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Examination of the Substrate Specificity of Cloned Rat Kidney Phenol UDP-Glucuronyltransferase Expressed in COS-7 Cells

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SUMMARY

A cDNA encoding a rat kidney UDP-glucuronyltransferase (UDPGT) was subcloned into the vector pKCRH2. Expression driven by the SV40 promoter produced enzymatically active UDPGT in COS-7 cells cultured *in vitro*. The appearance of enzyme activity was associated with an immunodetectable glycosylated UDPGT protein (M_r 53 kDa) in the cells. The expressed enzyme rapidly catalyzed the glucuronidation of 1-naphthol, 4-methylumbelliferone, and 4-nitrophenol. Studies using more than 20 compounds showed that the cloned UDPGT exhibited a restricted specificity towards planar phenols. A crude description of the molecular conformation of 4-alkylphenols accepted within the active site of the protein was obtained. The glucuronidation of morphine, thymol, menthol, testosterone, androsterone, or estrone was not catalyzed by this enzyme.

Kidney UDPGTs play an important role in glucuronidation and facilitated elimination of potentially toxic compounds in the urine (1). Variation in the complement of different UDPGT isoenzymes in different tissues may result in dramatic differences in the ability of the target organ to eliminate even simple therapeutic agents (2).

Many analgesic drugs, such as paracetamol, are inactivated during first-pass metabolism. A relatively large dose of these drugs has to be used every few hours to overcome the short pharmacological effect and poor bioavailability of these fairly water-soluble drugs (3). These analgesic compounds, which often contain a phenolic hydroxyl group as the pharmacologically active substituent, are attractive substrates for UDPGTs (4). Therefore, it is important to determine the specificity of glucuronidation, which effects safe elimination of potentially dangerous compounds, such as biogenic amines, but which can also be used as a guide for the design of improved drugs.

At least two UDPGT isoenzymes, one of which is inducible by 3-MC (5), catalyze the glucuronidation of phenolic compounds. The contribution of each isoenzyme and the complexity of the UDPGT family has been indicated from developmental studies in which rat fetal liver had a limited ability to glucuronidate alkyl phenols that were glucuronidated by the adult liver (4). An effective method to determine the substrate specificity of a single UDPGT, avoiding overlapping activities of the other isoenzymes, is to express a cDNA clone coding for the enzyme in a mammalian cell. The additional advantage of this approach is that the specificity of drug glucuronidation can be assessed in whole cells and presumably the reactions that occur *in vivo* can be reproduced.

A cDNA encoding a UDPGT has been cloned from a rat kidney cDNA library in λgt11.[1] The amino acid coding sequence of the enzyme predicted from the nucleotide sequence was identical to a 3-MC-inducible liver mRNA reported to code for 4-nitrophenol UDPGT (6).

In this study we have inserted a kidney UDPGTcDNA into an expresssion vector and transfected the recombinant plasmid to monkey kidney COS-7 cells. The expression of the transferase has facilitated the examination of the substrate specificity of the single cloned UDPGT isoenzyme, for which the activity can be measured in disrupted cultured cells. The results provide a rational basis for the description of the types of compound that can be glucuronidated by this enzyme.

Materials and Methods

Construction of recombinant plasmids for expression of UDPGT cDNAs. A rat kidney (K39) UDPGT cDNA[1] was subcloned into the *Hin*dIII site of expression vector pKCRH2 (7), kindly provided by S. Shibahara (Friedrich Meischer-Institut, Basel, Switzerland). Recombinant plasmids were grown in *Escherichia coli* DH1 and plasmids with the cDNA insert in the correct orientation with respect to transcription from the SV40 early gene were identified by restriction mapping.

Transfection of COS-7 cells and assay of UDPGT activities. COS-7 monkey kidney cells (8) were kindly provided by C. Madin (Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom) and cultured in Dulbecco's modified Eagle's Medium (Flow Laboratories, Irvine, Scotland) containing 10% fetal bovine

[1] D. Harding, M. R. Jackson, R. B. Corser, and B. Burchell, unpublished work.

ABBREVIATIONS: UDPGT, UDP-glucuronyltransferases; TLC, thin layer chromatography; 3-MC, 3-methylcholanthrene; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

serum (GIBCO Europe, Glasgow, Scotland) at 37° in 95% air/5% CO_2. Plasmid DNA (40 μg) was used to transfect each semiconfluent dish (150 cm^2) of COS-7 cells, using calcium phosphate/glycerol shock procedure (9). The cells were harvested 72 hr later and assayed for UDPGT activity.

Assay of UDPGT activities. Cell pellets were collected by centrifugation at 500 × *g* for 5 min and washed twice with 0.15 M NaCl, 5 mM Tris·HCl buffer, pH 7.4 (TBS), and stored frozen at −80°. Cell pellets were thawed and gently hand-homogenized in a ground glass homogenizer in TBS. Wistar rat liver and kidney microsomes were prepared as previously described (10). Assays were performed as previously described (11) and modified as follows. The routine incubation mixtures contained the following: cell homogenate (0.1 mg of protein) or microsomes (0.05 mg of protein), aglycone (0.5 mM), UDP-glucuronic acid (10 μM sodium salt; Sigma Chemical Co., Poole, Dorset, United Kingdom), 0.25 μCi of [^{14}C]UDP-glucuronic acid (272 mCi/mmol; Amersham plc, Amersham, United Kingdom), 100 mM Tris-maleate, 10 mM $MgCl_2$ buffer, pH 7.4, in a final volume of 100 μl. This low concentration of UDP-glucuronic acid was used to improve the sensitivity of the assay, allowing a greater proportion of the radioactive label to be incorporated into the glucuronides. The assay was observed to occur at linear rates with the range of protein concentrations used.

The reaction mixtures were incubated for 30 min at 37° and terminated by addition of 200 μl of absolute ethanol. These mixtures were centrifuged at 10,000 × *g* for 15 min and 200 μl of supernatant was reduced to 20 μl by vacuum centrifugation and applied to precoated silica gel 60 F_{254} plates (20 cm × 20 cm, 0.25 mm thick; Merck, Darmstadt, Federal Republic of Germany). Chromatography conditions were as previously described (11). The chromatography plates were dried and then sprayed with 1% (w/v) butyl [2-(4′-tert-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole] (BDH Ltd., Poole, Dorset, United Kingdom) in toluene and autoradiographed for 3 days. The spots containing the glucuronides (R_F 0.80) were scraped from the TLC plates and radioactivity counted as previously described (11). The following aglycone substrates were used: 1-naphthol, 2-naphthol, thymol, (−)-menthol, 4-hydroxybiphenyl, and 4-isopropylphenol from Sigma; 4-methylphenol, 4-ethylphenol, 4-*n*-propylphenol, 4-*t*-butylphenol, (*R*)-(−)-1,2,3,4-tetrahydro-1-naphthol, (*S*)-(+)-1,2,3,4-tetrahydro-1-naphthol, 5,6,7,8-tetrahydro-1-naphthol, and carvacrol from Aldrich (Gillingham, Dorset, United Kingdom); phenol, and phenolphthalein from BDH; (−)-morphine from Allardyce Chemists (Dundee, United Kingdom); and (+)-morphine from National Institute on Drug Abuse, Bethesda, MD.

Protein concentrations were determined by the method of Lowry *et al.* (12).

Labeling and immunoprecipitation of expressed UDPGT from transfected COS-7 cells. COS-7 cells in 20-cm^2 dishes were washed 72 hr after transfection with methionine-free RPMI 1640 containing 10% (w/v) dialyzed fetal bovine serum and then incubated with 7.5 ml of the same medium in the presence or absence of 2 μg/ml tunicamycin for 1 hr. This medium was subsequently removed and replaced by 1 ml of the same medium supplemented with 50 μCi of [^{35}S]methionine (1000 Ci/mmol) and incubated for 4 hr. Cells were rinsed twice with ice-cold 138 mM NaCl/2.7 mM KCl/1.5 mM KH_2PO_4/8 mM Na_2HPO_4 adjusted to pH 7.4 with HCl (phosphate buffered saline). Cells were harvested and centrifuged at 1500 × *g* for 5 min. The pellets were suspended and washed with phosphate-buffered saline by centrifugation. The pellet was solubilized with 0.5% (w/v) deoxycholate, 1% (w/v) Triton X-100, 0.1% (w/v) sodium dodecyl sulfate in 10 mM Tris·HCl, pH 7.4, by gentle hand homogenization. The mixture was centrifuged at 16,000 × *g* and the supernatant was incubated with preimmune serum and sheep anti-rat UDPGT antiserum as previously described (13), except that incubation with antiserum was overnight in ice. Immunoprecipitates were washed stringently as previously described (13). The final products were analyzed by sodium dodecyl sulfate gel electrophoresis on 7% gels (14), followed by autoradiography (15).

Results

Expression of UDPGT protein in transfected COS-7 cells. COS-7 cells were transfected with the vector containing UDPGT K39 cDNA as described in Materials and Methods. Proteins in the cells were labeled with [^{35}S]methionine and UDPGT proteins specifically immunoprecipitated from the transfected cells. Immunoprecipitates from control COS-7 cells and transfected cells are shown in Fig. 1. Preimmune isolates were compared with the immunoprecipitates to indicate nonspecific isolation of several proteins. A comparison of the nonspecifically isolated proteins in the controls (Fig. 1, *lanes 1* and *2*) with the immunoprecipitated proteins revealed that the antibody specifically identified a protein of 53 kDa (Fig. 1, *lane 3*). Tunicamycin, a selective inhibitor of the first stage of *N*-linked oligosaccharide biosynthesis and protein glycosylation at this concentration (16), was also added to some cell cultures

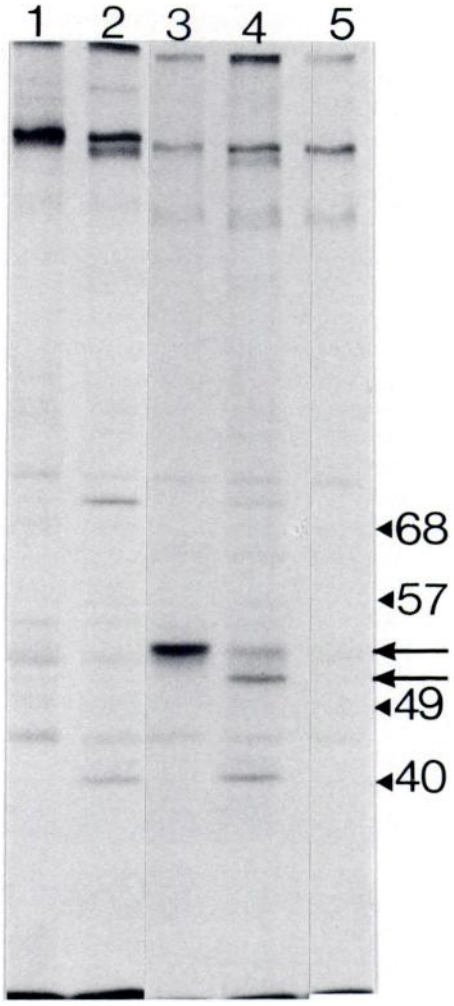


Fig. 1. Expression of rat kidney phenol UDPGT protein in transfected COS-7 cells. The proteins in COS-7 cells were labeled with [^{35}S]methionine for 72 hr after transfection in the presence (*lanes 2* and *4*) or absence (*lanes 1* and *3*) of tunicamycin. Immunoprecipitates containing UDPGT protein were analyzed by sodium dodecyl sulfate gel electrophoresis and autoradiography (see Materials and Methods). *Lanes 1* and *2*, proteins nonspecifically precipitated with preimmune serum; *lanes 3–5*, proteins isolated with ani-UDPGT antiserum. *Lanes 1–4*, cells were transfected with UDPGT K39 cDNA; *lane 5*, vector-transfected cells. The mobilities of the protein standards (albumin, 68 kDa; pyruvate kinase, 57 kDa; fumarase, 49 kDa; and aldolase, 40 kDa) are shown on the *right*.

during [^{35}S]methionine labeling. The presence of a smaller protein of 51 kDa was observed after tunicamycin treatment (Fig. 1, *lane 4*). The approximately 2 kDa downward shift in the molecular mass of the protein indicates the reduction in size to the nonglycosylated form of the enzyme.

Glucuronidation of a series of chemically related naphthols by UDPGT K39 expressed in COS-7 cells. Cellular homogenates (10%, w/v) from UDPGT K39-transfected and plasmid-transfected COS-7 cells were prepared in 0.25 M Sucrose/5 mM HEPES, pH 7.4. Aliquots (0.1 mg of protein) were used for assay of UDPGT activity towards a wide range of substrates as described in Materials and Methods. Fig. 2 shows an autoradiograph from a TLC plate, indicating the glucuronides produced from the incubation of several different substrates with COS-7 cells transfected with UDPGT K39 cDNA. The radioactive metabolites (R_F 0.8) were hydrolyzed by β-glucuronidase and this hydrolysis was inhibited by 10 mM saccharo-1,4-lactone, providing supportive evidence that these products were indeed glucuronides (data not shown). These radioactive products were not observed in the assay blank, incubated without the aglycone substrate.

Glucuronides formed during this assay must be identified carefully. Glucuronides of the more polar substrates may exhibit reduced mobility during separation on TLC. For example, morphine glucuronide was observed at R_F 0.25 in this TLC system.

Quantitative analysis of these results and many other data are reported in Fig. 2 and Table 1. UDPGT activities in control cells, either vector-transfected or transfected with UDPGT K39 cDNA in the wrong orientation, were very low. High levels of UDPGT activity, more than 80 times greater than the rate in control cells, were observed with 1-naphthol and 2-naphthol as substrates when using homogenates of cells transfected with UDPGT K39 cDNA (Table 1).

Fig. 2 also shows that (*R*)-(−)- or (*S*)-(+)-1,2,3,4-tetrahydro-1-naphthols were not substrates for the cloned UDPGT. 5,6,7,8-Tetrahydro-1-naphthol was only a very poor substrate, although the phenolic ring structure was maintained. This data suggested that the electronic configuration conferred by the unsaturated bonding system was essential for glucuronidation at the hydroxyl group.

Glucuronidation of a series of phenolic compounds substituted at the 4-position by UDPGT K39 expressed in COS-7 cells. Cells transfected with UDPGT K39 cDNA were also assayed with a series of structurally related phenolic compounds (see Table 1) to attempt to determine certain limits of chemical configuration accepted into the active site of the enzyme. Having established the essential requirements for the electronic configuration of the phenol ring, it was interesting to observe the effect of substitution at the 4-position of the 'phenol' as a substrate for this UDPGT (see Fig. 3).

The parent phenol was a relatively poor substrate, glucuronidated at only 5% of the rate of glucuronidation of 1-naphthol. The compound formed by substitution by a nitro-group at the 4-position of phenol, 4-nitrophenol, was a considerably better substrate, glucuronidated at a rate similar to 1-naphthol. 4-Methyl- and 4-ethyl-phenol were also good substrates, whereas 4-propylphenol and 4-*t*-butylphenol were poor substrates, glucuronidated approximately 10 times more slowly than 4-methylphenol. 4-Hydroxybiphenyl was glucuronidated at a low rate (see Table 1).

These data show that the cloned UDPGT cDNA encoded a transferase that catalyzed the glucuronidation of phenols, when expressed in COS-7 cells. The range and structure of the substrates glucuronidated were similar to that observed when rat kidney microsomes were used as the source of UDPGT. Studies with rat liver microsomes emphasized the presence of other UDPGTs catalyzing the glucuronidation of 4-isopropyl- and 4-*t*-butylphenols, thymol, and menthol.

Glucuronidation of a wide range of potential substrates by the cloned phenol UDPGT expressed in COS-7 cells. Glucuronidation of several drugs or endogenous compounds, e.g., clofibric acid, (−)- and (+)-morphine, testosterone, androsterone, and estrone were not glucuronidated by this cloned enzyme system. Phenolphthalein was also not a substrate for this enzyme. 4-Methylumbelliferone was rapidly glucuronidated, as predicted from assays using rat kidney microsomes, although more than one UDPGT enzyme was probably involved in 4-methylumbelliferone glucuronidation in liver.

Discussion

A rat kidney phenol UDPGT cDNA has been cloned and expressed in COS-7 cells. The protein product generated from

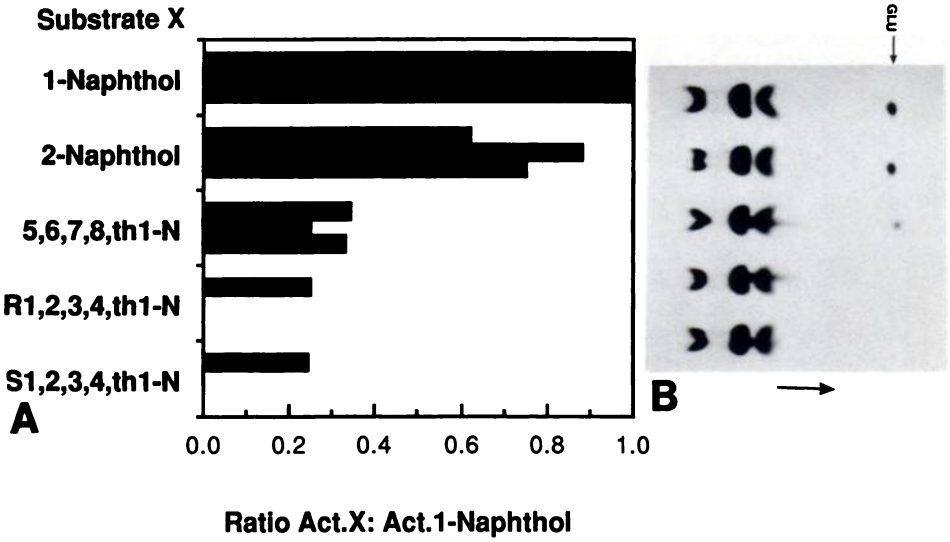


Fig. 2. Glucuronidation of a series of naphthols by phenol UDPGT expressed in COS-7 cells. A, Comparison of the rates of UDPGT activity towards five substrates using liver microsomes (■), kidney microsomes (▧), and cloned transferase in COS-7 cells (■). B, An autoradiograph of a TLC plate showing glucuronides formed during assay of the cloned UDPGT with five different substrates, as indicated in A. The glucuronide spots are indicated by the *arrow*. Direction of migration was from *left* to *right*.

TABLE 1

Glucuronidation of phenolic and other compounds by the cloned UDPGT expressed in COS-7 cells

The results were obtained from at least two independent batches of transfected cells. The specific activity towards 1-naphthol in the different batches of cells used was less than 10%. The numbers in parenthesis indicate the ratio of the UDPGT activity towards each substrate versus the UDPGT activity towards 1-naphthol.

Substrate	UDPGT activity			
	Control cells	UDPGT K39 cells	Rat kidney microsomes	Rat liver microsomes
	pmol/min/mg of protein			
1-Naphthol	0.6	49.7	36.8 (1.0)	81.5 (1.0)
Phenol	0.1	1.3	0.3 (0.01)	3.4 (0.04)
4-Methylphenol	0.5	29.0	19.6 (0.53)	38.4 (0.47)
4-Ethylphenol	ND[a]	30.5 (0.61)	20.3 (0.58)	43.2 (0.53)
4-*n*-Propylphenol	ND	2.7 (0.05)	2.2 (0.06)	82.8 (1.02)
4-Isopropylphenol	ND	7.6 (0.15)	8.2 (0.22)	45.6 (0.56)
4-*t*-Butylphenol	0.31	2.4 (0.05)	4.7 (0.13)	64.4 (0.79)
4-Nitrophenol	0.53	33.5 (0.67)	22.8 (0.62)	40.7 (0.50)
4-Hydroxy biphenyl		4.3 (0.09)	4.9 (0.13)	44.5 (0.55)
Thymol		ND	0.9 (0.02)	7.6 (0.09)
Menthol		ND	ND	20.1 (0.24)
Carvacrol		ND	4.8 (0.13)	28.2 (0.35)
4-Methylumbelliferone	0.01	46.2 (0.93)	36.4 (0.99)	55.9 (0.68)

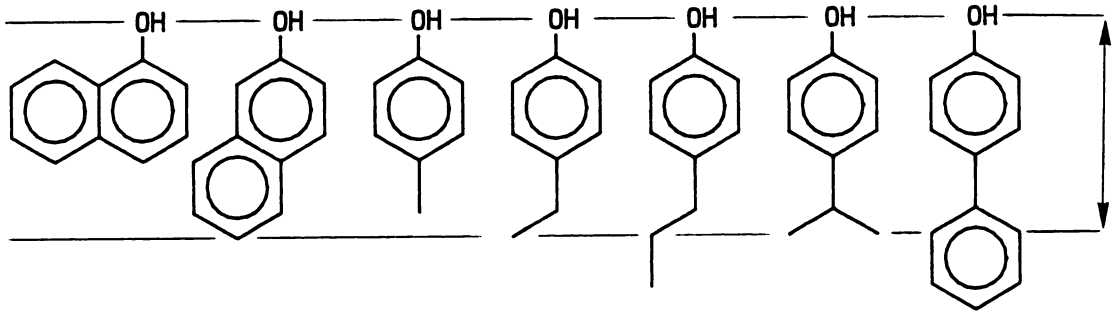
[a] ND, not detectable.

the expressed cDNA was a glycosylated protein of 53 kDa, similar to that purified from rat kidney (10) and identified immunochemically in rat liver especially after treatment of rats with 3-MC (17). Iyanagi *et al.* (6) have proposed that a rat liver UDPGT cDNA encoding a 3-MC-inducible rat mRNA was a 4-nitrophenol UDPGT, based on limited sequence comparisons. The rat kidney phenol UDPGT cDNA used here contains a coding sequence identical to that reported by Iyanagi *et al.* (6), but interesting 5′ sequence differences exist, which may be involved in the tissue-specific expression of this protein.[2] The enzyme is encoded by a unique gene and therefore differences in post-transcriptional processing probably give rise to the different tissue-specific mRNAs.

Studies of the substrate specificity of the UDPGT expressed in COS-7 cells from the cDNA provide direct evidence that the cDNA encoded a 'phenol' UDPGT. The enzyme catalyzed the glucuronidation of several phenolic compounds and 4-methylumbelliferone. The glucuronidation of many other UDPGT substrates was not catalyzed by this cloned isoenzyme. Testosterone and androsterone UDPGT cDNA sequences (18, 19) have been shown to be only 40% homologous to this phenol UDPGT cDNA and expression of these cloned cDNAs (20, 21) has demonstrated their specificity in steroid glucuronidation. Another cloned UDPGT cDNA encoding a phenobarbital-inducible mRNA, when expressed in COS-7 cells, produced an enzyme that catalyzed the glucuronidation of chloramphenicol and 4-hydroxybiphenyl at high rates (21). 4-Hydroxybiphenyl was only glucuronidated by the expressed phenol UDPGT at very low rates, and morphine, which is another substrate glucuronidated by phenobarbital-inducible UDPGTs, was not a substrate for the cloned phenol UDPGT, indicating the substrate specificity differences of the cloned expressed transferases. Phenol UDPGT and the phenobarbital-inducible UDPGT (21) are both capable of glucuronidating 4-methylumbelliferone at high rates, which indicates that 4-methylumbelliferone is a poor substrate for discrimination between different UDPGTs in crude enzyme preparations.

Many of the other compounds assessed as potential substrates are glucuronidated in rats (see Refs. 22 and 23) and glucuronide formation was easily detectable when kidney or hepatic microsomes were used as enzyme source (Table 1). These results showed that compounds such as 4-*n*-propylphenol and 4-hydroxybiphenyl were poorly glucuronidated by kidney microsomes and the expressed phenol UDPGT, in contrast to their rapid glucuronidation by liver microsomes. These data provide further evidence that these substrates must be glucuronidated in liver by other UDPGTs and not phenol UDPGT. In contrast, the substrate specificity found in the kidney was very similar to that observed with the expressed phenol

[2] D. Harding, M. R. Jackson, R. B. Corser, and B. Burchell, manuscript in preparation.



STRUCTURE							
SUBSTRATE	1-naphthol	2-naphthol	4-methyl-phenol	4-ethyl phenol	4-n-propyl-phenol	4-isopropyl-phenol	4-hydroxy-biphenyl
Glucuronidation in K39 COS cells	++++	++++	+++	+++	+	+	+
Length of the substrate	<6.3Å	=6.3Å	<6.3Å	=6.3Å	>6.3Å	=6.3Å	>6.3Å
Bulkiness > or < 4Å	planar	planar	planar	planar	planar	bulky	bulky

Fig. 3. Structural formulae and dimensions of a series of related phenols and naphthols.

UDPGT, suggesting that only this phenol UDPGT is present in this organ.

Examination of the ability of the expressed enzyme to glucuronidate saturated and unsaturated related naphthols showed that an aromatic ring is required to allow the transfer of glucuronic acid to the hydroxyl group. Previous attempts, based on indirect criteria, have been made to discriminate substrates conjugated by separate isoforms, according to their physicochemical structure (23, 24). Our report shows that the thickness of the molecule (4 Å) and its length are indeed critical structural features for its glucuronidation by this phenol UDPGT (Fig. 3). For example, 4-ethylphenol was conjugated at a high rate, in contrast to *n*-propylphenol, but was only 0.7 Å shorter in length. Further, 2-naphthol, which has exactly the same length between the oxygen and the farthest carbon in the same plane as does 4-isopropylphenol (6.3 Å), was glucuronidated approximately 7-fold more rapidly than the latter bulkier compound (>4 Å thick, see Ref. 24). More information on the structural features of compounds glucuronidated by this phenol UDPGT will be revealed by additional studies with this system.

Acknowledgments

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