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Cloning and characterisation of the first drug-metabolising canine UDP-glucuronosyltransferase of the 2B subfamily

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Abstract

Glucuronidation is a major route of clearance for a diverse set of both drug and endogenous substrates. The present study was undertaken to redress the lack of molecular information currently available on drug glucuronidation by the dog, a species widely used in metabolism studies by the pharmaceutical industry. A novel dog uridine diphosphate glucuronosyltransferase (UGT), designated UGT2B31 (GenBank Accession Number: AY135176), has been isolated from a dog cDNA library, expressed in V79 cells and characterised using various methods: (i) UGT2B31 sequence has been compared with mammalian UGT sequences using both sequence alignments and phylogenetic analysis; and (ii) the substrate specificity of UGT2B31 has been determined using functional analysis and compared with that obtained using UGT2B7 and dog liver microsomes. The following results were obtained: (i) sequence alignments between UGT2B31 and UGT2B15 gave the greatest degree of identity (76%); however, human UGT2B4, human UGT2B7, monkey UGT2B9 (all 75%), and rat UGT2B1 (73%) also gave a high degree of identity; (ii) phylogenetic analysis determined UGT2B31 to be most closely related to rat UGT2B1; (iii) UGT2B31 displayed a substrate specificity similar to human UGT2B7 and rat UGT2B1, catalysing the glucuronidation of phenols, opioids, and carboxylic acid-containing drugs; and (iv) UGT2B31 only formed morphine-3-glucuronide; however, kinetic analysis determined the K_m of this reaction to be similar to that observed with UGT2B7 (both approximately 1300 μM). The results suggest that UGT2B31 plays a crucial role in drug detoxification by the dog and may be the canine equivalent of human UGT2B7.

Keywords: UDP-glucuronosyltransferase; Dog UGT2B31; UGT activity; Rat UGT2B1; UGT2B7; Morphine glucuronidation

1. Introduction

UGTs are a super family of membrane-bound enzymes that catalyse the addition of a glucuronic acid moiety to a range of substrates resulting in the formation of a glucuronide that is more hydrophilic than the parent compound and hence more readily excreted in either urine or bile [1]. Glucuronidation is the primary route of metabolism for a diverse set of endogenous compounds and xenobiotics [2]. In fact, the role of glucuronidation as a clearance mechanism may well increase with the preselection of compounds stable to oxidative metabolism [3].

To date, fifteen functional human UGTs have been identified, and the majority of these have been expressed in recombinant cell lines [4]. Perhaps the most important isoform with regard to hepatic drug glucuronidation is UGT2B7. UGT2B7 catalyses the glucuronidation of several different classes of compounds ranging from opioid substrates (morphine, codeine, and naloxone [5], NSAIDs such as naproxen, ibuprofen, and ketoprofen [6], and carboxylic acid-containing drugs such as valproate [6]) to nucleoside analogues such as 3′-azido-3′-deoxythimidine [7]. Although human recombinant UGT cell lines can

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Abbreviations: DLM, dog liver microsomes; NSAIDs, nonsteriodal anti-inflammatory drugs; PCR, polymerase chain reaction; UDPGA, uridine diphosphate glucuronic acid; UGT, uridine diphosphate glucuronsyltransferase.

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be invaluable in reaction phenotyping drugs cleared primarily by glucuronidation [8], this process often occurs during the later stages of preclinical development.

Pharmaceutical companies often use animal species such as the rat, dog, or monkey in studies aimed at evaluating the pharmacodynamics, metabolism, pharmacokinetics, and safety of new chemical entities. Therefore, it is crucial that any potential inter-species differences in drug glucuronidation are well characterised. Recent studies have revealed quantitative and qualitative differences in drug glucuronidation between rats, dogs, and humans using hepatic microsomes [9,10]. However, the specific UGT isoforms that catalyse drug glucuronidation by the dog, in particular, remain relatively unknown [11].

The aim of this work was to isolate dog UGTs involved in drug glucuronidation from a dog liver cDNA library. In this study, dog UGT2B31 (GenBank Accession Number: AY135176) has been isolated and its role in canine detoxification determined using a variety of methods. The sequence of UGT2B31 has been compared with mammalian UGT sequences using both sequence alignments and phylogenetic analysis. UGT2B31 was then stably expressed in V79 cells, and its substrate specificity towards a range of endobiotics and drugs was examined. Catalytic activity was also compared to human UGT2B7, rat UGT2B1, and DLM.

2. Materials and methods

2.1. Materials

[α-^{32}P]dCTP (specific activity 1000 Ci/mmol) and [^{14}C]UDPGA (specific activity 293.6 mCi/mmol, 99.7% purity) were obtained from DuPont NEN. *Taq* and *pfu* DNA polymerase and restriction enzymes were purchased from Promega. Tissue-culture media and supplements, including G418 (geneticin), were obtained from Gibco-BRL Life Technologies. Aglycone substrates for glucuronidation assays were obtained from either Sigma-Aldrich or BDH and were of the highest grade available. V79 cells expressing human UGT2B7 were obtained from D. Smith.

2.2. cDNA cloning of a canine UGT

A canine liver cDNA library was provided by Dr. Steve Hood (then University of Surrey, now Glaxo-Welcome). PCR primers were chosen from the variable region (exon 1) of human UGT2B7 and were used to create a probe to screen the canine liver cDNA library for potential dog UGTs from the 2B subfamily. The sequences of the forward and reverse primers used were: 5′-GCTTATTCAGAGAGGTCATGAGG-3′ and 5′-CAGATAACGTAGTGGGTCTTCC-3′, respectively. PCR was performed at 94° for 1 min, at 52° for 1 min, and at 72° for 1.5 min for a total of 40 cycles using *Taq* DNA polymerase. The resulting 562 bp PCR product (representing the 5′ end of UGT2B7 between nucleotides 158 and 720) was purified using a spin column (CloneTech), subjected to DNA sequencing, and subsequently labelled with [α-^{32}P]dCTP using the random-primer procedure [12]. This ^{32}P-labelled cDNA probe was used to screen the dog cDNA library by plaque hybridisation. The phages were grown in XL1-Blue host cells and transferred from plates onto nitrocellulose filters (Hybond N$^+$, Amersham). Hybridisation of the filters was performed at 52° in hybridisation buffer containing 6× SSC (where 1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0), 5× Denhardt's reagent, 0.1% SDS, and the ^{32}P-labelled cDNA probe for 12 hr followed by a single washing step with 2× SSC/0.5% SDS at 52° for 5 min. Positive clones were selected, purified by secondary and tertiary screening, and finally amplified by PCR. The PCR products produced were purified using a spin column, subcloned into the pGEM®-T Easy vector (Promega), and subjected to DNA sequencing. Sequencing identified one particular clone of 2 kb (UGT2B31) as a full-length canine UGT from the 2B subfamily.

2.3. DNA sequencing

An ABI 377 sequencer (Perkin-Elmer ABI) was used in combination with a Big Dye Terminator Cycle Sequencing Kit (ABI). Sequencing of potential canine UGTs was performed in both directions in duplicate using double-stranded cDNA as a template.

2.4. Stable expression of UGT2B31 cDNA in mammalian cells

Chinese hamster lung fibroblast V79 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% Nu serum and 100 units/mL of penicillin/streptomycin. The cDNA insert of the isolated full-length dog clone was excised from the pGEM®-T Easy vector using *Not*I and was cloned into the *Not*I site of the mammalian expression vector pCI-neo (Promega). The correct orientation and sequence of the cDNA in the expression vector were assessed by restriction analysis and DNA sequencing of the cloning site. V79 cells were transfected with the pCI-neo cDNA construct using Lipofectin reagent (Gibco-BRL) according to the instructions of the manufacturer. After 48 hr, the cells were split, and stable transfections were selected using medium containing 1 mg/mL of G418. Resistant cells were screened at the 6-well stage using an *ex vivo* method. Cells were incubated for 24 hr with 20 μM androstanediol and 50 μM hyodeoxycholic acid. Then 0.5 mL of the dosed medium was mixed with 0.5 mL of ice-cold acetonitrile and centrifuged at 14,000 *g* for 10 min at room temperature; 90 μL of the resultant supernatant was analysed using mass spectrometry (see below).

2.5. Microsomal preparations from human and animal tissues

Human, dog, rat, and marmoset tissues were obtained and stored as stated previously [9,10]. All human and animal microsomes were prepared using a differential centrifugation method adapted from that of Coughtrie *et al.* [13]. Briefly, the liver was minced and homogenised in 4 vol. of ice-cold 0.25 M sucrose, 5 mM HEPES, pH 7.4. The resultant homogenates were then centrifuged at 10,000 *g* for 15 min at 4°, and the supernatants were centrifuged at 100,000 *g* for 60 min at 4°. Microsomal pellets were resuspended in 1 vol. (mL) of ice-cold 0.25 M sucrose, 5 mM HEPES, pH 7.4, equal to the wet weight (in grams) of tissue. Protein in microsomal samples was determined by the method of Lowry *et al.* [14], using bovine serum albumin as a standard. Aliquots were stored at −80°.

2.6. Gradient HPLC assay of UGTs using [^{14}C]UDPGA and radiochemical detection

V79 cells transfected with the pCI-neo/UGT2B31 construct were harvested after rinsing with ice-cold phosphate-buffered saline (pH 7.4), centrifuged (750 *g* for 2 min at 4°), resuspended in phosphate-buffered saline followed by a second centrifugation (2000 *g* for 5 min at 4°), and finally stored as cell pellets at −80°. Each frozen cell pellet was resuspended in approximately 200 μL of phosphate-buffered saline followed by sonication for 4 × 5 s bursts (Microson Ultrasonic Cell Disruptor, Heat Technologies), allowing at least 1 min on ice between bursts.

UGT assays were performed as described previously [15]. Briefly 100 mM Tris/maleate buffer, pH 7.4, containing 5 mM $MgCl_2$, 10 mM saccharic acid 1,4-lactone, typically 500 μM substrate, 50–300 μg cellular sonicate, and 2 mM UDPGA (0.1 μCi [^{14}C]UDPGA/assay) were combined in a total volume of 100 μL. Assays were pre-warmed at 37° for 5 min and started with the addition of [^{14}C]UDPGA. Reactions containing cell homogenates of recombinant UGT2B31 and human UGT2B7 were incubated at 37° for 4 h (incubations using DLM were incubated for 45 min) and then terminated by the addition of 100 μL of methanol that had been pre-chilled to −20°. The mixture was centrifuged at 14,000 *g* for 10 min at room temperature. The resulting supernatant was then transferred to an HPLC vial, and 150 μL of this volume was injected directly onto the HPLC column. Radioactive UDPGA and glucuronide were detected using Reeve 9701 radioactivity monitors fitted with a 200 μL solid scintillant flowcell packed with silanised cerium activated lithium glass. Preliminary studies (data not shown) determined that glucuronidation using UGT2B31 was linear with respect to time for up to at least 4 hr and with respect to protein up to 500 μg/assay.

The biosyntheses of androstanediol, gemfibrozil, hyodeoxychoic acid, ketoprofen, 1-naphthol, and naproxen glucuronides were performed using duplicate UGT assays in the presence of [^{14}C]UDPGA and unlabeled UDPGA [15]. Assays utilising [^{14}C]UDPGA were performed to quantify the glucuronide in an assay for each particular substrate. Assays containing unlabeled UDPGA were performed in tandem under identical conditions and were used subsequently as calibration standards to quantify assays analysed by mass spectrometry.

2.7. Analysis of glucuronide formation by mass spectrometry

Mass spectrometry was conducted using a Micromass Quattro LC triple quadrapole mass spectrometer with an HP1100 HPLC system for separation. All mass spectrometry methods used electrospray ionisation. Potential positive UGT2B31 transfectants were analysed using multiple reaction monitoring, and conditions were optimised for each glucuronide. By using the negative ion mode, androstanediol glucuronide was detected monitoring the transition 467.4 > 113 using a cone voltage of 30 V and a collision energy of 30 eV. Using the positive ion mode, hyodeoxycholic acid glucuronide was detected monitoring the transition 596.4 > 357.2 using a cone voltage of 30 V and a collision energy of 30 eV.

Glucuronides formed using UGT2B31 and UGT2B7 were detected by single reaction monitoring. The negative ion mode was used in the analysis of gemfibrozil glucuronide (*m/z* 425), ketoprofen glucuronide (*m/z* 429), morphine-3- and –6-glucuronides (*m/z* 460), 1-naphthol glucuronide (*m/z* 319), and naproxen glucuronide (*m/z* 405).

Chromatographic separation was obtained using a Spherisorb 5ODS2 column (2.1 × 150 mm) obtained from Waters. The mobile phase consisted of water containing 0.1% (v/v) formic acid, with the organic phase being acetonitrile containing 0.1% (v/v) formic acid. All chromatography was performed using a generic method ($t = 0$ min % organic = 0, $t = 6$ min % organic = 100, $t = 6.1$ min % organic = 0, total runtime = 9 min). The flow rate was set at 0.3 mL/min.

3. Results

A cDNA clone, designated UGT2B31, was isolated from a canine liver cDNA library using a probe created from the variable region of human UGT2B7. Sequence analysis determined that UGT2B31 has an open reading frame of 1590 bp, which encodes a protein of 530 amino acids. UGT2B31 contains both a potential UDP binding domain between amino acids 372 and 400 and a dilysine motif at positions −3 and −5 from the C-terminal end. Fig. 1 shows a sequence alignment between dog UGT2B31 and the major mammalian UGTs of the UGT2B subfamily. Dog UGT2B31 and human UGT2B15 displayed the greatest identity at the amino acid level (76%); however, a high

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human2B7        MSVKWTSVILLIQLSFCFSSGNCGKVLVWAAEYSHWMNIKTILDELIQRGHEVTVLASSA 60
monkey2B9       MSVKWTSVILLIQLSFYFSSGSCGKVLVWAAEYSHWMNMKTILEELVQRGHEVTVLASSA 60
human2B4        MSMKWTSALLLIQLSCYFSSGSCGKVLVWPTEFSHWMNIKTILDELVQRGHEVTVLASSA 60
human2B15       MSLKWTSVFLLIQLSCYFSSGSCGKVLVWPTEYSHWINMKTILEELVQRGHEVTVLTSSA 60
dog2B31         MSMKWISVLLGLQLSCYFSSGSCGKVLVWPTEYSHWINVKTILDELVQRGHEVTVLTSSA 60
rat2B1          MSMKQTSVFLLIQLICYFRPGACGKVLVWPTEYSHWINIKIILNELAQRGHEVTVLVSSA 60
                ** *  *  *  **   *  * *******  * *** * * ** ** ********* ***

human2B7        SILFDPNNSSALKIEIYPTSLTKTELENFIMQQIKRWSD-LPKDTFWLYFSQVQEIMSIF 119
monkey2B9       SILFDPNNSSALKIEVFPTSLTKTEFENISMQEVKRWIE-LPKDTFWLYFSQMQEIMWRF 119
human2B4        SISFDPNSPSTLKFEVYPVSLTKTEFEDIIKQLVKRWAE-LPKDTFWSYFSQVQEIMWTF 119
human2B15       STLVNASKSSAIKLEVYPTSLTKNDLEDSLLKILDRWIYGVSKNTFWSYFSQLQELCWEY 120
dog2B31         SILVDPNKLSAIKFEIYSAHLSRGDFEAFFIKLLNILIYDMPKDSFWTYFSLMQEFFWEF 120
rat2B1          SILIEPTKESSINFEIYSVPLSKSDLEYSFAKWIDEWTRDFETLSIWTYYSKMQKVFNEY 120
                *        *    *     *     *                      * * *  *

human2B7        GDITRKFCKDVVSNKKFMKKVQESRFDVIFADAIFPCSELLAELFNIPFVYSLSFSPGYT 179
monkey2B9       GDIIRNFCKDVVSNKKLMKKLQESRFDVVFADPIFPCSELLAELFNIPLVYSLRFTPGYI 179
human2B4        NDILRKFCKDIVSNKKLMKKLQESRFDVVLADAVFPFGELLAELLKIPFVYRPRFSPGYA 179
human2B15       YDYSNKLCKDAVLNKKLMMKLQESKFDVILADALNPCGELLAELFNIPFLYSLRFSVGYT 180
dog2B31         YECAQKLCKDVVLNKKLMTKLQESKFDLVLADTIIPCGELLAELLKIPLVYSLRFSPGYA 180
rat2B1          SDVVENLCKALIWNKSLMKKLQGSQFDVILADAVGPCGELLAELLKTPLVYSLRFCPGYR 180
                       **    **  * * * * **   **   *  ******   *  *   *  **

human2B7        FEKHSGGFIFPPSYVPVVMSELTDQMTFMERVKNMIYVLYFDFWFEIFDMKKWDQFYSEV 239
monkey2B9       FEKHCGGFLFPPSYVPVVMSELSDQMTFMERVKNMIYMLSFDFYFQMYDMKKWDQFYSEV 239
human2B4        IEKHSGGLLFPPSYVPVVMSELSDQMTFIERVKNMIYVLYFEFWFQIFDMKKWDQFYSEV 239
human2B15       FEKNGGGFLFPPSYVPVVMSELSDQMIFMERIKNMIHMLYFDFWFQIYDLKKWDQFYSEV 240
dog2B31         FEKHSGGLPLPPSYVPVILSELTDQMTFMERVKNMLYVLYFDFWFQTINEKSWDQFYSEV 240
rat2B1          CEKFSGGLPLPPSYVPVVLSELSDRMTFVERVKNMLQMLYFDFWFQPFKEKSWSQFYSDV 240
                 **  **   *******  *** * * * ** ***   * * * *     * * **** *

human2B7        LGRPTTLSETMGKADVWLIRNSWNFQFPHPLLPNVDFVGGLHCKPAKPLPKEMEDFVQSS 299
monkey2B9       LGRPTTLSETMGKADIWLIRNSWNFQFPHPLLPNVDFVGGLHCKPAKPLPKEMEEFVQSS 299
human2B4        LGRPTTLSETMAKADIWLIRNYWDFQFPHPLLPNVEFVGGLHCKPAKPLPKEMEEFVQSS 299
human2B15       LGRPTTLFETMGKAEMWLIRTYWDFEFPRPFLPNVDFVGGLHCKPAKPLPKEMEEFVQSS 300
dog2B31         LGRPTTLYELMRKADIWLIRTYWDFEYPHPLLPHFDFVGGLHCKPAKSLPTEMEEFVQSS 300
rat2B1          LGRPTTLTEMMGKADIWLIRTFWDLEFPHPFLPNFDFVGGLHCKPAKPLPREMEEFVQSS 300
                ******* * * **  ****  *    * * **   *********** ** *** *****

human2B7        GENGVVVFSLGSMVSNMTEERANVIASALAQIPQKVLWRFDGNKPDTLGLNTRLYKWIPQ 359
monkey2B9       GENGVVVFSLGSMVTNMEEERANVIASALAQIPQKVLWRFDGKKPDTLGLNTRLYKWIPQ 359
human2B4        GENGVVVFSLGSMVSNTSEERANVIASALAKIPQKVLWRFDGNKPDTLGLNTRLYKWIPQ 359
human2B15       GENGIVVFSLGSMISNMSEESANMIASALAQIPQKVLWRFDGKKPNTLGSNTRLYKWLPQ 360
dog2B31         GENGIVVFSLGSMVNNMTEERANVIASALAQIPQKVLWRFDGKKPDTLGPNTRLYKWLPQ 360
rat2B1          GEHGVVVFSLGSMVKNLTEEKANVVASALAQIPQKVVWRFDGKKPDTLGSNTRLYKWIPQ 360
                ** * ********  *  ** **  ***** ***** ***** ** *** ******* **

human2B7        NDLLGHPKTRAFITHGGANGIYEAIYHGIPMVGIPLFADQPDNIAHMKARGAAVRVDFNT 419
monkey2B9       NDLLGHPKTRAFITHGGANGIYEAIYHGVPMVGIPLFADQPDNIAHMKTKGAAVRLDFDT 419
human2B4        NDLLGHPKTRAFITHGGANGIYKAISPRIPMVGVPLFADQPDNIAHMKAKGAAVSLDFHT 419
human2B15       NDLLGHPKTKAFITHGGTNGIYEAIYHGIPMVGIPLFADQHDNIAHMKAKGAALSVDIRT 420
dog2B31         NDLLGHPKTKAFITHGGTNGIYEAIYHGIPMVGIPLFADQADNIVHMKAKGAAIRLDFST 420
rat2B1          NDLLGHPKTKAFVAHGGTNGIYEAIYHGIPIVGIPLFADQPDNINHMVAKGAAVRVDFSI 420
                ********* **  *** **** **    * ** ****** *** **   ***   *

human2B7        MSSTDLLNALKRVINDPSYKENVMKLSRIQHDQPVKPLDRAVFWIEFVMRHKGAKHLRVA 479
monkey2B9       MSSTDLANRLKTVINDPLYKENVMKLSRIQHDQPVKPLDRAVFWIEFVMRHKGAKHLRPA 479
human2B4        MSSTDLLNALKTVINDPLYKENAMKLSRIHHDQPVKPLDRAVFWIEFVMRHKGAKHLRVA 479
human2B15       MSSRDLLNALKSVINDPVYKENVMKLSRIHHDQPMKPLDRAVFWIEFVMRHKGAKHLRVA 480
dog2B31         MSSADLLNALRMVINDPSYKENAMKLSGIHHDQPIKPLDRAVFWIEYVMRHQGAKHLRPA 480
rat2B1          LSTTGLLTALKIVMNDPSYKENAMRLSRIHHDQPVKPLDRAVFWIEYVMRHKGAKHLRST 480
                 *   *   *  * *** **** * ** * **** *********** **** ******

human2B7        AHDLTWFQYHSLDVIGFLLVCVATVIFIVTKCCLFCFWKFARKAKKGKND 529
monkey2B9       AHDLTWFQYHSLDVIGFLLACVATVIFVIMKCCLFCFWKFARKGKKGKSD 529
human2B4        AHDLTWFQYHSLDVTGFLLACVATVIFIITK-CLFCVWKFVRTGKKGKRD 528
human2B15       AHNLTWIQYHSLDVIAFLLACVATVIFIITKFCLFCFRKLAKTGKKKKRD 530
dog2B31         SHDLTWFQYHSLDVIGFLLACVATAIFVTTQCCLFCCRKVAKTGKKIKKE 530
rat2B1          LHDLSWFQYHSLDVIGFLLLCVVGVVFIITKFCLFCCRKTANMGKKKKE- 529
                 * * * *******  *** **    *     ****  *     ** *
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Fig. 1. Sequence analysis of UGT2B31 with UGTs of the UGT2B subfamily. Key: (∗) conserved residue.

degree of identity was also observed between UGT2B31 and human UGT2B4, human UGT2B7, monkey UGT2B9 (all 75%), and rat UGT2B1 (73%). A phylogenetic analysis of the common mammalian UGTs for the UGT2B subfamily is shown in Fig. 2. However, dog UGT2B31 did not cluster with rat UGT2B1, UGT2B7, or monkey UGT2B9. Since recent species-specific gene duplications in the UGT2B subfamily make it impossible to make orthologue assignments based on sequence [4], the substrate specificity of dog UGT2B31 should aid in the determination of its role in detoxification.

The UGT activity of dog UGT2B31 was assessed using cell homogenates from a stably expressing cell line with over twenty aglycone substrates and ^{14}C-labelled UDPGA. Glucuronidation studies using DLM and cell homogenates from a stable cell line expressing human UGT2B7 were also performed as a comparison (see Table 1). UGT2B31 catalysed the glucuronidation of a range of compound classes including aliphatic alcohols, phenols, opioids, NSAIDs, and steroids. However, while UGT2B31, UGT2B7, and UGT2B1 glucuronidated the majority of the phenolic compounds studied, there were differences in

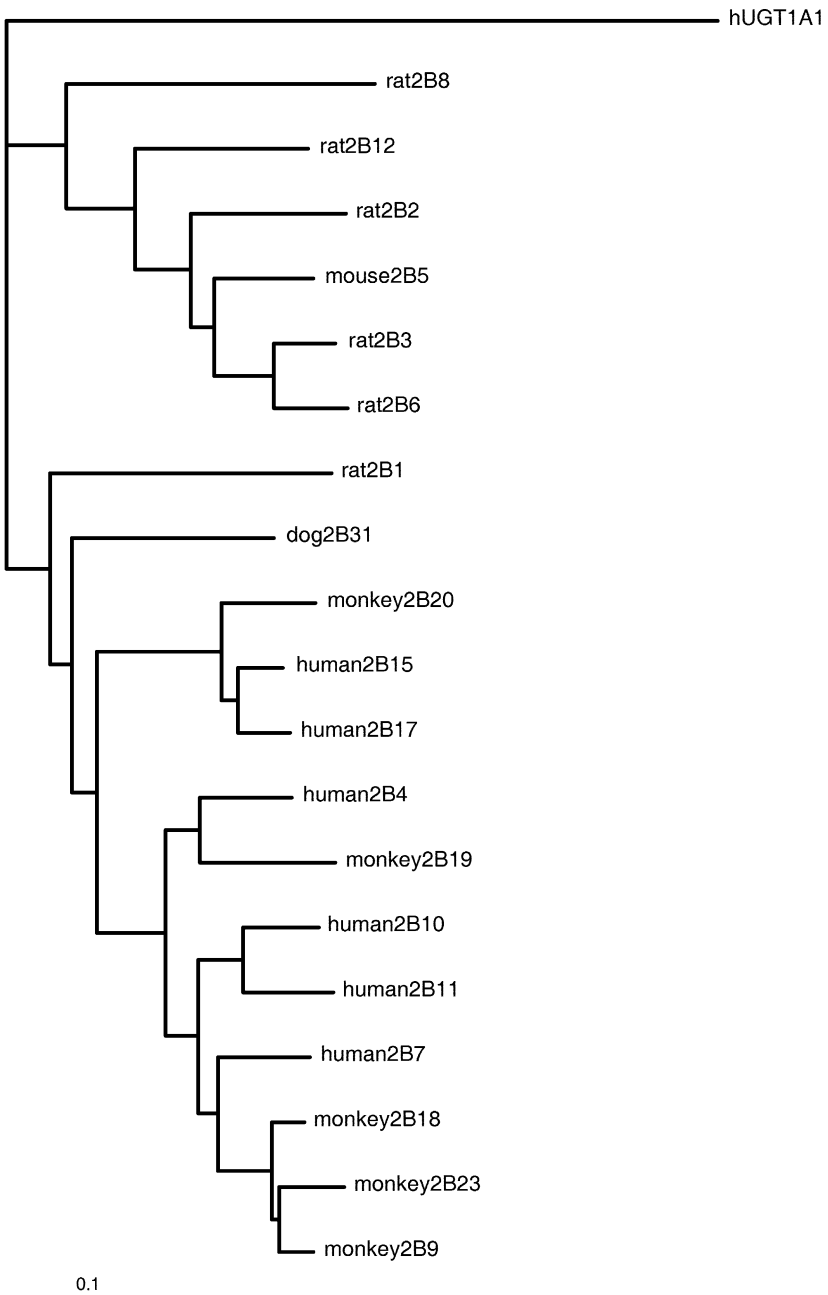

Fig. 2. Phylogenetic analysis of mammalian UGTs from the 2B subfamily. Amino acid sequences were aligned and then used to generate a distance-based phylogram with ClustalX. The tree was rooted with the distantly related human UGT1A1. UGT sequences were obtained from the UGT homepage (http://www.unisa.edu.au/pharm_medsci/gluc_trans/).

Table 1
Glucuronidation of structurally diverse compounds by dog liver microsomes (DLM), V79-UGT2B31 homogenates, V79-UGT2B7 homogenates, or V79-UGT2B1 homogenates

Compound	Activity (nmol/hr/mg)			
	DLM	UGT2B31	UGT2B1	UGT2B7
Aliphatic alcohols				
Lorazepam	180	3.6	<0.6	ND
Bile acids				
Hyodeoxycholic acid	73	ND	ND	74.4
Lithocholic acid	13	ND	0.18	ND
Carboxylic acids				
Diclofenac	87	ND		17
Furosemide	11	ND	ND	ND
Gemfibrozil	250	4		16
Ibuprofen	73	ND	8.4	6.3
Ketoprofen	48	0.54	9.6	3.6
Naproxen	38	0.66	4.2	7.7
Valproic acid	50	ND	8.4	6.1
Complex phenols				
4-Methylumbelliferone	1800	4.5	72	12.9
Opioids				
Codeine	59	ND	ND	0.5
Hydromorphone	330	ND		3.5
Morphine 3-glucuronide	580	1.7	700	3.5
Morphine 6-glucuronide		ND	ND	
Simple phenols				
4-Ethylphenol	130	4.2	23	12
Eugenol	2500	4.9		62
1-Naphthol	1500	4.8	1.8	14
4-Nitrophenol	210	1.9	9	24
Propofol	ND	ND		ND
Steroids				
Androstanediol	200	2.2		13
Ethinylestradiol	32	ND		ND
Testosterone	120	ND	0.6	ND

Substrates (0.5 mM) were incubated with DLM or cell homogenates of V79 cells transfected with either UGT2B31 or UGT2B7 in the presence of [^{14}C]UDPGA (2 mM) for 4 hr (45 min for DLM incubations). Glucuronide formation was detected by HPLC. Each value is the mean of at least three incubations. ND is below the level of detection (0.48 nmol/hr/mg). Activity values obtained with UGT2B1 were taken from Refs. [16,17]. Activities obtained with DLM were taken from Ref. [9].

substrate specificity for the other compound classes examined (see Table 1). For example, UGT2B31 catalysed the glucuronidation of gemfibrozil, ketoprofen, and naproxen, but other carboxylic acid-containing drugs such as ibuprofen and valproic acid (which were substrates for UGT2B7 and UGT2B1) were not glucuronidated. Differences were also observed in opioid glucuronidation. UGT2B7 catalysed the glucuronidation of all opioid substrates studied; however, UGT2B31 and UGT2B1 were only capable of forming morphine-3-glucuronide. Investigation into morphine glucuronidation using hepatic microsomes prepared from rats, dogs, monkeys, and humans is shown in Fig. 3. Analysis using a sensitive mass spectrometric method allowed the detection of morphine-6-glucuronide formation in all four species studied. However, the ratio of morphine-3-glucuronide:morphine-6-glucuronide was much greater using microsomes prepared from the animal species compared to those with human hepatic microsomes (see Fig. 3).

To further investigate the substrate specificity of dog UGT2B31, the kinetic parameters V_{max} and K_m were determined for six substrates using homogenates of both UGT2B31 and UGT2B7 (see Table 2). The maximal rate of glucuronidation for all of the substrates studied was greater using UGT2B7 than UGT2B31; however, this could be due in part to a different expression level between the two cell lines. Interestingly, the affinities of UGT2B31 and UGT2B7 for androstanediol, morphine, and 1-naphthol (as assessed by K_m) were similar. By contrast, the affinity of UGT2B7 towards the NSAIDs, gemfibrozil and ketoprofen was up to 7-fold greater than that observed using UGT2B31.

4. Discussion

The pharmaceutical industry often uses the dog to evaluate the metabolic fate of new chemical entities during early drug development. Although recent microsomal studies have highlighted potential inter-species differences in drug glucuronidation between humans and dogs [9], the specific canine UGTs involved remain to be identified [11]. The present study was undertaken to redress the lack of molecular information currently available on drug glucuronidation by the dog. A canine UGT, designated UGT2B31, has been isolated, stably expressed in V79 cells, and characterised against a range of drug substrates. The high degree

Table 2
Kinetic parameters for the glucuronidation of six compounds by V79-UGT2B31 and V79-UGT2B7 homogenates

Compound	UGT2B31			UGT2B7		
	V_{max} (pmol/min/mg)	K_m (μM)	CL_{int} (μL/min/mg)	V_{max} (pmol/min/mg)	K_m (μM)	CL_{int} (μL/min/mg)
Androstanediol	75, 53	14, 12	4.4, 5.4	148, 144	20, 15	9.9, 7.1
Gemfibrozil	112 ± 59	139 ± 27	0.9 ± 0.6	174, 262	23,16	7.6, 17
Ketoprofen	8.2, 5.4	1640, 1750	0.005, 0.003	68, 77	480, 480	0.1, 0.1
Morphine-3-glucuronide	0.92, 1.2	1140, 1710	0.001, 0.001	99, 232	930, 1370	0.11, 0.17
1-Naphthol	111 ± 27	220 ± 30	0.5 ± 0.06	240, 290	260, 320	0.93, 0.92
Naproxen	11.5 ± 3.5	580 ± 80	0.02 ± 0.01	53 ± 30	250 ± 160	0.34 ± 0.31

Each value represents single kinetic determinations or the mean ± SD of three determinations. Each set of kinetics was produced using six substrate concentrations (in duplicate).

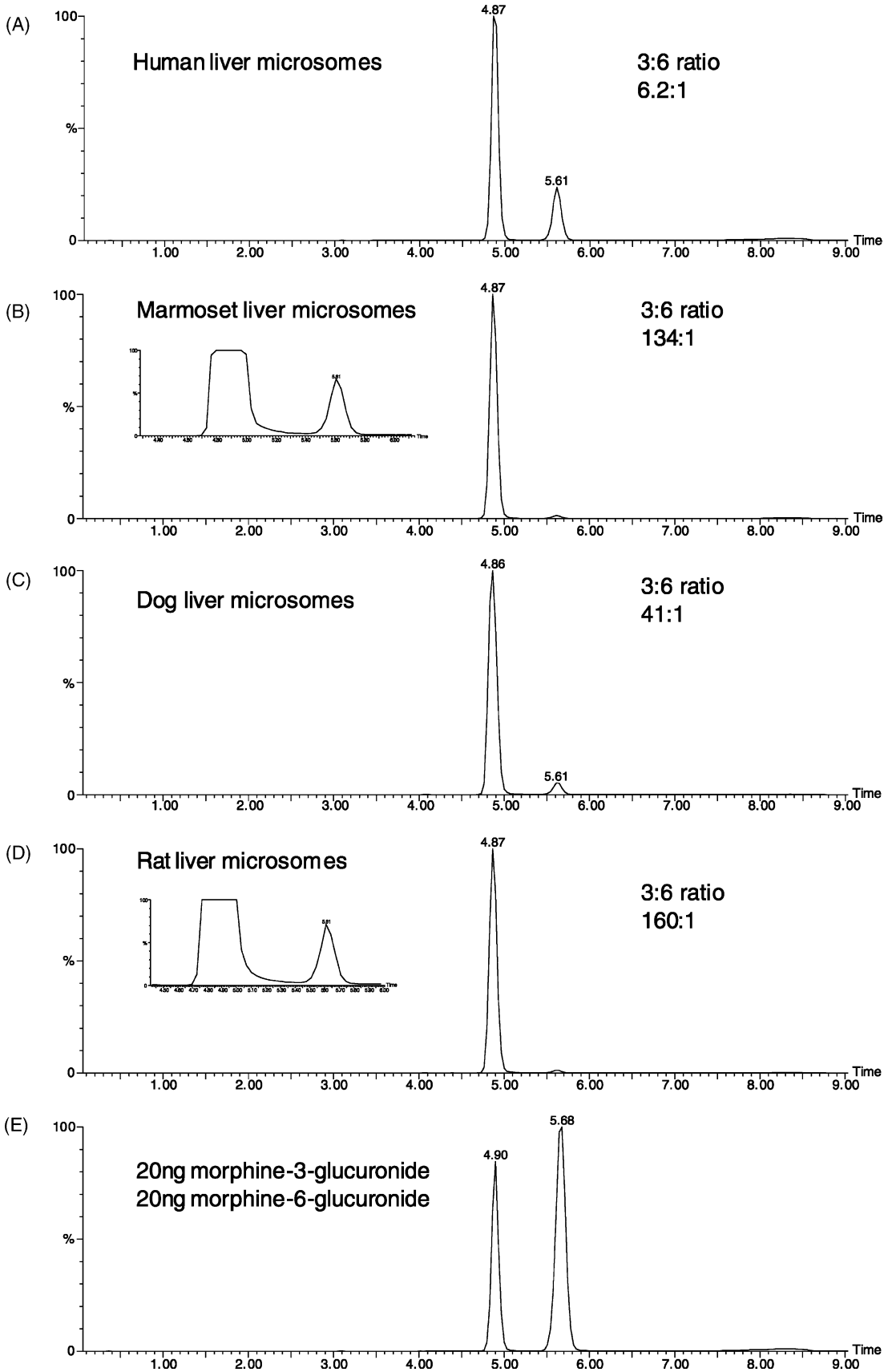

Fig. 3. Hepatic morphine glucuronidation determined using mass spectroscopy. Morphine glucuronidation was determined using human liver microsomes (A), marmoset liver microsomes (B), DLM (C), and rat liver microsomes (D). The analyses of 20 ng of both morphine-3-glucuronide and morphine-6-glucuronide are also shown (E). Inserts are included in (B) and (D) to clearly demonstrate morphine-6-glucuronidation.

of sequence identity observed between UGT2B31 and UGT2B4, UGT2B7, and UGT2B15 (see Fig. 1) suggests the designation of UGT2B31 as a member of the 2B subfamily to be accurate. More specifically, the sequence similarity to UGT2B1 and UGT2B9 [16,18] suggests that, based purely on sequence information, UGT2B31 may be the canine equivalent of human UGT2B7.

UGT2B31 expressed in V79 cells has been shown to glucuronidate a range of clinically used drugs. In particular, the glucuronidation of NSAIDs, phenols, and opioids was catalysed by UGT2B31. Interestingly, a similar substrate specificity has been observed for rat UGT2B1 [16], monkey UGT2B9 [18], and human UGT2B7 (Table 1; [6,19]). However, while UGT2B31, UGT2B1, and UGT2B7 have a similar broad substrate specificity, UGT2B31 was more limited in its catalytic activity towards particular compounds (see Table 1).

Perhaps the most clinically important drug glucuronidated by UGT2B31 is the analgesic morphine. Hepatic morphine glucuronidation exhibits both qualitative and quantitative differences across species. Both human and cynomolgous monkey glucuronidate morphine extensively at both the 3 and the 6 position, a reaction that is known to be catalysed by UGT2B7 [19] and UGT2B9 [18], respectively. Interestingly, strain differences may be apparent in morphine glucuronidation by the monkey as the present study detected only small amounts of the 6-glucuronide using marmoset liver microsomes (see Fig. 3), whereas more extensive levels were observed using the rhesus and cynomolgous monkey [18,20]. Rat liver microsomes have been shown previously to be capable of only forming morphine-3-glucuronide [10,21]. However, the use of mass spectrometry in the present study afforded the detection of small amounts of the 6-glucuronide (see Fig. 3). The formation of both morphine-3- and morphine-6-glucuronides using DLM has also been observed (see Fig. 3), which confirms the earlier work of King *et al*. [20] and Bock *et al*. [22]. However, UGT2B31, like UGT2B1 in the rat, was only capable of glucuronidating morphine at the 3-position (see Tables 1 and 2). This suggests that more than one UGT isoform may be involved in canine morphine glucuronidation as has been proposed for both rats and humans [17,23].

Previously, the intrinsic clearance of morphine (as well as other UGT2B7 substrates) was found to be an order of magnitude greater in dog than in human liver microsomes [9,20,22]. However, the affinities (as assessed by K_m) of both UGT2B31 and UGT2B7 for morphine were similar (see Table 2). This might suggest a higher hepatic expression level of UGT2B31 than UGT2B7. Unfortunately, the species-specific antibodies required to confirm this are currently unavailable.

UGT2B31, UGT2B7, and UGT2B1 also catalysed the glucuronidation of a range of phenolic substrates (see Table 1). However, in humans the majority of hepatic UGTs have been shown to glucuronidate phenols [24]. It is tempting to speculate that a similar situation might occur in dogs. Kinetic analysis of 1-naphthol with UGT2B31 determined the K_m to be 220 μM, whereas previous work with dog UGT1A6 exhibited a greater affinity towards naphthol ($K_m = 41$ μM) [11]. This suggests that although UGT2B31 is involved, UGT1A6 might be the predominant isoform involved in the detoxication of simple phenolic substrates by dogs.

This report has described the cloning, expression, and functional characterisation of a novel canine UGT, UGT2B31. UGT2B31 displayed a similar substrate specificity to UGT2B1 and UGT2B7 albeit more limited particularly towards certain carboxylic acid-containing compounds. However, UGT2B31 has been shown to play a key role in drug detoxification in the dog. The data obtained in this study will help determine the suitability of the dog as a comparative animal model for drug metabolism studies in addition to aiding the production of veterinary medicines.

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