DMD Fast Forward. Published on November 14, 2019 as DOI: 10.1124/dmd.119.088229
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Glucuronidation of Abiraterone and its Pharmacologically Active Metabolites by UGT1A4, Influence of Polymorphic Variants and their Potential as Inhibitors of Steroid Glucuronidation.

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Running title

Glucuronidation of abiraterone and its metabolites

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Number of words:

Abstract: 242

Introduction: 529

Discussion: 1247

References: 43

Number of:

Text pages: 17

Tables: 3

Figures: 6

Supplementary Tables: 1

Supplementary Figures: 8

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Non-standard abbreviations:

5α-Abi : 5α-abiraterone

A5-diol : androstanediol

AA : abiraterone acetate

Abi: abiraterone

ADT : androgen deprivation therapy

D4A : Δ^4-abiraterone

DHEA: dehydroepiendrosterone

DHT : dihydrotestosterone

G: glucuronide

Gal: galeterone

HEK: human embryonic kidney

HLM: human liver microsomes

HIM: human intestinal microsomes

HKM: human kidney microsomes

IC_{50}: half maximal inhibitory concentration

K_i: inhibitor constant

K_m: Michaelis constant

LC-MS/MS: liquid chromatography-tandem mass spectrometry

Testo: testosterone

UDP-GlcA: UDP-glucuronic acid

UGT: UDP-glucuronosyltransferases

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V_{max}: maximum velocity

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Abstract

Abiraterone acetate (AA) is a prodrug of abiraterone (Abi), a CYP17A1 inhibitor used to treat patients with advanced prostate cancer (PCa). Abi is a selective steroidal inhibitor that blocks the biosynthesis of androgens. It undergoes extensive biotransformation by steroid pathways leading to the formation of pharmacologically active Δ^4-abiraterone (D4A) and 5α-abiraterone (5α-Abi). This study aimed to characterize the glucuronidation pathway of Abi and its two active metabolites. We show that Abi, its metabolites and another steroidal inhibitor galeterone (Gal), undergo secondary metabolism to form glucuronides (G) in human liver microsomes with minor formation by intestine and kidney microsomal preparations. The potential clinical relevance of this pathway is supported by the detection by liquid chromatography-tandem mass spectrometry (LC-MS/MS) of Abi-G, D4A-G and 5α-Abi-G in patients under AA therapy. A screening of UGT enzymes reveals that UGT1A4 is the main enzyme involved. This is supported by inhibition experiments using a selective UGT1A4 inhibitor hecogenin. A number of common and rare nonsynonymous variants significantly abrogate the UGT1A4-mediated formation of Abi-G, D4A-G and 5α-Abi-G *in vitro*. We also identify Gal, Abi and its metabolites as highly potent inhibitors of steroid inactivation by the UGT pathway with submicromolar K_i values. They reduce the glucuronidation of both the adrenal precursors and potent androgens in human liver, prostate cancer cells and by recombinant UGTs involved in their inactivation. In conclusion, tested CYP17A1 inhibitors are metabolized through UGT1A4 and germline variations affecting this metabolic pathway may also influence drug metabolism.

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Significance statement

The antiandrogen abiraterone (Abi) is a selective steroidal inhibitor of the cytochrome P450 17α−hydroxy/17,20-lyase (CYP17A1), an enzyme involved in the biosynthesis of androgens. Abi is metabolized to pharmacologically active metabolites by steroidogenic enzymes. We demonstrate that Abi and its metabolites are glucuronidated in the liver and that their glucuronide derivatives are detected at variable levels in circulation of treated prostate cancer patients. UGT1A4 is the primary enzyme involved and nonsynonymous germline variations affect this metabolic pathway *in vitro*, suggesting a potential influence of drug metabolism and action in patients. Their inhibitory effect on drug and steroid glucuronidation raises the possibility that these pharmacological compounds might affect the UGT-associated drug-metabolizing system and pre-receptor control of androgen metabolism in patients.

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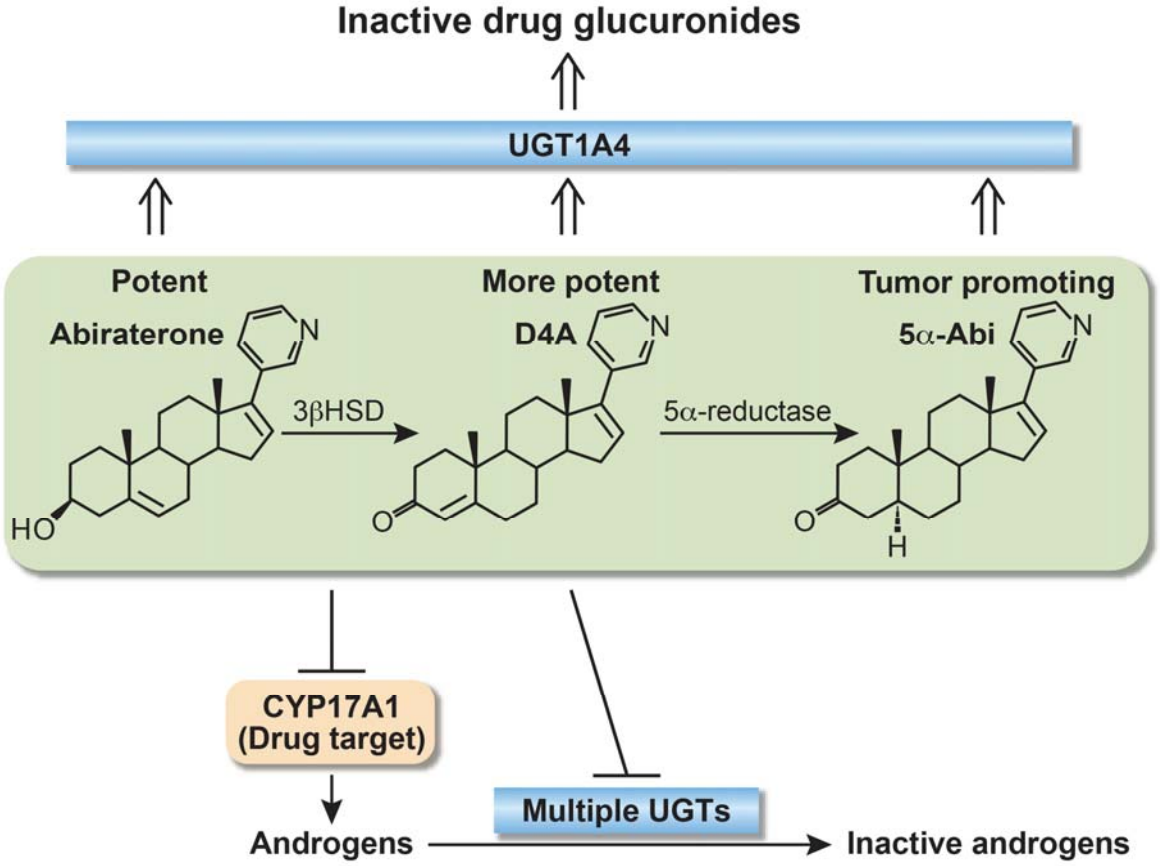
Visual abstract



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Introduction

Prostate cancer (PCa) is the leading cause of cancer in men and the second cause of death in Western countries (Miller et al., 2016). Much progress has been made in the last few years with new generation hormonal therapies. Abiraterone (Abi) and enzalutamide have significantly changed clinical management, increasing survival and quality of life of patients with metastatic PCa (Fizazi et al., 2012; Scher et al., 2012; Beer et al., 2014; Ryan et al., 2015; Tucci et al., 2015). In advanced disease, PCa cells remain dependent on androgens for proliferation (Montgomery et al., 2008). Therefore, Abi, orally administered as the prodrug abiraterone acetate (AA), is commonly used as part of the androgen deprivation therapy (ADT) in advanced disease clinical settings.

Abi is a selective steroidal inhibitor of the cytochrome P450 17α−hydroxy/17,20-lyase (CYP17A1), an enzyme involved in the biosynthesis of androgens. Abi is an androgen-receptor–axis targeted agent that blocks the formation of androgens in testes, adrenal, peripheral tissues and prostate tumor cells. Recently, new metabolites of Abi formed by steroidogenic enzymes have been described, with at least two exhibiting significant pharmacological activities, Δ^4-abiraterone (D4A) and 5α-abiraterone (5α-Abi) (Li et al., 2016). Abi is first converted to D4A by 3β-hydroxysteroid dehydrogenase (3β-HSD) and blocks CYP17A1, 3β–HSD and steroid-5α-reductase (SRD5A), whereas D4A also antagonizes the androgen receptor (AR) (Li et al., 2015). D4A is irreversibly converted to 3-keto-5α-Abi (5α-Abi) or 3-keto-5β-Abi (5β-Abi). Both metabolites may then be converted to their 3α-OH and 3β-OH derivatives for a total of 6 downstream metabolites of D4A. 5β-Abi metabolites were not reported to be active. On the other hand, the direct

product of D4A, 5α-Abi, acts as an agonist of the AR (Li et al., 2016). Likewise, the structurally related CYP17A1 inhibitor galeterone (Gal) yields analogous metabolites (Alyamani et al., 2017).

We hypothesized that, similar to endogenous steroids, Abi, its metabolites and the structurally related Gal (**Supp. Fig.1**), undergo direct metabolism by the glucuronidation pathway. A large family of 19 uridine 5′-diphosphate-glucuronosyltransferase (UGT) enzymes catalyze this phase II drug metabolic pathway (Guillemette et al., 2014). UGTs are involved in the addition of a glucuronic acid (GlcA) moiety to a large diversity of acceptor molecules including therapeutic drugs from all classes containing hydroxyl, carboxylic acid, thiol, or amine groups (Guillemette et al., 2014). This leads to the formation of glucuronide (G) derivatives that most often lack biological activity and that are readily excreted through bile and urine. This pathway also catalyzes the clearance of steroids such as the potent androgen dihydrotestosterone (DHT), and thereby regulates their bioavailability and the hormonal environment to which PCa cells are exposed (Beaulieu et al., 1996). The UGT pathway undergoes complex regulation. This involves regulatory loops characterized by the ability of UGT substrates to regulate the expression of genes encoding UGT enzymes involved in their metabolism (Hu et al., 2014). We further postulated that CYP17A1 inhibitors potentially influence endogenous steroid metabolism by affecting their conjugation by UGTs.

Herein, we report that Abi and its metabolites as well as Gal undergoes metabolism by the UGT pathway and that they inhibit inactivation of steroids. Results of these *in vitro*

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investigations are reinforced by the detection of glucuronide derivatives of Abi, D4A and 5α-Abi in PCa patients treated with AA.

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Materials and Methods

Chemicals and Reagents

All chemicals and reagents were of the highest grade commercially available. UDP-glucuronic acid (UDP-GlcA) and bilirubin were obtained from Sigma-Aldrich (Oakville, ON, Canada). All chemicals and solvents used for the mass spectrometry were HPLC grade. Milli-Q water was produced using the Millipore system. Abi and Gal were purchased from Toronto Research Chemical Inc. (Toronto, ON, Canada). Tacrolimus (FK-506) was purchased from Cell Signaling Technology (Danvers, MA). β-glucuronidase type VII from *Escherichia coli* was purchased from Sigma (St. Louis, MO). Hecogenin was obtained from Santa Cruz Biotechnology (Dallas, TX). The Organic Synthesis Service of the CHU de Québec Research Center (Quebec, QC, Canada) synthesized D4A and 5α-Abi based on a published method (Li et al., 2016). To identify D4A and 5α-Abi synthesized by the Medical Chemistry platform, nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Advance 400 digital spectrometer (Billerica, MA) at 400 MHz for ^{1}H NMR. Spectra were referenced relative to the central residual proton solvent resonance in 1H NMR (CDCl3 = 7.26 ppm, MeOH-d4 = 3.31 ppm). HPLC analyses for chemical purities (D4A 96% purity and 5α-Abi 98% purity) were performed on a Shimadzu Prominence instrument (Kyoto, Japan) using a diode array detector (wavelength detection = 190). Abi-G, D4A-G, 5α-Abi-G, and Gal-G analytical standards were produced from *in vitro* enzymatic assays as described (Caron et al., 2019). Briefly, the glucuronide formed was treated with β-glucuronidase type VII from *E. coli* (50 U) incubated at 37°C for 20 h in phosphate buffer (pH 6.5) to cleave the

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GlcA group and allow quantification with a calibration curve of the corresponding aglycone. Deuterated glucuronides were also produced and purified according to the same procedure. Microsomal preparations from human tissues (liver mixed gender pool of 50 individuals: catalog #H0620, lot #1410013 (24 females, 26 males), #1210267 (19 females, 31 males) and #1610016 (20 females, 30 males), kidney mixed gender pool of 8 individuals, catalog #H0610.R, lot #0810236 (4 females, 4 males), and colon mixed gender pool of 7, catalog #452210, lot #05886 (4 females, 3 males)) and commercial Supersomes (UGT1A1 (catalog #456411, lot #6320009), 1A3 (catalog #456413, lot #7065003), 1A4 (catalog #456414, lot #6334004, #7179004 and #8073001), 1A6 (catalog #456416, lot #6216001), 1A7 (catalog #456407, lot #4308001), 1A8 (catalog #456418, lot #7177002), 1A9 (catalog #456419, lot #7065002), 2B4 (catalog #456424, lot # 6244003), 2B7 (catalog #456427, lot #7101003), 2B15 (catalog #456435, lot #5348003) and 2B17 (catalog #456437, lot #6312001, #8038005 and #8177003)) were purchased from Xenotech (Kansas City, KS), BD Biosciences (Woburn, MA) and Corning (Corning, NY), respectively. Microsomal protein extracts from HEK293-UGT1A4 cells expressing variant enzymes as well as LNCaP microsomes were prepared by differential centrifugation and quantified by Western blot as described (Villeneuve et al., 2003; Laverdiere et al., 2011).

Glucuronidation Assays

Enzymatic assays were conducted with 10 µg of UGT membrane protein, 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM UDP-GlcA, pepstatin, leupeptin and 20 µg/mL alamethicin in a final volume of 100 µl. The reactions were terminated by adding 100 µl

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of methanol and were centrifuged at 14,000*g* for 10 min before analysis. Kinetics were conducted with microsomal fractions of a pool of 50 human livers, commercial UGT1A4 supersomes and HEK293 recombinant human UGT1A4*1 ($R^{11}P^{24}L^{48}$), UGT1A4*2 (T^{24}), UGT1A4*3 (V^{48}) and UGT1A4*4 (W^{11}). Kinetics were performed using substrate concentrations ranging from 0.1 to 400 µM for 30 min. Additional assays were done with 2 µM AA and HLM with an incubation time of 30 min and an overnight incubation to compare with Abi. Inhibition assays were performed using the following concentrations (0.5, 1, 2.5, 5, 10 µM) of a selective UGT1A4 inhibitor hecogenin and a constant concentration near K_m of substrates for 30 min. The half maximal inhibitory concentration (IC_{50}) was then calculated using GraphPad (7.0) (GraphPad Software Inc., La Jolla, CA). The inhibitor constant (K_i) values were evaluated using the following concentrations of hecogenin (0.5, 1, 2.5, 5, 10 µM) and three different concentrations of each substrate (Abi: 2, 100, 200 µM; D4A: 6, 100, 200 µM; 5α-Abi: 5, 100, 200 µM; Gal: 0.5, 2, 5 µM). The inhibition of steroid glucuronidation was performed using Abi, D4A, 5α-Abi and Gal as inhibitors and by monitoring glucuronidation of two potent androgens (testosterone (Testo), dihydrotestosterone (DHT)) and their adrenal precursors (dehydroepiandrosterone (DHEA) and androstanediol (A5-diol)). Those assays were performed for 30 min using three concentrations of inhibitors (5, 25, 200 µM) and three concentrations of substrates (5, 25, 200 µM), as indicated in the legends of figures.

Kinetics parameters were determined by non-linear regression using Sigma Plot 11.0 assisted by Enzyme Kinetics 1.3 (SSI, San Jose, CA) using the Marquardt-Levenberg algorithm, which solves non-linear least square equations. The best fitting enzyme

kinetics models were subsequently determined using goodness of fit criteria, including the coefficient of determination (r^2 values), Akaike information criteria (AIC), standard errors, and 95% confidence intervals, followed by a visual inspection of fitted functions. The enzyme kinetics were subsequently represented by Eadie-Hofstee plots for glucuronidation profiles or Lineweaver-Burk plots for inhibition models. Values are expressed as mean ± S.D. of triplicate determinations of at least two independent experiments. Enzymatic activities were considered statistically significant for *P* values < 0.05, according to Student's *t* test variance analysis.

Prostate cancer patients treated with AA

A study ongoing at our center recruits prostate cancer patients treated with AA at 1000 mg daily dose (CHU de Québec #2018-4125). The study is conducted in accordance to the declaration of Helsinki and all patients provided consent for this project. Plasma samples were collected at least one month after treatment initiation to assess steady state levels of Abi and its metabolites using a protocol described previously (Caron et al., 2019).

Mass spectrometry analyses

Detection of analytes was performed by high-performance liquid chromatography (LC) coupled to a Q-TRAP mass spectrometer (API6500 mass spectrometer (MS) - AB Sciex, Concord, ON, Canada) using a validated method as described (Caron et al., 2019). Briefly, the LC system consisted of a Nexera 30-AD (Shimadzu Scientific Instruments Inc., Columbia, MD) and controlled through Analyst Software, version 1.6.2. The MS was operated in multiple reactions monitoring mode (MRM) and equipped with

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a turbo spray source set at 500 °C. The resolution used for Q1 and Q3 was Unit/Unit and the voltage was held at 5500 V in positive mode. Mass transitions (m/z), ionisation mode, declustering potential (DP) and collision energy (CE) and lower limit of quantification (LLOQ) were reported in Caron *et al*. 2019 (Caron et al., 2019). For Gal, the chromatographic separation was identical as for Abi whereas the MRM transition for Gal-G1 and Gal-G2 was 565.2 → 389.2. The chromatographic separation was also the same for Gal. The detection of tacrolimus-G and bilirubin-G were performed as described (Levesque et al., 2007; Laverdiere et al., 2011). For the detection of DHT-G, Testo-G, DHEA-G, A5-diol-3G and A5-diol-17G, the chromatographic separation was achieved with an ACE 3 C18 HL 3.0 um packing material, 100 X 4.6 mm (Canadian Life Science, Peterborough, Ontario, Canada). The mobiles phases were water, 1 mM ammonium formate (solvent A) and methanol, 1 mM ammonium formate (solvent B) at a flow rate of 0.9 ml/min. DHT-G (484.4 →273.2) and DHEA-G (482.3 → 271.3) were detected in positive mode and eluted with 75% solvent B. Testo-G (463.1 → 75) was detected in negative mode and eluted with 75% solvent B. A5-diol-3G and A5-diol-17G (484.4 → 273.2) were detected in positive mode and eluted with the following gradient: 0-4.3 min, isocratic 50% B; 4.3-4.4 min, linear gradient 50-90% B; 4.4-5.4 min , isocratic 90% B; 5.4-5.5 min, 90-50%B; 5.5-8.0 min, isocratic 50%B.

Results

Glucuronidation of Abi, D4A, 5α-Abi and Gal in human liver microsomes and their detection in PCa patients treated with Abi

Enzymatic assays using pooled human liver microsomes (HLM) revealed the formation of secondary polar metabolites of Abi, D4A and 5α-Abi. One glucuronide derivative was observed upon D4A and 5α-Abi incubations and two glucuronides were observed from Abi, named G1 and G2 according to the order of elution (**Fig.1A**). The absence of polar metabolites in assays conducted without UDP-GlcA and their hydrolysis by β-glucuronidase treatment further supported that these metabolites correspond to glucuronide derivatives (**Fig.1B-C**). *In vitro* incubations in the same conditions, with the prodrug AA generated similar results compared to Abi with no difference in the nature of the peaks formed and their abundance in HLM, likely due to its rapid conversion to Abi (Bouhajib and Tayab, 2019). Based on the presence of possible reactive groups, one possibly corresponds to a N-linked glucuronide of Abi (G1) whereas the other to an O-linked glucuronide (G2). This is supported by our previous analysis (Caron et al., 2019), in which we observed a loss of signal for Abi-G1 peak when analyzed in negative mode whereas the signal for Abi-G2 remained. The loss of Abi-G1 peak is coherent with the quaternary amine being positively charged. Similarly, two glucuronides G1 and G2 were observed for the structurally related CYP17A1 inhibitor Gal. An NMR based analysis is required to clarify the identity of all glucuronide derivatives observed for Abi, D4A, 5α-Abi and Gal.

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A subset of PCa patients under AA therapy was analyzed to establish the presence of glucuronide derivatives of Abi and its metabolites *in vivo*, using a validated LC-MS/MS method recently published by our group (Caron et al., 2019). Abi-G, D4A-G and 5α-Abi-G were detected in circulation of patients at variable concentrations. Abi-G and 5α-Abi-G were measured in all patients whereas D4A-G was below LLOQ (>5 ng/ml) for 2 patients (**Table 1**). A larger study is required to help clarify the clinical significance of this pathway.

UGT1A4-mediated glucuronidation of Abi and its metabolites: kinetics characterization and UGT1A4 variants with impaired activity

Using recombinant human UGT enzymes, the hepatic UGT1A4 exhibited the highest glucuronidation activity for all four substrates while UGT1A3 displayed much lower activity (**Fig.2**). Screening experiments using 2 µM or 200 µM of substrate for 2 or 16-hour incubations with any UGT did not result in the formation of glucuronides besides with UGT1A4 and at a much lower extent with UGT1A3 (not shown). This is coherent with the expression profile of these two enzymes (Ohno and Nakajin, 2009; Margaillan et al., 2015), and the minor formation of glucuronide derivatives in the intestine and none detected in the kidney. Consistent with a major contribution of the UGT1A4 enzyme, kinetic parameters (apparent K_m, V_{max} and clearance values) of all four substrates were almost identical for HLM and UGT1A4 (**Table 2; Fig.3**). Eadie-Hofstee plots of HLM and recombinant UGT1A4 for all substrates are depicted in **Supp. Fig.2-4**, and suggest atypical and complex kinetics. Inhibition experiments with the selective

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UGT1A4 inhibitor hecogenin also led to highly comparable inhibition profiles and apparent inhibitory constant (K_i) values (0.29 to 1.05 μM) between HLM and UGT1A4 for all four substrates, further supporting its major involvement (**Fig.4; Supp. Fig.7**). Based on these results, we conclude that UGT1A4 is likely the only enzyme involved in the hepatic glucuronidation of Abi, D4A, 5α-Abi and Gal. We also observed that these compounds were effective at inhibiting their glucuronidation, leading to similar levels of inhibition by ~30-50% in assays performed at K_m values in the liver and with the UGT1A4 enzyme (**Supp. Fig.5)**. As an example, 2 μM of Abi proficiently inhibited the formation of D4A-G by 29% and 41% in HLM and UGT1A4 recombinant system, respectively.

The impact of common *UGT1A4* coding region variations was then evaluated using recombinant UGT1A4 isoenzymes expressed in HEK293 human cells, namely UGT1A4*1 ($R^{11}P^{24}L^{48}$), UGT1A4*2 (T^{24}), UGT1A4*3 (V^{48}) and UGT1A4*4 (W^{11}) (**Fig.5**). Compared to the UGT1A4*1 reference enzyme, the UGT1A4*4 isoenzyme presented a velocity reduced by nearly 50% for the conjugation of Abi, D4A and 5α-Abi (**Fig.5D**; **Supp. Table 1**). UGT1A4*2 and UGT1A4*3 had slightly higher affinities. Additional rare variants presented a significantly reduced activity compared with UGT1A4*1 by up to 90% at concentration based on the apparent K_m values, except for UGT1A4*9 (**Fig.5E**). This suggests that collectively, these variants UGT1A4 allozymes with coding variations may be associated with reduced hepatic glucuronidation of Abi and its metabolites.

Inhibition of steroid glucuronidation by Abi, D4A, 5α-Abi and Gal

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The ability of Abi, its metabolites and Gal to inhibit glucuronidation of androgens (Testo and DHT) and their adrenal precursors (DHEA and A5-diol) was also evaluated. Conjugation of these steroids is significant in human liver microsomes and by the UGT1A4 enzyme, except for Testo, which is a poor substrate of this enzyme. Steroid glucuronidation was inhibited by ≥ 40% in the presence of 2 µM Abi and reached nearly 90% at 200 µM (**Fig.6**). The inhibition pattern by D4A, 5α-Abi and Gal was similar for HLM and UGT1A4 (**Supp. Fig.6**). This inhibition potency was comparable to the effect of Abi on the hepatic conjugation of tacrolimus, a specific substrate of the UGT1A4 enzyme, whereas no inhibitory effect was observed using 2 µM of Abi for bilirubin-G formation performed by the UGT1A1 enzyme in HLM (data not shown). The estimated inhibitory K_i values for these drugs were in the low micromolar range, between 0.17 to 3.59 µM for HLM and UGT1A4 (**Table 3; Suppl. Fig.8**). Inhibition was with mixed characteristics according to goodness of fit statistics and the Lineweaver-Burk plot graphical representation of experimental data. We observed similar inhibitory effects for Testo-G formation in assays with the androgen-conjugating recombinant UGT2B15 and UGT2B17 enzymes, and the prostate cancer cell line LNCaP, expressing UGT2B15, UGT2B17 and UGT2B28 involved in local androgen inactivation. K_i values observed in the presence of Abi for Testo-G formation were 0.10 ± 0.04 µM, 0.73 ± 0.53 µM and 3.19 ± 3.74 µM for LNCaP, UGT2B17 and UGT2B15, respectively (**Table 3**).

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Discussion

Our study establishes that Abi, a steroidal CYP17A1 inhibitor, and its downstream active metabolites, are conjugated with GlcA in the liver, and that only the UGT1A4 enzyme expressed in human liver is involved. The structurally related androgen-receptor–axis targeted agent Gal is also a substrate of the UGT1A4 enzyme. Their glucuronidation *in vivo* is sustained by the detection of glucuronide derivatives of Abi, D4A and 5α-Abi in plasma samples of PCa patients treated with AA, with a predominance of Abi-G and 5α-Abi-G over D4A-G. We also demonstrate that a number of common and rare UGT1A4 variant allozymes with nonsynonymous substitutions exhibit significantly lower activity for these molecules compared to the reference UGT1A4 enzyme. Based on the results of our *in vitro* investigations, these pharmacological compounds further emerge as potent inhibitors of their conjugation in the liver by a mixed inhibition model. Abi, D4A and 5α-Abi also proficiently inhibit steroid glucuronidation catalyzed by UGT1A4 and other UGTs namely UGT2B15 and UGT2B17 expressed in the liver and PCa cells. This suggests that CYP17A1 inhibitors are conjugated by the glucuronidation pathway, with the potential to affect the UGT-associated drug-metabolizing system and potentially drug-endogenous molecule interactions.

Mass spectral analysis of purified glucuronide derivatives from these drugs suggests the formation of O- and N-glucuronide products of Abi, and N-glucuronide products of D4A and 5α-Abi, but this will require confirmation by NMR. Our work reveals that UGT1A4 catalyzes predominantly their glucuronidation with a potential minor role for UGT1A3.

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UGT1A4 is a main hepatic drug elimination pathway that participates in the N-glucuronidation of primary, secondary and aromatic amines, which include many pharmacologically important drugs such as lamotrigine, tamoxifen and tacrolimus (Argikar and Remmel, 2009; Zhou et al., 2010; Laverdiere et al., 2011). UGT1A4 is also capable of conjugating steroidal compounds with hydroxyl groups such as hecogenin, (Green and Tephly, 1996) a steroidal saponin aglycone found in the leaves of species from the *Agave* genus and a selective inhibitor of UGT1A4 (Paik et al., 2005). The glucuronidation efficiency of UGT1A4 for several steroids such as 3α-diol (5α-androstane-3α,17β-diol) and pregnanediol (5α-pregnane-3β,20α-diol) was demonstrated with K_m values of 16 μM and 7.3 μM compared to 18 μM for hecogenin (Green and Tephly, 1996). Zhou and collaborators also showed that UGT1A4 displays significant activity for the potent androgen DHT, also revealing the existence of multiple aglycone substrate binding sites in UGT1A4 (Zhou et al., 2010). Here, we observed inferior K_m values for Abi and its metabolites in the low micromolar range varying from 0.61 to 7.4 μM. Based on highly similar kinetic parameters observed for HLM and the UGT1A4 enzyme, we conclude that UGT1A4 is the major UGT contributing to the formation of both N- and O- glucuronides of Abi, its metabolites and Gal *in vitro*. This is also supported by the important loss of activity caused by the selective UGT1A4 inhibitor hecogenin that hinders drug-glucuronide formation by over 50 % at low micromolar concentration (e.g. at K_m value). The extent of the inhibition was comparable for UGT1A4 and liver microsomes, reinforcing the notion that this is likely the primary enzyme involved. Since UGT1A4 is not expressed in the prostate (Ohno and Nakajin,

2009), this reaction would take place primarily in the liver with a minor implication of the intestine. UGT1A3 is potentially responsible for the low activity observed in the human intestine microsomes based on its weak activity and its expression in this tissue (Ohno and Nakajin, 2009; Margaillan et al., 2015). Moreover, the lack of inhibition for UGT1A1-mediated bilirubin conjugation is also consistent with the low occurrence of hyperbilirubinemia observed in clinical trials (Fizazi et al., 2012; Smith et al., 2017; Fizazi et al., 2019). Besides, the microsomal assays exhibited complex glucuronidation kinetics, including substrate inhibition. Previous report showed that UGTs exhibit atypical kinetics in vitro, potentially explained by existence of two binding sites or substrate-induced changes in enzyme conformation (Kaivosaari et al., 2008; Uchaipichat et al., 2008; Zhou et al., 2010).

UGT1A4 displays significant variability in its coding sequence with three common reported allozymes, UGT1A4*2, UGT1A4*3 and UGT1A4*4, presenting frequencies of 1.8%, 13.8% and 1.4% respectively, according to 1000 genomes. These variants have been functionally characterized for different drugs *in vitro* and *in vivo* (Mori et al., 2005; Benoit-Biancamano et al., 2009a; Erickson-Ridout et al., 2011; Laverdiere et al., 2011; Zhou et al., 2011; Edavana et al., 2013; Reimers et al., 2016; Sutiman et al., 2016; Smith et al., 2018). These variant allozymes displayed kinetic parameters comparable to those of the reference UGT1A4*1 enzyme, except UGT1A4*4 that had a significantly lower velocity for Abi and its metabolites, consistent with the reported impact of this specific variant on tamoxifen (Benoit-Biancamano et al., 2009a; Zhou et al., 2011). We also explored the impact of additional rare polymorphisms with frequencies below 1%

that all presented much inferior activities, suggesting that collectively these UGT1A4 variant isoforms could potentially impact the hepatic glucuronidation of Abi and its metabolites. The impact of these UGT1A4 germline variants on the overall hepatic metabolism, response and hepatic toxicity will require additional studies.

The data reported here also provide insights into the potential inhibitory effect of Abi and its active metabolites on steroid glucuronidation. These observations are in line with the reported inhibitory activity of Abi and Gal on the sulfonation of DHEA (Yip et al., 2018). Abi, D4A, 5α-Abi and Gal exhibited strong inhibition towards glucuronidation of adrenal precursors (DHEA and A5-diol) and potent androgens (Testo and DHT) with comparable inhibitory profiles. The apparent Ki values (<10 μM for Testo-G formation) were in order of magnitude similar to the plasma drug concentrations achieved in prostate cancer patients (Alyamani et al., 2018). In comparison, the antiandrogen finasteride was shown to inhibit UGT1A4-mediated activity using trifluoperazine as a substrate with a K_i of 6.05 μM (Lee et al., 2015). Our results further suggest that Abi and its metabolites not only inhibit hepatic glucuronidation of androgens but also glucuronidation of androgens by UGT2B15 and UGT2B17 enzymes highly expressed in the prostate (Chouinard et al., 2004). This is reinforced by the inhibition of androgen glucuronidation in LNCaP cells that express UGT2B15, UGT2B17 and other UGTs. The inhibition potential R (R value = 1 + [I]/Ki) was calculated to estimate the potential of Abi to block androgen metabolism by UGTs as previously done (Oda et al., 2015) (Deb et al., 2014), using the peak plasma concentration (I) of 2.2 μM (Ryan et al., 2010), and K_i values from liver microsomes (K_i = 0.24 to 1.01 uM for Testo, DHT, DHEA and A5-diol)

and prostate cells (K_i = 0.10 μM for Testo). R values of Abi were all above the cut-off R value > 1.1 for having the potential to cause drug-drug interactions *in vivo* (Yu et al., 2017). R values ranged from 3.2 to 23.0, suggesting that the levels of Abi attained in humans could potentially inhibit the catabolism of androgens *in vivo*. However, as the metabolism of Abi is complex, involves multiple steroid biotransforming enzymes, also expressed locally in PCa cells, additional *in vivo* evidence is needed. Though Abi leads to a major depletion of circulating steroid hormones, it is tempting to speculate that this inhibitory impact on steroid inactivation pathways may potentially alter pre-receptor control of androgen metabolism in patients with incomplete suppression of adrenal androgens. It may also be relevant when PCa cells become Abi-resistant, where the glucuronidation inhibition of Testo and DHT might impact PCa growth.

In conclusion, we have demonstrated that the metabolism of CYP17A1 steroidal inhibitors continues downstream to the final glucuronidation steps as observed for steroid biotransformation pathways. We showed that the hepatic UGT1A4 enzyme is the primary enzyme involved in this conjugation process and that coding polymorphisms affecting this gene significantly reduced conjugation capacity *in vitro*. Additional translational pharmacogenomics studies are required to establish the potential clinical relevance of the UGT1A4 pathway to drug metabolism and clinical response.

Acknowledgments

The authors are thankful to all participating patients and clinical staff who have made this scientific contribution possible.

DMD Fast Forward. Published on November 14, 2019 as DOI: 10.1124/dmd.119.088229
This article has not been copyedited and formatted. The final version may differ from this version.

Financial disclosure

No conflict of interests was disclosed.

Authorship contributions

Participated in research design: Guillemette and Levesque

Conducted experiments: Vaillancourt, Turcotte, Villeneuve and Caron

Contributed new reagents or analytic tools: Turcotte and Caron

Performed data analysis: Vaillancourt, Turcotte, Caron, Villeneuve and Guillemette

Wrote or contributed to the writing and revision of the manuscript: All authors

Contributed to patients recruitment: Pouliot, Lacombe and Lévesque

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Footnotes

Part of this work was funded by the CHU de Québec - Université Laval foundation, Québec, Canada to EL and CG and the Canada Research Chair Program (grant no. 950-203962, to CG). E. Lévesque holds a CIHR Clinician-Scientist Award. C. Guillemette holds the Canada Research Chair in Pharmacogenomics (Tier I). JV holds a Fonds de recherche du Québec – Santé studentship award.

DMD Fast Forward. Published on November 14, 2019 as DOI: 10.1124/dmd.119.088229
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Figure legends.

Figure 1. Identification of two glucuronide metabolites of Abi (A) Left: Chromatogram of the two polar metabolites observed after a 30 min incubation of Abi with HLM at the transition 526.7 Da → 350.2 Da. Metabolites were named G1 and G2 based on the order of elution. Right: The MS/MS fragmentation profile of the glucuronides. Masses of the protonated parent ion Abi-G $[M+H]^+$ or $[M]^+$ and of the fragment ion Abi $[M-G+H]^+$ are consistent with the loss of the glucuronide moiety (represented by the dashed line in each Abi-G structure). (B) These metabolites are observed in assays with HLM in the presence of the UGT co-substrate, UDP-GlcA. (C) Polar metabolites disappear when incubated for 20 hours in presence of β-glucuronidase type VII from *Escherichia coli.* HLM: Human Liver Microsomes; G: Glucuronide.

Figure 2. Abi, D4A, 5α-Abi and Gal glucuronidation by human tissues and recombinant human UGT enzymes. Glucuronidation activity of human tissues and recombinant UGTs (commercial supersomes and microsomes isolated from HEK293 cells for UGT1A10, UGT2B10 and UGT2B11) was measured at 200 μM substrate concentration for 2 h. Results are expressed in pmol/min/mg protein and as mean ± S.D. of duplicate determinations. The expression of individual UGT was confirmed by western blotting and the relative UGT expression between UGT1A3 and UGT1A4 supersomes was comparable, consistent with one of our previous report using these protein preparations (Benoit-Biancamano et al., 2009b). HLM: Human Liver

Microsomes; HKM: Human Kidney Microsomes; HIM: Human Intestine Microsomes; S.D.: standard deviation; G: Glucuronide.

Figure 3. Kinetic profiles for the formation of glucuronides of Abi, D4A, 5α-Abi and Gal by human liver microsomes (HLM) and recombinant UGT1A4 enzyme. HLM and UGT1A4 were incubated with concentrations ranging from 0 to 400 μM of Abi, D4A, 5α-Abi or Gal for 30 min as described in Materials and Methods. Results are expressed as mean ± S.D. of triplicate determinations of one representative experiment. S.D.: standard deviation; G: glucuronide.

Figure 4. Dose-dependent inhibition of Abi-G1, Abi-G2, D4A-G, 5α-Abi-G, Gal-G1 and Gal-G2 formation by hecogenin in human liver microsomes (HLM) and recombinant UGT1A4 enzyme. (A-D) Inhibition profiles of Abi (A), D4A (B), 5α-Abi (C) and Gal (D) glucuronidation by hecogenin. Activity was measured at constant concentration of substrate (2 μM Abi, 6 μM D4A, 5 μM 5α-Abi or 0.5 μM Gal) with six concentrations of hecogenin ranging from 0 to 10 μM for 30 min as described in Materials and Methods. In the control, no hecogenin was added. (E) Inhibitory constant values (Ki) of Abi, D4A, 5α-Abi and Gal by hecogenin measured using three different concentrations of substrate ranging from 2 to 200 μM with six concentrations of hecogenin ranging from 0 to 10 μM for 30 min as described in Materials and Methods. Results are expressed as mean ± S.D. of triplicate determinations of at least two experiments. Inhibition models observed were mixed. S.D.: standard deviation; G: glucuronide.

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Figure 5. Formation of glucuronides of Abi, D4A and 5α-Abi by UGT1A4 isoenzymes. Kinetic profiles for the formation of Abi (A-B), D4A (C) and 5α-Abi (D) glucuronides by UGT1A4 common isoenzymes (UGT1A4*1 $R^{11}P^{24}L^{48}$, UGT1A4*2 T^{24}, UGT1A4*3 V^{48} and UGT1A4*4 W^{11}). UGT1A4 microsomes were incubated with increasing concentrations (up to 200 μM) of substrate for 30 min as described in Materials and Methods. Results are expressed as mean ± S.D. of triplicate determinations. (E) Formation of Abi, D4A and 5α-Abi glucuronides by additional UGT1A4 isoenzymes for 30 min using Abi (2 μM), 5α-Abi (5 μM) and D4A (6 μM). Results are expressed as mean ± S.D. of triplicate determinations of at least two experiments. UGT1A4*1 corresponds to $R^3R^{11}P^{24}L^{48}E^{50}H^{56}H^{68}I^{176}S^{250}I^{276}$. The level of UGT protein assessed by Western blot was used to calculated relative glucuronidation activities. S.D.: standard deviation; G: glucuronide.

Figure 6. Dose-dependent inhibition of adrenal precursors (DHEA and A5-diol) and androgens (Testo and DHT) glucuronidation by Abi in human liver microsomes (HLM) expressing a variety of steroid-conjugating UGTs (A) and recombinant UGT1A4 enzyme (B). Activity was measured at constant concentration of substrate (5 μM) with two concentrations of Abi (2 and 200 μM) for 30 min as described in Materials and Methods. In the control, no Abi was added. K_i values were derived from an additional set of experiments performed using three different concentrations of substrates and three different concentrations of inhibitor ranging from 0 to 200 μM. Results are expressed as mean ± S.D. of triplicate determinations of at least two

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experiments. Inhibition models observed were mixed. S.D.: standard deviation; G: glucuronide.

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Table 1 Detection of Abi, D4A and 5α-Abi and their glucuronide derivatives in five prostate cancer patients under AA treatment.

	Steady state blood levels													
Patients	**Abi**		**D4A**		**5α-Abi**		**Abi-G1**		**Abi-G2**		**D4A-G**		**5α-Abi-G**	
	ng/ml	**nM**	**ng/ml**	**nM**	**ng/ml**	**nM**	**ng/ml**	**nM**	**ng/ml**	**nM**	**ng/ml**	**nM**	**ng/ml**	**nM**
AA-009	6.71	19.23	0.33	0.95	1.07	3.07	0.41	0.78	0.26	0.49	0.04	0.08	0.06	0.11
AA-015	35.10	100.57	1.92	5.53	10.00	28.65	0.81	1.54	1.39	2.64	0.18	0.34	0.60	1.14
AA-024	15.90	45.56	1.11	3.20	6.29	18.02	0.47	0.89	0.94	1.79	0.10	0.19	0.46	0.87
AA-025	62.60	179.37	3.53	10.17	8.88	25.44	0.34	0.65	1.28	2.43	0.02	0.04	0.20	0.38
AA-026	58.80	168.48	4.93	14.21	4.66	13.35	1.12	2.13	1.23	2.34	0.10	0.19	0.37	0.70
Mean	35.82	102.64	2.36	6.81	6.18	17.71	0.63	1.20	1.02	1.94	0.09	0.17	0.34	0.64
S.D.	24.95	71.49	1.86	5.36	3.55	10.16	0.33	0.62	0.46	0.87	0.06	0.12	0.21	0.40
CV	70%		79%		57%		52%		45%		69%		62%	

S.D.: standard deviation; CV: coefficient of variation; G: glucuronide. Plasmatic concentrations correspond to C_{min} values and are similar to those observed in a previous group of five patients recruited at the same hospital (Caron et al., 2019).

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Table 2 Enzyme kinetics of Abi, D4A, 5α-Abi and Gal glucuronidation by human liver and the UGT1A4 enzyme

	Apparent K_m (μM)	K_i (μM)	*n*	V_{max} (pmol/min/mg protein)	*Clearance* (μl/min/mg)
Abiraterone					
Abi-G1					
HLM	2.86 ± 0.25	446 ± 34		40.96 ± 1.12	14.38 ± 0.85
UGT1A4	2.80 ± 0.14	645 ± 3		43.47 ± 6.54	15.51 ± 1.55
Abi-G2					
HLM	1.10 ± 0.12	551 ± 24		74.17 ± 3.00	67.89 ± 10.25
UGT1A4	0.72 ± 0.10	730 ± 216		66.75 ± 11.84	92.96 ± 3.53
D4A-G					
HLM	4.52 ± 0.09		0.93 ± 0.10	125.02 ± 27.18	27.62 ± 5.49
UGT1A4	7.40 ± 1.36		0.81 ± 0.01	138.65 ± 11.99	19.21 ± 5.15
5α-Abi-G					
HLM	5.07 ± 0.58	446 ± 52		206.19 ± 7.28	40.79 ± 3.24
UGT1A4	3.92 ± 0.02	1 712 ± 430		222.83 ± 5.87	56.82 ± 1.84
Galeterone					
Gal-G1					
HLM	3.52 ± 0.84	1755 ± 850		33.59 ± 1.90	9.89 ± 2.90
UGT1A4	4.84 ± 1.20	1741 ± 398		40.08 ± 6.22	8.39 ± 0.79
Gal-G2					
HLM	1.03 ± 0.25	538 ± 42		34.41 ± 0.80	34.50 ± 9.10
UGT1A4	0.61 ± 0.19	419 ± 75		34.90 ± 5.44	58.14 ± 8.80

Results are expressed as Mean ± SD of triplicate determinations of at least two independents experiments. Kinetic profiles observed were substrate inhibition for Abi-G1, Abi-G2, 5α-Abi-G, Gal-G1 and Gal-G2 and Hill for D4A-G. HLM: Human liver microsomes; S.D.: standard deviation; G: glucuronide.

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Table 3 Abi inhibits glucuronidation of adrenal precursors (DHEA and A5-diol) and potent androgens (Testo and DHT).

	Testo-G	DHT-G	DHEA-G	A5-diol-3G	A5-diol-17G
			K_i (μM) for Abi		
HLM	0.24 ± 0.05	0.49 ± 0.13	1.01 ± 0.36	0.90 ± 0.57	0.62 ± 0.52
UGT1A4	n.d.	1.18 ± 0.19	0.41 ± 0.10	0.50 ± 0.10	0.61 ± 0.10
UGT2B15	3.19 ± 3.74	-	-	-	-
UGT2B17	0.73 ± 0.53	-	-	-	-
LNCaP[1]	0.10 ± 0.04	-	-	-	-

[1]LNCaP cells are androgen-sensitive human prostate adenocarcinoma cells that express several UGTs namely UGT2B15, UGT2B17 and UGT2B28.

K_i values were derived from experiments using used three concentrations of Abi (5, 25, 200 μM) and three concentrations of Testo, DHT, DHEA and A5-diol (5, 25, 200 μM). For assays using UGT2B15, UGT2B17 and LNCaP cells, we used lower concentrations of Abi (0.1, 1, 5 μM) and testosterone (1, 5, 25 μM). Results are expressed with Mean ± S.D. of triplicate determinations of at least two independents experiments.

n.d.: not detected because UGT1A4 leads to minor formation of Testo-G;

– : not determined.

Inhibition of steroid glucuronidation by D4A and 5α-Abi is presented in Supp. Figure 6, and compared to Abi for HLM and UGT1A4. These graphical representations (Figure 6

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and Supplementary Figure 6) only illustrate percentage of inhibition observed in assays using 5 μM of androgen in the presence of 0, 2 and 200 μM of Abi. Inhibition models observed were mixed. HLM: Human liver microsomes; S.D.: standard deviation; G: glucuronide; L: liver; P: prostate.

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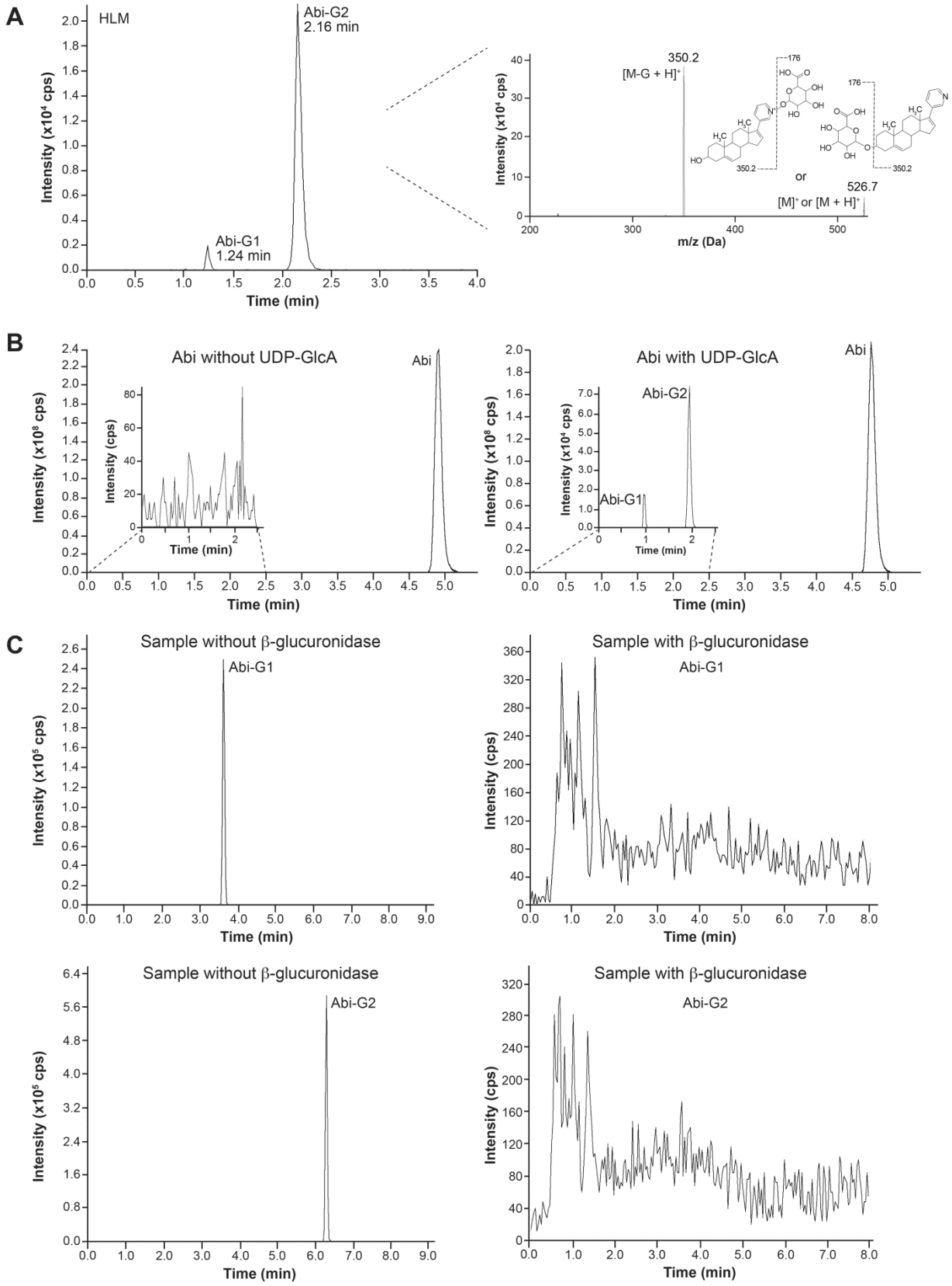

Figure 1.

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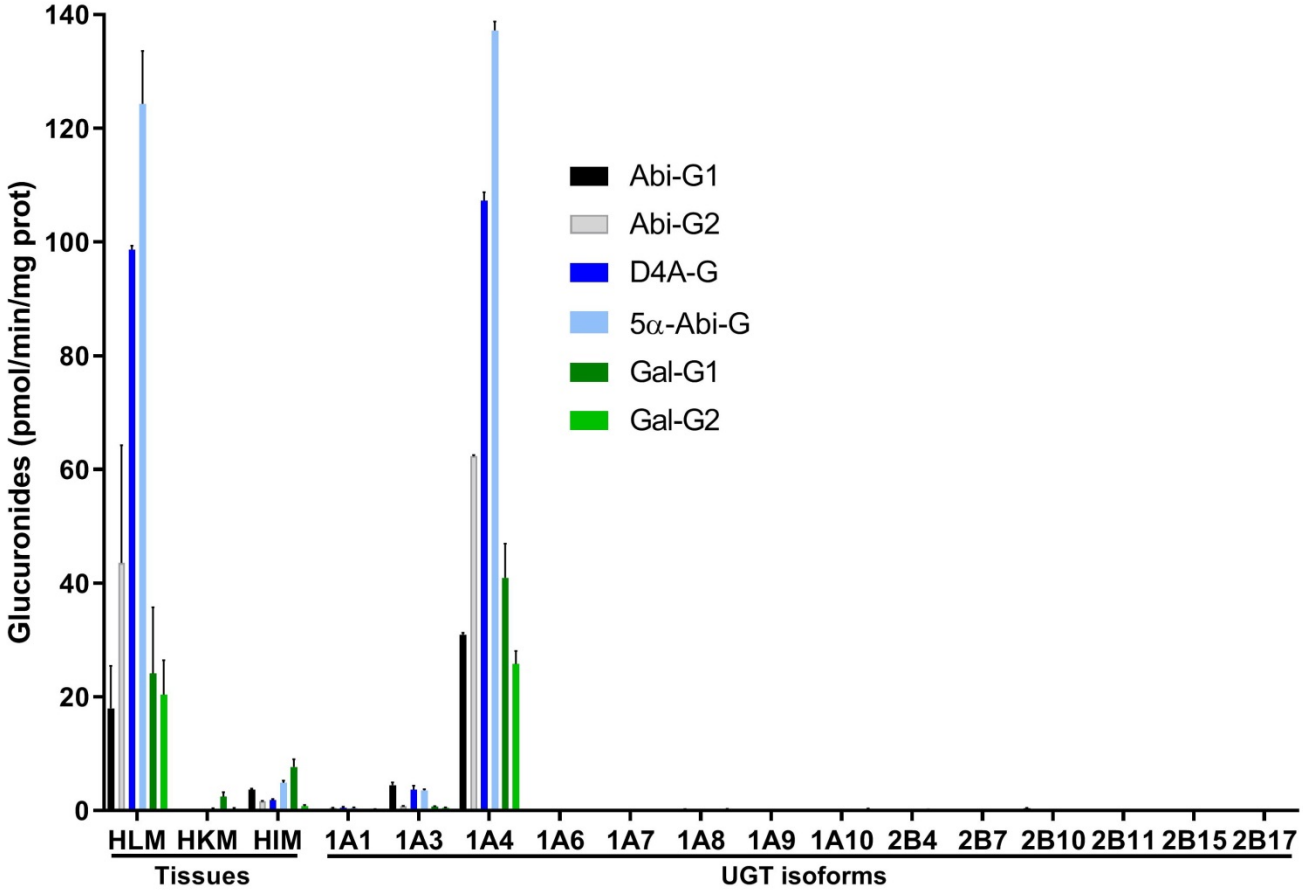

Figure 2.

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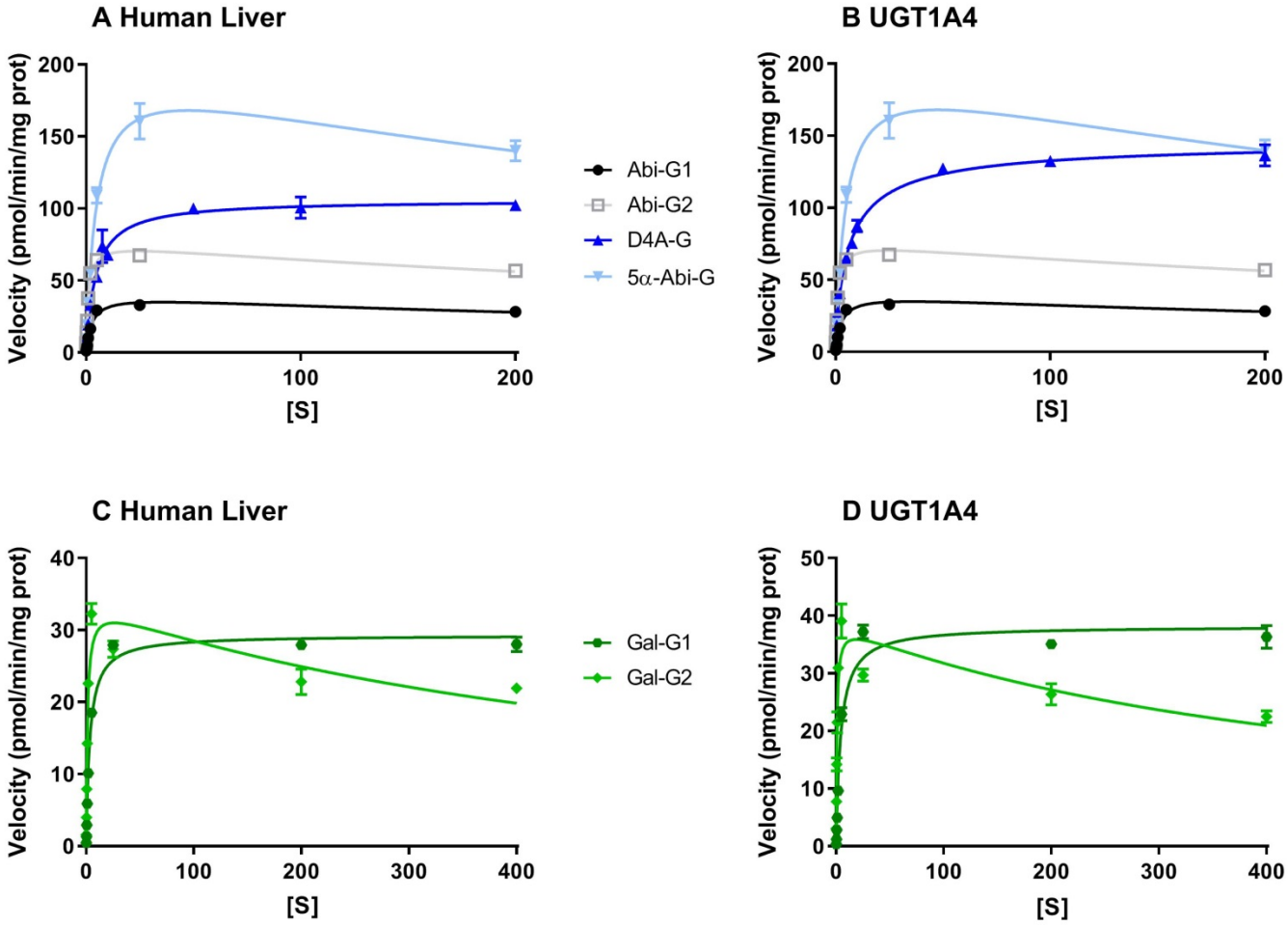

Figure 3.

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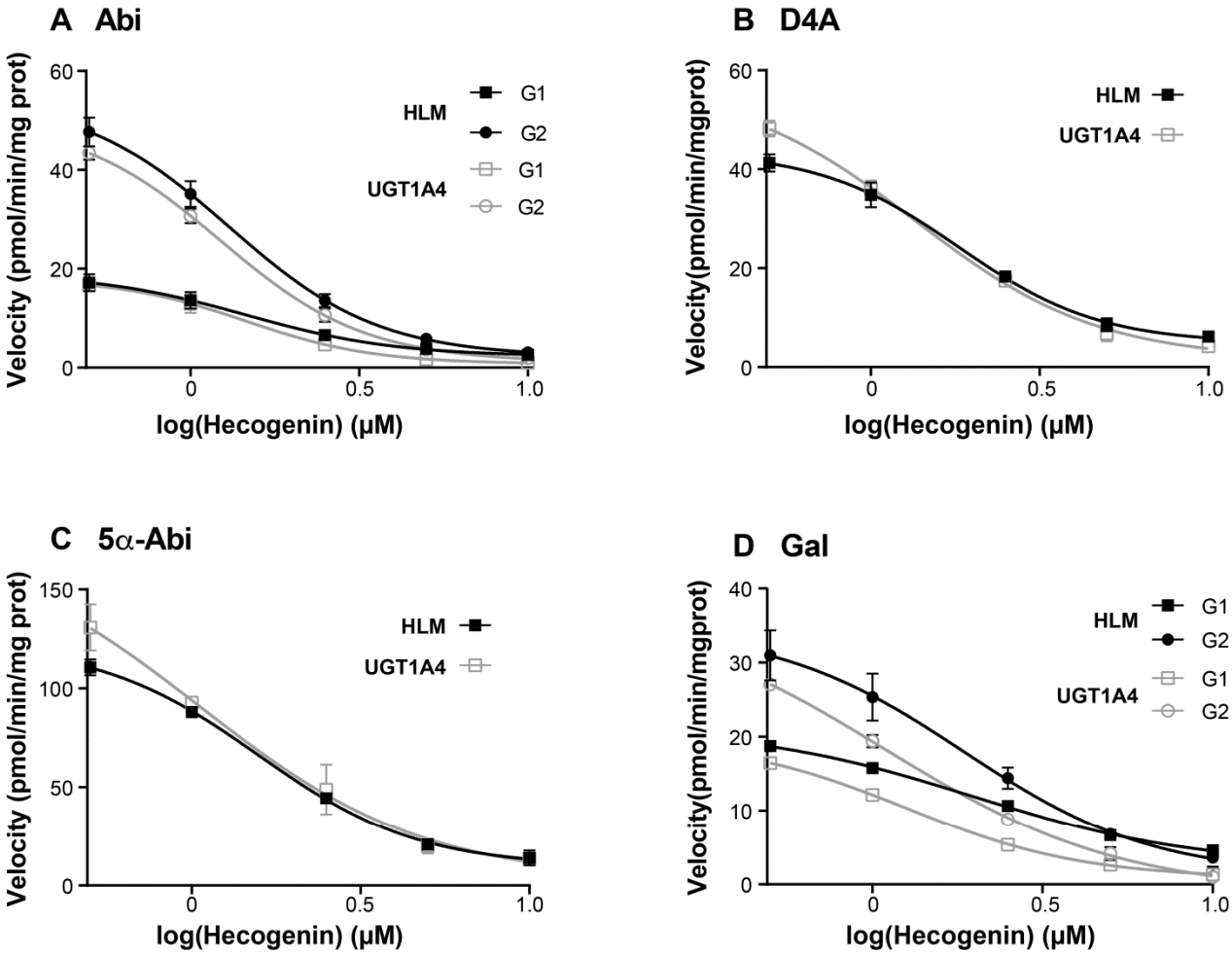

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K_i (μM)	Abi		D4A	5α-Abi	Gal	
	G1	G2	G	G	G1	G2
HLM	0.66 ± 0.10	0.12 ± 0.02	0.62 ± 0.18	0.52 ± 0.34	1.05 ± 0.04	0.63 ± 0.13
UGT1A4	0.36 ± 0.00	0.10 ± 0.04	0.44 ± 0.33	0.29 ± 0.06	0.81 ± 0.05	0.48 ± 0.25

Figure 4.

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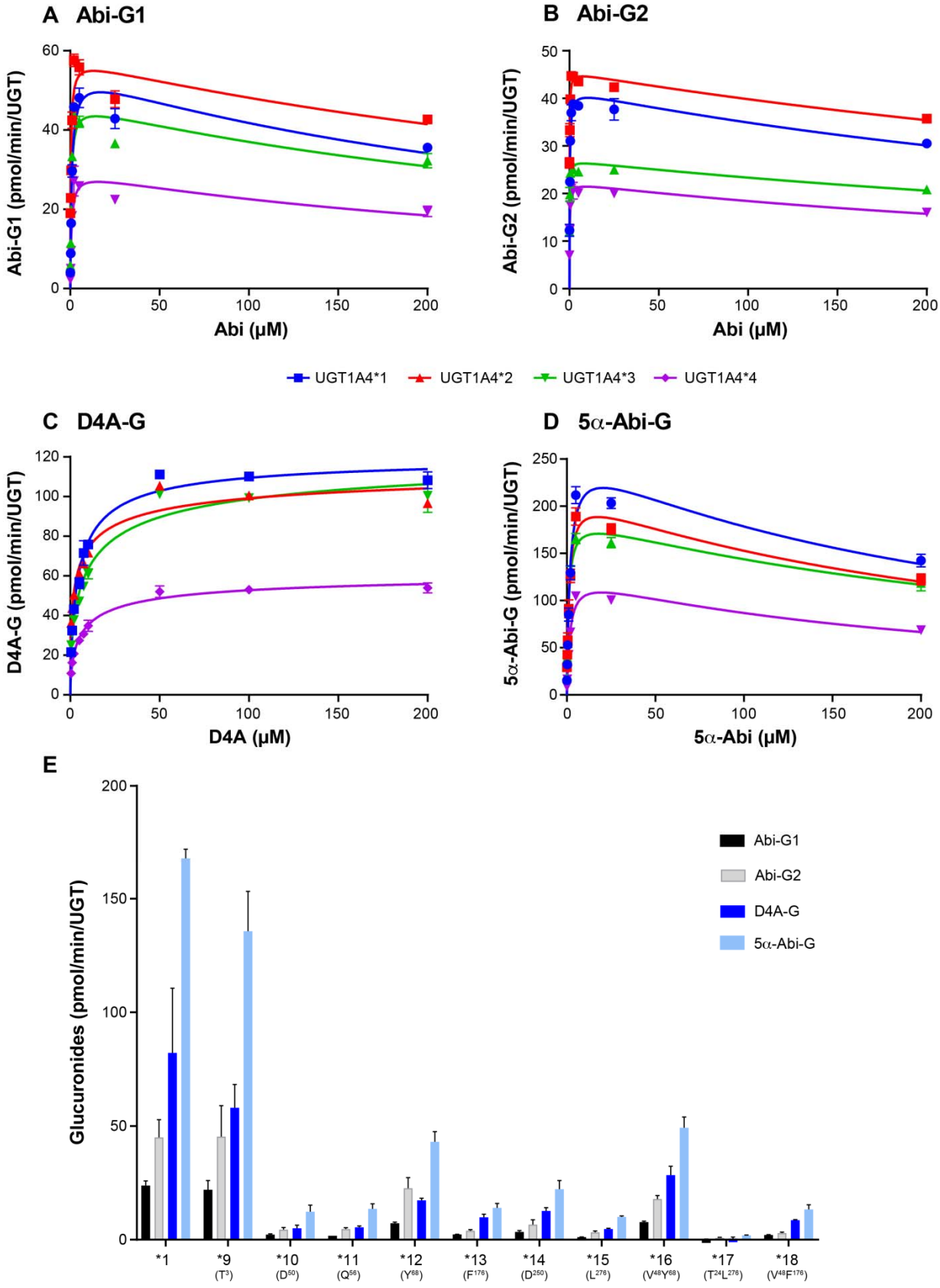

Figure 5.

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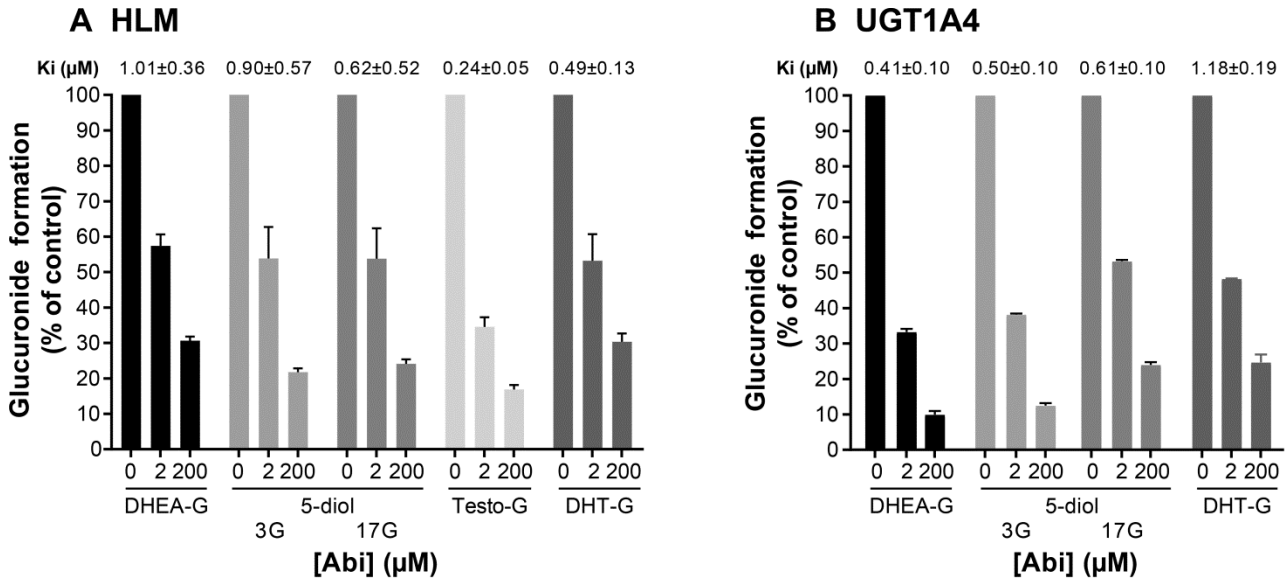


Figure 6.

Glucuronidation of Abiraterone and its Pharmacologically Active Metabolites by UGT1A4, Influence of Polymorphic Variants and their Potential as Inhibitors of Steroids Glucuronidation.

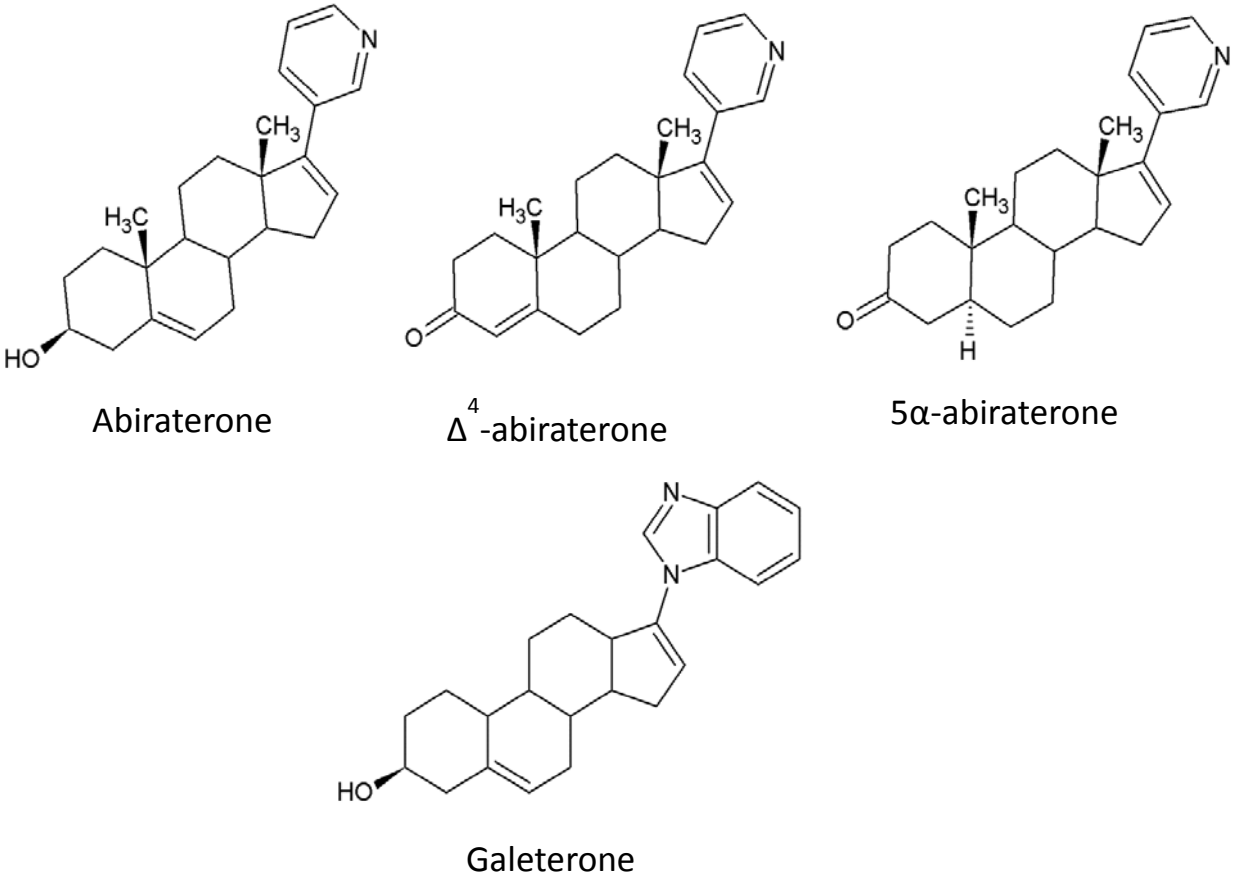
Joanie Vaillancourt, Véronique Turcotte, Patrick Caron, Lyne Villeneuve, Louis Lacombe, Frédéric Pouliot, Éric Lévesque, Chantal Guillemette

Supplemental tables and figures

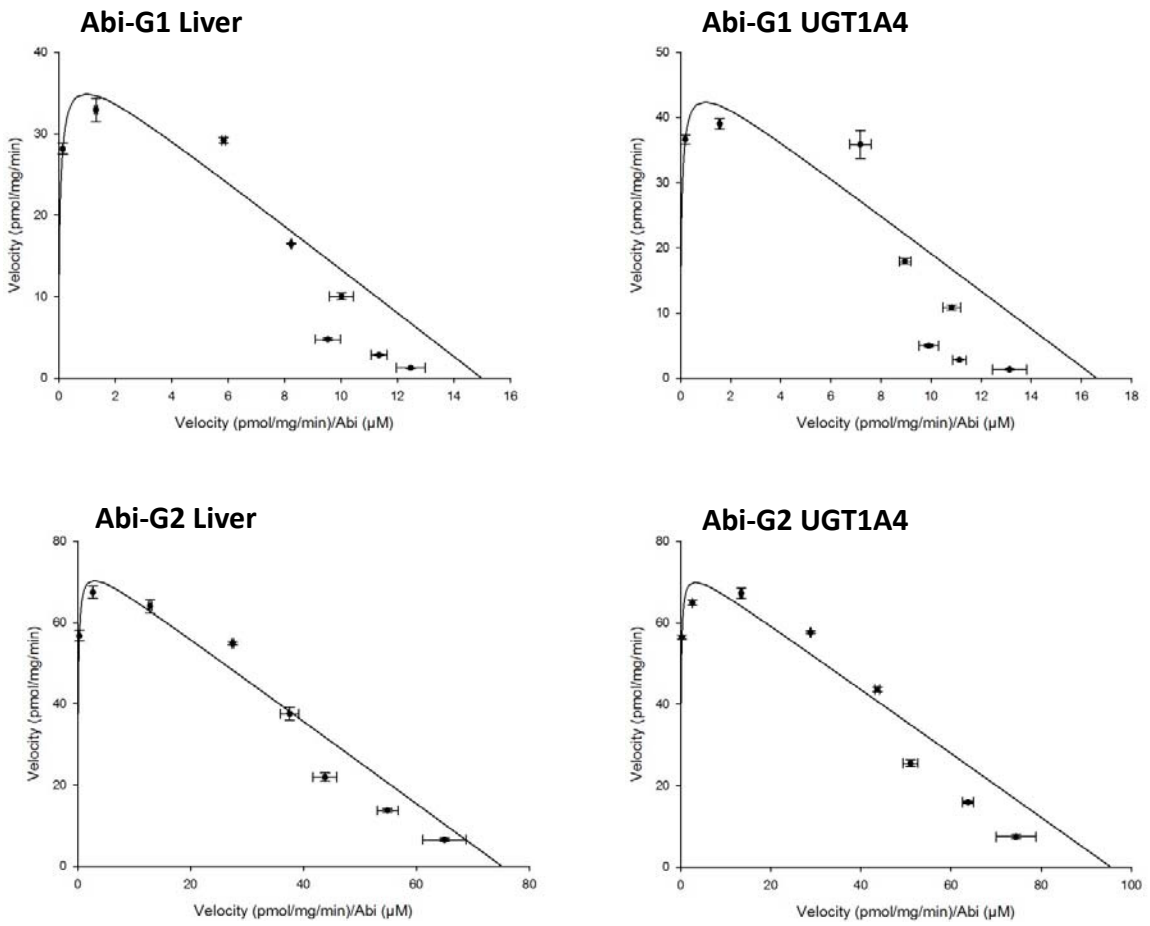
Supplementary Table 1 Enzyme kinetics of Abi, D4A and 5α-Abi glucuronidation by UGT1A4 alloenzymes

UGT1A4 alloenzyme	Apparent K_m (μM)		Relative V_{max} (pmol/min/mg protein)		Clearance (μl/min/mg)	
			Abi-G1			
UGT1A4*1	0.79 ± 0.05		43.13 ± 10.76		54.22 ± 11.38	
UGT1A4*2	0.22 ± 0.14	**	54.68 ± 4.25		307.20 ± 182.23	
UGT1A4*3	0.52 ± 0.00	***	42.10 ± 6.42		80.59 ± 11.63	
UGT1A4*4	0.71 ± 0.10		23.18 ± 5.96	*	33.49 ± 10.39	
			Abi-G2			
UGT1A4*1	0.20 ± 0.02		45.82 ± 7.91		231.40 ± 58.71	
UGT1A4*2	0.07 ± 0.01	***	47.77 ± 3.14		720.23 ± 161.71	*
UGT1A4*3	0.11 ± 0.02	*	26.01 ± 1.33		235.96 ± 62.64	
UGT1A4*4	0.18 ± 0.02		23.58 ± 4.24	*	133.48 ± 7.69	
			D4A-G			
UGT1A4*1	5.22 ± 0.78		112.28 ± 12.92		21.92 ± 5.75	
UGT1A4*2	4.87 ± 1.81		118.38 ± 1.45		26.15 ± 10.00	
UGT1A4*3	9.30 ± 1.31		120.49 ± 10.37		13.00 ± 0.71	
UGT1A4*4	5.80 ± 1.35		58.14 ± 6.80	*	10.17 ± 1.20	
			5α-Abi-G			
UGT1A4*1	1.85 ± 0.15		213.12 ± 40.09		116.49 ± 28.16	
UGT1A4*2	1.12 ± 0.12	**	179.04 ± 49.97		158.62 ± 27.98	
UGT1A4*3	0.99 ± 0.00	**	178.33 ± 16.27		179.30 ± 15.95	
UGT1A4*4	1.44 ± 0.27		107.86 ± 17.88	*	75.26 ± 1.58	

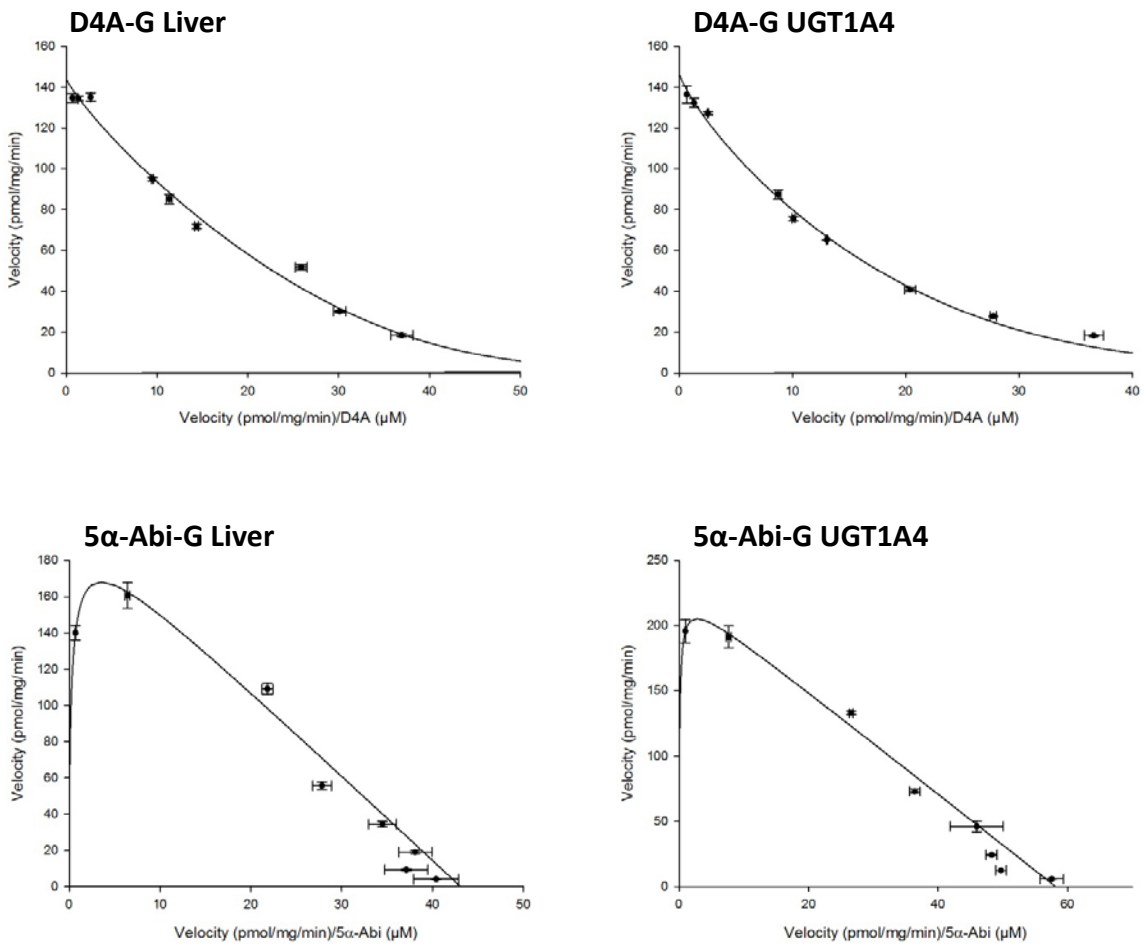
Results are expressed with Mean ± S.D. of triplicate determinations of at least two independent experiments. Relative V_{max} values were adjusted for UGT1A4 protein content assessed by western blotting (Laverdiere et al., 2011). UGT1A4*1 ($R^{11}P^{24}L^{48}$), UGT1A4*2 (T^{24}), UGT1A4*3 (V^{48}), UGT1A4*4 (W^{11}) * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. SD: standard deviation; G: glucuronide.



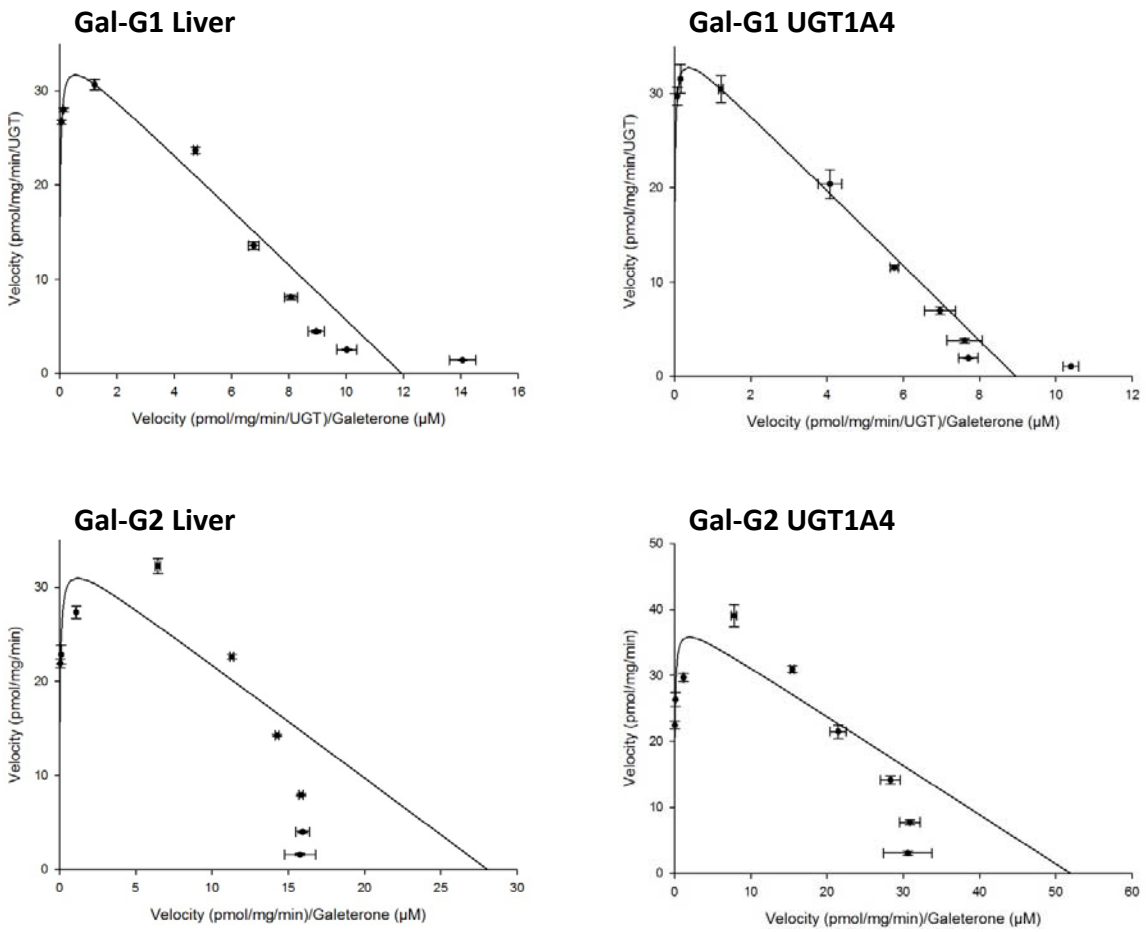
Supplementary Figure 1. Structures of compounds relevant to this study.



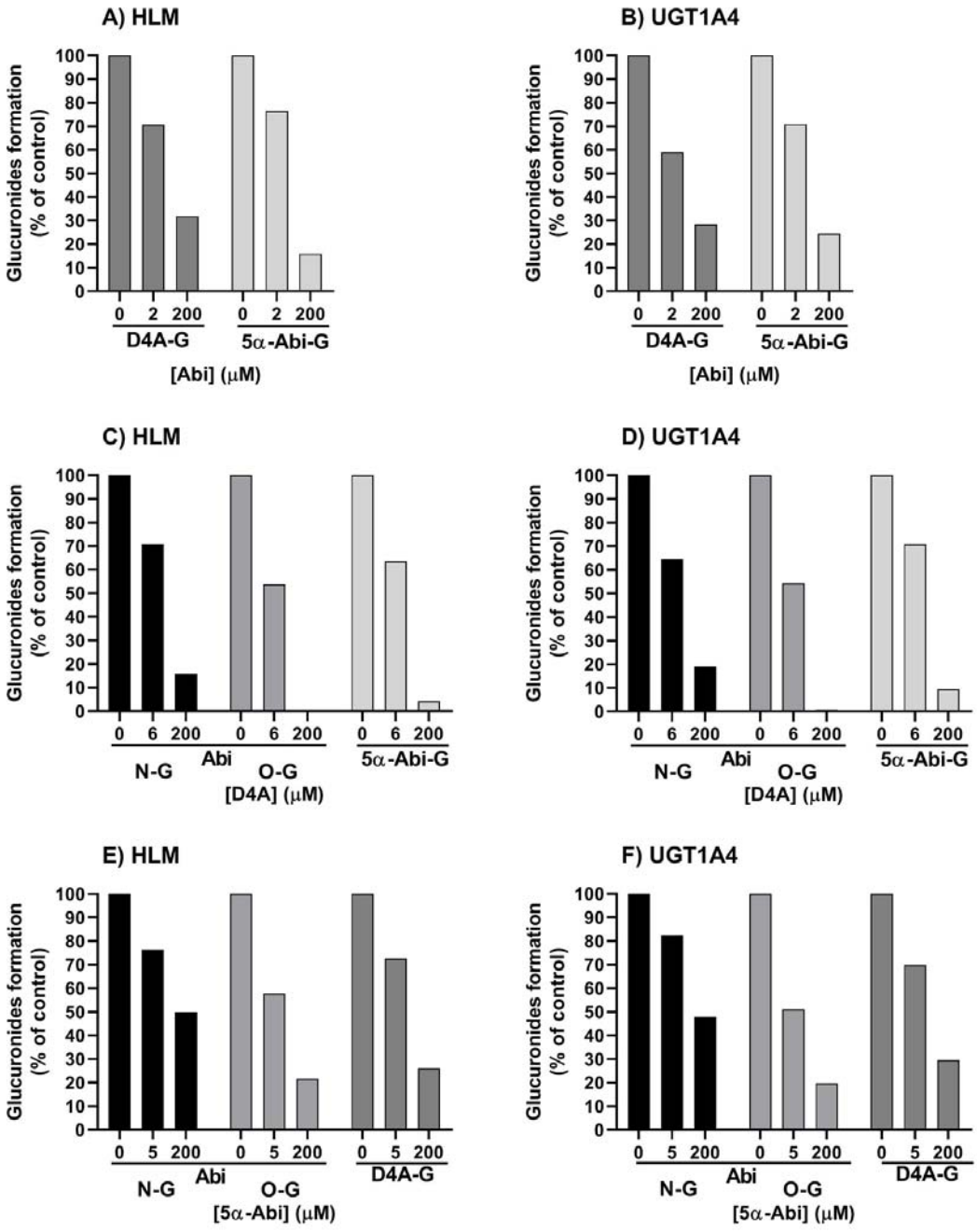
Supplementary Figure 2. Eadie-Hofstee regressions for the formation of glucuronides of Abi by human liver microsomes (HLM) and recombinant UGT1A4 enzyme. HLM and UGT1A4 were incubated in the presence of concentrations ranging from 0 to 200 µM of Abi for 30 min as described in Materials and Methods. Results are expressed as mean ± S.D. of triplicate determinations of one representative experiment. S.D.: standard deviation; G: glucuronide.



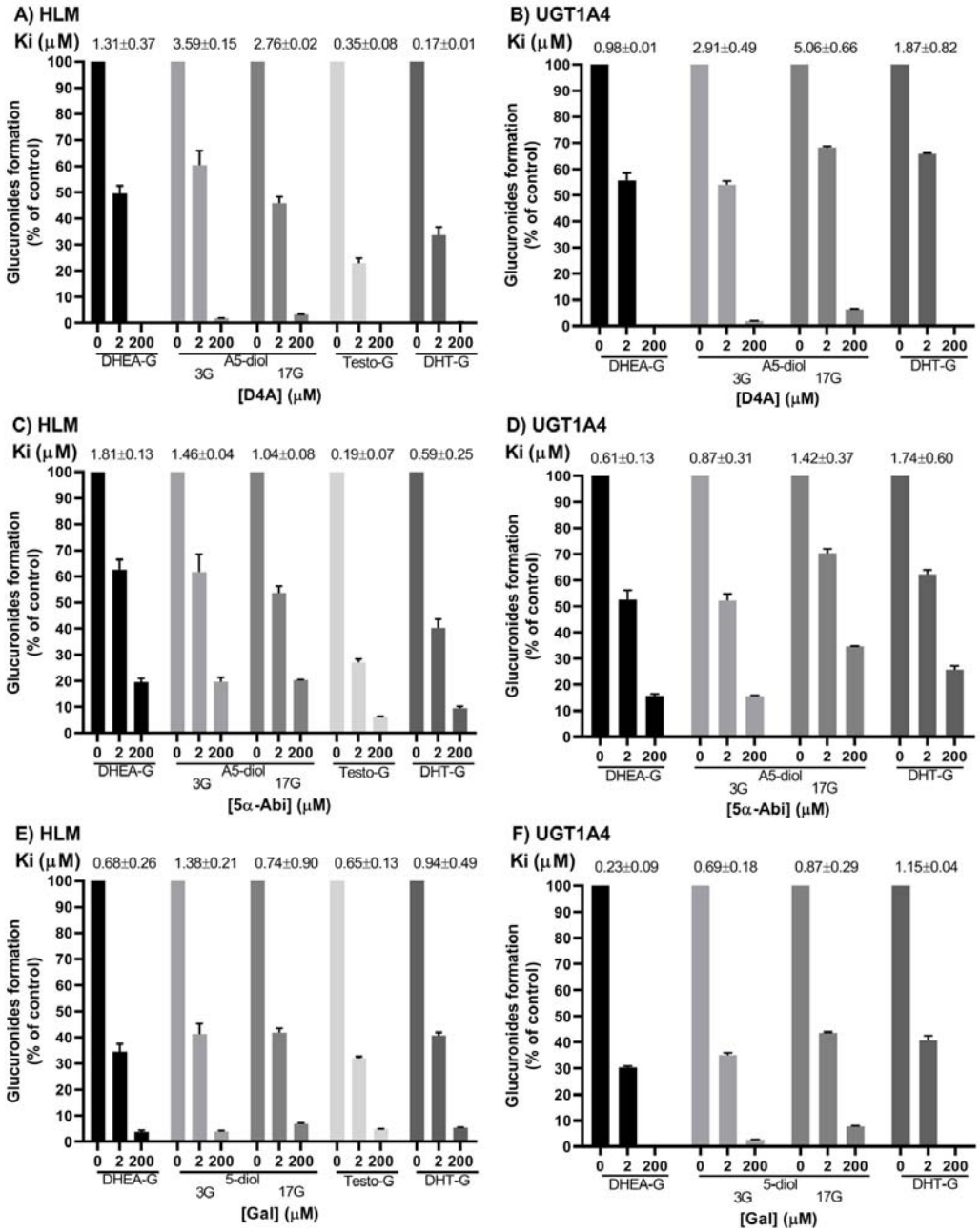
Supplementary Figure 3. Eadie-Hofstee regressions for the formation of glucuronides of D4A and 5α-Abi by human liver microsomes (HLM) and recombinant UGT1A4 enzyme. HLM and UGT1A4 were incubated in the presence of concentrations ranging from 0 to 200 μM of D4A or 5α-Abi for 30 min as described in Materials and Methods. Results are expressed as mean ± S.D. of triplicate determinations of one representative experiment. S.D.: standard deviation; G: glucuronide.



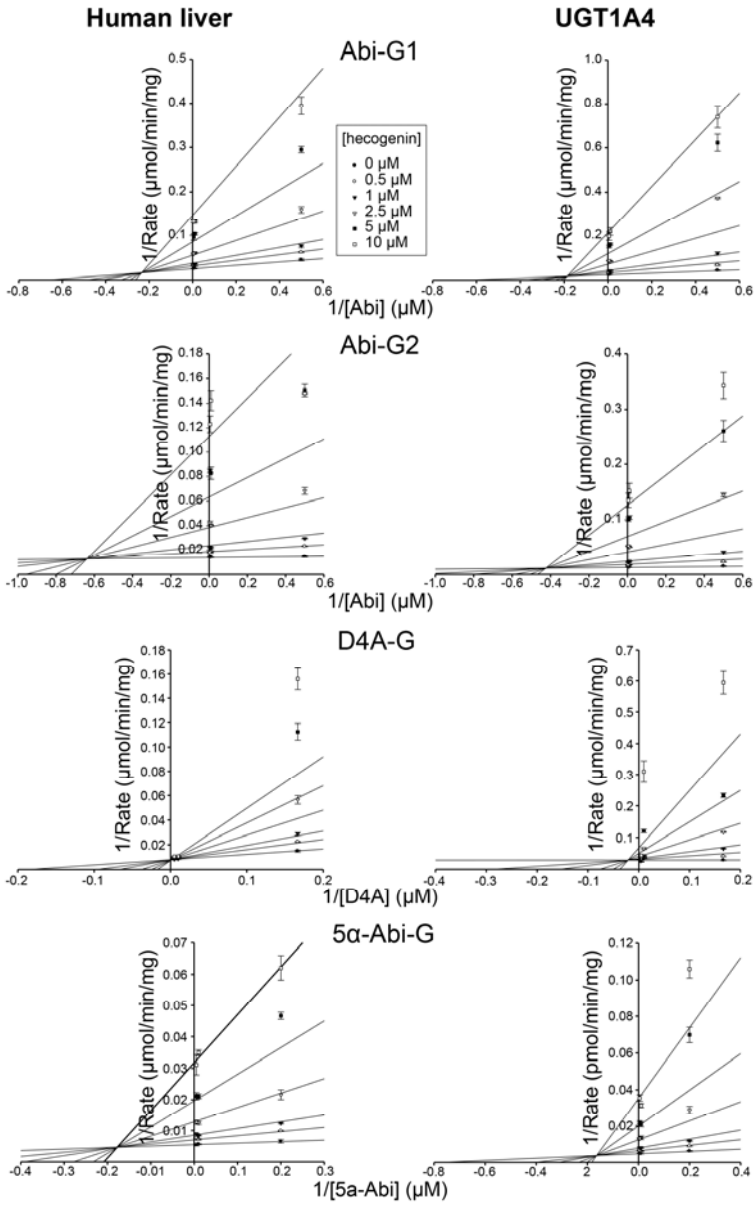
Supplementary Figure 4. Eadie-Hofstee regressions for the formation of glucuronides of Gal by human liver microsomes (HLM) and recombinant UGT1A4 enzyme. HLM and UGT1A4 were incubated in the presence of concentrations ranging from 0 to 400 μM of Gal for 30 min as described in Materials and Methods. Results are expressed as mean ± S.D. of triplicate determinations of one representative experiment. S.D.: standard deviation; G: glucuronide.



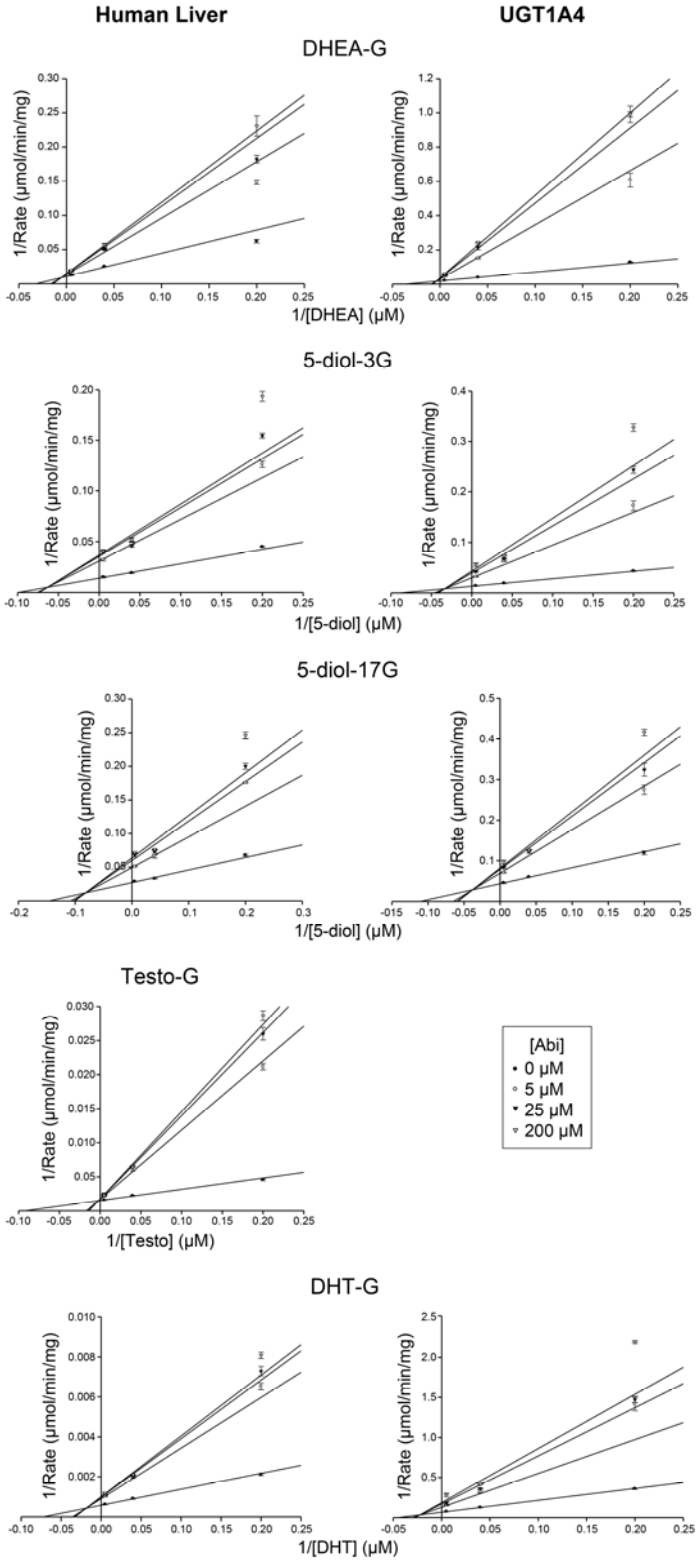
Supplementary Figure 5. Dose-dependent inhibition of drug glucuronide formation by Abi, D4A, 5α-Abi in human liver microsomes (HLM) (A, C, E) and recombinant UGT1A4 enzyme (B, D, F). Activity was measured at a constant concentration of substrate at K_m concentrations (Abi (2 μM), 5α-Abi (5 μM), and D4A (6 μM)) with different concentrations of inhibitor (0, K_m concentrations and 200 μM) for 30 min as described in Materials and Methods. Results are expressed as mean ± S.D. of triplicate determinations of at least two experiments. S.D.: standard deviation; G: glucuronide.



Supplementary Figure 6. Dose-dependent inhibition of glucuronidation of adrenal precursors (DHEA and A5-diol) and androgens (Testo and DHT) by D4A, 5α-Abi and Gal in human liver microsomes (HLM) (A, C, E) and recombinant UGT1A4 enzyme (B, D, F). Activity was measured at a constant concentration of substrate (5 μM) with different concentrations of inhibitor (0, 2 and 200 μM) for 30 min as described in Materials and Methods. K_i values were derived from experiments performed using three concentrations of Abi (5, 25, 200 μM) and three concentrations of Testo, DHT, DHEA and A5-diol (5, 25, 200 μM). For assays using UGT2B15, UGT2B17 and LNcaP cells, we used lower concentrations of Abi (0.1, 1, 5 μM) and testosterone (1, 5, 25 μM). Results are expressed as mean ± S.D. of triplicate determinations of at least two experiments. S.D.: standard deviation; G: glucuronide.



Supplementary Figure 7. Lineweaver-Burk plots for inhibition of drug glucuronidation by hecogenin in human liver microsomes (HLM) (left) and recombinant UGT1A4 enzyme (right). K_i values were derived from experiments performed using the following concentrations of hecogenin (0.5, 1, 2.5, 5, 10 µM) and three different concentrations of each substrate (Abi: 2, 100, 200 µM; D4A 6, 100, 200 µM; 5α-Abi: 5, 100, 200 µM; Gal: 0.5, 2 and 5 µM). Results are expressed as mean ± S.D. of triplicate determinations of at least two experiments. S.D.: standard deviation; G: glucuronide.



Supplementary Figure 8. Lineweaver-Burk plots for inhibition of glucuronidation of adrenal precursors (DHEA and A5-diol) and androgens (Testo and DHT) by Abi in human liver microsomes (HLM) (right) and recombinant UGT1A4 enzyme (left). K_i values were derived from experiments performed using three concentrations of Abi (5, 25, 200 µM) and three concentrations of Testo, DHT, DHEA and A5-diol (5, 25, 200 µM). Results are expressed as mean ± S.D. of triplicate determinations of at least two experiments. S.D.: standard deviation; G: glucuronide.