In Silico Modeling for the Prediction of Dose and Pathway-Related Adverse Effects in Humans From In Vitro Repeated-Dose Studies

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ABSTRACT

Long-term repeated-dose toxicity is mainly assessed in animals despite poor concordance of animal data with human toxicity. Nowadays advanced human in vitro systems, eg, metabolically competent HepaRG cells, are used for toxicity screening. Extrapolation of in vitro toxicity to in vivo effects is possible by reverse dosimetry using pharmacokinetic modeling. We assessed long-term repeated-dose toxicity of bosentan and valproic acid (VPA) in HepaRG cells under serum-free conditions. Upon 28-day exposure, the EC50 values for bosentan and VPA decreased by 21- and 33-fold, respectively. Using EC10 as lowest threshold of toxicity in vitro, we estimated the oral equivalent doses for both test compounds using a simplified pharmacokinetic model for the extrapolation of in vitro toxicity to in vivo effect. The model predicts that bosentan is safe at the considered dose under the assumed conditions upon 4 weeks exposure. For VPA, hepatotoxicity is predicted for 4% and 47% of the virtual population at the maximum recommended daily dose after 3 and 4 weeks of exposure, respectively. We also investigated the changes in the central carbon metabolism of HepaRG cells exposed to orally bioavailable concentrations of both drugs. These concentrations are below the 28-day EC10 and induce significant changes especially in glucose metabolism and urea production. These metabolic changes may have a pronounced impact in susceptible patients such as those with compromised liver function and urea cycle deficiency leading to idiosyncratic toxicity. We show that the combination of modeling based on in vitro repeated-dose data and metabolic changes allows the prediction of human relevant in vivo toxicity with mechanistic insights.

Key words: oral equivalent dose; reverse dosimetry; HepaRG; repeated-dose toxicity; metabolic pathway; IVIVE; VPA; Bosentan

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Research Article
Evaluation of long-term repeated-dose effects is still one of the most challenging areas in toxicity screening of compounds and risk assessment (Pfaller et al., 2001). Although for acute effects a variety of in vitro assays amenable to high-throughput applications and multiplexing are available, long-term repeated-dose toxicity assessment is heavily dependent on animal studies. This is despite the fact that animal studies fail to predict human relevant toxicity mainly due to inter species variances (Baillie and Rettie, 2011; Collins, 2011; Greaves et al., 2004).

Modern toxicology is focusing on designing in vitro assays for the identification of early perturbations of biological pathways in human in vitro systems and the use of these for the prediction of adverse effects (Sheldon and Cohen Hubal, 2009). However, extrapolation of in vitro results to human in vivo is sometimes limited due to the fact that nominal concentrations in the in vitro assays are used without consideration of the exposure magnitude, timing, and duration (Coecke et al., 2012). Other factors such as in vivo bioavailability, metabolic clearance, and in vitro-specific parameters such as plastic binding and evaporation (Groothuis et al., 2015) are often overlooked.

Reverse dosimetry is commonly used for the calculation of plausible exposure concentrations also called oral equivalent doses (OEDs). The OED represents the dose required to produce in vivo steady-state blood concentrations equivalent to a given in vitro effective concentration (Rotroff et al., 2010; Wetmore et al., 2012). This approach was used to compare acute effects of chemicals and drugs in humans by in vitro to in vivo extrapolation (IVIVE) using simple pharmacokinetic (PK) models (Chang et al., 2015; Hamon et al., 2015; Rotroff et al., 2010; Wetmore et al., 2012; Yoon et al., 2014). The PK knowledge is needed to relate the in vitro exposure to the in vivo target tissue concentration. Metabolic clearance and dose-response data, both derived from in vitro uptake, metabolism, and viability, can be used for the estimation of the OED by IVIVE.

Most compounds interfere with cellular carbon metabolism either directly or through effects at the transcriptional level. Determination of uptake and conversion rates of substrates (e.g., glucose) and cellular metabolites (amino acids, urea, lactate, etc.) gives insights into the pathways involved in central carbon metabolism and have been previously used to study drug effects (Niklas et al., 2009; Strigun et al., 2011).

The human hepatic cell line HepaRG finds increasing application in toxicity studies (Gunness et al., 2013; Jetten et al., 2013; Klein et al., 2014; Mueller et al., 2014) due to long-term functional stability and similarity to primary human hepatocytes (PHHs) in terms of drug metabolizing enzymes, i.e., the cytochrome P450 (CYP450) system (Josse et al., 2008; Lübbertstedt et al., 2011) and membrane transporters relevant for drug uptake and efflux (Kanebratt and Andersson, 2008).

In this study, using HepaRG cells, we investigated the hepatotoxic potential of two drugs namely bosentan and valproic acid (VPA); upon acute and repeated-dose exposure for 28 days (14 doses). Viability, stability of CYP450 activity, metabolites of test drugs, and drug efflux transporter activity were measured for the assessment of the quality of the test system. Using an IVIVE approach, we estimated the OED from our in vitro repeated-dose toxicity data taking EC10 values (effective concentration in 10% of cell population) as lowest threshold of in vitro toxicity for the prediction of in vivo human toxicity. EC10 values are considered as the lowest concentrations that cause statistically significant cytotoxic effects (Jover et al., 1992). For pathway relevant information, we investigated the alterations in the central carbon metabolism (uptake and secretion rates of selected metabolites) upon exposure to drug concentrations which are in the range of in vivo blood levels. The study aimed at combining in vitro repeated-dose data and in silico tools for the prediction of OEDs and pathway relevant information for the assessment of repeated-dose toxicity in humans in vivo. Our approach is an important step toward replacing animal studies in long-term repeated-dose toxicity evaluations for which no adequate in vitro methods exist.

**MATERIALS AND METHODS**

**Cell Culture**

The human hepatoma cell line HepaRG was obtained from Biopredic International (St. Grégoire, France). Before seeding, 96-well and 6-well plates (Greiner Bio-One, Frickenhausen, Germany) were incubated for 1 h with 50 μg/ml type I rat collagen (Roche Applied Sciences, Penzberg, Germany) in Dulbecco’s modified eagle medium (AMIMED BioConcept, Allschwil, Switzerland). Afterwards, wells were rinsed once with 0.1 or 2 ml phosphate-buffered saline (Gibco Invitrogen, Darmstadt, Germany), respectively, and equilibrated with the same volume of Williams Medium E (Pan Biotec, Aidenbach, Germany) overnight. Differentiated HepaRG cells were seeded in triplicates (n = 3) 4 days prior to treatment in collagen coated 96-well plates in a density of 72000 cells per well for viability and CYP450 activity assay, as well as for the quantification of the drug parent compounds and selected metabolites of central carbon metabolism. For the study of transporter activity (n = 1) and quantification of drug metabolites (n = 3), cells were seeded in 6-well plates with a density of 2.4 million cells per well. Seeding and maintenance was conducted in Williams Medium E supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin (c.c. pro, Oberdorf, Germany), 50 μM hydrocortisone 21-hemisuccinate, 5 μg/ml human insulin (Sigma Aldrich, St. Louis, MO), 10% (v/v) fetal bovine serum (FBS, PAA Laboratories, Pasching, Austria), and 0.5% (v/v) dimethyl sulfoxide (DMSO, Sigma Aldrich, St. Louis, MO). Medium was renewed 1 day after seeding and 1 day before treatment using serum-free seeding and maintenance medium, supplemented with 10 ng/ml human hepatocyte growth factor (Hemanzyme, Chicago, IL) and 2 ng/ml mouse epidermal growth factor (Sigma Aldrich, St. Louis, MO). Cells were kept in and a cell culture incubator (Memmert GmbH, Schwabach, Germany) at 37°C with 95% relative humidity and 5% CO2 supply. For the reduction of evaporation, outer wells of the 96-well culture plates were filled with sterile distilled water (Gibco Invitrogen, Darmstadt, Germany). Culture medium was replaced with fresh medium (with and without drugs) every 2nd day in the long-term cultures.

**Viability Assessment**

Viability was assessed using the CellTiter-Blue assay (Promega, Mannheim, Germany). The assay solution was mixed with medium in a ratio of 1:5. Prewarmed mixture (120 μl) was added to the cells which were incubated for 3 h at 37°C. Fluorescence was detected using a Fluoroskan Ascent CF fluorescence reader (Thermo Labsystems, Vantaa, Finland), measuring emission at 590 nm (excitation 540 nm). Medium without cells served as background control. The fluorescence signals were normalized to the signal of the untreated control at each investigated time point. Sigmoidal curves were fitted to dose-response data using OriginLab 9.0 (OriginLab Corporation, Northampton, MA), and
determined parameters were used for the calculation of the EC$_{10}$ applying equation 1:

$$\text{EC}_{10} = \text{EC}_{50} \times \left(\frac{100 - 10}{10}\right)^p$$

(1)

where $p$ represents the hill slope ($p < 0$). Contour graphs were plotted with OriginLab 9.0 to visualize time and dose dependency. From the acute and repeated-dose response curves, the viability at non-overlapping concentrations was estimated using equation 2:

$$\text{Viability} = \frac{\text{BA} - \text{TA}}{1 + 10^{\log_x - \log_C - p}}$$

(2)

BA and TA are the bottom and top asymptote, respectively. Log $x$ and log $C$ represent the common logarithm of the EC$_{10}$ and the drug concentration for which viability was to be calculated. All drug concentrations were corrected for the measured total concentration of the parent compound.

CYP450 Activity Assay

CYP1A2, CYP2B6, CYP3A4, and CYP2D6 activity was determined as described in our previous study (Klein et al., 2014). Additionally, the activity of CYP2E1 was assessed using chlorozoxazone (Sigma Aldrich, St. Louis, MO) as a substrate with a final concentration of 300 $\mu$M in medium. Cells in a 96-well plate were incubated with 100 $\mu$l of the substrate cocktail for 1 h at 37°C, after which the supernatants were mixed with an equal volume of acetonitrile and frozen immediately. Details of the method are given in the Supplementary material.

Assessment of Efflux Transporter Activity Using Fluorescence Microscopy

For the comparison of efflux transporter activity in standard serum and serum-free cultivation, HepaRG cells were either cultivated for 4 days in serum as described by Kanebratt and Andersson (2008) or in serum-free conditions as described before (cell culture). To assess activity of the multidrug resistance-associated protein 2 (MRP2), cells were incubated with 4$\mu$M of 5-chloromethylfluorescein diacetate (CMFDA, Thermo Fisher Scientific, MA) in Williams Medium E. CMFDA enters the cells and is cleaved by esterases into fluorescent thiol-reactive chloromethylfluorescein. The chloromethyl group reacts with glutathione yielding glutathione methylfluorescein (GSMF) which is a substrate of MRP2 (Förster et al., 2008; Gutmann et al., 1999). Additionally, nuclei were stained by the addition of 10$\mu$M Hoechst 33342 (Sigma Aldrich, Steinheim, Germany). After 15-min incubation at 37°C, cells were washed twice with 2.5 ml phosphate-buffered saline, and fluorescence was monitored using an Olympus IX70 fluorescence microscope (excitation at 494 nm for GSMF and 555 nm for Hoechst 33342).

Preparation of Drug Solutions and Exposure

For bosentan (Sequoia Research, Pangbourne, UK), a 200 mM stock solution in DMSO and for VPA (Sigma Aldrich, St. Louis, MO) an 80 mM stock solution in serum-free seeding and maintenance medium were prepared. All dilutions were performed in serum-free seeding and maintenance medium. The end concentration of DMSO in all tested dilutions was kept at 0.5%. All drug concentrations and the exposure protocol are depicted in the Supplementary Figure 1. A summary of the concentrations with the applied analyses is given in Table 1. In case of the acute toxicity study, the drug concentrations were applied for 24 h (1 dose). In the repeated-dose toxicity study, drug concentrations in fresh medium were applied every 2nd day for 28 days (14 doses in total). Concentrations for the repeated-dose toxicity study were chosen from the results of the acute study. These concentrations ranged from 0.05% (lower limit) to 20% (upper limit) of the acute EC$_{50}$ values of the respective drugs.

Quantification of Drug Metabolites

For the quantification of test drug metabolites in HepaRG cultures, cells were incubated with serum-free seeding and maintenance medium (control) or 5$\mu$M rifampicin (Sigma Aldrich, Steinheim, Germany) in serum-free seeding and maintenance medium (induced) for 24 h. Subsequently, control and induced cells were treated with bosentan and VPA with acute (24 h) EC$_{10}$ (namely, 1.2 mM bosentan and 14.8 mM VPA) of each drug separately. Supernatants were collected after 3 h and 24 h of incubation and stored at −80°C until quantification. The detailed method can be found in the Supplementary material.

Extraction and Quantification of Total Drug Parent Compound

For the determination of the in vitro drug removal, the total drug parent compound concentrations in medium supernatants were quantified at days 1, 6, 14, 20, and 28. Drugs were extracted from 25$\mu$l sample of cell culture supernatants. As reference, supernatants before and after incubation for 48 h at 37°C in cell-free collagen-coated 96-well plates at all sampling points were used.

Internal standards were used for the quantification. These were 15 ng bosentan-$^{13}$C$_2$-$^2$H$_4$ sodium salt (Alsachim, Illkirch-Graffenstaden, France) in case of bosentan and 5$\mu$g benzoic acid (Sigma Aldrich, Steinheim, Germany) for VPA. Bosentan was extracted in 75$\mu$l acetonitrile (Sigma Aldrich, Steinheim, Germany). For VPA extraction, acetonitrile was acidified with 0.1% formic acid (Sigma Aldrich, Steinheim, Germany). Samples were vortexed for 30 s, followed by centrifugation at 12 100 g for 10 min. The supernatant (25$\mu$l) was collected and diluted with 50$\mu$l MilliQ water.

| TABLE 1. List of Concentrations Used for the Quantification of Drug Clearance, Central Carbon Metabolite Analyses, and Drug Metabolite Quantification |
|---|---|---|---|---|---|---|
| | Bosentan ($\mu$M) | VPA (mM) |
| | Acute | Repeated | OBC | Acute | Repeated | OBC |
| Drug clearance | 1.5/38 | 4.8/32 | 19 to 38 | 11/53 | 0.13/1.6 | 0.5 to 4 |
| Metabolome analysis (high dose) | — | 65 | — | 1.6 |
| Metabolome analysis (low dose) | — | 32 | — | 0.13 |
| Drug metabolite quantification (EC$_{10}$) | 1297 | — | 14.8 | — | — |
| Drug metabolite quantification (EC$_{50}$) | — | — | — | — |

The orally bioavailable concentrations (OBC) calculated from the recommended daily dose (Gabbay et al., 2007; Stefan and Fraunberger, 2005; Suzuki et al., 1991) and bioavailability (Perruca et al., 1978; Weber et al., 1999) are indicated. VPA, valproic acid.
Quantification was performed with a Perkin Elmer series 200 HPLC connected to an Applied Biosystem-SCIEX API 3000 triple quadrupole mass spectrometer. Details of the method are given in the Supplementary material.

Reverse Dosimetry for the Estimation of the OED
Cytotoxicity and gene silencing

OED values were estimated following a published method based on the estimation of hepatic and renal clearance (Wetmore et al., 2012). We calculate OED values for experimentally determined time-dependent EC_{10} values from the acute and repeated-dose experiment using equation 3. All calculations are based on the simplifying assumption of well mixed blood (Supplementary Fig. 2).

\[
\text{OED} = \frac{\text{EC}_{10} \times \text{MW} \times 24}{\text{BW}} \times (\text{GFR} \times \text{fu}_P + \text{CL}_{\text{Hep}}) \times \frac{1}{\text{BA}_{\text{oral}}} \quad (3)
\]

OED is oral equivalent dose (mg/(kg BW * day)) necessary to produce a steady-state in vivo blood concentration equivalent to the measured in vitro EC_{10}. GFR is the glomerular filtration rate (l/h), fu_P the unbound fraction in plasma (−), and MW and BW the molecular weight (g/mol) and body weight (kg), respectively. BA_{oral} is the in vivo oral bioavailability of the compound (−). In vivo hepatic clearance CL_{Hep} was calculated following Poulin et al. (2012):  

\[
\text{CL}_{\text{Hep}} = \frac{Q_L \times R_{BP} \times \text{CL}_{\text{int}} \times \text{fu}_P}{Q_L \times R_{BP} + \text{CL}_{\text{int}} \times \text{fu}_P} \quad (4)
\]

Q_L is the total blood flow in the liver (arterial and portal vein) (l/h), R_{BP} is the blood to plasma concentration ratio of the test compound (Supplementary Table 2), CL_{int} the intrinsic clearance rate (l/h) and fu_P the fraction of unbound compound in the liver (−). fu_P,app is the unbound fraction of the compound in the in vitro experiment (−) accounting for plastic and protein binding. Since we did not detect plastic binding for the two compounds and due to the absence of serum proteins in our experiment, fu_P,app was set to 1.

CL_{int} was calculated from the measured in vitro removal rate constant of the compound (Obach et al., 1997). The calculation took into account the different cellular volumes of HepaRG and primary human hepatocytes (PHHs).

\[
\text{CL}_{\text{int}} = k_{i\text{in vitro}} \times \text{LW} \times \text{hc} \times \frac{V_{\text{Hep}}}{V_{\text{PHH}}} \quad (5)
\]

k_{i\text{in vitro}} is the in vitro first order rate constant calculated for a single cell (1/cell * h) in the case of first order reaction, LW the liver weight (kg), hc the hepatocellularity (cells/kg liver), V_{in vitro} is the volume used in the in vitro incubation (l), V_{PHH} the average volume of a primary human hepatocyte (PHH), and V_{Hep} is the average volume of a HepaRG cell. In the case of Michaelis–Menten kinetics, the corresponding value of k_{i\text{in vitro}} depends on the compound concentration (Supplementary material). The kinetics of removal of the compound in the in vitro experiment were determined at different time points of the acute and long-term in vitro experiment using a high and low concentration at each time point, including their values at the beginning and after incubation with HepaRG cells. Cell volumes (V_{PHH} and V_{Hep}) were estimated based on measured diameters of PHH and HepaRG cells using an automated cell counter (Supplementary material and Supplementary Fig. 4).

\[
f_uL = \frac{\text{PLR} \times \text{fu}_{P,\text{app}}}{1 + (\text{PLR} - 1) \times \text{fu}_{P,\text{app}}} \quad (6)
\]

with PLR as the plasma-to-whole-liver concentration ratio, taking into account the extracellular protein binding in plasma relative to liver. fu_{P,app} is the apparent unbound fraction in plasma, taking into account drug ionization, which is an important factor for ionizable compounds since extracellular and intracellular pH differs (7.4 vs. 7.0, respectively) (Poulin et al., 2012).

\[
f_uP = \frac{1 + 10^{(\text{pH}_{\text{plasma}} - \text{p}K_a)}}{1 + 10^{(\text{pH}_{\text{Hep}} - \text{p}K_a)}} \quad (7)
\]

All values applied in the calculation of the OED are given in Supplementary Table 2.

Quantification of the Metabolites of the Central Carbon Metabolism in Medium

Glucose and lactate were quantified by high-performance liquid chromatography (HPLC) as described previously (Klein et al., 2014). Quantification of branched-chain amino acids (BCAA) valine, leucine, and isoleucine was performed with an HPLC method as previously described in depth (Krömer et al., 2005). Urea concentrations were assessed using an HPLC method formerly reported in detail (Clark et al., 2007; Mueller et al., 2012).

Statistical Analysis

Unpaired Student’s t-tests were performed using MatLab 2007b. Differences in two measurements are considered significant at P < .001, P < .01, and P < .05.

RESULTS

Characterization of the Test System

Viability of HepaRG cells upon long-term cultivation. There was no loss of viability of untreated HepaRG cells upon long-term cultivation in serum-free conditions (Supplementary Fig. 5).

CYP450 activity in HepaRG cells. The CYP450 activity for untreated cultures over the course of 28 days remained stable for most isoforms (Supplementary Fig. 6). However, at day 28, CYP3A4 (64% remaining) and CYP2B6 (62% remaining) activities were significantly lower relative to day 6. The other isoforms (CYP2D6, CYP1A2, and CYP2E1) had activities in the same range as on day 6.

Drug efflux transporter activity in serum and serum-free HepaRG cultivation. Microscopical analysis shows that GSMF was concentrated in the bile pockets in both cultivations of HepaRG cells (Supplementary Fig. 7).

Quantification of drug metabolites. Drug metabolites were quantified to assess the system’s capacity to form major metabolites upon incubation with the parent compounds. In the case of bosentan, all major metabolites described in literature were detected (Supplementary Fig. 8A). As for Ro 64-1056, more than one peak was found, probably resulting from hydroxylation at different positions, and the compound was therefore referred to as Ro 47-8634-OH. Preincubation of the HepaRG cells with rifampicin significantly increased the amount of bosentan.
metabolites found in the supernatants, except for bosentan glucuronide. Between 3 and 24 h drug incubation, relative contribution of bosentan metabolites to the total drug molecules in the supernatant increased, while the proportion of the different bosentan metabolites was stable over time (Supplementary Table 3). The two primary metabolites Ro 48-5033 and Ro 47-8634 were the most abundant metabolites, with more than 98% contribution at both time points. In the case of VPA, OH-VPA, ene-VPA, and VPA glucuronide were detected (Supplementary Fig 8B). Contrary to bosentan, only the glucuronide conjugate concentration increased slightly, when cells were preincubated with rifampicin. From 3 to 24 h drug exposure, the amount of VPA glucuronide in the supernatant increased notably (Supplementary Table 4), whereas OH-VPA and ene-VPA metabolites remained below 2% of the drug metabolites. VPA-CoA, 2,4-diene-VPA and Keto-VPA could not be detected in the supernatants.

Quantification of the total drug parent compound concentration in vitro. For the evaluation of the concentrations of the tested drugs to which the cells were exposed to in our in vitro setup, parent compound drug concentrations were measured before and after 48 h cell-free incubation in 96-well plates at 37°C. No statistically significant differences in both bosentan and VPA concentrations upon incubation were observed.

**Viability of HepaRG Cells Upon Acute and Repeated-Dose Drug Treatment**

Dynamic and dose-dependent viability of HepaRG cells upon acute and repeated-dose treatment with bosentan and VPA is depicted in Figure 1. Additionally, for both drugs, EC10 and EC50 values at different time points are given in Table 2. For bosentan, EC10 values ranged from 1603 μM in case of acute toxicity to 75.9 μM after 4 weeks of repeated-dose application (14 doses in total), whereas the EC10 dropped from 1297 μM on day 1 to 45.3 μM after 4 weeks of treatment. EC20 values for VPA ranged from 26.1 to 0.79 mM. The EC10 after 4 weeks treatment was 0.42 mM compared to 14.8 mM at day 1. For both drugs, EC10 and EC50 values decreased constantly over time. The highest drop in the EC was observed within the first 6 days of treatment. Upon application of 14 doses, EC10 values were reduced 21-fold for bosentan and 33-fold for VPA, compared with values for acute toxicity. Concentrations of 40 μM or lower in case of bosentan and 0.2 mM or lower for VPA did not elicit observable cell death after 4 weeks of treatment (14 doses).

**Estimation of Drug Hepatotoxicity Potential in Humans by Using Reverse Dosimetry**

For both drugs, the OEDs over the course of the study are depicted in Figure 2. For bosentan (Fig. 2A), all individuals of the virtual population remained above the recommended daily dose (≥ 3.8 mg/(kg BW*day)) at all time points. In the case of VPA (Fig. 2B), all estimated OEDs were above the maximum recommended daily dose of 60 mg/(kg BW*day) for 2 weeks. At the 3rd week, 4% of the population had an OED below that threshold followed by 47% during week 4. The average calculated whole body clearances (renal and hepatic clearance, for all time points and all individuals of the virtual population) were 4.9 ± 2.6 and 43.6 ± 8.8 ml/(h/kg BW) for bosentan and VPA, respectively.

**Drug-induced Metabolic Alterations at Orally Bioavailable Doses**

Bosentan. Uptake and secretion rates for glucose, lactate, urea, and BCAA in HepaRG cells upon repeated-dose exposure to bosentan are shown in Figure 3. Viability and the lactate secretion to glucose uptake ratio are also depicted. During repeated-dose exposure to bosentan, glucose uptake was increased relative to the control except at the 4-week time point upon treatment with high dose (65 μM). Lactate secretion increased upon treatment with high dose until day 20 after which it fell to the level at low dose (32 μM) on day 28, both being significantly higher than the control. The ratio of lactate secretion and glucose uptake was affected only at the high dose (65 μM), for which it increased over time to a value of almost 2 on day 28. Uptake of BCAA and secretion of urea were significantly decreased when cells were treated with bosentan. Urea production was decreased to similar levels irrespective of doses. Viability was only affected upon repeated high-dose exposure from day 14 onward dropping to 66% of the control on day 28.

VPA. The rates for metabolite bidirectional transport in the cells upon exposure to VPA are illustrated in Figure 4. Until day 14 of repeated high dose (1.6 mM) VPA treatment, glucose uptake was reduced. After 3 weeks of treatment (10 doses), the uptake returned to the original level and eventually surpassed the uptake of glucose in the control. In the case of the low dose (0.13 mM), there was a significant increase in glucose uptake at the last sampling point on day 28. Upon repeated high-dose exposure, lactate secretion was decreased until day 14 and remained stable afterward until day 28. However, at repeated low-dose exposure, the lactate secretion was increased from day 14 onward. BCAA uptake rates were significantly lower in the presence of VPA at high dose. Urea secretion was significantly lower when compared with the control irrespective of the dose. Viability was only affected upon exposure to high dose whereby it dropped to roughly 15% after 4 weeks of repeated treatment (14 doses). Lactate secretion in relation to the glucose consumption was lower upon repeated treatment with high dose than in the control.

**DISCUSSION**

Reliable prediction of human relevant repeated-dose toxicity using in vitro systems and tools is of major interest not only in preclinical drug development in pharmaceutical industry but also in the prioritization and de-risking of chemicals in other industries. Recently, biokinetic/toxidynamic models were used to study the time course of test compound effects in HepaRG cultures upon acute and long-term exposure (Teng et al., 2015) emphasizing the necessity of long-term exposure in vitro data for the recapitulation of chronic hepatotoxic effects. In this study, we investigated the hepatotoxic potential of bosentan and VPA on HepaRG cells upon acute and repeated-dose exposure for 28 days and predicted their toxicity on humans in vivo by the estimation of the OEDs. At clinically
relevant concentrations, changes in central carbon metabolism upon drug exposure in vitro provided mechanistic information that will help understand some of the effects observed in vivo.

Estimation of Drug Hepatotoxicity Potential in Humans by Using Reverse Dosimetry

For bosentan and VPA several cases from mild to severe hepatotoxicity have been reported (Eriksson et al., 2011; Nanau and Neuman, 2013). However, their mechanisms of toxicity are only partially elucidated (Fattinger et al., 2001; Silva et al., 2008). In the case of bosentan, contradictory reports are available. Bosentan was reported to inhibit the bile salt export pump (BSEP) leading to hepatotoxicity (Fattinger et al., 2001). However, a recent study with patients showed that variants of the ABCB11 gene coding for the BSEP transporter are not associated with bosentan hepatotoxicity (Seyfarth et al., 2014). Another study linked the export of bosentan and its glucuronide conjugate to MRP2 rather than BSEP as clearance mechanism (Fahrmaier et al., 2012). CYP3A4 and CYP2C9 are involved in the metabolism of bosentan (Dingemanse and Van Giersbergen, 2004; Treiber et al., 2007). Recently, it was reported that besides metabolic bioactivation, the toxicity of bosentan is dose dependent (Kenna et al., 2014). As for VPA, complex and multiple effects are reported. Long-term VPA treatment is accompanied by intracellular accumulation of lipids occasionally resulting in dose-independent microvesicular steatosis leading to liver failure (Eadie et al., 1988; Fromenty and Pessayre, 1995). VPA causes elevation in liver transaminases in 5–10% of patients and more than 100 fatal cases linked to hepatotoxicity have been reported (livertox.nih.gov/Valproate.htm, accessed on July 24, 2015).

Bosentan causes mild hepatotoxicity in approximately 10% of patients (Eriksson et al., 2011) necessitating withdrawal of therapy in about 3% of patients (Humbert et al., 2007). In susceptible patients, there can be very severe consequences (Mulchey and Bshouty, 2009).
In our study, all members of the virtual population had an OED above the recommended daily dose predicting no adverse effects in these cases within 28-day treatment with bosentan. Comparing the whole body clearance of bosentan estimated from our in vitro experiment ($1.0 \pm 0.2$ ml/(h*kg BW) after 24 h and $7.4 \pm 1.6$ ml/(h*kg BW)) after 28-day exposure to the clearance observed in vivo (243–305 ml/(h*kg BW)) after 8–14 days (Dingemanse and Van Giersbergen, 2004; Markert et al., 2014b), the predicted clearance of bosentan in our study is underestimated. We observe an increase in the clearance of bosentan in our in vitro study. Bosentan is known to induce its own metabolism and the in vivo clearance is increased by 60–70% after 6–10 days of treatment (Markert et al., 2014a; van Giersbergen et al., 2002a,b). The OEDs calculated using the in vivo clearances (Supplementary Table 5) are above the recommended daily dose, as observed for OEDs estimated from our in vitro data. Under- and overestimation of clearances is a major problem in in vitro PK studies, and there are many attempts to overcome this obstacle by introducing further parameters into the estimation of clearances (Berezhkovskiy, 2011; Berezhkovskiy et al., 2009; Poulin et al., 2012). In the case of bosentan, underestimation of the hepatic clearance can be related to the absence of serum in our setup. A 14 times lower clearance for bosentan is reported when the incubation was performed in a serum-free instead of serum-supplemented environment (Blanchard et al., 2006). It is hypothesized that not
only the unbound fraction of a compound can be taken up into
the cells but compound-protein complexes can be similarly
involved in uptake processes (Blanchard et al., 2004).

Regarding VPA, the incidence of liver failure is estimated to be
below 0.02% (Nanau and Neuman, 2013). However, occurrence of
mild hepatotoxicity (elevation of liver enzymes) is around 11%
(Powell-Jackson et al., 1984). The doses upon which hepatotoxicity
occurs range from 0.1 to 80 mg/(kg BW * day). Infants and polyme-
dicated patients are more susceptible to VPA hepatotoxicity
(Nanau and Neuman, 2013). Moreover, inducers of CYP2E1 such
as ethanol have been reported to increase hepatotoxicity of VPA
(Neuman et al., 2001). In our study, after 4 weeks, 47% of the vir-
tual population had an OED below the maximum recommended
daily dose (60 mg/(kg BW * day) predicting hepatotoxic effects
for these cases. VPA has a very wide therapeutic dose range.
Thus, for better comparison, in vitro dosing should be adapted to
specific recommended dosing in patients for patient-specific
prediction. The whole body clearance for VPA in vivo is in the
range of 17.0 ± 6.10.6 ml/(h*kg BW) in
women (Birnbaum et al., 2004). Our estimations are above these
values with a clearance of 43.6 ± 6.8.9 ml/(h*kg BW). Taking in vivo
clearance in the OED estimation, the toxicity of VPA is predicted
to occur already on day 6 with an OED of 121 ± 675 mg/(kg BW *
day), which further decreases to 24 ± 615 mg/(kg BW * day)
on day 28. VPA follows non-linear PK in vivo (Fattore et al., 2006;
Lampon and Tutor, 2012; Vučićević et al., 2009) depending
on dosage, age, poly medication, and hepatic or renal
impairment.
Our in silico approach allows easy estimation of hepatic clearance and is amenable to high-throughput setups. However, as a result of its simplicity, there are some limitations and aspects like accumulation and distribution of compounds in tissues were not considered. In addition, many drugs show substantial serum binding and their uptake into the cells is complex. Therefore, these should be considered in the assessment and calculation of in vitro hepatic clearances. Moreover, incorporation of data on transport, permeability and metabolism is expected to improve predictions (Poulin, 2013).

Drug-Induced Metabolic Alterations at Orally Bioavailable Drug Doses

Many drugs interfere with central carbon metabolism of cells disturbing metabolic homeostasis. These changes can provide important insights on the adverse outcome pathways. In this study, we investigated key metabolites in the central carbon metabolism of HepaRG cells upon exposure to in vivo relevant concentrations of bosentan and VPA. These selected concentrations were derived from dosing recommendations and oral bioavailability. Bosentan has not been reported to have any direct interactions were derived from dosing recommendations and oral bioavailability. Bosentan has not been reported to have any direct effects on enzymes involved in the central carbon metabolism.

In our in vitro study we see an increased uptake of glucose with time. Collapse of glucose uptake (also lactate production) as observed after 4 weeks of treatment with a high dose could be the result of insulin resistance affected by repression of the GLUT4 gene, related to chronic PPAR activation (Finck et al., 2005; Said et al., 2005). There was a steady increase in lactate production until day 20 of the experiment. Lactate is reported to be a marker for cellular stress (Limoncier et al., 2011). We observed an increase in the lactate to glucose ratio which may be due to increased expression of LDH which is involved in conversion of pyruvate to lactate. Increase in LDH is reported to be due to the activation of HIF-1α (Kim et al., 2006) which is a known receptor for reactive oxygen species. Accumulation of bosentan and its metabolites probably results in oxidative stress in hepatocytes. These hypotheses need confirmation by additional experiments. Furthermore, urea formation in hepatocytes was shown to be impaired upon exposure to bosentan (Chatterjee et al., 2013). However, in that study, it was not evident whether this effect was only due to loss of cells or due to effects of bosentan on cellular metabolism. We show that bosentan severely reduces urea formation and BCAA uptake.

Effects of VPA on the central carbon metabolism are manifold (Nanau and Neuman, 2013; Wang et al., 2012). Our observations support previous studies that show that VPA-CoA and other metabolites influence the central carbon metabolism by inhibiting the dihydrolipoamide dehydrogenase, a subunit of the branched-chain α-ketoacid dehydrogenase complex (BCKDC) and pyruvate dehydrogenase complex (PDC) (Luís et al., 2007). VPA exposure resulted in an increase in the lactate to glucose ratio although the conversion of reactive oxygen species upon treatment is reported (Kiang et al., 2011). As VPA-CoA impairs the access of pyruvate to the TCA cycle by inhibiting the PDC, it is likely that pyruvate is converted into acetyl-CoA which then serves as a building block for fatty acid synthesis, contributing to the emergence of steatosis. Increasing concentrations of fatty acids are reported to upregulate the glycolytic genes (Kota et al., 2005). Indeed, chronic VPA treatment is linked to metabolic syndromes (Nanau and Neuman, 2013). In addition, we observed significantly decreased rates in urea secretion during repeated dosing. This is an indirect hint to urea cycle disruption upon long-term repeated-dose exposure to VPA. This effect may stem from the inhibition of N-acetylglutamate synthase which leads to hyperammonemia in certain patients (Aires et al., 2011). Again this hypothesis needs further investigation for verification. Nevertheless, patients with certain metabolic predispositions such as ornithine transcarbamylase deficiency resulting in hyperammonemia might be more susceptible to drug toxicity as a result of additional stress elicited by impaired ammonia removal.

CONCLUSION

Long-term repeated-dose in vitro toxicity data can be used for the prediction of human relevant toxicity using a simple in silico method of IVIVE. Metabolic changes upon drug exposure to in vivo relevant concentrations identify key players in cellular-stress related to idiosyncratic toxicity. This knowledge can help in the adjustment of individual dosing regimens in a step toward personalized medicine. The methods can be adapted to compound screenings for long-term repeated-dose effects and could significantly contribute to animal-free assessment of toxicity.

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SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

REFERENCES


