Human Efflux Transport of Testosterone, Epitestosterone and Other Androgen Glucuronides

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HIGHLIGHTS

- Androgens are conjugated in the liver to glucuronides that are excreted in urine
- Basolaterally located MRP3 is a high affinity transporter for androgen glucuronides
- Apically located MRP2 is a low affinity transporter for androgen glucuronides
- Apically located BCRP does not transport androgen glucuronides
- High MRP3-mediated transport agrees with urinary excretion of androgen glucuronides

ABSTRACT

Several drug-metabolizing enzymes are known to control androgen homeostasis in humans. UDP-glucuronosyltransferases convert androgens to glucuronide conjugates in the liver and intestine, which enables subsequent elimination of these conjugated androgens via urine. The most important androgen is testosterone, while other important ones are the testosterone metabolites androsterone and etiocholanolone, and the testosterone precursor dehydroepiandrosterone. Epitestosterone is another endogenous androgen, which is included as a crucial marker in urine doping tests. Since glucuronide conjugates are hydrophilic, efflux transporters mediate their excretion from tissues. In this study, we employed the membrane vesicle assay to identify the efflux transporters for glucuronides of androsterone, dehydroepiandrosterone, epitestosterone, etiocholanolone and testosterone. The human hepatic and intestinal transporters MRP2 (ABCC2), MRP3 (ABCC3), MRP4 (ABCC4), BCRP (ABCG2) and MDR1 (ABCB1) were studied in vitro. Of these transporters, only MRP2 and MRP3 transported the androgen glucuronides investigated. In kinetic analyses, MRP3 transported glucuronides of androsterone, epitestosterone and etiocholanolone at low K_m values, between 0.4 and 4 µM, while the K_m values for glucuronides of testosterone and dehydroepiandrosterone were 14 and 51 µM, respectively. MRP2 transported the glucuronides at lower affinity, as indicated by K_m values over 100 µM. Interestingly, the MRP2-mediated transport of androsterone and epitestosterone glucuronides was best described by sigmoidal kinetics. The inability of BCRP to transport any of the androgen glucuronides investigated is drastically different from its highly active transport of several estrogen conjugates. Our results explain the transporter mediated disposition of androgen

glucuronides in humans, and shed light on differences between the human efflux transporters MRP2, MRP3, MRP4, BCRP and MDR1.

KEYWORDS

ABCC2, ABCC3, ABCC4, ABCG2, ABCB1, androgen

ABBREVIATIONS

androsterone-G, androsterone glucuronide; ABC, ATP-binding cassette; dehydroepiandrosterone-G, dehydroepiandrosterone glucuronide; epitestosterone-G, epitestosterone glucuronide; etiocholanolone-G, etiocholanolone glucuronide; SULT, sulfotransferase; testosterone-G, testosterone glucuronide; UGT, UDP-glucuronosyltransferase.

1. INTRODUCTION

Testosterone is the main androgen in humans, but also a drug and doping substance [1]. Multiple enzymes from several families including cytochrome P450s, hydroxysteroid dehydrogenases and oxidoreductases, sulfotransferases (SULT) and UDP-glucuronosyltransferases (UGT) regulate androgen homeostasis in the human body following their biosynthesis from cholesterol [2,3]. SULTs and UGTs are particularly important for excretion of androgens, since they produce androgen sulfates and glucuronides, respectively [3,4]. These conjugates are mostly inactive and excreted as such via urine. The UGTs that catalyze androgen glucuronidation are mainly expressed in the liver and intestine [5-7]. Conjugated steroids are too hydrophilic to passively cross cell membranes, and thus require active transporters for their excretion from cells [8,9].

Testosterone is converted to androsterone and etiocholanolone via several intermediate steps [3]. The latter two steroids are the main end products of androgen metabolism, and after conjugation with glucuronic acid, the androsterone and etiocholanolone glucuronides (androsterone-G and etiocholanolone-G, respectively, Fig. 1), are excreted in urine. Dehydroepiandrosterone is the precursor for biosynthesis of testosterone and testosterone-derived androgens, whereas epitestosterone is biosynthesized via a different pathway and is neither a metabolite nor a precursor of testosterone [2-4,10,11]. Although the physiological function of epitestosterone is currently unknown, and its biosynthesis rate is low compared with testosterone, it is a pivotal androgen for doping testing [10,11]. Large deviation from the urinary ratio of testosterone glucuronide to epitestosterone glucuronide (testosterone-G and epitestosterone-G, respectively), which is typically between one and two in urine, is utilized to detect the use of prohibited testosterone [11-13]. However, these

glucuronides are excreted in varying amounts in the population, and ratios clearly below one are mostly found in individuals that lack the *UGT2B17* gene, which encodes UGT2B17, the main enzyme responsible for testosterone glucuronidation [5,12,14]. Nevertheless, ratios above four might be explained by decreased excretion of epitestosterone-G, although the comprehensive genetic basis for impaired excretion is unknown [5,15]. Currently, doping tests also include the androsterone-G to etiocholanolone-G ratio to accomplish a more comprehensive test for detecting the use of exogenous androgens [13].

Androgens are excreted in urine mainly as glucuronides, whereas free androgens are essentially absent from urine [4,12,14]. Androsterone-G and etiocholanolone-G are the main androgens in urine, while dehydroepiandrosterone glucuronide (dehydroepiandrosterone-G), epitestosterone-G and testosterone-G are about ten- to fifty-fold less abundant [12,16]. After administration of exogenous testosterone, androsterone-G and etiocholanolone-G are quantitatively the main testosterone metabolites in human urine and constitute almost the entire recovered dose [17]. The basal concentrations of androgen glucuronides in plasma are at nanomolar range [17-20]. After the administration of exogenous testosterone, androgen glucuronides, particularly androsterone-G and etiocholanolone-G, may reach micromolar concentrations. However, glucuronide conjugates are formed intracellularly, and therefore the plasma concentrations do not fully reflect the intracellular concentrations [9,21]. The plasma and intracellular concentrations may differ, because the cell membrane restricts access of glucuronides to the systemic circulation from cells [8].

ATP-binding cassette (ABC) and solute carrier transporters are involved in disposition of steroid conjugates in human tissues [9,22-24]. Multidrug resistance-associated proteins 2, 3 and 4 (MRP2-4, gene *ABCC2-4*), multidrug resistance protein 1 (MDR1,

P-gp, gene *ABCB1*) and breast cancer resistance protein (BCRP, gene *ABCG2*) are the major transporters that mediate efflux of endogenous and exogenous compounds, including their glucuronide and sulfate conjugates, in the liver, intestine and kidney [9,25-27]. The role of MDR1 in disposition of glucuronide and sulfate conjugates is limited, while MRP2, MRP3 and BCRP have significant contributions in vivo [9,24,27]. MRP2, BCRP and MDR1 are expressed in apical membranes of hepatocytes and enterocytes, while MRP3 and MRP4 are localized at the opposite, basolateral, membranes of the same polarized cells [25,26]. The aforementioned transporters are studied in vitro in cell-based or vesicle-based assays of which only the latter assays can be used for hydrophilic compounds [28]. The vesicle-based assays provide qualitative data and are used to identify the active transporters toward the compounds in question. Furthermore, these assays allow characterization and comparison of the transport kinetics of individual transporters. The apparent affinities derived for transporters in vitro, along with current knowledge on expression levels of each transporter in tissues, provide evidence of the most active transporters in vivo. The vesicles are derived from either mammalian or insect cells that express recombinant human transporters [28]. Although some differences might occur between mammalian and insect cell derived transporter preparations, these discrepancies are normally reported as quantitative [29, 30]. However, the qualitative data remain unaffected.

Estrogens and androgens share the same steroid backbone, while their A ring is different being planar and aromatic only in estrogens (Fig. 1 and 5). In addition, androgens have a second methyl group attached to carbon 10 and this group is not present in estrogens (Fig. 1). We have previously studied the transport of estrogen glucuronides by the aforementioned transporters and found high affinity transport by MRP3 and high active transport by BCRP [24]. However, due to the differences in their

structures, deducing transport activity for androgen glucuronides based on the knowledge of the transport of estrogen glucuronides is not feasible.

The main aims of this study were to identify and characterize the hepatic human ABC transporters that contribute to efflux of androsterone-G, dehydroepiandrosterone-G, epitestosterone-G, etiocholanolone-G and testosterone-G (Fig. 1) in order to explain the mechanisms behind their in vivo disposition. An additional aim was to derive information on the structural elements in steroid conjugate transport, which affect individual transporters studied here. To this end, we tested the transport of these androgen glucuronides in vitro by employing the vesicular transport assay for the human efflux transporters MRP2, MRP3, MRP4, BCRP and MDR1.

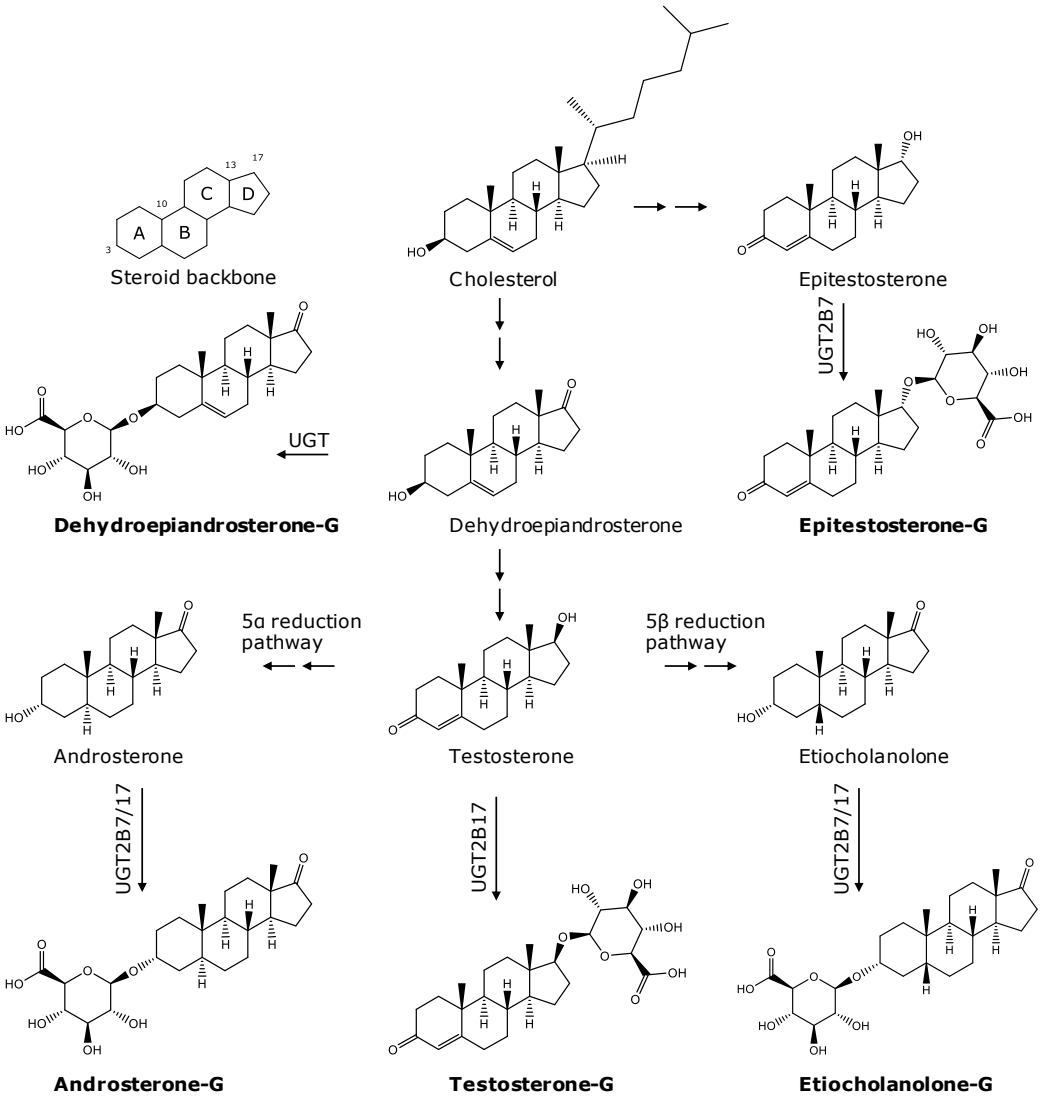

Figure 1. Structures of the androgen glucuronides investigated. Chemical structures and main biosynthesis pathways for the androgen glucuronides that were included in this study are presented [4-6].

2. MATERIALS AND METHODS

2.1 Chemicals and solvents

Androsterone-G (100% purity), dehydroepiandrosterone-G (>99% purity), epitestosterone (100% purity) and testosterone-G (99% purity) were from Sigma-Aldrich (St. Louis, MO, USA), while etiocholanolone-G was obtained from Steraloids (Newport, RI, USA). D_3-testosterone-G was from the National Analytical Reference Laboratory (Australia). Epitestosterone-G was biosynthesized in-house as reported below. Formic acid and methanol were of analytical quality and obtained from Sigma-Aldrich. Milli-Q water purification system including 0.22 µm filter (Merck Millipore, Darmstadt, Germany) was employed as the water source for analyses and assays.

2.2 Biosynthesis of epitestosterone-G

Recombinant human UGT2B7 expressed in Sf9 cells was applied for the biosynthesis of epitestosterone-G [5]. The final volume of enzyme reaction was 25 ml and contained 750 µM epitestosterone, 7.5% (v/v) DMSO, 100 mM Tris-HCl buffer pH 7.5, 5 mM $MgCl_2$, 4 mM uridine 5'-diphosphoglucuronic acid and 48.1 mg of protein from UGT2B7 membrane preparation. After incubation for 24 hours in a 37 °C thermo-shaker, the suspension was centrifuged for 10 min at 16 000 g, the resulting pellet was washed twice with water and all three resulting supernatants were combined. Liquid-liquid extraction was employed to remove the unreacted epitestosterone by alkalizing the supernatant and extracting it twice with equal amount of dichloromethane. The aqueous phase was acidified with formic acid and loaded onto two separate 6 ml Oasis HLB (200 mg) cartridges from Waters (Milford, MA, USA). The Cartridges were washed with 4 ml of 35% methanol containing 2% formic acid, both v/v, and the glucuronide

fraction was subsequently eluted with 75% methanol containing 5% ammonia, both v/v. After evaporation, the eluate was dissolved in a mixture of methanol and water, and subjected to high performance liquid chromatography purification. The fractions corresponding to epitestosterone-G were collected, combined and evaporated. The final yield of the reaction was 70% (n/n) resulting in 6.3 mg of epitestosterone-G. Nuclear magnetic resonance (NMR) spectra were obtained for epitestosterone-G in DMSO-d_6 employing Ascend 400 MHz spectrometer from Bruker (Billerica, MA, USA), and the chemical shifts of spectra were related to the residual DMSO peak (Fig. S1-S5). High-resolution mass spectrometric analysis at negative electrospray ionization of epitestosterone-G resulted in 463.231 ($[M-H]^-$) m/z (calculated 463.234).

2.3 Vesicular transport assays

Inside-out membrane vesicles from Sf9 insect cells expressing the human recombinant transporters MRP2, MRP3, MRP4, BCRP, MDR1 or a transport defective mutant of MRP3 for control preparations (CTRL) were prepared as reported previously [31,32]. BCRP and MDR1 vesicles were loaded with cholesterol to enhance the transport activity as reported and described previously [30,31,33]. We have previously reported the proper functionality of the aforementioned human transporters in our Sf9 vesicle-based assay [24,31,32].

The vesicular transport assays were carried out in 96-well plates as previously described [24,31]. Transporter vesicles, 40 µg of total membrane protein, were suspended in a total volume of 50 µl of the transport assay mixture (40 mM MOPS adjusted to pH 7.0 with Tris–HCl, 6 mM $MgCl_2$ and 60 mM KCl) containing the androgen glucuronide to be tested, on ice. The suspension was incubated for 10 min on a shaker adjusted to 500 rpm and at a temperature of +37 °C. The transport

reactions were initiated by adding 25 µl of the transport assay mixture containing ATP (+ATP samples) or no ATP (-ATP samples), bringing the reactions to a final volume of 75 µl. The final concentration of ATP was 4 mM, while the concentrations of androgen glucuronides are stated in each figure legend. The transport reaction was then incubated on the shaker for a pre-determined time (see figure legends). The transport reactions were terminated by adding 200 µl of cold wash buffer (40 mM MOPS adjusted to pH 7.0 with Tris HCl and 70 mM KCl) to each well and by transferring the samples to a filter plate that was kept under vacuum. Each well on the filter plate was then washed five times with 200 µl of the cold wash buffer. The filter plate was dried at room temperature, after which androgen glucuronides were eluted from the wells with 100 µl of 75% methanol containing 0.1% formic acid (both v/v), and an internal standard (Table 1). The eluted samples were analyzed by LC-MS/MS as described below (Section 2.4).

The stock solutions of the androgen glucuronides were prepared in DMSO at 50 mM and stored at -20 °C. Subsequent dilutions were prepared in the transport assay buffer so that the final DMSO concentration was below 0.1% (v/v) in the single substrate concentration assays and 1% (v/v) in the kinetic assays. Before conducting the kinetic assays, the solubility of the androgen glucuronides in the transport assay mixture, over the entire concentration range used in the assays, was ensured by employing high-performance liquid chromatography with UV detection. To exclude any artifacts during the assay such as disruption of the vesicles, -ATP values from the transport kinetic assays were examined against the concentrations of the androgen glucuronides. A linear fitting of these data was then performed, which resulted in R^2 values of 0.99 or higher for all the kinetic assays.

For the transport kinetic assays, the linear time range of transport was determined and the transport assay time was selected based on that range (Fig. S7). The transport kinetic data is presented as ATP-dependent values that are derived by subtracting the -ATP (passive and unspecific transport) from +ATP (transporter-mediated, passive and unspecific transport) values and combining the standard deviations. The data were fitted to either the Michaelis-Menten equation ($v = V_{max}[S]/([S] + K_m)$) or to the Hill equation ($v = V_{max}[S]^h/((S_{50})^h + [S]^h)$), where V_{max} represents the maximal transport rate, [S] is the substrate concentration, K_m represents the Michaelis constant, S_{50} is the concentration producing half-maximal reaction rate and h is the Hill coefficient.

2.4 Analytical methods

ACQUITY UPLC I Class Ultra Performance Liquid Chromatography connected to Xevo TQ-S triple quadrupole mass spectrometry, both from Waters, were employed for quantification of androgen glucuronides in the vesicle assay samples. Samples, 0.5-4 µl, were injected into Acquity UPLC BEH C18 column (2.1×100 mm, 1.7 µm from Waters) that was kept at 50 °C and operated at a flow rate of 0.4 ml/min. The chromatographic eluents were water (A) and methanol (B) both with 0.1% (v/v) formic acid. The method was 0-1 min (60% B), 1-2.5 min (60->95% B), 2.5-3.5 (95% B) and 3.5-4.5 min (60% B). Negative electrospray ionization was employed in the mass spectrometry with the following parameters: -2.5 kV capillary voltage, 1 V cone, 100 V source offset, 150 °C source temperature, 600 °C desolvation gas temperature, 150 and 900 l/hr cone and desolvation gas flow, 6 bar nebulizer gas flow and 0.09 ml/min collision gas flow. The ion source gas and collision gas were nitrogen and argon, respectively, both from Aga (Espoo, Finland). The mass transitions, collision energies, dwell times, retention times and internal standards for the analytes are presented in Table 1. The analyte to internal standard ratio was employed for quantification. Fresh

standard curve was prepared for each experiment, similarly to the actual samples, that is in 100 µl of the elution solvent, incubated on a pre-wetted filter plate and centrifuged to a collection plate. The standard range curve was adjusted to each experiment in order to cover all samples and was typically in the range of 0.5 nM to 1000 nM.

Table 1. **Mass spectrometry parameters, retention times and internal standards for the androgen glucuronides.**

Analyte	**Mass transition ($[M-H]^-$ m/z)**	**Collision energy (V)**	**Dwell time (ms)**	**Retention time (min)**	**Internal standard**
Androsterone-G	465.2 -> 85.2	35	200	2.34	Dehydroepiandrosterone-G
Dehydroepiandrosterone-G	463.2 -> 85.2	35	200	1.48	Androsterone-G
Epitestosterone-G	463.2 -> 85.2	30	200	2.03	d_3-testosterone-G
Etiocholanolone-G	465.2 -> 85.2	35	200	2.28	Dehydroepiandrosterone-G
Testosterone-G	463.2 -> 85.2	30	200	1.35	d_3-testosterone-G
d_3-testosterone-G	466.6 -> 290.2	30	40	1.34	-

3. RESULTS

3.1 MRP2, MRP3, MRP4, BCRP and MDR1 transport of androgen glucuronides

The transport of androsterone-G, dehydroepiandrosterone-G, epitestosterone-G etiocholanolone-G and testosterone-G (see structures in Fig. 1) by human MRP2, MRP3, MRP4, BCRP and MDR1 was first assayed at two single substrate concentrations, 1 µM (Fig. 2) and 50 µM (Fig. S6). In the presence of ATP (+ATP), the uptake of 1 µM androsterone-G, dehydroepiandrosterone-G, epitestosterone-G etiocholanolone-G and testosterone-G into MRP3 vesicles was 17-, 2-, 34-, 34- and 9-fold (Fig. 2), respectively, higher than in the absence of ATP (-ATP). For MRP2, the respective values were 1.4, 6, 3, 5 and 5 (Fig. 2). The -ATP data represent passive transfer of a compound into vesicles and unspecific binding, while the +ATP data also include the active transport. These data indicate that MRP3 transports efficiently all the glucuronides investigated, except dehydroepiandrosterone-G (Fig. 2B), while MRP2 transports similarly all the glucuronides with the exception of androsterone-G (Fig. 2A). Although MRP2-mediated transport of 1 µM androsterone-G was similar to CTRL vesicles (Fig. 2A), at 50 µM substrate concentration (Fig. S6A) this transport was two-fold higher in the +ATP samples in comparison to the –ATP samples. This suggests that androsterone-G is a low affinity substrate for MRP2.

The transport ratio (+ATP/-ATP) of the glucuronides studied into MRP4 vesicles was below two and similar to the ratio in CTRL vesicles, which indicates negligible contribution of MRP4 in the transport of androgen glucuronides (Fig. 2). Furthermore, the transport ratios of the androgen glucuronides investigated into BCRP and MDR1 vesicles were also close to two (Fig. 2) and similar to the transport observed into their control vesicles (CTRL+C). This indicates negligible contribution of these two

transporters as well. Higher endogenous transport in these cholesterol enriched Sf9-derived mock vesicles has been reported earlier [34].

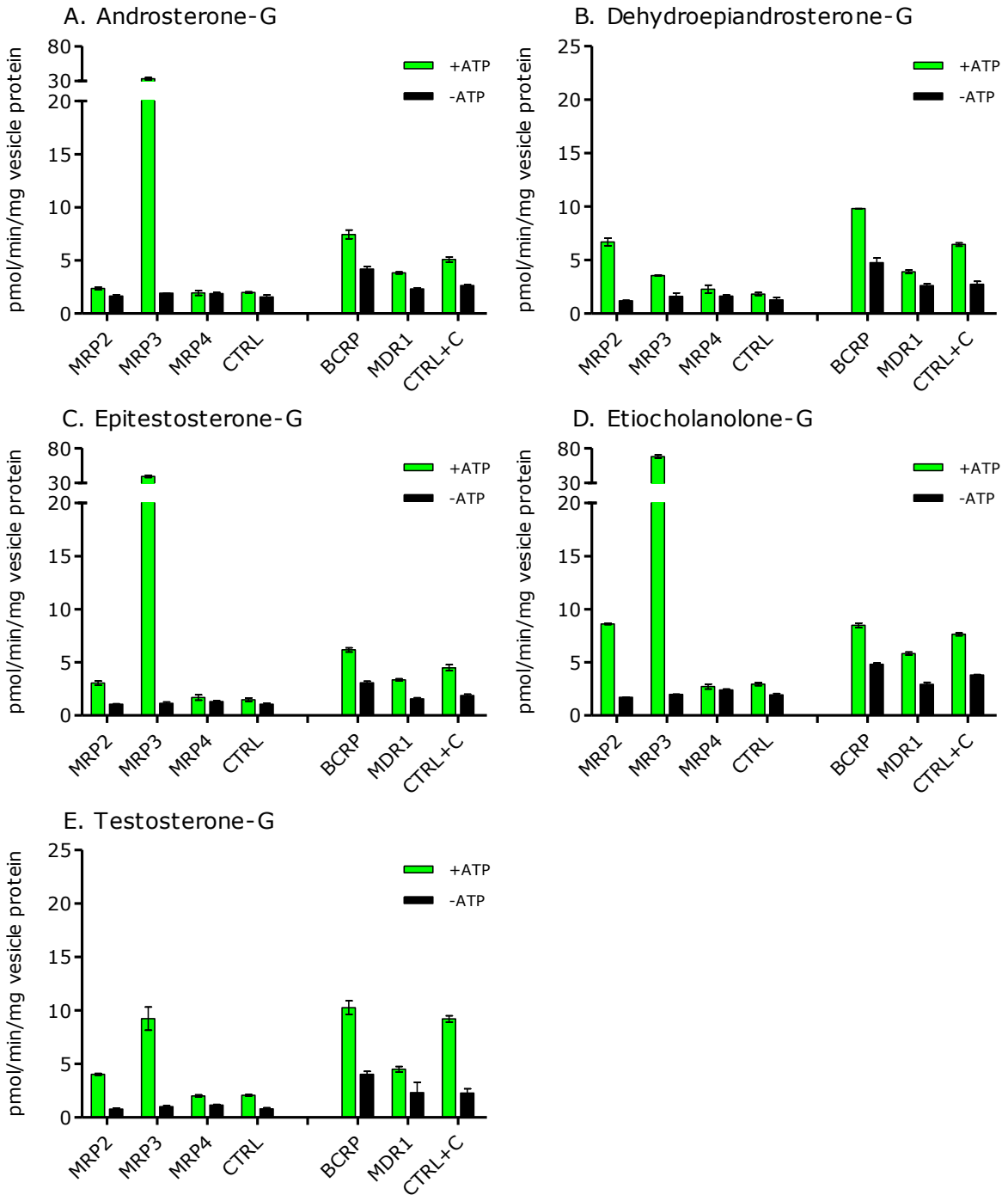

Figure 2. Human efflux transporters in the transport of androgen glucuronides. The assays were conducted at 1 µM substrate concentration for 3 min in the presence of ATP (+ATP) or in its absence (-ATP). The total protein amount of membrane vesicles was 40 µg per sample. BCRP and MDR1 as well as their control vesicles CTRL+C were supplemented with cholesterol as described in the Materials and Methods. The results are from an experiment in triplicate samples and the error bars represent ±S.D.

3.2. Characterization of MRP2 and MRP3 transport kinetics of androgen glucuronides

MRP2 and MRP3 transported all the glucuronides in a time-dependent matter (Fig. S7), and hence concentration-depend transport was characterized for all the glucuronides by both transporters. The kinetic analyses revealed that MRP3 transported androsterone-G, epitestosterone-G and etiocholanolone-G at low micromolar K_m values, while the transport of testosterone-G was at somewhat lower apparent affinity since its K_m value was above ten micromolar (Fig. 3A, 3B and Table 2). The MRP3-mediated transport of dehydroepiandrosterone-G was clearly weakest for this transporter in comparison to other androgen glucuronides tested, as seen from the high K_m and low V_{max} values (Fig. 3C and Table 2).

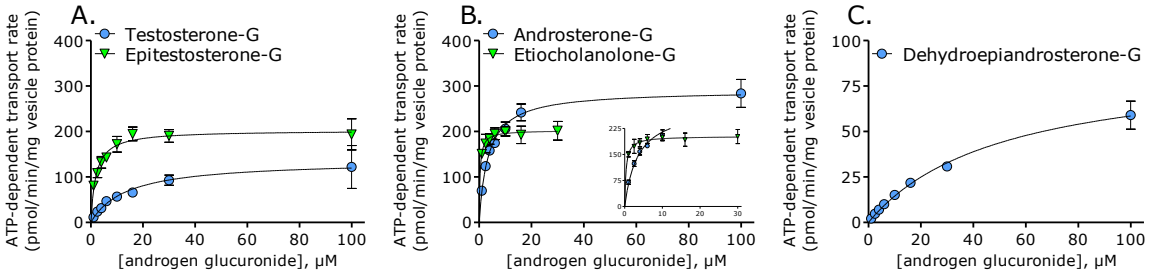

Figure 3. MRP3 transport kinetics of the androgen glucuronides. The Y-axis values were derived by subtracting the transport values without ATP (-ATP) from the values in the presence of ATP (+ATP), resulting in ATP-dependent transport rates. The lines represent the fitting of the data to the Michaelis-Menten equation (see the fitted parameters in Table 2). The inset of panel B presents transport kinetics of etiocholanolone-G at the lower concentrations. The transport reaction times were selected based on the time-linearity results (Fig. S7) and were 1 min, except 3 min for dehydroepiandrosterone-G. The total protein amount of membrane vesicles was 40 µg per sample. The results are from an experiment in triplicate samples and the error bars represent ±S.D.

In contrast to MRP3, the MRP2 transport of all the glucuronides tested occurred at clearly higher K_m values, over 100 micromolar, but also at higher V_{max} values (Fig. 4 and Table 2). The MRP2 transport of androsterone-G and epitestosterone-G exhibited

sigmoidal kinetics, and thus these data were fitted to the Hill equation (Fig. 4A, 4B and Table 2). MRP2 transport of dehydroepiandrosterone-G, etiocholanolone-G and testosterone-G were fitted to the Michaelis-Menten equation (Fig. 4 and Table 2). The kinetic curve for testosterone-G transport by MRP2 did not saturate within the concentration range tested (Fig. 4A). Hence, the fitted kinetic constants represent the best estimates based on the available data (Table 2).

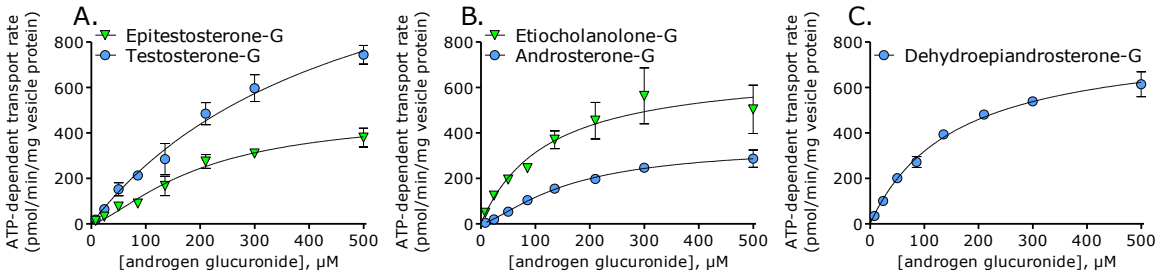

Figure 4. MRP2 transport kinetics of the androgen glucuronides. The Y-axis values were derived by subtracting the transport values without ATP (-ATP) from the values in the presence of ATP (+ATP), resulting in ATP-dependent transport rates. The lines represent the fitting of the data to the Michaelis-Menten equation or to the Hill equation in the case of androsterone-G and epitestosterone-G (see the fitted parameters in Table 2). The transport reaction times were selected based on the time-linearity results (Fig. S7) and were 2 min (etiocholanolone-G), 4 min (dehydroepiandrosterone-G, epitestosterone-G and testosterone-G) or 6 min (androsterone-G). The total protein amount of membrane vesicles was 40 µg per sample. The results are from an experiment in triplicate samples and the error bars represent ±S.D.

Table 2. **The derived transport kinetic parameters for MRP2 and MRP3.**

Androgen glucuronide	MRP2		MRP3	
	K_m (95% CI), µM	V_{max} (95% CI), pmol/min/mg vesicle protein	K_m (95% CI), µM	V_{max} (95% CI), pmol/min/mg vesicle protein
Androsterone-G	S_{50}=160 (114-206) h=1.41 (1.10-1.72)	344 (290-399)	3.53 (2.84-4.21)	290 (274-307)
Dehydroepiandrosterone-G	155 (130-180)	816 (761-871)	51.0 (39.1-62.9)	88.4 (78.0-98.8)
Epitestosterone-G	S_{50}=197 (107-287) h=1.44 (0.98-1.90)	484 (353-614)	1.93 (1.33-2.52)	202 (189-216)
Etiocholanolone-G	120 (57.7-183)	692 (555-828)	0.357 (0.188-0.527)	203 (193-212)
Testosterone-G	479 (300-659)	1490 (1150-1830)	13.7 (7.14-20.3)	136 (111-161)

The experimental data (Fig. 3 and 4) were fitted to the Michaelis-Menten equation, except in the cases of MRP2 transport of androsterone-G and epitestosterone-G where the Hill equation was employed. CI=confidence interval of the fitting

4. DISCUSSION

In this work, we investigated the five main ABC transporters that were expected to be involved in the transport of androgen glucuronide conjugates in the liver. Our results are significant for understanding molecular mechanisms in disposition of the androgen glucuronides studied. In addition, our results reveal new aspects on the specificity of MRP2, MRP3, MRP4 and BCRP to several androgen glucuronides.

4.1 Implications of structural features in androgen glucuronides for their efflux transport

Previously, we have shown that endogenous estrogen glucuronides are excellent substrates for BCRP and MRP3, while MRP2 is a low-affinity transporter and MRP4 transports selectively only some of the glucuronides [24,31]. Here, our results show that none of the androgen glucuronides tested are substrates for BCRP (Fig. 2), suggesting that the BCRP-mediated transport of steroid conjugates is dependent on the presence of a flat aromatic A ring in the substrate, which androgens lack in their steroid backbone (Fig. 1 and 5). In contrast, MRP2 and MRP3 transport both androgen and estrogen glucuronides, and thus exhibit lower selectivity in the transport of steroid glucuronides. This suggests that the aromatic A ring or its effect on the flatness of steroid structure are not essential for the transport activity of MRP2 and MRP3 (Fig. 2-4) [24]. We show here that MRP3 transports androgen glucuronides at even lower K_m values (Table 2) than estrogen glucuronides (the K_m values of estrogen glucuronides were between 3-18 µM [24]), while in the case of MRP2 the K_m values of both estrogen glucuronides (the K_m values were between 180-790 µM [24]) and androgen glucuronides (Table 2) are similar.

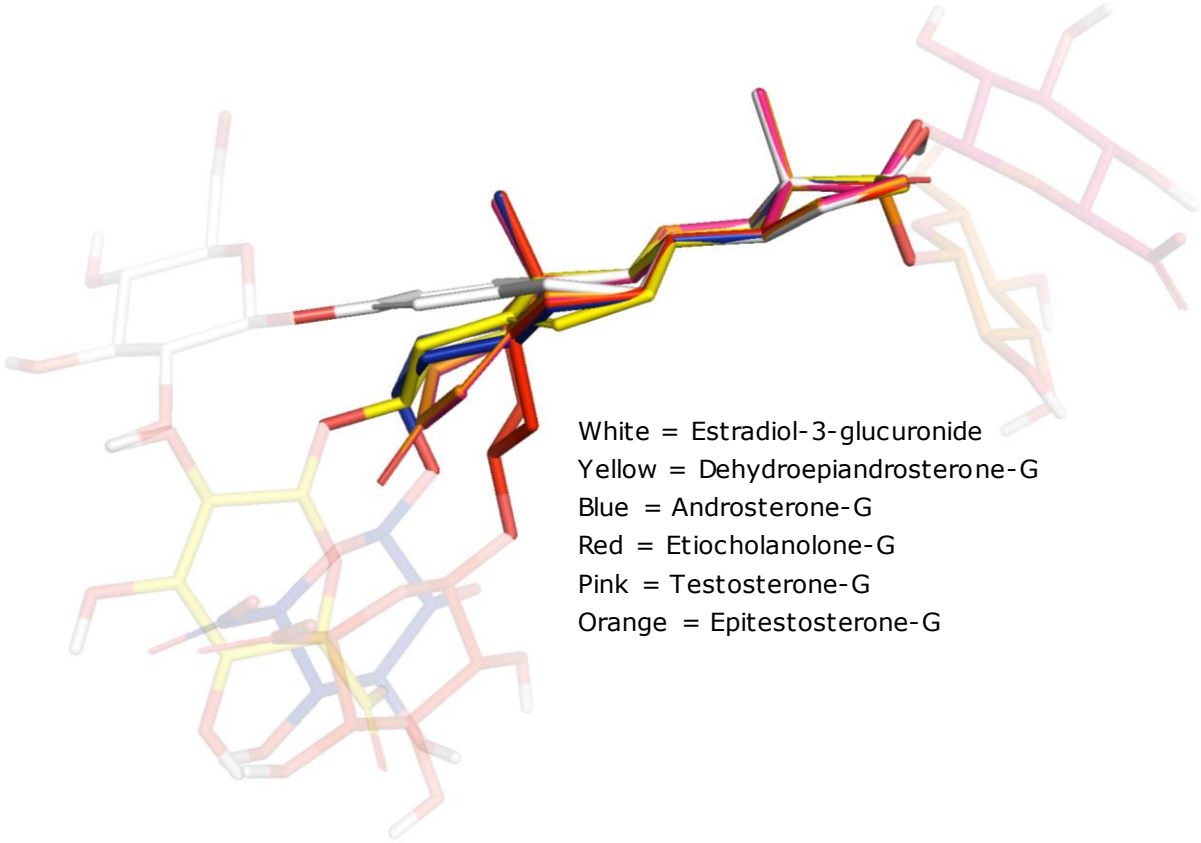

Figure 5. Three-dimensional conformations of androgen and estrogen glucuronides. Oxygens are colored in red, while non-polar hydrogens are omitted. The glucuronic acid moieties of the compounds are faint in relation to the rest of the structure for clarity. Estradiol-3-glucuronide is included to present a substrate of BCRP [24]. The structures were prepared with PyMOL [45].

The transport affinity of MRP3 is highest for androsterone-G, epitestosterone-G and etiocholanolone-G (Table 2). In these compounds, the glucuronic acid moiety points towards the opposite direction of the bulky methyl groups of the steroid backbone (Fig. 5). Furthermore, the A ring of etiocholanolone-G is at almost a right angle with respect to the steroid backbone, which results in even less flat structure and placing the glucuronide acid out of the plane at a higher angle (Fig. 5). This structure had the highest affinity to MRP3, resulting in a 10-fold lower K_m value than for androsterone-G that is otherwise structurally similar to etiocholanolone-G but with less bent A ring (Table 2, Fig. 5). The glucuronic acid moieties of dehydroepiandrosterone-G and

testosterone-G point towards the same direction as the bulky methyl groups and these two compounds have almost an order of magnitude higher K_m values for MRP3. MRP2, on the other hand, exhibited overall lower affinities for androgen glucuronides, and thus was less affected by differences in the steroid backbone. This might indicate a more flexible binding site and wide compound acceptance for MRP2. Interestingly, its transport of androsterone-G and epitestosterone-G showed sigmoidal kinetics (Fig. 4A, 4B and Table 2). Sigmoidal transport kinetics by MRP2 have been reported also for another steroid conjugate, estradiol-17-glucuronide [31,35]. Together, differences in the selectivity of MRP2, MRP3 and BCRP may have significant implications for the disposition and excretion of steroid metabolites in humans.

4.2 Efflux transporters in the disposition of androgen glucuronides

Androgens are mostly excreted as conjugates and the biliary excretions of androsterone, dehydroepiandrosterone and etiocholanolone are clearly below ten percent, while for testosterone it is slightly above ten percent [3,36-38]. On the other hand, the biliary excretion of the main estrogens is much higher and could comprise half of their total excretion [38]. Since glucuronide conjugates are the main metabolites for most androgens and estrogens, the difference in biliary excretion could partly be explained by the low BCRP and high MRP3 transport of androgen glucuronides, because BCRP efficiently transports only estrogen glucuronides (Fig. 2 and Table 2) [24]. For androgens, this appears to result in higher basolateral efflux transport to the systemic circulation compared with excretion to bile, while estrogens are more likely to be transported in bile than androgens.

Epitestosterone-G and testosterone-G are excreted physiologically in urine in similar amounts, resulting typically in testosterone-G to epitestosterone-G ratio between one and two, although among individuals this ratio varies from below one up to five [12]. Interestingly, the endogenous production of epitestosterone is only about 3% of the testosterone's biosynthesis, while the urinary excretion of their glucuronides (mass per day) is almost equal, indicating more efficient formation and excretion of epitestosterone-G to urine [10]. Rapid excretion of epitestosterone-G, the main metabolite of epitestosterone, in urine is found also after exogenous administration of epitestosterone [10,19,39]. Testosterone is also metabolized rapidly after exogenous administration and subsequently testosterone-G is excreted in urine quickly, but it comprises only a few percent of the total testosterone metabolites [17]. In addition, testosterone conjugates are excreted in bile to some extent, which leads to excretion of unconjugated testosterone via feces [36,38]. Our results indicate that apical (MRP2) and basolateral (MRP3) efflux transporters control the excretion of epitestosterone-G and testosterone-G differently (Fig. 2C, 2E and Table 2). MRP3 transports epitestosterone-G 10-fold more efficiently than testosterone-G, while MRP2 transports both glucuronides similarly but at much lower apparent affinity than MRP3 (Fig. 3A, 4A and Table 2). These results suggest that MRP3 contributes to systemic excretion of epitestosterone-G and testosterone-G from the liver and intestine, while MRP2 may transport particularly some testosterone-G in bile. These differences may affect the excretion of testosterone-G and epitestosterone-G, and their ratio in urine.

Androsterone-G and etiocholanolone-G are the end metabolites of androgens and the main metabolites of testosterone [3,4,17]. Only a few percent of androsterone and etiocholanolone are excreted in bile and none in feces after exogenous administration of these steroids, while neither is excreted in bile after testosterone administration [37].

The low biliary and intestinal excretion of androsterone-G and etiocholanolone-G could be explained by our results showing that MRP2 has a low affinity towards these glucuronides (Fig. 2A, 2D, 4B, Table 2). On the other hand, androsterone and etiocholanolone are excreted rapidly in urine within a few hours as glucuronides following their intravenous administration or after administration of oral testosterone [17,20,37]. In particular, etiocholanolone-G appears more rapidly in the plasma and is eliminated faster than androsterone-G. The rapid urinary excretion of these glucuronides is explained by the high affinity transport of MRP3 in comparison to MRP2, and the high affinity MRP3-mediated transport of etiocholanolone-G in comparison to androsterone-G that appears to correspond to the faster excretion of the former metabolite (Fig. 3B, 4B, Table 2). To the best of our knowledge, the renal clearances of androgen glucuronides have not been comprehensively investigated. However, one study suggests that androsterone-G and etiocholanolone-G are excreted by filtration in the kidney [40]. It should be noted that UGT2B7 is also expressed in the kidney, and thus this enzyme might contribute to the glucuronidation of androsterone and etiocholanolone, alongside the hepatic and intestinal UGT2B7 and UGT2B17 [6,7]. However, quantitative contribution of different tissues to systemic glucuronidation of androsterone and etiocholanolone are not currently understood.

Dehydroepiandrosterone-G is present in the plasma and urine at much lower levels than the corresponding sulfate conjugate [16,18,41]. Interestingly, for androsterone the opposite is true, although the same enzyme, SULT2A1, catalyzes sulfation of both compounds with similar efficiency [16,41-43]. The reasons behind the small fraction of dehydroepiandrosterone-G in plasma and urine in comparison to dehydroepiandrosterone sulfate are unknown, particularly since the UGTs that catalyze dehydroepiandrosterone glucuronidation have not been identified, yet. Based

on the results of our current study, the hepatic basolateral efflux for dehydroepiandrosterone-G may be low (Table 2). In comparison to androsterone-G and epitestosterone-G, the in vitro clearance value of MRP3 is 50-fold lower for dehydroepiandrosterone-G, which might contribute to low systemic and urinary concentrations of dehydroepiandrosterone-G (Table 2).

While our manuscript was under preparation, Li and colleagues (2019) published transport data on androsterone-G, dihydrotestosterone glucuronide, etiocholanolone-G and testosterone-G [44]. Our results are in line with their observations that MRP2 is a low affinity and MRP3 a high affinity transporter for androgen glucuronides, while MRP4 and BCRP do not transport these conjugates. Our study also includes dehydroepiandrosterone-G and epitestosterone-G and thereby enhances significantly the understanding of androgen glucuronide disposition and provides further insights into structural features that affect steroid conjugate transport by the important human efflux transporters. In addition, our results reveal new aspects on the formation of urinary testosterone-G to epitestosterone-G ratio, which is commonly close to one despite a large difference in the biosynthesis rate of these two androgens. This ratio is crucial for detecting testosterone-doping abuse and it is important to understand its basis in order to better interpret large individual variation in it.

5. CONCLUSIONS

We studied here the efflux transport of important androgen conjugates androsterone-G, dehydroepiandrosterone-G, epitestosterone-G, etiocholanolone-G and testosterone-G. Our results reveal that MRP3 transports these conjugates at low K_m values, while the corresponding values for MRP2 are over a magnitude higher. In particular, the MRP3 transport of androsterone-G, epitestosterone-G and etiocholanolone-G resulted in K_m values of 3.5, 1.9 and 0.36 µM, which indicates a major role for this hepatic and intestinal basolateral transporter in the disposition of these androgen glucuronides in humans. In addition, we show that BCRP does not transport androgen glucuronides, which is in sharp difference to its high transport of estrogen conjugates.

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DECLARATIONS OF INTEREST

None

REFERENCES

[1] C.J. Bagatell, W.J. Bremner, Androgens in men - Uses and abuses. New Engl. J. Med. 334 (11) (1996) 707-714.

[2] A.H. Payne, D.B. Hales, Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones. Endocr. Rev. 25 (6) (2004) 947-970.

[3] F.T.G. Prunty, Androgen Metabolism in Man—Some Current Concepts. Brit. Med. J. 2 (5514) (1966) 605-613.

[4] L. Schiffer, W. Arlt, K.-. Storbeck, Intracrine androgen biosynthesis, metabolism and action revisited. Mol. Cell. Endocrinol. 465 (2018) 4-26.

[5] T. Sten, I. Bichlmaier, T. Kuuranne, A. Leinonen, J. Yli-Kauhaluoma, M. Finel, UDP-glucuronosyltransferases (UGTs) 2B7 and UGT2B17 display converse specificity in testosterone and epitestosterone glucuronidation, whereas UGT2A1 conjugates both androgens similarly. Drug Metab. Dispos. 37 (2) (2009) 417-423.

[6] T. Sten, M. Kurkela, T. Kuuranne, A. Leinonen, M. Finel, UDP-glucuronosyltransferases in conjugation of 5a- and 5ß-androstane steroids. Drug Metab. Dispos. 37 (11) (2009) 2221-2227.

[7] Y. Sato, M. Nagata, K. Tetsuka, K. Tamura, A. Miyashita, A. Kawamura, T. Usui, Optimized methods for targeted peptide-based quantification of human uridine 59-diphosphate-glucuronosyltransferases in biological specimens using liquid chromatography-tandem mass spectrometry. Drug Metab. Dispos. 42 (5) (2014) 885-889.

[8] E.P. Giorgi, W.D. Stein, The transport of steroids into animal cells in culture. Endocrinology 108 (2) (1981) 688-697.

[9] M.J. Zamek-Gliszczynski, 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.

[10] H. Wilson, M.B. Lipsett, Metabolism of epitestosterone in man. J. Clin. Endocrinol. Metab. 26 (8) (1966) 902-914.

[11] L. Stárka, Epitestosterone. J. Steroid Biochem. Mol. Biol. 87 (1) (2003) 27-34.

[12] P. Van Renterghem, P. Van Eenoo, H. Geyer, W. Schänzer, F.T. Delbeke, Reference ranges for urinary concentrations and ratios of endogenous steroids, which can be used as markers for steroid misuse, in a Caucasian population of athletes. Steroids 75 (2) (2010) 154-163.

[13] P.-. Sottas, N. Robinson, O. Rabin, M. Saugy, The athlete biological passport. Clin. Chem. 57 (7) (2011) 969-976.

[14] J. Jakobsson, L. Ekström, N. Inotsume, M. Garle, M. Lorentzon, C. Ohlsson, H.-. Roh, K. Carlström, A. Rane, Large differences in testosterone excretion in Korean and Swedish men are strongly associated with a UDP-glucuronosyl transferase 2B17 polymorphism. J. Clin. Endocrinol. Metab. 91 (2) (2006) 687-693.

[15] J.J. Schulze, M. Lorentzon, C. Ohlsson, J. Lundmark, H.-. Roh, A. Rane, L. Ekström, Genetic aspects of epitestosterone formation and androgen disposition: Influence of polymorphisms in CYP17 and UGT2B enzymes. Pharmacogenet. Genomics 18 (6) (2008) 477-485.

[16] F. Badoud, J. Boccard, C. Schweizer, F. Pralong, M. Saugy, N. Baume, Profiling of steroid metabolites after transdermal and oral administration of testosterone by ultra-

high pressure liquid chromatography coupled to quadrupole time-of-flight mass spectrometry. J. Steroid Biochem. Mol. Biol. 138 (2013) 222-235.

[17] S.-. Peng, J. Segura, M. Farré, J.C. González, X. De La Torre, Plasma and urinary markers of oral testosterone undecanoate misuse. Steroids 67 (1) (2002) 39-50.

[18] M. Brochu, A. Bélanger, Comparative study of plasma steroid and steroid glucuronide levels in normal men and in men with benign prostatic hyperplasia. Prostate 11 (1) (1987) 33-40.

[19] L. Dehennin, G. Pérès, Plasma and urinary markers of oral testosterone misuse by healthy men in presence of masking epitestosterone administration. Int. J. Sports Med. 17 (5) (1996) 315-319.

[20] A. Basit, J.K. Amory, B. Prasad, Effect of Dose and 5a-Reductase Inhibition on the Circulating Testosterone Metabolite Profile of Men Administered Oral Testosterone. Clin. Transl. Sci. 11 (5) (2018) 513-522.

[21] X. Chu, K. Korzekwa, R. Elsby, K. Fenner, A. Galetin, Y. Lai, P. Matsson, A. Moss, S. Nagar, G.R. Rosania, J.P.F. Bai, J.W. Polli, Y. Sugiyama, K.L.R. Brouwer, Intracellular drug concentrations and transporters: Measurement, modeling, and implications for the liver. Clin. Pharmacol. Ther. 94 (1) (2013) 126-141.

[22] 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.

[23] E. Cho, R.B. Montgomery, E.A. Mostaghel, Minireview: SLCO and ABC transporters: A role for steroid transport in prostate cancer progression. Endocrinology 155 (11) (2014) 4124-4132.

[24] E. Järvinen, F. Deng, H. Kidron, M. Finel, Efflux transport of estrogen glucuronides by human MRP2, MRP3, MRP4 and BCRP. J. Steroid Biochem. Mol. Biol. 178 (2018) 99-107.

[25] 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-Gliszczynski, L. Zhang, Membrane transporters in drug development. Nat. Rev. Drug Discov. 9 (3) (2010) 215-236.

[26] 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.

[27] M.J. Zamek-Gliszczynski, X. Chu, J.W. Polli, M.F. Paine, A. Galetin, Understanding the transport properties of metabolites: Case studies and considerations for drug development. Drug Metab. Dispos. 42 (4) (2014) 650-664.

[28] K.L.R. Brouwer, D. Keppler, K.A. Hoffmaster, D.A.J. Bow, Y. Cheng, Y. Lai, J.E. Palm, B. Stieger, R. Evers, In vitro methods to support transporter evaluation in drug discovery and development. Clin. Pharmacol. Ther. 94 (1) (2013) 95-112.

[29] R. Elsby, V. Smith, L. Fox, D. Stresser, C. Butters, P. Sharma, D.D. Surry, Validation of membrane vesicle-based breast cancer resistance protein and multidrug resistance protein 2 assays to assess drug transport and the potential for drug-drug interaction to support regulatory submissions. Xenobiotica 41 (9) (2011) 764-783.

[30] 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.

[31] 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. 14 (10) (2017) 3299-3311.

[32] E. Järvinen, N. Sjostedt, J.B. Koenderink, H. Kidron, M. Finel, Efflux Transport of Nicotine, Cotinine and Trans-3'-hydroxycotinine glucuronides by Human Hepatic Transporters. Basic Clin. Pharmacol. Toxicol. (2019).

[33] 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.

[34] N. Sjöstedt, T.A. Salminen, H. Kidron, Endogenous, cholesterol-activated ATP-dependent transport in membrane vesicles from Spodoptera frugiperda cells. Eur. J. Pharm. Sci. 137 (2019).

[35] 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.

[36] A.A. Sandberg, W.R. Slaunwhite Jr., Metabolism of 4-C14-testosterone in human subjects. I. Distribution in bile, blood, feces and urine. J. Clin. Invest. 35 (12) (1956) 1331-1339.

[37] W.R. Slaunwhite Jr., A.A. Sandberg, Metabolism of 4-C14-testosterone in human subjects. III. Fate of androsterone and etiocholanolone. J. Clin. Endocrinol. Metab. 18 (10) (1958) 1056-1066.

[38] W.R. Slaunwhite Jr., M.J. Burgett, A.A. Sandburg, Disposition of dehydroepiandrosterone and its sulfate in human subjects. J. Clin. Endocrinol. Metab. 27 (5) (1967) 663-670.

[39] L. Dehennin, On the origin of physiologically high ratios of urinary testosterone to epitestosterone: Consequences for reliable detection of testosterone administration by male athletes. J. Endocrinol. 142 (2) (1994) 353-360.

[40] A.E. Kellie, E.R. Smith, Renal clearance of 17-oxo steroid conjugates found in human peripheral plasma. Biochem. J. 66 (3) (1957) 490-495.

[41] L. Dehennin, M. Ferry, P. Lafarge, G. Pérès, J.-. Lafarge, Oral administration of dehydroepiandrosterone to healthy men: Alteration of the urinary androgen profile and consequences for the detection of abuse in sport by gas chromatography-mass spectrometry. Steroids 63 (2) (1998) 80-87.

[42] H.-. Chang, R. Shi, P. Rehse, S.-. Lin, Identifying androsterone (ADT) as a cognate substrate for human dehydroepiandrosterone sulfotransferase (DHEA-ST) important for steroid homeostasis: Structure of the enzyme-ADT complex. J. Biol. Chem. 279 (4) (2004) 2689-2696.

[43] J.W. Mueller, L.C. Gilligan, J. Idkowiak, W. Arlt, P.A. Foster, The regulation of steroid action by sulfation and desulfation. Endocr. Rev. 36 (5) (2015) 526-563.

[44] C.Y. Li, A. Basit, A. Gupta, Z. Gáborik, E. Kis, B. Prasad, Major glucuronide metabolites of testosterone are primarily transported by MRP2 and MRP3 in human liver, intestine and kidney. J. Steroid Biochem. Mol. Biol. 191 (2019).

[45] Schrödinger LLC. (2019) The PyMOL Molecular Graphics System, Version 2.1.0

Supplementary materials for

Human Efflux Transport of Testosterone, Epitestosterone and Other Androgen Glucuronides

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Figures S1-S5 present nuclear magnetic resonance (NMR) analyses of epitestosterone-G that was biosynthesized in our laboratory (see Materials and Methods of the main text)

Figure S6 shows transport results for all the tested androgen glucuronides at 50 µM concentration, whereas in the main text the results for 1 µM substrate concentration are shown. The experimental details are presented in Figure 2 and Materials and Methods section of the main text .

Figure S7 presents results of time-dependent transport assays, for both MRP2 and MRP3, each one with all the tested substrates. The experimental details are same as are presented in Figure 2 and Materials and Methods section of the main text, except the concentration in the assays was 10 µM.

FIGURE S1. ^{1}H NMR spectrum of epitestosterone-G

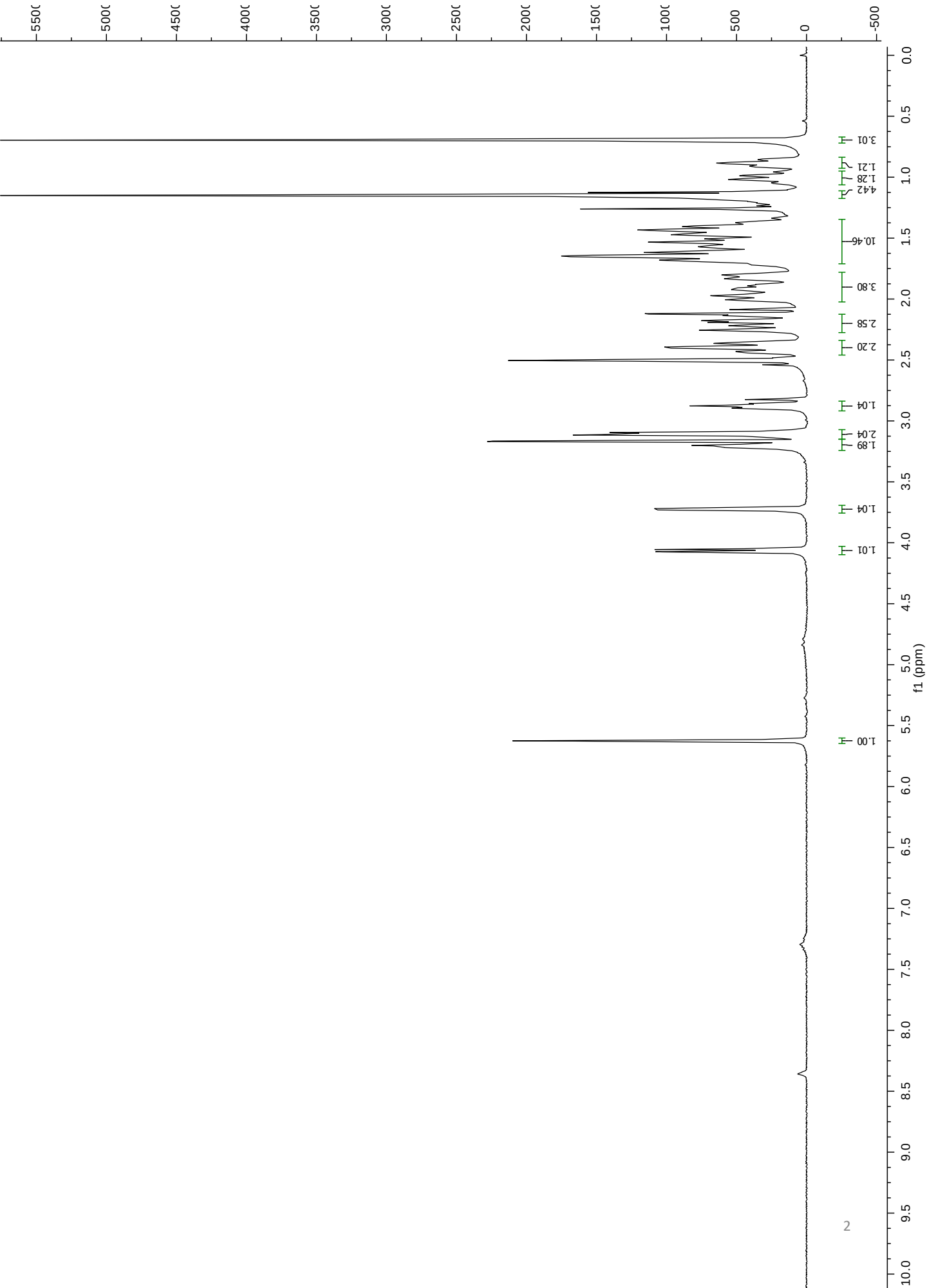

FIGURE S2. ^{13}C NMR spectrum of epitestosterone-G

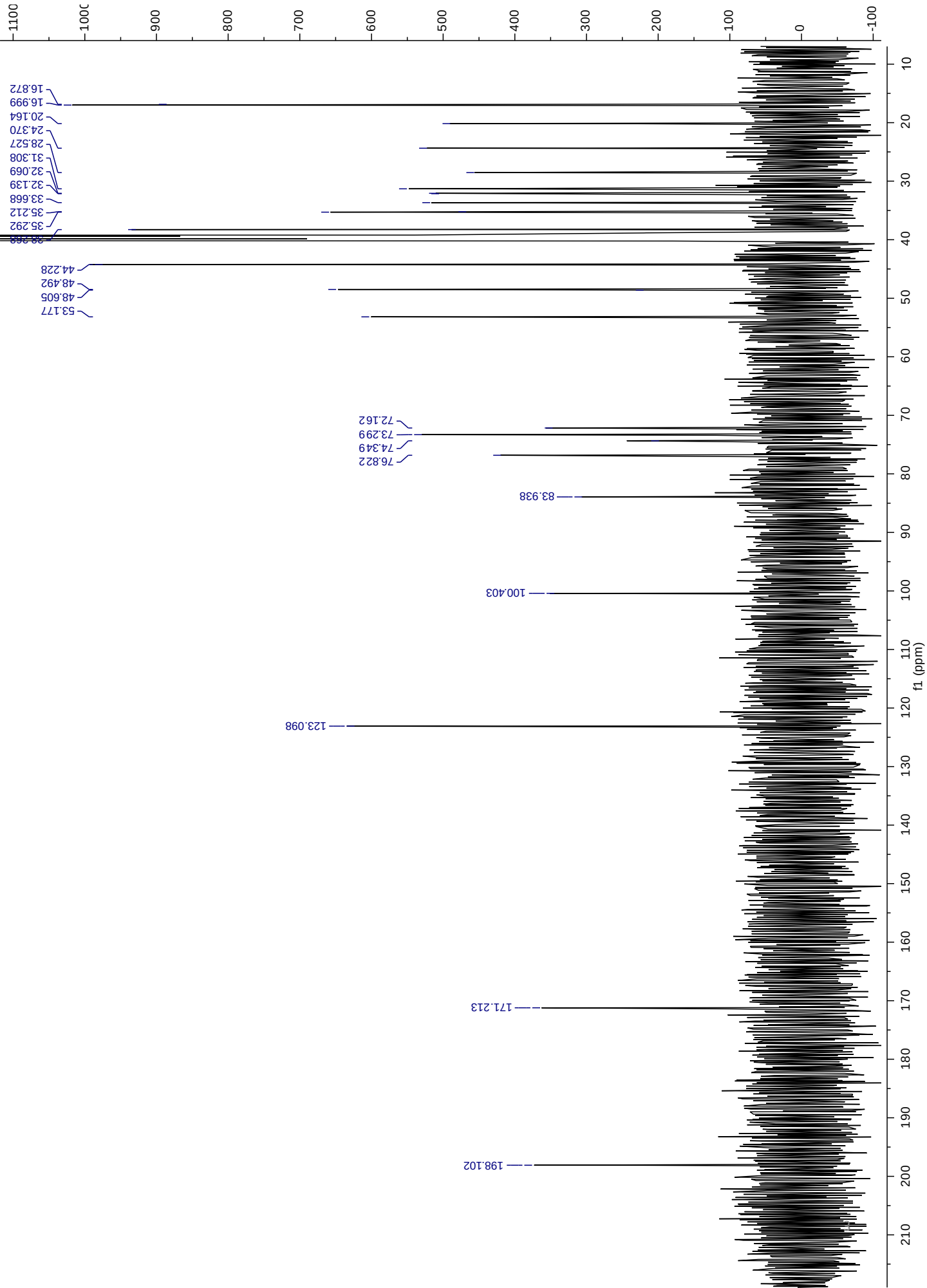

FIGURE S3. ^{1}H-^{13}C Heteronuclear Single Quantum Correlation - Distortionless Enhancement by Polarization Transfer (HSQC-DEPT) NMR spectrum of epitestosterone-G

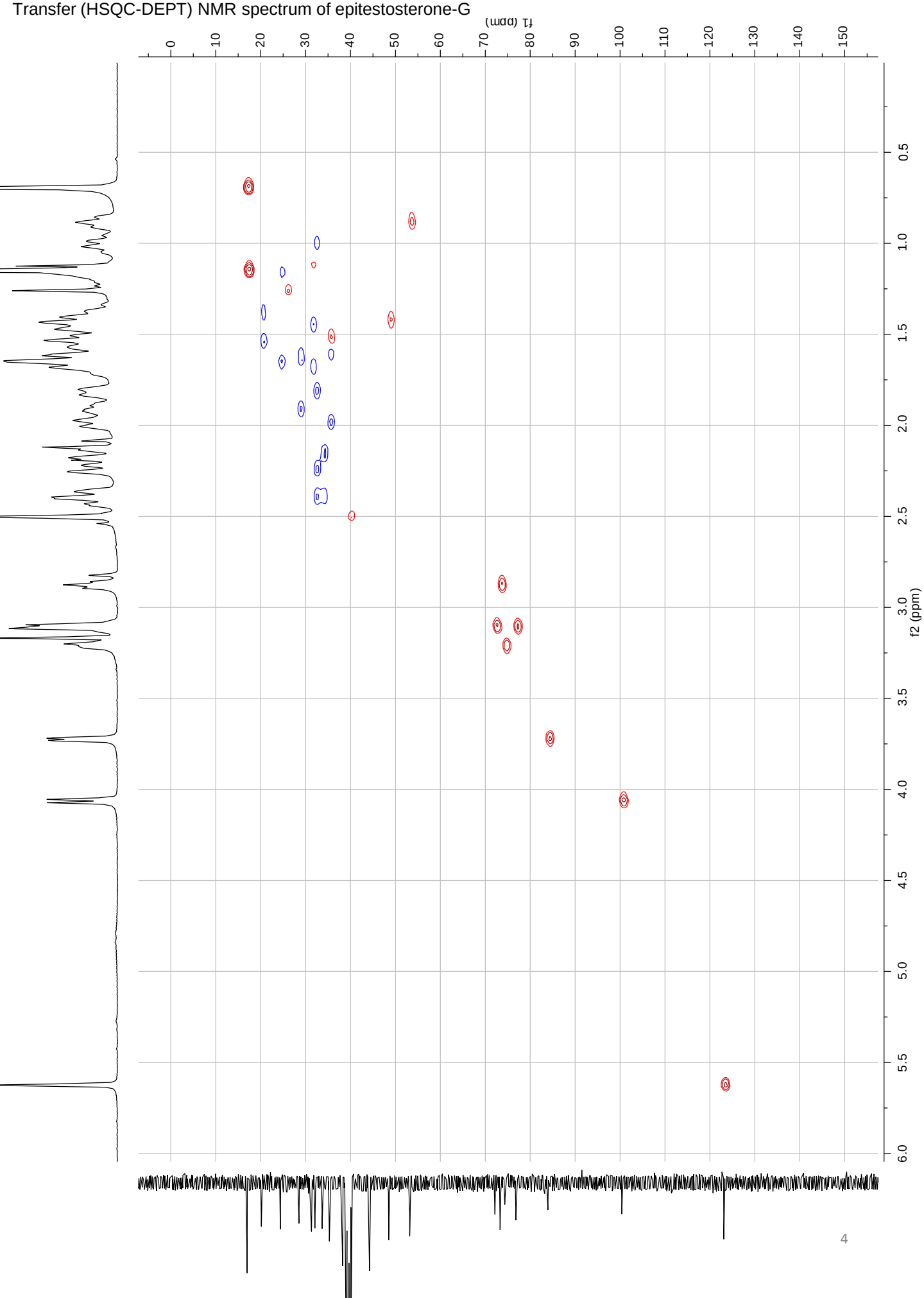

FIGURE S4. ^{1}H-^{13}C Heteronuclear Multiple Bond Correlation (HMBC) NMR spectrum of epitestosterone-G

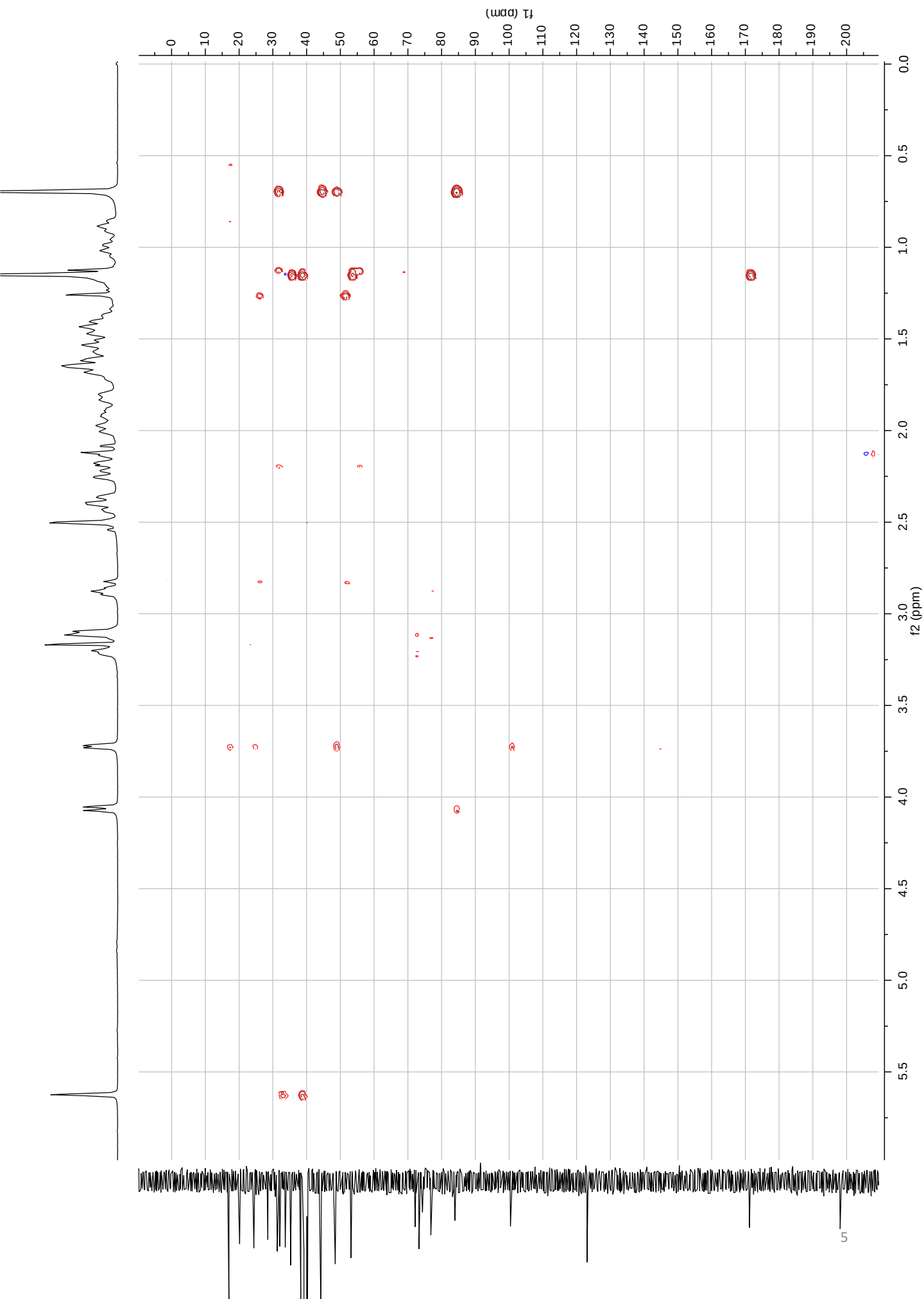

FIGURE S5. ^{1}H-^{1}H Correlation Spectroscopy (COSY) NMR spectrum of epitestosterone-G

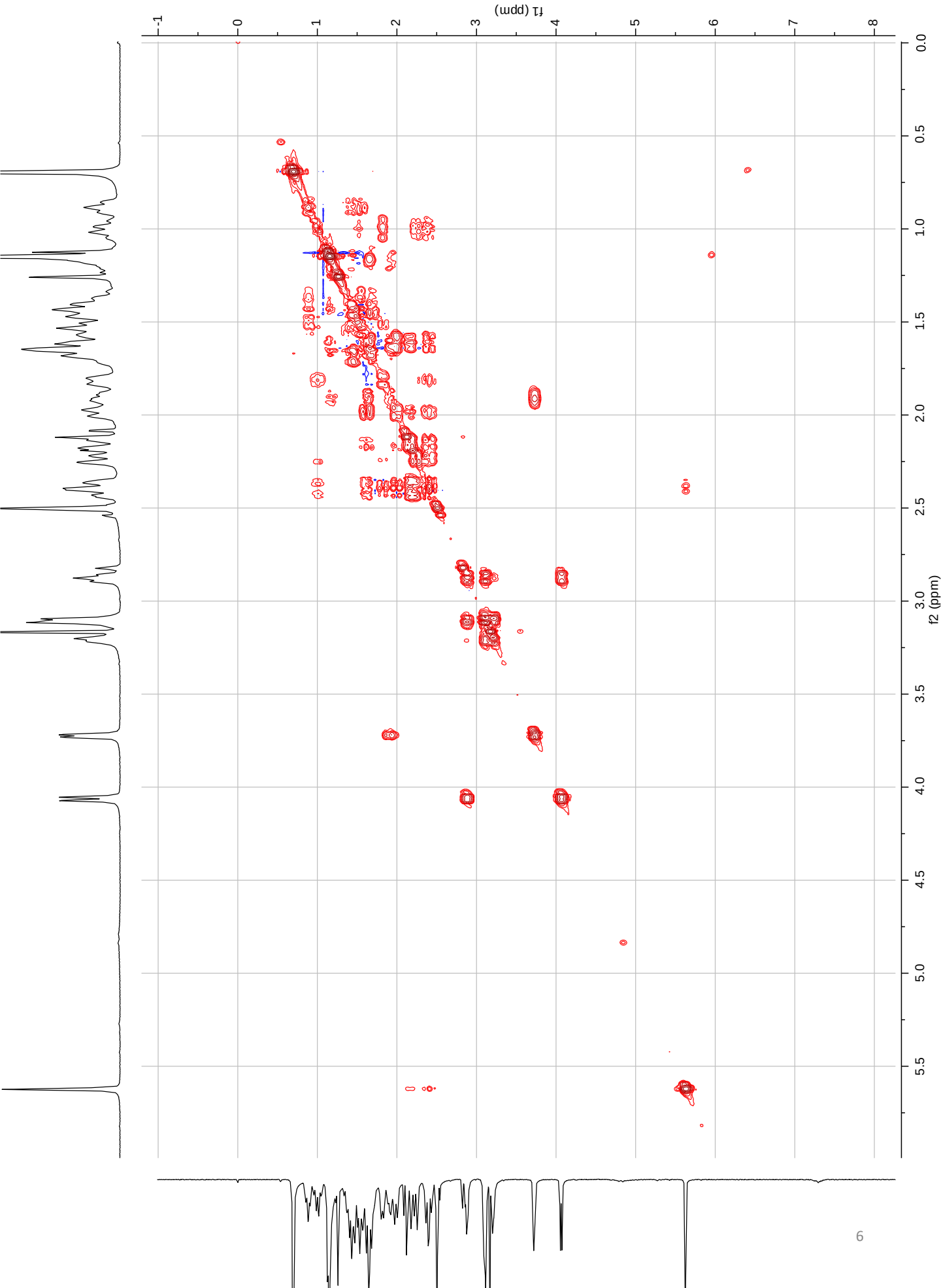

FIGURE S6. Human efflux transporters in the transport of 50 µM androgen glucuronides.

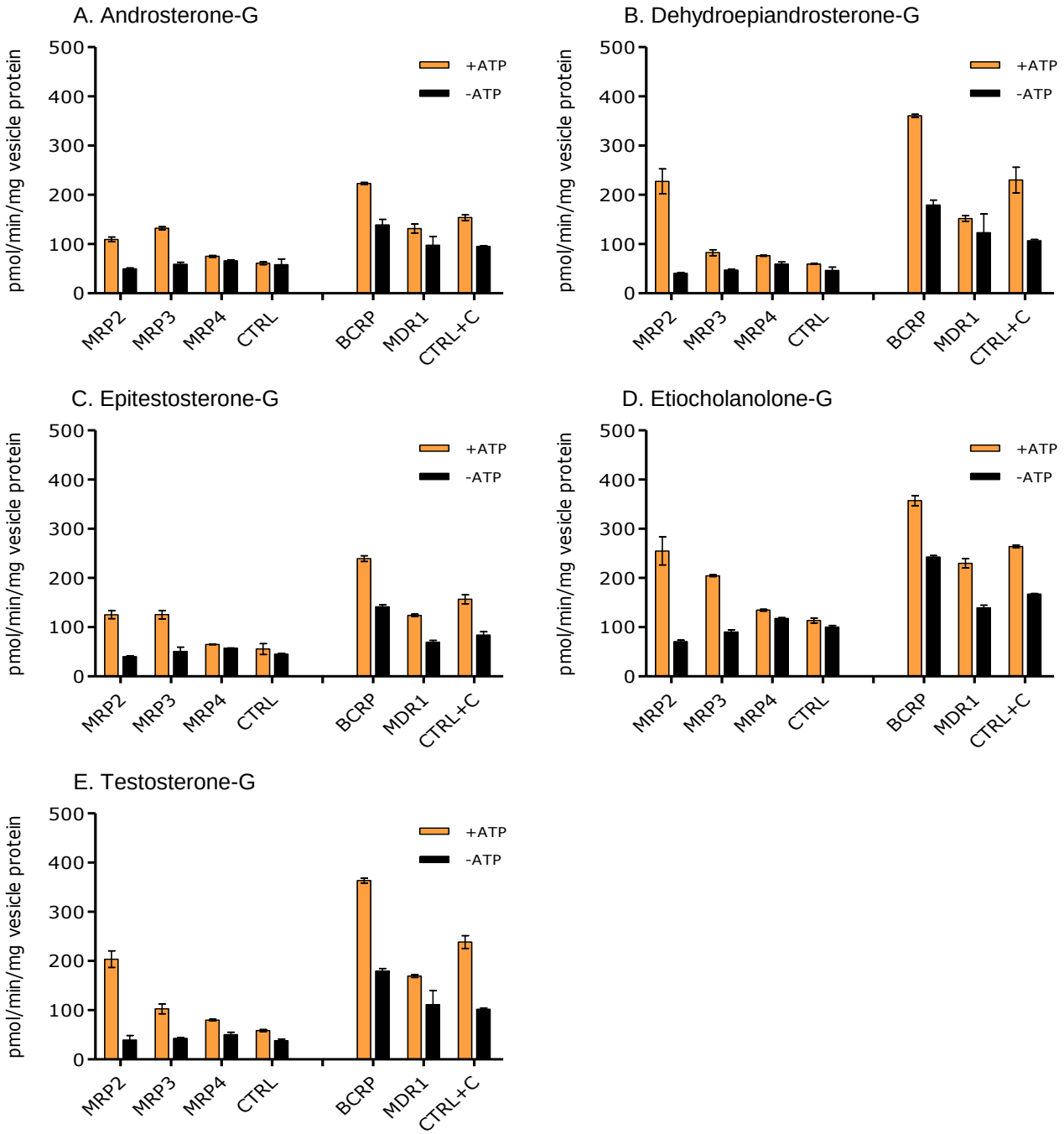

FIGURE S7. MRP2 and MRP3 time-dependent transport of 10 µM androgen glucuronides