were used during multiple reaction monitoring (MRM) to semiquantitatively detect compounds in biologic matrices of WT and *Mrp3*$^{(-/-)}$ mice that specifically lost 176 daltons. The MRM list was based on neutral loss scans on control or deglucuronidated plasma samples of WT mice: compounds detected in control treated samples but absent after deglucuronidation were included in the MRM list. Enhanced product spectra of glucuronides found to be differentially present in plasma or urine of WT and *Mrp3*$^{(-/-)}$ mice were made using information-dependent acquisition and enhanced product ion generation and compared with enzymatically synthesized phytoestrogen-glucuronide standards (see below) for identification.

Generation of Phytoestrogen-Glucuronide Standards

Phytoestrogens (100 μmol/L) were glucuronidated in vitro using mouse liver microsomes as described previously for morphine-3-glucuronide,[11] with 1 modification: the addition of 1% fatty acid-free bovine serum albumin (BSA). For large-scale synthesis of phytoestrogen-glucuronides, an aglycon concentration of 1 mmol/L and overnight incubation were used. Enzymatically synthesized phytoestrogen glucuronides were isolated by SPE and, when used in vesicular transport experiments, purified by preparative high-pressure liquid chromatography (see Supplementary Materials and Methods), concentrated by freeze-drying and dissolved in 10 mmol/L Tris/HCl, pH 7.4.

[^{3}H]enterodiol-glucuronide, [^{3}H]enterolactone-glucuronide, [^{3}H]seicoisolariciresinol-glucuronide, and [^{3}H]glyciteinglucuronide were synthesized as described above but replacing 5 mmol/L nonlabeled UDP-glucuronic acid with 10 μCi [^{3}H]UDP-glucuronic acid and using 50 μmol/L of the aglycone. [^{3}H]genistein-glucuronide and [^{3}H]equolglucuronide were synthesized by incubating [^{3}H]genistein and [^{3}H]equol in the presence of 5 mmol/L UDP-glucuronic acid as described above for nonlabeled synthesis. [^{3}H]phytoestrogen-glucuronides were purified by high-pressure liquid chromatography as described in the Supplementary online Materials and Methods, freeze-dried, and dissolved in 10 mmol/L Tris/HCl, pH 7.4.

Cell Lines, Preparation of Membrane Vesicles, and Vesicular Transport Assay

Culture conditions and Human Embryonic Kidney cells (HEK) 293 cells expressing a human MRP3 complementary DNA (cDNA) construct have been described.[12] HEK293 cells were tranfected with pBabeCMVpuro-mMrp3 by calcium phosphate precipitation, and standard procedures were used to select clones that expressed mouse MRP3 (detected by immunoblot analysis and immunofluorescence microscopy using the A66 antibody,[5] not shown). Preparation of membrane vesicles[12] and vesicular transport experiments with phytoestrogen-glucuronides in HEK293 control and human or mouse MRP3 containing vesicles was done as described earlier.[11]

Results

Development of a Screen for the Detection of Glucuronidated Compounds in Mouse Plasma

Glucuronides are formed by linking a glucuronic acid moiety to a compound via a glycosidic bond.[7] During tandem MS, this glycosidic bond is easily broken during CID, resulting in the loss of an uncharged fragment of 176 daltons, the glucuronate moiety, thereby generating charged ions with a mass-to-charge (m/z) ratio of $[M\text{-}H^+\text{-}176]^-$ if measured in the negative ion mode.[10] In short, during neutral loss scans, the first mass analyzer (first quadrupole) sequentially determines the masses (m/z ratios) of the molecules that enter the mass spectrometer. In the second quadrupole, the molecules are fragmented by CID, and the mass of these fragments is determined in the second mass analyzer (third quadrupole). This allows the detection of compounds with a mass that is 176 daltons lower in the second than in the first mass analyzer, ie, scanning for a neutral loss of 176 daltons. Applying these neutral loss scans to plasma samples of WT mice, fractionated by LC, resulted in chromatograms with many peaks (Figure 1*A*) that are absent after treatment with β-glucuronidase, an enzyme with preference for O-linked glucuronides (Figure 1*B*). This shows that scanning for a neutral loss of 176 daltons allows the detection of glucuronidated compounds in plasma.

Neutral loss scans are not very quantitative, are not sensitive, and have a poor chromatographic resolution because of the long time needed to scan from 200 to 800 daltons. To overcome these limitations, we focused on all the masses detected in control plasma, but absent after deglucuronidation, in an MRM list, adding up to 80 specific mass transitions in which 176 daltons were lost after CID (Supplementary Table 1). Each mass was scanned for 15 ms, resulting in shortened cycling time (1.2 seconds), improved sensitivity, higher selectivity, and better chromatographic resolution (compare Figures 1*A* and 2*A*).

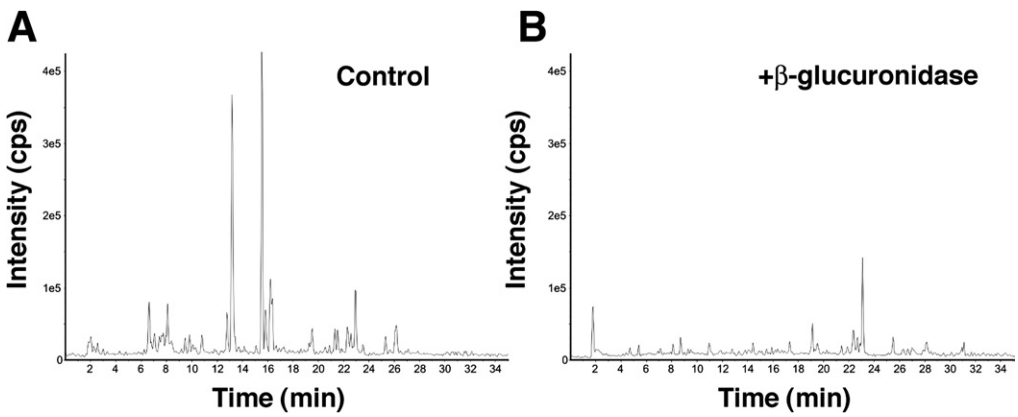

Figure 1. Survey scans for a neutral loss of 176 daltons of compounds present in mouse plasma. Shown are base peak chromatograms (m/z 300–800) from control (*A*) or β-glucuronidase-treated (*B*) plasma samples.

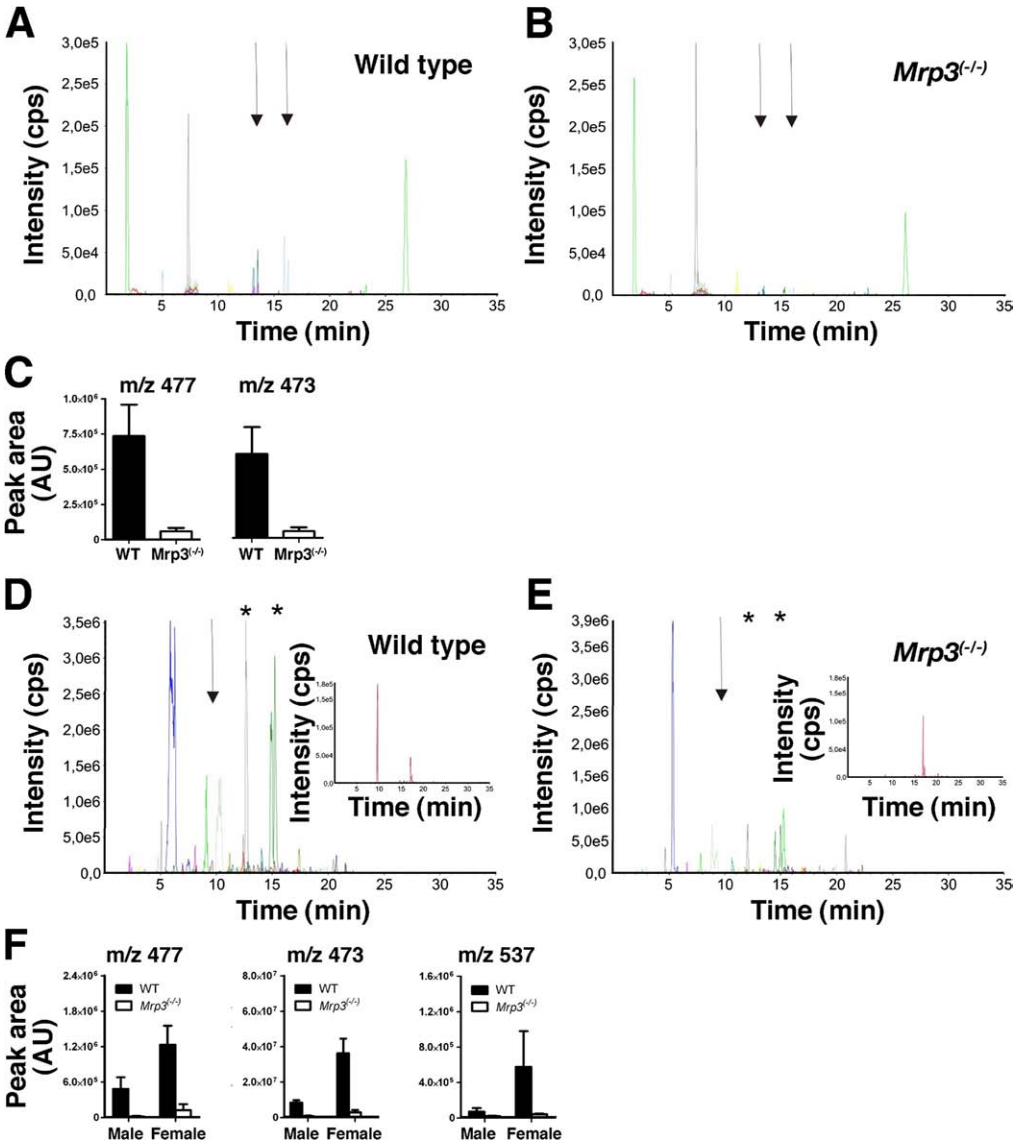

Figure 2. Relative concentration of selected glucuronides of unknown nature in plasma and urine of WT and *Mrp3*$^{(-/-)}$ mice. Plasma and urine samples were analyzed by LC/MS/MS for the relative abundance of several unknown glucuronides specifically losing 176 daltons using MRM. Plasma of a wild-type (*A*) and *Mrp3*$^{(-/-)}$ (*B*) mouse. Every color represents the chromatogram of a specific mass transition in which 176 daltons were lost after CID. Note that especially compounds with a retention time between 12 and 18 minutes (*arrows*) are lower in plasma of the *Mrp3*$^{(-/-)}$ mouse. (*C*) Determination of the peak areas (which is a measure for their relative abundance) of the unknown glucuronides with m/z ratios of 473 and 477 in plasma of WT and *Mrp3*$^{(-/-)}$ mice (n = 3 or 4), respectively. Urine of a WT (*D*) and *Mrp3*$^{(-/-)}$ (*E*) mouse analyzed by MRM. The *insets* represent magnifications of the MRM tracer with an m/z ratio of 537. The *arrow* points at a low abundant MRM tracer with a mass of 537 (*red line*), which is absent in urine of the *Mrp3*$^{(-/-)}$ mouse. *Asterisks* mark peaks corresponding to the unknown glucuronides with m/z 477 and 473. (*F*) Relative quantification of the glucuronides with m/z ratios of 477, 473, and 537 in urine of WT and *Mrp3*$^{(-/-)}$ mice (n = 4).

Screening for Unknown Glucuronides in Plasma and Urine of WT and Mrp3$^{(-/-)}$ *Mice*

We found several peaks present in plasma of WT mice that were highly reduced in plasma of mice that lack MRP3 (Figure 2*A* and *B*). The most dominant peaks had m/z ratios of 477 and 473 (see *arrows* in Figure 2*A*) and eluted after 13 and 16 minutes, respectively. Chromatograms of both mass transitions showed 2 major peaks eluting within 30 seconds of each other. Determination of the peak area of the 2 major peaks for each m/z ratio (473 and 477) showed that the plasma concentrations of these glucuronides in *Mrp3*$^{(-/-)}$ mice were more than 10-fold lower than in WT mice (Figure 2*C*).

The amounts of the unknown glucuronides with m/z 473 and 477 excreted via urine were also lower in *Mrp3*$^{(-/-)}$ mice compared with WT controls (Figure 2*D–F*, *asterisks* mark peaks corresponding to the unknown glucuronides with m/z 477 and 473). In urine, we found another glucuronide with a lower abundance in the absence of MRP3 (Figure 2*D–F*). The *arrow* in Figure 2*D* points to a small red peak in urine of a WT mouse. This glucuronide had an m/z ratio of 537 and a retention time of approximately 10 minutes, and it eluted as a single peak in urine of WT mice (Figure 2*D*) but was virtually absent in urine of *Mrp3*$^{(-/-)}$ mice (Figure 2*E*). The concentrations of glucuronidated compounds were much higher in urine than in plasma (10- to 20-fold), explaining why we missed the glucuronide with a m/z ratio of 537 in our initial screen in plasma. Interestingly, all 3 unknown glucuronides had a higher abundance in female than in male urine.

Identification of the Unknown Glucuronides With m/z Values of 473, 477, and 537

The unknown glucuronides that were lower in plasma and/or urine of *Mrp3*$^{(-/-)}$ mice (Figure 2) were

expected to be MRP3 substrates and further characterized. Based on their mass, the composition of our standard rodent diet, and a search of a compound database (http://chembiofinderbeta.cambridgesoft.com), we hypothesized that the unknown peaks with m/z ratios of 473, 477, and 537 could be the glucuronides of enterolactone, enterodiol, and secoisolariciresinol, respectively. All these compounds are enterolignans and extensively glucuronidated in vivo.[13,14] To verify this tentative identification, we enzymatically generated glucuronic acid conjugates of these compounds from their respective aglycons for identification. The chromatogram of in vitro generated enterodiol-glucuronide is shown in Figure 3*A* (for clarity only the MRM tracer for the mass transition 477/301 is shown). The retention time exactly matched that of the unknown glucuronide with m/z 477 in mouse urine (Figure 3*B*, only the chromatogram of MRM tracer 477/301 is shown), and both chromatograms showed 2 separate peaks. We attribute these peaks to 2 different glucuronide conjugates of enterodiol that can be formed because of the presence of 2 phenolic hydroxyl groups.[15] By the same procedure, we identified the glucuronide with a m/z ratio of 473 as enterolactone-glucuronide (Figure 3*C* and *D*) and the unknown glucuronide with m/z 537 as secoisolariciresinol-glucuronide (Figure 3*E* and *F*). The aglycones of these compounds (for structures, see Supplementary Figure 1*A*) are bacterial breakdown products of lignans present in several plants and are all known to be extensively glucuronidated in the gut.[13]

Transport of Enterodiol-, Enterolactone-, and Secoisolariciresinol-Glucuronide in Vesicular Transport Experiments

Decreased plasma and urinary excretion levels in *Mrp3*$^{(-/-)}$ mice could in theory also be due to compensatory alterations in the expression of other (efflux) transporters and/or glucuronidating enzymes in this mouse strain. To link directly the lower levels of enterolactone-,

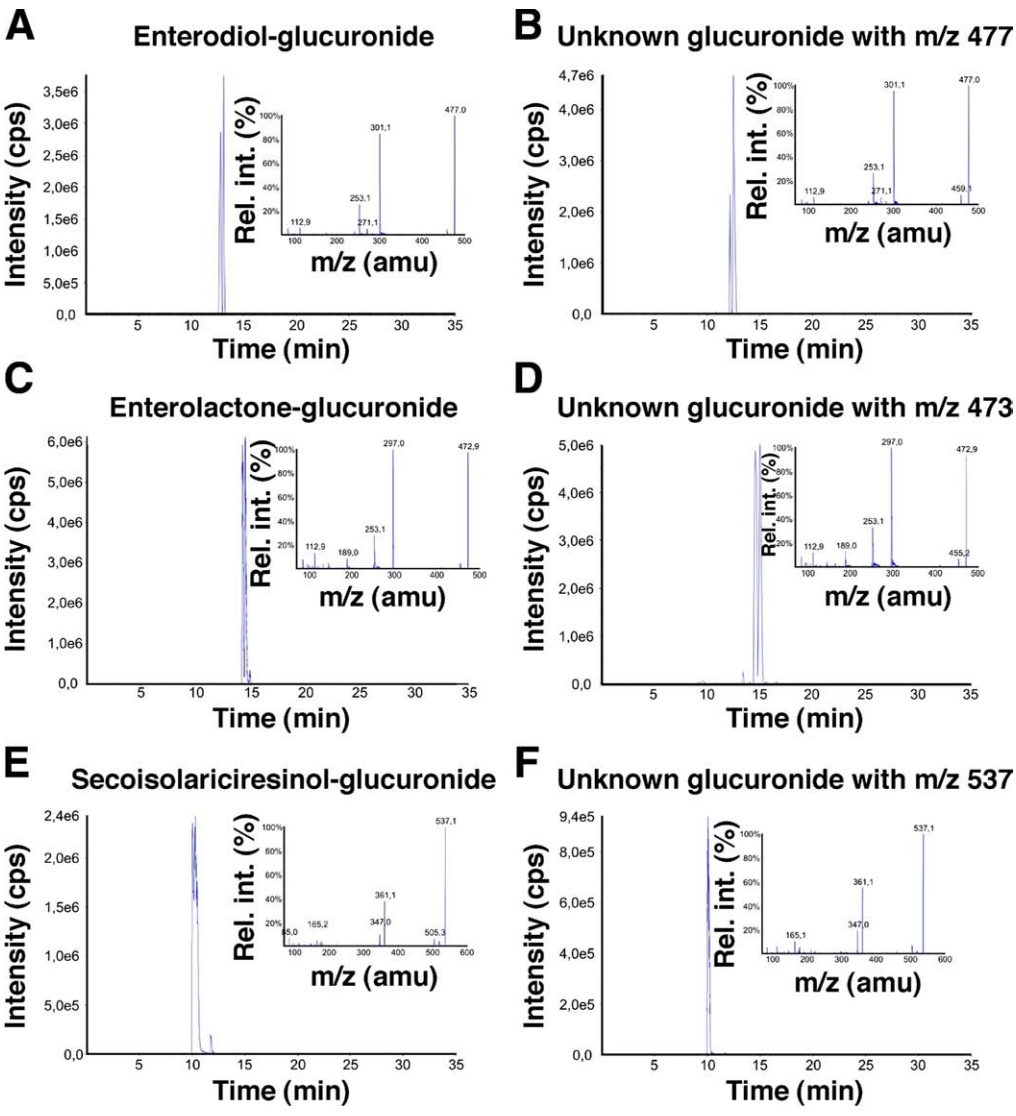

Figure 3. Identification of the unknown glucuronides in plasma and urine that are lower in the absence of MRP3. Urine samples of WT mice and in vitro generated enterolignan-glucuronides were analyzed by LC/MS employing information-dependent acquisition for the generation of fragmentation spectra (*insets*). Chromatograms of the MRM tracer for the unknown glucuronide with a m/z ratio of 477 using in vitro generated enterodiol-glucuronide (*A*) and pooled WT mouse urine (*B*) are shown. Chromatograms of the MRM tracer for the unknown glucuronide with a m/z value of 473 using in vitro generated enterolactone-glucuronide (*C*) and pooled WT mouse urine (*D*). Chromatograms of the MRM tracer for the unknown glucuronide with a m/z value of 537 using in vitro generated secoisolariciresinol-glucuronide (*E*) and pooled WT mouse urine (*F*).

enterodiol-, and secoisolariciresinol-glucuronide in plasma and urine of *Mrp3*$^{(-/-)}$ mice to reduced MRP3-mediated transport activity, we used these as substrates of mouse and human MRP3 in vesicular transport experiments. Both mouse and human MRP3-containing vesicles showed clear time- and ATP-dependent transport of all 3 glucuronidated enterolignans, whereas transport in control vesicles was virtually absent (Figure 4*A*, *C*, and *E*). In general, mouse and human MRP3 had similar affinities for the substrates tested, all being in the low micromolar range (Figure 4*B*, *D*, and *F*), with mouse MRP3 having a somewhat higher affinity for enterodiol- and secoisolariciresinol-glucuronide than its human orthologue (Figure 4*B* and *F*). Both the affinity and the catalytic efficiency (V_{max}/K_m) for the 3 glucuronidated phytoestrogens belong to the highest reported for MRP3 substrates to date, ranging for mouse MRP3 between enterolactone-glucuronide (68[μL/mg/min]) > enterodiol-glucuronide (28[μL/mg/min]) ≈ secoisolaricirecinol-glucuronide (25[μL/mg/min]) and for human MRP3 between enterolactone-glucuronide (63[μL/mg/min]) > enterodiol-glucuronide (9.8[μL/mg/min]) ≈ secoisolariciresinol-glucuronide (8.5[μL/mg/min]).

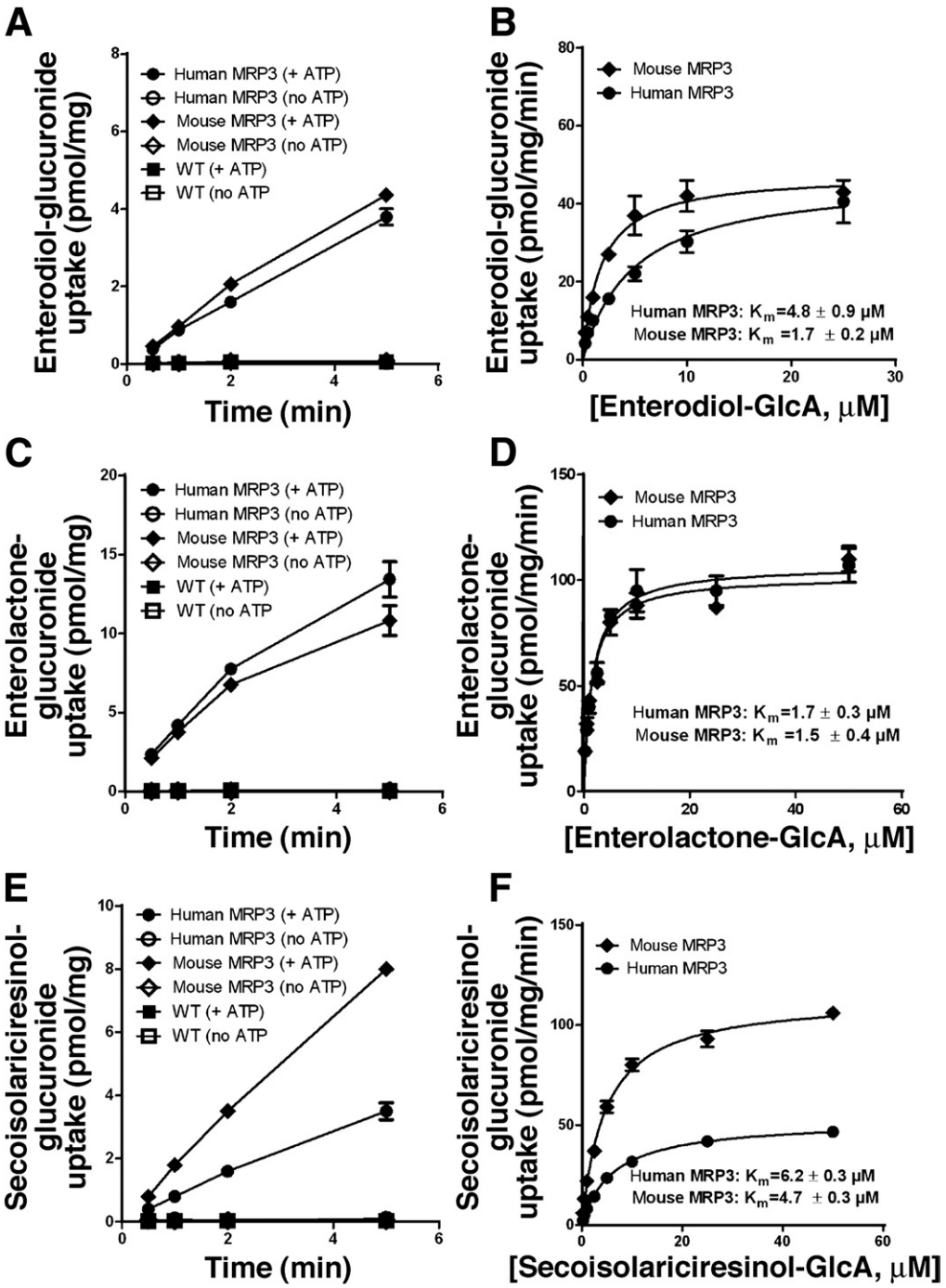

Figure 4. Transport of (entero)-lignan-glucuronides by human and mouse MRP3 in in vitro vesicular transport experiments. Membrane vesicles containing human MRP3 (*circles*), mouse MRP3 (*diamonds*), or control membranes (*squares*) from HEK293 cells were incubated at 37°C in the presence of 4 mmol/L ATP (*closed symbols*) or without ATP (*open symbols*). (*A*, *C*, and *E*) Time-dependent transport of enterodiol-glucuronide (22 nmol/L), enterolactone-glucuronide (27 nmol/L), and secoisolariciresinol-glucuronide (25 nmol/L), respectively. (*B*, *D*, and *F*) Concentration-dependent transport of enterodiol-glucuronide, enterolactone-glucuronide, and secoisolariciresinol-glucuronide, respectively. MRP3-dependent transport was calculated by subtracting ATP-dependent transport in control vesicles from ATP-dependent transport in MRP3-containing vesicles. Note that, in *A*, *C*, and *E*, the *lines* representing transport in WT vesicles and in MRP3-containing vesicles in the absence of ATP overlap and are therefore not separately visible. Values are presented as means ± SD of triplicate (+ ATP) or duplicate values (no ATP).

Identification of MRP3 as a More General Phytoestrogen-Glucuronide Transporter

We have previously shown that MRP3 transports the glucuronic acid conjugate of the stilbene resveratrol,[12] a phytoestrogen found in foods such as grapes.[16] Together with our current results, this suggested that MRP3 might be a more general transporter of phytoestrogen-glucuronides. Because soy is known to be a rich source of phytoestrogens,[13] we put WT and *Mrp3*$^{(-/-)}$ mice on a diet containing 20% soy meal and screened their urine by LC/MS for compounds that specifically lost 176 daltons. The change in diet dramatically changed the spectrum of urinary glucuronides (compare Supplementary Figure 2*A* and Figure 2*E*) with many additional glucuronides detected in WT mouse urine. Several of these glucuronides had a lower abundance in urine samples of mice lacking MRP3 (Figure 5). We analyzed some of these compounds using the technique developed for the enterolignan-glucuronides. Apart from enterodiol-glucuronide and enterolactone-glucuronide, we found less daidzein-, genistein-, glycitein-, and equol-glucuronide (for structures of the respective aglycones, see Supplementary Figure 1*B*) in urine of *Mrp3*$^{(-/-)}$ than WT mice. MRP3-mediated transport of genistein-, glycitein-, and equol-glucuronide was tested in vesicular transport experiments (Figure 6), and both human and mouse MRP3 were found to transport these isoflavone-glucuronides in a time- and ATP-dependent manner (Figure 6*A*, *C*, and *E*). However, human MRP3 transported genistein-glucuronide and glycitein-glucuronide at lower rates than its mouse orthologue (Figure 6*A* and *C*). Apart from human MRP3-mediated transport of glycitein-glucuronide, which we could not saturate with the concentrations available, the K_m values for isoflavone-glucuronide transport by mouse and human MRP3 were similar and ranged from 1.5 to 14 μmol/L (Figure 6*B*, *D*, and *F*). This shows that, although there are some minor species-specific differences in transport kinetics between the human and mouse MRP3 orthologues, in general the data obtained in the *Mrp3*$^{(-/-)}$ mouse can be extrapolated to the function of MRP3 in humans.

We also fed *Mrp3*$^{(-/-)}$ and WT mice a diet that contained 300 mg/kg daidzein or 300 mg/kg genistein. Unexpectedly, the differences in phytoestrogen-glucuronide concentrations in urine between *Mrp3*$^{(-/-)}$ and WT mice were largely lost (not shown). Only equol-glucuronide and the glucuronic acid conjugates of some other daidzein metabolites were found to have a lower abundance in urine of *Mrp3*$^{(-/-)}$ mice under these circumstances. We will revisit this result in the Discussion section.

Discussion

MRP3 transports its substrates over the basolateral membrane of liver and gut toward the circulation for subsequent urinary excretion.[2,3] We have used a targeted metabolomics approach to identify substrates of MRP3 transported under normal physiologic conditions, taking advantage of the facts that (1) MRP3 has a preference for glucuronidated compounds,[2,3] (2) glucuronidated compounds have a specific fragmentation spectrum following CID during MS, and (3) substrates of MRP3 should be decreased in plasma and/or urine of *Mrp3*$^{(-/-)}$ mice. This approach allowed us to screen for nonobvious MRP3 substrates, and we identified glucuronidated phytoestrogens as a major class of MRP3 substrates transported under normal physiologic conditions in mice. Many dietary components of plant origin contain phytoestrogens,[13] and humans (and mice) are, therefore, exposed to these compounds via their diet.

Our approach identifies physiologic substrates that are encountered by MRP3 in vivo under normal conditions. Because the approach is relatively unbiased, it can find nonobvious substrates. Its potential can be further increased by developing screens for other conjugates than glucuronosyl conjugates, such as compounds containing a sulphate- or glutathione moiety. Employing a full metabolomics approach instead of the targeted metabolomics approach (ie, screening for glucuronidated or sul-

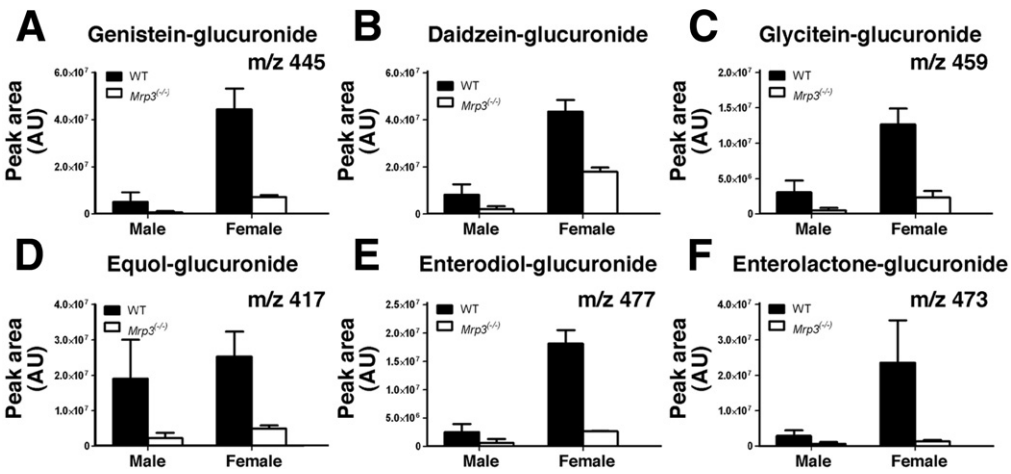

Figure 5. Relative concentrations of several phytoestrogen-glucuronides in 24-hour urine samples of WT and *Mrp3*$^{(-/-)}$ mice. The relative concentration of the indicated phytoestrogen-glucuronides were determined in 24-hour urine samples of WT and *Mrp3*$^{(-/-)}$ mice on a diet containing 20% soy. (*A*) Genistein-glucuronide, (*B*) daidzein-glucuronide, (*C*) glycitein-glucuronide, (*D*) equol-glucuronide, (*E*) enterodiol-glucuronide, (*F*) enterolactone-glucuronide.

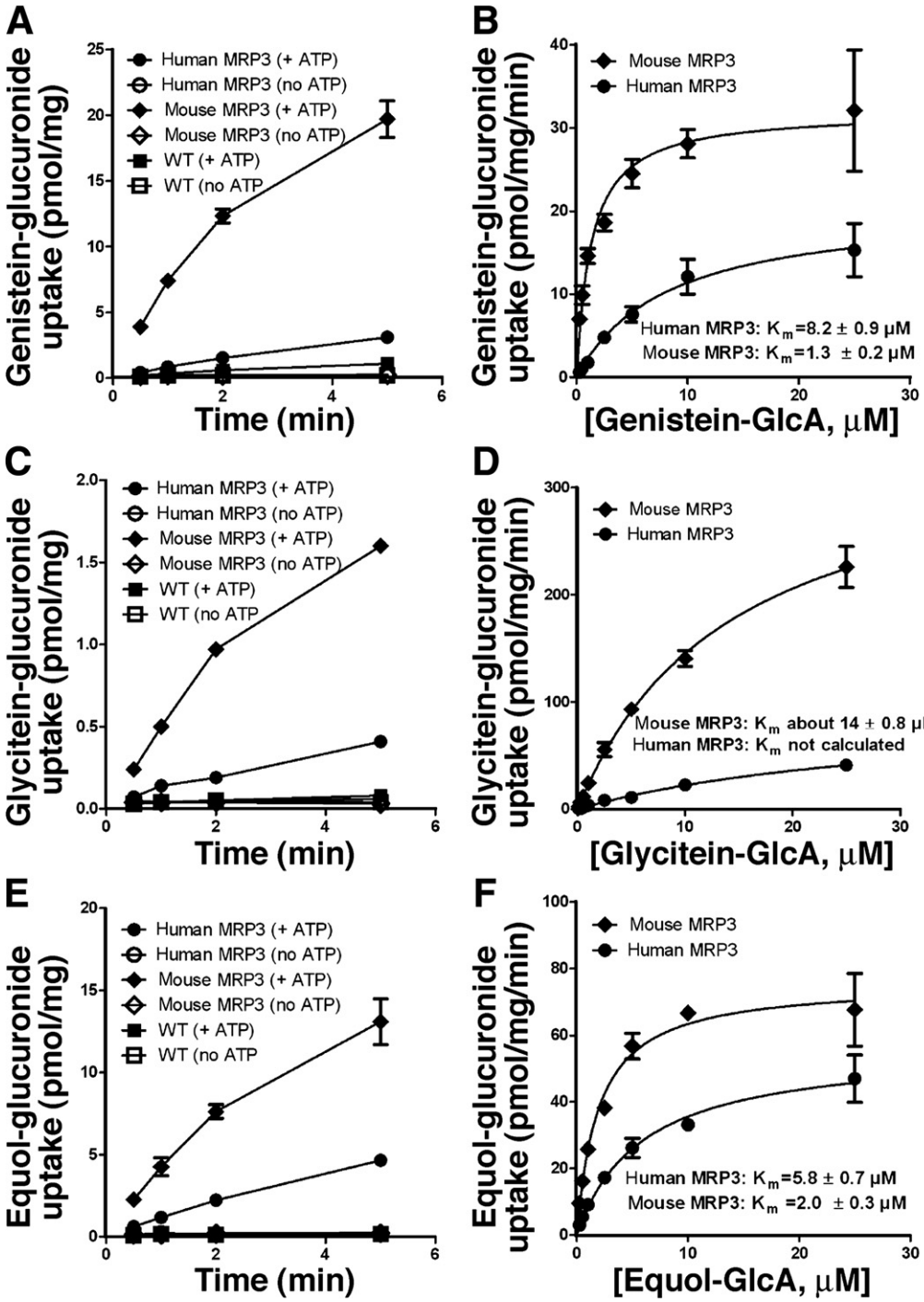

Figure 6. Transport of isoflavone-glucuronides by human and mouse MRP3 in in vitro vesicular transport experiments. Membrane vesicles containing human MRP3 (*circles*), mouse MRP3 (*diamonds*), or control membranes (*squares*) from HEK293 cells were incubated at 37°C in the presence of 4 mmol/L ATP (*closed symbols*) or without ATP (*open symbols*). (*A*, *C*, and *E*) Time-dependent transport of respectively genistein-glucuronide (104 nmol/L), glycitein-glucuronide (19 nmol/L), and equol-glucuronide (75 nmol/L). (*B*, *D*, and *F*) Concentration-dependent transport of genistein-glucuronide, glycitein-glucuronide, and equol-glucuronide, respectively. MRP3-dependent transport was calculated by subtracting ATP-dependent transport in control vesicles from ATP-dependent transport in MRP3-containing vesicles. Note that, in *A*, *C*, and *E*, *lines* representing transport in WT vesicles and in MRP3-containing vesicles in the absence of ATP overlap and are therefore not visible separately. Values are presented as means ± SD of triplicate (+ ATP) or duplicate values (no ATP).

phated compounds) could even further increase the spectrum of identifiable substrates, although with reduced sensitivity and selectivity. Modifying the diet can be another way to expand the range of substrates to be found: A high soy diet allowed us to detect several additional new MRP3 substrates. Hypothesis-driven and in vivo screening approaches can, therefore, complement each other in the identification of the key physiologic substrates of MRP3.

Phytoestrogens are a structurally diverse group of plant derived-compounds.[13] Because of their structural similarity to estradiol, they (weakly) interact with estrogen receptors, thereby exerting estrogenic or anti-estrogenic effects.[13] In vitro, nonconjugated phytoestrogens can modulate a wide range of cellular processes.[13] In vivo, high doses of phytoestrogens have been found to reduce fertility[17,18] and to have toxic effects on the urogenital system.[19,20] Mammals have extensive detoxification mechanisms that prevent the interference of phytoestrogens with normal cellular processes. Glucuronidation is one of these.[21] Nonmetabolized phytoestrogens are hydrophobic in nature and passively diffuse over the plasma mem-

brane. Phytoestrogen-glucuronides, however, are too hydrophilic for passive diffusion, and MRP3 and other transporters are needed for their removal from the cell for subsequent excretion via urine or feces.[14] Both the affinity (K_m) and catalytic efficiency (V_{max}/K_m) of MRP3-mediated transport of the phytoestrogen-glucuronides belong to the highest described for MRP3 so far and are much higher than for the prototype MRP substrate estradiol-17 β-glucuronide.[22,23] This should allow MRP3 to efficiently dispose of phytoestrogen-glucuronides under normal conditions. In addition, MRP3 and several glucuronidating enzymes are encoded by pregnane-X-receptor (PXR) target genes.[24] PXR activation by lignans and other phytoestrogens[25] could, therefore, provide a coordinated pathway for the regulation of the detoxification machinery for these compounds during an increase in exposure.

The main glucuronidating organs in the body are liver, kidney, and the gastrointestinal tract, all organs in which MRP3 is present at considerable levels.[5,6] The gut wall represents the first barrier that phytoestrogens will encounter upon ingestion.[26] Intestinal perfusion experiments have shown that genistein is predominantly glucuronidated in the gut.[27] In addition, the phytoestrogen resveratrol is absorbed through the rat jejunum conjugated to glucuronic acid.[28] These data indicate that orally administered phytoestrogens are predominantly glucuronidated in the gut wall. We infer that the higher plasma and urinary levels of phytoestrogen-glucuronides in WT mice compared with *Mrp3*$^{(-/-)}$ mice are due to the MRP3 present in the enterocytes of the colon. This inference is supported by the following data: (1) enterolactone, enterodiol, and secoisolariciresinol are formed from a lignan precursor molecule by microorganisms in the colon[29]; (2) in the gastrointestinal tract, the highest MRP3 levels are found in the colon[5,30]; (3) human epithelial cell lines of colon origin have been shown to conjugate rapidly these compounds to glucuronic acid[31]; (4) enterolactone and enterodiol are predominantly found conjugated to glucuronic acid in venous portal blood of rats[32]; (5) lignan-glucuronides accumulate in colon (and cecum) tissue extracts of *Mrp3*$^{(-/-)}$ mice (Supplementary Figure 3), indicating their impaired efflux.

In soy, the major part of genistein, glycitein, and daidzein is found as glycoside conjugates, which are too hydrophilic to diffuse into the enterocytes.[21] Removal of this glycoside moiety occurs predominantly in the colon by resident bacteria thereby releasing genistein, glycitein, and daidzein, which can then diffuse into the enterocytes of the colon. After the formation of genistein-glucuronide, glycitein-glucuronide, and daidzein-glucuronide in the enterocytes of the colon, these conjugates are transported toward the circulation by MRP3 for excretion in the urine. In contrast, when mice were fed a diet containing free daidzein or genistein (not containing a glycoside moiety), we did not find lower levels of daidzein-glucuronide and genistein-glucuronide in plasma and urine of *Mrp3*$^{(-/-)}$ mice than of WT mice (not shown). This apparent discrepancy can be explained by the fact that these free phytoestrogens are predominantly absorbed and conjugated to glucuronic acid in the small intestine, from where they are transported over the basolateral membrane toward the circulation apparently without the help of MRP3. This explanation also nicely fits the fact that we found lower levels of equol-glucuronide in *Mrp3*$^{(-/-)}$ mice after feeding of free daidzein (not shown): equol is

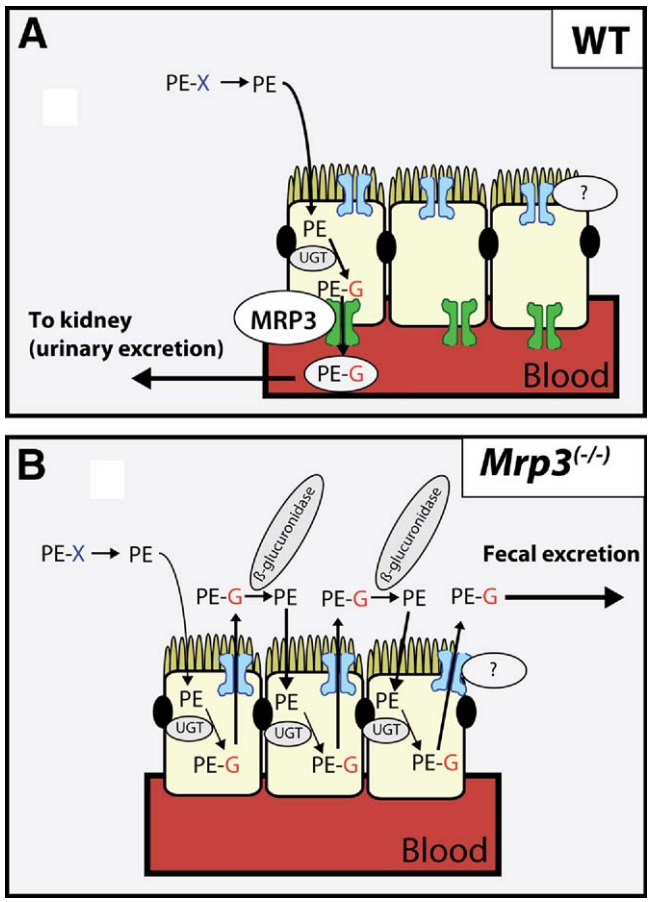

Figure 7. Schematic overview of phytoestrogen (*PE*) handling in enterocytes of the colon of WT (*A*) and *Mrp3*$^{(-/-)}$ mice (*B*). *PE-X* represents PE precursors that are converted into PE in the gut. PEs pass the plasma membrane of the enterocytes of the colon, most probably via passive diffusion. Inside the enterocytes, PEs are subject to glucuronidation by UDP-glucuronosyl transferases (*UGT*). In WT mice, the PE-glucuronides (*PE-G*) formed are predominantly transported over the basolateral membrane toward the blood circulation for urinary excretion. In *Mrp3*$^{(-/-)}$ mice, this transport of PE-G is impaired, leading to accumulation of PE-G in enterocytes and an increased fraction of the PE-G being transported over the apical membrane by an as yet unknown transporter. Once present in the intestinal lumen, bacterial β-glucuronidases can de-glucuronidate PE-G thereby releasing PE, which is subject to another futile cycle of diffusion into enterocytes, conjugation to glucuronic acid, and transport over the apical membrane and de-conjugation. This futile cycle will lead to increased exposure of the intestinal epithelium to PEs and an increased part of PE being excreted via the feces in mice that lack MRP3.

a secondary daidzein metabolite formed in the colon by resident bacteria.[13]

It makes sense that phytoestrogen-glucuronides formed intracellularly, in the enterocytes, are transported toward the circulation for subsequent urinary excretion, instead of being transported over the apical membrane into the gut for fecal excretion. Transport into the gut lumen will probably lead to deglucuronidation by resident bacteria, which especially occurs in the large intestine (ie, colon and cecum). This would initiate a wasteful futile cycle of re-uptake into enterocytes, glucuronidation and transport out of the cell. Avoiding this futile cycle could be especially important for dietary carcinogens, such as the extensively glucuronidated heterocyclic amines, because this would lead to prolonged exposure of the epithelial cells of the gut to carcinogens. We are testing this possibility in *Mrp3*$^{(-/-)}$ mice. In contrast, disposal of glucuronide conjugates via the urine does not result in a futile cycle because urine is sterile and does not contain bacterial glucuronidases. In line with this interpretation, the apical efflux pump MRP2, which also transports many glucuronides,[33] is not present in the colon.[30] A schematic representation of the differences in disposition of phytoestrogen-glucuronides in the colon of WT and *Mrp3*$^{(-/-)}$ mice is given in Figure 7.

Because several of the members of the ABCC subfamily have overlapping substrate specificities, our approach can be applied to any other transporter for which a knockout mouse model is available. The approach can be broadened by using other biologic matrices (for instance, bile, liquor cerebrospinalis, and others) and by including screens for other classes of compounds (such as sulpho- and glutathione conjugates) or even a full metabolomics approach. This should provide more insight into the physiologic function of transport proteins.

Supplementary Data

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2009.06.052.

References

1. Deeley RG, Westlake C, Cole SP. Transmembrane transport of endo- and xenobiotics by mammalian ATP-binding cassette multidrug resistance proteins. Physiol Rev 2006;86:849–899.
2. Borst P, de Wolf C, van de Wetering K. Multidrug resistance-associated proteins 3, 4, and 5. Pflugers Arch 2007;453:661–673.
3. Kruh GD, Belinsky MG, Gallo JM, et al. Physiological and pharmacological functions of Mrp2, Mrp3, and Mrp4 as determined from recent studies on gene-disrupted mice. Cancer Metastasis Rev 2007;26:5–14.
4. Belinsky MG, Dawson PA, Shchaveleva I, et al. Analysis of the in vivo functions of Mrp3. Mol Pharmacol 2005;68:160–168.
5. Zelcer N, van de Wetering K, de Waart R, et al. Mice lacking Mrp3 (Abcc3) have normal bile salt transport, but altered hepatic transport of endogenous glucuronides. J Hepatol 2006;44:768–775.
6. Scheffer GL, Kool M, de Haas M, et al. Tissue distribution and induction of human multidrug resistant protein 3. Lab Invest 2002;82:193–201.
7. Tukey RH, Strassburg CP. Human UDP-glucuronosyltransferases: metabolism, expression, and disease. Annu Rev Pharmacol Toxicol 2000;40:581–616.
8. Manautou JE, de Waart DR, Kunne C, et al. Altered disposition of acetaminophen in mice with a disruption of the Mrp3 gene. Hepatology 2005;42:1091–1098.
9. Zelcer N, van de Wetering K, Hillebrand M, et al. Mice lacking multidrug resistance protein 3 show altered morphine pharmacokinetics and morphine-6-glucuronide antinociception. Proc Natl Acad Sci U S A 2005;120:7274–7279.
10. Qu J, Wang Y, Luo G, et al. Identification and determination of glucuronides and their aglycones in *Erigeron breviscapus* by liquid chromatography-tandem mass spectrometry. J Chromatogr A 2001;928:155–162.
11. van de Wetering K, Zelcer N, Kuil A, et al. Multidrug resistance protein 2 and 3 provide alternative routes for hepatic excretion of morphine-glucuronides. Mol Pharmacol 2007;72:387–394.
12. van de Wetering K, Burkon A, Feddema W, et al. Intestinal BCRP/Bcrp1 and MRP3/Mrp3 are involved in the pharmacokinetics of resveratrol. Mol Pharmacol 2008;75:876–885.
13. Dixon RA. Phytoestrogens. Annu Rev Plant Biol 2004;55:225–261.
14. D'Alessandro TL, Boersma-Maland BJ, Peterson TG, et al. Metabolism of phytoestrogen conjugates. Methods Enzymol 2005;400:316–342.
15. Knust U, Hull WE, Spiegelhalder B, et al. Analysis of enterolignan glucuronides in serum and urine by HPLC-ESI-MS. Food Chem Toxicol 2006;44:1038–1049.
16. Baur JA, Sinclair DA. Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 2006;5:493–506.
17. Setchell KD, Gosselin SJ, Welsh MB, et al. Dietary estrogens—a probable cause of infertility and liver disease in captive cheetahs. Gastroenterology 1987;93:225–233.
18. Adams NR. Permanent infertility in ewes exposed to plant oestrogens. Aust Vet J 1990;67:197–201.
19. Whitten PL, Lewis C, Naftolin F. A phytoestrogen diet induces the premature anovulatory syndrome in lactationally exposed female rats. Biol Reprod 1993;49:1117–1121.
20. Lewis RW, Brooks N, Milburn GM, et al. The effects of the phytoestrogen genistein on the postnatal development of the rat. Toxicol Sci 2003;71:74–83.
21. Setchell KD, Brown NM, Desai P, et al. Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J Nutr 2001;131:S1362–S1375S.
22. Akita H, Suzuki H, Hirohashi T, et al. Transport activity of human MRP3 expressed in Sf9 cells: comparative studies with rat MRP3. Pharm Res 2002;19:34–41.
23. Zeng H, Liu G, Rea PA, et al. Transport of amphipathic anions by human multidrug resistance protein 3. Cancer Res 2000;60:4779–4784.
24. Teng S, Piquette-Miller M. Hepatoprotective role of PXR activation and MRP3 in cholic acid-induced cholestasis. Br J Pharmacol 2007;151:367–376.
25. Jacobs MN, Nolan GT, Hood SR. Lignans, bacteriocides and organochlorine compounds activate the human pregnane X receptor (PXR). Toxicol Appl Pharmacol 2005;209:123–133.
26. Gregory PA, Lewinsky RH, Gardner-Stephen DA, et al. Regulation of UDP glucuronosyltransferases in the gastrointestinal tract. Toxicol Appl Pharmacol 2004;199:354–363.
27. Chen J, Wang S, Jia X, et al. Disposition of flavonoids via recycling: comparison of intestinal versus hepatic disposition. Drug Metab Dispos 2005;33:1777–1784.

28. Kuhnle G, Spencer JP, Chowrimootoo G, et al. Resveratrol is absorbed in the small intestine as resveratrol glucuronide. Biochem Biophys Res Commun 2000;272:212–217.
29. Eeckhaut E, Struijs K, Possemiers S, et al. Metabolism of the lignan macromolecule into enterolignans in the gastrointestinal lumen as determined in the simulator of the human intestinal microbial ecosystem. J Agric Food Chem 2008;56:4806–4812.
30. Maher JM, Slitt AL, Cherrington NJ, et al. Tissue distribution and hepatic and renal ontogeny of the multidrug resistance-associated protein (Mrp) family in mice. Drug Metab Dispos 2005;33:947–955.
31. Jansen GH, Arts IC, Nielen MW, et al. Uptake and metabolism of enterolactone and enterodiol by human colon epithelial cells. Arch Biochem Biophys 2005;435:74–82.
32. Axelson M, Setchell KD. The excretion of lignans in rats—evidence for an intestinal bacterial source for this new group of compounds. FEBS Lett 1981;123:337–342.
33. Borst P, Zelcer N, van de Wetering K. MRP2 and 3 in health and disease. Cancer Lett 2005;234:51–61.

Received April 3, 2009. Accepted June 25, 2009.

Correspondence

Address correspondence to: Piet Borst, MD, Division of Molecular Biology (H8), Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands. e-mail: p.borst@nki.nl; fax: (31) 20-669 1383.

Acknowledgments

The authors thank their colleagues Alfred Schinkel and Hein te Riele (The Netherlands Cancer Institute) for critical reading of the manuscript and Noam Zelcer (HHMI, Department of Pathology and Laboratory Medicine, University of California, Los Angeles) for providing the mouse MRP3 cDNA.

Conflicts of interest

The authors disclose no conflicts.

Funding

Supported by ZonMw for the experimental work in our laboratory (TOP grant number 40-00812-98-07-028, to K.vdW. and P.B.).