

<https://helda.helsinki.fi>

Efflux transport of nicotine, cotinine and trans-3 '-hydroxycotinine glucuronides by human hepatic transporters

Jarvinen, Erkka

2019-12

Jarvinen , E , Sjöstedt , N , Koenderink , J B , Kidron , H & Finel , M 2019 , ' Efflux transport of nicotine, cotinine and trans-3 '-hydroxycotinine glucuronides by human hepatic transporters ' , Basic & Clinical Pharmacology & Toxicology , vol. 125 , no. 6 , pp. 490-498 . <https://doi.org/10.1111/b>

<http://hdl.handle.net/10138/311406>

<https://doi.org/10.1111/bcpt.13281>

cc_by_nc_sa

acceptedVersion

Downloaded from Helda, University of Helsinki institutional repository.

This is an electronic reprint of the original article.

This reprint may differ from the original in pagination and typographic detail.

Please cite the original version.

EFFLUX TRANSPORT of ESTROGEN GLUCURONIDES by HUMAN MRP2, MRP3, MRP4 and BCRP

Erkka Järvinen¹, Feng Deng², Heidi Kidron² and Moshe Finel^{1*}

¹Division of Pharmaceutical Chemistry and Technology, ²Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland

Corresponding Author:

*Moshe Finel, Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki, 00014 University of Helsinki, Finland,

moshe.finel@helsinki.fi

Abbreviations:

estrone sulfate (E₁-S), estrone glucuronide (E₁-G), estradiol-3-glucuronide (E₂-3G), estradiol-17β-glucuronide (E₂-17G), estriol-3-glucuronide (E₃-3G), estriol-16α-glucuronide (E₃-16G), breast cancer resistance protein (BCRP, ABCG2), multidrug resistance associated proteins (MRP, ABCC), multidrug resistance protein 1 (MDR1, P-glycoprotein, P-gp), UDP-glucuronosyltransferases (UGT), ATP-binding cassette transporters (ABC)

ABSTRACT

Estrone, estradiol and estriol are endogenous human estrogens that are rapidly conjugated with glucuronic acid in both intestinal and hepatic epithelial cells. The resulting glucuronides, estrone-3-glucuronide (E_1 -G), estradiol-3- and 17-glucuronides (E_2 -3G and E_2 -17G), as well as estriol-3- and 16-glucuronides (E_3 -3G and E_3 -16G) are found in human plasma and urine. Unlike E_2 -17G, the efflux transport of other estrogen glucuronides by human transporters has not yet been investigated comprehensively. We have studied the transport of E_1 -G, E_2 -3G, E_3 -3G, E_3 -16G and estrone-3-sulfate (E_1 -S), another important estrogen conjugate, using the vesicular transport assay with recombinant human MRP2, MRP3, MRP4, MDR1 and BCRP that were expressed in insect cells. The transport screening assays revealed that whereas E_1 -S was a good and specific substrate for BCRP, the less transporter-specific conjugates, E_1 -G and E_2 -3G, were still transported by BCRP at 10-fold higher rates than E_1 -S. BCRP also transported E_3 -16G at higher rates than the studied MRPs, while it transported E_3 -3G at lower rates than MRP3. MRP2 exhibited lower or equal transport rates of E_1 -G, E_2 -3G, E_3 -3G and E_3 -16G in comparison to MRP3 and BCRP in the screening assays, mainly due to its high K_m values, between 180 and 790 μ M. MRP3 transported all the tested glucuronides at rather similar rates, at K_m values below 20 μ M, but lower V_{max} values than other transporters. In the case of E_3 -3G, MRP3 was the most active transporter in the screening assay. MRP4 transported only E_3 -16G at considerable rates, while none of the tested estrogen conjugates was transported by MDR1 at higher rates than control vesicles. These new results, in combination with previously reported in vivo human data, stimulate our understanding on the substrate specificity and role of efflux transporters in disposition of estrogen glucuronides in humans.

Keywords: drug transporters, steroid disposition, steroid transport, estrogen, glucuronides, steroid excretion

1. INTRODUCTION

Estrogens are important endogenous steroids that play fundamental roles in numerous body functions [1, 2]. In addition, estrogens are widely used as drugs, in both contraception and hormone replacement therapies. Homeostasis and metabolism of estrogens are complex processes that are regulated by oxidative and reductive metabolism (phase I), as well as conjugative metabolism (phase II), resulting in over hundred different biotransformation products in human [3, 4]. The most important naturally occurring estrogens in women are estriol (E_3), estradiol (E_2) and estrone (E_1). Conjugation of estrogens with glucuronic acid (i.e. glucuronidation), is catalyzed by several UDP-glucuronosyltransferase enzymes (UGTs) and results in different estrogen glucuronides. These glucuronides are regarded as end products of estrogen metabolism and they are mostly excreted from the body without further biotransformation. Glucuronidation of estrogens takes place in various tissues, mainly in the prominent metabolizing tissues, namely in the liver, intestine and kidney [4]. The estrogen metabolizing UGTs, such as UGT1A1, UGT1A10 and UGT2B7, are expressed at different levels and in a tissue-specific manner, resulting in variable glucuronidation rates and specificities among the tissues.

Disposition of estrogen glucuronides has a characteristic feature of bile excretion, followed by extensive enterohepatic circulation, which highlights not only the role of metabolizing enzymes but also the role of active efflux transport of the conjugated estrogens in the liver [5, 6]. However, despite extensive bile excretion and enterohepatic circulation of estrogen conjugates, high amounts of conjugated estrogens are also found in the human blood circulation and are excreted, eventually, via urine [7, 8]. Estrone-3-glucuronide (E_1 -G), estradiol-3- and estradiol-17-glucuronide (E_2 -3G and E_2 -17G), estriol-3 and estriol-16-glucuronide (E_3 -3G and E_3 -16G) are the glucuronosyl conjugates of estrone, estradiol and estriol, respectively (Fig. 1). Estrone sulfate (E_1 -S) is the most abundant estrogen conjugate in the blood

circulation and, possibly, acting as a reservoir for free estrogens [3, 4, 7]. The concentration of E₁-S varies in healthy individuals between 0.5-5 nM, but up to 180 nM has been measured during pregnancy [7, 8, 9]. The plasma concentration of estrogen glucuronides is generally over ten-fold lower, with increased concentrations during pregnancy similar to E₁-S.

(Figure 1)

Human ATP-binding cassette (ABC) transporters are a family of multiple efflux transporters that utilize ATP to actively transport compounds across biological membranes [10, 11]. ABC transporters include several pharmacologically, but especially pharmacokinetically, relevant transporters that are localized on plasma membranes of polarized cells [11]. Among them are the multidrug resistance associated proteins 2, 3 and 4 (MRP2-4, *ABCC2-4*) and the breast cancer resistance protein (BCRP, *ABCG2*), which are localized on either the apical or basolateral membranes of human enterocytes, hepatocytes and proximal tubular cells [12, 13]. They contribute to systemic exposure and biliary, intestinal and urinary excretion of their substrates that are mostly anionic and include glucuronide conjugates of drugs, other xenobiotics, as well as endogenous compounds [14]. MRP2 and BCRP are expressed on apical membranes in both hepatocytes and enterocytes, where they restrict systemic exposure of their substrates [12, 13]. MRP3 is localized on the opposite membranes, basolateral, in the same polarized cells, contributing to systemic exposure of its substrates. MRP4 is also expressed on basolateral membranes of hepatocytes and enterocytes [12, 13, 15, 16]. However, it may have a more prominent role in the kidneys, where it is expressed on luminal membranes of the proximal tubular cells, by contributing to active secretion of its substrates into urine [12, 13].

Not much is currently known about the interactions of E₁-G, E₂-3G, E₃-3G and E₃-16G with the human transporters MRP2, MRP3, MRP4, MDR1 and BCRP. Contrary to the aforementioned glucuronides, the transport of E₂-17G has been well characterized and it is a prominent substrate for MRP2, MRP3, MRP4

and BCRP, as well as for several uptake and other efflux transporters [14, 17]. In addition, the transport of E₂-17G by the multidrug resistance protein 1 (MDR1, P-glycoprotein, P-gp) has been reported, although this transporter is rarely considered to contribute significantly to the transport of phase II conjugates [14, 18]. On the other hand, E₁-S is a known, good and widely used substrate for BCRP, as well as for several uptake transporters [14, 17]. Of the four estrogen glucuronides included in this study, E₂-3G was previously reported to be a rather good substrate for MRP2, but to lack the distinctive cooperative transport kinetics which is typical for the transport of E₂-17G by MRP2 [19].

We have now studied the efflux transport of physiologically important estrogen conjugates E₁-G, E₁-S, E₂-3G, E₃-3G and E₃-16G (Fig. 1) by recombinant human transporters MRP2, MRP3, MRP4, MDR1 and BCRP, using the membrane vesicle transport assay. Our aims were to explore the substrate specificity and kinetic differences between these transporters, *in vitro*, in order to improve the understanding of estrogen conjugate disposition *in vivo*. The results add to the scarce knowledge of steroid conjugates transport by human efflux transporters, an important topic in cancer and drug research.

2. MATERIALS and METHODS

2.1 Chemicals and solvents

Sodium salts of E₂-17G, E₃-3G, and E₁-S, as well as E₃-16G were from Sigma Aldrich (St. Louis, MO, USA), sodium salt of E₁-G was from Toronto Research Chemicals (Toronto, ON, Canada) and sodium salt of E₂-3G was from Cayman Chemical (Ann Arbor, MI, USA). Tritium-labelled E₁-S (6, 7-³H, as ammonium salt, specific activity 54 mCi/μmol) and the liquid scintillation cocktail (Optiphase Hisafe 3) were from Perkin Elmer (Waltham, MA, USA). All solvents and formic acid were of analytical grade or better higher and were obtained from Sigma-Aldrich. Water for the analyses and assays was purified using Milli-Q water purification system and filtered through 0.22 μM filter (Merck Millipore, Darmstadt, Germany).

2.2 Expression and vesicle preparation of human MRP2, MRP3, MRP4, MDR1 and BCRP

The human recombinant transporters MRP2, MRP3, MRP4, MDR1 and BCRP were expressed in baculovirus-infected *Sf9* insect cells and inside-out membrane vesicles were prepared from them and used for the vesicular transport assays (see section 2.3) as previously described [20-24]. In addition, control vesicles (Ctrl^M for MRP2-MRP4 and Ctrl^{+C} for MDR1 and BCRP, see below) were prepared from *Sf9* insect cells that were transfected with baculovirus containing no human cDNA.

MDR1 and BCRP vesicles were supplemented with additional cholesterol to enhance their transport activity, as reported previously [25, 26] and carried out in our laboratory [21-24]. Accordingly, also the control vesicles for MDR1 and BCRP assays were cholesterol loaded (Ctrl^{+C}).

2.3 Vesicular transport assays

The vesicular transport assays were carried out in 96-well polystyrene plates at a final volume of 75 μl per well, as previously described [19, 20, 21, 22]. The assay mixture consisted of 40 mM MOPS (adjusted

to pH 7.0 with Tris-HCl), 6 mM MgCl₂, 60 mM KCl, 7 mM Tris-HCl, 7 mM mannitol and 0.3 mM EGTA. The total vesicle protein amount in the assays were either 40 µg (MRP2, MRP3, MRP4, MDR1 and Ctrl^M) or 20 µg (BCRP and Ctrl^{+C}) per well. The substrate stock solutions were prepared in DMSO, at 50 mM concentration, and stored at -20 °C. Subsequent substrate dilutions were done in the assay buffer (MOPS-MgCl₂-KCl), resulting in a final DMSO concentration of either 0.02% (initial screening assays) or 1.0% (kinetic assays) in the transport assay.

Transport assay mixtures were prepared on ice prior the pre-incubation at 37 °C for 10 min. Transport reactions were initiated by the addition of either Mg-ATP to a final concentration of 4 mM (+ATP samples) or blank reaction mixture (-ATP samples), both were pre-incubated at 37 °C. The transport assays, following initiation, were carried out for pre-determined times (1-6 min, see figure legends for the incubation time of each experiment) at 37 °C and constant shaking at 500 rpm. For the kinetic assays, the incubation times were selected based on prior linear transport assays of each substrate-transporter combination (See data in the supplementary material, Fig. S1). The transport reactions were quenched by adding 200 µl of cold buffer (70 mM KCl and 40 mM MOPS pH 7.0) and were transferred to a 96-well filter plate (pore size 1.0 µm, glass fiber filters, from Merck Millipore, Darmstadt, Germany). The samples were then filtered and washed with five aliquots of the same cold buffer under vacuum filtration. The filter plate was subsequently dried at room temperature, after which 100 µl of 1:1 acetonitrile: 0.2% formic acid in water, containing E₂-17G as internal standard, was applied to each well. The plate was then incubated at room temperature for 30-60 min under gentle shaking. Finally, the filter plate was centrifuged for 2 min at 3000 g to collect the samples (filtrate) into a new well plate and the samples were subjected to analysis by LC-MS/MS (see section 2.4).

Unlike the estrogen glucuronides, the transport of E₁-S was assayed using a radioactively labelled compound. In this case, the transport assays included 1-150 nCi of tritium labelled E₁-S per well. The

transport reactions were carried out as described above for the estrogen glucuronides, but quantification of E₁-S was done by the addition of 50 µl scintillation cocktail to each well, followed by incubation of the plate at room temperature for 30 min before radioactivity counting using a Microbeta 1450 Trilux scintillation counter (from Wallac, Turku, Finland). In addition, when labelled E₁-S was used, the filter-plate was pre-soaked, before transferring the assay samples, with 100 µl of 50 µM unlabeled E₁-S to decrease the unspecific binding of the labelled compound.

Transport assays were conducted in triplicate samples for each time and concentration point, including both +ATP and –ATP samples. The experimental data are reported as means ± SD of retained compound within the vesicles per amount of total vesicle protein per incubation time, resulting in pmol/min/mg protein values. Kinetic assays were conducted using at least six different substrate concentrations and the data are reported as means of ATP-dependent transport ± SD after subtracting the -ATP values from the +ATP values. Kinetic data were fitted to the Michaelis-Menten equation ($v = V_{\max} [S] / ([S] + K_m)$), using least squares fit in GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA), that was used also for data visualization. The goodness of fit was inspected in each case both visually, using Eadie-Hofstee transformation of the experimental data (Suppl. Fig. 2), as well as by the coefficient of determination (R^2) value of the fit (Table 1). In addition, linear substrate transport versus concentration, in the absence of ATP (passive), was inspected to exclude artifacts, such as solubility limitations, during the assays. The substrate solubility in the reaction mixtures, at the used concentrations, was tested before the transport assays by HPLC analyses, visual inspection and nephelometer analyses (Nepheloskan Ascent, Labsystems, Finland).

2.4 Analytical methods

The amounts of E₁-G, E₂-3G, E₃-3G and E₃-16G that were retained in the vesicles at the end of the transport assays, were quantified by triple quadrupole mass spectrometry (Xevo TQ-S), connected to an

Ultra Performance Liquid Chromatography (ACQUITY UPLC I Class), both from Waters (Milford, MA, USA). Samples on a 96-well plate (kept at 15 °C) were injected (1-5 µl) into Acquity UPLC BEH C18 (2.1x100 mm, 1.7 µm from Waters) column that was kept at 30 °C and operated at a flow rate of 0.4 ml/min. The chromatography eluents were water (A) and acetonitrile (B), both containing 0.1% formic acid. The gradient program (0-2.5 min 10% B to 65% B, 2.5-4 min 95% B followed by at least 1 min equilibrium at 10% B) was used to elute E₃-3G, E₃-16G, E₂-3G, E₂-17G (internal standard) and E₁-G at retention times of 1.33, 1.72, 1.95, 2.08 and 2.16 min, respectively.

The mass spectrometry was operated in negative electrospray ionization mode, using nitrogen as ion source gas and argon as collision gas, both from Aga (Espoo, Finland). The operation parameters were set as following: capillary voltage at -2.0 kV, cone at 1.0 V, source offset at 50 V, source temperature at 150 °C, cone gas flow at 150 l/h, nebulizer gas pressure at 7.0 bar, as well as desolvation gas temperature and flow rate at 650 °C and 1100 l/h, respectively. Quantification was done using MS/MS mode, by selecting deprotonated precursor ions [M-H]⁻ at the first quadrupole (463.2 m/z for E₃-3G and E₃-16G, 447.2 m/z for E₂-3G and E₂-17G and 445.2 m/z for E₁-G), fragmenting them at the second quadrupole (collision energies were 45 V, 30 V, 35 V, 28 V and 38 V for E₃-3G, E₃-16G, E₂-3G, E₂-17G and E₁-G, respectively) and monitoring the product ions resulting from the loss of glucuronic acid [M-H-176]⁻ (287.2 m/z for E₃-3G and E₃-16G, 271.2 m/z for E₂-3G and E₂-17G, as well as 269.2 m/z for E₁-G). An additional product ion at 113.0 m/z, for qualitative confirmation, was monitored for all the analytes using 20-30 V collision energies. Dwell time was 100 ms for all the monitored reactions.

The ratio of analyte to internal standard (E₂-17G) was used for the quantification. The standard curve samples were prepared similarly to the test samples, namely in 100 µl of 1:1 acetonitrile: 0.2% formic acid in water containing E₂-17G as internal standard, and filtered through pre-wetted and dried filter well plates. The linear range of quantification ($R^2 \geq 0.99$ in each case) was adjusted for each assay and

compound, usually the lower limit for quantification was 1 nM and the upper limit of quantification was 1000-5000 nM, depending on the analyte.

3. RESULTS

3.1 Screening of estrogen conjugate transport

The transport of estrogen glucuronides and E₁-S (see structures in Fig. 1) was first tested using a single substrate concentration of 10 μM (for time-dependent transport of all the tested estrogen conjugates and transporters, also when no transport activity was found, see Supplementary Figure S1). The results of the initial screening experiment clearly showed that E₁-S was not transported by any of the tested MRPs or by MDR1 (Fig. 2A). Even the addition of 5 mM glutathione (GSH) to the transport assays with MRPs did not change this (results not shown). In sharp contrast to the MRPs, BCRP transported E₁-S at high rates (Fig. 2A), in agreement with previous reports [14, 27].

BCRP was also highly active in the transport of E₁-G (Fig. 2B) and E₂-3G (Fig. 2C). In the case of estriol glucuronides, however, BCRP exhibited much lower activity toward E₃-3G in comparison to E₁-G and E₂-3G (Fig. 2D). On the other hand, BCRP transported E₃-16G at higher rates than each of the other studied transporter (Fig. 2E), but the rate was still clearly lower than the transport rates of E₁-S, E₁-G and E₂-3G by BCRP (Fig. 2A-C).

(Figure 2)

The transport activity of MRP2 in the initial screening toward E₁-G and E₃-3G was lower in comparison to E₂-3G and E₃-16G (Fig. 2B-E). However, even in both latter cases the activity of MRP2 was clearly lower than the activity of BCRP and similar to the rates exhibited by MRP3 (Figs. 2B and 2C).

MRP3 transported all the tested estrogen glucuronides at rather similar rates, at least under the conditions of the initial screening experiments where the substrate concentration was 10 μM (Figs. 2B-2E). MRP4, on the other hand, transported only E₃-16G at considerable rates (Fig. 2B-E), exhibiting quite clear and narrow selectivity in the transport of the studied estrogen glucuronides. Of the transporters included in

this study, MDR1 was the only one not transporting any of the tested estrogen conjugates at higher rates than the control vesicles (Fig. 2 and Supplementary figure S1).

It should be noted here that precise rate comparisons between different efflux transporters in this study, particularly when the differences are not very large, should be considered with care. Presently, we do not have a method to accurately measure the amount of active transporter in the vesicle preparations. On the other hand, the transport rates of different substrates by the same transporter could be compared reliably in this study, since they were done with the same vesicle preparation.

3.2 Kinetic analysis of estrogen conjugate transport

Kinetic analyses were carried out for all the tested transporters that exhibited substantial activity in the initial screening assay (Fig. 2). The kinetic curves are presented for each transporter separately (Figs. 3-5) and the derived kinetic constants of the fitted model are listed in Table 1.

The kinetic assay results of BCRP transport of E₁-G and E₂-3G (Fig. 3A) revealed that this transporter reaches higher V_{max} values with these glucuronides than any other studied transporter while its K_m values are in the moderate range, below 100 μM (Table 1). In the case of E₁-S, the K_m value for BCRP was very low, 1.2 μM (Fig. 3B and Table 1). However, the V_{max} of E₁-S transport by BCRP was about 10-fold lower than for E₁-G and E₂-3G, whereas the K_m values for these estrogen glucuronides, although in the moderate range, were over 60-fold larger than for E₁-S transport by BCRP (Table 1). The transport kinetics of E₃-3G by BCRP was not saturable at the studied concentrations (Fig. 3C), a result that is in agreement with the initial screening result that revealed lower transport rates of this glucuronide by BCRP (Fig. 2D). Contrary to E₃-3G, the transport of E₃-16G by BCRP was saturable at low concentrations, yielding a low K_m value of 29 μM (Fig. 3C and Table 1), but also a V_{max} value in the

same range as for the transport of E₁-S, namely much lower than the V_{max} values of BCRP for the transport of E₃-3G or E₁-G and E₂-3G (Table 1).

(Figure 3)

MRP2 exhibited lower transport rates of E₁-G, E₂-3G, E₃-3G and E₃-16G in the initial screening assays (Figs. 2B-E) and the kinetic assays (Fig. 4, Table 1) suggest that the prime reason for this was higher K_m values in comparison to the other transporters, particularly in the cases of estriol glucuronides, not lower V_{max} values (Table 1). The transport kinetics of the studied estrogen glucuronides by MRP2 followed the Michaelis-Menten equation (Fig. 4, Table 1 and Suppl. Fig 2) and no indication of cooperative kinetics for MRP2 was found, when the data was analyzed with Eadie-Hofstee transformations (Suppl. Fig. 2). This is a clear difference between the tested glucuronides and E₂-17G, an estrogen glucuronide of which transport by MRP2 is well described with cooperative kinetics [14].

(Figure 4)

The transport of estrogen glucuronides by MRP3 differed from the other tested transporters by its nearly similar rates for all the tested glucuronides in the initial screening assays (Fig. 2B-E). The kinetic analyses (Figs. 5A and 5B) further revealed that in the case of MRP3, there were no large differences between the transport kinetics of the studied estrogen glucuronides. The maximal transport velocities varied by no more than two-fold and the K_m values ranged from 2.8 to 18.2 μM (Table 1), demonstrating similar transport activity for E₁-G, E₂-3G, E₃-3G and E₃-16G by MRP3. These results also indicate that the affinity of MRP3 for the transport of all the tested estrogen glucuronides is higher than the corresponding values for BCRP and MRP2, even if the V_{max} values of MRP3 are generally lower.

(Figure 5)

Among the tested transporters, only MRP4 exhibited selective transport of only one of the studied compounds, E₃-16G, at substantial rates (Fig. 2). Kinetic analysis revealed that this transport follows the Michaelis-Menten equation and both the K_m and the V_{max} values of MRP4 are higher than the corresponding values for E₃-16G transport by MRP3 (Fig. 5C and Table 1).

4. DISCUSSION

The most frequently used substrate for efflux transporters, E₂-17G, is an estrogen glucuronide [14]. In this study, however, we have examined the transport of four other estrogen glucuronides and an estrogen sulfate, by the ATP-dependent efflux transporters MRP2, MRP3, MRP4, MDR1 and BCRP. Our results reveal new information on the substrate specificity of the transporters and differences between them in this respect. It is essential to take into account the location of each transporter in polarized epithelial cells of human intestine, liver and kidney when considering the new results (Fig. 6), since this location could determine and affect the disposition of the studied estrogen conjugates in vivo.

While we cannot directly compare the transport rates and V_{max} values by the recombinant transporters to each other, since expression levels may differ somewhat among vesicle preparations, the changes in these values from one substrate to another and the magnitude of the values are clearly informative. In addition, the K_m values of the kinetic analyses (Table 1) provide indications on respective affinity to the substrates and how it differs among transporters and between substrates.

(Figure 6)

4.1. Transport of E₁-S

No transport of E₁-S was observed by any other transporter studied here except BCRP that transported E₁-S at a high affinity, as suggested by the low K_m value (Figs. 2A and 3B, and Table 1). The E₁-S transport results are in line with previously published findings for BCRP, MRP2 and MRP3 [27-29]. Like MRP2 and MRP3, the additional efflux transporters included in this study, MRP4 and MDR1, also did not exhibit transport activity toward E₁-S (Fig. 2A). It may be interesting that similar results were previously found for the sulfate metabolite of another estrogen, ethinylestradiol sulfate that is transported by BCRP, but not by MRP2, MRP3 or MRP4 [30, 31].

Since E₁-S is found at high amounts in the bile [32], apical excretion in the liver could be explained by BCRP that solely transported this compound (Figs. 2A and 3B, and Table 1). Nonetheless, E₁-S is also found in the human blood circulation, suggesting that basolateral transport from the liver also takes place, alongside the apical transport into bile [4, 7]. Hence, an interesting question is how E₁-S crosses the basolateral membranes of the liver. MRP4 could have been a candidate transporter for this activity since it carries steroid sulfates such as dehydroepiandrosterone sulfate [14]. However, under our experimental conditions no E₁-S transport by MRP4, neither by the other basolateral transporter MRP3, was observed (Fig. 2A). Thus, it is likely that other basolateral transporters are involved in the systemic excretion of E₁-S in human. It has actually been reported that MRP1 and OST α/β transport E₁-S in vitro and this might explain the hepatic in vivo basolateral transport of E₁-S, although the former transporter may be expressed at low levels in healthy human livers [33-36]. Further studies are needed to fully clarify this issue.

4.2. Transport and disposition of E₁-G and E₂-3G

In the human intestine, estrone undergoes direct glucuronidation and sulfation, whereas in the liver only estrone sulfation is catalyzed at high rates [37, 38]. Estrone glucuronidation is catalyzed almost only by the extrahepatic UGT1A10 [38], while its sulfonation is primarily catalyzed by the high-affinity sulfotransferase SULT1E1 that is expressed in both the liver and small intestine [37]. Obviously, the expression level of UGTs and SULTs in different tissues have an effect on the over 10-fold higher concentrations of E₁-S than E₁-3G in human plasma [7, 39]. However, intestinal and hepatic efflux transporters, including their localization in the plasma membranes may also contribute to relative plasma levels of estrone glucuronide and sulfate. Unlike estrone, estradiol is mainly glucuronidated in the liver resulting in E₂-17G as the main glucuronide and E₂-3G as a minor product [40, 41]. In the intestine, however, estradiol is almost exclusively glucuronidated to E₂-3G [40].

The rapid metabolism of exogenously administered estradiol to estrone, and partly to estriol, complicates the determination of glucuronidation contribution to total estradiol metabolism and the subsequent impact of different efflux transporters on the disposition of estradiol glucuronides [1, 39]. In addition, particularly in the case of E₂-17G, the hepatic uptake transporters may also play important roles in its disposition, as indicated by the fact that there is only minor direct urinary excretion of E₂-17G when it was administered, as such, via parenteral route, which is in line with findings using in vitro expressed hepatic uptake transporters [17, 42]. E₂-17G is a substrate for the three MRPs, included in this study, and BCRP [14], while the other glucuronide of estradiol, E₂-3G, was transported in this study by the same transporters as E₂-17G, with the exception of MRP4 (Fig 2C).

Interestingly, none of the transporters appear to differ between the transport of E₁-G and E₂-3G. However, the affinity of MRP3 toward E₁-G and E₂-3G, as suggested by its K_m values for these glucuronides, is 10-fold higher than in the case of BCRP, while the K_m values of MRP2 for both glucuronides are rather high, suggesting poor affinity (Figs. 3A, 4A, 5A and Table 1). Contrary to E₂-17G, in the cases of E₁-3G and E₂-3G the hepatic uptake transporters might play less prominent roles and these glucuronides have been reported to be excreted mainly into urine from the blood circulation, without further enterohepatic circulation [42, 43]. Thus, systemic excretion of E₁-G and E₂-3G may be controlled by MRP3 in the basolateral membranes of the intestine. Nevertheless, also apically expressed MRP2 and BCRP could contribute to the disposition of E₁-G and E₂-3G (Figs. 2-6 and Table 1), especially in the intestine where these glucuronides are formed and both the above transporters are expressed [12, 38, 40]. It should be noted that UGT1A10 and MRP3 have higher and BCRP lower expression in the large intestine than in the small intestine, which may mean that more E₁-3G and E₂-3G are formed in the distal parts of the intestine from unconjugated estrogens and subsequently these glucuronides are transported to the blood circulation by MRP3 [44, 45].

(Table 1)

4.3. Transport and disposition of E₃-3G and E₃-16G

Estriol is assumed to be an end product of estrogen metabolism (endogenous and exogenously administered) and it is extensively conjugated directly to E₃-3G and E₃-16G in the intestine, or only to E₃-16G in the liver [1, 5, 46]. After oral administration of estriol, the glucuronides circulate at relatively high levels, almost 1000-fold higher than the parent compound, until they are finally excreted into urine [1, 47, 48]. The enterohepatic circulation of estriol conjugates does not seem to be as extensive as for estradiol and estrone conjugates [5, 6, 49]. In the urine, E₃-16G is the major metabolite of estriol and the intestinal specific metabolite E₃-3G is present at about 10-20% of the total estriol [50, 51].

The observations above suggest that efflux transporters are responsible for most of the estriol glucuronide disposition. The predominant role of the basolateral efflux of estriol glucuronides is in line with our results (Figs. 2D and 2E and Table 1). We found that of the tested transporters, E₃-3G has a high affinity only to MRP3 (Fig. 5B and Table 1). This result supports the significance of basolateral excretion of this glucuronide from the human intestine. Based on our results, E₃-3G seems to be a rather specific substrate for MRP3 in the human small intestine. This may mean that oral administration of estriol, followed by plasma profiles of E₃-3G, could serve as a marker for intestinal MRP3 function. Especially, because there is no indication of active uptake of E₃-3G into the liver or other tissues and this glucuronide is rapidly excreted into urine when it is administered to humans, as such [52]. In addition, the large intestine could be exposed to higher amounts of unconjugated estriol, part of which could be conjugated into E₃-3G, because of the enzymes catalyzing estriol glucuronidation, only UGT1A10, is expressed in this tissue [45, 46]. The significance of MRP3 in the disposition of E₃-3G may also be higher in the large intestine, because its higher expression in comparison with the small intestine [44].

Although estriol carries two hydroxyl groups in the D ring, hepatic glucuronidation occurs only at the hydroxyl in the 16, not 17 position, resulting in the formation of E₃-16G [46]. We found a high affinity transport of E₃-16G by the basolateral transporter MRP3, whereas the affinity for E₃-16G by the other rather highly expressed hepatic transporter, the apical MRP2, as far as suggested by the K_m value, was low (Figs. 4B and 5B, Table 1). In addition, BCRP and MRP4 transported E₃-16G with moderate K_m values (Figs. 2C and 2B, Table 1). Thus, it is likely that MRP3 is the main contributor to the disposition of E₃-16G from the liver, due to its lower K_m value and higher expression level in comparison to MRP4 and BCRP [36]. Our results and the latter suggestion are supported by reported findings in humans that have indicated the predominant excretion route of E₃-16G to be into the circulation and subsequently to the urine, even if some E₃-16G is also excreted into bile [5, 6, 49-51]. However, how much each transporter contributes to the disposition of E₃-16G in the liver, is difficult to determine or predict accurately, not least due to variability in expression levels of the different transporters in this tissue [36]. Especially MRP4 and BCRP are reported to be expressed at low levels in healthy human livers in comparison to MRP2 and MRP3 [35, 36]. In addition, hepatic uptake transporters might also contribute to disposition of E₃-16G, as indicated by some biliary excretion following its administration, as such, to humans via parenteral route [49, 53].

In humans, active renal secretion of E₃-16G has been reported [47, 48]. Our in vitro results are in agreement with this, since E₃-16G was transported by both MRP2 and MRP4, two important kidney transporters [12, 13] (Fig. 2E and Table 1). The renal clearance of E₃-16G exceeds inulin clearance by 3-8 times, whereas the renal clearance of E₃-3G (not transported by MRP4, Fig. 2D) is only 1-2 times that of inulin clearance, when both conjugates are formed from endogenous estriol [47, 48]. However, estriol may also be glucuronidated, in vivo, in the human kidney to E₃-16G, but not to E₃-3G, a factor that complicates full comparison between the renal clearances of these two glucuronides [54]. On the

other hand, the ratio between estriol glucuronides and the parent compound in the blood circulation is remarkably high, almost 1000, suggesting that the renal glucuronidation may only have a small impact, and the high excretion of E₃-16G is a result of uptake from the circulation and subsequent efflux transport in the kidney [1]. While MRP2 is also expressed on the apical membranes of proximal tubule cells [12], the results of this study showed that the K_m value of MRP2 for the transport of E₃-16G is more than 10-fold higher than the corresponding value of MRP4 (Table 1), suggesting that MRP4 is likely to play a major role in the renal excretion of E₃-16G.

It may be interesting that while each of the tested transporters exhibited rather similar activity and kinetics toward both E₁-3G and E₂-3G (Figs. 3-5), two of the estrogens that carry glucuronic acid in ring A, this similarity does not extend to E₃-3G, even though its glucuronic acid is in the exact same position as in E₁-3G and E₂-3G (Fig. 1). The differences between transport of E₁-3G, E₂-3G and E₃-3G are most obvious in the case of BCRP (Figs. 2D and 3C, and Table 1), but are also seen in MRP2. MRP3, in contrast to BCRP and MRP2, was only little affected by the substrate change to E₃-3G, even if its K_m value in this case was somewhat higher than for any other of the glucuronides in this study, including E₃-16G (Table 1). While it is currently unclear why the differences between estradiol and estriol have strong effect on BCRP and MRP2, this might provide a tool to explore the structure of the binding sites of these transporters and understand similarities and differences between them.

Summary

We have studied here the efflux transport of E₁-G, E₂-3G, E₃-3G, E₃-16G and E₁-S by recombinant human transporters MRP2, MRP3, MRP4, BCRP and MDR1, using inside-out membrane vesicles. Among these transporters, BCRP exhibited the highest transport rates of E₁-G and E₂-3G, while E₁-S was a good, specific and high affinity substrate for it. MRP2 exhibited low affinity and MRP3 rather high transport affinity toward all the tested estrogen glucuronides, but at moderate rates. As a result, E₃-3G

was efficiently transported almost only by MRP3. From the tested estrogen glucuronides MRP4 transported only E₃-16G, and MDR1 did not transport any of the aforementioned estrogen conjugates at detectable rates. Our results provide new details and in vitro explanations for most of the already known in vivo disposition data of estrogens, thereby improving our understanding of how these estrogen glucuronides are disposed in humans and what are the underlying molecular mechanisms.

ACKNOWLEDGEMENTS

We would like to thank Johanna Mosorin for her skillful help in the transport vesicle preparations and Noora Sjöstedt for preliminary transport experiments.

The funding from the University of Helsinki Doctoral Program in Drug Research, Sigrid Juselius Foundation (grant no. 4704583) and the Academy of Finland (grant no. 292779) is acknowledged.

CONFLICTS OF INTEREST: NONE

Table 1. Kinetic constants for the studied estrogen conjugates and transporters.

Compound	K_m	V_{max}	R^2
	μM (95% CI)	pmol/mg/min (95% CI)	
MRP2			
E₁-G	241 (210-273)	884 (830-938)	0.99
E₂-3G	180 (145-216)	1700 (1560-1840)	0.97
E₃-3G	791 (640-942)	1800 (1560-2030)	0.99
E₃-16G	773 (596-949)	6440 (5330-7540)	0.99
MRP3			
E₁-G	7.3 (5.6-8.9)	182 (171-194)	0.92
E₂-3G	2.8 (2.0-3.6)	260 (245-274)	0.86
E₃-3G	18 (16-21)	441 (419-463)	0.98
E₃-16G	4.8 (3.4-6.3)	195 (180-211)	0.90
MRP4			
E₃-16G	65 (53-77)	522 (491-554)	0.96
BCRP			
E₁-G	74 (65-82)	9310 (8940-9690)	0.99
E₂-3G	81 (50-112)	7910 (7010-8810)	0.88
E₃-3G	1020 (736-1300)	4410 (3500-5320)	0.99
E₃-16G	29 (21-37)	1080 (1000-1170)	0.92
E₁-S	1.2 (0.78-1.7)	817 (745-889)	0.83

The kinetic parameters are derived from the experimental data, presented in Figures 3-5, fitted in the Michaelis-Menten equation. The 95% confidence intervals (CI) for the derived kinetic values are presented in the parentheses. For the experimental details, see Figures 3-5.

FIGURES

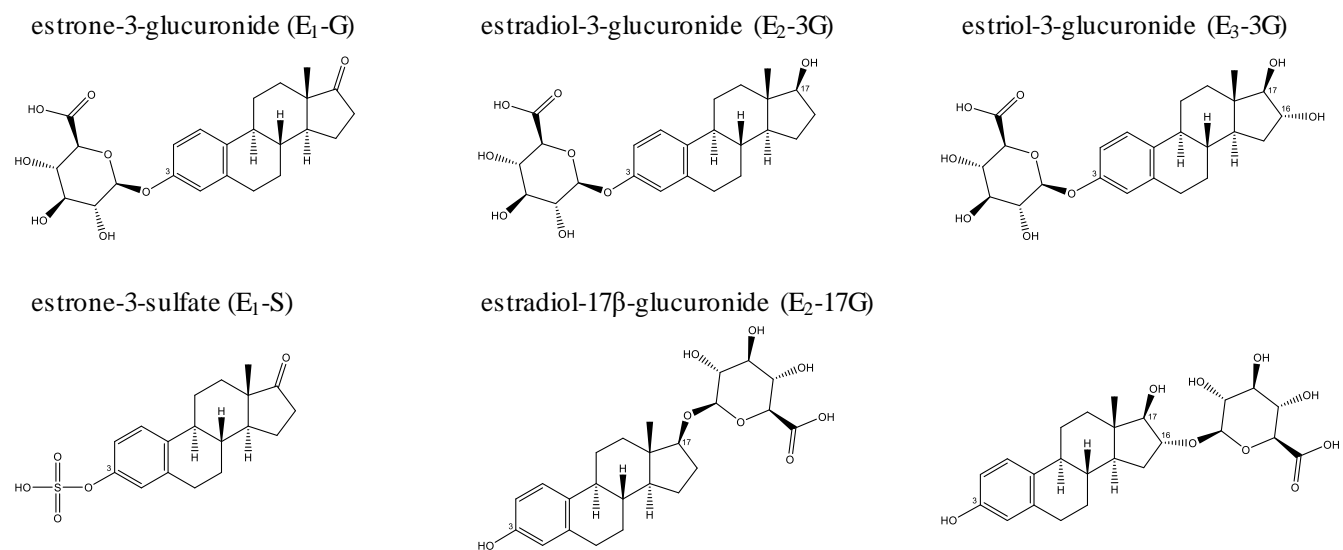


Fig. 1. Structures of the estrogen conjugates.

Structures of the studied glucuronides of estrone, estradiol and estriol, estrone sulfate and E₂-17G.

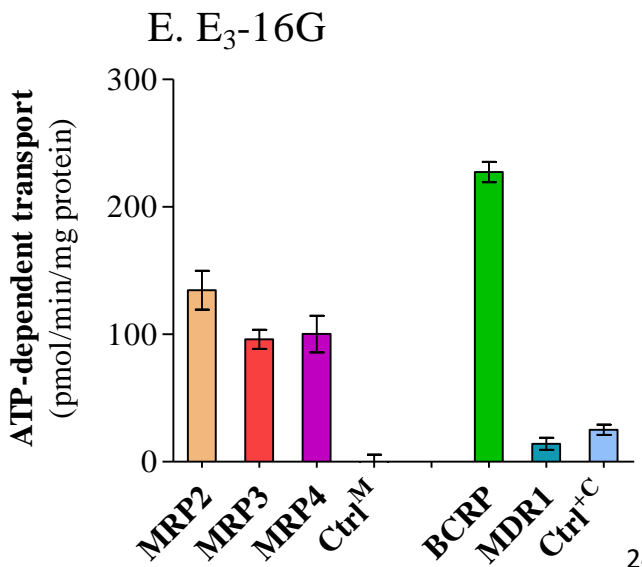
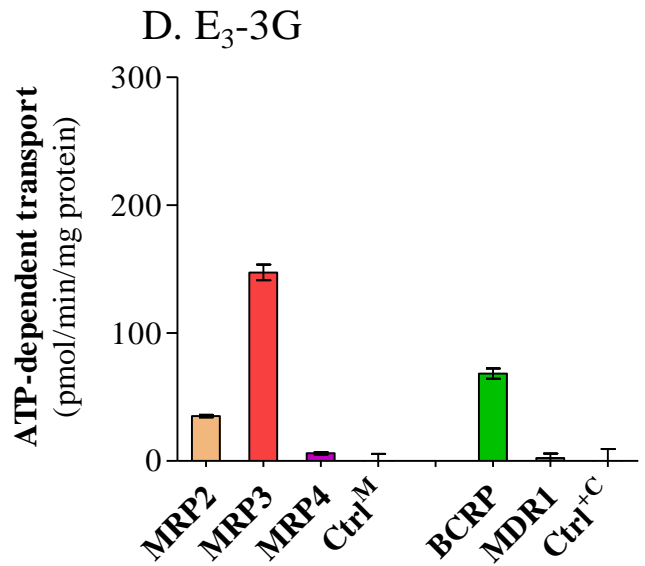
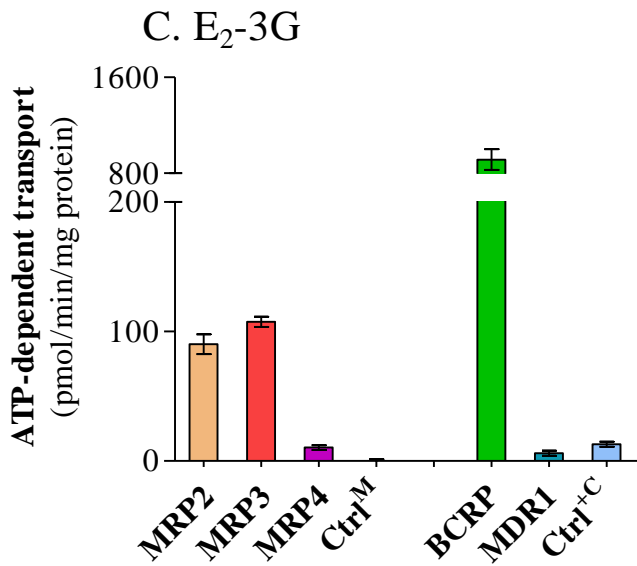
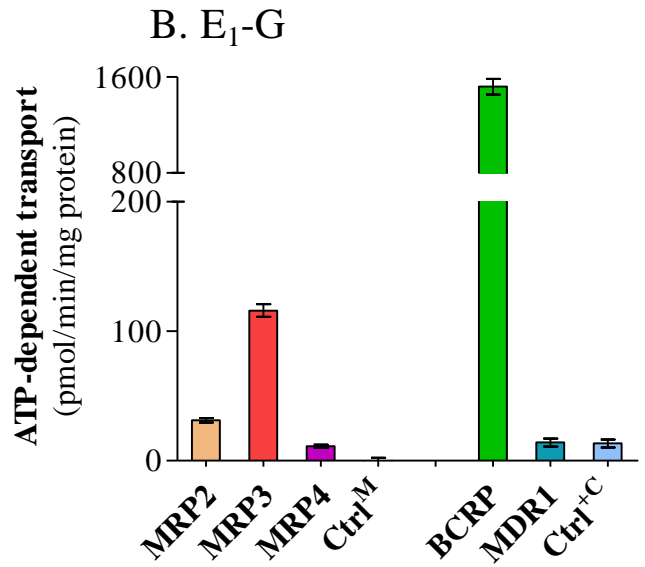
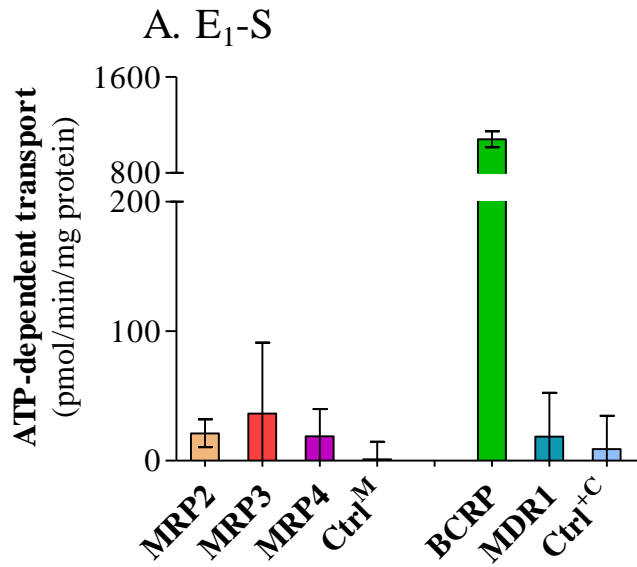


Fig. 2. Screening results of E₁-S, E₁-G, E₂-3G, E₃-3G and E₃-16G transport.

Transport of E₁-S (A), E₁-G (B), E₂-3G (C), E₃-3G (D) and E₃-16G (E) by MRP2, MRP3, MRP4, MDR1 and BCRP was studied using 10 μ M substrate concentration and 2 min incubation. The transport assays contained 40 μ g (MRPs, MDR1 and Ctrl^M) or 20 μ g (BCRP and Ctrl^{+C}) of total vesicle protein per sample. Control vesicles, containing no human transporter, were included in all assays and are presented as Ctrl^M for MRPs, or Ctrl^{+C} for MDR1 and BCRP (MDR1, BCRP and Ctrl^{+C} vesicles were supplemented with cholesterol, see section 2.2). The columns represent ATP-dependent transport and the data are from a single experiment that was conducted in triplicate samples, and the error bars represent \pm SD.

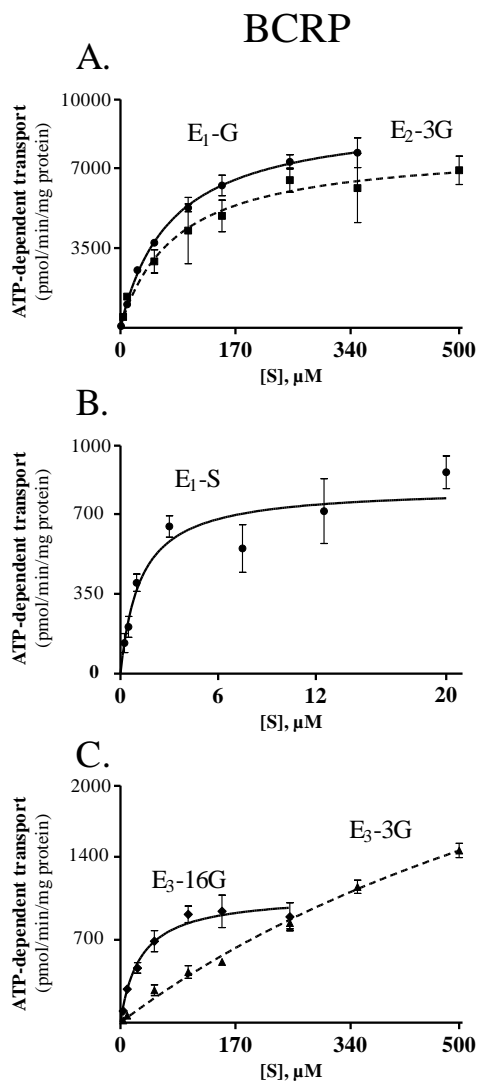


Fig. 3. BCRP transport kinetics of E₁-S, E₁-G, E₂-3G, E₃-3G and E₃-16G.

The ATP-dependent BCRP transport kinetics of E₁-G and E₂-3G (A), E₁-S (B) and E₃-3G and E₃-16G (C) were studied during 1 min (E₁-S), 2 min (E₁-G, E₂-3G and E₃-16G) or 6 min (E₃-3G) incubations. In each sample, the total vesicle protein amount was 20 μg. The fitted model was the Michaelis-Menten equation and the fitting is presented by the lines. The data points represent means of the ATP-dependent values ± SD, from a single experiment conducted in triplicate samples.

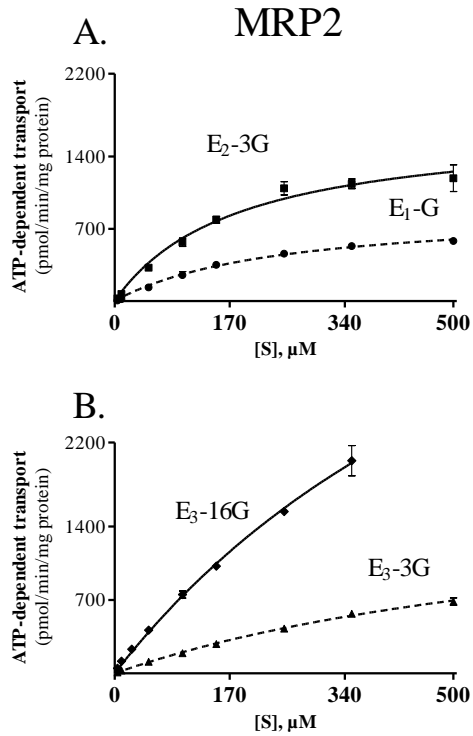


Fig. 4. MRP2 transport kinetics of E₁-G, E₂-3G, E₃-3G and E₃-16G.

The ATP-dependent MRP2 transport kinetics of E₁-G and E₂-3G (A), as well as E₃-3G and E₃-16G (B), were assayed either for 2 min (E₂-3G and E₃-16G) or 6 min (E₁-G and E₃-3G). In each sample, the total vesicle protein amount was 40 μg . The fitted model was the Michaelis-Menten equation and the fitting is presented by the lines. The data points represent means of the ATP-dependent values \pm SD from a single experiment conducted in triplicate samples.

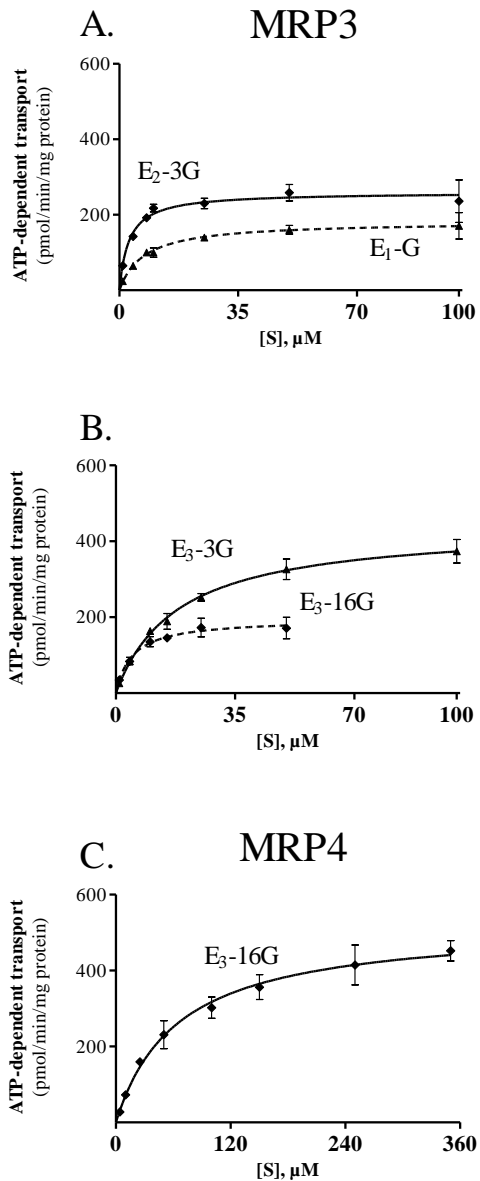


Fig. 5. MRP3 transport kinetics of E₁-G, E₂-3G, E₃-3G and E₃-16G, and MRP4 transport kinetics of E₃-16G.

The ATP-dependent MRP3 transport kinetics of E₁-G and E₂-3G (A), as well as E₃-3G and E₃-16G (B), were assayed for either 1 min (E₂-3G) or 2 min (E₁-G, E₃-3G and E₃-16G). The ATP-dependent MRP4 transport kinetics of E₃-16G (C) was studied using 2 min incubation. In each sample, the total vesicle protein amount was 40 μg . The fitted model was the Michaelis-Menten equation and the fitting is

presented by the lines. The data points represent means of the ATP-dependent values ± SD from a single experiment conducted in triplicate samples.

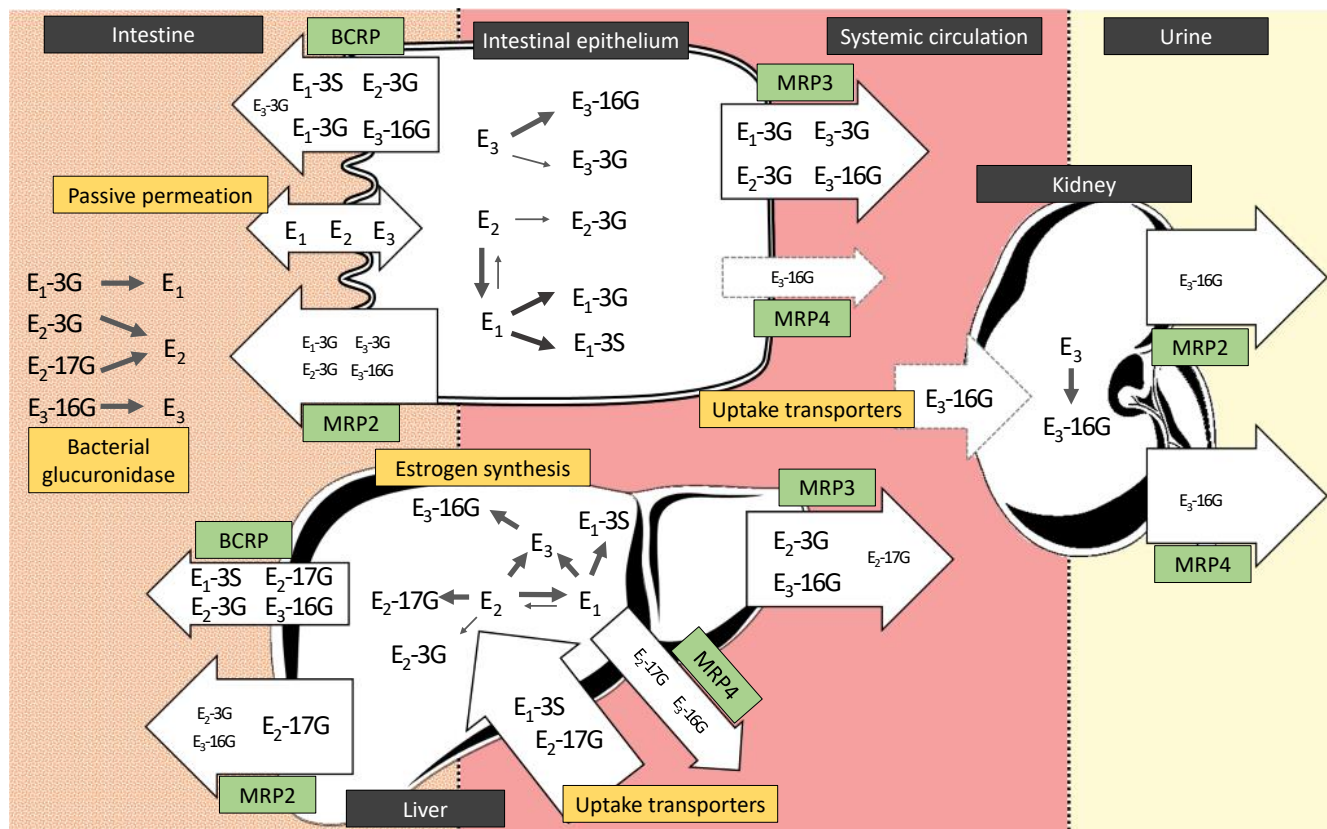


Fig. 6. Disposition of human estrogen conjugates; a schematic presentation based on a combination of new and previous results.

Transporters are represented as white arrows, the width of which indicates whether it is relatively highly or lowly expressed in the tissue and dashed outlines stand for speculative transporters or mechanisms that have limited evidence. The liver schema stands for a typical hepatocyte. Conjugated and unconjugated estrogens are represented by abbreviations containing numbers and letters. The abbreviation of a compound denotes the type of estrogen (E₁, E₂, and E₃ standing for estrone, estradiol, and estriol, respectively) and the latter part describes the conjugation position and the type of conjugate, as in the main text. Larger compound names indicate higher transport rates of the given substrate by the

specific transporter. Black arrows inside the cells represent estrogen biotransformation reactions and the relative extent of these reactions. The figure is based on results from the present study and from previously published works [1, 6, 15-17, 22, 36-38, 40, 41, 44, 46-48, 54, 55].

Supplementary materials:

Figure S1: Eadie-Hofstee transformations of the kinetic data presented in Figures 3-5

Figure S2: Transport versus time curves for each of the studied estrogen conjugate and transporter

REFERENCES

- [1] H. Kuhl, Pharmacology of estrogens and progestogens: Influence of different routes of administration. *Climacteric* 8 (SUPPL. 1) (2005) 3-63.
- [2] R.J. Ruggiero, F.E. Likis, Estrogen: Physiology, pharmacology, and formulations for replacement therapy. *J. Midwifery Women's Health* 47 (3) (2002) 130-137.
- [3] B.T. Zhu, A.H. Conney, Functional role of estrogen metabolism in target cells: Review and perspectives. *Carcinogenesis* 19 (1) (1998) 1-27.
- [4] R. Raftogianis, C. Creveling, R. Weinshilboum, J. Weisz, Estrogen metabolism by conjugation. *J. Natl. Cancer Inst. Monographs* (27) (2000) 113-124.
- [5] H. Adlercreutz, F. Martin, P. Järvenpää, T. Fotsis, Steroid absorption and enterohepatic recycling. *Contraception* 20 (3) (1979) 201-223.
- [6] H. Adlercreutz, F. Martin, Biliary excretion and intestinal metabolism of progesterone and estrogens in man. *J. Steroid Biochem.* 13 (2) (1980) 231-244.
- [7] É. Audet-Walsh, J. Lépine, J. Grégoire, M. Plante, P. Caron, B. Têtu, P. Ayotte, J. Brisson, L. Villeneuve, A. Bélanger, C. Guillemette, Profiling of endogenous estrogens, their precursors, and metabolites in endometrial cancer patients: Association with risk and relationship to clinical characteristics. *J. Clin. Endocrinol. Metab.* 96 (2) (2011).
- [8] Y. Zhao, J.M. Boyd, M.B. Sawyer, X.-. Li, Liquid chromatography tandem mass spectrometry determination of free and conjugated estrogens in breast cancer patients before and after exemestane treatment. *Anal. Chim. Acta* 806 (2014) 172-179.

- [9] F. Andreolini, C. Borra, F. Caccamo, A.D. Corcia, R. Samperi, Estrogen Conjugates in Late-Pregnancy Fluids: Extraction and Group Separation by a Graphitized Carbon Black Cartridge and Quantification by High-Performance Liquid Chromatography. *Anal. Chem.* 59 (13) (1987) 1720-1725.
- [10] V. Vasiliou, K. Vasiliou, D.W. Nebert, Human ATP-binding cassette (ABC) transporter family. *Hum. Genomics* 3 (3) (2009) 281-290.
- [11] A.H. Schinkel, J.W. Jonker, Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: An overview. *Adv. Drug Deliv. Rev.* 55 (1) (2003) 3-29.
- [12] K.M. Giacomini, S.-. Huang, D.J. Tweedie, L.Z. Benet, K.L.R. Brouwer, X. Chu, A. Dahlin, R. Evers, V. Fischer, K.M. Hillgren, K.A. Hoffmaster, T. Ishikawa, D. Keppler, R.B. Kim, C.A. Lee, M. Niemi, J.W. Polli, Y. Sugiyama, P.W. Swaan, J.A. Ware, S.H. Wright, S. Wah Yee, M.J. Zamek-Gliszczyński, L. Zhang, Membrane transporters in drug development. *Nat. Rev. Drug Discov.* 9 (3) (2010) 215-236.
- [13] K.M. Hillgren, D. Keppler, A.A. Zur, K.M. Giacomini, B. Stieger, C.E. Cass, L. Zhang, Emerging transporters of clinical importance: An update from the international transporter consortium. *Clin. Pharmacol. Ther.* 94 (1) (2013) 52-63.
- [14] M.J. Zamek-Gliszczyński, K.A. Hoffmaster, K.-. Nezasa, M.N. Tallman, K.L.R. Brouwer, Integration of hepatic drug transporters and phase II metabolizing enzymes: Mechanisms of hepatic excretion of sulfate, glucuronide, and glutathione metabolites. *Eur. J. Pharm. Sci.* 27 (5) (2006) 447-486.
- [15] D.R. De Waart, K. Van De Wetering, C. Kunne, S. Duijst, C.C. Paulusma, R.P.J. Oude Elferink, Oral availability of cefadroxil depends on ABCC3 and ABCC4. *Drug Metab. Dispos.* 40 (3) (2012) 515-521.

- [16] X. Ming, D.R. Thakker, Role of basolateral efflux transporter MRP4 in the intestinal absorption of the antiviral drug adefovir dipivoxil. *Biochem. Pharmacol.* 79 (3) (2010) 455-462.
- [17] M. Roth, A. Obaidat, B. Hagenbuch, OATPs, OATs and OCTs: The organic anion and cation transporters of the SLCO and SLC22A gene superfamilies. *Br. J. Pharmacol.* 165 (5) (2012) 1260-1287.
- [18] L. Huang, T. Hoffman, M. Vore, Adenosine triphosphate-dependent transport of estradiol-17 β (β -D- glucuronide) in membrane vesicles by MDR1 expressed in insect cells. *Hepatology* 28 (5) (1998) 1371-1377.
- [19] P.M. Gerk, W. Li, V. Megaraj, M. Vore, Human multidrug resistance protein 2 transports the therapeutic bile salt tauroursodeoxycholate. *J. Pharmacol. Exp. Ther.* 320 (2) (2007) 893-899.
- [20] H. Kidron, G. Wissel, N. Manevski, M. Häkli, R.A. Ketola, M. Finel, M. Yliperttula, H. Xhaard, A. Urtili, Impact of probe compound in MRP2 vesicular transport assays. *Eur. J. Pharm. Sci.* 46 (1-2) (2012) 100-105.
- [21] F. Deng, N. Sjostedt, H. Kidron, The Effect of Albumin on MRP2 and BCRP in the Vesicular Transport Assay. *PLoS One* 11 (10) (2016).
- [22] E. Järvinen, J. Troberg, H. Kidron, M. Finel, Selectivity in the efflux of glucuronides by human transporters MRP4 is highly active toward 4-methylumbelliferone and 1-naphthol glucuronides, while MRP3 exhibits stereoselective propranolol glucuronide transport. *Mol. Pharm.* (2017).
- [23] N. Sjöstedt, K. Holvikari, P. Tammela, H. Kidron, Inhibition of Breast Cancer Resistance Protein and Multidrug Resistance Associated Protein 2 by Natural Compounds and Their Derivatives. *Mol. Pharm.* 14 (1) (2017) 135-146.

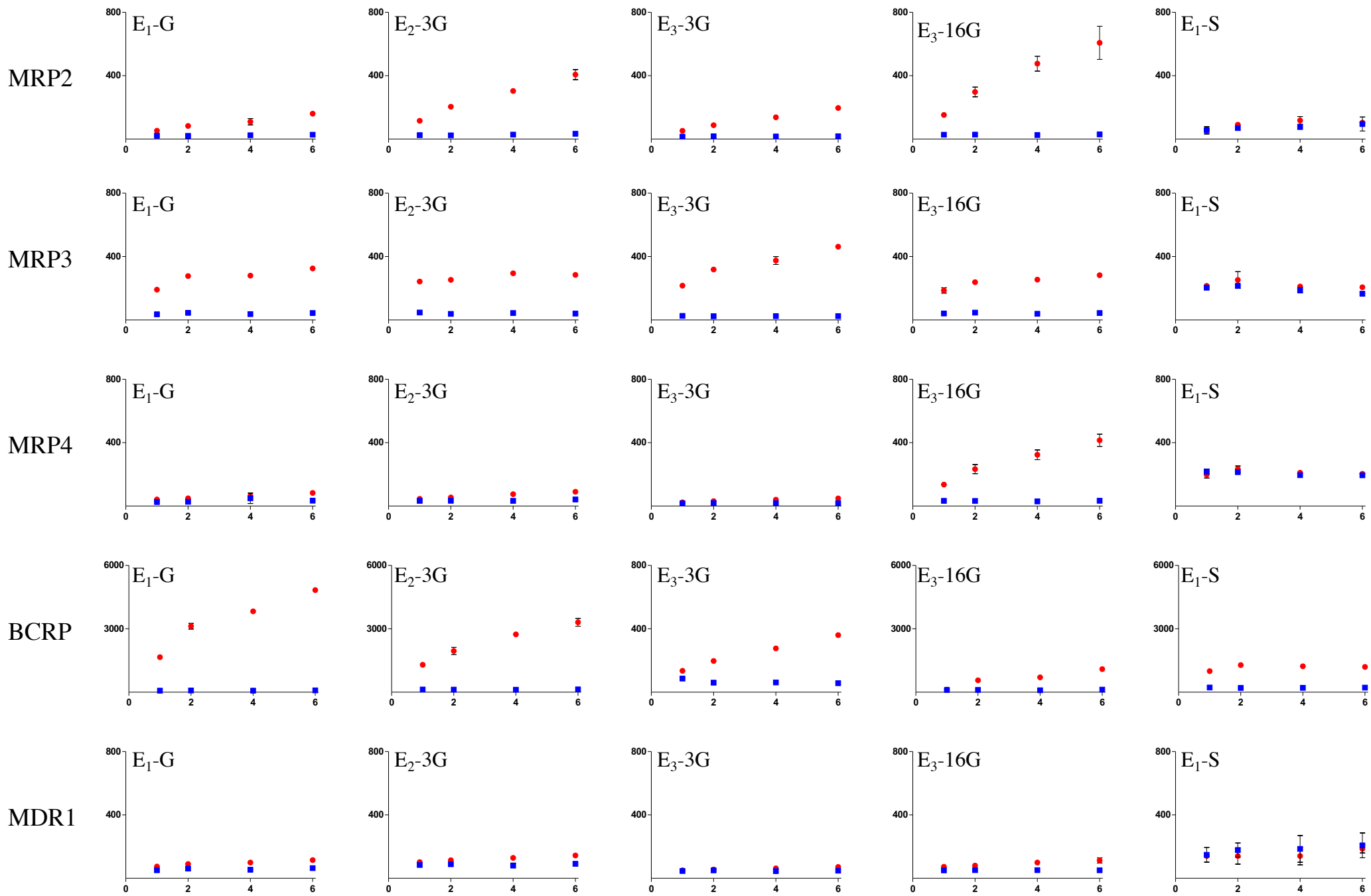
- [24] N. Sjostedt, F. Deng, O. Rauvala, T. Tepponen, H. Kidron, Interaction of Food Additives with Intestinal Efflux Transporters. *Mol. Pharm.* (2017).
- [25] Á. Telbisz, M. Müller, C. Özvegy-Laczka, L. Homolya, L. Szente, A. Váradi, B. Sarkadi, Membrane cholesterol selectively modulates the activity of the human ABCG2 multidrug transporter. *Biochim. Biophys. Acta Biomembr.* 1768 (11) (2007) 2698-2713.
- [26] K. Herédi-Szabó, J.E. Palm, T.B. Andersson, Á. Pál, D. Méhn, Z. Fekete, E. Beéry, K.T. Jakab, M. Jani, P. Krajcsi, A P-gp vesicular transport inhibition assay - Optimization and validation for drug-drug interaction testing. *Eur. J. Pharm. Sci.* 49 (4) (2013) 773-781.
- [27] Y. Imai, S. Asada, S. Tsukahara, E. Ishikawa, T. Tsuruo, Y. Sugimoto, Breast cancer resistance protein exports sulfated estrogens but not free estrogens. *Mol. Pharmacol.* 64 (3) (2003) 610-618.
- [28] P.E. Bandler, C.J. Westlake, C.E. Grant, S.P.C. Cole, R.G. Deeley, Identification of regions required for apical membrane localization of human multidrug resistance protein 2. *Mol. Pharmacol.* 74 (1) (2008) 9-19.
- [29] C.E. Grant, M. Gao, M.K. DeGorter, S.P.C. Cole, R.G. Deeley, Structural determinants of substrate specificity differences between human multidrug resistance protein (MRP) 1 (ABCC1) and MRP3 (ABCC3). *Drug Metab. Dispos.* 36 (12) (2008) 2571-2581.
- [30] Y.-. Han, D. Busler, Y. Hong, Y. Tian, C. Chen, A.D. Rodrigues, Transporter studies with the 3-O-sulfate conjugate of 17 α - ethinylestradiol: Assessment of human kidney drug transporters. *Drug Metab. Dispos.* 38 (7) (2010) 1064-1071.
- [31] Y.-. Han, D. Busler, Y. Hong, Y. Tian, C. Chen, A.D. Rodrigues, Transporter studies with the 3-O-sulfate conjugate of 17 α - ethinylestradiol: Assessment of human liver drug transporters. *Drug Metab. Dispos.* 38 (7) (2010) 1072-1082.

- [32] H. Jirku, M. Levitz, Biliary and urinary metabolites of estrone-6,7-3H-sulfate-35S in a woman. *J. Clin. Endocrinol. Metab.* 29 (5) (1969) 615-637.
- [33] Y.-. Qian, W.-. Song, H. Cui, S.P.C. Cole, R.G. Deeley, Glutathione Stimulates Sulfated Estrogen Transport by Multidrug Resistance Protein 1. *J. Biol. Chem.* 276 (9) (2001) 6404-6411.
- [34] D.J. Seward, A.S. Koh, J.L. Boyer, N. Ballatori, Functional complementation between a novel mammalian polygenic transport complex and an evolutionarily ancient organic solute transporter, OST α -OST β . *J. Biol. Chem.* 278 (30) (2003) 27473-27482.
- [35] V.R. More, Q. Cheng, A.C. Donepudi, D.B. Buckley, Z.J. Lu, N.J. Cherrington, A.L. Slitt, Alcohol cirrhosis alters nuclear receptor and drug transporter expression in human livers. *Drug Metab. Dispos.* 41 (5) (2013) 1148-1155.
- [36] H.J. Burt, A.E. Riedmaier, M.D. Harwood, H.K. Crewe, K.L. Gill, S. Neuhoff, Abundance of hepatic transporters in Caucasians: A meta-analysis. *Drug Metab. Dispos.* 44 (10) (2016) 1550-1561.
- [37] N. Gamage, A. Barnett, N. Hempel, R.G. Duggleby, K.F. Windmill, J.L. Martin, M.E. McManus, Human sulfotransferases and their role in chemical metabolism. *Toxicol. Sci.* 90 (1) (2006) 5-22.
- [38] R.A. Kallionpää, E. Järvinen, M. Finel, Glucuronidation of estrone and 16 α -hydroxyestrone by human UGT enzymes: The key roles of UGT1A10 and UGT2B7. *J. Steroid Biochem. Mol. Biol.* 154 (2015) 104-111.
- [39] C. Longcope, S. Gorbach, B. Goldin, M. Woods, J. Dwyer, A. Morrill, J. Warram, The Effect of a Low Fat Diet on Estrogen Metabolism. *J. Clin. Endocrinol. Metab.* 64 (6) (1987) 1246-1250.

- [40] R. Hobkirk, M. Nilsen, Metabolism of 17 β -estradiol to 17 β -estradiol- 3-glucosiduronate and 17 β -estradiol-17-glucosiduronate by the normal human female. *J. Clin. Endocrinol. Metab.* 32 (6) (1971) 779-785.
- [41] M.B. Fisher, K. Campanale, B.L. Ackermann, M. Vandenbranden, S.A. Wrighton, In vitro glucuronidation using human liver microsomes and the pore- forming peptide alamethicin. *Drug Metab. Dispos.* 28 (5) (2000) 560-566.
- [42] P.I. Musey, R.N. Green, R. Hobkirk, The role of an enterohepatic system in the metabolism of 17 β -estradiol-17-glucosiduronate in the human female. *J. Clin. Endocrinol. Metab.* 35 (3) (1972) 448-457.
- [43] R. Hobkirk, M. Nilsen, Metabolism of estrone-3-glucosiduronate and 17 β -estradiol-3-glucosiduronate in the human female. *Steroids* 15 (5) (1970) 649-667.
- [44] M. Drozdziak, C. Gröer, J. Penski, J. Lapczuk, M. Ostrowski, Y. Lai, B. Prasad, J.D. Unadkat, W. Siegmund, S. Oswald, Protein abundance of clinically relevant multidrug transporters along the entire length of the human intestine. *Mol. Pharm.* 11 (10) (2014) 3547-3555.
- [45] G.N. Asher, J.K. Fallon, P.C. Smith, UGT concentrations in human rectal tissue after multidose, oral curcumin. *Pharmacol. Res. Perspect.* 4 (2) (2016).
- [46] N. Sneitz, M. Vahermo, J. Mosorin, L. Laakkonen, D. Poirier, M. Finel, Regiospecificity and stereospecificity of human udp-glucuronosyltransferases in the glucuronidation of estriol, 16-epiestriol, 17-epiestriol, and 13-epiestradiols. *Drug Metab. Dispos.* 41 (3) (2013) 582-591.
- [47] B.K. Young, H. Jirku, S. Kadner, M. Levitz, Renal clearances of estriol conjugates in normal human pregnancy at term. *Am. J. Obstet. Gynecol.* 126 (1) (1976) 38-42.

- [48] M. Levitz, S. Kadner, B.K. Young, Intermediary metabolism of estriol in pregnancy. *J. Steroid Biochem.* 20 (4 PART 2) (1984) 971-974.
- [49] A.A. Sandberg, W.R. Slaunwhite Jr., Studies on phenolic steroids in human subjects. VII. Metabolic fate of. *J. Clin. Invest.* 44 (1965) 694-702.
- [50] U. Goebelsmann, K. Sjöberg, N. Wiquist, E. Diczfalusy, Oestriol-3-glucosiduronate, a major urinary metabolite of oestriol and oestriol-16(17?)-glucosiduronate. *Acta Endocrinol (Copenh)* 50 (2) (1965) 261-272.
- [51] K.F. Stoa, M. Levitz, Comparison of the conjugated metabolites of intravenously and intraduodenally administered oestriol. *Acta Endocrinol (Copenh)* 57 (4) (1968) 657-668.
- [52] R.Y. Kirdani, W.R. Slaunwhite Jr., A.A. Sandberg, Studies on phenolic steroids in human subjects. XIV. Studies on the fate of injected 6,7-H³-oestriol-3-C¹⁴-glucosiduronate. *J. Steroid Biochem.* 1 (4) (1970) 265-278.
- [53] N. Inoue, A.A. Sandberg, J.B. Graham, W.R. Slaunwhite Jr., Studies on phenolic steroids in human subjects. 8. Metabolism of estriol-16 alpha-glucosiduronate. *J. Clin. Invest.* 48 (2) (1969) 380-389.
- [54] R.Y. Kirdani, D. Sampson, G.P. Murphy, A.A. Sandberg, Studies on phenolic steroids in human subjects. XVI. role of the kidney in the disposition of estriol. *J. Clin. Endocrinol. Metab.* 34 (3) (1972) 546-557.
- [55] B. Prasad, K. Johnson, S. Billington, C. Lee, G.W. Chung, C.D.A. Brown, E.J. Kelly, J. Himmelfarb, J.D. Unadkat, Abundance of drug transporters in the human kidney cortex as quantified by quantitative targeted proteomics. *Drug Metab. Dispos.* 44 (12) (2016) 1920-1924.

Fig S1. Transport versus time of the different combinations of the studied estrogen conjugates and transporters. The concentration of each compound was 10 μ M and transport was measured after incubation times of 1- 6 min. The Y- and X- axes represent transport rate (pmol/mg total protein) and incubation time after ATP addition (min), respectively. Red circles and blue squares represent values in the presence of and absence of ATP during the incubation, respectively. Each data point represent mean \pm SD of triplicate samples in a single experiment. For further details see the main text.



Suppl. Fig. S1

MRP2

MRP3

BCRP

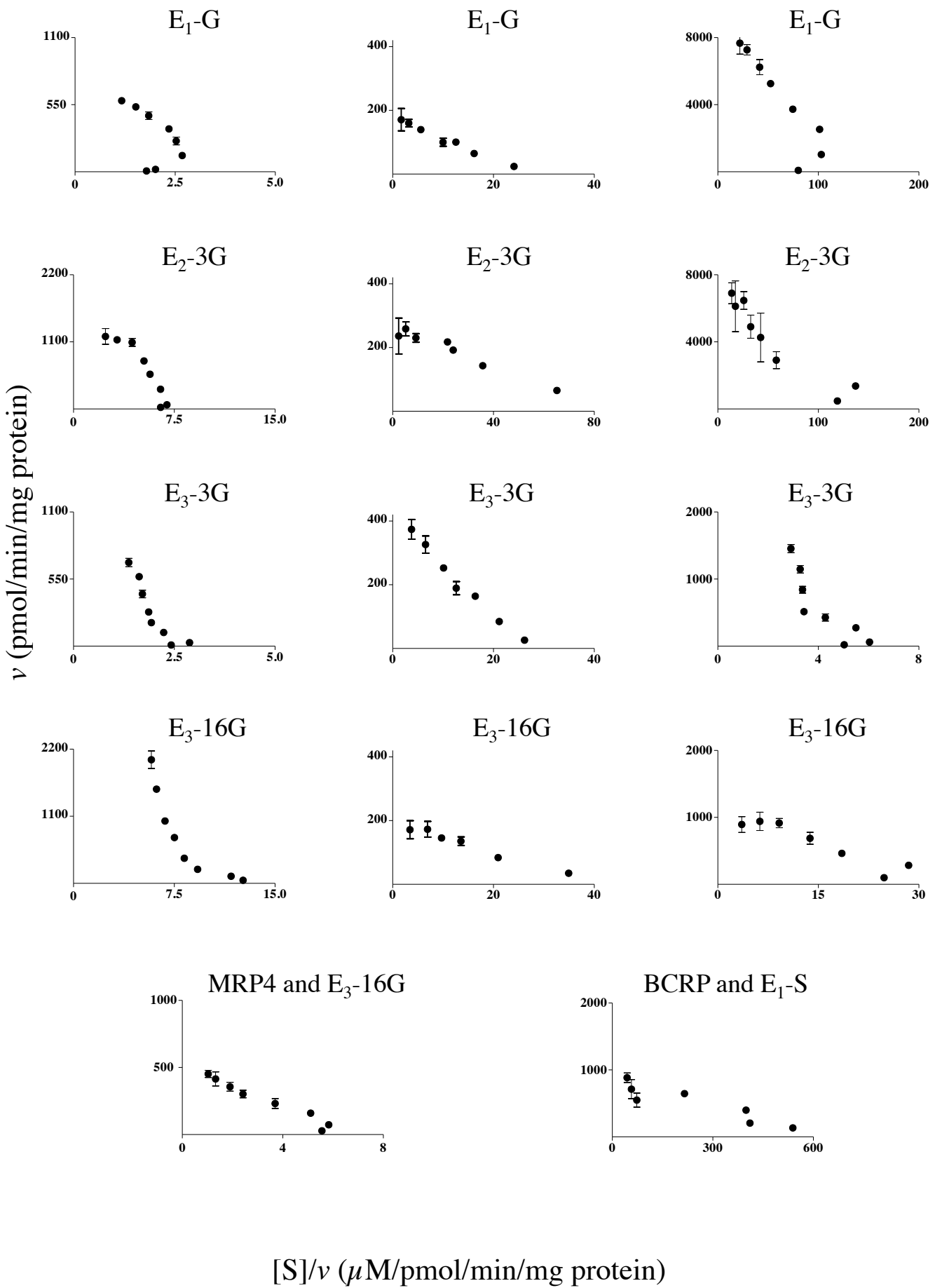


Fig S2. Eadie-Hofstee transformations of the data presented in Figures 3-5. For further details see Figures 3-5 in the main text.