Biochemical Pharmacology, Vol. 45, No. 9, pp. 1809–1814, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00

PARACETAMOL GLUCURONIDATION BY RECOMBINANT RAT AND HUMAN PHENOL UDP-GLUCURONOSYLTRANSFERASES

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(*Received* 17 *November* 1992; *accepted* 1 *February* 1993)

Abstract—Stably expressed human and rat phenol UDP-glucuronosyltransferases (UGTs) of the UGT1 complex (HlugP1, HlugP4 and 3-methylcholanthrene-inducible rat UGT1A1, the latter considered to be an orthologous enzyme to HlugP1) have been used to investigate the role of UGTs in paracetamol glucuronidation. Kinetic analysis of recombinant UGTs was compared to that of total UGT activities in liver microsomes. Paracetamol was found to be an overlapping substrate of several UGTs. It shows higher affinity for HlugP1 and rat UGT1A1 (apparent K_m values of 2 and 3 mM, respectively) than for HlugP4 (K_m = 50 mM) and other UGTs present in liver microsomes (K_m values of >12 mM). Glucuronidation of paracetamol with HlugP1 contrasts with that of 6-hydroxychrysene and of 4-methylumbelliferone, which are conjugated with higher affinity by HlugP4 than by HlugP1. Due to the wide tissue distribution of rat UGT1A1, paracetamol glucuronidation was also investigated in extrahepatic rat and human tissues. Paracetamol UGT activity was present and inducible by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in rat kidney, lung and spleen. It was also detected in human kidney. A selective cDNA probe for exon 1 of HlugP1 cross-reacted with mRNA from both human liver and kidney. The results demonstrate that paracetamol is conjugated by HlugP1 and its rat orthologue UGT1A1 with higher affinity than by HlugP4 and other UGTs.

Paracetamol (acetaminophen) is a widely used non-prescription analgesic/antipyretic drug which, at an oral dose of 1000 mg, is mostly excreted as glucuronide (55%) and sulphate conjugate (30%) in urine [1]. Safety at therapeutic doses led to its use as a model drug for investigating the regulation of glucuronidation in humans. Paracetamol glucuronidation has been found to be increased by phenobarbital-type inducers [2–4], by 3-methylcholanthrene-type inducers present in cigarette smoke [3, 5] and in Brussels sprouts and cabbage [6], and by the use of oral contraceptive steroids [7–9].

Little is known about UDP-glucuronosyltransferase (UGT‖) isozymes responsible for paracetamol glucuronidation. Previous kinetic studies in human liver microsomes did not lead to conclusive answers [4, 10]. Recently, two UGT families have been characterized both in rats and humans [11, 12]. Family 1 consists of at least four isozymes of the UGT1A or phenol/bilirubin UGT gene complex [13]. Due to inconsistencies in the proposed nomenclature of the UGT1A gene complex [11] trivial names (HlugP1, HlugP4) are used in the present report. The rat orthologue of HlugP1 is termed UGT1A1. The isozymes are formed by differential splicing of this large gene complex located on human chromosome 2. Two phenol UGTs of this complex have been characterized, HlugP1 conjugating planar phenols [14] and HlugP4 conjugating a wide variety of planar and bulky phenols [15]. Family 2 consists of multiple steroid UGTs with broad substrate specificity.

In the present study, paracetamol glucuronidation was investigated utilizing human phenol UGT isozymes HlugP1 and HlugP4, stably expressed in eukaryotic cells, and the results were compared with findings in liver microsomes. Similar studies were carried out with a stably transfected rat phenol UGT, UGT1A1 [16], in comparison with liver microsomes from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated rats and untreated controls. Because of evidence for extrahepatic distribution of HlugP1 and rat UGT1A1, paracetamol glucuronidation was also studied in human kidney as well as in rat kidney, lung and spleen. The studies aimed at elucidating the role of individual UGT isozymes in paracetamol metabolism.

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‖ Abbreviations: UGT, UDP-glucuronosyltransferase (EC 2.4.1.17); TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

MATERIALS AND METHODS

Chemicals. 6-Hydroxychrysene was synthesized by Dr A. Seidel (Institute of Toxicology, University of Mainz, Germany) by a route previously described

for other phenolic metabolites of chrysene [17]. CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulphonate) was obtained from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Brij 58 (a condensate of hexadecyl alcohol with 20 mol of ethylene oxide/mol) was obtained from Serva (Heidelberg, F.R.G.).

Primers for exon 1 of HlugP1 were obtained from Appligene (Illkirch, France), 2'-deoxynucleotide 5'-triphosphates from Pharmacia (Uppsala, Sweden) and the AmpliTaq DNA Polymerase from Perkin Elmer Cetus (Norwalk, CT, U.S.A.). The random primed labelling DNA kit was purchased from Boehringer (Mannheim, F.R.G.).

Human tissues. Human livers were obtained shortly after death from kidney transplant donors. Characteristics of patients were as follows: HL17 was a sample obtained from a 70-year-old male smoker (ethanol abusus). Samples HL15 and HL18 were obtained from normal livers of a boy (5 years) and a woman (25 years), and sample HL16 from a fatty liver of unknown etiology. Human kidney samples (HK1, HK2, HK3, HK10) were obtained from kidneys removed from non-smokers (50–67 years) because of kidney tumours, and HK4 and HK5 from patients with hydronephrosis (56 and 82 years, respectively). Tissue homogenates and microsomes were prepared as described previously [4, 18] and stored at −80°.

Animal tissues. Wistar rats were treated with TCDD (10 μg/kg; dissolved in olive oil) once i.p. and tissues were removed after 10 days. Tissue microsomes were prepared as described previously [19]. Protein was determined according to the method of Lowry *et al.* [20] using bovine serum albumin as protein standard.

Recombinant UGT isozymes. Stable transfection of eukaryotic cells with cDNA of HlugP1 [14], HlugP4 [15] and rat UGT1A1 [16] was carried out, cells were cultured, and homogenates and microsomes were prepared as described. The cell lines stably expressed UGT activities over 10 passages, up to the described experiments. V79 cells (a Chinese hamster lung fibroblast cell line) were chosen for transfection because of low endogenous UGT activities. Endogenous UGT activities toward 4-methylumbelliferone and 6-hydroxychrysene comprised 5–10% of those of the recombinant enzyme activities. Therefore, endogenous UGT activity toward these substrates was subtracted. No appreciable endogenous paracetamol UGT activity could be detected in V79 cells.

UGT assays. Described methods were used for the assay of UGT activity toward 4-methylumbelliferone [21] and 6-hydroxychrysene [16]. For reasons of standardization and comparison, the assays were performed at 37° in the presence of 0.1 M Tris–HCl, pH 7.4 and 5 mM $MgCl_2$. Liver microsomes or homogenates of livers and recombinant cells were fully activated by addition of Brij 58 (0.5 or 0.05 mg/mg protein, respectively), with the exception of 6-hydroxychrysene which was activated in recombinant cell microsomes by 0.5 mg Brij 58/mg protein. Paracetamol UGT activity was fully activated by adding various CHAPS concentrations: 0.2% for rat microsomes, 0.05% for human homogenates and microsomes, and 0.01% for cell homogenates of transfected V79 cells. Addition of the detergent led to an 8-, 1.3- and 1.2-fold increase of paracetamol UGT activity in rat and human microsomes, and homogenates from UGT-transfected cells, respectively. The reaction was started by the addition of UDP-glucuronic acid (3 mM).

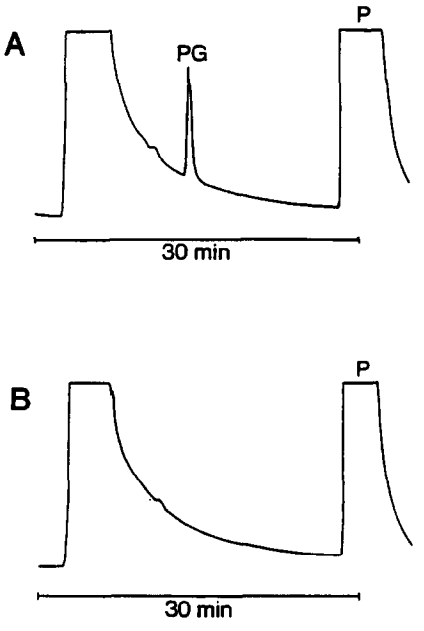

Fig. 1. HPLC isolation of paracetamol glucuronide (PG) formed by transfected HlugP1 (A). Analysis of incubation mixtures with untransfected V79 cells is shown for comparison (B). The reaction was stopped by addition of perchloric acid and 250 μL of the deproteinized supernatant was injected into the HPLC system. UV absorption was monitored at 243 nm. P, paracetamol. PG was identified as described in Materials and Methods.

Paracetamol UGT was measured by modification of a previously published procedure at 5 mM paracetamol [4]. The modifications led to a clear separation of paracetamol glucuronide from other UV-absorbing material (Fig. 1). Paracetamol was incubated for 30 min in 0.3 mL of the incubation mixture described above containing 0.2 mg microsomal protein. The reaction was stopped by the addition of perchloric acid (5% final concentration). The precipitated protein was centrifuged and filtered through a Millipore filter Millex HV 13, 0.45 μm. The supernatant was injected into a Waters HPLC system (Millipore, Bedford, MA, U.S.A.) with variable UV detector. Separation of metabolites was achieved on a 4.6 × 250 mm steel column filled with Spherisorb ODS2 (5 μm). It was eluted with 10 mM perchloric acid, adjusted to pH 2, containing 3.2% acetonitrile at a flow rate of 0.7 mL/min. UV absorption was monitored at 243 nm. Retention time for paracetamol glucuronide was 15 min (Fig. 1). It was identified by adding authentic paracetamol glucuronide isolated from human urine after ingestion

of 1000 μg paracetamol [3] and by treatment with β-glucuronidase.

Kinetic studies. In the experiments performed to determine the apparent K_m and V_{max} for paracetamol glucuronidation the UDP-glucuronic acid concentration was 30 mM. Activity was measured over the range of 0.05–50 mM paracetamol. 4-Methylumbelliferone and 6-hydroxychrysene glucuronidation was measured over ranges of 0.005–0.5 and 0.01–0.5 mM, respectively. Apparent K_m and V_{max} were determined using statistical programs to fit experimental data to the Michaelis–Menten equation. Estimations were obtained using Lineweaver–Burk, Eadie–Hofstee and Hanes plots, which differed by less than 30%.

Northern blot analysis. Total RNA was extracted from human liver and kidney by the guanidine thiocyanate method of Chirgwin *et al.* [22]. mRNA samples (20 μg) were denatured with 2.2 M formaldehyde, 50% formamide and 1 × MOPS-buffer (20 mM 3-*N*-morpholinopropane sulphonic acid, 5 mM sodium acetate, 1 mM EDTA, pH 7.0) by heating for 3 min, electrophoresed on a 1% agarose gel containing 2.2 M formamide, transferred onto a Nylon membrane (Hybond N, Amersham Buchler) in 20 × SSC (3 M NaCl, 0.3 M sodium citrate buffer, pH 7.0) by capillary blotting and covalently bound to the membrane by UV radiation. Gels were stained with ethidium bromide for comparison of ribosomal RNA bands (28S and 18S). The membrane was then prehybridized in 6 × SSC, 50% deionized formamide, 10 × Denhardt's solution, 0.5% sodium dodecyl sulphate, 0.4 mg/mL denatured herring sperm DNA at 44° for 12 hr. Hybridization was performed at 44° for 48 hr using a selective cDNA probe for exon 1 of HlugP1, similar to the procedure adopted to prepare a cDNA probe for rat UGT1A1 [23]. The human cDNA probe was prepared by polymerase chain reaction using two oligonucleotides (nucleotides 1–23 and 328–350) as primers and genomic DNA from human liver HL17. Reactions were performed in a thermocycler. Amplified cDNAs corresponded to the expected 350 bp fragment (nucleotide 1–350) of exon 1 of HlugP1. Synthetic cDNAs of HlugP1 were labeled with [^{32}P]dCTP using the random primed labeling system. Washing of the membrane was carried out twice in 2 × SSC, 0.1% sodium dodecyl sulphate at 50° for 20 min. The membrane was exposed for 7 days at −70° to Kodak XAR-5 film with intensifying screens. The relative amounts of mRNA were estimated by densitometric scanning of autoradiograms of northern blots.

The size of HlugP1 mRNA was determined using a mRNA ladder (Gibco, BRL). Loading of equal amounts of total RNA and intactness of the mRNA were checked by ethidium bromide staining of the gels, from optical densities of 260 nm and from rehybridization with a β-actin-like pseudogene [24], as described for the HlugP1 cDNA probe.

Table 1. Paracetamol UGT activity in human tissues and recombinant UGTs

	UGT activity (nmol/min/mg protein)		
	Liver		Kidney
Microsomes		Microsomes	
HL15	0.28 ± 0.02	HK1	0.14 ± 0.01
HL16	0.27 ± 0.02	HK2	0.26 ± 0.02
HL17	0.69 ± 0.03	HK3	0.15 ± 0.06
HL18	0.26 ± 0.07	HK4	0.09 ± 0.01
		HK5	0.05 ± 0.01
		HK10	0.07 ± 0.01
Homogenates			
HL15	0.14 ± 0.02		
HL17	0.35 ± 0.04		
HlugP1	0.30 ± 0.02		
HlugP4	0.03 ± 0.01		

Data represent means ± SD (N = 4)

RESULTS

Paracetamol glucuronidation was detectable in homogenates of HlugP1-transfected cells (Fig. 1). Enzyme activity measured in homogenates of transfected cells was in the range found in homogenates of human livers such as HL15 and HL17 (Table 1). Liver microsomal paracetamol UGT activity was comparable to previous data from livers of patients with no known exposure to inducers [4]. Enzyme activity of sample HL17, obtained from a smoker with ethanol abusus, was 2-fold higher. Paracetamol glucuronidation was also detectable in

Table 2. Kinetic analysis of transfected human and rat UGT isozymes

Isozyme substrate	K_m (mM)	V_{max} (nmol/min/mg protein)
HlugP1		
4-Methylumbelliferone	0.15	14
6-Hydroxychrysene	0.14	2.5
Paracetamol	2.0	0.4
Paracetamol, HL17*	3.0	0.7
HlugP4		
4-Methylumbelliferone	0.02	1.80
6-Hydroxychrysene	0.02	1.68
Paracetamol	50	1.9
Paracetamol, HL17*	20	2.6
Rat UGT1A1		
4-Methylumbelliferone	0.18	25.5
6-Hydroxychrysene	0.1	0.6
Paracetamol	2.7	0.2
Paracetamol, RL/untreated†	12.4	12
Paracetamol, RL/TCDD-treated†	5.2	27

UGT activities with human recombinant isozymes were measured in homogenates whereas those with rat UGT1A1 were assayed in microsomes.

* Data from the homogenate of HL 17. They represent high- and low-affinity components of its biphasic kinetics, shown in Fig. 3.

† Data from liver microsomes of TCDD-treated rats and untreated controls (RL).

Data represent means of three experiments.

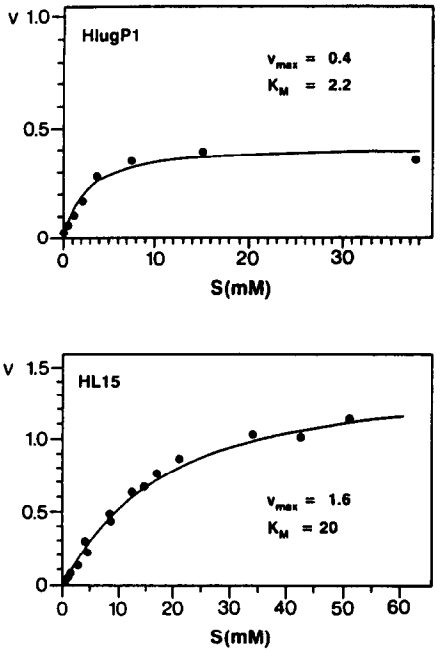

Fig. 2. Kinetic analysis of paracetamol glucuronidation with transfected HlugP1 and with HL15. K_m, mM; V, nmol/min/mg protein.

human kidney (Table 1). K_m and V_{max} values could not be determined with the kidney enzymes since paracetamol UGT activity increased linearly up to 50 mM, suggesting the presence of UGT isozymes with low affinity for paracetamol in this tissue.

UGT activity in transfected cells obviously depends upon the number of integrated gene copies. Therefore V_{max} determined with transfected HlugP1 and HlugP4 cannot be compared without information on the levels of the expressed proteins. However,

Table 3. Paracetamol UGT activity in microsomal fractions from various tissues of TCDD-treated rats and untreated controls

Organ	UGT activity (nmol/min/mg protein) Untreated controls	TCDD-treatment
Females		
Liver	4.91 ± 0.80	10.49 ± 0.98
Kidney	0.18 ± 0.04	0.36 ± 0.05
Lung	0.02 ± 0.01	0.17 ± 0.07
Spleen	0.08 ± 0.04	0.28 ± 0.16
Males		
Liver	3.38 ± 0.54	5.57 ± 0.78
Kidney	0.20 ± 0.05	0.33 ± 0.09
Lung	0.03 ± 0.01	0.06 ± 0.01
Spleen	0.06 ± 0.01	0.18 ± 0.11

Data represent means ± SD (N = 4).

K_m values may be characteristic of the integrated UGT isozymes. Interestingly, kinetic analysis of paracetamol glucuronidation with transfected HlugP1 revealed apparent K_m values of 2–3 mM, which were much lower than those calculated with transfected HlugP4 (*ca.* 50 mM) and with most livers investigated (Table 2; Figs 2 and 3). Sample HL17 revealed biphasic kinetics with apparent K_m values of 3 and 20 mM, suggesting the presence of UGTs with differing affinity for paracetamol. Paracetamol glucuronidation was compared to that of standard substrates such as 4-methylumbelliferone and 6-hydroxychrysene (the latter used as a standard phenolic metabolite of polycyclic aromatic hydrocarbons [16]. It was found that, in contrast to paracetamol, 4-methylumbelliferone and 6-hydroxychrysene show higher affinity for HlugP4 than for HlugP1 (Table 2).

Transfected rat UGT1A1 also exhibited a relatively low apparent K_m value of 2.7 mM for paracetamol

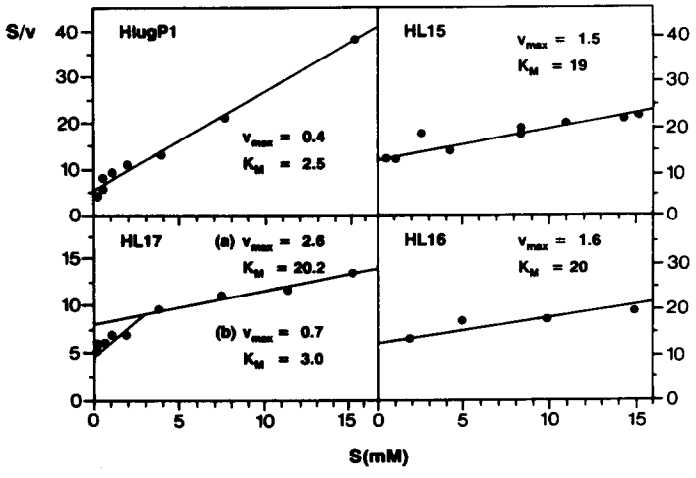

Fig. 3. Hanes plots of paracetamol glucuronidation with transfected HlugP1 and with human liver microsomes. K_m, mM; V, nmol/min/mg protein.

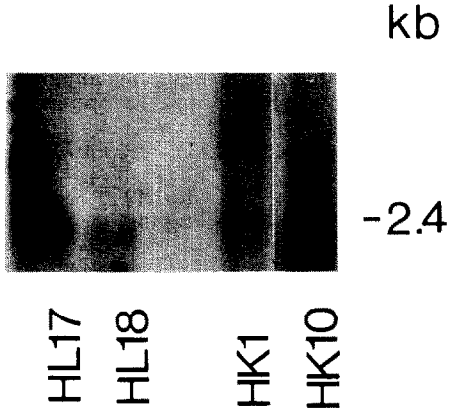

Fig. 4. Northern blot analysis of HlugP1 mRNA with HL17, HL18, HK1 and HK10. Each lane contained equal amounts of total RNA (20 μg) as judged from ethidium bromide staining of ribosomal RNA bands (28S and 18S) and from rehybridization of the filters with a β-actin pseudogene ([24] data not shown).

(Table 2). Again this value was lower than that found with microsomes from untreated rats (12.4 mM). However, the K_m value calculated with microsomes from TCDD-treated rats approached the K_m value found with UGT1A1. The marked increase of V_{max} indicated that paracetamol glucuronidation was induced by TCDD treatment.

Indirect evidence obtained previously suggested that UGT1A1 is widely distributed in rat tissues and cell lines [19, 25]. Therefore, paracetamol glucuronidation was also studied in the kidneys, lungs and spleen from TCDD-treated rats and untreated controls (Table 3). Paracetamol UGT activity was induced in all tissues investigated. Induction tended to be higher in female rats.

Using a selective cDNA probe of HlugP1 exon 1, mRNA of HlugP1 could be clearly detected in human livers (Fig. 4). Densitometric tracings suggested that mRNA was approximately 5-fold higher in HL17 (obtained from a smoker) than in HL18. The size of the HlugP1 mRNA (2.4 kb) is in good agreement with the published size of its cDNA [26]. HlugP1 mRNA is also clearly detectable in two out of 10 kidneys, for example in HK10. The higher molecular mass band is probably a longer transcript of HlugP1 because it hybridizes with the selective exon 1 probe of HlugP1. This putative longer transcript was not included in the calculations. In contrast to HlugP1 mRNA, paracetamol UGT activity was lower in HK10 than in HK1 (Table 1) suggesting the presence of other UGT isozymes responsible for glucuronidation of the drug.

DISCUSSION

The results of the present study demonstrate that paracetamol shows higher affinity for HlugP1 than for HlugP4. The apparent K_m value for HlugP1 (2 mM) is close to that obtained for the rat orthologue (K_m 2.7 mM). The findings for paracetamol glucuronidation are in contrast to those obtained for the glucuronidation of two standard substrates (4-methylumbelliferone and 6-hydroxychrysene) which appear to be high affinity substrates for HlugP4. The findings with HlugP4 extend previous investigations on the substrate specificity of HlugP4 showing that HlugP4 is able to conjugate a variety of planar and non-planar phenols, anthraquinones, flavones, aliphatic alcohols, aromatic carboxylic acids, steroids and many drugs of varied structure [27]. Comparison of the metabolic capacity or intrinsic clearance (V_{max}/K_m) of different UGT isozymes needs additional information on the level of enzyme protein in transfected cells. For example, monospecific antibodies to the constant region of HlugP1 and HlugP4 are needed which are not yet available.

The results obtained with rat liver, kidney, lung and spleen indicate that paracetamol glucuronidation is inducible by 3-methylcholanthrene-type inducers such as TCDD. Paracetamol appears to be an overlapping substrate of several rat UGTs. This is suggested by the observation that the K_m of microsomal UGT activity from TCDD-treated rats is lower than that obtained with microsomes from untreated rats and becomes close to that seen with the transfected rat UGT1A1 (Table 2).

Paracetamol glucuronidation is also detectable in human kidney. The difficulty in reaching substrate saturation for paracetamol glucuronidation with human kidney UGTs may be due to the contribution of low affinity UGTs at high substrate concentrations. The latter may be of doubtful significance *in vivo* since only low concentrations of paracetamol are reached in blood therapeutically. The plasma concentration after an oral dose of 20 mg/kg paracetamol reaches only 20 μg/mL = 0.13 mM [1]. Northern blot analysis revealed the expression of HlugP1 mRNA in HK1 and HK10 (Fig. 4). However, this isozyme may not markedly contribute to paracetamol glucuronidation in the kidney. On the other hand, HlugP4 is highly expressed in human kidney, higher than in the livers of patients treated with phenytoin or phenobarbital [28]. This may be one of the reasons why a low affinity component of the K_m value for paracetamol glucuronidation could not be detected in this tissue.

The results demonstrate that paracetamol is conjugated by HlugP1 and its rat orthologue UGT1A1 with higher affinity than by HlugP4 and other UGTs. It is conceivable that HlugP1, similar to its rat orthologue [29], may be inducible by 3-methylcholanthrene-type inducers. HlugP1 mRNA was higher in HL17 obtained from a smoker than in HL18 obtained from a non-smoker (Fig. 4). Recently, a population study in a subgroup of male smokers revealed a significant correlation ($r = 0.85$) between urinary metabolic ratios for paracetamol glucuronidation [3] and caffeine oxidation [30], the latter mainly representing P4501A2 activity), suggesting coordinate induction of the two enzymes under these conditions (Bock KW, Schrenk D, Forster A, Brockmeier D and Eichelbaum M, unpublished results). This may explain the small but significant increase in paracetamol glucuronidation in humans exposed to 3-methylcholanthrene-type inducers [3, 5, 6].

Acknowledgements—This work was supported by the

Schwerpunkt Umwelttoxikologie Baden-Württemberg, the Deutsche Forschungsgemeinschaft and The Wellcome Trust and Cancer Research Campaign, U.K.

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