IDENTIFICATION OF THE RABBIT LIVER UDP-GLUCURONOSYLTRANSFERASE CATALYZING THE GLUCURONIDATION OF 4-ETHOXYPHENYLUREA (DULCIN)

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Received June 28, 2004; accepted September 22, 2004

ABSTRACT:

Dulcin (DL), 4-ethoxyphenylurea, a synthetic chemical about 200 times as sweet as sucrose, has been proposed for use as an artificial sweetener. DL is excreted as a urinary ureido-N-glucuronide after oral administration to rabbits. The phenylurea N-glucuronide is the only ureido conjugate with glucuronic acid known at present; therefore, DL is interesting as a probe to search for new functions of UDP-glucuronosyltransferases (UGTs). Seven UGT isoforms (UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT2B13, UGT2B14, and UGT2B16) have been identified from rabbit liver, but these UGTs have not been investigated using DL as a substrate. In this work, the identities of UGT isoforms catalyzing the formation of DL glucuronide were investigated using rabbit liver microsomes (RabLM) and cloned/expressed as rabbit UGT isoforms. DL-N-glucuronide (DNG) production was determined quantitatively in RabLM and homogenates of COS-7 cells expressing each UGT isoform by using electrospray liquid chromatography-tandem mass spectrometry. Analysis of DNG formation using RabLM, by Eadie-Hofstee plot, gave a \( V_{\text{max}} \) of 0.911 nmol/min/mg protein and the \( K_{i} \) of 1.66 mM. DNG formation was catalyzed only by cloned expressed rabbit UGT1A7 and UGT2B16 (\( V_{\text{max}} \) of 3.98 and 1.16 pmol/min/mg protein and a \( K_{i} \) of 1.23 and 1.69 mM, respectively). Substrate inhibition of UGT1A7 by octylgallate confirmed the significant contribution of UGT1A7 to the formation of DNG. Octylgallate was further shown to competitively inhibit DNG production by RabLM (\( K_{i} = 0.149 \) mM). These results demonstrate that UGT1A7 is the major isoform catalyzing the N-glucuronidation of DL in RabLM.

DL tastes about 200 times as sweet as sugar and previously has been used as an artificial sweetener (Siedler, 1916). Metabolic studies have demonstrated that it has a unique conjugation reaction in rabbits (Akagi et al., 1962). It was reported that DNG, one of the main metabolites of DL in rabbit urine, is produced by glucuronidation of the primary amino group of the ureido residue (Fig. 1) (Akagi et al., 1963). Phenylureas, such as DL, are the only type of ureido compound that has shown evidence of glucuronidation, in vivo (Akagi et al., 1966). This fact suggests that DL might become an important probe for elucidation of substrate recognition mechanisms of UGTs. Publications related to the DL glucuronidation ceased in the 1960s when use of DL as a sweetener was terminated due to its toxicity.

Rabbits are an important animal model of N-glucuronidation since rodents do not possess UGT1A4, the major N-glucuronidating UGT. Characterization of UGT(s) catalyzing the glucuronidation of DL or its analogs has not been reported. At present, seven UGT isoforms from RabL have been sequenced (Tukey et al., 1993; Bruck et al., 1997; Li et al., 1997, 2000; Nguyen and Tukey, 1997). Typical substrates of each isoform were also investigated. In this study, these UGTs were expressed in a mammalian cell system to examine the specificity of the glucuronidation of DL. The DNG produced in the enzyme reaction with the homogenate of each different UGT-expressing cell line was measured by a novel, highly sensitive LC/MS-MS detection system. The results of these investigations are described in this article.

Materials and Methods

Materials. Alamethicin, 2-aminobiphenyl, 1-naphthol, octylgallate, phenolphthalein, propofol, saccharolactone (\( \beta \)-saccharic acid 1,4-lactone), scopolin, and \( \beta \)-s UDP-glucuronic acid (UDPGA) were purchased from Sigma-Aldrich (Gillingham, UK). [Glucuronyl-\( ^{14} \)C(U)]UDPGA (specific activity 293.6 mCi/mmol, 99.7% purity) was purchased from PerkinElmer (Beaconsfield, UK). Formic acid and acetonitrile (HPLC grade) and all other chemicals (highest grade available) were purchased from Merck Eurolab (Poole, UK). Tissue culture media and supplements, including G418 (geneticin), were obtained from Invitrogen (Paisley, UK). Anti-rat liver UGT antibody (RAL) was raised in sheep against glucuronosyltransferase protein purified from rat, as described previously (Coughtrie et al., 1987). RabL UGT1A3, UGT1A4, UGT1A6, UGT1A7, UGT2B13, UGT2B14, and UGT2B16 cDNAs were subcloned into an expression vector, pSVL, kindly provided by Dr. Robert H. Tukey (Departments of Pharmacology, Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA), and the recombinant plasmids were grown in Escherichia coli JM109. DL was synthesized from 4-ethoxyaniline according to the method of Kurzer (1951). The structure of DL was confirmed by nuclear magnetic resonance (NMR) and electron ionization mass spectrometry (data not shown). DNG as a standard
sample was purified from urine of rabbits administered dulcin orally (Akagi et al., 1986). Briefly, rabbits were kept in a metabolism cage designed to permit separate collection of urine and feces. DL (1.5 g) was administered orally to rabbits. The collected 48-h urine was adjusted to pH 4 with glacial acetic acid and treated with saturated lead acetate solution until no further precipitation occurred, and then was removed by filtration. The filtrate was adjusted to pH 7.5 to 8.0 with ammonium hydroxide, and saturated lead acetate solution was added in excess. The lead salt was suspended in 10 ml of hot MeOH. After standing for 2 days at room temperature, the mixture was divided into the solid and mother liquor. To the solid was added 3 ml of water, and then the insoluble materials were discarded by filtration and MeOH was added to the filtrate until a slight turbidity developed. After standing overnight in a refrigerator, 1.4 g of white crystals were obtained. Structure of DNG was confirmed previously by NMR and fast atom bombardment mass spectrometry (see below). The collected 48-h urine was adjusted to pH 4 with glacial acetic acid and treated with saturated lead acetate solution until no further precipitation occurred, and then was removed by filtration. The filtrate was adjusted to pH 7.5 to 8.0 with ammonium hydroxide, and saturated lead acetate solution was added in excess. The lead salt was suspended in 10 ml of hot MeOH. After standing for 2 days at room temperature, the mixture was divided into the solid and mother liquor. To the solid was added 3 ml of water, and then the insoluble materials were discarded by filtration and MeOH was added to the filtrate until a slight turbidity developed. After standing overnight in a refrigerator, 1.4 g of white crystals were obtained. Structure of DNG was confirmed previously by NMR and fast atom bombardment mass spectrometry (see below).

**LC/MS-MS Analysis of DNG.** Confirmation of the formation of DL glucuronide was made with a 100 μM DNG sample in 50% acetonitrile, which was directly infused into the electrospray interface (Quattro LC; Micromass, Manchester, UK) by a syringe pump at a flow of 5 μl/min. The [M + H]⁺ ion was recorded in positive ionization mode (m/z 357.2) at a cone voltage of 25 eV and capillary voltage of 3.0 eV with nebulizing and desolvation gas set at 100 l/h and 500 l/h, respectively. A daughter ion scan was performed of the parent ion (m/z 357.2), over a mass range of 50 to 357 m/z. The fragmentation pattern was compared with predicted structure.

**Dulcin N-Glucuronidation Assay Conditions.** Optimal pH for DL N-glucuronidation was 8.2 in RabLM (data not shown). Microsomes and homogenates of cell lines expressing rabbit UGT isoforms were activated by alamethicin (50 μg/mg protein). Dulcin was incubated with 0.2 mg of microsomes or recombinant UGTs in 100 mM Tris-maleate buffer (pH 8.2), containing 10 mM magnesium chloride, and 10 mM UDPGA for 1 h at 37°C, in a final volume of 100 μl. The reactions were terminated by the addition of 200 μl of chloroform, and 150 μl of supernatant was removed for analysis by LC/MS-MS. Samples were diluted 10-fold in 50% (v/v) acetonitrile/water before analysis by LC/MS-MS using an HP1100 LC system (Agilent Technologies, Stockport, UK), connected to a Micromass Quattro LC with a 10-μl injection per run. The LC separation used a mobile phase of 0.1% formic acid (buffer A) and acetonitrile containing 0.1% formic acid (buffer B). LC separation and elution were achieved using a 2-min isocratic portion at 95% buffer A followed by a short step to 40% buffer A for 3 min, followed by another short step to 95% buffer B. This was followed by a 2-min wash at 95% buffer B and a 3-min re-equilibration step at 80% buffer A. Chromatography was performed on a Waters Spherisorb (ODS2) 2-μm, 2.1 mm × 150 mm column (Waters Milford, MA) at a flow rate of 0.3 ml/min with a 2-cm Hypersil (ODS2) guard column. Mass spectral analysis was performed with direct infusion into the electrospray source, with column diversion during the first 3 min to protect the source from excessive salt. The glucuronide peak from the LC column was analyzed using a multiple reaction monitoring (MRM) method in positive ion mode. Optimized MRM conditions used three transitions from 357.3 → 176 m/z, 357.3 → 158 m/z, 357.3 → 194 m/z at a cone voltage of 40 eV and capillary voltage of 3.0 eV; collision energy was 15, 20, and 10 eV, respectively, and the collision gas was at 3 mbar. The nebulizing gas was set at 100 l/h and the desolvation gas at 700 l/h. To quantify the glucuronide produced, two identical DNG standards, one radiolabeled and one nonradiolabeled, were generated by parallel incubation of DL with RabLM as described above (performed in triplicate). The amount of DNG produced was calculated by analysis of the [14C]DNG using a HPLC radiochemical detection assay (see below). The nonradiolabeled samples were generated in parallel; therefore, they contained the same amount of DNG as measured value for the radiolabeled samples. The nonradioactive standard was then used in conjunction with all analysis to quantify the amount of DNG present.

**Preparation and Treatment of RabLM.** Liver microsomes were prepared from male New Zealand White rabbits as previously described (Coughtrie et al., 1987). Briefly, the liver was minced and homogenized in 4 volumes 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°C; the resulting supernatants were centrifuged at 100,000 g for 60 min at 4°C. Microsomal pellets, 1,000 × g weight compared with the wet weight (in grams) of liver, were resuspended in 1 volume of ice-cold 50 mM HEPES buffer, pH 7.4. Aliquots were stored at −80°C.

**Expression of RabL UGT Isoforms in Transfected COS-7 Cells.** COS-7 cells were maintained in Dulbecco’s minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum and 50 units/ml penicillin/streptomycin. The recombinant plasmid (70 μg/140-mm dish) was transfected into 70% confluent cells by the calcium phosphate/glycerol shock procedure (Parker and Stark, 1979). HEPES buffer, pH 7.2 (50 mM), was added to the medium to maintain constant pH. Cells were harvested 2 days after transfection, washed in 138 mM NaCl, 2.7 mM KCl, 1.5 mM potassium dihydrogen phosphate, 8 mM disodium hydrogen phosphate, pH 7.4, and pelleted by centrifugation at 2000 g for 5 min at 4°C, then stored at −80°C.

**Gradient HPLC Assay of UGTs Using [14C] UDPGA and Radiochemical Detection.** To ease quantification when no glucuronide standard is available and when high activities are observed, it may be preferable to use radiochemical detection (e.g., for probe substrates). Each frozen cell pellet was resuspended in approximately 200 μl of phosphate-buffered saline, followed by homogenization with alamethicin (50 μg/mg protein). UGT assays with the substrates listed in Table 1 were performed as described previously (Ethell et al., 1998). Briefly, 100 mM Tris-maleate buffer, pH 7.4, containing 5 mM magnesium chloride, 10 mM saccaric acid 1,4-lactone, 1 mM concentration of each substrate, approximately 1 mg of cellular homogenate protein, and 2 mM UDPGA (0.1 Ci of [14C]UDPGA/assay) were combined in a total volume of 100 μl. Assays were prewarmed at 37°C for 5 min and the reaction was started with the addition of [14C]UDPGA. Reactions containing cell homogenates of each recombinant UGT isoform were incubated at 37°C for 2 h, and then the reaction was terminated by the addition of 100 μl of methanol that had been prechilled to −20°C. 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

**Fig. 1. Schematic of the formation of DNG from DL.**
TABLE 1
Catalytic activities of COS-7-expressed RabL UGTs

<table>
<thead>
<tr>
<th>Isoform</th>
<th>Substrate</th>
<th>Activities (nmol/min/mg protein)</th>
</tr>
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<tbody>
<tr>
<td>UGT1A3</td>
<td>Scopoletin</td>
<td>0.23 ± 0.02</td>
</tr>
<tr>
<td>UGT1A4</td>
<td>2-Aminobiphenyl</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>UGT1A6</td>
<td>1-Naphthol</td>
<td>5.56 ± 0.81</td>
</tr>
<tr>
<td>UGT1A7</td>
<td>1-Naphthol</td>
<td>0.37 ± 0.01</td>
</tr>
<tr>
<td>UGT2B13</td>
<td>2-Aminobiphenyl</td>
<td>0.08 ± 0.01</td>
</tr>
<tr>
<td>UGT2B14</td>
<td>Phenolphthalein</td>
<td>0.05 ± 0.01</td>
</tr>
<tr>
<td>UGT2B16</td>
<td>2-Aminobiphenyl</td>
<td>0.08 ± 0.01</td>
</tr>
</tbody>
</table>

Fig. 2. Immunoblot analysis of COS-7 cell extracts. pSVL expression plasmids containing UGT1A1, UGT1A3, UGT1A4, UGT1A6, and UGT1A7 (A) and UGT2B (UGT2B13, UGT2B14 and UGT2B16) (B) subfamilies from RabL were transfected into COS-7 cells. Controls were transfected with only the pSVL plasmid. Each aliquot of the cell homogenate (20 μg of protein) was analyzed using SDS-polyacrylamide gel electrophoresis and immunoblotting as described under Materials and Methods.

Discussion

It was reported that DNG was one of the major metabolites of DL in urine of rabbits that received a single dose orally (Akagi et al., 1963). Subsequently, it was demonstrated that the responsive enzyme(s) recognizes an amino group in phenylurea, a basic skeleton of DL, in investigations using analogs of the compound (Akagi et al., 1966). Phenylurea-N-glucuronides resulted from the glucuronidation of DL, and the analogs are the only known ureido glucuronate at present. Ureido glucuronides are also important since methyurea has recently been shown to be a carcinogen (Kitano et al., 1997) and ureido glucuronidation in humans has only recently been demonstrated (Y. Uesawa, manuscript in preparation).

In this study, DL N-glucuronidation activities were measured to characterize the UGT isoform(s) related with this unique conjugation. Typical Michaelis-Menten enzyme kinetics was observed in the RabL microsomal DNG formation reaction (Fig. 4A). The velocity of the glucuronidation was saturated at a concentration of 2 mM DL in the reaction mixture. Eadie-Hofstee plots of DL N-glucuronidation of RabL support an intrinsic clearance measurement (Vmax/Km) in vitro of 0.549 μmol/min/mg protein. In reports of N-glucuronidations of amitriptyline, diphenhydramine, and imipramine in human liver microsomes, the contribution of multiple UGT isoforms was suggested because Eadie-Hofstee plots of the reaction were biphasic (Breyer-
Pfaff et al., 1997; Nakajima et al., 2002); however, the Eadie-Hofstee plots of DL\textsubscript{N}-glucuronidation reported here were monophasic. This observation suggests that one major enzyme is responsible for DL\textsubscript{N}-glucuronidation in RabL. Seven known isoforms from RabL [UGT1A3 (Li et al., 2000), UGT1A4 (Bruck et al., 1997), UGT1A6 (Nguyen and Tukey, 1997; Li et al., 2000), UGT1A7 (Bruck et al., 1997), UGT2B13 (Tukey et al., 1993; Li et al., 1997), UGT2B14 (Tukey et al., 1993), and UGT2B16 (Li et al., 1997)] were transitionally expressed in COS-7 cells to ascertain which UGTs are responsible for DL\textsubscript{N}-glucuronidation. The expression of each isoform was confirmed by immunoblotting with a specific anti-UGT antibody (RAL) (Fig. 2) (Coughtrie et al., 1987). The electrophoretic mobility of each isoform corresponded well to reported values (Tukey et al., 1993; Bruck et al., 1997; Li et al., 1997, 2000; Nguyen and Tukey, 1997). UGT2B13 and UGT2B16 show relatively weak bands (by Western blotting against RAL) compared with the other rabbit isoforms; however, the glucuronidation activities in the cell homogenates expressing these isoforms were similar to those of the other UGTs studied here (Table 1). Both UGT2B13 and UGT2B16 were able to glucuronidate 2-aminobiphenyl equally as well as UGT1A4, which appeared to be better expressed on the immunoblot, as shown in Fig. 2. These observations could be because the anti-UGT antibody has lower affinities to both these UGT2B isoforms or that UGT2B13 and UGT2B16 have better relative activities toward 2-aminobiphenyl.

DNG generation from DL in the incubation mixture with each cell homogenate was measured by LC/MS-MS, a sensitive and selective detection method. As a result, significant DL\textsubscript{N}-glucuronidation activities in UGT1A7 and UGT2B16 were observed; there was no observable activity in UGT1A4, which has been indicated to be important in N-glucuronidations (Bruck et al., 1997). Investigations using Eadie-Hofstee plot analyses of DL\textsubscript{N}-glucuronidation revealed that the active isoforms, UGT1A7 and UGT2B16, had \(K_m\) values of 1.23 and 1.69 mM, respectively (Fig. 5, B and C). The conjugation with RabLM also showed a similar \(K_m\) (1.66 mM) for these results (Fig. 4A). It was reported that bulkier, nonplanar phenols such as 4-\textit{tert}-butylphenol and propylgallate were mainly conjugated by UGT1A7 (Bruck et al., 1997). This enzyme was also capable of generating quaternary amine glucuronides such as imipramine glucuronide (Bruck et al., 1997). However, a clear demonstration of the ureido group N-glucuronidation by UGT1A7 is reported in this study for the first time. It was previously reported that UGT2B16 catalyzes the glucuronidation of bulky phenols such as 2- and 4-hydroxybiphenyl, as well as smaller phenolic compounds like 2-naphthol (Li et al., 1997). The N-glucuronidation activity of UGT2B16 has never been established. As shown in Table 1, UGT1A7 and UGT2B16 have capabilities of N-glucuronidation of 2-aminobiphenyl; this aromatic amine was also conjugated by UGT1A4. DL has been shown here to be an unusual substrate in that the free nitrogen is not glucuronidated by UGT1A4 but is instead formed by UGT1A7 and UGT2B16. Since glucuronidation of the ureido group by any specific UGT has not been previously measured, it is possible that the less basic nature of the

**Fig. 3.** Mass spectrum of DNG run from 100 to 400 m/z in positive ion mode; the spectrum was derived from a daughter ion scan at the dulcin-N-glucuronide mass (m/z 357), from an incubation of DL with RabLM and UDPGA. Peaks correspond to DNG (m/z 357), dehydroxylated DNG (m/z 339), and DL (m/z 181). Peak at m/z 194 represents the amino glucuronic acid fragment, with consequential dehydroxylations at m/z 176 and m/z 158.
nitrogen prevents glucuronidation by UGT1A4, which exhibits high activity toward basic amine nitrogens.

An inhibition experiment was performed with octylgallate to confirm the contribution of UGT1A7 and UGT2B16 to the microsomal DL N-glucuronidation. The N-glucuronidation activity with 1 mM DL was almost completely inhibited by 1 mM and 2 mM octylgallate (94.7% and 99.3% inhibition, respectively). Dixon plot analysis illustrated that DL N-glucuronidation was competitively inhibited by octylgallate ($K_i = 0.143$ mM) (Fig. 5). Such observations suggest that octylgallate might be a substrate of a major UGT isoform catalyzing DL N-glucuronidation. It has been reported that octylgallate was a substrate for UGT1A7, but not UGT2B16 (Bruck et al., 1997; Li et al., 1997). Therefore, the current observations clearly demonstrated that UGT1A7 is a major UGT that glucuronidates DL.

In conclusion, it was discovered in this investigation that UGT1A7 and UGT2B16 from RabL catalyze the conjugation reaction on the amino group of DL, which is a unique ureido-N-glucuronidation. In particular, it was suggested that UGT1A7 is a major enzyme for the conjugation in RabL. Interestingly, DL was not glucuronidated with UGT1A4, the most important UGT1A isoform for N-glucuronidations. Information about interactions between the ureido group of DL and UGT1A7 or UGT2B16 will contribute to elucidation of recognition mechanisms of UGT isoforms with substrates.

Acknowledgments. We acknowledge Dr. Takashi Uesugi and Dr. Kazumi Sano at Meiji Pharmaceutical University for introduction to the study of dulcin. We thank Dr. Robert H. Tukey at University of California for providing cDNAs from RabL. We are grateful to members of the Division of Pathology and Neuroscience at University of Dundee for skillful technical assistance.

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