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Mono- and Diglucuronide Formation from Chrysene and Benzo(*a*)pyrene Phenols by 3-Methylcholanthrene-Inducible Phenol UDP-Glucuronosyltransferase (UGT1A1)

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SUMMARY

Mono- and diphenols of chrysene and benzo(*a*)pyrene are suspected substrates of a 3-methylcholanthrene (MC)-inducible phenol UDP-glucuronosyltransferase (UGT1A1). Mono- and diglucuronide formation from these compounds was studied in two systems, (a) livers of MC-treated rats (homologous expression) and (b) a Chinese hamster lung fibroblast cell line (V79) containing rat UGT1A1 cDNA and stably expressing this isozyme (heterologous expression). In liver microsomes of MC-treated rats, glucuronidation of 6-hydroxychrysene was stimulated 11-fold by MC treatment. With 3,6-dihydroxychrysene, formation of its 3-hydroxy-6-monoglucuronide and of the diglucuronide was increased 24- and 310-fold, respectively. Induction factors obtained for monoglucuronide formation (but not for diglucuronide formation) were in line with published data on the increase of immunodetectable UGT1A1 protein and of its mRNA. It is suggested that the high induction factors for diglucuronide formation are the result of a combination of the induction of UGT1A1 and facilitated interaction of neighboring UGT1A1 molecules in endoplasmic reticulum membranes. Glucuronidation of 6-hydroxychrysene, 3-hydroxybenzo(*a*)pyrene, and 3,6-dihydroxybenzo(*a*)pyrene to their mono- and diglucuronides was clearly detectable in V79 cells expressing UGT1A1. However, conjugation of 3,6-dihydroxychrysene to its monoglucuronides was low and diglucuronide formation was not detectable, suggesting that UGT isozymes other than UGT1A1 are responsible for these reactions.

UGT represents a family of isozymes located in endoplasmic reticulum membranes of liver and various extrahepatic tissues, such as intestine, kidney, and olfactory epithelium (1, 2). With regard to the function of rat liver UGT isozymes, considerable overlap of substrate specificity has been observed with both endogenous and exogenous substrates (3–5). For example, widely used standard substrates such as 1-naphthol are efficiently conjugated both by MC-inducible phenol UGT (4NP UGT, UGT1*06, or UGT1A1) and by testosterone/phenol UGT (UGT2B3) (3). Phenolic metabolites of PAH have been suggested to be more selective substrates of UGT1A1 (6–8). This suggestion was based on markedly higher induction factors, after MC treatment, with PAH phenols such as 3-hydroxybenzo(*a*)pyrene (10-fold), compared with standard substrates such as 1-naphthol (4-fold). The induction of UGT activity toward 3-hydroxybenzo(*a*)pyrene roughly corresponds to the increase of UGT1A1 mRNA (about 10–15-fold) (9, 10). However, an unusually high induction factor for diglucuronide formation was found in studies of mono- and diglucuronide formation from 3,6-dihydroxybenzo(*a*)pyrene (40-fold) (11). Glucuronidation of these diphenols was of interest because it prevents toxic redox cycles between quinones and the diphenols. To further investigate the selectivity of PAH phenols as substrates of UGT1A1, mono- and diglucuronide formation was studied with 3- and 6-hydroxychrysene and with 3,6-dihydroxychrysene (Fig. 1). These compounds are chemically more stable, easier to synthesize, and safer to handle than the corresponding benzo(*a*)pyrene derivatives (12, 13). Glucuronidation of the PAH phenols was also studied using a Chinese hamster fibroblast cell line (V79) containing rat UGT1A1 cDNA and stably expressing this isozyme (14). V79 cells are widely used for stable transfection of drug-metabolizing enzymes, with the aim of characterizing their substrate specificity.

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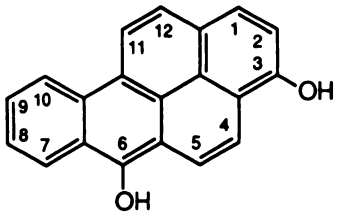
Materials and Methods

Chemicals. 3-Hydroxybenzo(*a*)pyrene and 3,6-dihydroxybenzo(*a*)pyrene were obtained from the Chemical Carcinogen Reference Standard Repository, National Institutes of Health (Bethesda,

ABBREVIATIONS: UGT, UDP-glucuronosyltransferase (EC 2.4.1.17); PAH, polycyclic aromatic hydrocarbons; MC, 3-methylcholanthrene; M6, 3,6-dihydroxychrysene 3-hydroxy-6-glucuronide; M3, 3,6-dihydroxychrysene 6-hydroxy-3-glucuronide; HPLC, high performance liquid chromatography.

3,6-Dihydroxybenzo(a)pyrene



3,6-Dihydroxychrysene

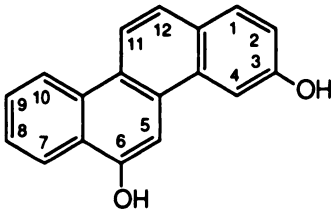


Fig. 1. Chemical structures of 3,6-dihydroxybenzo(*a*)pyrene and 3,6-dihydroxychrysene.

MD). 6-Hydroxy- and 3,6-dihydroxychrysene were synthesized by a route previously described for other phenolic metabolites of chrysene (13). The identity of the compounds was confirmed by high-field proton NMR spectroscopy [6-hydroxychrysene (^{1}H NMR, 400 MHz, acetone-d_6/D_2O): δ 8.85 (1, d, H_{10}, $J_{9,10}$ = 8.5 Hz), 8.72 (1, d, H_{11}, $J_{11,12}$ = 9.0 Hz), 8.68 (1, d, H_4, $J_{3,4}$ = 8.2 Hz), 8.42 (1, dd, H_7, $J_{7,8}$ = 7.7 Hz, $J_{7,9}$ = 1.1 Hz), 8.18 (1, s, H_5), 7.99 (1, dd, H_1, $J_{1,2}$ = 8.4 Hz, $J_{1,3}$ = 1.5 Hz), 7.86 (1, d, H_{12}), 7.76–7.58 (4, m, H_2, H_3, H_8, H_9); 3,6-dihydroxychrysene (^{1}H NMR, 400 MHz, acetone-d_6/D_2O): δ 8.79 (1, d, H_{10}, $J_{9,10}$ = 8.4 Hz), 8.48 (1, d, H_{11}, $J_{11,12}$ = 9.0 Hz), 8.39 (1, d, H_7, $J_{7,8}$ = 8.0 Hz), 8.00 (1, s, H_5), 7.97 (1, d, H_4, $J_{2,4}$ = 2.2 Hz), 7.83 (1, d, H_1, $J_{1,2}$ = 8.6 Hz), 7.75 (1, d, H_{12}), 7.68–7.64 (2, m, H_9, H_8), 7.21 (1, dd, H_2)] and mass spectrometry. Their purity was found to be >98%, based on HPLC analysis. UDP-[^{14}C]glucuronic acid (260 mCi/mmol) was obtained from Amersham Buchler (Braunschweig, FRG) and Brij 58 (a condensate of hexadecyl alcohol with 20 mol of ethylene oxide/mol) from Atlas (Essen, FRG).

Treatment of animals and preparation of liver microsomes. Male Wistar rats (200 g) were used. MC (40 mg/kg, dissolved in olive oil) was given once intraperitoneally, and the animals were killed after 4 days. Phenobarbital, sodium salt (100 mg/kg, dissolved in saline), was given once intraperitoneally, followed by 0.1% (w/v) of the drug in the drinking water for 4 days. Liver microsomes were prepared as described (15). Microsomal protein was determined according to the method of Lowry *et al.* (16).

Stable transfection of UGT1A1 into V79 cells. Chinese hamster V79 cells were grown in Dulbecco's modified Eagle's medium (GIBCO, UK) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 units/ml streptomycin, and 100 units/ml penicillin, in a humidified atmosphere of 5% CO_2, at 37°.

V79 cells were stably transfected by the calcium phosphate glycerol shock procedure (17), as previously described (14). Briefly, 20 μg of linearized eukaryotic expression vector pKCRH2 containing the rat UGT1A1 clone and 5 μg of linearized SFVneo plasmid DNA were applied to approximately 30% confluent V79 cells as calcium phosphate DNA co-precipitate. The cells were split 1:5 after 2 days of culture and were grown in medium containing G418 (Geneticin; 800 μg/ml; Sigma, UK). The selective medium was changed every 2–3 days, to remove dead cells and to allow colonies of resistant cells to grow. Resistant colonies were picked individually and propagated for assay.

Cell monolayers were washed with cold 0.9% sodium chloride and harvested by scraping with 0.25 M sucrose/0.01 M Tris·HCl, pH 7.4 (buffered sucrose). The cells were then centrifuged for 5 min at 1000 × *g*, resuspended with buffered sucrose, and hand-homogenized with a Dounce homogenizer. The homogenate was centrifuged for 10 min at 10,000 × *g*. The resulting supernatant was centrifuged for 60 min at 100,000 × *g* to prepare microsomes, which were suspended in buffered sucrose and stored at −80°. The yield of UGT activity toward 4-methylumbelliferone in the microsomal fraction was about 70% of the homogenate activity.

V79 cells express low levels of their own phenol UGT. Endogenous UGT activities toward 4-methylumbelliferone, 1-naphthol, 1-naphthylamine, and 6-hydroxychrysene comprised 5–10% of those of the expressed rat UGT1A1. Therefore, endogenous UGT activity toward these substrates had to be subtracted. The cell line stably expressed UGT activity over 10 passages, up to the described experiments.

Immunoblot analysis. Analysis was carried out as previously described (18). Anti-rat UGT1A1 antibodies (broad spectrum) were used.

UGT assays. Described methods were used for the assay of UGT activity toward 1-naphthol (15), 4-methylumbelliferone (19), 1-naphthylamine (20), 3-hydroxybenzo(*a*)pyrene (6), and 3,6-dihydroxybenzo(*a*)pyrene (11). UGT activities were determined in the presence of Brij 58 (0.5 mg/mg of protein).

UGT activity toward 3- and 6-hydroxychrysene and toward 3,6-dihydroxychrysene. Chrysene phenols (0.2 mM) and microsomal protein (0.2 mg) were incubated in the presence of 0.2 M Tris·HCl, pH 7.4, 5 mM $MgCl_2$, and Brij 58 (0.5 mg/mg of protein), in a total volume of 500 μl. The reaction was started by the addition of 3 mM UDP-glucuronic acid. With microsomes of V79 cells, 0.4 mg of protein was used. After incubation for 2 min at 37° (or for 10 min when diglucuronide formation from 3,6-dihydroxychrysene was measured), the reaction was stopped by the addition of 50 μl of $HClO_4$. After centrifugation of the denatured protein, the supernatant was filtered through a Millex-HV_{13} (0.45-μm) filter (Millipore, Bedford, MA) for HPLC analysis.

HPLC determination of mono- and diglucuronides of chrysene and benzo(*a*)pyrene phenols. Glucuronides were separated by reverse phase HPLC. The system consisted of a Waters HPLC system (Millipore) equipped with two 501/510 pumps, a variable-wavelength spectrophotometer (model 481) to measure UV absorbance at 270 nm, a fluorescence detector (model S 3400; Sykam, Gilding, FRG), and an IBM XT computer (model 286). Separation of the glucuronides was carried out on a Latek Spherisorb C_{18}-2 column (250 × 4.6 mm, 5 μm). The mobile phase was composed of two solvents. Solvent A consisted of 0.05 M ammonium acetate, pH 6.4, and solvent B of 0.05 M ammonium acetate, pH 6.4/acetonitrile (70/30, v/v). A linear gradient of 100% solvent A to 100% solvent B was used up to 15 min, followed by isocratic elution with 100% solvent B. Glucuronides of 6- and 3-hydroxychrysene eluted at retention times of 17.3 and 18 min. Mono- and diglucuronides of 3,6-dihydroxychrysene eluted at retention times of 13, 17.2, and 17.9 min for the diglucuronide, M6, and M3, respectively. Glucuronides were identified in UGT incubation mixtures containing UDP-[^{14}C]glucuronic acid. Glucuronides were first concentrated on SEP-PAK C_{18} cartridges and then separated using the HPLC system described above. Radioactivity was determined by liquid scintilation counting. A linear relationship was observed between radioactivity, fluorescence, and UV absorption and was used to calibrate glucuronide formation. Assignment of M3 and M6 was based on the retention times of the corresponding 3- and 6-hydroxychrysene monoglucuronides. 3,6-Dihydroxybenzo(*a*)pyrene mono- and diglucuronides were formed and identified using the UGT incubation mixture and the HPLC system described above. Di- and monoglucuronides eluted with retention times of 12 and 16 min, respectively.

UGT activity toward 6-hydroxychrysene. The incubation mixture was the same as described above. The reaction was stopped by rapid mixing with chloroform (2 ml) and centrifugation. An aliquot of the aqueous supernatant (200 μl) was added to 1.6 M glycine-NaOH

buffer, pH 10.3 (500 μl). Fluorescence was determined using a Perkin Elmer LS 5B spectrophotometer, using 330 nm and 390 nm as excitation and emission wavelengths, respectively. Fluorescence intensity was calibrated using glucuronides isolated by HPLC.

Results

Glucuronidation of hydroxychrysenes. UGT activity toward 6-hydroxychrysene proved to be selectively induced by MC treatment (11-fold), whereas 3-hydroxychrysene appeared to be an overlapping substrate of both MC- and phenobarbital-inducible UGTs (Table 1). These observations confirm the regioselectivity of UGT1A1 described previously (7). When glucuronidation of 3,6-dihydroxychrysene was studied, the two monoglucuronides (M6 and M3) and the diglucuronide were well separated by HPLC (Fig. 2). Formation of the diglucuronide was barely detectable in microsomes of untreated controls but was markedly stimulated (together with the formation of M6) after MC treatment. The influence of substrate concentration on the formation of the three glucuronides was complex (Fig. 3). At low substrate concentration, rapid formation of M6 and of the diglucuronide was observed. However, formation of the diglucuronide appeared to be inhibited at concentrations of >0.2 mM 3,6-dihydroxychrysene. In contrast, the formation of M3 steadily increased with increasing substrate concentration. Time-course studies at 0.2 mM 3,6-dihydroxychrysene also showed rapid formation of M6, which appeared to be subsequently converted to the diglucuronide in a precursor-product relationship (Fig. 4). Differential induction of mono- and diglucuronide formation was striking with 3,6-dihydroxychrysene (Table 2). After MC treatment, formation of M6 and of the diglucuronide was increased 24- and 310-fold, respectively.

TABLE 1

Differential induction by MC and phenobarbital of UGT activities toward 6-hydroxy- and 3-hydroxychrysene

Data represent the means ± standard deviations of four experiments.

Treatment	UGT activity	
	6-Hydroxychrysene	3-Hydroxychrysene
	nmol/min/mg of protein	
Untreated control	3.0 ± 0.7 (1)[a]	6.0 ± 0.9 (1)
Phenobarbital	5.0 ± 1.4 (1.7)	14.4 ± 2.2 (2.4)
MC	32.4 ± 6.4 (10.8)	18.7 ± 2.6 (3.1)

[a] Induction factor, i.e., ratio of enzyme activities obtained from inducer-treated rats and from untreated controls.

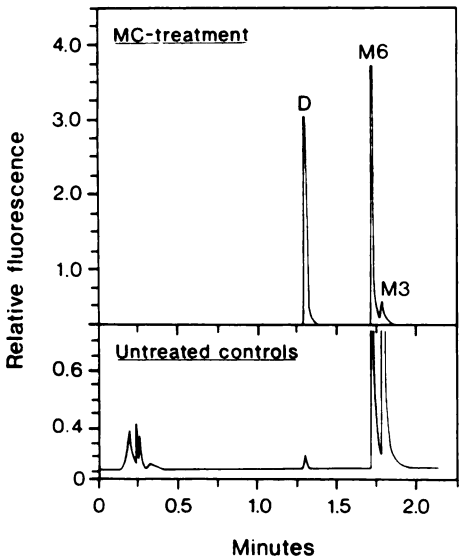


Fig. 2. HPLC separation of 3,6-dihydroxychrysene mono- and diglucuronides. Glucuronides were generated in the UGT assay mixture, which was incubated for 10 min with microsomes from MC-treated rats or untreated controls. Incubations were stopped with perchloric acid, and 20 or 100 μl of the supernatants were injected into the HPLC system, respectively. UV absorption and radioactivity of [^{14}C]glucuronic acid-labeled glucuronides corresponded to their fluorescence. *D*, diglucuronide.

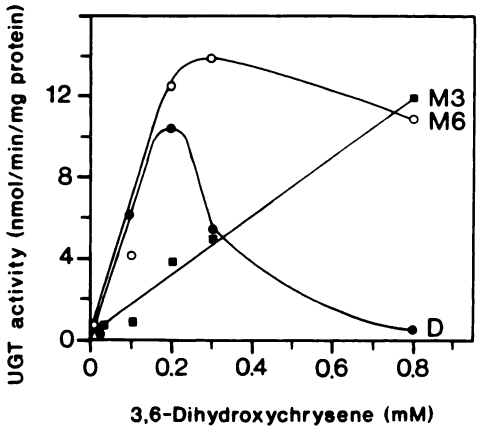


Fig. 3. Influence of 3,6-dihydroxychrysene concentration on the formation of its mono- and diglucuronides. 3,6-Dihydroxychrysene was incubated with liver microsomes from MC-treated rats. Incubation time was 2 min for monoglucuronide and 10 min for diglucuronide formation. *Points*, means of three experiments. *D*, diglucuronide.

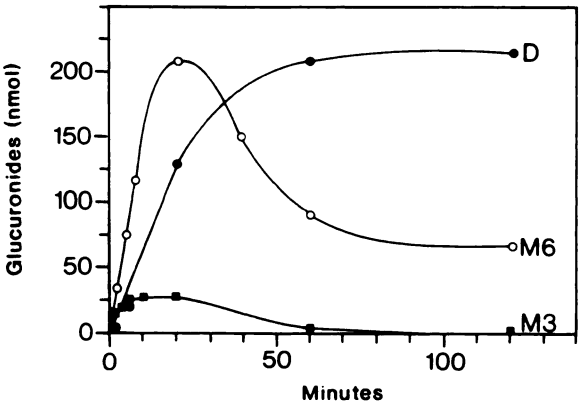


Fig. 4. Time-course of the formation of 3,6-dihydroxychrysene mono- and diglucuronides. 3,6-Dihydroxychrysene (0.2 mM) was incubated with liver microsomes from MC-treated rats. *Points*, means of two experiments. *D*, diglucuronide.

TABLE 2

Differential induction by MC and phenobarbital of UGT activities toward 3,6-dihydroxychrysene

Data represent the means ± standard deviations of four experiments.

Treatment	UGT activity		
	M6	M3	Diglucuronide
	nmol/min/mg of protein		
Untreated control	0.6 ± 0.1 (1)[a]	2.2 ± 0.5 (1)	0.02 ± 0.01 (1)
Phenobarbital	1.7 ± 0.3 (3)	5.6 ± 1.6 (3)	0.14 ± 0.01 (7)
MC	14.6 ± 1.0 (24)	6.2 ± 0.4 (3)	6.20 ± 0.30 (310)

[a] Induction factor, i.e., ratio of enzyme activities obtained from inducer-treated rats and from untreated controls.

Mono- and diglucuronide formation from PAH phenols by V79 cell-expressed UGT1A1. Immunoblot analysis with anti-rat UGT1A1 antibodies (broad spectrum) was used to detect the UGT1A1 protein expressed in transfected cells. For comparison, rat liver microsomes from MC-treated and untreated rats were blotted in parallel (Fig. 5). The molecular weight (55,000) of the protein was identical to that of the MC-induced polypeptide in liver, indicating synthesis of UGT1A1 protein of the correct size. It is not clear whether the smaller band (M_r 54,000–55,000) results from differential processing of the mRNA or differential maturation of the same protein.

With fresh homogenates of UGT1A1-transfected V79 cells, UGT activity toward 4-methylumbelliferone and 6-hydroxychrysene was activated by detergent 8-fold and 3-fold, respectively (data not shown). Maximal activation was obtained with the addition of 0.5 mg of Brij 58/mg of protein. Detergent activation of the enzyme suggests that UGT1A1 has been incorporated into the endoplasmic reticulum membranes of V79 cells, as in liver, presumably with the active site on the luminal face of the membrane (2).

With UGT1A1-transfected V79 cell microsomes, the formation of both mono- and diglucuronides of 3,6-dihydroxybenzo(*a*)pyrene could be clearly demonstrated, in contrast to studies with V79 cells without integrated UGT1A1 (Fig. 6). The formation of the diglucuronide in fluorescence tracings is overemphasized, because the relative fluorescence of the diglucuronide was found to be 7-fold higher than that of 3-hydroxy-6-monoglucuronide (11). Formation of the 6-hydroxy-3-monoglucuronide of 3,6-dihydroxybenzo(*a*)pyrene has not been detected (11).

UGT activities with a number of standard substrates and PAH phenols were studied and compared with those of liver microsomes from MC-treated rats (Table 3). With standard substrates, similar ratios of UGT activities were found in the two systems. However, with PAH phenols, and in particular with 0.2 mM 3,6-dihydroxychrysene, relative UGT activities were low in microsomes from V79 cell-expressed UGT1A1. Diglucuronide formation from 3,6-dihydroxychrysene was below the detection limit. Lower concentrations did not lead to higher UGT activity, suggesting that the lack of diglucuronide formation was not due to substrate inhibition. When intact cells were incubated for 20 hr with 3,6-dihydroxychrysene in sulfate-free medium containing 5 mM glucose (conditions preventing the formation of sulfate esters) (11), the two monoglucuronides were clearly formed, whereas diglucuronide formation was undetectable (data not shown). Kinetic analysis was carried out with 6-hydroxychrysene as substrate. Similar apparent K_m values of 0.1 mM were determined both with microsomes from MC-treated rats and with microsomes of V79 cell-expressed UGT1A1 (data not shown).

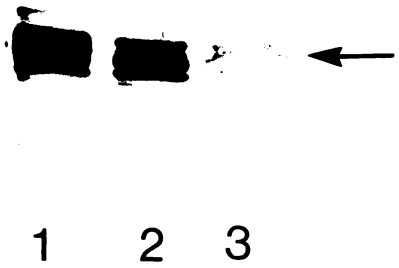


Fig. 5. Immunoblot analysis of expressed rat UGT1A1 protein in transfected V79 cells. Microsomes were electrophoresed on sodium dodecyl sulfate-7.5% polyacrylamide gels and were probed with anti-rat UGT1A1 antibodies (broad spectrum). *Lane 1*, liver microsomes of MC-treated rats (5 μg); *lane 2*, liver microsomes of untreated rats (5 μg); *lane 3*, microsomes of V79 cell-expressed rat UGT1A1 (75 μg). With microsomes from untransfected V79 cells (mock-transfected cells), no immunochemical reaction could be detected (not shown).

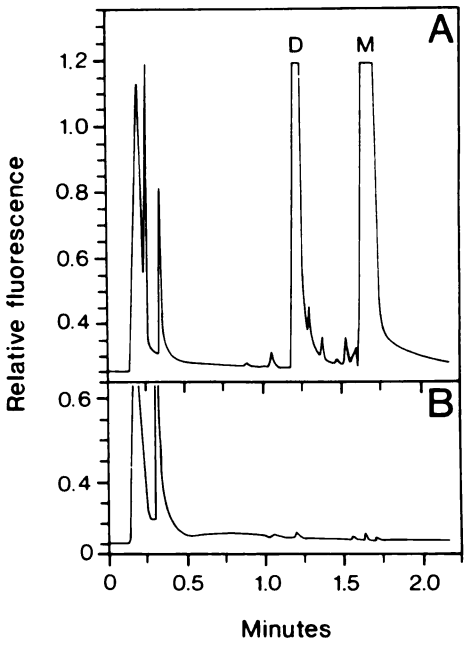


Fig. 6. HPLC separation of 3,6-dihydroxybenzo(*a*)pyrene mono- and diglucuronides. Glucuronides were generated in the UGT assay mixture, which was incubated for 10 min with microsomes (0.4 mg of protein) of V79 cell-expressed UGT1A1 (A). Analysis of incubation mixtures with untransfected V79 cells is shown for comparison (B). The reaction was stopped by mixing with $CHCl_3$, and 200 μl of the aqueous supernatant were injected into the HPLC system. Fluorescent peaks were identified by comparison with the corresponding glucuronides generated with liver microsomes from MC-treated rats. *M*, 3-hydroxy-6-monoglucuronide + traces of 6-hydroxy-3-monoglucuronide; *D*, diglucuronide.

Discussion

Glucuronidation of 6-hydroxychrysene and diglucuronide formation from 3,6-dihydroxychrysene have been shown to be markedly increased by MC treatment (11- and 310-fold, respectively), suggesting that these compounds represent selective substrates of MC-inducible UGTs. They appear to be promising marker substrates, because they are stable, easy to synthesize, and not genotoxic (12).

PAH phenols appear also to be substrates of other UGT isozymes. This is suggested by glucuronidation of PAH phenols with liver microsomes from untreated rats, which contain little UGT1A1 (18). Moreover, MC-inducible UGT shows regioselectivity for certain planar molecules, suggesting a distinct geometry of its binding site (7). Nevertheless, PAH phenols with high induction factors for UGT activities (about 10-fold) have been shown to be substrates of V79 cell-expressed UGT1A1, confirming and extending earlier findings with conventionally purified UGT1A1 (18). When mono- and diglucuronide formation from chrysene and benzo(*a*)pyrene phenols is compared, both similarities and differences are recognized. In liver

TABLE 3

UGT activities in microsomes of V79 cell-expressed UGT1A1 and of livers of MC-treated rats

Data represent the means ± standard deviations of at least four determinations. In parenthesis, ratios of UGT activities are listed, based on those toward 4-methylumbelliferone.

Substrate	UGT activity	
	Liver (MC-treated)	UGT1A1 (V79-expressed)
	nmol/min/mg of protein	
Standard substrates		
4-Methylumbelliferone	159 ± 18 (1)	12.1 ± 0.4 (1)
1-Naphthol	142 ± 26 (0.9)	9.0 ± 0.4 (0.7)
1-Naphthylamine	74 ± 19 (0.5)	7.2 ± 1.0 (0.6)
PAH phenols		
3-Hydroxybenzo(*a*)pyrene	37 ± 3 (0.2)	0.07 ± 0.01 (0.006)
6-Hydroxychrysene	32 ± 6 (0.2)	0.27 ± 0.02 (0.02)
3,6-Dihydroxybenzo(*a*)pyrene	64 ± 6 (0.4)	1.5 ± 0.2 (0.1)
3,6-Dihydroxybenzo(*a*)pyrene 6-monoglucuronide	10 ± 4 (0.06)	0.09 ± 0.03 (0.007)
3,6-Dihydroxychrysene	14.6 ± 1[a] (0.09)	0.016 ± 0.005[a] (0.001)
	6.2 ± 0.4[b] (0.04)	0.014 ± 0.008[b] (0.001)
3,6-Dihydroxychrysene monoglucuronides[c]	6.2 ± 0.3 (0.04)	ND[d]

[a] Formation of M6.
[b] Formation of M3.
[c] Initial rates of diglucuronide formation were measured starting from 3,6-dihydroxychrysene, as indicated in Fig. 4.
[d] ND, not detectable.

microsomes, both mono- and diglucuronide formation from the two diphenols is strongly inducible by MC treatment, with induction factors being higher with the chrysene derivative (discussed below). As far as regioselectivity of the glucuronidation of 3- and 6-monophenols is concerned, conjugation of 3- and 6-hydroxybenzo(*a*)pyrene is selectively MC inducible (7). In contrast, with the chrysene derivatives only 6-hydroxychrysene glucuronidation is selectively MC inducible, whereas 3-hydroxychrysene appears to be an overlapping substrate of several UGTs.

It is intriguing that the formation of diglucuronides is strikingly stimulated by MC treatment [with 3,6-dihydroxybenzo(*a*)pyrene, 40-fold; with 3,6-dihydroxychrysene, 310-fold]. The induction factor for monoglucuronide formation appears to be in line with the increase of the immunodetectable UGT1A1 protein (10-fold, based on densitometry of immunoblots, as published previously) (18) and the increase of its mRNA level (10–15-fold) (9, 10). Diglucuronide formation from 3,6-dihydroxybenzo(*a*)pyrene was clearly detectable with the V79 cell-expressed UGT1A1 (Fig. 6). Diglucuronide formation from 3,6-dihydroxybenzo(*a*)pyrene was also efficiently catalyzed by the conventionally purified enzyme (18). Similarly, bilirubin diglucuronide formation is catalyzed by cell-expressed human bilirubin UGTs (21) and by the conventionally purified rat enzyme (22). Hence, additional UGT isozymes may not be necessary for diglucuronide formation of diphenols and dicarboxylic acids such as bilirubin. An explanation for the unusually high induction factors for diglucuronide formation may be a combination of the effects of the induction of UGT1A1 isozymes and of their facilitated interaction by lateral movement within the plane of the membrane. When the monoglucuronide of a diphenol is formed at the active site of UGT1A1, the molecule has to be shifted for attachment of the second glucuronic acid moiety. This shift may not be easy, because a bulky polar group has already been attached. It may be easier to release the monoglucuronide, which then binds to neighboring UGT1A1 molecules for diglucuronide formation. Interaction between different UGT1A1 molecules may also explain the marked substrate inhibition at high concentrations of 3,6-dihydroxychrysene (Fig. 3). At high substrate concentrations, all neighboring UGT1A1 molecules may be occupied while being involved in monoglucuronide formation. It remains to be determined whether this interaction of UGT1A1 molecules may be viewed as a collision of enzyme molecules by lateral movement within the plane of the endoplasmic reticulum membrane or as a result of the formation of oligomeric complexes. Radiation-inactivation analysis represents a method that determines the target size of the radiation-inactivated enzyme, i.e., the molecular mass of the membrane-bound enzyme *in situ* (23). Previous studies using this method suggest, for bilirubin UGT, a model composed of four subunits. Whereas the formation of the monoglucuronide was found to be mediated by a single subunit, for bilirubin diglucuronide formation the complete tetrameric enzyme appeared to be required (24). Hence, radiation-inactivation analysis of 3,6-dihydroxychrysene glucuronidation may be useful to substantiate the concept that neighboring UGT isozymes have to interact in diglucuronide formation.

V79 cell-expressed UGT1A1 clearly catalyzed glucuronidation of certain PAH phenols, in addition to standard substrates (Table 3). However, UGT activities toward PAH phenols were relatively low with microsomes from cell-expressed UGT1A1, in comparison with those found in microsomes of MC-treated rats. In particular, diglucuronide formation from 3,6-dihydroxychrysene was not detectable with UGT1A1-transfected cells (both microsomal fractions and intact cells), although formation of the two monoglucuronides was clearly detectable. Formation of a UGT1A1 protein of the correct size was indicated by immunoblot analysis. Furthermore, similar apparent K_m values (0.1 mM) for 6-hydroxychrysene glucuronidation with microsomes of MC-treated rats and the cell-expressed UGT1A1 point to the correct integration of the isozyme into endoplasmic reticulum membranes. Taken together, these observations suggest that UGT isozymes other than UGT1A1 are involved in diglucuronide formation of 3,6-dihydroxychrysene.

Comparative studies of more than one UGT isozyme have to be carried out to understand fully their substrate specificity. This notion is supported by recent comparative studies of two

similarly transfected human phenol UGTs, HlugP1 (14, 25), which is considered to be an orthologue of rat UGT1A1, and HlugP4 (26), which, in addition to planar phenols, conjugates bulky phenols. 6-Hydroxychrysene appeared to be a high affinity substrate of HlugP4 but not of HlugP1.[1] Apparent K_m values of 0.1 and 0.02 mM have been determined with microsomes of V79 cells transfected with HlugP1 and HlugP4, respectively. Interestingly, diglucuronide formation from 3,6-dihydroxychrysene was clearly detectable with HlugP4 but not with HlugP1, the putative orthologue of rat UGT1A1. The relatively high apparent K_m value of 0.1 mM suggests that 6-hydroxychrysene is not a high affinity substrate of UGT1A1. The discrepancy between cell-expressed UGT1A1 activities and those found in liver microsomes of MC-treated rats points to the existence of undiscovered high affinity UGT isozymes for the investigated substrates. A gene and mRNA for a novel MC-inducible rat liver UGT (A-10) have been found (27). However, it remains to be elucidated to what extent this or other MC-inducible UGTs are expressed in rat tissues.

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