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**Transcriptomic hepatotoxicity signature of Chlorpromazine after short- and long-term exposure
in primary Human sandwich cultures**

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CPZ: Chlorpromazine

CYP450: Cytochrome P450

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DMSO: Dimethyl Sulfoxide

EGTA: ethylene glycol tetraacetic acid

FBS: Fetal Bovine Serum

IPA: Ingenuity Pathways Analysis

LPS: Lipopolysaccharide

PHH: Primary Human Hepatocytes

RT: Room Temperature

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Abstract

Drug induced liver injury is the most frequent reason for market-withdrawal of approved drugs and is difficult to predict in animal models. Here, we analyzed transcriptomic data derived from short- and long-term cultured primary human hepatocytes (PHH) exposed to the well-known human hepatotoxin Chlorpromazine (CPZ). Samples were collected from five PHH cultures after short-term (1 and 3 days) and long-term (14 days) repeat daily treatment with 0.1 or 0.2 μ M CPZ, corresponding to C_{max}. Two PHH cultures were additionally treated with 1 μ M CPZ, and the three others with 0.02 μ M CPZ. Differences in the total number of gene changes were seen between donors and over time of treatment. Specific transcriptomic hepatotoxicity signatures were created for CPZ and consisted of inflammation/hepatitis, cholestasis and liver proliferation in all 5 donors, as well as of fibrosis and steatosis that were observed in 4/5 donors. Necrosis was present in 3/5 donors, and an indicative signature of cirrhosis was observed after long-term 14-day repeat treatment also in 3/5 donors. The inter-donor variability in the inflammatory response to CPZ treatment was associated with variability in the strength of the response of the transcriptomic hepatotoxicity signatures, suggesting that features of inflammation could be related to the idiosyncratic hepatotoxic effects of CPZ in humans.

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Introduction

Drug induced liver injury is the most frequent reason cited for market-withdrawal of approved drugs (Lee, 2003). In order to detect hepatotoxic effects as early as possible in the process of drug development, *in vitro* liver models, such as primary hepatocytes, are increasingly being used (Tuschl et al., 2008; Gomez-Lechon et al., 2010). Primary hepatocytes have been shown to be the most relevant model for predicting *in vivo* xenobiotic metabolism (Blanchard et al., 2005; Foster et al., 2011), drug-drug interaction through a reversible or time-dependent inhibition of CYP450s (Zhao et al., 2005; Soars et al., 2007; Gomez-Lechon et al., 2010), interference with transporters (Soars et al., 2007; De Bruyn et al., 2011) and for drug toxicity (Richert et al., 2003; Liguori et al., 2005; Li, 2009).

Microarray analysis has great utility for the elucidation of underlying mechanisms of hepatotoxicity (Richert et al., 2008; Lauer et al., 2009). In addition, large inter-individual variability has been observed in both the basal expression (Rogue et al., 2012) and in response to treatment of many genes related to drug metabolism: Liguori et al., (2005) showed that after 24 hours treatment of trovofloxacin, gene changes varied across four different primary human hepatocytes (PHH) cultures. We (Abadie-Viollon et al., 2010) have shown an important inter-donor variability in the response of three PHH cultures to a 72-hour exposure to reference CYP450 inducers, and Goyak et al. (2008) reported that only a small set of genes was consistently deregulated across ten PHH cultures, whereas relatively large gene sets were regulated uniquely in a given PHH culture in response to 24 hours exposure to reference inducers.

To date mainly short-term exposure *in vitro* toxicity studies have been reported (Lauer et al., 2009; Gerets et al., 2012), but effects in humans during long-term medication needs also to be evaluated and thus long-term repeat-dose toxicity assessments in *in vitro* systems need to be performed. The expression of many liver-specific genes decrease during the initial stage of cultivation of PHHs (Boess et al., 2003; Richert et al., 2006), often associated with declines in metabolic capacities (Richert et al., 2002; Binda et al., 2003; Madan et al., 2003). They are however maintained at acceptable levels over time in long-term cultures using sandwich configuration and appropriate medium (Hewitt et al., 2007;

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Tuschl et al., 2009; Mueller et al., 2012). Prolonged exposure studies of PHH to drugs affecting major toxicity and metabolism pathways, such as ethanol during 14 days (van Pelt et al., 2003) or diclofenac during 23 days (Mueller et al., 2012) have indicated a good *in vivo* / *in vitro* correlation.

Chlorpromazine (CPZ) is the most widely used phenothiazine neuroleptic for the treatment of schizophrenic patients (Wojcikowski et al., 2010). CPZ clearance was demonstrated to be dependent on the patient's bodyweight and lifestyle which is a major problem for its therapeutic use (Pantuck et al., 1982; Chetty et al., 1994). An inter-donor CYP450 polymorphism is also recognized for CPZ pharmacokinetics, since it is extensively metabolized by CYP2D6 and CYP1A2 (Yeung et al., 1993; Yoshii et al., 2000). CPZ has been reported to be cytotoxic and several of its metabolites, generated by both rat and human metabolism, are known to be equally or more cytotoxic than CPZ itself (Abernathy et al., 1977; Tavoloni and Boyer, 1980; Yeung et al., 1993). A species-difference in the *in vitro* sensitivity of hepatocytes in monocultures has been reported, with an EC₅₀ as low as $9 \pm 6 \mu\text{M}$ CPZ in human (Gerets et al., 2012) compared to 110 μM in rat (Zhang et al., 2011) hepatocytes. An incidence of 1-2 % cholestasis has been reported in patients treated with CPZ at therapeutic doses of 2 to 5 mg/kg (Boelsterli et al., 1987; Horikawa et al., 2003; de Abajo et al., 2004). In contrast, CPZ was not hepatotoxic in pre-clinical toxicity studies: for example, CPZ did not cause cholestasis after 3 week repeated-dosing at the highest dose level of 20 mg/kg in rats (Tsao et al., 1983). More recently, Buchweitz et al. (2002) demonstrated the appearance of cholestasis in rats after one dose of CPZ (70mg/kg bodyweight) given 2 hours after LPS administration. Involvement of an inflammatory process could indeed underlie the idiosyncrasy of CPZ since, in humans, CPZ-induced cholestasis has been associated with an infiltration of inflammatory cells, such as neutrophils and lymphocytes (Moradpour et al., 1994).

In the present study the mRNA expression profile of PHH's was analyzed after single- and repeat-dose treatment of CPZ, with the aim to address mechanisms as well as inter-individual variability of PHH.

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Material and methods

Chemicals and solutions

William's E medium GlutaMAXTM, fetal bovine serum (FBS), insulin-transferrin-selenium (ITS), sodium pyruvate, gentamycin and GeltrexTM were purchased from Invitrogen (Fisher, Illkirch, France). Insulin, penicillin/streptomycin, dexamethasone, bovine serum albumin, dimethyl sulfoxide (DMSO), Percoll®, collagenase and chlorpromazine hydrochloride were from Sigma-Aldrich (St-Quentin-Fallavier, France). Hepatocyte maintenance medium (HMMTM) was from Lonza (Verviers, Belgium).

Isolation of primary human hepatocytes (PHH)

Human liver tissue was obtained from resections from patients undergoing partial liver hepatectomy for therapy of hepatic tumors, with permission of the national ethics committees and regulatory authorities (Table 1). Biopsies (20-100 g) were removed from the safety margin of the tissue resected near the tumor. Human hepatocytes were isolated using a two-step perfusion technique as previously described by Richert et al. (2004) and Lecluyse and Alexandre (2010). Briefly, two to four hepatic vessels were cannulated and perfused with 0.5 mM EGTA-containing buffer for 10 min and then with a 0.02 % collagenase-containing buffer for 20 min at 37°C. At the end of the perfusion, the resection was removed from the perfusion system and immersed into warm HBSS containing 10 % FBS (suspension buffer). Using tissue forceps and scissors, the Glisson's capsule was gently torn and the hepatocytes released into the medium following gentle shaking and passing the tissue between the tissue forceps, leaving behind the connective tissue and any undigested material. The cells were filtered through 850, 400 and 100 µm meshes and then centrifuged at 80 g for 5 min at room temperature (RT). Cells were washed once in suspension buffer and once in Percoll®-containing buffer (final concentration 28.8 %) at 168 g for 20 min at RT. The hepatocytes were washed again in suspension buffer at 80 g for 5 min at RT and cell viability and number were determined using the Trypan blue (0.4 %) exclusion method. Only preparations with more than 70 % cell viability were used for further experiments.

Exposure to CPZ for cell viability

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To determine the toxicity of CPZ, PHH suspensions obtained after isolation was seeded onto collagen I coated 96-well plates (Biocoat[®], Dutscher, France) at 0.05×10^6 viable cells per well in 100 μ L William's E medium supplemented with 10 % FBS, 4 μ g/mL insulin, 1 μ M dexamethasone and gentamycin (50 μ g/mL). After overnight culturing, the monolayer was overlaid with 350 μ g/mL Geltrex[™] in seeding medium without FBS for 24 hours. Seven concentrations of CPZ were tested; 0 – 1 – 10 – 20 – 30 – 40 and 50 μ M and either a resazurin or a MTT test was performed after 1, 3 and 14 days of treatment. For the resazurin assay, cell monolayers were incubated for 30 min with 450 μ M resazurin and fluorescence was measured with an excitation wavelength at 530-560 nm and an emission wavelength at 590 nm. For the MTT assay, cell monolayers were incubated for 30 min with thiazolyl blue tetrazolium bromide (1 mg/mL), the supernatant was removed and 100 μ L per well of DMSO was added. After gentle shaking, the absorbance was measured at 595 nm. For the two assays, results were expressed as a percentage versus the vehicle control.

Culture and exposure to CPZ for transcriptomic analysis

The PHH suspension obtained after isolation was seeded as described in “*Exposure to CPZ for cell viability*” onto collagen I coated 6-well plates (Biocoat[®], Dutscher, France) at 2×10^6 viable cells per well. Forty eight hours after seeding, treatment with CPZ was started. The culture medium consisted of Hepatocyte Maintenance Medium (HMM[™]) supplemented with 1 % ITS, 100 nM dexamethasone and 50 μ g/mL gentamycin. Medium was renewed every day and a new overlay with Geltrex[™] (350 μ g/mL) was applied 6, 9 and 13 days after seeding.

Stock solutions of CPZ were made in DMSO: 0.01, 0.05, 0.1 and 0.5 mM CPZ. Stock solutions of CPZ were diluted in appropriate medium to obtain 0.02, 0.1, 0.2 and 1 μ M. Compound containing medium was renewed every day until day 13. Cells not treated with CPZ but receiving only the vehicle (DMSO) served as the control.

RNA extraction

After 1, 3 and 14 days of CPZ treatment, RNA was extracted for whole transcriptome analysis. Cells were washed with PBS 1X and scraped in RNA protect Cell Reagent (Qiagen). After centrifugation,

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cells were lysed in RLT Buffer plus (Qiagen) complemented with 1 % 2-mercapto-ethanol, pipetted onto a QIAshredder spin column (Qiagen) and centrifuged for 2 min at 13800 *g* to be sure of full homogenization. The lysates were kept at -80°C until RNA extraction.

Total RNA was extracted using the RNeasy Plus Mini Kit from Qiagen (Hilden, Germany) on the automated QIAcube™ robotic workstation (Qiagen), both according to the manufacturer's protocols. The RNA quantity was assessed with the NanoDrop 2000 (Thermo Fisher Scientific, Waltham (MA), USA), while the quality was determined with the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

Transcriptomics Processing

Biotinylated cRNA was amplified with the Illumina® TotalPrep™-96 RNA Amplification kit (Ambion®) according to the manufacturer's protocol. After assessing the quantity (NanoDrop 2000) and the quality (Agilent 2100 Bioanalyzer) of the labeled cRNA it was hybridized onto the appropriate Illumina® BeadChips (HumanHT-12_v4). The BeadChips were washed and stained using the Little Dipper® Microarray Processor (SciGene Corporation, Sunnyvale (CA), USA) and subsequently scanned with the Illumina® BeadArray Reader (Illumina Inc., San Diego (CA), USA). The GenomeStudio® Data Analysis Software (Illumina) was used to check the quality of the transcriptomic raw data and to make it accessible for further processing (Illumina, 2010). Transcriptomic raw data were log₂ transformed and quantile normalized before statistical analysis. Quantile normalization was performed as outlined in Bolstad et al. (2003) using the application package 'preprocessCore' (<http://www.bioconductor.org>) written for the open-source statistical software R (<http://www.R-project.org>). Microarray data are available in the ArrayExpress database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-1747.

Data processing and Analysis

Due to a large donor-to-donor variability the transcriptomics results of the five PHH preparations were analyzed separately. Fold change values were calculated to compare gene expression measurements between corresponding time-matched vehicle controls and CPZ treatment conditions. Genes with a

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fold change value larger than 2 were regarded as up-regulated while genes with a fold change value smaller than - 2 were regarded as down-regulated. The total number of deregulated genes was plotted against treatment time and PHH donor, to visualize time and donor effects on these numbers.

For each PHH donor and each time point, hepatic pathway hypotheses were generated using the Ingenuity Pathway Analysis[®] v.7.0 (IPA, Ingenuity System, CA) software. A pathway was selected when at least 2/3 donors treated with 0.02 μ M CPZ, 3/5 donors with 0.1 or 0.2 μ M CPZ or 1/2 donors with 1 μ M have a p-value \leq 0.05 (this IPA[®]-derived p-value is calculated via Fisher's Exact Test and reflects the likelihood that the association between a set of genes and a certain pathway is due to random chance).

A heatmap illustrating fold change values of the genes involved in the identified hepatic signatures were created within the Analyst[™] software (Genedata, Basel, Switzerland). To classify deregulated genes in hepatic signatures, the genes assigned to the different hepatic pathways according to IPA[®] (supplemental table 1) were validated manually using current literature. In addition, these hepatic signatures were expanded by further additional deregulated genes known to be involved in hepatotoxicity, taken from literature (NCBI – Gene and PubMed, Weizmann Institute of Science - The GeneCards Human Gene Database).

The number of deregulated genes per hepatic signature was calculated separately for each hepatic signature, PHH donor, CPZ treatment concentration and treatment time. Dot plots created with R, version 2.14.1, were used to compare numbers of deregulated genes across hepatic signatures, donors and treatment times.

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Results

a. Treatment concentrations for transcriptomic analysis

Within the European Seventh Framework Program Predict-IV (FP7/2007-2013) the CPZ repeat-treatment protocol of PHH was set to a daily exposure over a 14-day period with a concentration causing a maximum of 10 % decrease in cell viability (TC_{10}) and with a presumed non-cytotoxic concentration being $1/10^{\text{th}}$ of TC_{10} .

To determine the TC_{10} of CPZ in PHH, a preliminary cytotoxicity experiment was performed using a range of CPZ concentrations. Cell viability was assessed after short-term (1 day and 3 days) and long-term (14 days) CPZ repeat daily exposure. A large inter-donor variability in the response to CPZ cytotoxicity was observed, since the TC_{10} varied between 1 and 18 μM and TC_{50} between 3 and 35 μM from 13 donors after 3 days of treatment (Figure 1) and ranged between 0.2 and 1 μM in three donors treated daily up to 14 days. As this range of concentrations corresponded to reported C_{max} in patients (0.16 μM) (Borges et al., 2011), PHH (n = 5) were treated with 0.1 or 0.2 μM , i.e. TC_{10} , and in addition either with 1 μM (n = 2), i.e. ten-fold TC_{10} or with 0.02 μM (n = 3) CPZ, i.e. $1/10^{\text{th}}$ of TC_{10} .

b. Hepatotoxicity signatures of CPZ

A total of 34,693 genes were included in the transcriptomic analysis. Figure 2 shows a large variability between donors as well as between treatment times with respect to the total number of gene expression changes. With respect to short-term treatment, the total number of gene expression changes for a given donor ranged between 8 and 1062 genes after 1 day of treatment and between 22 and 188 genes after 3 days of treatment. After 14 days repeated treatment, the total number of gene expression changes per donor varied between 32 and 1499. The number of common genes deregulated at each time point for a given donor varied between 0 and 9, suggesting little biological significance for these genes. Indeed, these were pseudogenes and genes involved in cellular homeostasis (e.g. PCK1 and HNRNPA2B1) that were not included in the hepatic signatures described hereafter. This large variability indicates that a global analysis of gene expression data only makes sense when looking at each donor in isolation.

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Therefore, when looking into specific pathways of hepatotoxicity, we analyzed the five donors and the three CPZ treatment times individually.

Firstly, functional analysis with Ingenuity Pathway Analysis[®] (IPA) software was conducted on the whole gene expression profiles. Several hepatotoxicity signatures of CPZ at 0.1 or 0.2 μM were identified (table 2). Cholestasis, fibrosis, hepatitis, inflammation, liver proliferation, necrosis, GSH depletion and steatosis were identified both after short-term (1 or 3 days) and long-term (14 days) CPZ treatment, while genes related to the pathway giving rise to a signature of cirrhosis were deregulated only after 14 days.

These IPA[®]-derived hepatic signatures were thereafter expanded by further deregulated genes known to be involved in liver toxicity as illustrated in Figure 3. Signatures of liver inflammation/hepatitis, liver proliferation and cholestasis were present in all donors. Cholestasis-related genes were observed as early as the first exposure and in 5/5 donors, this effect was maintained over the entire treatment time. Signatures of fibrosis/cirrhosis, and steatosis were present in 4/5 donors and steatosis was represented with a minimum of one deregulated gene after short-term treatment and two deregulated genes after prolonged treatment. Necrosis/GSH depletion/oxidative stress was present in 3/5 donors.

Even though the hepatotoxicity signatures were to some degree present in all donors, differences could be highlighted: for example, PHH donor S251110 showed signs of necrosis only after short-term treatment with CPZ but no signs of fibrosis/cirrhosis, indicating only transient effects. PHH donor S301110 showed signs of fibrosis only after short-term treatment and no signs of cirrhosis, whereas liver inflammation appeared only after long-term treatment and no signs of necrosis were seen. PHH donor S1045 gave no signs of steatosis or necrosis. PHH donor B1050 showed no induction of steatosis after long-term treatment. PHH donor M291110 was the only donor showing all hepatic signatures both in short- and long-term CPZ treatment.

In order to better capture the time-dependent and donor-dependent hepatotoxicity response to CPZ treatment, we compared the number of gene expression changes across hepatic signatures, donors and

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treatment times (Figure 4). The number of gene expression changes after 3 days of treatment was less pronounced in all of the 5 PHH donors as compared to 1 or 14 days of treatment.

Of particular relevance was the fact that at 1 and 14 days of CPZ treatment, donors with a large number of gene changes related to liver inflammation/hepatitis (M291110 and B1050) also showed large numbers of gene changes related to other specific hepatotoxic signatures.

With treatment concentrations of 0.02 μM (i.e. $1/10^{\text{th}}$ of C_{max} ($n = 3$)) fewer gene changes were observed and with 1 μM (i.e. 10-fold C_{max} ($n = 2$)) more gene changes were observed, but 85 % and 66 % of those genes were also deregulated with C_{max} (0.1 or 0.2 μM) treatment concentrations, respectively (data not shown). Therefore, we can conclude that whatever the concentration used the hepatic signatures were overall similar (Table 2) indicating that we could not define a concentration that did not induce adverse effects. Genes related to necrosis, GSH depletion, steatosis, fibrosis, cholestasis, inflammation, hepatitis and liver proliferation pathways were deregulated both after short- and long-term treatment. Signs of cirrhosis were observed both after short- and long-term of treatment with 1 μM of CPZ, while at 0.1 and 0.2 μM signs of cirrhosis appeared only after 14 days of treatment.

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Discussion

After a 14 day treatment period with increasing concentrations of CPZ, the highest non-cytotoxic concentration observed in PHH was between 0.2 and 1 μM . These results are in accordance with the TC_{50} ($9 \pm 6 \mu\text{M}$ after 48 hours of treatment in PHH) reported by Gerets et al. (2012). The fact that these concentrations of CPZ are close to the reported C_{max} levels in humans (Borges et al., 2011) prompted us to choose PHH repeat-treatment concentrations of 0.1 or 0.2 μM ($n = 5$), as well as $1/10^{\text{th}}$ (0.02 μM , $n = 3$) and the 10-fold concentration (1 μM , $n = 2$) for transcriptomics analysis.

In the present study, we found that after 3 days of treatment fewer gene changes were induced compared to days 1 and 14. We (Richert et al., 2009) have previously described that genes deregulated after 24 hours treatment are not automatically deregulated after 72 hours, and this could represent an adaptation phase after the primary acute response on day 1. Nevertheless, repeat exposure for up to 14 days provided valuable additional information in some donors (S251110, M291110 and S1045) and gave further insight into the mechanism of long-term CPZ induced hepatotoxicity.

The CYP1A gene, one of the main CYP450 involved in CPZ metabolism (Wojcikowski et al., 2010) and producing a metabolite more toxic than the parent (Tavoloni and Boyer, 1980), was found to be up-regulated in the present study after the first day and up to 14-days of treatment at 0.1 and 0.2 μM (2-fold) and 1 μM (3-fold) in PHH. However, other mechanisms than increased CYP1A1/2-reactive metabolite formation are involved in CPZ cytotoxicity towards human hepatocytes, since a similar increase was observed in rat hepatocytes exposed to 1 μM CPZ without any overt signs of cytotoxicity (unpublished data). Inter-individual CYP2D6 dependent reactive metabolite formation seems not to be directly related to toxicity. Indeed, donor B1050 displayed the highest expression in CYP2D6 (between 3- to 9-fold that of the 4 other donors) and a CYP2D6 activity of 1.5 pmol/min/mg cell protein compared to 0.5 pmol/min/mg cell protein for donor S1045, but was not more susceptible to CPZ toxicity than the latter (see also discussion below).

The global hepatic signature obtained after 0.1 or 0.2 μM CPZ treatment generated using the Ingenuity Pathway Analysis[®] (IPA) software was comparable in the five PHH cultures. Cholestasis is the main

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form of hepatotoxicity described in patients treated with CPZ, with an incidence of 1-2 % after treatment with therapeutic doses (Boelsterli et al., 1987; Regal et al., 1987). In 5/5 donors the gene expression changes are indicative of, according to IPA analysis, the cholestasis pathway. The hepatocyte uptake transporter NTCP (SLC10A1) has been described to be down-regulated during cholestasis (Lee and Boyer, 2000; Zollner et al., 2001). In the present study, NTCP (SLC10A1) was down-regulated in 3/5 donors, after the first day of treatment at 0.02 and 0.2 μ M (M291110 and S301110) and after 14-day of treatment at 0.1 μ M (S1045). OCT (SLC22A1) was down-regulated in 2/5 PHH donor exposed to short- (M291110) and long-term (S1045) 0.1 or 0.2 μ M CPZ. The expression of the hepatocyte efflux transporter BSEP (ABCB11) has been reported to be either decreased (Zollner et al., 2001) or not affected (Lee and Boyer, 2000; Demeilliers et al., 2006) in patients with liver cholestasis. In the present study, BSEP, p-gp (ABCB1) and BCRP (ABCG2) were not transcriptionally deregulated at any treatment time in any of the donors after CPZ treatment. Since CPZ is known to inhibit uptake transporters, such as OCT1 (SLC22A1) and also efflux transporters such as BSEP (ABCB11), p-gp (ABCB1) and BCRP (ABCG2) (Pedersen et al., 2008), the results of the present study suggest a cellular feed-back regulation at the transcriptional level of the uptake but not secretory transporter proteins to counteract the inhibition of canalicular transport of bile acids. Down-regulation of CYP7A1, which is a major CYP450 involved in bile acid synthesis (Demeilliers et al., 2006), has also been described in cholestatic patients. CYP7A1 was deregulated in 3/5 donors, up-regulated in two donors, after short-term (B1050) or long-term treatment (S251110) and down-regulated in M291110 after long-term treatment. Taken together, these data highlight feed-back regulations at the transcriptional level by human hepatocytes exposed to CPZ reflecting their attempts to reverse disturbances in bile acid homeostasis.

Fibrosis degenerating into cirrhosis was also described in patients with a lower sulfoxidation capacity (Larrey and Pageaux, 1997; Brind, 2007). Indeed, sulfoxidation mediates CPZ detoxification (Wojcikowski et al., 2010) and a poor capacity of sulfoxidation leads to an accumulation of toxic metabolites that could cause fibrosis and, eventually cirrhosis. From the five studied PHH donors, two (M291110 and S301110) displayed transcriptional signs of fibrosis at both 0.02 and 0.2 μ M, with no

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signs of cirrhosis whereas in two other donors (S1045 and B1050) fibrosis and cirrhosis were highlighted both after short and long-term treatment, which might suggest a poor capacity of sulfoxidation in these donors. Our data did not indicate expression changes of genes involved in sulfoxidation, suggesting that changes at the post-translational level, not measurable by transcriptomics, might possibly lead to these adverse effects.

Signs of liver steatosis were also evident in PHH cultures treated with CPZ: up-regulation of the CYP2E1 gene in 3/5 donors (M291110, S301110, S1045) and of the CYP4A11 gene in 2/5 donors (M291110 and S301110), two markers involved in lipid metabolism and known to be induced in patients with steatohepatitis (Jansen, 2004).

In the present study, transcriptomic data showed gene expression changes related to signs of necrosis, possibly due to the treatment with a concentration close to the TC_{10} , thus causing cell damage. S251110 PHH culture showed signs of necrosis only after short-term treatment whereas M291110 and B1050 showed these signs both after short- and long-term treatment. Liver regeneration has been described to be a defense mechanism of the liver to cytotoxicity (Lee, 2003; Hinson et al., 2010). The early signaling components $TNF\alpha$ and IL-6 secretion are known to cause up-regulation of genes involved in liver regeneration, such as STAT1, MAPK, or IGFBP1 and also up-regulation of vascular endothelial growth factor gene (VEGF) (Li et al., 2002). In the present study a strong up-regulation of IL-6 was accompanied by an up-regulation of MAPK9, IGFBP1, IL1RN and VEGFA (M291110) or STAT1 (S1045), while down-regulation of $TNF\alpha$ and IL-6 genes was accompanied by a down-regulation of IGFBP1, IL1RN and STAT1 genes (B1050). All of these genes involved in liver regeneration were hardly affected when early signaling was not or only slightly deregulated (S251110 and S301110).

IL-6 and $TNF\alpha$ are also widely described to promote inflammatory events through the expansion and activation of T-cells, differentiation of B-cells, and the induction of acute-phase reactants by hepatocytes (Jones et al., 2001). $TNF\alpha$ gene polymorphisms are known to result in variable TNF production which leads to inter-individual drug toxicity in human (Louis et al., 1998). Interestingly,

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when an inflammatory event was induced prior to CPZ treatment, cholestasis could be induced in rats (Buchweitz et al., 2002). In the present study, up-regulation of IL-6 gene expression (M291110 and S1045) was accompanied by an up-regulation of the IL-8 gene, another interleukin induced during liver inflammation, as well as several chemokines and chemokine receptors, such as CCL2 or CXCL2 genes. When the IL-6 gene was down-regulated (B1050), many chemokine and receptor genes were also down-regulated and when the IL-6 gene was not deregulated (S251110 and S301110), no other genes of this IL-6 dependent inflammatory pathway were deregulated. It is also noteworthy that donors with TNF/IL-6 deregulated pathways (M291110, S1045 and B1050) presented more genes deregulated in other pathways, including cholestasis and fibrosis/cirrhosis. Taken together, these results support the hypothesis that features of inflammation could be related to the idiosyncratic effects of CPZ (Roth et al., 2003)

In summary, short- and long-term *in vitro* exposure of PHH to CPZ at concentrations close to human C_{max} (0.1 or 0.2 μM) induced six hepatotoxicity-related transcriptomic signatures: cholestasis, fibrosis/cirrhosis, liver proliferation, necrosis, liver inflammation/hepatitis and steatosis. Inter-donor variability in the inflammatory response of human hepatocyte cultures to CPZ treatment was associated with variability in the strength of these transcriptomic hepatotoxicity signatures. Taken together, these results further suggest that features of inflammation could be related to the idiosyncratic hepatotoxic effects of CPZ in humans and that pathway-specific analysis of gene expression profiles in individual human donor hepatocytes are associated with increased susceptibility to toxic effects of drugs.

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Autorship Contributions

Participated in research design: Parmentier, Truisi, Stanzel, Richert

Conducted experiments: Parmentier, Truisi and Alexandre

Contributed analytic tools: Moenks, Lukas and Truisi

Performed data analysis: Parmentier, Truisi, Stanzel, Kopp-Schneider and Moenks

Wrote or contributed to the writing of the manuscript: Parmentier, Richert, Truisi, Mueller, Stanzel,
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Footnotes

Céline Parmentier and Germaine L Truisi contributed equally to the work presented in this manuscript.

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Figure legends

Fig.1: Toxicity of CPZ after 3 days of treatment. The black line represents mean viability of 13 donors (in % of untreated control). The grey area reflects variability between the 13 donors, with area borders illustrating minimum and maximum viability of the donors. Viability was assessed by MTT or resazurin assays (see Material and Methods).

Fig.2 : Barplot representing the total number of genes which changed at least a 2-fold (as compared to untreated control) for the five PHH cultures after 1, 3 and 14 days at 0.1 or 0.2 μ M of CPZ treatment.

Fig.3 : Heatmap illustrating fold change values (red: up-regulation; green: down-regulation; black: [-1.3, 1.3]) of genes involved in hepatic signatures that were generated for five PHH donors treated after 1, 3 and 14 days at 0.1-0.2 μ M of CPZ.

Fig.4 : Number of up- or downregulated genes per hepatic signature (● = inflammation & hepatitis, ■ = cholestasis, ▲ = fibrosis & cirrhosis, ○ = steatosis, □ = necrosis & GSH depletion & oxidative stress, × = general cellular metabolism, + = DME) obtained for the five PHH cultures (A) after 1 day, (B) after 3 days and (C) after 14 days of treatment with 0.1 or 0.2 μ M of CPZ .

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Table.1: characteristics of the 5 human donors used for the transcriptomics analysis.

M = male, F = female.

Donor ID	Sex	Age (years)	Pathology
S251110	M	63	Colorectal adenocarcinoma
M291110	F	66	Colorectal adenocarcinoma
S301110	M	60	Colorectal adenocarcinoma
S1045	M	75	Hepatic Tumor
B1050	M	63	Hydatid cyst

Table. 2: Transcriptomic signature of PHH after short- (1 or 3 days) and long-term (14 days) treatment with (a) 0.02 μM (3 donors), (b) 0.1-0.2 μM (5 donors) or (c) 1 μM (2 donors) CPZ. These signatures were obtained with Ingenuity Pathway Analysis[®] software, a specific pathway was selected when highlighted with a p-value < 0.05 in at least 2 donors (a), 3 donors (b) or one donor (c).

(a) 0.02 μM			(b) 0.1 or 0.2 μM			(c) 1 μM		
Short-term treatment		Long-term treatment	Short-term treatment		Long-term treatment	Short-term treatment		Long-term treatment
Day 1	Day 3	Day 14	Day 1	Day 3	Day 14	Day 1	Day 3	Day 14
Cholestasis	Cholestasis	Cholestasis	Cholestasis	-	Cholestasis	Cholestasis	Cholestasis	Cholestasis
Fibrosis	Fibrosis	Fibrosis	Fibrosis	Fibrosis	Fibrosis	Fibrosis	Fibrosis	Fibrosis
-	Cirrhosis	Cirrhosis	-	-	Cirrhosis	Cirrhosis	Cirrhosis	Cirrhosis
GSH depletion	GSH depletion	GSH depletion	-	GSH depletion	GSH depletion	GSH depletion	GSH depletion	GSH depletion
Necrosis	Necrosis	Necrosis	-	-	Necrosis	Necrosis	Necrosis	Necrosis
Hepatitis	Hepatitis	Hepatitis	Hepatitis	-	Hepatitis	Hepatitis	Hepatitis	Hepatitis
-	-	-	-	-	Inflammation	Inflammation	Inflammation	Inflammation
Liver proliferation	Liver proliferation	Liver proliferation	Liver proliferation	Liver proliferation	Liver proliferation	Liver proliferation	Liver proliferation	Liver proliferation
-	Steatosis	Steatosis	Steatosis	Steatosis	Steatosis	Steatosis	Steatosis	Steatosis

Figure 1

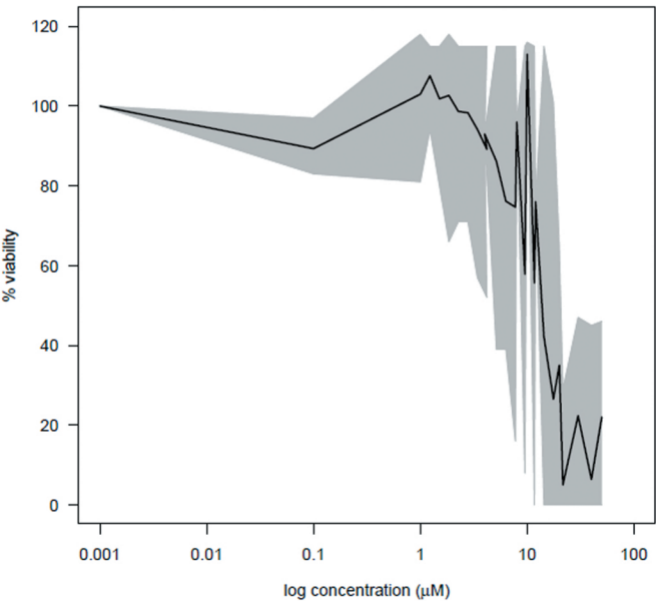


Figure 2

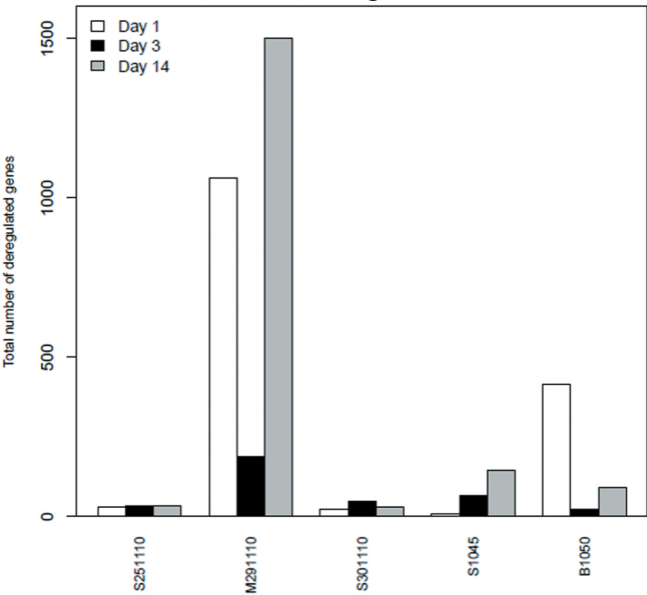


Figure 3

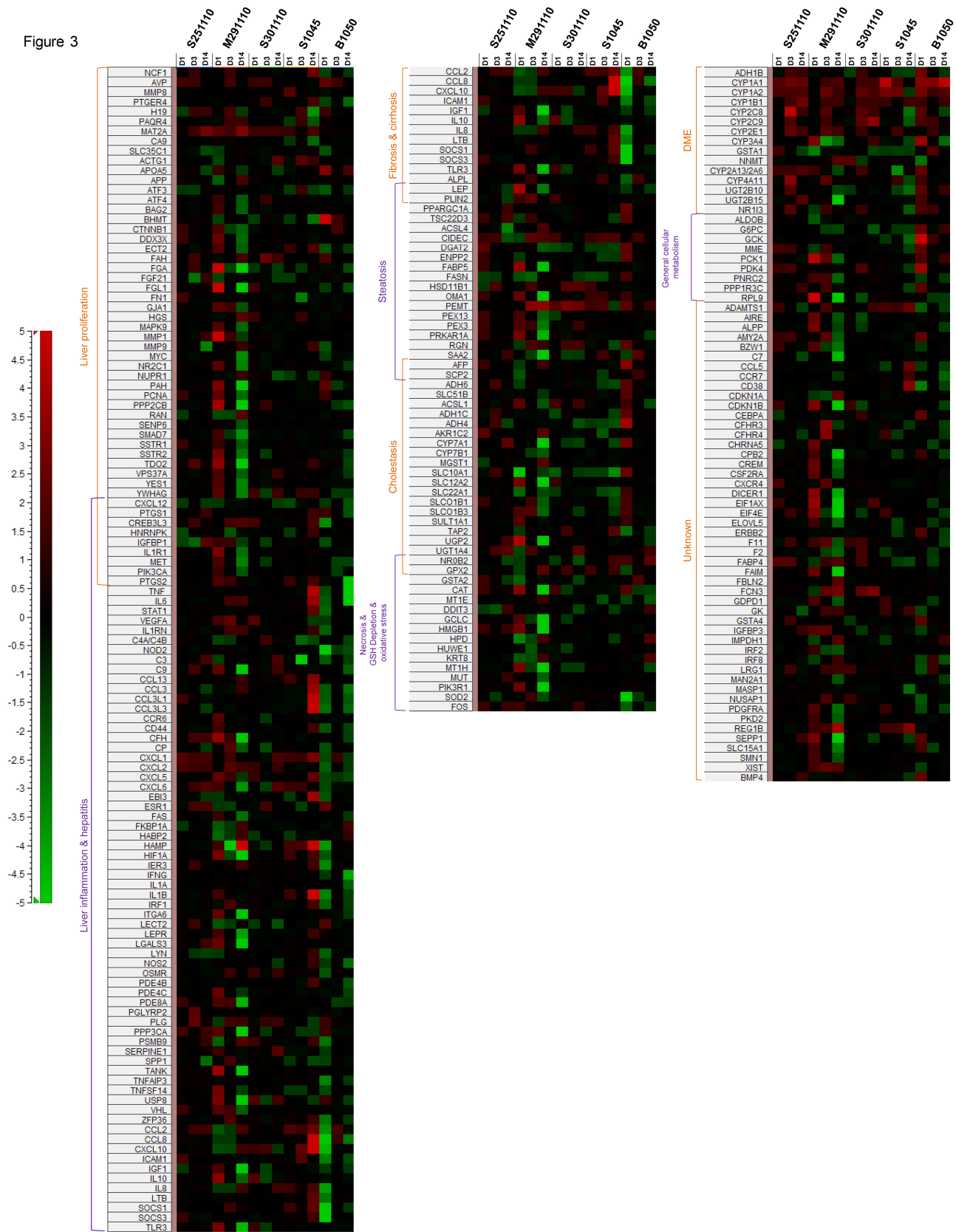


Figure 4

