INDUCTION OF UDP-GLUCURONOSYLTRANSFERASES IN PRIMARY CULTURES OF FRESH AND IMMORTALIZED HUMAN HEPATOCYTES

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Florence Ndikum-Moffor*, Maggie McMullen, Remi Gibson, Paul Toren, Cody Gartner, Jason Neat, Christa Linsenmeyer, Martin Perry, Maciej Czerwinski, Peter Opdam and Andrew Parkinson*

XENOTECH
16825 W 116th Street Lenexa, KS 66219

ABSTRACT

We have examined the induction of UDP-glucuronosyltransferases (UGTs) in primary cultures of human hepatocytes and in immortalized human hepatocytes (Fa2N-4 cells) under conditions that result in the induction of cytochrome P450 (CYP) enzymes. Primary cultures of human hepatocytes were treated daily for three days with either vehicle (0.1% DMSO or saline) or one of several prototypical human CYP inducers, namely β-naphthoflavone, omeprazole, phenobarbital, rifampin and isoniazid. Microsomes prepared from the primary hepatocyte cultures were analyzed by LC/MS/MS for thyroxine (T4) and triiodothyronine (T3) glucuronidation, and cell lysates were analyzed for UGT1A1, UGT1A6, UGT1A9, UGT2B4 and UGT2B7 mRNA expression by the branched DNA (bDNA) assay. UGT1A1 mRNA levels increased (> 2 fold) following treatment of cultures with β-naphthoflavone, omeprazole, phenobarbital, rifampin or isoniazid. UGT1A6 mRNA expression was induced by β-naphthoflavone and isoniazid. UGT1A9 mRNA expression was induced by rifampin. UGT2B4 mRNA expression was induced by β-naphthoflavone and rifampin. UGT2B7 mRNA expression was induced by β-naphthoflavone and phenobarbital.

Fa2N-4 cells were treated for 3 days with DMSO (vehicle), omeprazole, phenobarbital, rifampin or 3-methylcholanthrene. Cells were analyzed for UGT1A1, 1A6, 1A9, 2B4 and 2B7 mRNA expression. Omeprazole induced UGT1A1 mRNA (8.8 fold) and UGT1A9 mRNA (4.4 fold) whereas β-naphthoflavone caused a 4.7-fold increase in UGT1A1 mRNA levels. The other inducers had little or no effect on UGT mRNA expression in Fa2N-4 cells. Microsomal UGT activity toward triiodothyronine and thyroxine was not induced in immortalized human hepatocytes treated with β-naphthoflavone or rifampin. Although statistically different from samples treated with vehicle, treatment of cultured human hepatocytes with rifampin caused less than a 2-fold increase in UGT activity toward T3. These results suggest that human UGTs are not as highly inducible as certain human CYP enzymes, nor as inducible as certain UGTs in rats.

INTRODUCTION

There is increasing evidence that induction of UGTs plays a role in drug-drug interactions. Unlike the CYP enzymes (Madan *et al.*, 2003), there is relatively little information on the inducibility of UGTs, and on the mechanism of UGT induction. A few reports indicate that certain UGTs are inducible by prototypical CYP enzyme inducers (Li *et al.*, 1999; Soars *et al.*, 2004). However, results from these studies are inconsistent or conflicting. This may be because different probe substrates were used to measure UGT activity. The objective of the current study was to determine whether treatment of primary cultures of human hepatocytes and immortalized human hepatocytes (Fa2N-4 cells) with prototypical inducers of CYP enzymes causes an increase in the expression of specific UGT mRNAs and/or induction of microsomal UGT activity toward thyroxine (T4) and triiodothyronine (T3).

MATERIALS AND METHODS

Induction of UGTs in cultured human hepatocytes

Human hepatocytes were isolated using the two-step collagenase perfusion method and plated in 60 mm dishes essentially as described by Madan *et al.* (2003). Plated cells were treated for three consecutive days with vehicle (0.1% DMSO or 0.1% saline), β-naphthoflavone (33 µM), omeprazole (100 µM), phenobarbital (250 µM), rifampin (20 µM) or isoniazid (100 µM). The medium was changed every 24 h, and the cells were maintained at 37°C in an atmosphere of 95% relative humidity, 5% CO2. Following treatment, cells were harvested for the preparation of microsomes or disrupted with Lysis Buffer (see below) for analysis of mRNA expression.

UGT mRNA analysis

Cell lysates were analyzed for UGT1A1, 1A6, 1A9, 2B4 and 2B7 mRNA expression by branched DNA (bDNA) analysis with the Quantigene® High Volume kit (Genospectra, Freemont, CA), based on methods described in Czerwinski et al. (2002). Cell lysates were incubated in a 96-well plate with the appropriate probe sets at 53°C for 20 h, after which excess probe was removed, and samples were incubated with Amplifier Working Reagent at 53°C for 60 ± 3 min. The wells were washed with Wash Buffer, and incubated with Substrate Working Reagent at 53°C for 30 ± 3 min. Chemiluminescence of the reaction was measured on a Lumistar Galaxy microplate reader (BMG Labtechnologies, Offenburg, Germany).

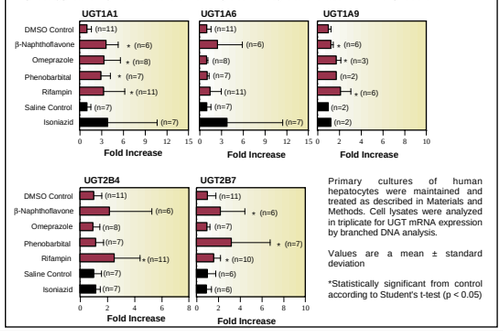
Fold increase = % of GAPDH ÷ % of GAPDH of 0.1% DMSO control of appropriate human hepatocyte preparation. Appropriate control for the treatments is 0.1% DMSO except for isoniazid which is 0.1% saline.

Table 1. Fold Induction (treatment/control) of UDP-glucuronosyltransferase mRNA in primary cultures of human hepatocytes

Treatment	UGT1A1	UGT1A6	UGT1A9	UGT2B4	UGT2B7
DMSO (0.1%, v/v)	1.00 ± 0.58 (n=11)	1.00 ± 0.55 (n=11)	1.00 ± 0.22 (n=6)	1.00 ± 0.57 (n=11)	1.00 ± 0.75 (n=11)
β-Naphthoflavone (33 µM)	3.64 ± 1.63 (n=6)	2.47 ± 3.39 (n=6)	1.23 ± 0.19 (n=6)	2.15 ± 3.10 (n=6)	2.19 ± 2.22 (n=6)
Omeprazole (100 µM)	3.34 ± 2.23 (n=8)	0.971 ± 0.142 (n=7)	1.72 ± 0.40 (n=3)	0.950 ± 0.486 (n=8)	0.953 ± 0.252 (n=7)
Phenobarbital (250 µM)	2.92 ± 1.27 (n=7)	1.05 ± 0.24 (n=7)	1.72 (n=2)	1.14 ± 0.56 (n=7)	3.20 ± 3.54 (n=7)
Rifampin (20 µM)	3.28 ± 2.89 (n=11)	1.45 ± 1.51 (n=11)	2.11 ± 0.93 (n=6)	2.51 ± 1.88 (n=11)	1.57 ± 0.61 (n=10)
Saline (0.1%, v/v)	1.00 ± 0.50 (n=7)	1.00 ± 0.53 (n=7)	1.00 (n=2)	1.00 ± 0.55 (n=7)	1.00 ± 0.74 (n=6)
Isoniazid (100 µM)	3.85 ± 6.78 (n=7)	3.78 ± 7.64 (n=7)	1.23 (n=2)	1.55 ± 0.31 (n=7)	0.900 ± 0.446 (n=6)

Values are mean ± standard deviation. Fold increase = % of GAPDH of treated sample ÷ % of GAPDH of 0.1% DMSO control of appropriate human hepatocyte preparation. Appropriate control for the treatments is 0.1% DMSO except for isoniazid which is 0.1% saline.

Figure 1. The effect of treating primary cultures of human hepatocytes with prototypical enzyme inducers on UDP-glucuronosyltransferase mRNA expression



Primary cultures of human hepatocytes were maintained and treated as described in Materials and Methods. Cell lysates were analyzed in triplicate for UGT mRNA expression by branched DNA analysis.

Values are a mean ± standard deviation

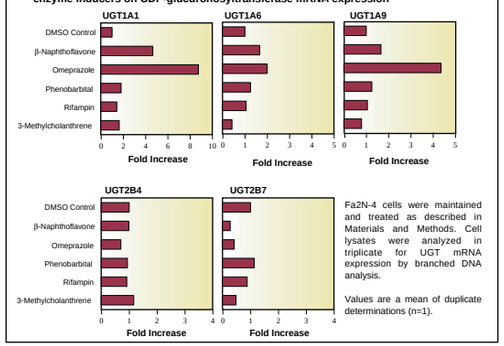
*Statistically significant from control according to Student's t-test ($p < 0.05$)

Table 2. Fold Induction (treatment/control) of UDP-glucuronosyltransferase mRNA in immortalized human hepatocytes (Fa2N-4 cells)

Treatment	UGT1A1	UGT1A6	UGT1A9	UGT2B4	UGT2B7
DMSO (0.1%, v/v)	1.00	1.00	1.00	1.00	1.00
β-Naphthoflavone (33 µM)	4.66	1.74	1.66	0.99	0.27
Omeprazole (100 µM)	8.79	2.00	4.36	0.70	0.41
Phenobarbital (750 µM)	1.81	1.25	1.73	0.94	1.13
Rifampin (20 µM)	1.42	1.05	1.45	0.91	0.88
3-MC (5 µM)	1.64	0.42	0.79	1.16	0.48

Values are mean of triplicate determinations. Fold increase = % of GAPDH of treated sample ÷ % of GAPDH of samples treated with vehicle (0.1% DMSO)

Figure 2. The effect of treating immortalized human hepatocytes with prototypical enzyme inducers on UDP-glucuronosyltransferase mRNA expression



Fa2N-4 cells were maintained and treated as described in Materials and Methods. Cell lysates were analyzed in triplicate for UGT mRNA expression by branched DNA analysis.

Values are a mean of duplicate determinations (n=1).

UGT Activity

UGT activity toward T4 and T3 was measured in microsomes prepared from the cultured hepatocytes. T4 and T3 glucuronidation were determined by the method of Ducrotoy *et al.* (1991) and DeSandro *et al.* (1992) with minor modifications. Microsomes (2.5 or 1.25 mg/incubation) were pre-incubated at room temperature in an equal volume of microsomes and CHAPS (3-[(3-cholamidopropyl)-dimethyl-ammonio]-1-propane sulfonate) solubilization buffer (400 mM Tris-HCl, pH 8.0; 250 mM sucrose; 10 mM CHAPS) for approximately 25 min. Microsomes (0.0625 or 0.03125 µg) were then incubated at 37 ± 1°C in 0.5 mL (final volume) incubations containing Tris-HCl (200 mM, pH 8.0), EDTA (1.0 mM, pH 7.4), $MgCl_2$ (10 mM), saccharic acid-1,4-lactone (100 µM), CHAPS (500 µM), UDP-glucuronic acid (UDP-GA, 4 mM) and L-thyroxine (10 µM) or triiodothyronine (10 µM). Reactions were started by the addition of UDP-GA and stopped after 80 or 160 min by the addition of 0.5 mL methanol (acidified 2% with formic acid) containing internal standard (10 ng/mL fexofenadine). Precipitated protein was removed by low-speed centrifugation, and aliquots (10 µL) of the supernatant fractions were analyzed by LC/MS/MS. The analyte peak area ratio of thyroxine or triiodothyronine glucuronide to internal standard was determined (integrated with Applied Biosystems (Foster City, CA) Analyst® data system, version 1.3.1).

Induction of UGTs in immortalized human hepatocytes

Fa2N-4 cells were propagated in multifunction-enhancing (MFE®) medium (MultiCell Technologies, Providence, RI) and plated on 6-well plates coated with Vitrogen (Cohesion Technologies, Palo Alto, CA) and maintained under normal cell culture conditions. The Fa2N-4 cells were grown to confluency and treated for 72 hours with 0.1% DMSO (vehicle), β-naphthoflavone (33 µM), omeprazole (100 µM), phenobarbital (750 µM), rifampin (20 µM) or 3-methylcholanthrene (5 µM).

UGT mRNA analysis

Following treatment (as described above), Fa2N-4 cells were disrupted with Lysis Buffer, and lysates were analyzed for mRNA expression of UGT1A1, 1A6, 1A9, 2B4 and 2B7 by bDNA analysis.

UGT Activity

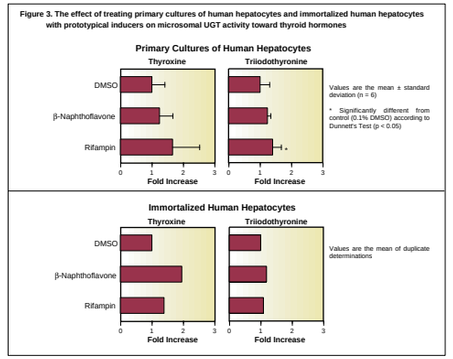
Microsomes were prepared from Fa2N-4 cells treated with 0.1% DMSO (vehicle), β-naphthoflavone (33 µM) or rifampin (20 µM), and analyzed for T4 and T3 glucuronidation by LC/MS/MS as described above.

Table 3. Fold Induction (treatment/control) of T4 and T3 glucuronidation in primary cultures of human hepatocytes and immortalized

Treatment	Thyroxine (T4) Glucuronidation		Triiodothyronine (T3) Glucuronidation	
	Human Hepatocytes	Immortalized Human Hepatocytes	Human Hepatocytes	Immortalized Human Hepatocytes
DMSO	1.00 ± 0.41 (n=6)	1.00	1.00 ± 0.30 (n=6)	1.00
β-Naphthoflavone	1.24 ± 0.42 (n=6)	1.95	1.23 ± 0.10 (n=6)	1.19
Rifampin	1.66 ± 0.86 (n=6)	1.39	1.40 ± 0.27 (n=6)	1.09

Values are mean ± standard deviation for primary cultures of human hepatocytes
Values are mean of duplicate measurements for immortalized human hepatocytes

Figure 3. The effect of treating primary cultures of human hepatocytes and immortalized human hepatocytes with prototypical inducers on microsomal UGT activity toward thyroid hormones



Values are the mean ± standard deviation (n = 6)

* Significantly different from control (0.1% DMSO) according to Dunnett's Test ($p < 0.05$)

Values are the mean of duplicate determinations

RESULTS

Human hepatocytes

The effect of treating cultured human hepatocytes with prototypical inducers on UGT mRNA expression was investigated and the results are shown in Table 1 and Fig. 1. UGT1A1 mRNA expression was increased (> 2-fold) by treatment of human hepatocytes with β-naphthoflavone, omeprazole, phenobarbital, rifampin and isoniazid. UGT1A6 mRNA expression increased following treatment with β-naphthoflavone and isoniazid, but the increases were not statistically significant. UGT1A9 mRNA expression was generally not induced (less than 2-fold) following treatment with prototypical inducers although a statistically significant difference (Student's t-test) was observed in samples treated with β-naphthoflavone, omeprazole and rifampin. UGT2B4 mRNA expression increased in human hepatocytes treated with β-naphthoflavone and rifampin. UGT2B7 mRNA expression was induced in human hepatocytes treated with β-naphthoflavone and phenobarbital. Although statistically different (Student's t-test) from samples treated with vehicle, treatment of cultured human hepatocytes with rifampin caused less than a 2-fold increase in UGT2B7 mRNA expression.

Immortalized human hepatocytes

The effect of treating immortalized human hepatocytes with prototypical inducers on UGT mRNA expression was investigated and the results are shown in Table 2 and Fig. 2. Except for UGT1A1 and UGT1A9, treatment of immortalized human hepatocytes with prototypical enzyme inducers did not cause an increase in mRNA expression of the UGT enzymes examined. UGT1A1 mRNA expression was increased following treatment of Fa2N-4 cells with β-naphthoflavone and omeprazole.

T4 and T3 glucuronidation

UGT activity toward T4 and T3 was measured in microsomes prepared from the cultured human hepatocytes or immortalized human hepatocytes. Treatment of immortalized human hepatocytes with prototypical enzyme inducers did not cause an increase in microsomal UGT activity toward the thyroid hormones T4 and T3 (Table 3, Fig. 3). Although statistically different from samples treated with vehicle, treatment of cultured human hepatocytes with rifampin caused less than a 2-fold increase in microsomal UGT activity toward T3.

CONCLUSIONS

Treatment of cultured human hepatocytes with prototypical enzyme inducers caused only slight increases in mRNA expression of some of the UGT enzymes examined. Microsomal UGT activity toward thyroxine (T4) and triiodothyronine (T3) was not induced in primary cultures of human hepatocytes or immortalized human hepatocytes treated with prototypical enzyme inducers. These results suggest that human UGTs are not as highly inducible as certain human CYP enzymes, nor as inducible as certain UGTs in rats and other species.

REFERENCES

Czerwinski M, Opdam P, Madan A, Carroll K, Mudra DR, Gan LL, Luo G, and Parkinson A. 2002. Analysis of CYP mRNA expression by branched DNA technology. *Methods Enzymol.* **357:** 170-179.

De Sandro V, Catinot R, Kriszt W, Cordier A, and Richter L. 1992. Male hepatic UDP-glucuronosyltransferase activity toward thyroxine, activation and induction properties - Relation with thyroxine plasma disappearance rate. *Biochem. Pharmacol.* **43:**1563-1569.

Ducrotoy C, Richert L, Lurier D, and Pacaud E. 1991. Determination of 125I-labeled thyroxine glucuronide by reverse-phase high performance liquid chromatography using on-line radiochemical detection to determine UDP-glucuronosyltransferase activity. *J.Chromatogr.* **566:**3859-3866.

Li AP, Hartman NR, Lu C, Collins JM, and Strong JM. 1999. Effects of cytochrome P450 inducers on 17α-ethinyloestradiol (EE2) conjugation by primary human hepatocytes. *Br. J. Clin. Pharmacol.* **48:**733-742.

Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, Downey AD, Czerwinski M, Forster J, Ribadeneira MD, Gan L, LeCluyse EL, Zech K, Robertson Jr. P, Koch P, Antonian L, Wagner G, Yu L, and Parkinson A. 2003. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. *Drug Metab. Disp.* **31:**421-431.

Soars MG, Petullo DM, Eckstein JA, Kasper SC, and Wrighton SA. 2004. An assessment of UDP-glucuronosyltransferase induction using primary human hepatocytes. *Drug Metab. Disp.* **32:**140-148.

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