DMD Fast Forward. Published on October 9, 2014 as DOI: 10.1124/dmd.114.059303
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The dog UGT enzymes of subfamily 1A; cloning, expression and activity

Johanna Troberg, Erkka Järvinen, Maria Muniz, Nina Sneitz, Johanna Mosorin, Marja Hagström, Moshe Finel

Division of Pharmaceutical Chemistry and Technology (JT, EJ, MM, NS, JM, MF) and Centre for Drug Research (MH), Faculty of Pharmacy, University of Helsinki, Finland

Running title: Recombinant dog UGT1As

Corresponding author: Moshe Finel

Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy

P.O.Box 56 (Viikinkaari 5)

00014 University of Helsinki

Finland

E-mail address: moshe.finel@helsinki.fi; Tel.: +358 504480748; Fax: +358 9 19159556

Number of text pages: (Abstract+Methods+Results+Discussion) 22

Number of words in Abstract: 245

Number of words in Introduction: 716

Number of words in Discussion: 1165

Number of References: 36

Number of Tables: 3

Number of Figures: 18

Abbreviations

DMSO, Dimethylsulfoxide; DIM, dog intestine microsomes; DLM, dog liver microsomes; HIM, human intestine microsomes; HLM, human liver microsomes; E_2, 17-β-estradiol; epi-E_2, 17-α-estradiol; 4-MU, 4-Methylumbelliferone; PCA, perchloric acid; UDPGA, UDP-α-D-glucuronic acid; UGT, UDP-glucuronosyltransferase

Abstract

Understanding drug glucuronidation in the dog, a preclinical animal, is important but currently poorly characterized at the level of individual enzymes. We have constructed cDNAs for the 10 dog UDP-glucuronosyltransferases of subfamily 1A (dUGT1As), expressed them in insect cells and assayed their activity, as well as the activity of the 9 human UGT1As, toward 14 compounds. The goal was to find out if individual dUGT1As and individual human UGT1As have similar substrate specificities. The results revealed similarities, but also many differences. For example, similarly to the human UGT1A10, dUGT1A11 exhibited high glucuronidation activity toward the 3-OH of 17β-estradiol, 17α-estradiol and ethinylestradiol, and also conjugated the drug entacapone. Unlike the human UGT1A10, however, it failed to catalyze considerable rates of R-propranolol, diclofenac and indomethacin glucuronidation. The estrogens glucuronidation assays revealed that dUGT1A8 and dUGT1A10 have a capacity to catalyze the formation of (linked) diglucuronides, an activity no human UGT1A exhibited. dUGT1A2-dUGT1A4 are homologues of the human UGT1A4, but none of them catalyzed *N*-glucuronidation of dexmedetomidine. Contrary to the human UGT1A4, however, dUGT1A2-dUGT1A4 catalyzed indomethacin and diclofenac glucuronidation. It may be concluded that, perhaps with the exception of UGT1A6, high similarities in substrate specificity between individual dog and human UGTs of subfamily 1A are rare or partial. Activity assays with liver and intestine microsomes, of both dog and human, further revealed interspecies differences, particularly in glucuronidation rates. In the dog, the microsomes assays also strongly suggested important roles for dUGTs of other subfamilies, mainly in the liver.

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Introduction

Safety evaluation of a new drug candidate in preclinical animals before first human exposure is a necessary stage in the drug development process. These tests include at least two preclinical species, a rodent and a non-rodent; the latter is often a dog (http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM078933.pdf). While these tests are important, differences in drug metabolism between preclinical animal species and human might affect considerably safety evaluation in different ways. Better knowledge of the enzymes that catalyze the tested reactions in different preclinical animals may allow improved interpretation of the results and, at the same time, contribute to the development of veterinary drugs for different domestic animals.

Glucuronidation of endogenous compounds and xenobiotics, including drugs, either directly or of their phase I metabolites, is catalyzed by UDP-glucuronosyltransferase enzymes (UGTs) and plays important roles in their biotransformation and pharmacokinetics (for reviews see King et al., 2000; Tukey and Strassburg, 2000). The UGTs catalyze the transfer of the glucuronic acid moiety from the cosubstrate, UDP-glucuronic acid (UDPGA) to a nucleophilic group on the acceptor molecule, mostly a hydroxyl or different amino groups. There are 19 different human UGTs that are divided into subfamilies 1A, 2A and 2B (Mackenzie et al., 2005). In addition, there are few other, closely related, sugar transferases that mainly catalyze the transfer of other sugar moieties (Rowland et al., 2013), but they will not be further discussed in this work.

About half of the human UGTs belong to subfamily 1A, which is encoded by a single large gene, *ugt1a*. Due the exon sharing, the mature mRNA for each UGT1A contains a different (but homologous) exon 1 and the same 4 exons, 2-5, which are shared by all the UGTs of this subfamily (splice variants among exons 2-5 are beyond the scope of this study). The first exon encodes the N-terminal half of the protein, whereas the shared exons, together, encode the C-terminal half of the UGT (Mackenzie et al., 2005).

Glucuronidation activity in dog liver and intestine microsomes (DLM and DIM, respectively) was previously examined and revealed similarities, alongside many differences, in substrate specificity and glucuronidation rates, in comparison to human liver and intestine microsomes microsomes (HLM and HIM, respectively) (Soars et al., 2001a; Kaivosaari et al., 2011; Furukawa et al., 2014; and other studies). The dog UGT1A6 was earlier cloned, sequenced, expressed and examined for the glucuronidation of many compounds, revealing high similarity to the human UGT1A6 (Soars et al., 2001b). Another dog UGT, named dUGT2B31, was later cloned, expressed and characterized by the same research group, but it was difficult to identify a clear and single human UGT2B enzyme as its homologue (Soars et al., 2003).

The complete dog genome was earlier sequenced (Kirkness et al., 2003; Lindblad-Toh et al., 2005) and that data, in combination with the knowledge of exon sharing in the *ugt1a* gene, guided the bioinformatic analysis of the dog genome, yielding cDNA sequences for the different dUGT1A enzymes (Li and Wu, 2007). There is, however, a minor mix-up in the names of individual human and dog UGT1As due to differences in pseudogenes content and exon 1s naming order (Fig. 1). In addition, the sequence identities between individual exon 1s of the dog and human UGT1As are not high enough to "pair" individual UGT1As among the groups dUGT1A2-dUGT1A4 and the human UGT1A3-UGT1A5, as well as dUGT1A7-dUGT1A11 and the human UGT1A7-UGT1A10 (Fig. 2). Therefore, we have followed the names given by Li and Wu (2007) for the dog UGT1As, but the common names for the human UGT1As.

The main objective of this study was to explore interspecies specificity in UGT1As at the level of individual enzymes. To this end, we have cloned, expressed and assayed the activity of the 10 dUGT1As, using 14 substrates that are known to be human UGTs substrates, even if some of them, like testosterone and epitestosterone, are mainly substrates of human UGTs of subfamilies 2A and 2B (Sten et al., 2009). The dUGT1As activities were compared to the

activities of the 9 human UGT1As that were expressed and assayed in the same way. In addition, the glucuronidation of the same compounds in liver and intestine microsomes from both dog and human was determined in order to get a broader picture of the glucuronidation capacity in these tissues and the probable contribution of UGTs of other subfamilies to this activity.

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Materials and Methods

Materials

UDP-α-D-glucuronic acid (UDPGA, ammonium salt), 4-methylumbelliferone (4-MU), 4-MU-β-D-glucuronide, 17-α-estradiol (epi-E_2), 17-β-estradiol (E_2), E_2-β-D-3-glucuronide (E_2-3-glucuronide), E_2-β-D-17-glucuronide (E_2-17-glucuronide), 17-α-ethinylestradiol (ethinylestradiol), epitestosterone, bilirubin, diclofenac, indomethacin, R-propranolol, S-propranolol, sodium phosphate monobasic dihydrate and disodium hydrogen phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Entacapone, dexmedetomidine and levomedetomidine were kindly provided by Orion Pharma Corporation (Espoo, Finland). Entacapone-β-D-glucuronide was synthesized in our laboratory (Luukkanen et al., 1999). Testosterone was from TCI Europe (Zwijndrecht, Belgium), magnesium chloride hexahydrate and perchloric acid (PCA) were from Merck (Darmstadt, Germany). Formic acid was from Riedel-deHaën (Seelze, Germany). Dimethyl sulfoxide (DMSO), acetonitrile, methanol and acetic acid were HPLC grade. Alamethicin was obtained from A.G. Scientific (San Diego, CA, USA) and oligonucleotide primers (Table 1) were from Oligomer, Helsinki, Finland.

Pooled human liver microsomes (HLM), human intestinal microsomes (HIM) and pooled dog liver microsomes (DLM, male beagle) were bought from BD Biosciences (Franklin Lakes, NJ USA), whereas dog pooled intestinal microsomes (DIM, male beagle) were from Xenotech, LLC (Lenexa, KS, USA). Dog liver cDNA (C1734149) was purchased from Biochain (Newark, CA, USA). The genomic DNA of beagle dogs was a generous gift from Dr. Hannes Lohi, University of Helsinki.

Cloning and expression of recombinant dUGT1As

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The cloning strategy for constructing complete cDNAs for the 10 different dog UGT1A enzymes was based on amplifying one "shared segment", made of correctly spliced exons 2-5, from hepatic cDNA, using oligonucleotides no. 1 and 2 (Table 1), and ligating to it the different exons 1s of each dUGT1A. To facilitate an *in frame* ligation of the 3'-end of the amplified exon 1 to the 5'-end of the "shared segment", an EcoR1 restriction site was generated, without changing an amino acid (silent mutagenesis) at the 5'-end of exon 2 (no internal EcoR1 site was found in exons 2-5).

Each of the 10 full-length first (variable) exons was amplified from genomic beagle DNA, by itself or as a group (see below), using 5' PCR primers (indicated by F in Table 1) that were designed based on the upstream flanking regions sequences in gene bank (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?PAGE_TYPE=BlastSearch&PROG_DEF=blastn &BLAST_PROG_DEF=megaBlast&BLAST_SPEC=OGP__9615__10726) and 3' primers (indicated by R, Table 1) that were designed using the sequences in Li and Wu (2007). Most of the R primers also contained an EcoR1 restriction site for *in frame* ligation to the EcoR1 site at the beginning of the shared segment (see Table 1s' for PCR primers sequences). An upstream restriction site was included in the F primers for the first exons to make subsequent subcloning simpler (the selected restriction site in each case is indicated in the primer's name, Table 1).

Two groups of exon 1s of the dUGTs, d1A2-d1A4 and d1A8-d1A11, have very high degree of sequence identity within their exon 1s segments (see also Fig. 2). Due to this, in these cases, instead of designing specific oligonucleotides for each of these UGT genes, we used F primers that are suitable for some or all the members in these very-high-homology groups (Table 1). The resulting mixed amplified PCR products were subcloned and single colonies were picked and cultured for plasmid purification without knowing which dUGT1a they encode. Only then, following DNA sequencing, it became clear which clone contains which cDNA. There was another complication in the case of dUGTs 1A2-1A4, the presence of an internal EcoR1 site at

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about the middle of exon 1. To overcome this internal site we have inserted an Nde1 restriction site downstream the internal EcoR1 site (using oligonucleotides no. 8 and 9, Table 1) and subcloned the two parts of these exons, the BamH1-Nde1 fragment and the Nde1-EcoR1 fragment together, as 3-fragments ligation in which the 3rd was the vector made from exons 2-5 segment in pFastBac-XHC (the vector with the fusion peptide prior to the insertion of the C-terminal of dUGT1A is described in Kurkela et al., 2003) that was digested with BamH1 and EcoR1.

Following cDNA construction within the modified pFast-Bac vector, pFB-XHC (Kurkela et al., 2003), the shuttle vectors (bacmids) and recombinant baculoviruses were prepared using the Bac-to-Bac system (Invitrogen, Carlsbad, CA, USA). The different recombinant His-tagged dog UGTs were produced in sf9 insect cells and cell homogenates were used as the study material in the activity assays. Total protein concentrations and relative expression levels of each recombinant dUGT were determined as described previously (Zhang et al., 2012a). Since the first batch of cell homogenates for each dUGT was not sufficient for all the assays, we have prepared a second one and determined the relative expression level of each dUGT in that batch, too. The 2nd batches of dUGTs cell homogenates were mainly used for the assays of indomethacin, diclofenac, R- and S-propranolol glucuronidation. The relative expression levels for the two batches of cell homogenates expressing recombinant dog UGTs were as follow (the expression levels in the 2nd batches are given square brackets): d1A1, 5.4 [2.9]; d1A2, 7.9 [4.9]; d1A3, 12.6 [3.4]; d1A4, 12.3 [7.4]; d1A6, 9.3 [6.4]; d1A7, 14.2 [6.1]; d1A8, 3.7 [1.0]; d1A9, 9.1 [14.1]; d1A10, 1.6 [1.6]; and d1A11, 3.4 [8.1]. The human recombinant UGTs were expressed and prepared as enriched insect cell membranes as previously described (Zhang et al., 2012a) and their relative expression levels were 1A1, 2.1; 1A3, 19.5; 1A4, 5.2; 1A5, 17.0; 1A6, 2.5; 1A7, 21.5; 1A8, 11.0; 1A9, 5.2; and 1A10, 1.3. These relative expression values for both the dog and human UGTs were determined using the same reference values to allow direct

comparisons between them and they were used to "normalize" the activity of different UGTs, or to correct the measured glucuronidation rates, per mg protein, according to the relative expression level of each enzyme.

Glucuronidation assays

The substrates (see Table 2) in methanol were added to the incubation tubes, followed by evaporation of the methanol and dissolution of the residue in the reaction mixture, containing 50 mM phosphate buffer pH 7.4, 10 mM $MgCl_2$ and the enzyme source. The assay conditions were adjusted to avoid consumption of more than 30% of the substrate during the incubation. To this end, in assays using recombinant dog UGTs cell homogenates, the total protein concentration was 1.0-2.2 mg/mL, while in assays using recombinant human UGTs in membrane preparations the total protein concentration was lower, 0.1-0.2 mg/ml. When pooled liver or intestine microsomes of either dog or human were the enzyme source, the protein concentration was 0.1-0.2 mg/mL and the reaction mixtures also contained 50 µg/mg alamethicin per total protein (Walsky et al., 2012). In the cases of indomethacin and diclofenac glucuronidation reactions, the pH of the phosphate buffer was adjusted to 6.0 in order to reduce acyl migration, as previously reported (Zhang et al., 2012b). The bilirubin glucuronidation assays were carried out avoiding light exposure to minimize substrate isomerization.

The ready mixtures were first pre-incubated at 0°C for either 15 min (recombinant dog UGTs) or 30 min (dog and human pooled microsomes), followed by 5 min at 37°C, before starting the reaction by the addition of UDPGA to a final concentration of 5 mM, in 100 µl reaction volume. Enzyme reactions were carried out at 37°C for 5–120 min and terminated as described in Table 2 and in the Supplementary materials (including Fig. S1). Following reaction termination, the samples were transferred to -20°C for an hour and then centrifuged at 16000 *g*

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for 10 min. Aliquots of the supernatants were analyzed by HPLC. The assays were done in triplicates and included control samples without UDPGA.

As presented below, in assays of estrogens glucuronidation by dUGT1A8 and dUGT1A10 a new glucuronide peak was detected that was not seen in assays with the other dUGT1As or the human UGT1As. For further examination of this glucuronide, that turned out to be estrogens diglucuronides (or linked glucuronides, Argikar, 2012), dUGT1A8 and dUGT1A10 were also incubated with either of the two E_2-monoglucuronides, namely E_2-3-glucuronide or E_2-17-glucuronide as the sole aglycone substrate. These incubation reactions contained either 75 µM of E_2-3-glucuronide or 125 µM of E_2-17-glucuronide, without DMSO. Otherwise, the reaction conditions and HPLC-analyses were carried out as described for estradiol (Tables 2 and 3).

Analytical methods

HPLC analyses were performed using Agilent 1100 series equipment with multiple wavelengths UV detector and fluorescence detector, connected to fraction collector (Agilent Technologies, Palo Alto, CA, USA). A Poroshell 120 EC-C18 column (4.6 × 100 mm, 2.7 µm; Agilent Technologies, Palo Alto, CA, USA) was used for glucuronide(s) and substrate separations. Details of the gradient HPLC runs are presented in Table 3. The resulting chromatograms were examined with Agilent ChemStation software and further analyzed with GraphPad Prism version 5.04 for Windows (GraphPad Software Inc., San Diego, CA, USA).

Quantification of the glucuronides in the assays was based on standard curves generated with authentic standards, when such standards were available, i.e. entacapone-glucuronide, 4-MU-glucuronide, E_2-3-glucuronide and E_2-17-glucuronide. In other cases, a close estimate of the generated glucuronide was employed by using the UV absorbance of the aglycone to prepare the standard curve (Court, 2005). If an authentic standard was not available and when

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fluorescence was used to detect low glucuronidation rates, namely in the cases of epi-E_2, ethinylestradiol, R- and S-propranolol, the standard curve was generated for the glucuronide by correlating its fluorescence to its UV-signal (in reactions were much of it was produced) and to a standard curve prepared with the UV absorbance of the aglycone. The limits of determination and quantification were determined by visual evaluation from chromatograms of the samples and controls without UDPGA or without substrate.

LC-MS and LC-MS/MS analyses were performed in few cases in order to further characterize the glucuronide (see below). Fractions from the peaks of interest were subjected to Waters Acquity UPLC equipped with Waters Acquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm; Waters, MA, USA). The chromatographic separation was carried out using 0.1% formic acid in water as eluent A and 0.1% formic acid in acetonitrile as eluent B, at constant flow of 0.4 mL/min, column temperature of 26°C and the following gradient: 0–0.5 min, 5% B; 0.5–4 min, 5→95% B; 4–4.6 min, 95→5% B; 4.6–6 min, 5% B. Mass spectrometric measurements were carried out using Waters Xevo Q-TOF mass spectrometer (Waters, MA, USA) with negative mode electrospray ionization. The scanning modes were MS scan and product ion scan, with mass range of 100-1000 m/z. The parameters for capillary voltages, sample cone, and extraction cone were 2100 V, 21.0 V, 2.2 V, respectively. The source temperature was 100°C and the desolvation temperature was 200°C. Nitrogen (Aga, Helsinki, Finland) was used as desolvation gas at a flow rate of 800 l/h and 0 l/h for cone, and the collision energy was 10-30 eV.

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Results

dUGT1As cloning and expression

In order to clone and express all the dUGT1As as individual recombinant proteins, even when having their DNA sequences (from Li and Wu, 2007), one should obtain the respective mRNA, total RNA or cDNA from a tissue in which each gene is expressed. Hepatic beagle dog cDNA is likely to contain cDNAs for some of the dUGT1As, but not for the extrahepatic enzymes. Nevertheless, relying on the exon sharing system in this subfamily, having beagle dog hepatic cDNA and genomic DNA is a suitable source for constructing cDNAs for all the dUGT1As, including the extrahepatic ones, following the approach we previously used to construct full-length cDNAs for the extrahepatic human UGTs 1A7, 1A8, 1A10 without having RNA from the tissues in which they are expressed (Kuuranne et al., 2003). Since it was previously shown that full-length dUGT1A6 is expressed in the liver (Soars et al., 2001b), we used hepatic dog cDNA library to clone the "shared (or constant) segment" (Fig. 1), a correctly spliced exons 2-5 that, together, encodes the C-terminal domain of all the dUGT1As. The variable exons 1 were amplified from genomic DNA and ligated, in frame, to the shared segment, as described in the Methods section. In addition, a fusion peptide, ending with a His-tag, was added to the C-terminal end of the shared segment to enable determination of relative expression level of the dUGT1As and to correct (normalize) their glucuronidation rates according to it. Similar normalization of rates, using the same references was carried out for the human UGT1As, allowing direct comparisons of activity rates between all the tested UGTs in this study.

The names of the individual dUGT1As in this study are according to Li and Wu (2007) (except the use of "A" here also for the dog instead of the "a", following recommendation of Dr. P. Mackenzie), but it should be noted that they do not fully correspond to the human UGT1As name. As shown in Fig. 1, there are differences in the number and location of pseudogene in the large *ugt1a* gene, as well as differences in the order in which the first exons were

numbered, once their numeric value is larger than 7 (Fig. 1). In addition, and perhaps more important than the names inconsistency between the exons 1 of dUGT1As and the human UGT1As, while there is a degree of protein sequence identity between corresponding dog and human UGT1As, it is not high enough to assume identical activities. As can be seen from the phylogenetic analysis of the dog and human UGT1As (Fig. 2), the degree of sequence identity among UGTs d1A2-d1A4 is higher than between any of them to any of the human UGTs 1A3-1A5, and vice versa. A similar situation is seen between UGTs d1A7-d1A11 on one hand to the human 1A7-1A10 on the other hand (Fig. 2). Taken together, these differences prompted us to examine activity similarity, mainly qualitatively, by testing all the dog and human UGT1As in each assay, regardless their name, location in the *ugt1a* gene, or degree of sequence identity.

Glucuronidation activity assays

The recombinant dUGT1As, in enriched insect cell homogenates, were subjected to glucuronidation activity assays toward 14 compounds, including several diastereomers and enantiomers that were assayed as a pure stereoisomer, not as a mixture. The human UGT1As, in enriched insect cells membranes, were subjected to assays with the same 14 compounds. The glucuronidation rates of all the recombinant UGTs were "normalized" or "corrected" according to their relative expression levels per mg total protein in the sample (see Methods). Each substrate was assayed at a single concentration (Table 2) and the determined glucuronidation rates are presented for both the dog (upper panel in the figures) and the human (lower panel) UGT1As in each case.

Bilirubin and estrogens

Bilirubin glucuronidation, an important activity of the human UGT1A1 (Bosma et al., 1992; Zhang et al., 2007), is also specifically catalyzed by dUGT1A1 among the dUGT1As (Fig. 3).

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The results suggest that the bilirubin glucuronidation activity of dUGT1A is lower than the corresponding activity of the human UGT1A1 (Fig. 3), a results that is supported by the bilirubin glucuronidation in microsomes (see below).

Another activity that was largely regarded as UGT1A1-specific, as far as the human UGTs are concern, is ethinylestradiol glucuronidation (Zhang et al., 2007), although later we showed that UGT1A10 also catalyzes this activity (Höglund et al., 2011). Contrary to the bilirubin glucuronidation assay results, dUGT1A1 was nearly inactive in ethinylestradiol glucuronidation (Fig. 4), while the results for the human UGT1As were in line with previous reports. The inactivity of dUGT1A1 in ethinylestradiol glucuronidation does not mean that this drug is not glucuronidated in the dog, however. The results demonstrate that d1A10 and d1A11 catalyze ethinylestradiol glucuronidation at the 3-OH at much higher rates than either the human 1A1 or 1A10 (Fig. 4). It is also likely that one or more dUGT(s) of other subfamilies catalyze ethinylestradiol glucuronidation at the 17-OH (see below).

Estradiol (17-β-estradiol, E_2) glucuronidation at the 3-OH is often used as a probe activity for human hepatic UGT1A1 (Court, 2005) and we have previously shown that the human intestinal UGT1A10 catalyzes this activity at much higher rates (Itäaho et al., 2008) (note that the activity of the commercially available human UGT1A10 is low; Zhu et al., 2012). The current results suggest that the E_2 glucuronidation rate of dUGT1A1 is very low and that the dog does not have a single UGT1A with a much higher E_2 glucuronidation activity as the other dUGT1As, like the activity of the human UGT1A10 in comparison to the E_2 glucuronidation activity of the other human UGT1As (Fig. 5). On the other hand, many dUGT1As catalyze the formation of E_2-3-glucuronide at considerable rate and a clear outcome of this study is that most dUGT1As catalyze E_2 preferably at the 3-OH. In addition, low rates of E_2-17-glucuronide formation (high rates of this reaction, at least in human, are mainly catalyzed by UGTs of subfamily 2B, Itäaho et al., 2008) were detected in some cases, which may be interesting since

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they reveal some similarity between the dog dUGT1A2 and dUGT1A3 to the human UGT1A3 and UGT1A4 (Fig. 5).

Epiestradiol, 17-α-estradiol (epi-E_2), is a diastereomer of E_2 and the different configuration at C17 was shown to strongly affect the activity of several human UGTs, mainly of subfamily 2B, in several ways (Itäaho et al., 2008). The present results show that it also affects the activity of some of the dog UGT1As, particularly decreases the activity of dUGT1A10 with respect to dUGT1A7 and dUGT1A11 (Fig. 6) in comparison to the corresponding E_2 glucuronidation rate of each of these dUGT1As (Fig. 5).

Estrogens linked diglucuronides formation

While carrying out the chromatographic analyses of the different activity assays of the recombinant dUGT1As, as well as dog liver and intestine microsomes, with one of the 3 estrogen substrates, E_2, epi-E_2 and ethinylestradiol, we have noticed that when the tested enzyme was dUGT1A8 or dUGT1A10, another small and earlier eluting peak appeared that was not detected in the control samples that were incubated in the absence of UDPGA. It was previously shown that, under suitable assay conditions, dog liver microsomes could produce diglucuronides of several steroids (Murai et al., 2005). In that study they presented a detailed description, using mass spectrometry, for determining whether the product is a diglucuronide (linked diglucuronide according to the terminology of Argikar, 2012), or a bis-glucuronide. Our LC-MS/MS analysis yielded the value previously assigned for two glucuronic acid moieties linked to each other, indicating that the first eluting peak was a linked diglucuronide. Hence, in a clear and significant advance with respect to the earlier studies that were carried out using dog liver microsomes that contain many different UGTs (Murai et al., 2005), we have now used

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recombinant dUGT1As and identified two individual enzymes that can catalyze such reactions, dUGT1A8 and dUGT1A10 (the results in Fig. 7 were obtained with dUGT1A8).

It was also previously shown that the true substrates for estrogens diglucuronides synthesis by dog liver microsomes were the respective monoglucuronides, not the aglycone (Murai et al., 2005). We have examined this with the recombinant dUGT1A8 and dUGT1A10, using E_2-glucuronide as the substrate, in the absence of E_2. The results were interesting and rather surprising since when E_2-3-glucuronide was the substrate, a single linked diglucuronide was generated (Fig. 8A), a diglucuronide with the same retention time as the diglucuronide that was formed when the substrate was E_2 (Fig. 7A). However, when E_2-17-glucuronide was the substrate, the results revealed a single new glucuronide peak with a higher retention time (Fig. 8C). LC-MS and LC-MS/MS analyses of this new glucuronide peak (Fig. 8C) clearly showed that it, too, is a linked estradiol diglucuronide, a result that is in full agreement with the elution order of the different diglucuronides in the previous study (Murai et al., 2005), strongly suggesting that the peak in Fig. 8C was linked E_2-17-diglucuronide.

Medetomidines, Propranolols, Diclofenac and Indomethacin

Turning from estrogens and bilirubin to different types of molecules, we have examined the glucuronidation of dexmedetomidine and levomedetomidine by dUGT1As. These compounds are substrates for the human UGT1A4 and UGT2B10, enzymes that mostly catalyzes *N*-glucuronidation reactions (Kaivosaari et al., 2008). While glucuronidation activity toward both medetomidines was found in dog liver microsomes (see below), in agreement with the previous report (Kaivosaari et al., 2008), none of the dUGT1As appeared to catalyze the glucuronidation of either dexmedetomidine or levomedetomidine (Figs. 9 and 10, respectively). These results

DMD Fast Forward. Published on October 9, 2014 as DOI: 10.1124/dmd.114.059303
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may suggest that in the dog, both dexmedetomidine and levomedetomidine are catalyzed by one or more UGTs of subfamily 2, as discussed below.

Diclofenac and indomethacin carry a carboxylic acid that undergo glucuronidation and we have previously reported that carrying the diclofenac and indomethacin glucuronidation reactions under conditions of lower pH, pH 6.0 rather than pH 7.4, strongly stimulates the glucuronidation activity of the human UGT1A10 toward both drugs, making it by far the most active human UGT of subfamily 1A in diclofenac and indomethacin glucuronidation (Zhang et al., 2012b). Testing diclofenac and indomethacin glucuronidation by the dUGT1As was expected both to reveal their activity toward carboxylic acid-containing compounds, as well as to support or reject the similarity between the dog dUGT1A11 to the human UGT1A10, a similarity that appeared from its high activity toward the 3-OH of different estrogens (Figs. 4-6). The results with diclofenac and indomethacin suggest, however, that as far as the glucuronidation of these drugs is concern, there is a significant difference between dUGT1A11 to the human UGT1A10 (Figs. 11 and 12). In the dog, at least under the assay condition (pH 6.0), it is UGTs d1A2 and d1A3 that exhibit relatively higher activity toward both these drugs, while dUGT1A4 also catalyzes indomethacin glucuronidation a relatively high rate (Fig. 12). It may be added here that the low but clearly detectable activity of the human UGT1A3 in diclofenac and indomethacin glucuronidation (Figs. 11 and 12, respectively; Zhang et al., 2012b) may suggest of also minor similarities among the differences between the dog and human UGT1As that catalyze these reactions.

4-methylumbelliferone, entacapone, R- and S-propranolol, testosterone and epitestosterone

In the first study on dUGT1A6 it was shown that it catalyzes 4-MU, but at much lower rate than the human UGT1A6 (Soars et al., 2001b). In our hands, using different expression system,

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and lower substrate concentration, the glucuronidation rates of 4-MU by dUGT1A6 and the human UGT1A6 were similar (Fig. 13). Interestingly, among the dog UGT1As, dUGT1A6 is significantly more active than any other dUGT1A in 4-MU glucuronidation, whereas in the human the 4-MU glucuronidation activity of UGT1A10 is as high, if not somewhat higher, than the activity of UGT1A6 (Fig. 13).

Entacapone is a very good substrate for the human UGT1A9 (Lautala et al., 2000), but is also glucuronidated by the human UGTs 1A7, 1A8 and 1A10 (Luukkanen et al., 2005). Testing the dUGT1As for entacapone glucuronidation revealed that one enzyme, dUGT1A11, catalyzes this reaction at relatively high rate, even if still much lower than the rate of entacapone glucuronidation by the human UGT1A9 (Fig. 14).

As a final attempt, in this study, to support or reject similarity in substrate specificity between dUGT1A11 and the human UGT1A10, or any of the human UGT1As in the 1A7-1A10 group, we have tested the glucuronidation of R-propranolol and S-propranolol by the dog and human UGT1As. The propranolol enantiomers were previously shown to be useful in exposing opposite stereoselectivity in the human UGT1A9 and UGT1A10 (Sten et al., 2006) and we anticipated that dUGT1A10 and dUGT1A11 will glucuronidate one of them, if not both. The results were different, however. Only dUGT1A7 exhibited meaningful glucuronidation rate and only toward R-propranolol, with dUGTs 1A1-1A3 exhibiting low but measurable rates of this activity (Fig. 15). In the case of S-propranolol, the preferred propranolol enantiomer of the human UGT1A9, only barely detectable activity was exhibited by the dUGT1As (Fig. 16).

Since this study focused on the dog (and human) UGTs of subfamily 1A, we have tested their glucuronidation activity using compounds that were known to be primarily glucuronidated by one or more human UGTs of subfamily 1A. The activities of the human UGTs toward the selected compounds were in full agreement with the previously published studies that are cited

here, confirming the analysis methods. The results with the dUGT1As, however, mostly differed considerably from the results with human UGT1As.

We finally tested the recombinant dUGT1As with two substrates, testosterone and epitestosterone that, in human, are mainly glucuronidated by two different UGTs of subfamily 2B, UGT2B17 and UGT2B7, respectively (Sten et al., 2009). The results revealed very low dUGT1A toward either androgen, rather similar to the very low testosterone glucuronidation activity that was exhibited by some human UGT1As that, in turn, was similar to what previously reported (Supplementary materials, Fig. S2). Nevertheless, this low activity is an unlikely contributor to the very high testosterone glucuronidation activity that was exhibited by dog liver microsomes (see below).

Glucuronidation activities in dog and human liver and intestine microsomes

In addition to testing the activities of dUGT1As and human UGT1As toward 14 compounds, we have also tested the activity of pooled microsomes from dog liver and intestine, as well as pooled human liver and intestine microsomes, for the glucuronidation of the same compounds (Figs. 17 and 18). The results show that in both species the glucuronidation rates, per mg microsomal protein, are mostly much higher in liver than in intestine microsomes, but not always. Examples for the latter are the rate of entacapone glucuronidation in DIM that is higher than in DLM, or the higher rate of E_2-3-glucuronide formation in the human intestine microsomes (HIM) than in HLM (Figs. 17 and 18). As for differences between DLM and HLM, perhaps the most striking are the differences in the glucuronidation rates of steroids at position 17, both the much higher rates of E_2-17-glucuronide and testosterone glucuronide formation in DLM than in HLM (Fig. 17). It is also worth noting that ethinylestradiol-17-glucuronide was clearly detected in DLM, whereas practically no ethinylestradiol-17-

glucuronide was detected in HLM (Fig. 17). Few more differences and similarities between the different microsomal preparations, e.g. in the glucuronidation of bilirubin and entacapone, will be discussed below.

Discussion

The beagle dog UGT enzymes of subfamily 1A were cloned and expressed as recombinant enzymes in baculovirus-infected insect cells. They were named according to Li and Wu (2007), rather than in full agreement with the exons 1 order in the large human *ugt1a* gene (Fig. 1). It is noteworthy that the degree of amino acids sequence similarity among the human UGT1As and dog dUGT1As, as revealed by phylogenetic tree analysis (Fig. 2), is lower than among some of the UGTs of the same species. This may be very important for substrate specificity, considering that even the high degree of sequence identity between the human UGTs 1A9 and 1A10 does not exclude large differences in their activities (Itäaho et al., 2010). Therefore, it may be wrong to assume that dog and human UGTs with similar names will have similar activities. On the other hand, the phylogenetic analysis provides an overview of the entire N-terminal halves (the domains encoded by exon 1 in the UGTs) of the sequence, while some specific residues within them may be much more important for a given activity than other ones. Since our current knowledge on the 3-D structure of the substrate-binding domain of the UGTs is still poor, the best way to examine the substrate specificity of the dog UGT1As and to compare it to the activities of the human UGT1As is to clone, express and assay the activities of the enzymes.

Bilirubin glucuronidation in human is, perhaps, the most important activity of the UGTs and it is (almost) specifically catalyzed by UGT1A1. The results suggest that also in the dog it is dUGT1A1 that catalyzes this activity (Fig. 3). Interestingly, the rate of bilirubin glucuronidation by DLM was clearly lower than in HLM, in agreement with the lower bilirubin glucuronidation activity (corrected for relative expression level) of the recombinant dUGT1A1 in comparison to the human UGT1A1 (Fig. 3).

Racemic medetomidine was used as a veterinary drug, also to treat dogs (Kuusela et al., 2000), and the results with DLM indicate that both medetomidine enantiomers can be glucuronidated in this tissue, as previously reported (Kaivosaari et al., 2008). Nevertheless, while human

UGT1A4 was the most active enzyme in dexmedetomidine glucuronidation (Kaivosaari et al., 2008; Fig. 9) none of the 3 dUGT1As that are closely related to it, dUGT1A2, dUGT1A3 and dUGT1A4, catalyze dexmedetomidine glucuronidation (Fig. 9). It is likely that they failed to catalyze this *N*-glucuronidation reaction since they have a His residue at the "catalytic His" position, position 39 of the protein precursor sequence (Li and Wu, 2007), while the human UGT1A4 has a Pro residue at this position (Kerdpin et al., 2009). It may be further suggested that both medetomidine enantiomers are glucuronidated in the dog by one or two UGTs of family 2, particularly if an equivalent of the human UGT2B10 is present there (Kaivosaari et al., 2008).

One of the large differences in activity between HLM to DLM was seen in entacapone glucuronidation rates, very high in human microsomes and low in dog liver microsomes (Fig. 17). Interestingly, entacapone glucuronidation is almost the only activity of those tested in this study (the other one was ethinylestradiol-3-glucuronide formation) that was catalyzed at significantly higher rate in the dog intestine than in the liver (Figs. 18 *vs*. 17). It is tempting to suggest that the relatively high entacapone glucuronidation rate in DIM, in comparison to DLM, indicates that dUGT1A11 is expressed in the dog intestine, but not in the dog liver. The latter suggestion turned out to be in agreement with a new study that was published while this manuscript was under revision (Heikkinen et al., 2014). The line of reasoning, or speculation, on the tissue of dUGTs expression based on observed glucuronidation activities differences between DLM to DIM (Fig. 17), particularly differences at the positions at which estrogens are conjugated, might be extended. Since most of the dUGT1As almost selectively catalyzed glucuronidation of the tested estrogens at the 3-OH (Figs. 4-6), the results may not only suggest that dUGT1A11 is an intestinal enzyme, but also that in the dog, UGTs of subfamily 2B, and perhaps other subfamilies, are significantly more important in the liver than in the intestine.

DMD Fast Forward. Published on October 9, 2014 as DOI: 10.1124/dmd.114.059303
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An interesting finding of this study was the identification of individual dUGTs, d1A8 and d1A10, that are able to catalyze the formation of linked diglucuronides of several estrogens, all the 3 estrogens that were examined in this study. Both dUGT1A8 and dUGT1A10 produce only the E_2-3-glucuronide when the substrate is E_2, suggesting that E_2 binds within their active site in a specific way that favors the production of E_2-3-glucuronide (Fig. 5). However, when a much larger substrate binds, either E_2-3-glucuronide or E_2-17-glucuronide, it is not the estrogen backbone structure that determines the binding orientation in the enzyme's substrate binding site, but the site at which the glucuronic acid moiety is attached to the estrogen backbone (Fig. 8). This suggests that in order to generate such linked diglucuronides, the 2'-hydroxyl of the first bound glucuronic acid should bound close to the "catalytic His" of the enzyme, since this is the hydroxyl group previously found to be conjugated in such diglucuronides by the second glucuronic acid moiety (Murai et al., 2005; Argikar, 2012). The new finding, the dUGT1A8 (or dUGT1A10) could bind either E_2-3-glucuronide or E_2-17-glucuronide in a "productive" way, that gives rise to linked diglucuronides, may help future modeling of its binding site with bound E_2-glucuronide in either orientation. This may be particularly interesting in the case of E_2-17-glucuronide since, unlike E_2-3-glucuronide, it is not a natural product of the enzyme.

It was known for a long time that the human UGT1A1 can catalyze both mono- and diglucuronidation of bilirubin (see Zhou et al., 2010, for kinetic analysis of the sequential reaction) and we have also noticed indications for this in dUGT1a1 (results not shown). Nevertheless, estrogens are different molecules than bilirubin, for example in their rigidity and lack of pseudo-symmetry. In addition, unlike the estradiol diglucuronides described here, the bilirubin diglucuronides are not linked diglucuronides. Hence, there seems to be a significant difference between the human UGT1A1 and dUGT1A1 on one hand, to dUGT1A8 and dUGT1A10 on the other hand, in the mechanism leading to the formation of diglucuronides.

Finally, in this study we have cloned and expressed the 10 dog UGTs of subfamily 1A, examined their glucuronidation activity and compared it to the activities of the 9 human UGTs of subfamily 1A that were expressed in the same system. In addition, we have examined the same activities in pooled liver and intestine microsomes from both dog and human. The results revealed many differences, alongside several similarities, both at the levels of individual enzymes and of microsomes. Nevertheless, the results provide many insights, both on the structure and function of the UGTs, as well as on the expression of individual UGTs in specific tissues. The results may also provide ground for further considerations of interspecies differences and its practical values, even if this issue has not been discussed here explicitly.

Acknowledgements

We would like to thank Orion Pharma Corporation, Espoo, Finland, for the generous gifts of Entacapone, Dexmedetomidine and Levomedetomidine, as well as Prof. Hannes Lohi, University of Helsinki, Finland, for a sample of beagle dog genomic DNA. We would also like to thank Dr. Peter I. Mackenzie, Flinders University, Adelaide, Australia, for verifying the correctness of the dog UGT1As sequences and for his useful comments about the way to name them. Thanks also to Dr. Linda Ahonen for her contribution to the LC-MS/MS analyses.

Authorship contribution

Participated in research design: Finel, Troberg

Conducted experiments: Järvinen, Troberg, Sneitz, Hagström, Muniz, Mosorin

Performed data analysis: Troberg, Järvinen, Finel

Wrote or contributed to the writing of the manuscript: Troberg, Järvinen, Finel

References

Argikar UA (2012) Unusual glucuronides. *Drug Metab Dispos* 40:1239-1251.

Bosma PJ, Chowdhury NR, Goldhoorn BG, Hofker MH, Oude Elferink RPJ, Jansen PLM, and Chowdhury JR (1992) Sequence of exons and the flanking regions of human bilirubin-UDP-glucuronosyltransferase gene complex and identification of a genetic mutation in a patient with Crigler-Najjar syndrome, type I. *Hepatology* 15:941-947.

Court MH (2005) Isoform-selective probe substrates for in vitro studies of human UDP-glucuronosyltransferases. *Methods Enzymol* 400:104-116.

Furukawa T, Naritomi Y, Tetsuka K, Nakamori F, Moriguchi H, Yamano K, Terashita S, Tabata K, and Teramura T (2014) Species differences in intestinal glucuronidation activities between humans, rats, dogs and monkeys. *Xenobiotica* 44:205-216.

Heikkinen AT, Friedlein A, Matondo M, Hatley OJ, Petsalo A, Juvonen R, Galetin A, Rostami-Hodjegan A, Aebersold R, Lamerz J, Dunkley T, Cutler P, and Parrott N (2014) Quantitative ADME proteomics – CYP and UGT enzymes in the beagle dog liver and intestine. *Pharm Res*, in press.

Höglund C, Sneitz N, Radominska-Pandya A, Laakonen L, and Finel M (2011) Phenylalanine 93 of the human UGT1A10 plays a major role in the interactions of the enzyme with estrogens. *Steroids* 76:1465-1473.

Itäaho K, Laakkonen L, and Finel M (2010) How many and which amino acids are responsible for the large activity differences between the highly homologous UDP-glucuronosyltransferases (UGT) 1A9 and UGT1A10? *Drug Metab Dispos* 38:687-696.

Itäaho K, Mackenzie PI, Ikushiro S-, Miners JO, and Finel M (2008) The configuration of the 17-hydroxy group variably influences the glucuronidation of ß-estradiol and epiestradiol by human UDP-glucuronosyltransferases. *Drug Metab Dispos*36:2307-2315.

Kaivosaari S, Finel M, and Koskinen M (2011) N-glucuronidation of drugs and other xenobiotics by human and animal UDP-glucuronosyltransferases. *Xenobiotica* 41:652-669.

Kaivosaari S, Toivonen P, Aitio O, Sipilä J, Koskinen M, Salonen JS, and Finel M (2008) Regio- and stereospecific N-glucuronidation of medetomidine: The differences between UDP glucuronosyltransferase (UGT) 1A4 and UGT2B10 account for the complex kinetics of human liver microsomes. *Drug Metab Dispos* 36:1529-1537.

Kerdpin O, Mackenzie PI, Bowalgaha K, Finel M, and Miners JO (2009) Influence of N-terminal domain histidine and proline residues on the substrate selectivities of human UDP-glucuronosyltransferase 1A1, 1A6, 1A9, 2B7, and 2B10. *Drug Metab Dispos* 37:1948-1955.

King CD, Rios GR, Green MD, and Tephly TR (2000) UDP-Glucuronosyltransferases. *Curr Drug Metab* 1:143-161.

Kirkness EF, Bafna V, Halpern AL, Levy S, Remington K, Rusch DB, Delcher AL, Pop M, Wang W, Fraser CM, and Venter JC (2003) The dog genome: Survey sequencing and comparative analysis. *Science* 301:1898-1903.

Kurkela M, Garcia-Horsmant JA, Luukkanen L, Mörsky S, Taskinen J, Baumann M, Kostiainen R, Hirvonen J, and Finel M (2003) Expression and characterization of recombinant human UDP-glucuronosyltransferases (UGTs): UGT1A9 is more resistant to detergent inhibition than the other UGTs and was purified as an active dimeric enzyme. *J Biol Chem* 278:3536-3544.

Kuuranne T, Kurkela M, Thevis M, Schänzer W, Finel M, and Kostiainen R (2003) Glucuronidation of anabolic androgenic steroids by recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* 31:1117-1124.

Kuusela E, Raekallio M, Anttila M, Falck I, Mölsä S, and Vainio O (2000) Clinical effects and pharmacokinetics of medetomidine and its enantiomers in dogs. *J Vet Pharmacol Ther* 23:15-20.

Lautala P, Ethell BT, Taskinen J, and Burchell B (2000) The specificity of glucuronidation of entacapone and tolcapone by recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* 28:1385-1389.

Li C and Wu Q (2007) Adaptive evolution of multiple-variable exons and structural diversity of drug-metabolizing enzymes. *BMC Evol Biol* 7:69.

Lindblad-Toh K, Wade CM, Mikkelsen TS, Karlsson EK, Jaffe DB, Kamal M, Clamp M, Chang JL, Kulbokas III EJ, Zody MC, Mauceli E, Xie X, Breen M, Wayne RK, Ostrander EA, Ponting CP, Galibert F, Smith DR, DeJong PJ, Kirkness E, Alvarez P, Biagi T, Brockman W, Butler J, Chin C-, Cook A, Cuff J, Daly MJ, DeCaprio D, Gnerre S, Grabherr M, Kellis M, Kleber M, Bardeleben C, Goodstadt L, Heger A, Hitte C, Kim L, Koepfli K-, Parker HG, Pollinger JP, Searle SMJ, Sutter NB, Thomas R, Webber C, and Lander ES (2005) Genome sequence, comparative analysis and haplotype structure of the domestic dog. *Nature* 438:803-819.

Luukkanen L, Taskinen J, Kurkela M, Kostiainen R, Hirvonen J, and Finel M (2005) Kinetic characterization of the 1A subfamily of recombinant human UDP-glucuronosyltransferases. *Drug Metab Dispos* 33:1017-1026.

Luukkanen L, Kilpeläinen I, Kangas H, Ottoila P, Elovaara E, and Taskinen J (1999) Enzyme-assisted synthesis and structural characterization of nitrocatechol glucuronides. *Bioconjug Chem* 10:150-154.

Mackenzie PI, Bock KW, Burchell B, Guillemette C, Ikushiro S-, Iyanagi T, Miners JO, Owens IS, and Nebert DW (2005) Nomenclature update for the mammalian UDP glycosyltransferase (UGT) gene superfamily. *Pharmacogenet Genomics* 15:677-685.

Murai T, Iwabuchi H, and Ikeda T (2005) Repeated glucuronidation at one hydroxyl group leads to structurally novel diglucuronides of steroid sex hormones. *Drug Metab Pharmacokinet* 20:282-93.

Rowland A, Miners JO, and Mackenzie PI (2013) The UDP-glucuronosyltransferases: Their role in drug metabolism and detoxification. *Int J of Biochem and Cell Biol* 45:1121-1132.

Soars MG, Riley RJ, Findlay KAB, Coffey MJ, and Burchell B (2001a) Evidence for significant differences in microsomal drug glucuronidation by canine and human liver and kidney. *Drug Metab Dispos* 29:121-126.

Soars MG, Smith DJ, Riley RJ, and Burchell B (2001b) Cloning and characterization of a canine UDP-glucuronosyltransferase. *Arch Biochem Biophys* 391:218-224.

Soars MG, Fettes M, O'Sullivan AC, Riley RJ, Ethell BT, and Burchell B (2003) Cloning and characterisation of the first drug-metabolising canine UDP-glucuronosyltransferase of the 2B subfamily. *Biochem Pharmacol* 65:1251-1259.

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

Sten T, Qvisen S, Uutela P, Luukkanen L, Kostiainen R, and Finel M (2006) Prominent but reverse stereoselectivity in propranolol glucuronidation by human UDP-glucuronosyltransferases 1A9 and 1A10. *Drug Metab Dispos* 34:1488-1494.

Tukey RH and Strassburg CP (2000) Human UDP-glucuronosyltransferases: Metabolism, expression, and disease. *Annu Rev Pharmacol Toxicol* 40:581-616.

Walsky RL, Bauman JN, Bourcier K, Giddens G, Lapham K, Negahban A, Ryder TF, Obach RS, Hyland R, and Goosen TC (2012) Optimized assays for human UDP-glucuronosyltransferase (UGT) activities: Altered alamethicin concentration and utility to screen for UGT inhibitors. *Drug Metab Dispos* 40:1051-1065.

Zhang H, Patana AS, Mackenzie PI, Ikushiro S, Goldman A, and Finel M (2012a) Human UDP-glucuronosyltransferase expression in insect cells: Ratio of active to inactive recombinant proteins and the effects of a C-terminal his-tag on glucuronidation kinetics. *Drug Metab Dispos* 40:1935-1944.

Zhang H, Soikkeli A, Tolonen A, Rousu T, Hirvonen J, and Finel M (2012b) Highly variable pH effects on the interaction of diclofenac and indomethacin with human UDP-glucuronosyltransferases. *Toxicol in Vitro* 26:1286–1293.

Zhang H, Cui D, Wang B, Han YH, Balimane P, Yang Z, Sinz M, and Rodrigues AD (2007) Pharmacokinetic drug interactions involving 17a-ethinylestradiol: A new look at an old drug. *Clin Pharmacokinet* 46:133-157.

Zhou J, Tracy TS, and Remmel RP (2010) Bilirubin glucuronidation revisited: Proper assay conditions to estimate enzyme kinetics with recombinant UGT1A1. *Drug Metab Dispos* 38:1907-1911.

Zhu L, Ge G, Zhang H, Liu H, He G, Liang S, Zhang Y, Fang Z, Dong P, Finel M, and Yang L (2012) Characterization of hepatic and intestinal glucuronidation of magnolol: application of

the RAF approach to decipher the contributions of multiple UGT isoforms. *Drug Metab Dispos* 40:529-538.

Footnotes

[1]This study was supported by grants from the Academy of Finland [12600101] and the Sigrid Juselius Foundation [47033421].

[2]Johanna Troberg and Erkka Järvinen contributed equally to this study.

DMD Fast Forward. Published on October 9, 2014 as DOI: 10.1124/dmd.114.059303
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Tables

Table 1. Oligonucleotides for the dUGT1As cloning (inserted restriction site sequences are underlined)

No.	Name	DNA sequence (5'->3')
1.	d1A-Eco-F	ttgaattcgaagcctatgttaatgcttctg
2.	d1A-Sal-R	tttgtcgacgtgtgccttggatttgtgag
3.	d1A1-Bam-F	aaggatccgtagcaaagacaagggcacca
4.	d1A1-Eco-R	tcgaattccccggggatcttggatag
5.	d1A2-Eco-R2	ttcgaattccttggatagtgggtttttgctg
6.	d1A2/3/4-Bam-F	aaggatccggtgctttgggcttctacag
7.	d1A2/3/4-Eco-R	tcgaattcctgagacaatggcttc
8.	d1A2/3/4-Nde-F	agaccatatgacgttcctgcaaagggtca
9.	d1A2/3/4-Nde-R	acgtcatatggtctgaattccttgttaatctg
10.	d1A6-Bam-F	gcggatcctgtcctctcccccagatg
11.	d1A6-Eco-R	tcgaattcctgaggcaggactcccttca
12.	d1A7/11-Bgl-F	caaagatctggattgccctggactgcag
13.	d1A8/9/10-Bgl-F	caaagatctggattgccctgggctgcag
14.	d1A7-Eco-R2	ccgaattcctttggcaatgtctttcc
15.	d1A8/9/10/11-Eco-R	ccgaattcctttggcaatggctttcc

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Table 2. Glucuronidation assays conditions

substrate, concentration (μM)	**DMSO (%)**	**incubation time (min)**	**stopping reagent (μL)**
4-MU (200)	-	5-30	4M PCA (10)
17α-estradiol (50)	1	5-60	5% acetic acid in methanol (100)
estradiol (50)	1	5-60	5% Acetic acid in methanol (100)
ethinylestradiol (50)	5	10-60	5% Acetic acid in methanol (100), 1:5 4M PCA:Methanol (60)
testosterone (50)	5	10-60	4M PCA (10)
epitestosterone (50)	5	30-60	1:5 4M PCA:Methanol (60), 4M PCA (10)
bilirubin (50)	10	15-60	Methanol (100)
entacapone (200)	-	5-60	1:5 4M PCA:Methanol (60), 4M PCA (10)
indomethacin (100)	5	10-60	Methanol (100)
diclofenac (100)	-	15-60	Methanol (100)
dexmedetomidine (1000)	-	60-120	5% Acetic acid in methanol (100)
levomedetomidine (500)	-	60-120	5% Acetic acid in methanol (100)
S-propranolol (500)	-	30-60	Methanol (100)
R-propranolol (500)	-	30-60	Methanol (100)

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Table 3. Analytical conditions and methods for HPLC glucuronide separation. The eluents were acetonitrile (B) and 50 mM phosphate buffer pH 3 (A) in all methods, except that in the R- and S-propranolol and bilirubin chromatographic methods 0.1 % formic acid in water was used as eluent A. The column temperature and the flow rate were 40 °C and 1 ml/min, respectively.

Analyte glucuronide	**Gradient**	**Injection volume** (μL)	**Detection parameters** (wavelength, nm)	**Retention time of glucuronide(s)** (min)
4-methylumbelliferone	0–3 min, 20→50% B; 3–3.1 min, 50→20% B; 3.1–5 min, 20% B	5-25	Fluorescence, λ_{ex} 316, λ_{em} 382	1.7
17α-estradiol	0–2 min, 20→40% B; 2–5 min, 40→50% B; 5–8 min, 50% B; 8–8.1 min, 50→20% B; 8.1–11 min, 20% B	40-50	UV, 281; Fluorescence, λ_{ex} 281, λ_{em} 306	3.1, 3.8 and 4.2
estradiol	0–2 min, 20→40% B; 2–5 min, 40→50% B; 5–8 min, 50% B; 8–8.1 min, 50→20% B; 8.1–11 min, 20% B	40-50	Fluorescence, λ_{ex} 281, λ_{em} 306	2.8, 3.4, 3.6 and 3.9
ethinylestradiol	0–6 min, 25→50% B; 6–9 min, 50%B; 9–9.1 min, 50→25% B; 9.1–11 min, 25% B	40	UV, 281; Fluorescence, λ_{ex} 281, λ_{em} 306	2.3, 4.0 and 4.6
testosterone	0–1.5 min, 27% B; 1.5–5 min, 27→50% B; 5–8 min, 50% B; 8–8.1 min, 50→27% B; 8.1–10 min, 27% B	40	UV, 244	4.6
epitestosterone	0–2 min, 32% B; 2–5 min, 32→50% B; 5–9 min, 50% B; 9–9.1 min, 50→32% B; 9.1–11 min, 32% B	40-60	UV, 244	4.7
bilirubin	0–6 min, 35→80% B; 6–6.3 min, 80→95% B; 6.3–12.5 min, 95% B; 12.5–12.7 min, 95→35% B; 12.7–15 min, 35% B	15-25	Visible, 449	3.2 and 4.8
entacapone	0–1 min, 15→45% B; 1–3 min, 45→50% B; 3–4.5 min, 50% B; 4.5–4.6 min, 50→15% B; 4.6–6 min, 15% B	20-25	UV, 309	2.9
indomethacin	0–4 min, 40→50% B; 4–9 min, 50% B; 9–9.1 min, 50→40% B; 9.1–11 min, 40% B	50	UV, 319	3.3
diclofenac	0–4 min, 35→50% B; 4–9 min, 50% B; 9–9.1 min, 50→35% B; 9.1–11 min, 35% B	40	UV, 276	3.8
dexmedetomidine	0–7 min, 10→45% B; 7–7.1 min, 45→10% B; 7.1–10 min, 10% B	50	UV, 215	4.8 and 5.0
levomedetomidine	0–7 min, 10→45% B; 7–7.1 min, 45→10% B; 7.1–10 min, 10% B	50	UV, 215	4.7 and 5.0
R-propranolol	0–1.5 min, 23.5% B; 1.5–4 min, 23.5→28% B; 4–5.5 min, 28% B; 5.5→5.65 min, 28→50% B; 5.65–7 min, 50% B; 7–7.15 min, 50→23.5% B; 7.15–10 min, 23.5% B	10	UV, 227; Fluorescence , λ_{ex} 227, λ_{em} 344	3.5
S-propranolol	0–1.5 min, 23.5% B; 1.5–4 min, 23.5→28% B; 4–5.5 min, 28% B; 5.5→5.65 min, 28→50% B; 5.65–7 min, 50% B; 7–7.15 min, 50→23.5% B; 7.15–10 min, 23.5% B	10	UV, 227; Fluorescence , λ_{ex} 227, λ_{em} 344	4.0

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Figure legends

Fig. 1. Schematic view of the arrangement of the exons in the dog and human *ugt1a* genes. The constant exons, 2-5, are included in each dUGT1A/UGT1A, whereas different UGT1As contain different exons 1. The first human exon 1s A2, A13, A11 and A12 are pseudogenes. Note the differences in the order/names of the variable exons between the two different *ugt1a* genes.

Fig. 2. A phylogenetic tree, based on the amino acid sequence identity degree of the N-terminal domain, the UGTs segments that are encoded by the respective exons 1, between the human and dog UGT1As. The tree was drawn using the free online Phylogeny.fr service (http://www.phylogeny.fr; Dereeper A, Guignon V, Blanc G, Audic S, Buffet S, Chevenet F, Dufayard JF, Guindon S, Lefort V, Lescot M, Claverie JM, and Gascuel O (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. Nucleic Acids Res 36(Web Server Issue):W465-469.). The blue-colored branches highlight the dog UGT1As whereas the red-colored branches show human UGT1As, indicated here as hUGTs.

Fig. 3. Bilirubin glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). The rates are corrected for relative expression level (see Methods), using the 2nd batch of dUGT1A8 as a reference for both the dog and human UGT1As.

Fig. 4. Ethinylestradiol glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details.

Fig. 5. Estradiol (17β-estradiol, E_2) glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). The bars mostly represent the rate of estradiol-3-glucuronide formation. Bars representing the formation of estradiol-17-glucuronide are marked by "17" on their top. See legend to Fig. 3 for further details.

Fig. 6. Epiestradiol (17α-estradiol, epi-E_2) glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 5 for further details.

Fig. 7. HPLC chromatograms (fluorescence detection) showing the results of E_2 glucuronidation by dUGT1A8 and control incubation carried out in the absence of UDPGA (see Methods and Table 3).

Fig. 8. HPLC chromatograms (fluorescence detection) showing the results of incubations of dUGT1A8 with E_2-3-glucuronide, in either the presence or absence of UDPGA (panels A and B, respectively), or its incubation with E_2-17-glucuronide, in either the presence or absence of UDPGA (panels C and D, respectively). See Methods and Table 3 for further details.

Fig. 9. Dexmedetomidine glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details. The G1 and G2 marks in the human samples indicate two different glucuronides (Kaivosaari et al., 2008).

Fig. 10. Levomedetomidine glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 9 for further details.

Fig. 11. Diclofenac glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details and Methods for the incubation conditions.

Fig. 12. Indomethacin glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details and Methods for the incubation conditions.

Fig. 13. 4-methylumbelliferone (4-MU) glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details.

Fig. 14. Entacapone glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details.

Fig. 15. R-propranolol glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details

Fig. 16. S-Propranolol glucuronidation rates by dog UGT1As (upper panel) and human UGT1As (lower panel). See legend to Fig. 3 for further details.

Fig. 17. Glucuronidation rates of the 14 test compounds used in this study by dog liver microsomes (DLM, upper panel) and human liver microsomes (HLM, lower panel). In the cases of estrogens, dexmedetomidine and levomedetomidine two different glucuronides were detected and they are both indicated by marks on the top of the columns (see legends to figs. 5 and 9).

Fig. 18. Glucuronidation rates of the 14 test compounds used in this study by dog intestine microsomes (DIM, upper panel) and human intestine microsomes (HIM, lower panel). See legend to Fig. 17 for further details.

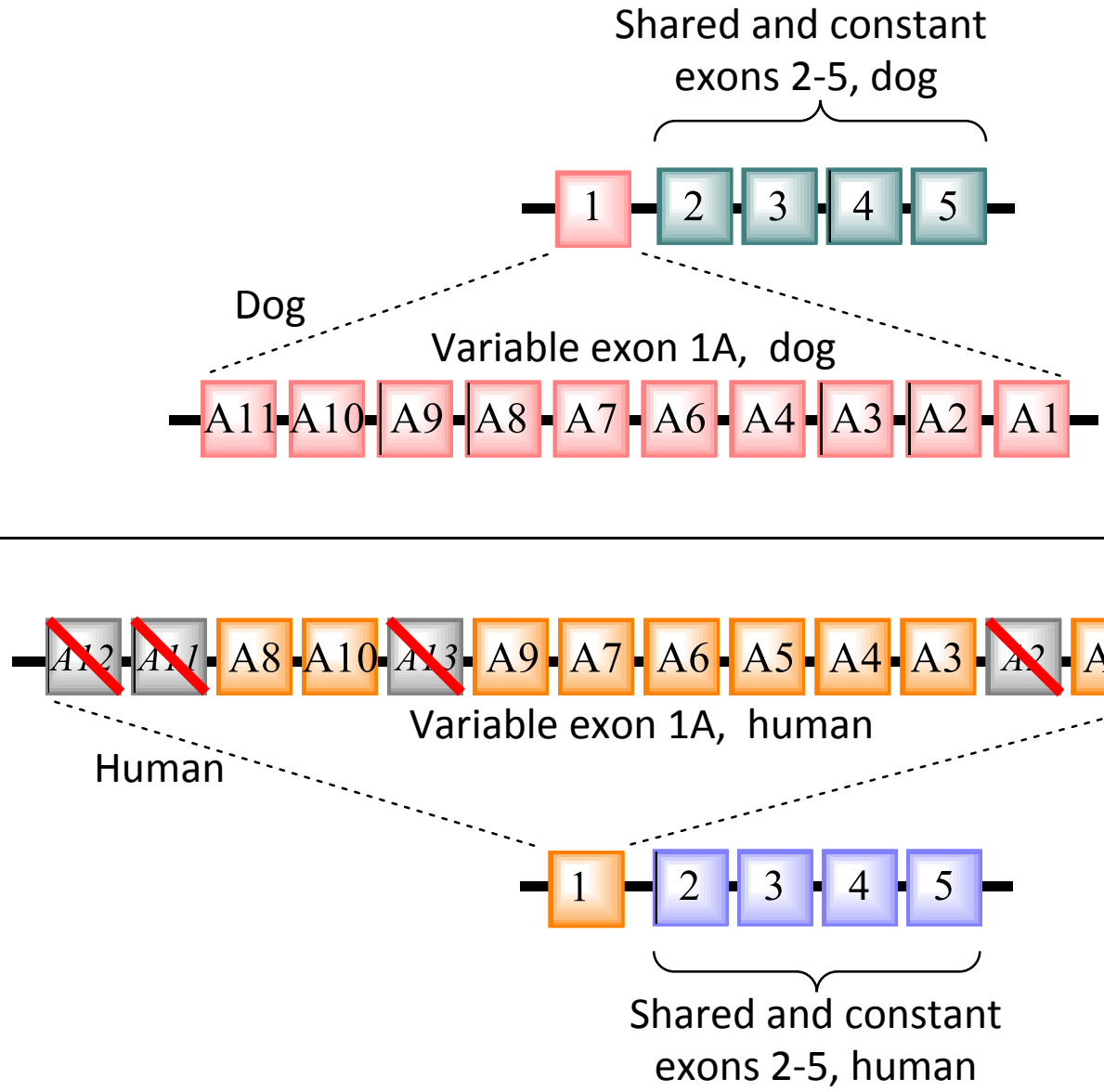
Shared and constant
exons 2-5, dog
1
2
3
4
5
Dog
Variable exon 1A, dog
A11
A10
A9
A8
A7
A6
A4
A3
A2
A1
A12
A11
A8
A10
A13
A9
A7
A6
A5
A4
A3
A2
A1
Variable exon 1A, human
Human
1
2
3
4
5
Shared and constant
exons 2-5, human

Fig. 1

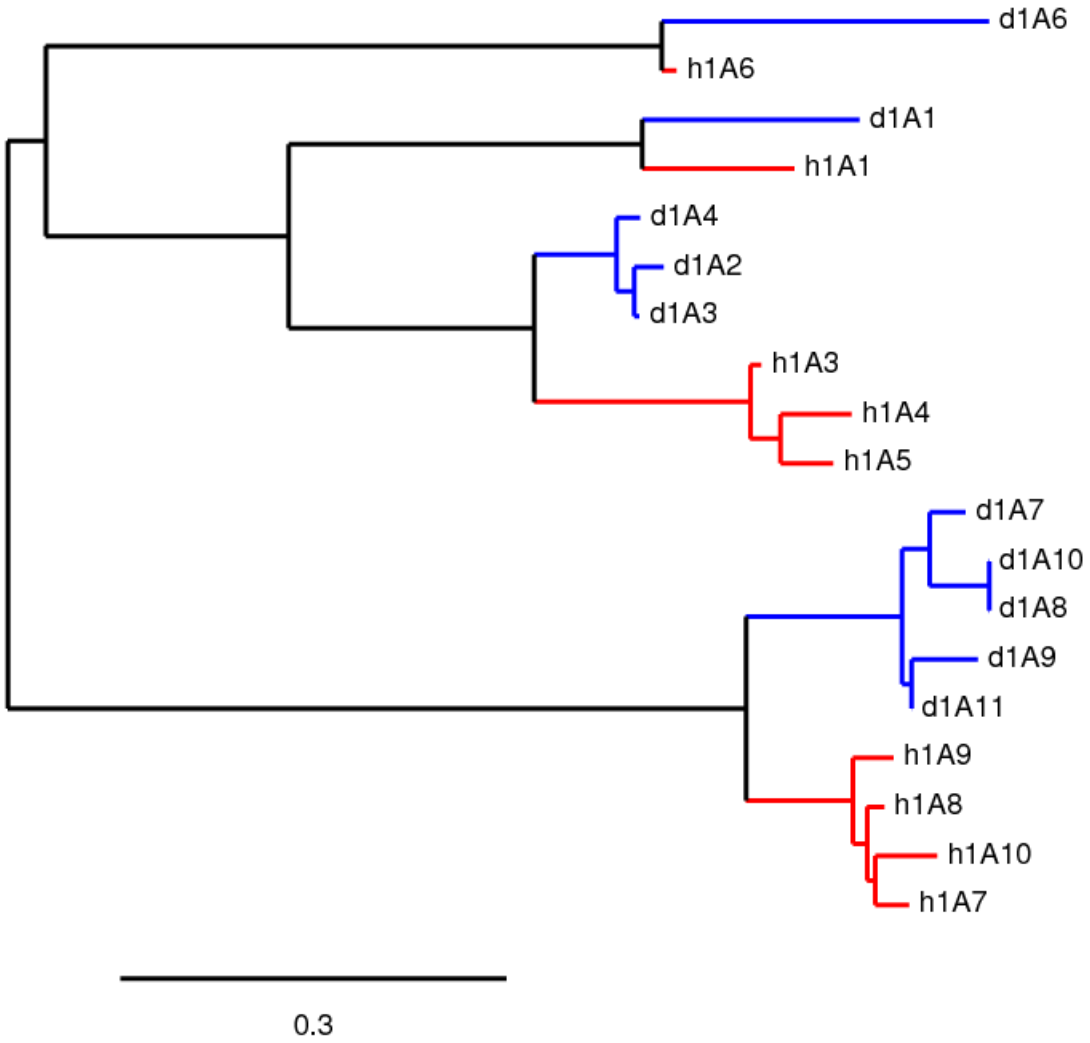

Fig. 2

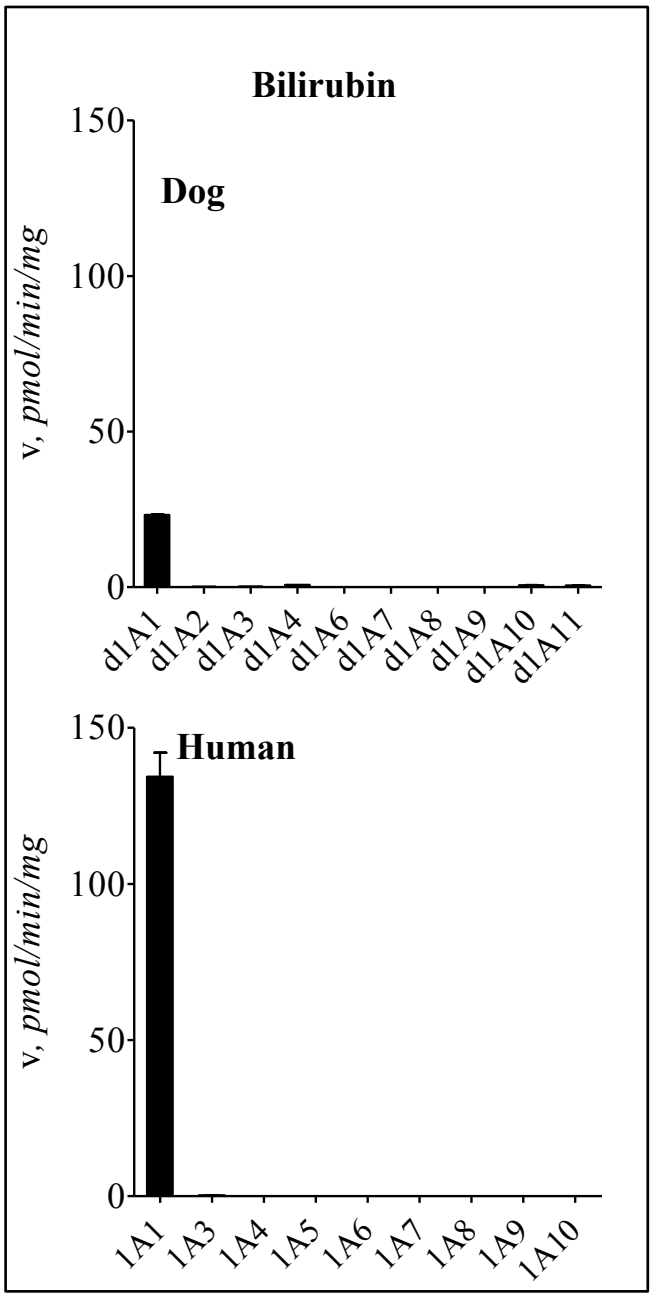
Bilirubin
Dog
v, pmol/min/mg
150
100
50
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
150
100
50
0
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 3

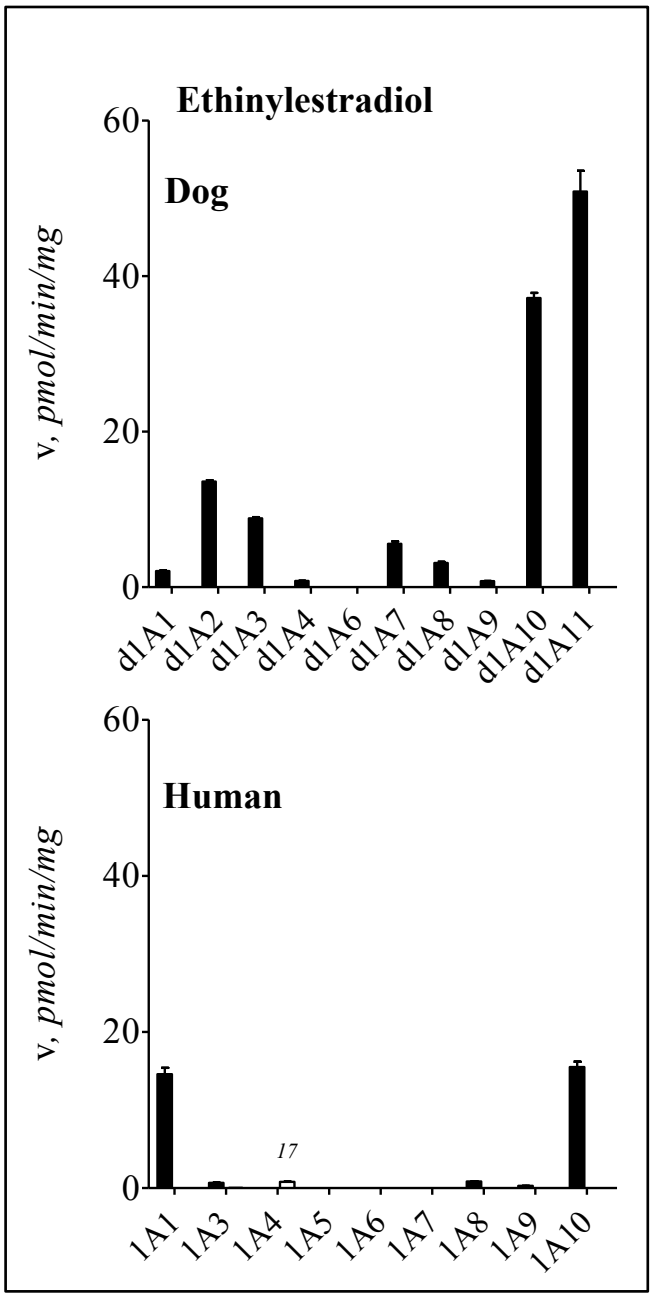
Ethinylestradiol
Dog
v, pmol/min/mg
60
40
20
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
60
40
20
0
17
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 4

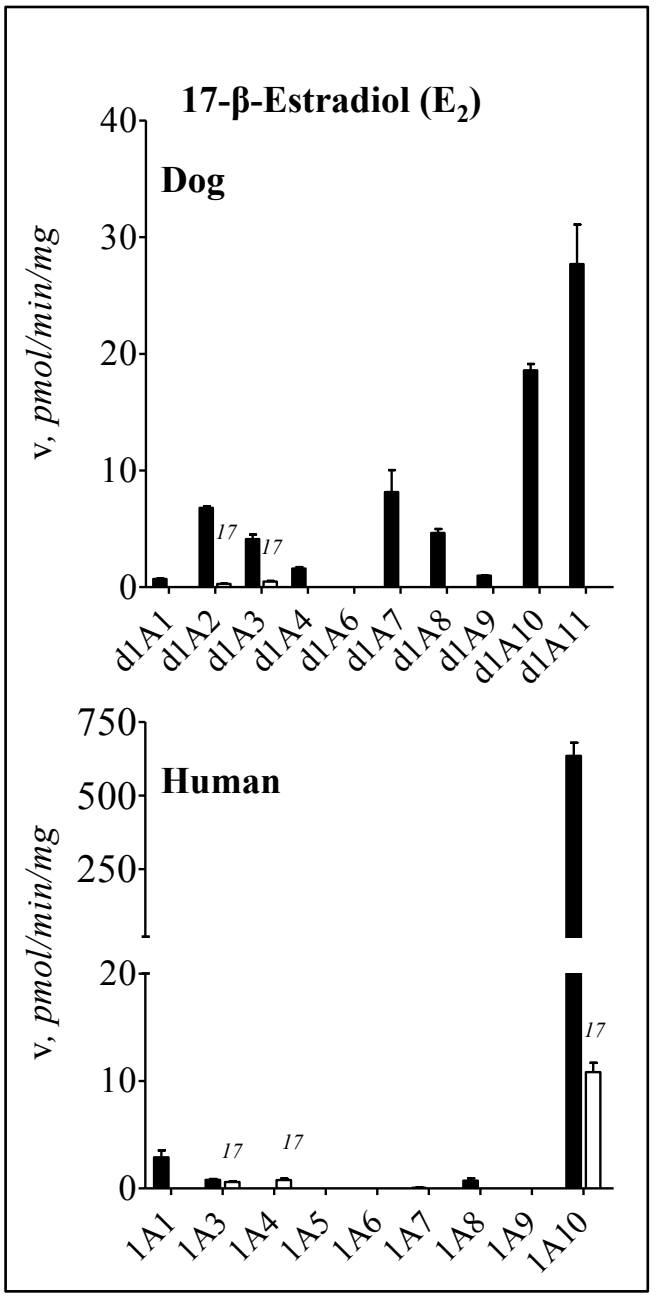
17-β-Estradiol (E2)
Dog
v, pmol/min/mg
40
30
20
10
0
17
17
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
750
500
250
20
10
0
17
17
17
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 5

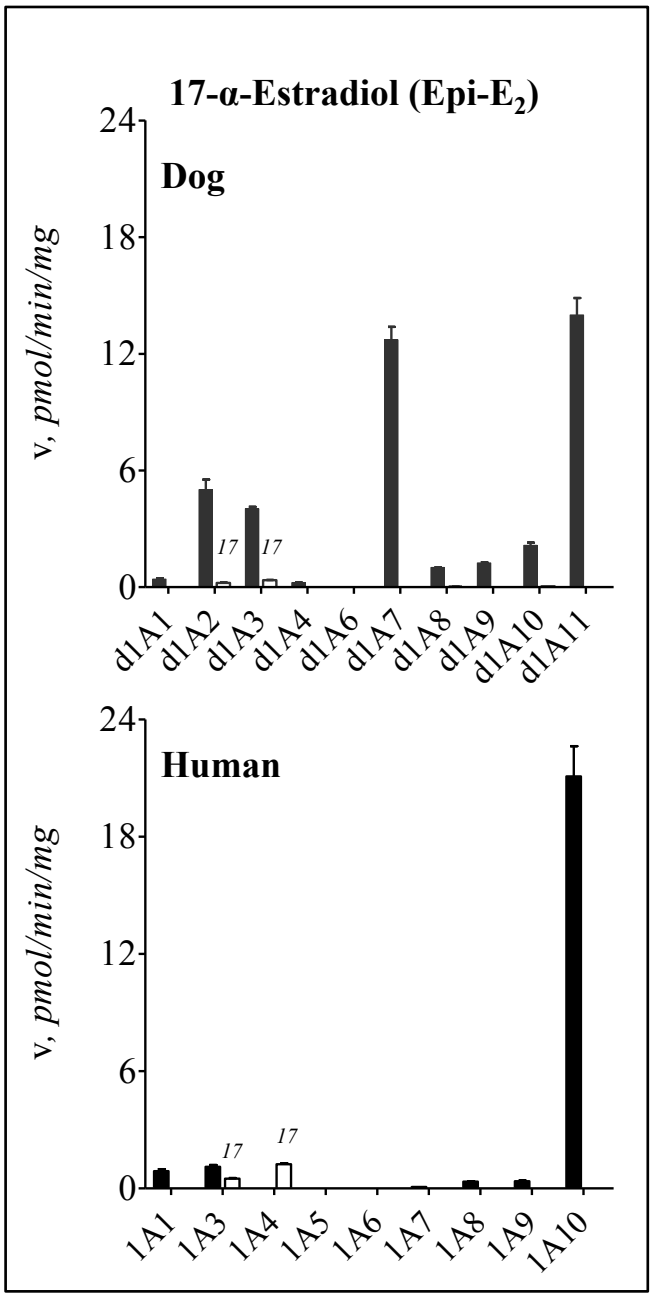
17-α-Estradiol (Epi-E2)
Dog
v, pmol/min/mg
24
18
12
6
0
17
17
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
24
18
12
6
0
17
17
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 6

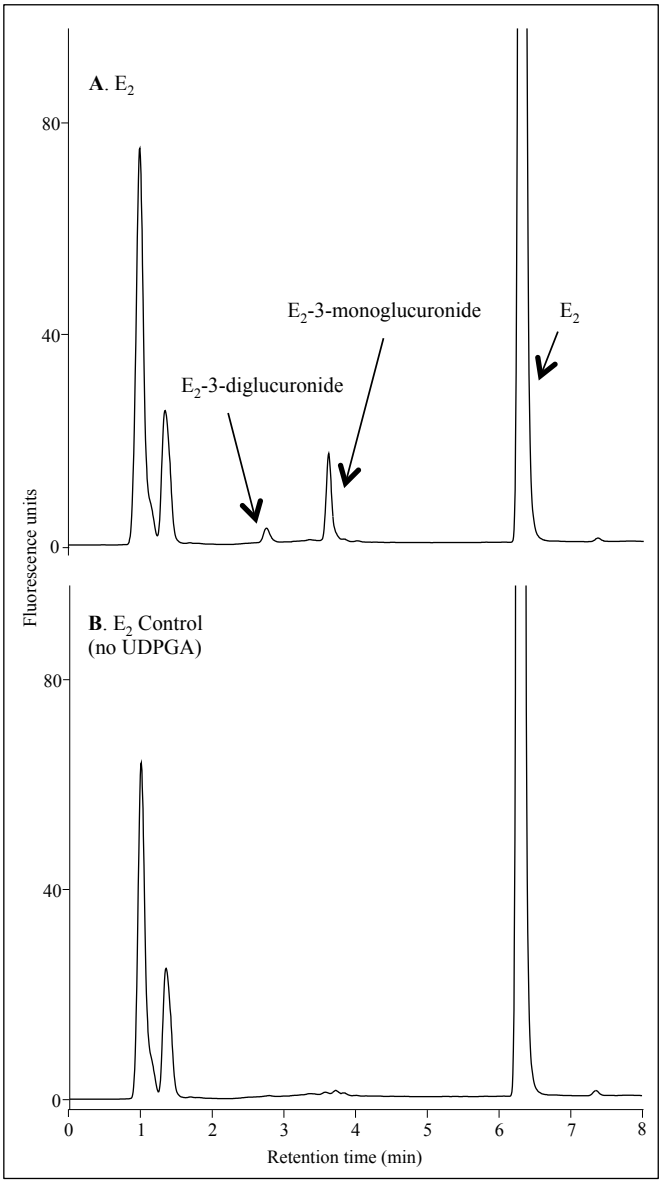
A. E2
80
40
0
E2-3-monoglucuronide
E2
E2-3-diglucuronide
Fluorescence units
B. E2 Control
(no UDPGA)
80
40
0
0
1
2
3
4
5
6
7
8
Retention time (min)

Fig. 7

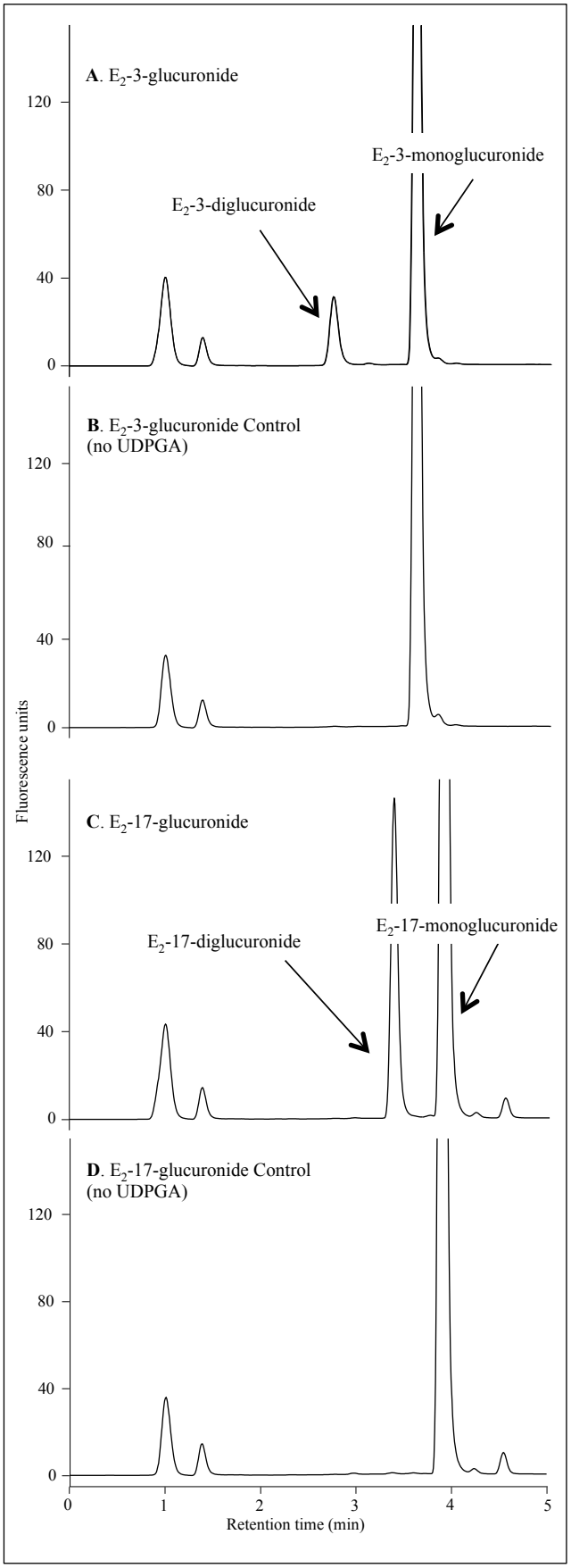
A. E2-3-glucuronide
E2-3-monoglucuronide
E2-3-diglucuronide
B. E2-3-glucuronide Control
(no UDPGA)
C. E2-17-glucuronide
E2-17-monoglucuronide
E2-17-diglucuronide
D. E2-17-glucuronide Control
(no UDPGA)
Fluorescence units
120
80
40
0
0
1
2
3
4
5
Retention time (min)

Fig. 8

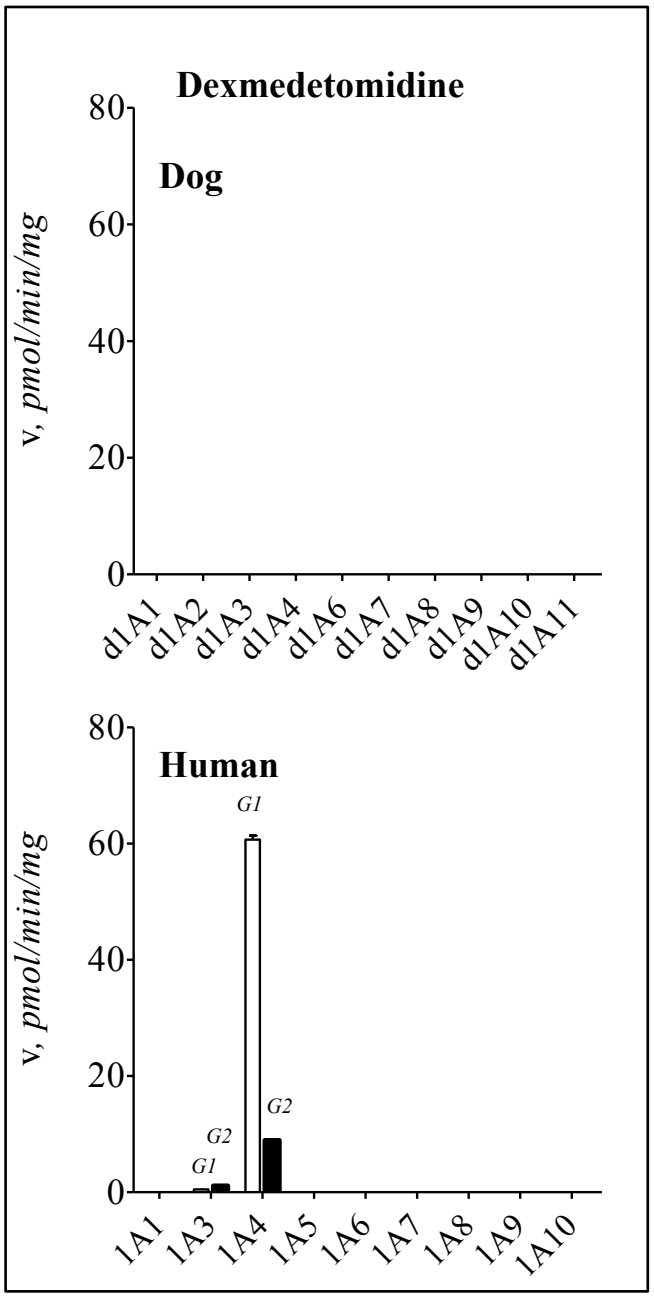
Dexmedetomidine
Dog
v, pmol/min/mg
80
60
40
20
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
80
60
40
20
0
G1
G2
G1
G2
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 9

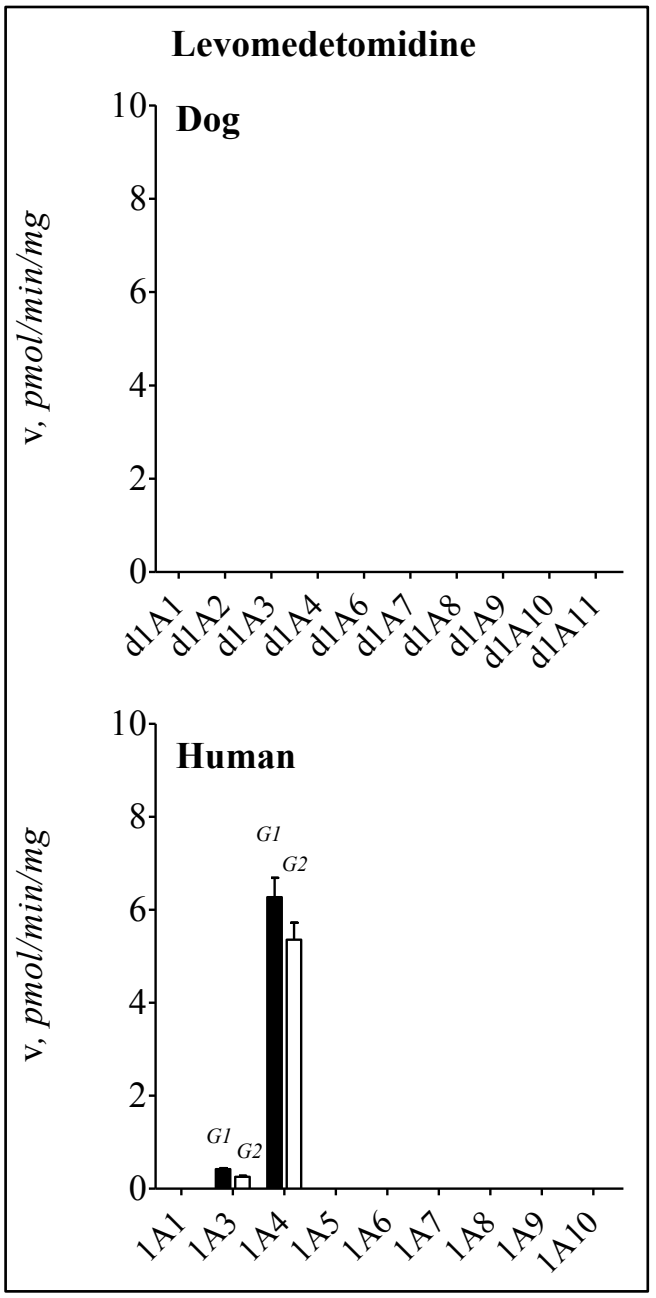
Levomedetomidine
Dog
v, pmol/min/mg
10
8
6
4
2
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
10
8
6
4
2
0
G1
G2
G1
G2
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 10

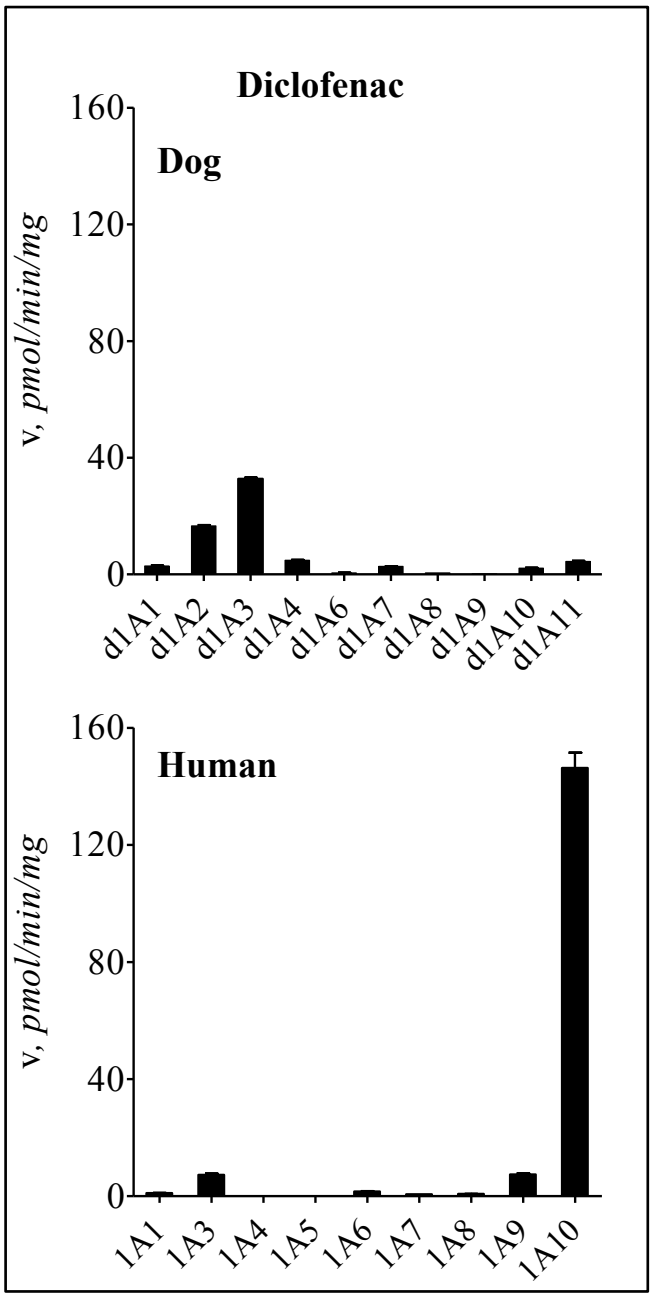
Diclofenac
Dog
v, pmol/min/mg
160
120
80
40
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
160
120
80
40
0
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 11

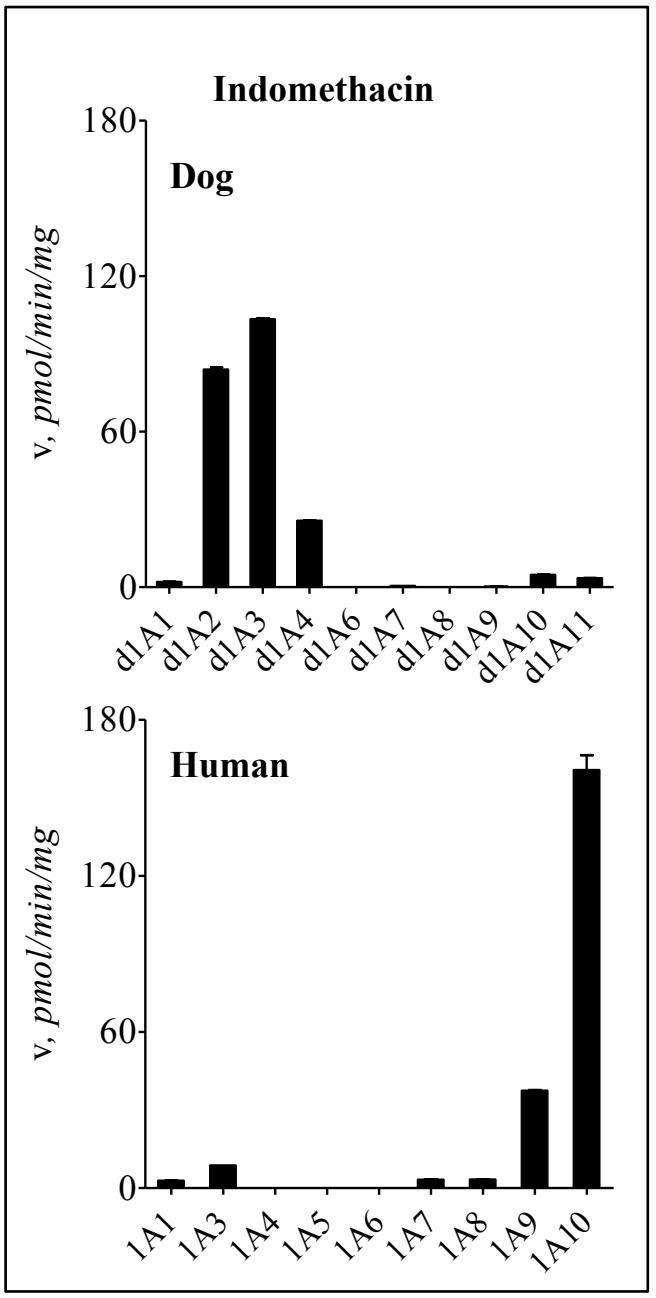
Indomethacin
Dog
v, pmol/min/mg
180
120
60
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
180
120
60
0
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 12

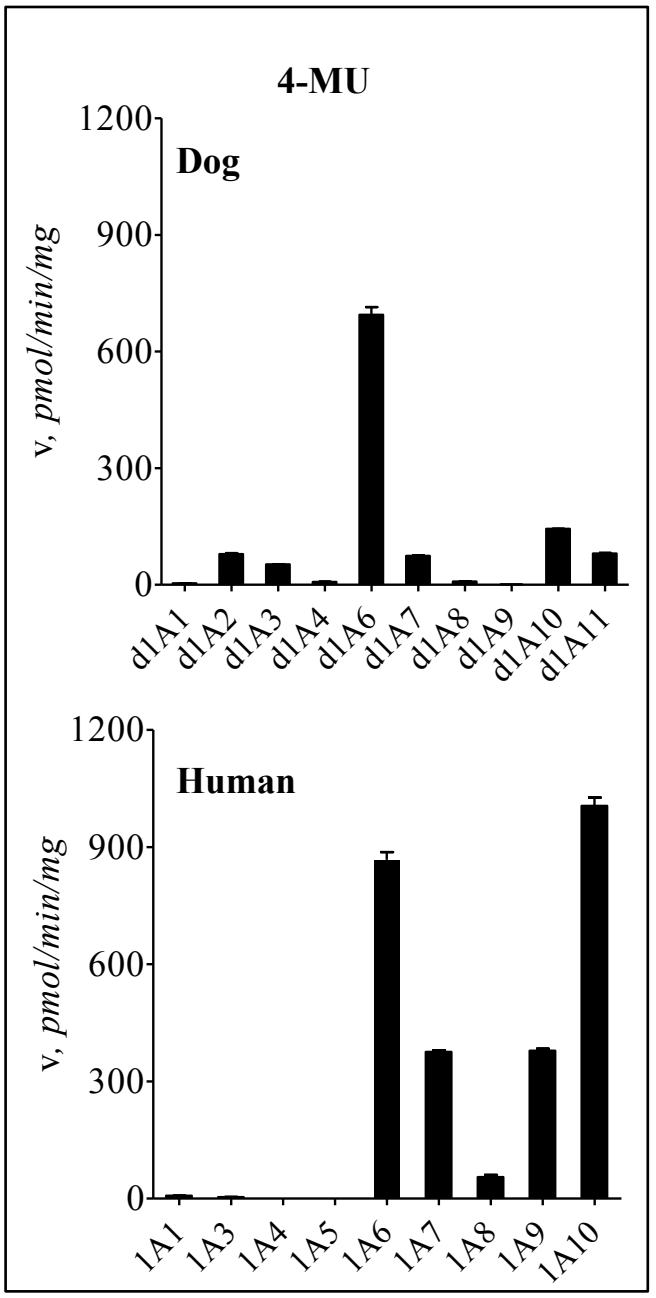
4-MU
Dog
v, pmol/min/mg
1200
900
600
300
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
1200
900
600
300
0
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 13

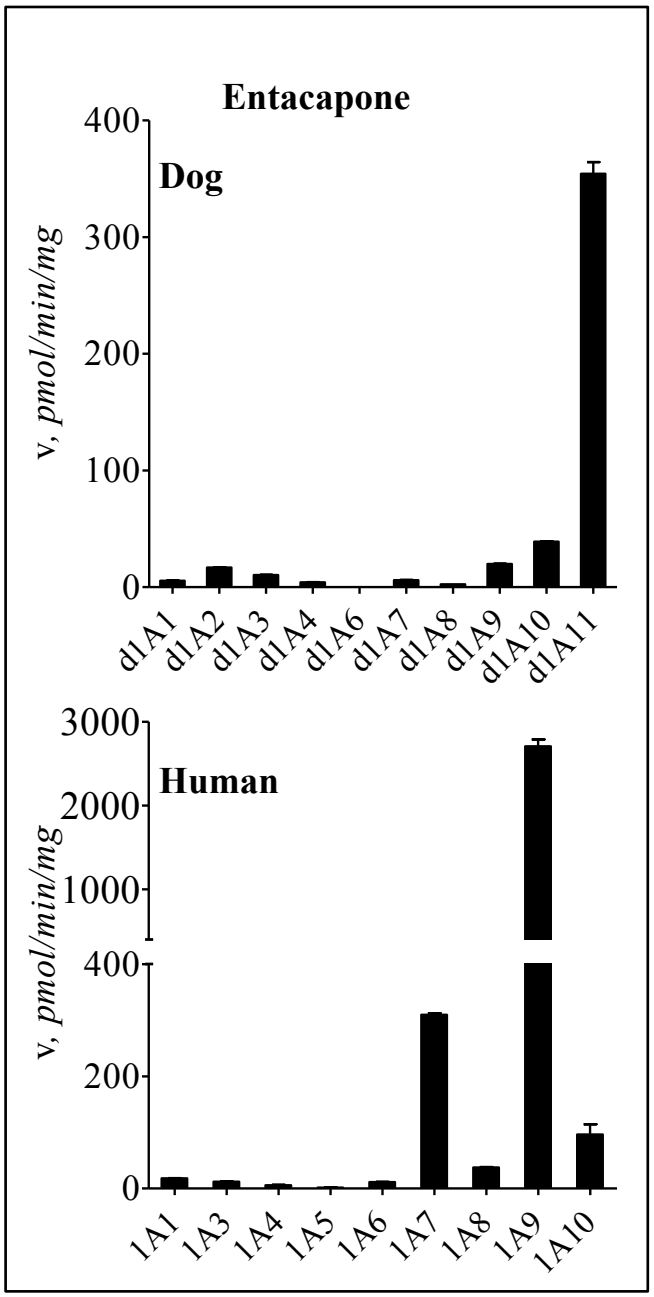
Entacapone
Dog
v, pmol/min/mg
400
300
200
100
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
3000
2000
1000
400
200
0
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 14

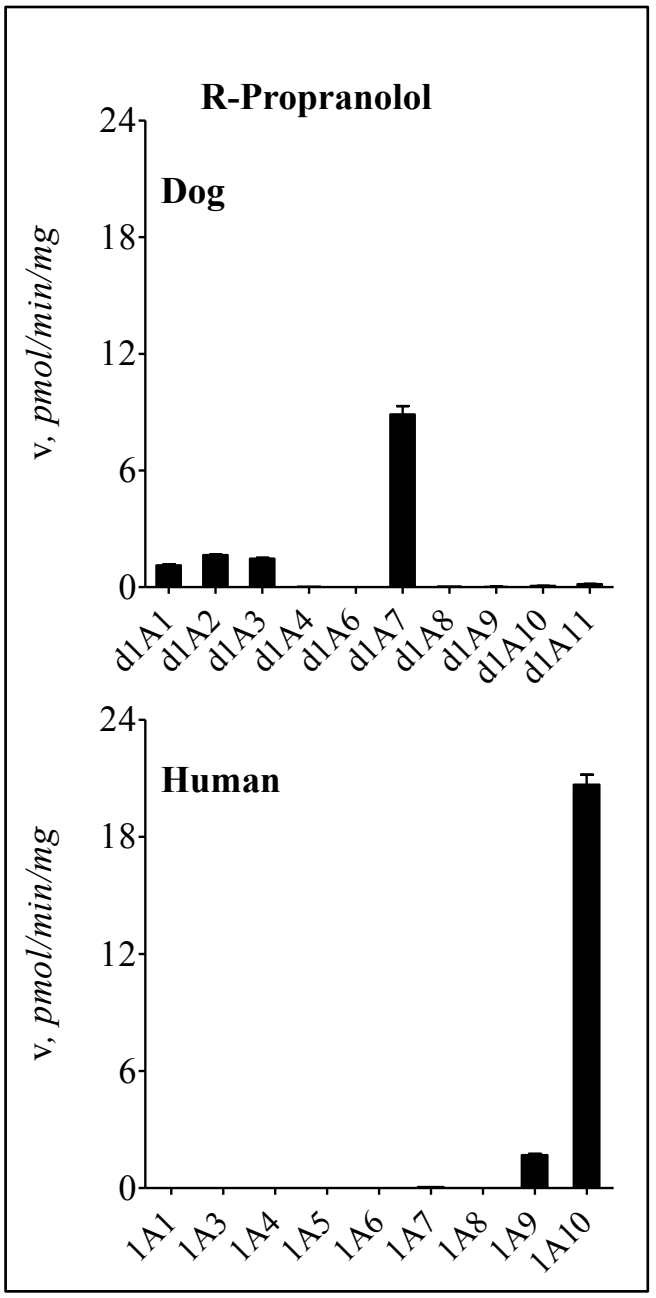
R-Propranolol
Dog
v, pmol/min/mg
24
18
12
6
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
24
18
12
6
0
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 15

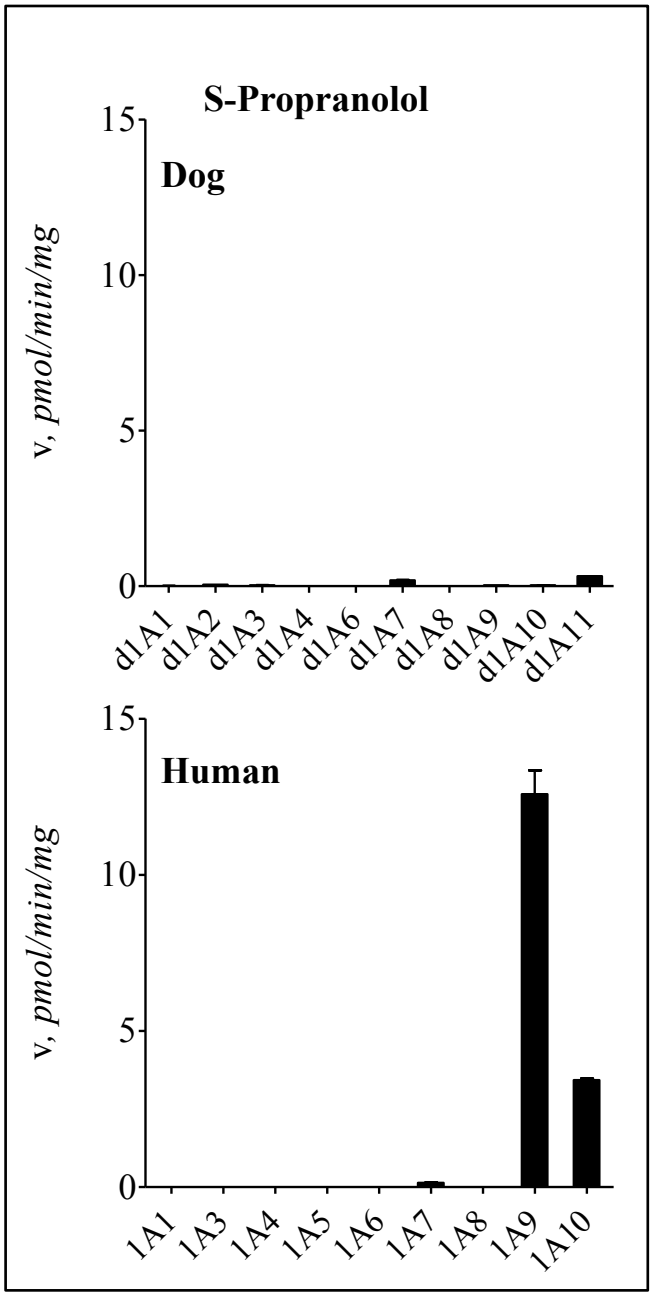
S-Propranolol
Dog
v, pmol/min/mg
15
10
5
0
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
v, pmol/min/mg
15
10
5
0
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10

Fig. 16

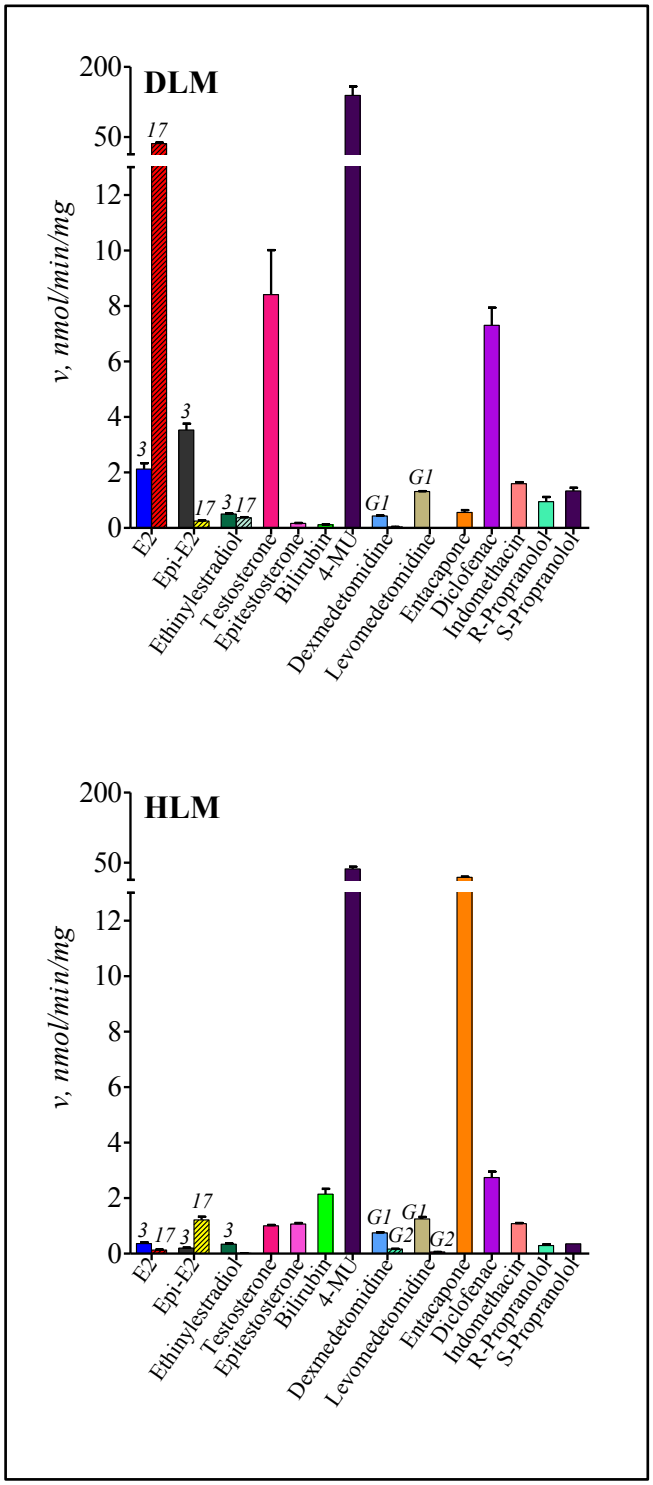
DLM
v, nmol/min/mg
200
50
12
10
8
6
4
2
0
17
3
3
17
3
17
G1
G1
E2
Epi-E2
Ethinylestradiol
Testosterone
Epitestosterone
Bilirubin
4-MU
Dexmedetomidine
Levomedetomidine
Entacapone
Diclofenac
Indomethacin
R-Propranolol
S-Propranolol
HLM
v, nmol/min/mg
200
50
12
10
8
6
4
2
0
3
17
3
17
3
G1
G2
G1
G2
E2
Epi-E2
Ethinylestradiol
Testosterone
Epitestosterone
Bilirubin
4-MU
Dexmedetomidine
Levomedetomidine
Entacapone
Diclofenac
Indomethacin
R-Propranolol
S-Propranolol

Fig. 17

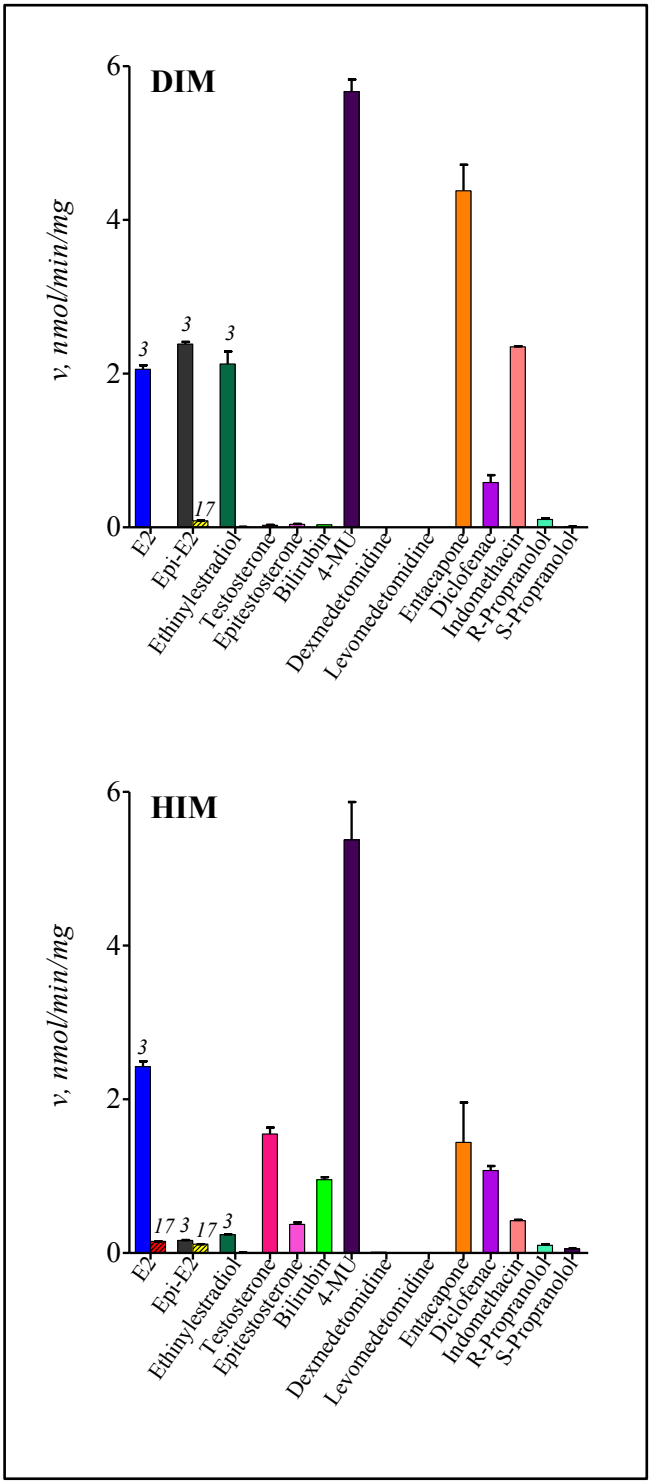
DIM
v, nmol/min/mg
6
4
2
0
3
3
17
3
E2
Epi-E2
Ethinylestradiol
Testosterone
Epitestosterone
Bilirubin
4-MU
Dexmedetomidine
Levomedetomidine
Entacapone
Diclofenac
Indomethacin
R-Propranolol
S-Propranolol
HIM
v, nmol/min/mg
6
4
2
0
3
17
3
17
3
E2
Epi-E2
Ethinylestradiol
Testosterone
Epitestosterone
Bilirubin
4-MU
Dexmedetomidine
Levomedetomidine
Entacapone
Diclofenac
Indomethacin
R-Propranolol
S-Propranolol

Fig. 18

The dog UGT enzymes of subfamily 1A; cloning, expression and activity

Johanna Troberg, Erkka Järvinen, Maria Muniz, Nina Sneitz, Johanna Mosorin, Marja Hagström, Moshe Finel

Supplementary text

Some of the incubation reactions, for example the entacapone glucuronidation measurements, were earlier terminated by the addition of 60 µl of cold 1:5 mixture of 4M perchloric acid and methanol. However, we have now noticed that when such samples stayed at room temperature for several hours, e.g. before injection to HPLC, a small peak appeared between the authentic glucuronide and the aglycone peaks (Fig. S1). LC-MS analyses of this fraction yielded a mass of deprotonated entacapone glucuronide plus 14 Da (m/z 494), while the major fragment in LC-MS/MS analysis of the same fraction was m/z 304, the mass of deprotonated entacapone. These results indicate that the additional 14 Da is probably due to methylation at the glucuronic acid moiety, not at the aglycone and the suggestion is in agreement with previous findings (Kuuranne et al. 2002). Similar additional peaks were also detected in assays with diclofenac, ethinylestradiol and epitestosterone.

An experiment was subsequently conducted in which the same incubation reaction was terminated either by the addition of 100 µl of ice-cold 5 % acetic acid in methanol, of 10 µl of 4 M perchloric acid without methanol, or by the addition of a ice-cold 1:5 mixture of 4M perchloric acid and methanol. Based on the results of this experiment, it became obvious that the additional peak appeared only when a mixture of methanol and perchloric acid, a strong acid, was used as a terminating reagent. It was also found out that the area of the additional peak increased the longer the samples were kept at room temperature, but the sum of the areas

of the correct glucuronide and the methylated remained constant, regardless the increase in the area of the methylated glucuronide peak (Fig. S1). In this case, glucuronidation of entacapone by dUGT1a11, the areas of entacapone glucuronide and methylated entacapone glucuronide, after 2 hours at room temperature post reaction stoppage, were 84 and 2, respectively, whereas after 20 hours at room temperature the areas of the same peaks were 76 and 9, respectively. The same phenomenon, no change in the combined area of the glucuronide and methylated glucuronide, was observed for the other substrates listed above as well. Due to this, we used the combined area to calculate glucuronidation rates and found no need to repeat the experiment using another termination method. Nevertheless, to avoid unnecessary complications in the future, we later tried to avoid the combination of a strong acid and methanol. Instead, we terminated the incubations either with a strong acid (4M perchloric acid) alone, or methanol with a weak acid, such as acetic acid.

Reference:

Kuuranne T, Aitio O, Vahermo M, Elovaara E, and Kostiainen R. (2002) Enzyme-assisted synthesis and structure characterization of glucuronide conjugates of methyltestosterone (17a-methylandrost-4-en-17ß-ol-3-one) and nandrolone (estr-4-en-17ß-ol-3-one) metabolites. Bioconjug Chem 13:194-199.

Legend to Fig. S1. The effect of time at room temperature on the appearance of the methylated entacapone glucuronide peak following entacapone glucuronidation reaction termination by the addition of a mixture of strong acid and methanol (a 1:5 mixture of cold 4M PCA and methanol). Both samples were from the same incubation reaction that was terminated as described above and subjected to centrifugation as described in the Methods section. Sample A

was subjected to HPLC following 2 hours at room temperature, whereas Sample B was 20 hours at room temperature before injecting to the column.

Legend to Fig. S2. Testosterone (A) and epitestosterone (B) glucuronidation rates by dog UGT1As (upper panels) and human UGT1As (lower panels). See legend to Fig. 3 in the main text for further details.

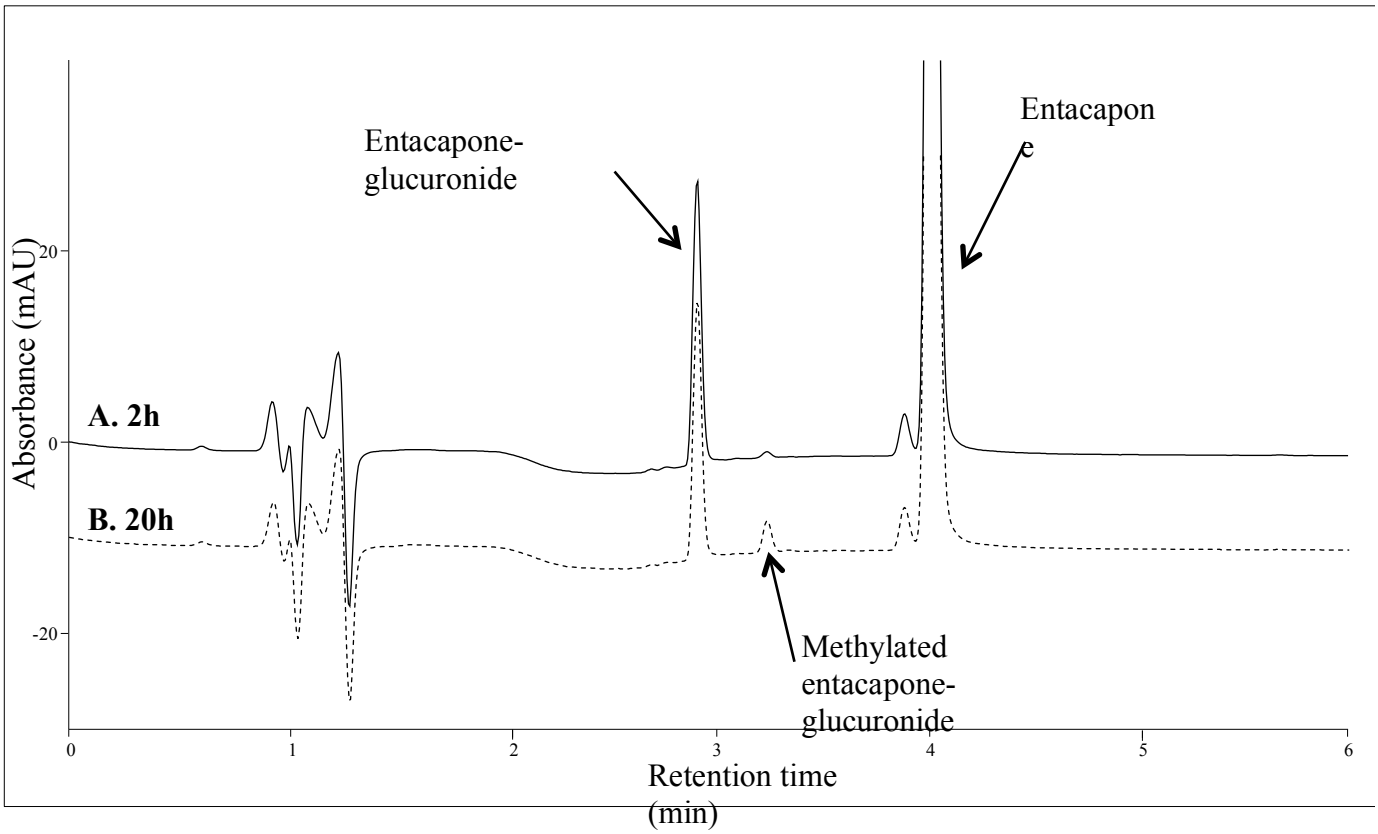

Fig. S1

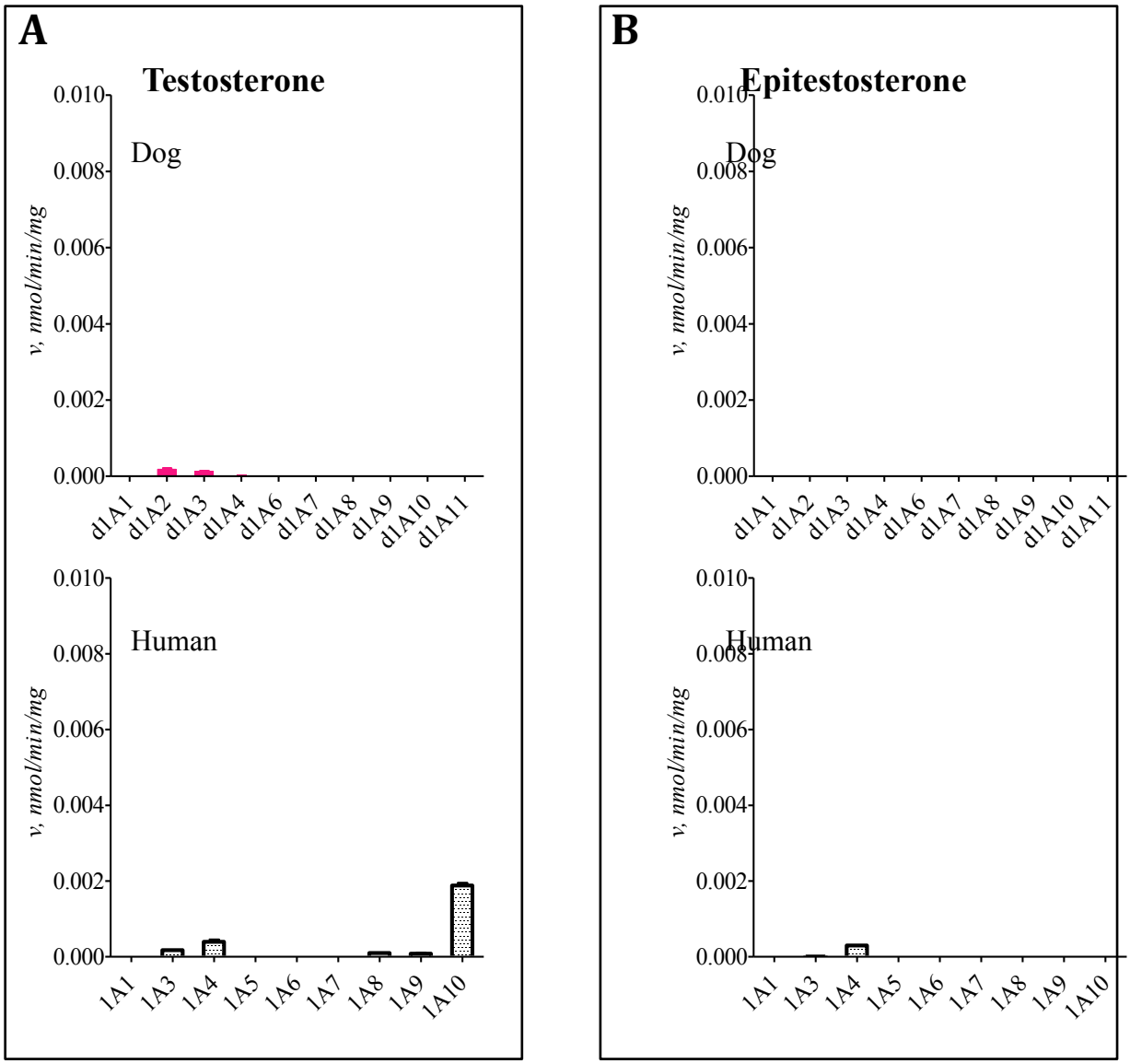
A
Testosterone
Dog
v, nmol/min/mg
0.010
0.008
0.006
0.004
0.002
0.000
d1A1
d1A2
d1A3
d1A4
d1A6
d1A7
d1A8
d1A9
d1A10
d1A11
Human
1A1
1A3
1A4
1A5
1A6
1A7
1A8
1A9
1A10
B
Epitestosterone
Dog
Human

Fig. S2