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THE ENANTIOSELECTIVE GLUCURONIDATION OF MORPHINE IN RATS AND HUMANS

EVIDENCE FOR THE INVOLVEMENT OF MORE THAN ONE UDP-GLUCURONOSYLTRANSFERASE ISOENZYME

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Abstract—The formation of morphine glucuronides is enantio- and regioselective in rats and humans. In rat liver microsomes, natural (−)-morphine formed only the 3-O-glucuronide, whereas the unnatural (+)-morphine formed glucuronides at both the 3-OH and 6-OH positions, with the 6-O-glucuronide being the principal product. In human liver microsomes, both the 3-OH-and 6-OH positions were glucuronidated with each of the enantiomers, with the 3-O-glucuronide being the major product with (−)-morphine, and the 6-OH position preferred with the (+)-enantiomer. By using a series of biochemical and biological situations such as induction by xenobiotics, ontogeny, selective inhibition and genetic deficiencies, which are considered to be diagnostic of UDP-glucuronosyltransferase heterogeneity, we determined that two UDP-glucuronosyltransferase isoenzymes were responsible for the glucuronidation of morphine in rat liver. One isoenzyme (the so-called "morphine UDP-glucuronosyltransferase") was responsible for the glucuronidation at the (−)-3-OH and (+)-6-OH positions of morphine, whereas the other formed only the (+)-morphine-3-glucuronide. Evidence from enzyme induction and the genetically deficient deficient Gunn rat suggested that bilirubin UDPGT may be responsible for the (+)-morphine-3-UDP-glucuronosyltransferase activity. In human kidney, glucuronidation of both (−)- and (+)-enantiomers at the 6-OH position was deficient, whereas the activity at the 3-OH positions was still present, which indicated the involvement of two UDP-glucuronosyltransferases in the glucuronidation of morphine in man, as well as rats.

Morphine is subject to enantioselective glucuronidation in rats, catalysed by microsomal UDP-glucuronosyltransferase (UDPGT)‖ [1]. There are two possible sites of glucuronidation on the morphine molecule—the phenolic 3- and the alcoholic 6-hydroxyl groups (Fig. 1). Using the natural (−)- and unnatural (+)-enantiomers of morphine, Rane *et al.* [1] reported the stereo- and regioselectivity of rat liver morphine glucuronidation. With (−)-morphine as aglycone, conjugation was unique to the 3-position of the molecule, however when the (+)-enantiomer was investigated, glucuronidation at the 6-OH position was favoured over the 3-position in the ratio 3:1. Upon treatment of rats with phenobarbital, the ratio of (+)M6G/(+)M3G increased to 7.4 to 1, suggesting that more than one UDPGT isoenzyme was involved in the metabolism of (+)-morphine. Further evidence for this was obtained from chro-

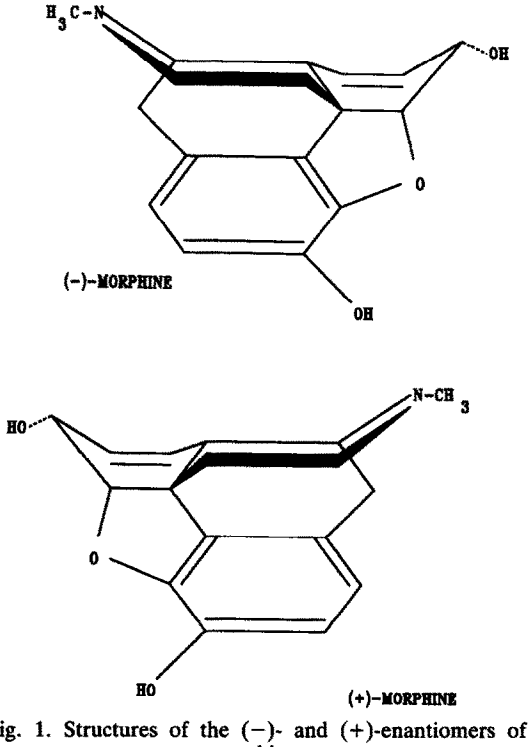

Fig. 1. Structures of the (−)- and (+)-enantiomers of morphine.

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‖ Abbreviations used: UDPGT, UDP-glucuronosyltransferase; M3G, morphine-3-β-D-glucuronide; M6G, morphine-6-β-D-glucuronide; UDPGA, uridine diphosphoglucuronic acid; β-NF, β-naphthoflavone; 3-MC, 3-methylcholanthrene; PB, phenobarbitone; SPI, spironolactone; SDS, sodium dodecyl sulphate; 1-NAA, 1-naphthylacetic acid.

Table 1. Clinical data on human liver samples

Sample no.	Sex	Age	Disease/cause of death
87/02	M	79	Road Accident
87/03	M	20	?
87/04	F	44	PBC*
87/07	M	48	HC
87/10	M	55	PBC
87/11	M	21	CAH
87/13	F	56	PBC
87/15	M	60	Cerebral haemorrhage

* (PBC) primary biliary cirrhosis, (HC) haemochromatosis, (CAH) chronic active hepatitis.

matofocusing of Emulgen 911-solubilised Sprague–Dawley rat liver microsomes on FPLC/MonoP [2]. One peak of activity resulting in the formation of (+)-M3G eluted at pH 8.2, and a second, broad peak of UDPGT activity catalysing the formation of (+)-M6G and (−)-M3G eluted between pH 7.7 and 6.7. Preliminary experiments have shown that human fetal liver shows a marked selectivity of glucuronidation of the 6-OH position of (+)-morphine (approx. 12 to 1) [3].

In the present study we have utilised some of the many biological situations which result in the differential expression of individual UDPGT isoenzymes—namely the effects of xenobiotic inducing agents [4]; the inherited deficiency of UDPGTs in the Gunn rat [5]; ontogeny [6] and tissue distribution—to further investigate the possible involvement of more than one UDPGT in the regio- and enantioselective glucuronidation of morphine in rats and humans. In addition we describe a novel selective inhibitor of morphine glucuronidation.

MATERIALS AND METHODS

Chemicals. (−)-Morphine sulphate was purchased from a local pharmacy, (+)-morphine and (+)-morphine-6-glucuronide were provided by the National Institute on Drug Abuse (Rockville, MD). UDPGA, Lubrol PX, 3-methylcholanthrene, β-naphthoflavone, spironolactone and (−)-morphine-3-glucuronide were purchased from Sigma Chemical Co. (Poole, U.K.). HPLC solvents were from Rathburn Chemicals (Walkerburn, U.K.). 1-Naphthylacetic acid was from Ega Chemie (Strasbourg, France) and phenobarbitone sodium from BDH (Poole, U.K.). All other chemicals were of analytical grade, and were purchased from commonly used suppliers.

Animals. Adult male Wistar and Gunn rats (6–8 weeks old, 120–170 g) were from colonies maintained in the Medical Sciences Institute, University of Dundee. For induction studies, animals received either phenobarbitone sodium, 100 mg/kg intraperitoneally in 0.9% NaCl (1 ml) daily for 4 days; spironolactone, administered by gavage as an aqueous suspension (125 mg/kg in 0.5 ml) daily for 4 days; 3-methylcholanthrene or β-naphthoflavone (100 mg/kg) injected intraperitoneally dissolved in 1 ml corn oil on day 1 and day 3; or clofibrate (200 mg/kg) i.p. in 0.5 ml corn oil twice daily for 4 days. Animals were killed 24 hours after the last injection. For ontogeny studies, WAG rats (Harlam-Olac Ltd, Blackthorn, U.K.) were used. This is an inbred strain of Wistar rats, genetically deficient in androsterone UDPGT. Male and female WAG rats were time mated, and at the relevant developmental age offspring were decapitated, the livers removed, immediately frozen in liquid nitrogen and stored at −80° until used (within 2 months). Only male animals were used, except for fetal rats, where sexing was not possible.

Human tissue samples. Samples of human liver and kidney were obtained at autopsy, immediately frozen in liquid nitrogen and stored at −80° until use. Available clinical data on samples is given in Table 1.

Preparation of microsomes. Samples were used immediately, or after thawing from −80°. Tissue was finely chopped with scissors, and homogenised in 250 mM sucrose, 5 mM HEPES, pH 7.4 to give a 20% (w/v) homogenate. Homogenates were centrifuged at 10,000 *g* for 15 min, and the resulting supernatants centrifuged at 105,000 *g* for 1 hr. Microsomal pellets were resuspended in the same buffer to give a protein content of approximately 20 mg/ml, and stored frozen at −80° until use (within 1 month).

Assays for UDPGT enzyme activity. UDPGT enzyme activities were determined with bilirubin [7] and androsterone [8] as acceptor substrates.

Morphine UDPGT assay. UDPGT activity was determined by a modification of the reverse phase, ion pair HPLC method described by Svensson *et al.* [9]. Incubation mixture (in 1.5 ml microcentrifuge tubes) comprised 167 mM Tris–Cl^-, pH 7.4, 8.3 mM $MgCl_2$, 3 mM morphine, and microsomal suspensions (0.02–0.2 mg protein) in a volume of 100 μl. The non-ionic detergent Lubrol PX was included, in a volume of 5 μl, in order to counteract the latency of UDPGT [10]. Detergent to protein ratios giving a maximal activation were determined for each tissue sample in pilot experiments. Incubations were started by the addition of UDPGA (4 mM), and after 15–30 min at 37°, reactions were terminated by heating for 2 min at 95°. This treatment has been shown by us to have no significant effect on the recovery of morphine glucuronide by the use of blank incubation mixtures spiked with authentic standards. After centrifuging at 16,000 *g* for 5 min in an Eppendorf microcentrifuge, 75 μl of the supernatants were mixed with 75 μl of acetonitrile and, after standing in ice for 10 min, centrifuged for a further 2 min to remove any further precipitated protein. Samples (25 μl) of supernatants were then subjected to HPLC on an ultratechsphere C_{18} (5 μ) column (HPLC Technology Ltd, Macclesfield, U.K.) connected to a Beckman model 334 liquid chromatograph. The mobile phase comprised 10 mM sodium dihydrogen orthophosphate, pH 2.1 (with phosphoric acid) containing 1 mM SDS and 28% acetonitrile flowing at 1.8 ml/min. UV detection of resolved reaction components was at 210 nm. Retention time of (−)-M3G and (+)-M3G was 4.0 min, and of (+)-M6G was 5.0 min. Incubations performed without morphine or without UDPGA produced no peaks of morphine glucuronide. Morphine glucuronides were quan-

Table 2. UDP-Glucuronosyltransferases enzyme activities towards morphine in liver microsomes prepared from Wistar rats treated with various xenobiotic inducing agents

Treatment	UDPGT Activity (nmol/min/mg)			
	(−)M3G	(+)M3G	(+)M6G	(+)M6G/(+)M3G
None	34 ± 6	3.7 ± 0.8	13 ± 2	3.5
SPI†	74 (76, 71)	4.0 (4.4, 3.5)	26 (30, 22)	6.5
3-MC	39 ± 6	3.4 ± 0.2	13 ± 3	3.8
β-NF	49 ± 10	4.4 ± 0.7	18 ± 4	4.1
PB	219 ± 42	7.7 ± 1.6	79 ± 12	10.3

Activities were determined on microsomal fractions in the presence of optimally-activating concentrations of Lubrol PX, determined for each tissue sample and morphine enantiomer.

* Activities represent the mean ± SD for at least three pools of liver (except for SPI, when only two pools were available).

† (SPI) Spironolactone 125 mg/kg p.o. daily for 4 days; (3-MC) 3-methylcholanthrene 100 mg/kg i.p. on days 1 and 3; (β-NF) β-naphthoflavone 100 mg/kg i.p. on days 1 and 3; (PB) phenobarbitone 100 mg/kg i.p. daily for 4 days.

titated by constructing standard curves (based on peak heights) with authentic standards.

Protein determination. Protein in microsomal samples was estimated by the method of Lowry *et al.* [11], with bovine serum albumin as standard.

RESULTS AND DISCUSSION

The conjugation of morphine with UDPGT has previously been shown to be enantio- and regioselective in the rat [1], and the authors suggested that more than one UDPGT isoenzyme might be involved in these reactions, based on the differential induction of (+)-M3G, and (+)-M6G/(−)-M3G formation with phenobarbitone. In this report we have examined various biological parameters which illustrate UDPGT heterogeneity in order to demonstrate the contribution of more than one UDPGT isoenzyme towards the glucuronidation of morphine enantiomers.

Morphine UDPGT activities in Wistar rat liver—the effect of administered xenobiotics

In Wistar rats, the overall rate of glucuronidation of (−)-morphine was twice that of (+)-morphine, and there was marked regioselectivity with both enantiometers (Table 2). With (−)-morphine as aglycone, no glucuronidation at the 6-OH position was observed. Conversely, with (+)-morphine the 6-OH position was favoured over the 3-OH by a ratio of 3.5 to 1. These data confirm previous findings [1]. When liver microsomes prepared from Wistar rats pretreated with various xenobiotics assumed to elicit isoenzyme-specific induction of UDPGTs were assayed for morphine UDPGT activity (Table 2), it was observed that treatment with phenobarbitone resulted in a marked induction of (−)-M3 UDPGT and (+)-M6 UDPGT enzyme activity (6.4- and 6.1-fold, respectively). Hepatic (+)-M3 UDPGT activity was only stimulated 2-fold upon treatment with phenobarbitone. Thus phenobarbitone treatment increased the ratio of (+)-M6G/(+)-M3G formation from 3.5 to 10.3. The pathways of (−)-M3G and (+)-M6G formation in rat liver microsomes therefore seem to share a common regulation with respect to phenobarbitone induction, whereas the formation of (+)-M3G was under separate control. Similarly, spironolactone had no effect on the rate of (+)-M3G formation, but did stimulate (−)-M3G and (+)-M6G formation 2-fold. Therefore spironolactone can be regarded as an inducer of (−)-M3 UDPGT and (+)-M6 UDPGT activity, but not of (+)-M3 UDPGT (spironolactone also induces UDPGT enzyme activity towards digitoxigenin monodigitoxoside [12] and bilirubin [13]). These data strongly suggested the involvement of more than one UDPGT isoenzyme in the glucuronidation of morphine. With this hypothesis in mind, we investigated the effects of 3-MC and β-NF (specific inducers of phenol UDPGT—see Refs 4 and 5), in order to determine if phenol UDPGT was involved in conjugating (+)-morphine at the 3-OH position. 3-MC and β-NF pretreatment of rats had no significant effect on the formation of (+)-M3G (Table 2), thereby eliminating phenol UDPGT as a candidate for the (+)-M3 UDPGT activity. The complete lack of conjugation at the 6-OH position of (−)-morphine was not altered by any of the inducers used.

When Wistar rats were treated with the hypolipidaemic drug clofibrate, a well known inducer of bilirubin UDPGT enzyme activity (see Ref. 14), the glucuronidation of (−)-morphine at the 3-OH position and (+)-morphine at the 6-OH position was suppressed, to about 50% of the control value (Table 3). However, the (+)-M3 UDPGT activity was stimulated upon treatment with clofibrate, to a similar extent as bilirubin (1.8- and 1.6-fold, respectively). The range of values obtained in this experiment was broad, but when data from individual rats were compared, the level of (+)-M3 UDPGT activity correlated exactly with the activity towards bilirubin. This suggests bilirubin UDPGT may be the major isoform contributing to the glucuronidation of (+)-morphine at the 3-OH position. This is discussed further in the context of the Gunn rat (see below).

Further, in order to eliminate the involvement of androsterone (3α-hydroxysteroid) UDPGT in the formation of (+)-M3G, we assayed liver microsomes

Table 3. Induction of morphine and bilirubin UDP-glucuronosyltransferase activities upon treatment of rats with clofibrate

	UDPGT Activity (nmol/min/mg)				
	(−)-Morphine		(+)-Morphine		
Treatment	M3G	M6G	M3G	M6G	Bilirubin
Corn oil	38.2 ± 4.7	0	1.6 ± 0.3	9.3 ± 1.1	1.4 ± 0.4
Clofibrate	19.7 ± 1.2	0	2.5 ± 0.6	5.9 ± 1.8	2.4 ± 0.6

Data represents the mean ± SD of determinations on three separate samples of liver microsomes, assayed in the presence of optimally-activating concentrations of Lubrol PX, determined for both substrates and treatments.

Table 4. Glucuronidation of morphine and androsterone by LA and HA Wistar rat liver microsomes

	UDPGT Activity (nmol/min/mg)			
	(−)M3G	(+)M3G	(+)M6G	Androsterone
LA	4.25	0.98	2.98	0.015
HA	5.74	1.47	3.10	0.248

Data represent mean of determinations performed in duplicate on two separate microsomal preparations.

prepared from LA and HA* Wistar rats [15], and determined that no morphine glucuronidation reaction was significantly decreased in the androsterone UDPGT deficient LA rat (Table 4).

Inhibition of morphine UDPGT activity by 1-naphthylacetic acid

1-NAA is glucuronidated by rat liver microsomes, and the UDPGT enzyme activity is inducible 6-fold by phenobarbital [16]. This compound was therefore a potential substrate for morphine UDPGT. We examined the effect of 1-NAA on (−)- and (+)-morphine UDPGT enzyme activities (Fig. 2). 1-NAA selectively inhibited (−)-M3 and (+)-M6 UDPGT activity, whereas the formation of (+)-M3G was unaffected except at the highest concentrations of 1-NAA. The effect on (+)-M3 and (+)-M6 UDPGT activities was competitive, with a K_i of approximately 1 mM, with maximally activated Wistar rat liver microsomes (not shown). The lack of inhibition of (+)-M3 UDPGT enzyme activity by 1-NAA strongly supports the hypothesis that a separate UDPGT is responsible for this activity. The slight (25%) inhibition of (+)-M3 UDPGT activity at high concentrations of 1-NAA may reflect an overlapping substrate specificity of the (−)-M3/(+)-M6 UDPGT resulting in a small contribution to the glucuronidation of (+)-morphine at the 3-OH position (see below).

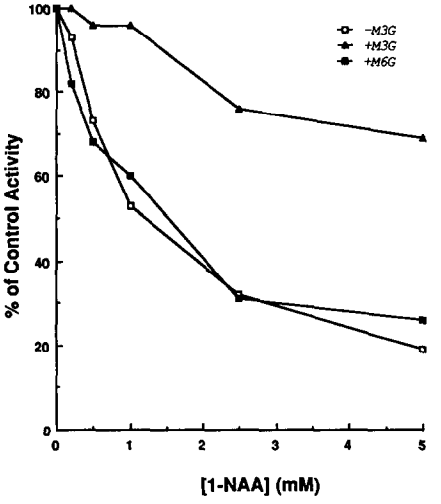

Fig. 2. Inhibition of morphine UDP-glucuronosyltransferase activities by 1-naphthylacetic acid. Adult male Wistar rat liver microsomes (75 μg) were assayed for morphine UDPGT enzyme activity with (−)- and (+)-morphine (3 mM) as aglycones. Incubations (100 μl) were performed in the presence of various concentrations of 1-naphthylacetic acid, included in the incubation dissolved in 5 μl of methanol. Control incubations contained 5 μl of methanol. Assays were performed in the presence of optimally-activating concentrations of Lubrol PX. □ = (−)-morphine-3-glucuronide; ▲ = (+)-morphine-3-glucuronide; ■ = (+)-morphine-6-glucuronide. Data points represent the mean of determinations on two to four samples of microsomes.

Development of morphine UDPGT enzyme activities

Further evidence for the separation of morphine UDPGT activities into (−)-M3G/(+)-M6G and (+)-M3G, and for the phenol UDPGT isoenzyme not being involved in the glucuronidation of morphine at the (+)-3-OH position came from ontogenic studies. These assays were performed on only one pool of Wistar rat livers for each developmental age, due to the scarcity of (+)-morphine. Different UDPGT enzyme activities in the rat have been shown to

* Most colonies of Wistar rats exhibit a discontinuous variation in androsterone UDPGT activity (see Ref. 15), whereas other UDPGT isoenzymes are expressed normally. The phenotypes are denoted LA (low androsterone) and HA (high androsterone). The colony maintained in this Institute exhibits such a variation, and the animals used in this study were typed LA or HA according to the expression of UDPGT enzyme activity towards androsterone. WAG rats are simply an inbred strain of Wistar rat, derived from animals expressing the LA phenotype.

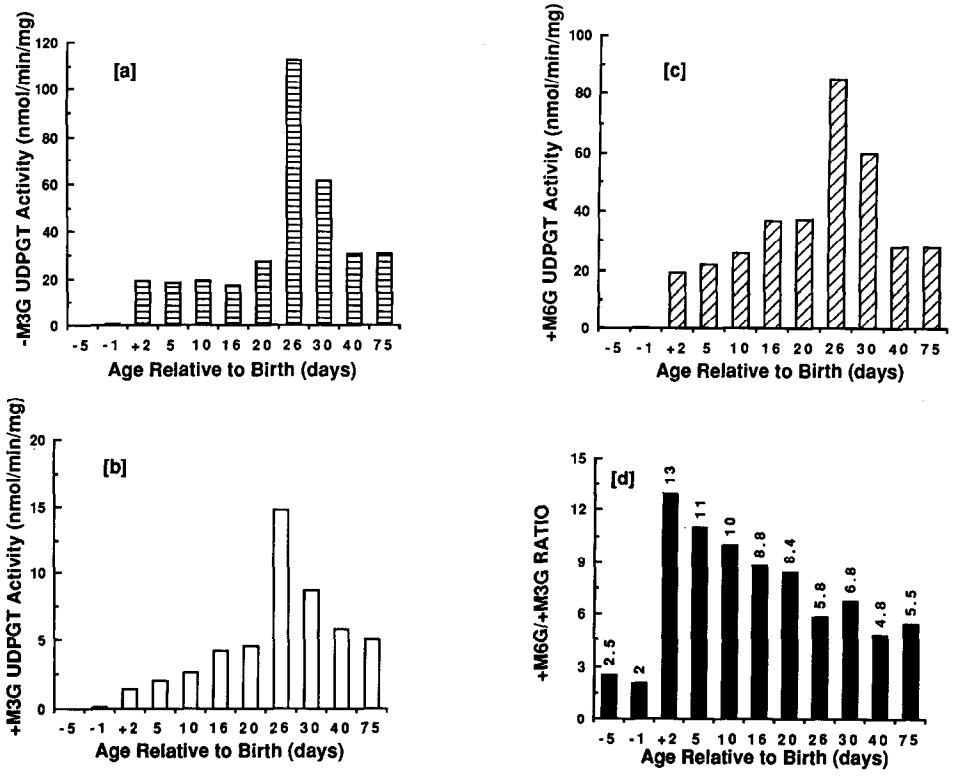

Fig. 3. Development of morphine UDP-glucuronosyltransferase enzyme activities in Wistar rat liver microsomes. Microsomes from livers of Wistar rats of different developmental ages were assayed for (−)- and (+)-morphine UDPGT enzyme activity, in the presence of optimally-activating concentrations of Lubrol PX, determined for each developmental age. (a) (−)-morphine-3-glucuronide; (b) (+)-morphine-3-glucuronide; (c) (+)-morphine-6-glucuronide; (d) ratio of (+)M6G/(+)M3G.

develop at different perinatal stages, resulting in the concept of isoenzyme "clusters" [6], with enzyme activity towards planar phenols (1-napthhol, 2-aminophenol) developing late-fetally, and bilirubin and testosterone UDPGTs developing after birth [6]. During this study we determined the UDPGT enzyme activities towards (−)- and (+)-morphine in rats of various developmental ages (Fig. 3). Formation of all three glucuronides [(−)-M3G, (+)-M3G and (+)-M6G)] was very low in fetal liver microsomes, and the activities developed postnatally, reaching a peak at 26 days *post partum* before falling away into adulthood (Fig. 3a–c). On first examination, the developmental pattern for glucuronidation at the (+)-3-OH and (+)-6-OH positions appeared to be very similar. However, when a plot of (+)-M6G/(+)-M3G ratio as a function of age was made, it was obvious that the development of the two activities followed different patterns (Fig. 3d), with the (+)-M6G/(+)-M3G ratio being the highest 2 days *post partum* [13], and falling to about 5 in adult liver microsomes. This is suggestive of the UDPGT activity towards (+)-morphine at the 3-OH and 6-OH positions being under separate regulation during development in the rat. The rate of formation of the (−)-M3G and (+)-M6G developed simultaneously over the period examined, suggesting coordinate regulation in the developing rat. It is difficult to say conclusively that the ontogeny of morphine glucuronidation represents the actions of two different UDPGT isoenzymes, but similar subtle differences in the postnatal development of other known UDPGTs (e.g. bilirubin and testosterone) have been observed [17]. However, when considered with the other evidence presented here, the differential development of morphine UDPGT activities supports the hypothesis that there are at least two different UDPGTs involved in the enantioselective glucuronidation of morphine.

Glucuronidation of morphine in the Gunn rat

Gunn rats are genetically deficient in bilirubin UDPGT enzyme activity [18], and this has recently been shown to be due to the absence of the bilirubin UDPGT isoenzyme [14]. In addition, UDPGT activity towards certain planar phenols is also deficient (about 5–20% of Wistar levels) in the Gunn rat, the result of the absence of immunoreactive phenol UDPGT as determined by immunoblot analysis [5]. Gunn rat liver microsomes catalysed the glucuronidation of morphine at the (−)-3-OH and (+)-6-OH positions at about the same rate as Wistar rat liver microsomes (Table 5). However, conjugation at the (+)-3-OH position of morphine was severely reduced, to about 20% of the level observed in Wistar rats, resulting in a (+)-M6G/(+)-M3G ratio of 13. When hepatic microsomes from Gunn rats treated with phenobarbitone were analysed, the

Table 5. UDP-Glucuronosyltransferase activity towards (+)- and (−)-enantiomers of morphine in Gunn rat liver microsomes

Treatment	UDPGT Activity (nmol/min/mg)			
	(−)M3G	(+)M3G	(+)M6G	(+)M6G/(+)M3G
None	27.7 ± 1.6	0.73 ± 0.11	9.75 ± 0.86	13
3-MC	28.7 ± 5.4	1.01 ± 0.18	11.4 ± 2.0	11
PB	253 ± 15	3.95 ± 0.27	72.1 ± 7.6	18

* Data are quoted as mean ± SD for determinations on three separate pools of liver. Measurements were made using optimally-activating concentrations of Lubrol PX.

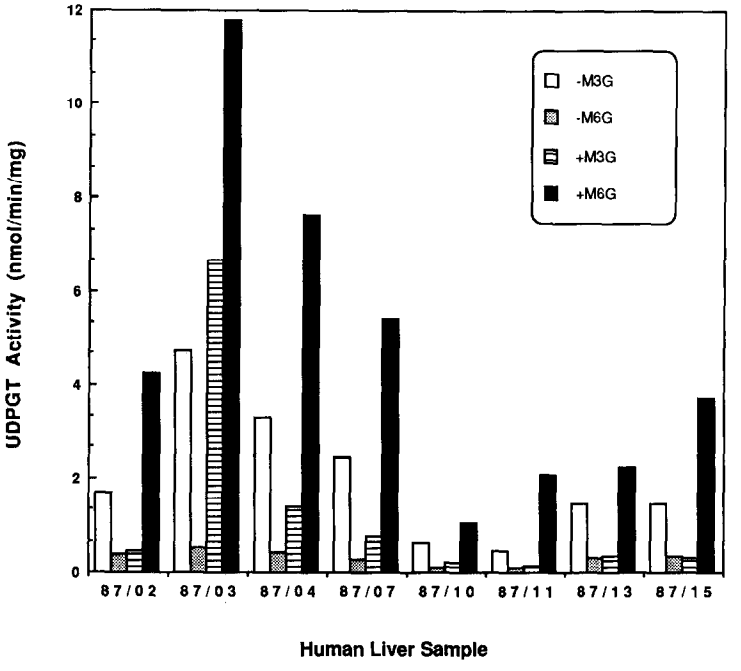

Fig. 4. Morphine UDP-glucuronosyltransferase enzyme activities in human liver microsomes. The formation of glucuronides of (−)- and (+)-morphine was determined with liver microsomes prepared from eight human liver samples of different clinical origin (see Table 1). The optimal concentration of detergent required to give maximum activity was determined for each sample. 125 μg of microsomal protein was included in each incubation.

(−)-M3G and (+)-M6G formation was induced 9- and 7.4-fold, respectively, and the formation of (+)-M3G was induced 5.4-fold (Table 5). No significant effect on the glucuronidation of either enantiomer of morphine was observed following treatment of Gunn rats with 3-methylcholanthrene. Therefore, the Gunn rat was deficient in the glucuronidation of (+)-morphine at the 3-OH position. The induction of Wistar rat (+)-M3 UDPGT activity by clofibrate (Table 3) suggested that bilirubin UDPGT may be responsible for this glucuronidation reaction, and the deficiency of the activity in the Gunn rat tends to support this assumption. The residual activity of formation of (+)-M3G in the Gunn rat was more inducible by phenobarbitone than the 'normal' Wistar (+)-M3 UDPGT activity, suggesting that the (−)-M3/(+)-M6 UDPGT activity was responsible for the residual (+)-M3 UDPGT activity in the Gunn rat.

These data demonstrate the involvement of two UDPGT isoenzymes in the enantioselective glucuronidation of morphine in rats. One activity catalysed the formation of (−)-M3G and (+)-M6G, and the second the glucuronidation of (+)-morphine at the 3-OH position. This second activity may be bilirubin UDPGT.

Enantioselective glucuronidation of morphine in man

When glucuronidation of the morphine enantiomers by human liver microsomes was investigated, it was found that there was conjugation at the 3-OH and 6-OH positions with both aglycones. Overall, (+)-morphine was a better substrate than its antipote, with (+)-M6G being the principal product, in contrast to (−)-morphine where the (−)-M3G was preferentially formed (Fig. 4). The M6G/M3G ratios, calculated from the mean value determined from the eight samples analysed were 0.16 and 3.72

Table 6. Formation of morphine glucuronides by human liver and kidney microsomes

	UDPGT Activity (nmol/min/mg)					
	(−)M3G	(−)M6G	M6G/M3G	(+)M3G	(+)M6G	M6G/M3G
Human Liver	1.31 (1.58, 1.03)	0.232 (0.307, 0.157)	0.18	0.308 (0.353, 0.262)	1.94 (2.62, 1.26)	6.3
Human Kidney	0.387 (0.296, 0.472)	0.019 (0, 0.037)	0.05	0.105 (0.083, 0.127)	0 (0, 0)	0

Activities were determined in the presence of optimally-activating amounts of Lubrol PX.

for the (−)- and (+)-enantiomers respectively. There was a marked inter-individual variation in the amount of product formed among the various liver samples employed in this study. Such variations have been reported for other UDPGT substrates [19]. The values reported in Fig. 4 for human liver morphine UDPGT varied with SD values up to >100% of the mean. However, no significant correlation between variation in morphine UDPGT activity and age, sex, or disease state was observed. It is likely that the variation exists as a result of the condition of the tissue sample (e.g. post-mortem time), genetic factors or exposure to drugs and environmental contaminants, or a combination of these factors. It must be noted that the sample population was not large enough to detect any of these.

Evidence that the glucuronidation of morphine in man is also the result of the action of more than one UDPGT came from examining the formation of the various enantiomeric glucuronides by human kidney microsomes. The two liver and kidney samples used in this study (Table 6) were matched—liver and kidney microsomes prepared from tissue samples obtained from the same individual. A different pattern of metabolism from the liver was observed when human kidney microsomes were incubated with either (−)- or (+)-morphine (Table 6). Little or no conjugation at the 6-OH position of either enantiomer of morphine was observed, suggesting that the human kidney is deficient in at least one UDPGT isoenzyme responsible for the glucuronidation of (+)- [and probably (−)-] morphine at the 6-OH position, and indicating the involvement of more than one UDPGT isoenzyme in the regio- and enantioselective glucuronidation of morphine in man.

These preliminary investigations suggest that there are two morphine UDPGTs in man, one conjugating at the 3-OH position, and the other at the 6-OH position. The isoenzyme selective for the 6-OH position appears to be absent from the kidney. Bilirubin UDPGT enzyme activity is also absent from human kidney [20] and preliminary immunoblot analysis of human kidney microsomes indicated that the complement of anti-UDPGT immunoreactive polypeptides was significantly reduced when compared with human liver microsomes (M. Coughtrie, unpublished work). Thus the regio- and enantio-selectivity of morphine UDPGT differs between human and rat liver microsomes. This emphasises the need for drug metabolism studies to be performed on human samples *in vitro* to identify differences in the metabolic pathways that exist between man and laboratory animals.

CONCLUDING REMARKS

We believe that the data presented in this study coupled with the reported separation of (+)-M3 UDPGT activity from (+)-M6 UDPGT and (−)-M3 UDPGT activity on chromatofocusing of solubilised rat liver microsomes [2] strongly suggests the existence of two UDPGTs in rats involved in the regio- and enantioselective glucuronidation of morphine. The isoenzyme responsible for the formation of (−)-M3G and (+)-M6G is probably the morphine

UDPGT purified by Puig and Tephly [21], i.e. highly phenobarbitone-inducible, $M_r = 56{,}000$. The identity of the (+)-M3 UDPGT has not been unequivocally determined, although the induction by clofibrate and the deficiency of this activity in the Gunn rat suggest bilirubin UDPGT is a candidate. However, the (+)-M3 UDPGT activity is not the 3-MC-inducible phenol UDPGT, nor androsterone UDPGT, and the fact that it is deficient in the Gunn rat means it is unlikely to be the testosterone isoenzyme, which is present at normal levels in the Gunn rat. The possibility that it represents a previously undescribed isoform cannot be dismissed at this stage. Similarly, the preliminary evidence reported here suggests the existence of two UDPGTs in man, with different regioselectivity for morphine glucuronidation. One of these activities, catalysing the formation of 6-O-glucuronides of (−)- and (+)-morphine appeared to be absent from human kidney. Expression of cloned cDNAs encoding these isoenzymes will ultimately allow the determination of the substrate specificities of the enzymes, and the characterization of the isoform catalysing the formation of (+)-M3G.

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