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Biokinetics of chlorpromazine in primary rat and human hepatocytes and human HepaRG cells after repeated exposure

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ABSTRACT

Since drug induced liver injury is difficult to predict in animal models, more representative tests are needed to better evaluate these effects in humans. Existing *in vitro* systems hold great potential to detect hepatotoxicity of pharmaceuticals. In this study, the *in vitro* biokinetics of the model hepatotoxicant chlorpromazine (CPZ) were evaluated in three different liver cell systems after repeated exposure in order to incorporate repeated-dose testing into an *in vitro* assay. Primary rat and human hepatocytes, cultured in sandwich configuration and the human HepaRG cell line were treated daily with CPZ for 14 days. Samples were taken from medium, cells and well plastic at specific time points after the first and last exposure. The samples were analysed by HPLC-UV to determine the amount of CPZ in these samples. Based on cytotoxicity assays, the three models were tested at 1–2 μM CPZ, while the primary rat hepatocytes and the HepaRG cell line were in addition exposed to a higher concentration of 15–20 μM. Overall, the mass balance of CPZ decreased in the course of 24 h, indicating the metabolism of the compound within the cells. The largest decrease in parent compound was seen in the primary cultures; in the HepaRG cell cultures the mass balance only decreased to 50%. CPZ accumulated in the cells during the 14-day repeated exposure.

Possible explanations for the accumulation of CPZ are a decrease in metabolism over time, inhibition of efflux transporters or binding to phospholipids. The biokinetics of CPZ differed between the three liver cell models and were influenced by specific cell properties as well as culture conditions. These results support the conclusion that *in vitro* biokinetics data are necessary to better interpret chemical-induced cytotoxicity data.

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1. Introduction

Liver injury is one of the most frequent causes for withdrawal of approved drugs from the market (Abboud and Kaplowitz, 2007; Lasser et al., 2002). Drug induced liver injury is difficult to predict with existing animal models and only approximately half of the drugs that cause human hepatotoxicity can be identified during preclinical testing in rodents, dogs and monkeys (Olson et al., 2000). Therefore, new or improved tests are needed that can better predict these unwanted effects in humans.

To determine if a compound has hepatotoxic effects, different *in vitro* models can be used. The most widely used *in vitro* systems

Abbreviations: BSA, bovine serum albumin; CPZ, chlorpromazine; CYPs, cytochrome P450 enzymes; DMEM, Dulbecco's modified eagle medium; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetra acetic acid; HPLC, high pressure liquid chromatography; ITS, insulin–transferrin–selenium; PBS, phosphate buffered saline; TC10, 10% toxic concentration.

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are primary hepatocyte cultures (Farkas and Tannenbaum, 2005; Guillouzo and Guguen-Guillouzo, 2008; Tuschl et al., 2008). Although primary human hepatocytes are considered the gold standard model for xenobiotic metabolism, drug–drug interactions and drug toxicity (Abadie-Viollon et al., 2010; Hewitt et al., 2007), the limited availability of freshly isolated human hepatocytes has long diminished their use. Improvements in cryopreservation processes in recent years enabled the use of human hepatocytes retaining the same properties as freshly isolated cells with regard to phase I and II enzyme expression (Richert et al., 2006) and activities (Alexandre et al., 2012; Li, 2007) as well as transporter expression and activities (Bi et al., 2006; De Bruyn et al., 2011; Richert et al., 2006).

Primary hepatocytes contain metabolically active enzymes at their physiological levels immediately after isolation, but most liver-specific gene expressions decrease during the initial stages of cultivation. The metabolic capacities of primary hepatocytes can thereafter be maintained at acceptable levels over time when cultured in a sandwich configuration and with appropriate medium, as has been shown for primary rat (Tuschl et al., 2009) and human (Hewitt et al., 2007; Mueller et al., 2012) hepatocytes.

Improvements in the field of immortalised cell lines have also been made. Three major immortalised human hepatocyte cell lines have been reported: HepG2, Fa2N-4 and HepaRG cells (LeCluyse et al., 2012). The HepaRG cell line, as described by Gripon et al. (2002), could be an alternative to primary human hepatocytes. After reaching confluence, these cells form hepatocyte-resembling colonies surrounded by biliary epithelial-like cells (Guillouzo et al., 2007). The HepaRG cells express different cytochrome P450-dependent monooxygenases (CYPs), phase II enzymes and transporters at levels that are comparable to cultured primary human hepatocytes and *in vivo* (Aninat et al., 2006; Le Vee et al., 2006). Studies have shown that the patient from whom the HepaRG cells were isolated was probably a poor CYP2D6 metaboliser (Aninat et al., 2006; Turpeinen et al., 2009).

Even though all of these *in vitro* liver cell systems are useful in the determination of drug-induced liver injury, mainly acute *in vitro* toxicity studies are performed (Gerets et al., 2012; Zhang et al., 2011). However, drugs are usually taken for a longer period of time, which makes it necessary to assess repeated-exposure effects in *in vitro* systems both for animal and human models, with the aim to better predict the effects in humans after repeated-dosing. Recent developments in the field of repeated-dose toxicity testing *in vitro* have been made by Jossé et al. (2008) for HepaRG cells. Also some studies report on the effects of long-term exposure on primary human (Mueller et al., 2012; Van Pelt et al., 2003) and rat (Tuschl et al., 2009) hepatocytes.

Major differences in cytotoxicity profiles between *in vitro* test systems from human origin, i.e. primary hepatocytes, HepaRG and HepG2 cells have been reported (Gerets et al., 2012). These differences can be explained by differences in expression of certain liver-specific functions (Gerets et al., 2012; Le Vee et al., 2006), as well as differences in culture conditions such as sandwich culture, medium composition (i.e. presence or absence of serum), number of cells and the ratio between medium and cells (Gerets et al., 2012; Gülden et al., 2001; LeCluyse et al., 2000; Kramer et al., 2012). One has to keep in mind that the bioavailability of a compound in an *in vitro* system is dependent on several processes, including protein binding, evaporation and sorption to plastic (Heringa et al., 2004; Kramer et al., 2012; Seibert et al., 2002; Tanneberger et al., 2013). Too often, these processes are not taken into account. Thus, it is not known to which amount of compound the cells are actually exposed and how this differs from the exposure of cells *in vivo*.

CPZ is indicated for the treatment of psychotic disorders and attained market approval in 1953 (López-Muñoz et al., 2005). This drug was chosen as one of the compounds to be studied for its *in vitro* biokinetics in the European Seventh Framework Programme Predict-IV (FP7/2007–2013) because of its species-species variability in hepatotoxicity (rat versus human). *In vivo*, CPZ induces cholestasis in rats following three weeks repeated-dosing only at high dose levels of 20 mg/kg bodyweight (Tsao et al., 1982), while an incidence of 1–2% of cholestasis was reported in patients treated with the therapeutic dose of 2–5 mg/kg bodyweight (Boelsterli et al., 1987).

The aim of the present study was to compare the *in vitro* biokinetics of CPZ after daily repeated exposure in two different species: rat and human. Primary rat hepatocytes were isolated, seeded and cultured in collagen/collagen sandwich configuration according to Tuschl et al. (2009), primary human hepatocytes were isolated according to LeCluyse and Alexandre (2010), cryopreserved and thawed according to Alexandre et al. (2012) and both fresh and cryopreserved hepatocytes were cultured in a collagen/Geltrex™ sandwich configuration with renewal of Geltrex™ every 3–4 days. The human HepaRG cell line was cultured according to Jossé et al. (2008).

After the first (day 0/1) and last (day 13/14) exposure to CPZ at non-cytotoxic concentrations, samples were taken at specific time points to determine the amount of test compound in the different compartments of the three *in vitro* cell systems. The aim was to assess species- and culture system-differences of the *in vitro* biokinetics and its relationship with the sensitivity to CPZ cytotoxicity.

2. Material and methods

2.1. Chemicals and solutions

Parts of culture media and supplements used for the primary rat and human hepatocytes and for the HepaRG cell line were purchased from Invitrogen (Darmstadt, Germany; Saint-Aubin and Illkirch, France). These include Dulbecco's modified eagle medium/nutrient mixture F-12 (DMEM/F12), GlutaMAX™, Williams' E medium, sodium pyruvate, insulin–transferrin–selenium (ITS), gentamycin, Geltrex™, glutamine, phosphate-buffered saline (PBS) and trypsin/EDTA.

Collagenase, insulin, penicillin/streptomycin, dexamethasone, bovine serum albumin (BSA), Dimethyl sulfoxide (DMSO), Percoll® and chlorpromazine hydrochloride (CPZ) were from Sigma–Aldrich (Munich, Germany; St. Quentin-Fallavier, France; Zwijndrecht, The Netherlands). Human hepatocyte maintenance medium (HMM) was purchased from Lonza (Verviers, Belgium) and HepaRG cell Williams' E medium from Laboratoires Eurobio (Les Ulis, France), FBS was from Hyclone® UK Ltd. (Cramlington, UK) or Perbio (Brebières, France) or Invitrogen (Fischer, Illkirch, France) and hydrocortisone hemisuccinate was from Upjohn Pharmacia (Guyancourt, France).

For the isolation of the primary rat hepatocytes, the liver perfusion, liver digest and hepatocyte wash buffer were prepared in-house using chemicals purchased from Merck, Applichem (Darmstadt, Germany), Roche (Mannheim, Germany) and Sigma (Steinheim, Germany).

For the chemical analysis, methanol HPLC-grade 99.9% purity was from Labscan (Dublin, Ireland) and orthophosphoric acid was from VWR Prolabo (Amsterdam, The Netherlands). Pure deionised water was prepared with a Millipore water purification system (Millipore Water, Amsterdam, The Netherlands).

2.2. Isolation and culture of cells

2.2.1. Primary rat hepatocytes

The primary rat hepatocytes were isolated from male Wistar rats by a two-step liver perfusion method (Seglen, 1976). The

primary rat hepatocytes were seeded at a density of $1.5 * 10^6$ cells per well of a 6-well plate and cultured in a collagen/collagen sandwich configuration. The collagen solution was prepared as previously described by Tuschl et al. (2009) using rat tail collagen type I (Roche). The cell seeding medium consisted of DMEM/F12 with 10% FBS, 100 units/mL penicillin; 100 μg/mL streptomycin, 1 mM sodium pyruvate and 5 μg/mL human recombinant insulin, while the culture medium consisted of DMEM/F12 GlutaMAX™ with penicillin/streptomycin, sodium pyruvate, ITS, 100 nM dexamethasone and 0.44 mg/mL bovine serum albumin. Exposure of the cells to CPZ was started three days after cell seeding.

2.2.2. Primary human hepatocytes

2.2.2.1. Isolation of primary human hepatocytes. 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. Hepatocytes were isolated using a two-step liver perfusion method described by LeCluyse and Alexandre (2010). Only preparations with more than 70% viability were used for further experiments.

2.2.2.2. Cryopreservation and thawing. Cells were cryopreserved as described by Alexandre et al. (2012). For the cell thawing, the vials were placed in a 37 °C water bath. As soon as the content was thawed (60–90 s), the hepatocyte suspension was transferred into a 50 mL centrifuge tube containing a pre-warmed Percoll® containing buffer (final concentration 28.8%) and centrifuged at 168 g for 20 min at room temperature. The resulting pellet was resuspended in adequate culture medium and viability was determined by the Trypan blue exclusion method.

2.2.2.3. Seeding and exposure to CPZ. The cell suspension was seeded onto collagen I coated 6-well plates (Biocoat®, Dutscher, France) at $2 * 10^6$ viable cells per well in Williams' E medium supplemented with 10% FBS, 4 μg/mL insulin, 1 μM dexamethasone and antibiotics (Penicillin/Streptomycin or Gentamycin). After overnight culturing, the monolayer was overlayed with 350 μg/mL Geltrex™ in seeding medium without FBS for 24 h. The culture medium consisted of Hepatocyte Maintenance Medium (HMM) supplemented with 1% ITS, 100 nM dexamethasone and antibiotics. The medium was renewed every day and exposure of the cells to CPZ was started at day 2 after seeding. A new overlay with Geltrex™ (350 μg/mL) was performed every 3-4 days.

2.2.3. HepaRG cells

HepaRG cells were cultured in Williams' E medium supplemented with 10% FBS, 2 mM glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin and $5 * 10^{-5}$ M hydrocortisone hemisuccinate according to Jossé et al. (2008). The cells were passaged every two weeks by washing with PBS and trypsinization. For the repeated exposure experiments, the cells were seeded into 6-well plates at a seeding density of $2.5 * 10^5$ cells per well. The cells were cultured for four weeks and medium was refreshed twice a week. After 14 days of culture, 2% DMSO was added to the culture medium to initiate differentiation of the cells. After these four weeks of culturing in the well plates, exposure to CPZ was started.

Table 1
Donor characteristics.

	Sex	Race	Age
Donor 1	Male	Caucasian	75
Donor 2	Male	Caucasian	63
Donor 3	Female	Caucasian	59

2.3. Repeated exposure and sampling

In previous cytotoxicity experiments, the 10% toxic concentration (TC_{10}) of CPZ was determined in the three cell systems after 14-day repeated exposure using the MTT cell viability assay. For the final experiments, two test concentrations were used: the TC_{10} and 1/10th of the TC_{10}. These nominal TC_{10}-values differed considerably between the systems. Stock solutions of CPZ were made in DMSO: 1 and 10 mM CPZ for the primary rat hepatocytes, 0.05 and 0.5 mM CPZ for the primary human hepatocytes and 1.5 and 15 mM CPZ for the HepaRG cells. Test solutions for the primary hepatocytes were prepared in corresponding culture medium: 2 and 20 μM CPZ for the rat hepatocytes, 0.1 and 1 μM for the human hepatocytes. The primary rat hepatocyte medium contained a final concentration of 0.44 mg/mL albumin. For the HepaRG cells, the test solutions containing 1.5 and 15 μM CPZ were prepared in medium containing 1% DMSO and 2% FBS. Samples were taken from the stock and test solutions for HPLC analysis and stored at −80 °C.

On day 0 of the experiment, the test solutions were added to the wells (1.5 mL/well for the rat hepatocytes and 2 mL/well for the human hepatocytes and the HepaRG cells) and test medium was refreshed daily for 14 days. Samples for kinetics analysis were taken at five different time points after the first (day 0/1) and last exposure (day 13/14). These time points were: 2 min, 30 min, 1 h, 3 h and 24 h. At these time points, the medium was collected from the wells. Next, the wells were washed twice with PBS and the cells were collected. For the primary rat and human hepatocytes (cultured in a sandwich), the cells were scraped from the well and transferred to a LoBind tube (Eppendorf, Hamburg, Germany) or glass vial, respectively, followed by a wash step with 250 μL methanol which was added to the same vial. After in-probe sonication, the volume of the vial was filled up to 1 mL with methanol. For the HepaRG cells, 200 μL PBS was added and the cells were scraped and removed from the wells and added to a glass vial containing 600 μL methanol. The well was washed with 200 μL PBS and this was also added to the vial. The sample was sonicated to homogenise the sample.

After removal of the cells, the wells were washed twice with PBS. Next, enough methanol was added to attain the same height as the culture medium (2 mL/well for all three systems). The plate was incubated under gentle shaking for 2 h at room temperature. After incubation, the methanol was sampled to determine plastic binding of CPZ.

All samples were stored at −80 °C until completion of the biological replicates and HPLC analysis. Experiments with rat hepatocytes and HepaRG cells were performed in triplicate and in each experiment each test condition was performed in duplicate. For the human hepatocytes, one technical replicate within each experiment and 3 biological replicates were performed.

2.4. Sample preparation and HPLC analysis

The samples were prepared for analysis by HPLC-UV. The stock solutions were diluted in 75% methanol: 25% Millipore water. For the test solutions and medium samples, 3 volumes of cold methanol were added to one volume of sample. This was mixed on an IKA MS 1 minishaker (IKA, Staufen, Germany) and subsequently centrifuged for 5 min at 2500 rpm (1455*g*) and 4 °C (Beckman Coulter Allegra X12-R, Beckman Coulter, Woerden, The Netherlands) to precipitate the proteins (96% recovery CPZ). The supernatant was transferred into a glass insert and used for analysis.

For the cell lysate and the samples for the well plastic binding, one volume of Millipore water was added to 3 volumes of sample. This was mixed on a vortex and the cell lysate samples were

centrifuged for 5 min at 2500 rpm to remove the cells. All samples were stored at −20 °C until HPLC analysis.

The HPLC method used to measure CPZ in the samples has been described previously by Broeders et al. (2011). The system consisted of a solvent delivery system (Varian), a C18 column with an ID length of 4.6 × 150 mm and a particle size of 5 μm (GraceSmart), a UV–VIS detector (set at 254 nm), a degasser (Shimadzu) and a Basic Marathon auto sampler (Spark Holland). The mobile phase contained 25% phosphate buffer (pH = 3) and 75% methanol. The limit of detection was 0.05 μM and the limit of quantification was 0.16 μM. Calibration standards with CPZ were prepared in mobile phase and in 25% culture medium: 75% methanol with concentrations ranging from 0.16 to 117 μM (R^2 = 0.999).

A paired *t*-test was performed on the data found in the medium and in the cell lysate samples to determine significant ($p < 0.05$) differences between the two sampling days within each cell system.

2.5. Modelling biokinetic behaviour of CPZ

ACSL-11.5.2 (Aegis Technologies Group, Huntsville) was used to model the biokinetic behaviour of CPZ in the HepaRG cell system during the 14-day repeated exposure to 15 μM CPZ. The model consists of a medium- and cell-compartment and metabolism of the parent compound was included. Moreover, metabolism rate was allowed to decrease during the experiment due to cell viability. The model is depicted in Fig. 1.

The model equations and best fitted parameter values can be found in supplementary material. Although the value for clearance decrease was obtained with a large error, the final amount in cells would be 1.6 fold of the nominal amount if clearance was assumed to decrease totally and 0.6 fold of the nominal amount if it was assumed to be constant.

The model parameter values for apparent permeability, clearance (initial clearance and final clearance as fraction of initial clearance) and cells-medium partition were fitted to the experimental data using ACSL-Math and ACSL-Optimize 2.1 (Aegis Technologies Group, Huntsville). We assumed that the partitioning between medium-cells and cells-medium was constant.

3. Results

3.1. Preliminary cytotoxicity results

To determine concentrations (expressed as a TC_{10}) to be used during the repeated exposure treatment, preliminary cytotoxicity studies were performed with the three cell systems. After 14 days of repeated daily exposure, the TC_{10} and 1/10th of the TC_{10} were selected as high and low test concentrations, respectively. For the primary rat hepatocytes, the TC_{10} was 20 μM, for primary human hepatocytes 1 μM and for the HepaRG cells 15 μM CPZ.

In vitro biokinetic assessments were thus performed at comparable (1–2 μM) concentrations in the three cell systems: in primary human hepatocytes where it corresponded to the TC_{10} and in primary rat hepatocytes and the human HepaRG cell line where it corresponded to 1/10th of TC_{10}. In addition, *in vitro* biokinetic comparison was made at 15–20 μM in the human HepaRG cell line and the primary rat hepatocytes (TC_{10} in both systems).

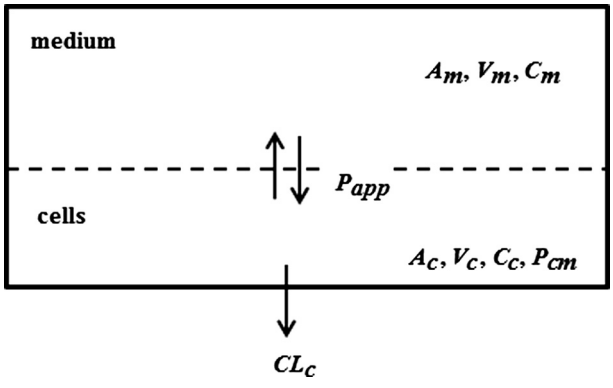

Fig. 1. Model scheme. Transfer of CPZ between medium and cells is described by an apparent permeability, P_{app}, at the medium-cells interface. Clearance of CPZ, CL_c, is from the cells through CPZ-metabolism. Medium and cells, with volumes *V*, contain amounts *A* (nmol) of CPZ with corresponding concentrations $C = A/V$. The chemical potential of CPZ in cells with respects to medium is C_c/P_{cm} with P_{cm} the cells-medium partition coefficient.

3.2. Test solutions and medium

The amount of chlorpromazine was measured in the medium of the primary rat and human hepatocytes and the HepaRG cell line at specific time points on the first and last exposure day (Fig. 2). The low concentration (1/10th of TC_{10}) for the human hepatocytes (0.1 μM) was under the limit of quantification of the HPLC method and could therefore not be measured.

In all three test systems, the amount of CPZ in the medium decreased over time. Two minutes after the addition of the test medium, the amount of CPZ in the medium decreased 15–25% in the primary rat hepatocytes, 13–18% in the primary human hepatocytes and 30–40% in the HepaRG cell line. After 24 h, almost all CPZ had disappeared from the medium: 0.5–18% was found back in the primary rat hepatocyte cultures exposed to both 2 and 20 μM and 0–14% in the HepaRG cell cultures exposed to both 1.5 and 15 μM. In the primary human hepatocytes, the amount in the medium 24 h after exposure was below the limit of detection (LOD). The data point shown in Fig. 2 indicates 1/2 ∗ LOD as a reference value for further calculations.

No significant differences were found between the first and last exposure day, except for the 15 μM CPZ test concentration in the HepaRG cell culture, where the amount in the medium decreased faster on the last exposure day.

3.3. Cell lysate

The amount of CPZ in the primary rat and human hepatocytes and HepaRG cells was measured over time (Fig. 3). For all three cell types, the amount of CPZ in the cells showed an initial increase followed by a decrease over time. After 24 h, almost all CPZ had disappeared from the primary rat (0–3%) and human (0%) hepatocytes. In the HepaRG cells, 30–40% of CPZ added was still present in the cells after 24 h.

Furthermore, significant differences were seen between the two sampling days in all three cell systems. In the primary rat hepatocytes exposed to 20 μM CPZ, the amounts measured on the last sampling day were much higher (6- to 20-fold) than the amounts found for the first sampling day. The same observation was made with the 2 μM CPZ concentration (3- to 5-fold) for the early sampling points of 30 min, 1 h and 3 h. In the primary human hepatocytes exposed to 1 μM of CPZ, higher amounts of CPZ were also found on the last sampling day for the 30 min, 1 h and 3 h sampling points (2-fold). In the HepaRG cell line, higher amounts in the cell lysate on the last sampling day compared to the first were found at all sampling time points and for both the 1.5 μM (4- to 8-fold) and 15 μM (7- to 15-fold) CPZ concentration.

The primary rat and human hepatocytes were cultured in a sandwich configuration and with the collection of cell lysate samples, the cells could not be separated from the extracellular sandwich material. To determine the sequestration of CPZ in the collagen/collagen and collagen/Geltrex™ sandwich, additional experiments were performed. Wells containing the gelatinised

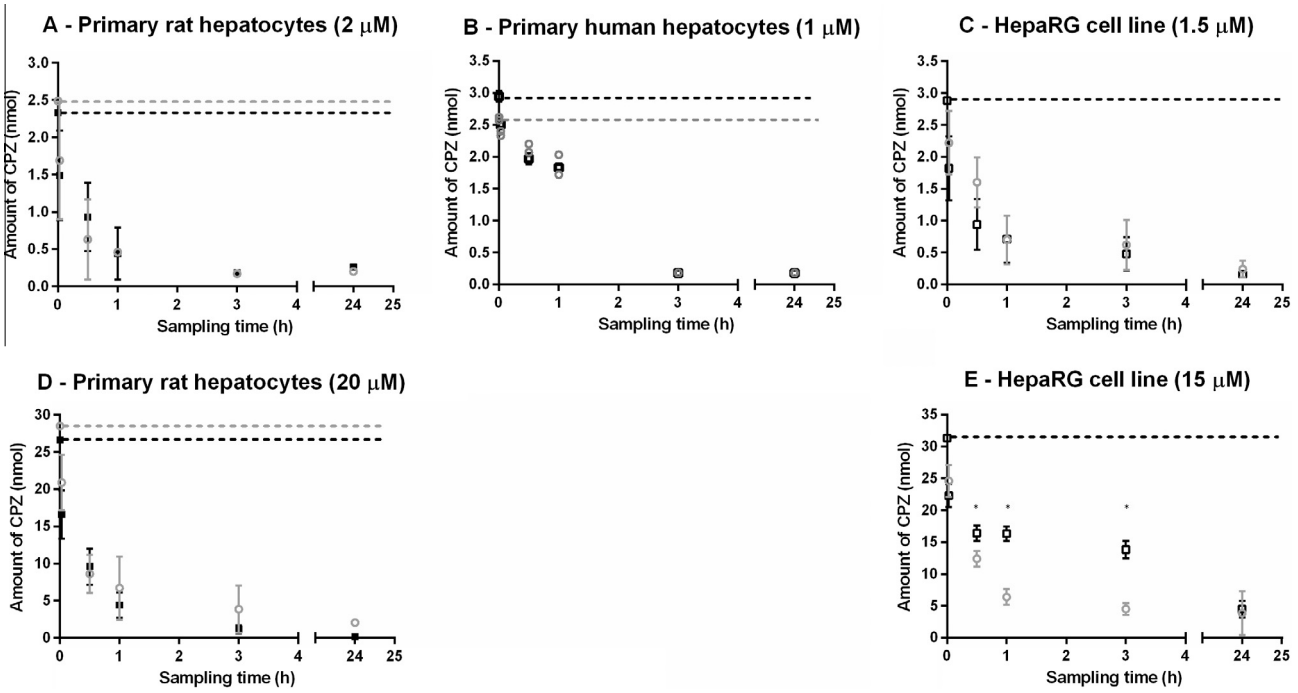

Fig. 2. Amount of CPZ in medium samples on the five time points of the first (□) and last (○) exposure day. (A) 2 μM CPZ in primary rat hepatocytes, (B) 1 μM CPZ in primary human hepatocytes, (C) 1.5 μM CPZ in HepaRG cells, (D) 20 μM CPZ in primary rat hepatocytes and (E) 15 μM CPZ in HepaRG cells. Mean and SD are shown for 3 biological replicates; amount added to the systems is indicated by the dashed lines (black for the first and grey for the last exposure day). Only for the primary human hepatocytes (B) on the last exposure day, data from two replicates are shown as single data points. Significant differences are indicated with an * above the corresponding data point.

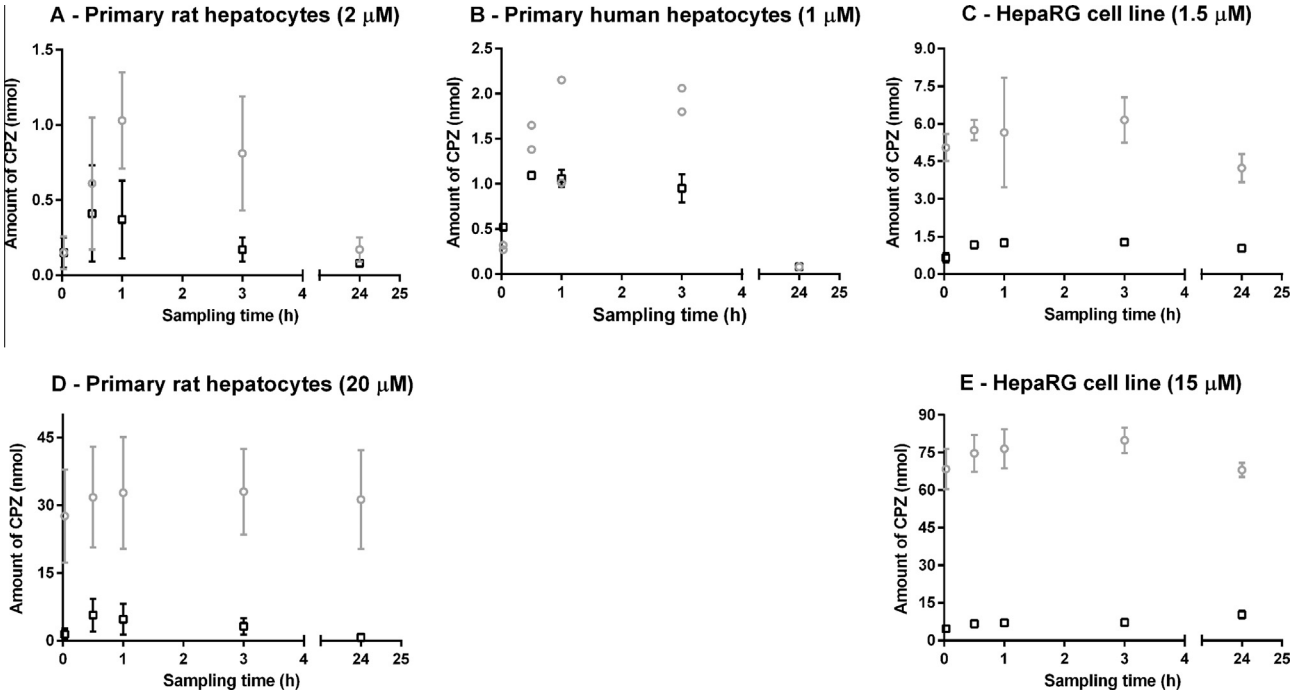

Fig. 3. Amount of CPZ in cell lysate samples taken on the five time points on the first (□) and last (○) exposure day. (A) 2 μM CPZ in primary rat hepatocytes, (B) 1 μM CPZ in primary human hepatocytes, (C) 1.5 μM CPZ in HepaRG cells, (D) 20 μM CPZ in primary rat hepatocytes and (E) 15 μM CPZ in HepaRG cells. Mean an SD are shown for 3 biological replicates; for the primary human hepatocytes (B) individual data points are shown for the last exposure day.

collagen without cells were exposed to CPZ for 14 days and the collagen was sampled after the first and last exposure. Measurements showed that Geltrex™ used for the primary human hepatocyte culture did not influence the experiment, but the collagen used for the primary rat hepatocyte culture did possibly influence the results. The amount of CPZ found in the collagen was higher on

the last sampling day compared to the first sampling day: twice as high for the low CPZ concentration (2 μM) and 9 times as high for the high CPZ concentration (20 μM).

3.4. Plastic

No CPZ was found in the well plastic samples of the primary rat and human hepatocyte cultures (data not shown), but CPZ was detected in the well plastic samples of the HepaRG cell cultures with marked differences between the three biological replicates. The percentage of CPZ in the plastic samples ranged from 0% to 25% (Fig. 4). The amount of CPZ in the well plastic samples remained relatively stable over time within the three replicates.

3.5. Mass balance and distribution

The mass balance of the parent compound chlorpromazine was calculated by adding the amounts found in the different compartments (supernatant, cell lysate, plastic) and comparing this to the amount added (as measured in the test solutions at 0 min, i.e. not given to the cells). The mass balance on the first exposure day is shown in Table 2 for all three liver *in vitro* systems and the different CPZ concentrations tested.

Over time, the amount of the parent compound CPZ decreased in all three cell systems. A large variability was found for the primary rat hepatocytes, especially for the 2 μM CPZ concentration. In the first and third biological replicates, the mass balance ranged from 40% after 2 min to 0% after 24 h. This was in contrast to the second biological replicate, where the mass balance was much higher: above 100% after 2 min to 50% after 24 h. In the primary human hepatocytes, the mass balance was around 100% after 2 and 30 min and decreased over time. After 24 h, no CPZ was found in the primary human hepatocyte cultures. In the HepaRG cell cultures, the mass balance decreased to about 50% at 24 h after addition of CPZ to the cell system. The variability in this experiment is in fact too high to draw clear conclusion from these data at these low concentrations (1–2 μM). However we decided to show these data because such a high variability can be characteristic for these kinds of experiments.

Because the amount of CPZ was measured in all compartments of the culture systems, the distribution of CPZ could be calculated. The percentage of CPZ in medium, cells and well plastic was determined by comparison to the amount added (as measured in the test solution). The results for the first exposure day are shown in Fig. 4 for the three test systems (mean of $n = 3$). A clear decrease was seen in the supernatant (open column) over time in all cell systems. The cell lysate (grey) showed an initial increase followed by a decrease over time. CPZ in the well plastic samples (black–white blocked) was only found in the HepaRG cells. Finally, the percentage of CPZ that was lost/metabolised (black) increased over time in all three liver cell systems. A higher variability was seen with the primary human hepatocytes exposed to 1 μM CPZ and with the primary rat hepatocytes exposed to 2 μM CPZ and we want to emphasise that no clear conclusions can be drawn from these data. In both conditions, the amounts of CPZ found were around the limit of quantification of the analytical method.

Comparable CPZ concentrations were tested in the primary human hepatocytes (1 μM CPZ, TC_{10}), primary rat hepatocytes (2 μM CPZ, 1/10th TC_{10}) and the HepaRG cell line (1.5 μM CPZ, 1/10th TC_{10}). When looking at these CPZ concentrations, it is clear that in the HepaRG cells the fastest decrease in medium and as a consequence the fastest increase in the amount of CPZ in the cells was observed. In the HepaRG cells, the percentage of CPZ in the cells was the highest and remained high until the 24 h sampling time. This was in contrast to the primary hepatocytes, where the percentage of CPZ in the cells went down after 24 h to below the detection limit in the human cells and to 3% in the rat hepatocytes. The mass balance of CPZ was comparable for the three cell systems for the first hour after exposure. Thereafter, the mass balance decreased the most for the primary human hepatocytes, followed

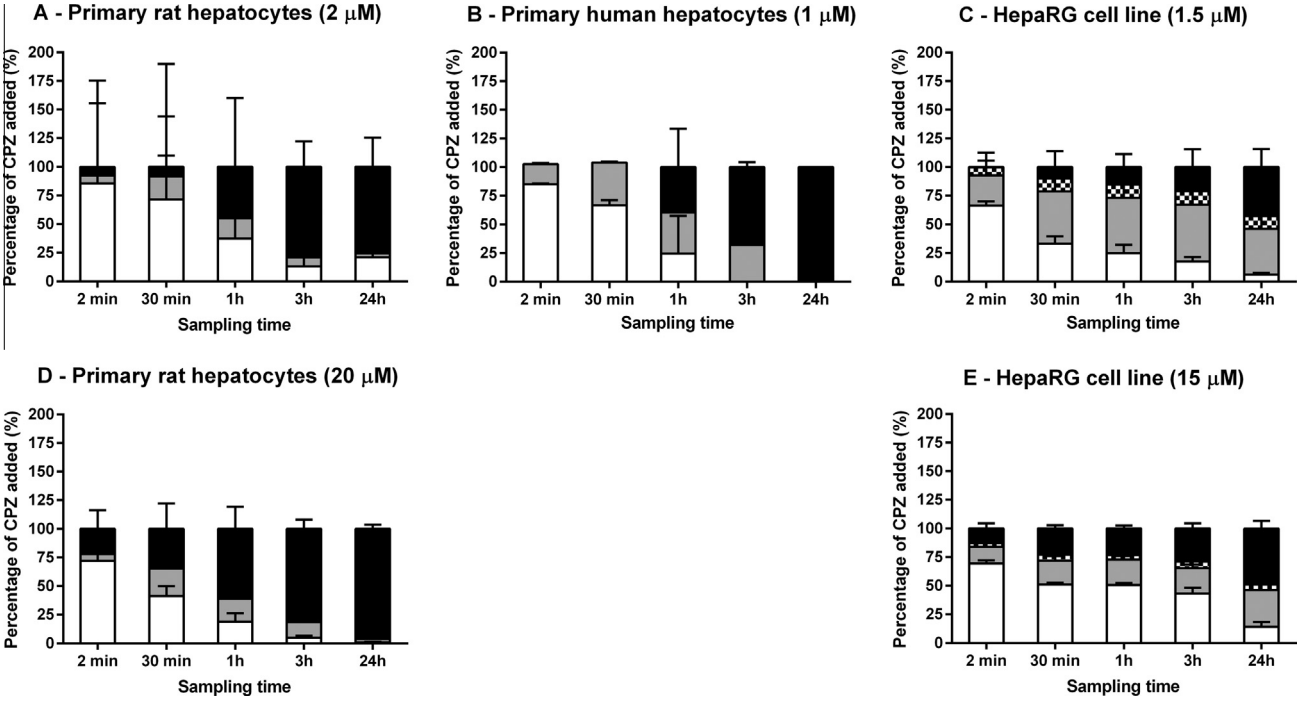

Fig. 4. Distribution of CPZ: percentage of CPZ added is shown for the medium (clear), cells (grey), plastic (white-black blocked) and lost/metabolised (black). Mean and SD are shown for 3 independent experiments. (A) 2 μM CPZ in primary rat hepatocytes, (B) 1 μM CPZ in primary human hepatocytes, (C) 1.5 μM CPZ in HepaRG cells, (D) 20 μM CPZ in primary rat hepatocytes and (E) 15 μM CPZ in HepaRG cells.

Table 2
Mass balance (%) of chlorpromazine in primary rat and human hepatocyte and HepaRG cell cultures on the first exposure day.

Sampling time	Rat hepatocytes		Human hepatocytes	HepaRG cells	
	2 μM CPZ	20 μM CPZ	1 μM CPZ	1.5 μM CPZ	15 μM CPZ
2 min	90.8 (76.5)	106.4 (36.9)	102.6 (1.5)	98.3 (14.8)	87.3 (4.5)
30 min	89.7 (91.2)	76.1 (25.9)	103.9 (4.1)	88.5 (16.0)	77.1 (2.9)
1 h	52.4 (62.6)	41.4 (17.5)	56.8 (36.5)	83.3 (13.5)	79.0 (3.4)
3 h	16.2 (24.5)	18.2 (8.7)	32.2 (4.5)	77.6 (17.8)	73.5 (6.2)
24 h	17.8 (27.7)	2.2 (2.1)	<LOD[a]	49.6 (16.9)	53.6 (9.2)

Mean (SD) in percentages (%) are shown for $n = 3$.
[a] Below limit of detection.

Table 3
Mass balance (%) of CPZ in HepaRG cell cultures after 24 h exposure in medium without FBS and DMSO.

Sampling time	Low CPZ concentration (1.5 μM)	High CPZ concentration (15 μM)
2 min	88.7	90.9
30 min	93.2	79.0
1 h	80.8	69.5
3 h	49.7	69.8
24 h	26.9	43.7

by the rat hepatocytes and lastly the HepaRG cells. In this cell line, the mass balance was still around 50% after 24 h.

In the primary rat hepatocytes and the HepaRG cells, higher CPZ concentrations were tested as well (20 and 15 μM, respectively). For these concentrations, the uptake of CPZ from the medium was faster in the rat hepatocytes. In the cell samples, the amount of CPZ showed an initial increase followed by a decrease over time, although this decrease was smaller in the HepaRG cells. Again, the mass balance remained higher in the HepaRG cells compared to the primary rat hepatocytes.

An additional experiment with the HepaRG cell line was performed by exposing cultures to the same two CPZ concentrations (1.5 and 15 μM) for 24 h in medium that did not contain FBS and DMSO. The results for the mass balances are shown in Table 3 and for the distribution in Fig. 5. This experiment was only performed once (one biological replicate), therefore no standard deviations are shown.

In the absence of serum, slightly higher percentages of CPZ were found in the cells and lower percentages in the medium, indicating a possible faster uptake of CPZ into the HepaRG cells. The mass balances were comparable for HepaRG cells exposed to CPZ in medium with and without 2% FBS, except for the 3 h and 24 h time points, which were lower in the exposure without serum.

3.6. Modelling biokinetic behaviour of CPZ

Experimental data is available for five specific time points on the first and last exposure days. ACSL was used to model the days in between these two experimental sampling days. The modelled data for the medium and cells for the HepaRG cell line is shown in Fig. 6. The modelled data specifically showed that the amount of CPZ in the cells accumulated over time with repeated exposure. The model indicated that a plateau was not yet reached within this 14-day exposure. Compared to the accumulation in cells, the modelled uptake of CPZ from the medium remained relatively stable over time in accordance with the experimental data. Initially, the amount in medium was better estimated by the model and at the final day the amount in cells was better estimated.

4. Discussion

In this study, the *in vitro* biokinetics of chlorpromazine were studied in repeated exposure experiments in primary rat and human hepatocytes and HepaRG cell cultures. This study shows that chlorpromazine distributed differently in the three *in vitro* systems, which inevitably will lead to differences in the interpretation of the results in cytotoxicity studies.

Two different CPZ concentrations were tested: the TC_{10} for cytotoxicity and 1/10th of the TC_{10}. These values were determined in separate repeated exposure cytotoxicity assays. The TC_{10}-values differed between the three cell models. The interpretation of differences in sensitivity of these three cell systems is not feasible; the TC_{10}-values are all based on nominal concentrations. Actual concentrations can be different and also the change in concentration over time is different in these assays (this study).

Another unknown factor that may influence the effect concentrations is the bioavailability. The presence of proteins in the medium may affect the bioavailability of CPZ as this compound is known to bind to proteins (Broeders et al., 2011; Sawada et al.,

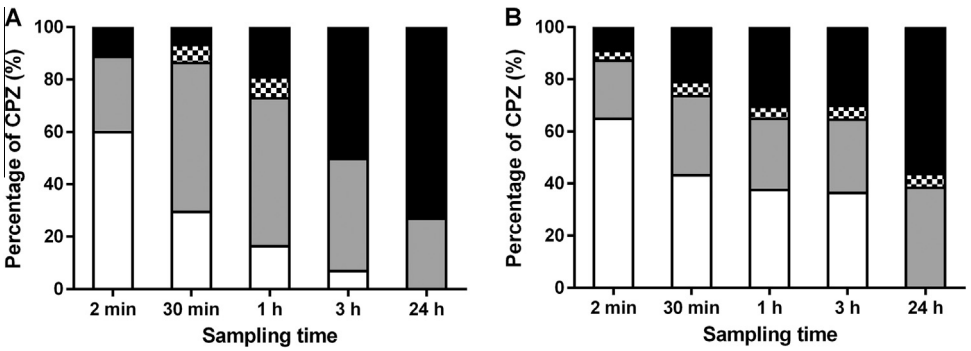

Fig. 5. Distribution of CPZ in the HepaRG cells for 1.5 μM (A) and 15 μM (B) CPZ after exposure for 24 h in medium without FBS and DMSO. Percentage of CPZ added is shown for the medium (clear), cells (grey), plastic (white-black blocked) and lost/metabolised (black). The mean from three technical replicates is shown.

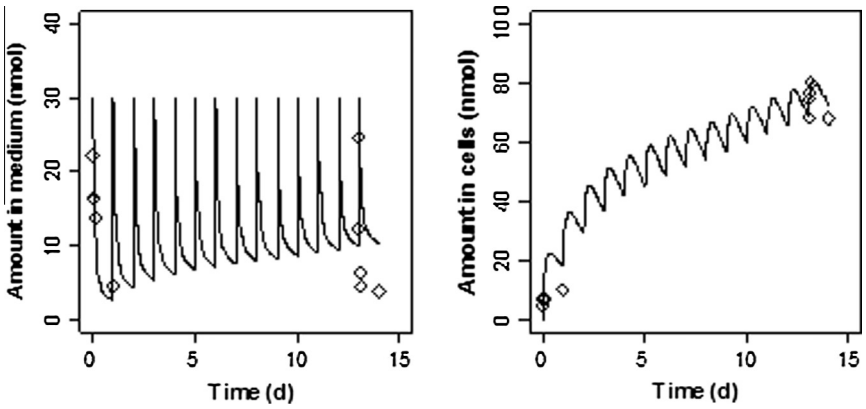

Fig. 6. Comparison of fitted model (line) with data (◇) for the amount in medium (left panel) and the amount in HepaRG cells (right panel) for the 14-days (d) repeated exposure to 15 μM CPZ.

1994). For the HepaRG cell medium, 2% FBS (corresponding to about 0.8–0.9 mg/mL albumin, Kramer et al., 2012) was present in the medium. Also in the primary rat hepatocyte exposure medium, 0.44 mg/mL albumin was present. CPZ is known to bind to proteins, and therefore, the freely available concentration is likely lower than the total medium concentration in these two cell systems. An additional experiment without serum in the medium was performed with HepaRG cells. The results showed that HepaRG cells were equally sensitive to CPZ compared to the experiment performed with serum in the medium. Accordingly, only small differences in uptake of CPZ were observed. Therefore, it might be concluded that serum in the medium did not markedly influence the biokinetics of CPZ. Besides binding to proteins, the bioavailability of CPZ might have been influenced by the culture set-up in a sandwich configuration. Although no sequestration of CPZ was found in the Geltrex™ used for the primary human hepatocytes sandwich culture, sequestration of CPZ in the collagen used for the primary rat hepatocyte culture was found (data not shown) and this complicates the interpretation of the amounts found in these cell samples. Because the partitioning of CPZ to collagen might be different in the presence of cells, it is difficult to correct for the adsorption of CPZ to the collagen.

Plastic bound CPZ was only found in the HepaRG cell cultures. The variability was high between the three biological replicates, possibly because in some samples cell remnants may have been present. The presence of collagen or Geltrex™ in the rat and human hepatocyte cultures may be responsible for not finding any plastic binding in these cell cultures.

The decreases over time in the amount of CPZ in the medium resulted from uptake of the compound into the cells. At treatment concentrations of CPZ in the range of 1–2 μM, the uptake was fastest in the HepaRG cell line, followed by the primary human and rat hepatocytes. The collagen sandwich and collagen/Geltrex™ sandwich used for the primary rat and human hepatocyte cultures, respectively, could delay the diffusion of CPZ to the cells, while the HepaRG cells were in direct contact with the medium.

Once inside the cells, CPZ was retained longest in the HepaRG cells. After 24 h on the first exposure day, 40% of the CPZ added (1.5 and 15 μM) was still present in the cells, in the presence or absence of serum in the medium. The lowest relative amounts of CPZ were found in the primary rat hepatocytes, while uptake from the medium was comparable to the primary human hepatocytes. The variability for this low CPZ concentration in these test systems was high, since the concentrations were around the limit of detection of the analytical method. The slower disappearance of CPZ from the HepaRG cells compared to human hepatocytes could be due to their lower CYP2D6 expression (Aninat et al., 2006; Gripon et al., 2002) since CYP2D6 is one of the enzymes responsible for the metabolism of CPZ in humans (Shin et al., 1999; Yoshii et al., 2000).

In the cell lysate samples of the HepaRG cells and primary rat hepatocytes a significant increase in the amount of CPZ was measured during the 14-day repeated exposure: for the CPZ concentration range of 1–2 μM, 3–5 times higher amounts were found in the primary rat hepatocytes, 4–8 times for the HepaRG cells and only up to twice as high in primary human hepatocytes. For the 15-20 μM CPZ concentrations, 7–15 times higher amounts were found in the HepaRG cells and 6–20 times for the primary rat hepatocytes. New test medium containing CPZ was added to the cell systems every 24 h. If after 24 h the compound is still inside the cells and new compound is added, the compound slowly accumulates inside the cells. Indeed, the accumulation of CPZ inside the cells was relatively important in primary rat hepatocytes exposed to 20 μM CPZ and in HepaRG cells exposed to 15 μM CPZ. Chlorpromazine has been shown to induce phospholipidosis *in vivo* (Anderson and Borlak, 2006; Tavoloni and Boyer, 1980) and *in vitro* (Anthérieu et al., 2013; Bachour-El Azzi et al., 2014) and to bind to lamellar bodies that are formed during phospholipidosis (Joshi et al., 1989). In our experiments, lamellar bodies were observed after the 14-day repeated exposure of HepaRG cells to 15 μM CPZ and of primary rat hepatocytes to 20 μM of CPZ (not shown). Thus, the accumulation of CPZ seen over the 14-day exposure in these two liver cell systems might be related to the formation of lamellar bodies. No such effects were seen in the three cell systems exposed to 1–2 μM CPZ.

Another possible explanation for the accumulation of CPZ in the cells after repeated exposure could be due to inhibition of efflux transporters. CPZ has been reported to be an inhibitor of various efflux transporters such as P-glycoprotein (Feng et al., 2008; Wang et al., 2006), MDR3, MRP2 and BSEP (Anthérieu et al., 2013), transporters which are impaired in human and rat cholestasis (Arrese and Trauner, 2003; Zollner et al., 2001). It should be pointed out that if inhibition of efflux transporters could participate in the accumulation of CPZ in all three repeat-treated cell systems, major differences in the effects on transporters and related manifestation of CPZ toxicity occur. CPZ has been reported to induce features of cholestasis in HepaRG cells at 50 μM (Anthérieu et al., 2013) and CPZ induced features of cholestasis in human hepatocytes at concentrations as low as 1 μM (Parmentier et al., 2013).

The modelled data shown in Fig. 6 closely resembles the experimental data for the first and last exposure day. A similar model for repeated exposure in kidney cells was described by Wilmes et al. (2012). To have a better fit, additional experiments with sampling

points on intermediate days are necessary. This model was included to have an indication of the trends of the behaviour of our test compound in the cell systems. We took the highest CPZ concentration (15 μM) in the HepaRG cell system, since these were the most reliable data and in this culture no sandwich layer was present. Similar data are expected for the other two cell systems. The model clearly shows a gradual accumulation of CPZ in the cells over the 14-day repeated exposure. Possible explanations for this accumulation include an inhibition of metabolism over time, inhibition of efflux transporters and the formation of lamellar bodies and binding of CPZ. We want to emphasise that the estimates of the parameters should probably not be interpreted as accurate estimates, because more data are needed for that. Our major aim of the modelling exercise was to show that the observed trends (in particular the ongoing increase in the amount of CPZ in the cells) may have realistic reasons that could be modelled in this way.

In conclusion, although the biokinetics of CPZ in the cell systems were influenced by specific cell properties, the possible functional changes (alterations) with time in culture, the presence of a sandwich configuration and the medium composition, making the calculations of actual free CPZ concentrations complicated, they confirm that rat and human primary hepatocytes present a different sensitivity to CPZ. Both the rate of metabolism as well as the formation of lamellar bodies resulting from binding of CPZ to the phospholipids influences the results. All this information should be taken into account for successful interpretation of cytotoxicity data and extrapolation of *in vitro* toxicity data to the *in vivo* situation.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2014.08.012.

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