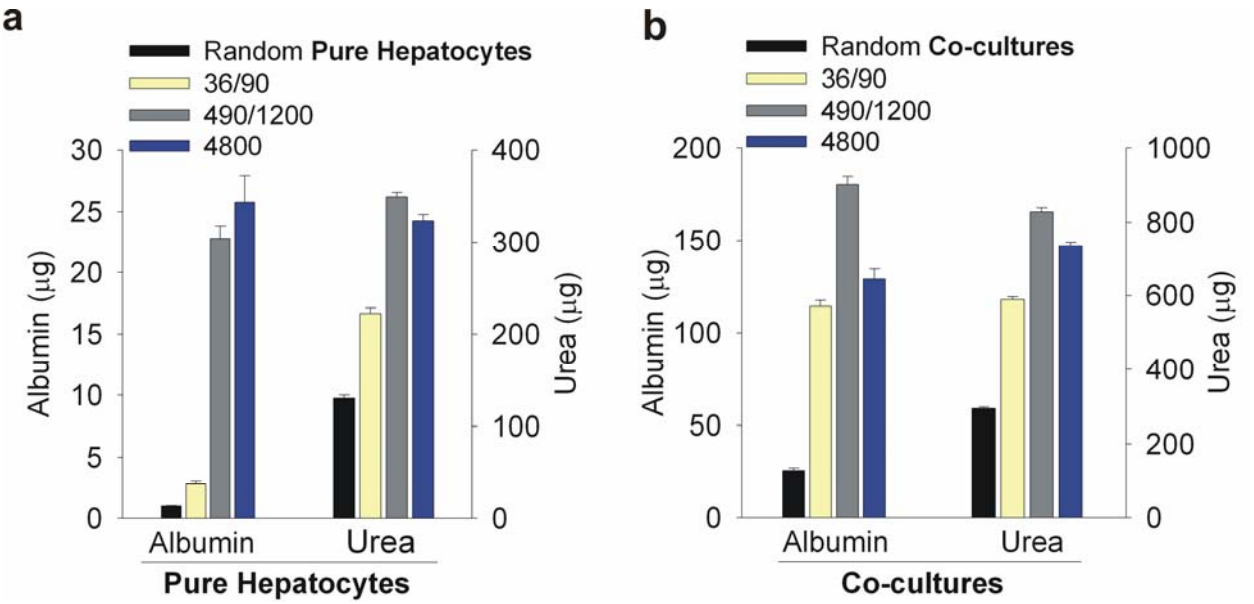
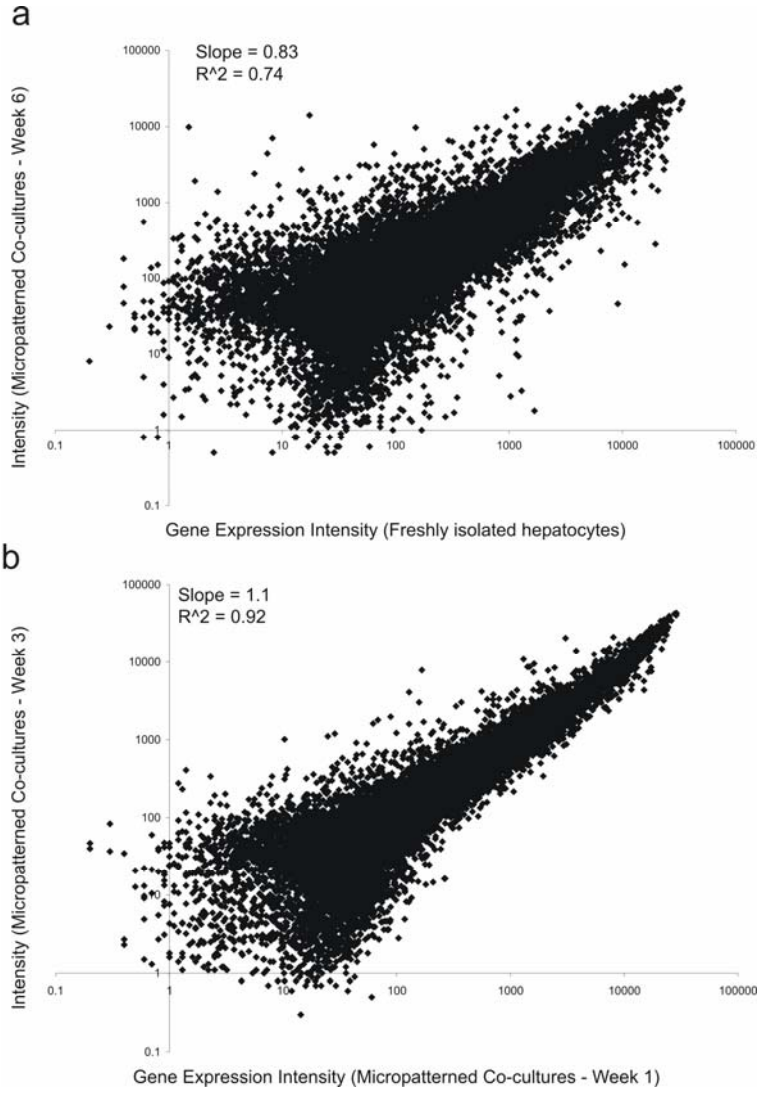
Supplementary Information for "Microscale Human Liver Tissue for Drug Development" by S. R. Khetani and S. N. Bhatia. ***Nature Biotechnology.***

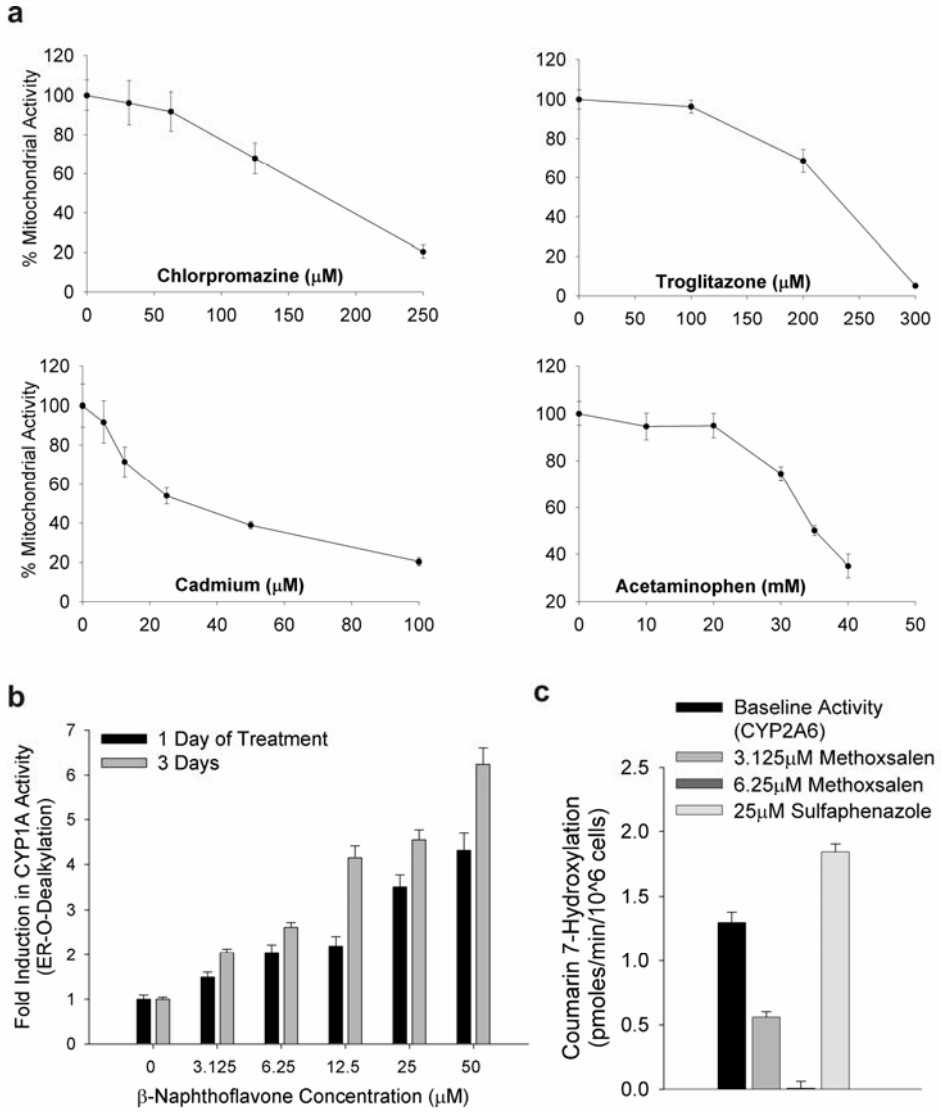
SUPPLEMENTARY FIGURES



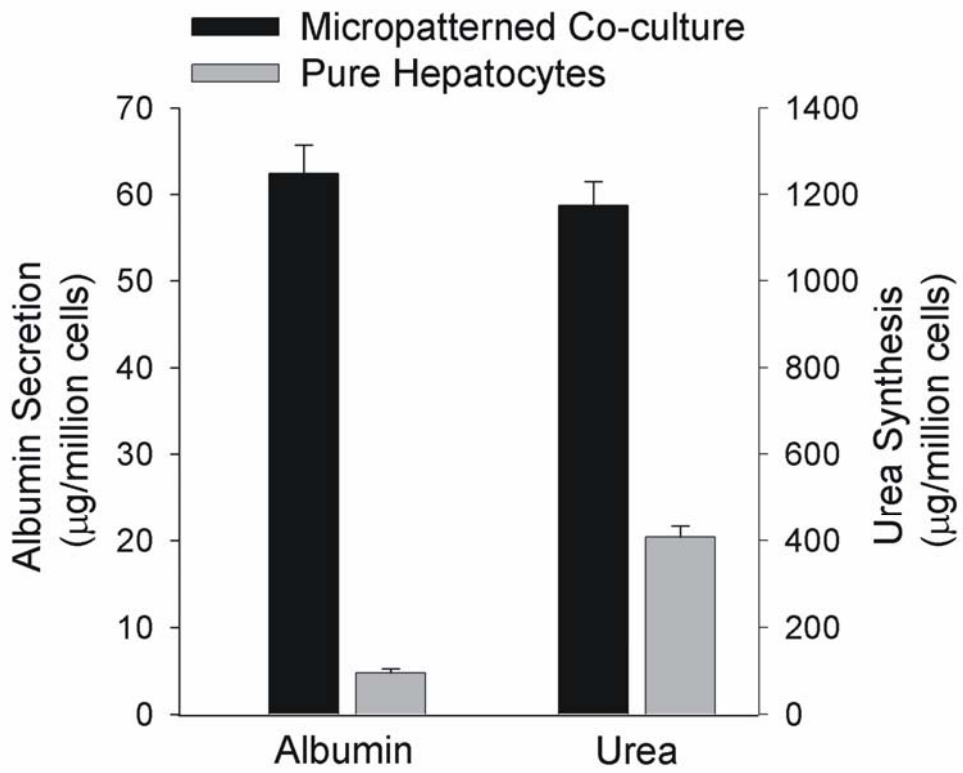
Supplementary Figure 1. **Functional optimization of human hepatocyte cultures and co-cultures via micropatterning.** Primary human hepatocytes were spatially organized onto collagen-coated islands of prescribed dimensions using photolithography. Island size (36, 490, 4800μm) and center-to-center spacing (i.e. 90μm for 36μm islands) between islands for each configuration were selected to keep total cell numbers constant. Dimensions were also chosen to enable comparisons with our previous work using primary rat hepatocytes[1]. In order to create micropatterned co-cultures, hepatocytes were surrounded by 3T3-J2 fibroblasts 24 hours after attachment and spreading. Total cell numbers and ratios of two cell types were kept constant across configurations. Randomly distributed control co-cultures ('Random') on collagen were also generated to enable comparisons. Cumulative liver-specific functions (albumin and urea secretion) over 2 weeks were compared in micropatterned *pure* human hepatocyte cultures (**panel a)** and in micropatterned co-cultures **(panel b)**. All error bars represent SEM (n = 3).



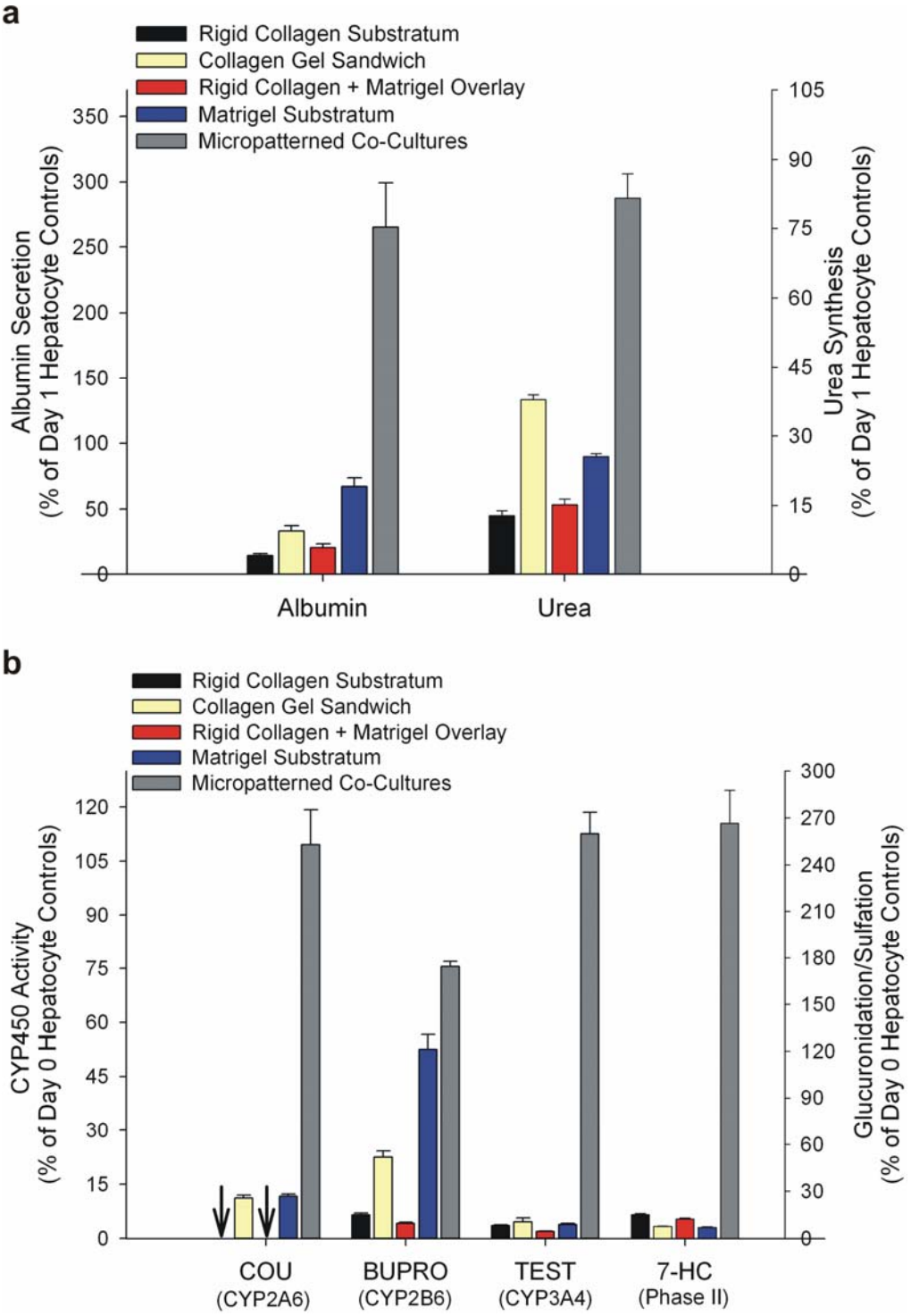
Supplementary Figure 2. **Global gene expression profiling of hepatocytes in microscale liver tissues. a.** Global scatter plot comparing gene expression intensities in human hepatocytes purified from 6 week old micropatterned co-cultures to expression intensities in freshly isolated hepatocytes in suspension prior to plating. Similar results were obtained when expression intensities from hepatocytes purified from micropatterned co-cultures were compared to intensities in a fresh mixture of all cell types of the liver (R^2 = 0.73, Slope = 0.87). **b.** Global scatter plot comparing expression intensities in human hepatocytes purified from micropatterned co-cultures 1 and 3 weeks after plating.



Supplementary Figure 3. **Case studies demonstrating utility of microscale human liver tissues in drug development. a**. Dose-dependent acute toxicity profiles of model hepatotoxins after acute exposure (24 hrs). Mitochondrial activity was measured via the MTT assay. All data was normalized to vehicle controls. **b**. Dose and time-dependent induction in CYP1A activity upon incubation of micropatterned co-cultures for 1 or 3 days with ß-Naphthoflavone. ER, Ethoxy-resorufin. **c**. Dose-dependent inhibition of CYP2A6 activity upon treatment of micropatterned co-cultures with Methoxsalen. Sulfaphenazole (CYP2C9 inhibitor) did not inhibit CYP2A6 activity even at a 25μM dose. All error bars represent SEM (n = 3).



Supplementary Figure 4. **Functional comparison of culture models created using cryopreserved human hepatocytes**. Plateable (or inducible) cryopreserved hepatocytes were thawed and plated according to manufacturer's instructions (Celsis In Vitro Technologies, Baltimore, MD). Cumulative albumin and urea secretion over the course of two weeks is shown for micropatterned co-cultures (500μm circular hepatocyte islands with 1200μm center-to-center spacing) and micropatterned pure hepatocytes. Error bars represent SEM (n = 3).



Supplementary Figure 5. **Functional comparison of microscale human liver tissues to well-established *in vitro* liver models utilized in the pharmaceutical industry.** Randomly distributed cultures were created in multi-well plates (12- and 24-well formats) and compared in

different formats: rigid type-I collagen coating, type-I collagen gel sandwich, rigid collagen coating with Matrigel overlay, Matrigel gel substratum, and micropatterned co-cultures (500μm circular hepatocyte islands with 1200μm center-to-center spacing). See 'Supplementary Methods' for additional details. **a.** Rates of albumin secretion and urea synthesis in the various culture models expressed as a percentage of the first 24 hour secretion values (day 1). Values from a representative day 17 are shown. **b.** Activities of CYP450 and Phase II enzymes in the various hepatocytes culture models expressed as a percentage of activities in a pure hepatocyte monolayer on day 0. Values from representative days (end of week 1 for COU, BUPRO, 7-HC and end of week 2 for TEST) are shown. COU, Coumarin; BUPRO, Bupropion HCL; TEST; Testosterone; 7-HC, 7-Hydroxycoumarin. CYP3A4 activity was assessed by measuring production of 6beta-hydroxytestosterone from testosterone, CYP2B6 activity by measuring production of Hydroxybupropion from Bupropion HCL, and CYP2A6 was assessed using the Coumarin 7-hydroxylation reaction. Phase II activity was assessed by measuring the amount of 7-hydroxycoumarin that was glucuronidated and sulfated. Arrows pointing to the x-axis indicate undetectable substrate metabolism in corresponding culture model. Error bars are SEM (n = 3).

Donor#	Age (years)	Sex	Cause of Death	Vendor
1	4	N/A	Anoxia	ADMET Technologies
2 *	5	M	Anoxia	BD-Gentest
3	5	M	Near Drowning	ADMET Technologies
4	7	F	N/A	Lonza
5	14	F	Gun shot wound	ADMET Technologies
6	19	M	Motor vehicle accident	In Vitro Technologies
7	20	M	Gun shot wound	In Vitro Technologies
8 **	23	F	Intracerebral hemorrhage	ADMET Technologies
9 **	23	M	N/A	CellzDirect
10 **	27	F	N/A	CellzDirect
11 *	41	M	Intracranial Hemorrhage	BD-Gentest
12 *	46	M	Motor vehicle accident	ADMET Technologies
13	51	F	Pneumonia	BD-Gentest
14	51	M	N/A	CellzDirect
15	52	M	Aortic dissection	In Vitro Technologies
16	53	M	Brain stem hemorrhage	Tissue Transformation Tech
17	54	F	Cardiac arrest	In Vitro Technologies
18	55	M	Seizure	Tissue Transformation Tech
19	55	F	Stroke	BD-Gentest
20	56	F	N/A	Lonza
21	58	M	Stroke	BD-Gentest
22**	59	M	N/A	CellzDirect
23	60	M	N/A	CellzDirect
24	61	M	Motor vehicle accident	BD-Gentest
25	63	M	N/A	CellzDirect
26	65	F	Cardiac Arrest	BD-Gentest
27 *	69	M	Intracranial bleeding	In Vitro Technologies
28	78	F	N/A	CellzDirect

* African-American Donors. ** Hispanic Donors. All other donors were of Caucasian descent. ‘N/A’ - not available at time of purchase.

Supplementary Table 1. Liver donor information. Reported here is specific information (age, sex, cause of death) on liver donors whose freshly isolated hepatocytes were purchased in suspension from multiple vendors for use in experiments of this study.

SUPPLEMENTARY METHODS

Fibroblast Culture

3T3-J2 fibroblasts were the gift of Howard Green (Harvard Medical School)[2]. Cells were cultured at 37ºC, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) with high glucose, 10% (v/v) calf serum, and 1% (v/v) penicillin-streptomycin.

Hepatocyte Culture under Different Conditions

Randomly distributed cultures were created in standard tissue culture multi-well plates (12 and 24-well formats). Extracellular matrix substratum included rigid type-I collagen (12.5 $\mu g/cm^2$), collagen gel (112.5 $\mu g/cm^2$) or Matrigel (1125 $\mu g/cm^2$). Hepatocytes were seeded at a density of 150,000-170,000 cells/cm^2 in FBS (10%)-supplemented hepatocyte culture medium. Culture medium (250$\mu L/cm^2$) was replaced daily. For the 'collagen gel sandwich' configuration, hepatocytes that were attached to a gelled substratum of collagen were overlaid with a second layer of collagen gel within 24 hours after seeding. For 'Matrigel overlay' condition, hepatocytes that were attached to a rigid collagen substratum were overlaid with a layer of Matrigel (62.5 or 83.5 $\mu g/cm^2$) within 24 hours after seeding.

Microscopy

Specimens were observed and recorded using a Nikon Diaphot microscope equipped with a SPOT digital camera (SPOT Diagnostic Equipment, Sterling Heights, MI), and MetaMorph Image Analysis System (Universal Imaging, Westchester, PA) for digital image acquisition.

Gene Expression Profiling

Micropatterned hepatocyte-fibroblast co-cultures were washed with phosphate buffered saline (PBS) followed by treatment with Trypsin/EDTA (Invitrogen) for 2-3 minutes at 37ºC. We found that fibroblasts were much more sensitive to trypsin-mediated detachment than hepatocytes arranged in clusters via micropatterning. Following incubation with trypsin, plates were shook mildly to remove loosely attached fibroblasts, the supernatant was aspirated and the attached hepatocytes (~95% purity) were washed with serum-supplemented hepatocyte medium. Hepatocyte RNA was extracted via TRIzol (Invitrogen) and purified using the RNeasy kit (Qiagen) as per manufacturer's instructions. The RNA was labeled, hybridized to an Affymetrix (Santa Clara, CA) Human U133 Plus 2.0 Array, and scanned as described previously[3]. Briefly, double-strand cDNA was synthesized using a T7- (dt)24 primer (Oligo) and reverse transcription (Invitrogen), cDNA was then purified with phenol/chloroform/isoamyl alcohol in Phase Lock Gels, extracted with ammonium acetate and precipitated using ethanol. Biotin-labeled cRNA was synthesized using the BioArray™ HighYield™ RNA Transcript Labeling Kit, purified over RNeasy columns (Qiagen), eluted and then fragmented. The quality of expression data was assessed using the manufacturer's instructions which included criteria such as low background values and 3'/5' actin and GAPDH (Glyceraldehyde-3-phosphate dehydrogenase) ratios below 2. All expression data was imported to GCOS (GeneChip Operating System v1.4) and scaled to a target intensity of 2500 to enable comparison across conditions.

Gene expression profiles of hepatocytes in micropatterned co-cultures were compared to gene expression in several models, which included: 1) all cell types of the human liver immediately after tissue disruption but prior to hepatocyte purification; 2) freshly isolated pure

hepatocytes in suspension prior to plating (day 0); and, 3) unorganized pure plated hepatocytes after 1 week of culture as a model for deteriorating functions.

Phase I & II Enzyme Activity Assays

Chemicals were purchased from Sigma: Coumarin (CM), 7-Hydroxycoumarin (7-HC), Ethoxyresorufin (ER), Resorufin (RR), Ketoconazole (KC), Sulfaphenazole (SP), Methoxsalen (MS), Salicylamide (SC), Testosterone (TS), 6ß-hydroxytestosterone (6ß-HTS) or purchased from BD-Gentest: 7-methoxy-4-trifluoromethylcoumarin (MFC), 7-benzyloxy-4-trifluoromethylcoumarin (BFC), 7-hydroxy-4-trifluoromethylcoumarin (7-HFC), Bupropion HCL (BUP), Hydroxybupropion (H-BUP). Cultures were incubated with substrates (CM, MFC, BFC and 7-HC at 50µM, ER at 5µM, TS at 200µM, and BUP at 500 µM) for 1 hour at 37ºC. For inhibition studies, cultures were incubated with substrates in the presence of specific inhibitors (MS at 25µM with CM, SP at 50µM with MFC, KC at 50µM with BFC, SC at 3mM with 7-HC)[4-7]. The reactions were stopped by collection of the incubation medium. Potential metabolite conjugates formed via Phase II activity were hydrolyzed by incubation of supernatants with ß-glucuronidase/arylsulfatase (Roche, IN) for 2 hours at 37° C. Samples were diluted 1:1 in quenching solution and metabolite formation was quantified with a fluorescence micro-plate reader (Molecular Devices, Sunnyvale, CA) as described elsewhere[8]. The amounts of 6ß-HTS and H-BUP in supernatants were quantified via Liquid Chromatography/Mass Spectrometry (Integrated Analytical Services, Berkeley, CA, and Apredica, Watertown, MA). Production of 6ß-HTS from TS is mediated by CYP3A4 in humans[9, 10], production of 7-HC from CM is mediated by CYP2A6[8], production of H-BUP from BUP is mediated by CYP2B6[11], production of 7-HFC from BFC or MFC is mediated by many CYP450s[12], and production of RR

from ER is mediated by CYP1A2[9]. Conjugation of 7-HC with glucuronic acid and sulfate groups is mediated by Phase II enzymes (UPD-Glucuronyl-transferase, Sulfo-transferase)[13].

Drug-Drug Interaction Studies

Co-cultures were first treated with Phenobarbital (PB, 1 mM) dissolved in culture medium for 3 consecutive days to induce CYP450 levels. A separate set of co-cultures were treated with 2 mM Probenecid for 24 hours. Next, co-cultures were incubated for with fresh medium supplemented with either Acetaminophen (30 mM APAP) alone or APAP in combination with PB or Probenecid. Following the 24 hour incubation period with compounds, viability was assessed using the MTT assay as described in 'Methods'.

Staining of Functional Bile Canaliculi

Co-cultures were washed three times with phenol-red free DMEM, then incubated with 2 µg/mL CDF [5-(and-6)-carboxy-2',7'-dichlorofluorescein diacetate, Molecular Probes] for 10 minutes, and washed three times again prior to examination with fluorescence microscopy (excitation/emission wavelengths: 495/520 nm). CDF gets internalized by hepatocytes, cleaved by intracellular esterases and excreted into the bile canaliculi between hepatocytes by transporters[14].

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