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Determination of liver specific toxicities in rat hepatocytes by high content imaging during 2-week multiple treatment

Davide Germano, Marianne Uteng, Francois Pognan, Salah-Dine Chibout, Armin Wolf*

Discovery and Investigative Safety, Preclinical Safety, Novartis Institutes for Biomedical Research, CH-4057 Basel, Switzerland

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ABSTRACT

DILI is a major safety issue during drug development and one of the leading causes for market withdrawal. Despite many efforts made in the past, the prediction of DILI using *in vitro* models remains very unreliable. In the present study, the well-established hepatocyte Collagen I-Matrigel™ sandwich culture was used, mimicking chronic drug treatment after multiple incubations for 14 days. Ten drugs associated with different types of specific preclinical and clinical liver injury were evaluated at non-cytotoxic concentrations. Mrp2-mediated transport, intracellular accumulation of neutral lipids and phospholipids were selected as functional endpoints by using Cellomics™ Arrayscan® technology and assessed at five timepoints (day 1, 3, 7, 10, 14). Liver specific functional impairments after drug treatment were enhanced over time and could be monitored by HCI already after few days and before cytotoxicity. Phospholipidosis-inducing drugs Chlorpromazine and Amiodarone displayed the same response as *in vivo*. Cyclosporin A, Chlorpromazine, and Troglitazone inhibited Mrp2-mediated biliary transport, correlating with *in vivo* findings. Steatosis remained difficult to be reproduced under the current *in vitro* testing conditions, resulting into false negative and positive responses. The present results suggest that the repeated long-term treatment of rat hepatocytes in the Collagen I-Matrigel™ sandwich configuration might be a suitable tool for safety profiling of the potential to induce phospholipidosis and impair Mrp2-mediated transport processes, but not to predict steatosis.

1. Introduction

Drug-induced liver injury (DILI) is one of the most common adverse event leading to drug attrition during pharmaceutical development (Kola and Landis, 2004) and to drug withdrawals (Wilke et al., 2007) after market introduction. There are many clinical situations and mechanisms leading to DILI. Intracellular accumulation of lipids (steatosis) or phospholipids (phospholipidosis) and inhibition of biliary clearance (cholestasis and hyperbilirubinemia) are regarded as severe pathological features affecting the liver.

Following impairment of multiple mechanisms such as mitochondrial β-oxidation, *de novo* fatty acid synthesis (lipogenesis) or fatty acid release from adipose tissues (lipolysis), neutral lipids can accumulate in hepatocytes leading to micro- and macrovesicular steatosis (Begriche et al., 2011; Labbe et al., 2008). Long-term treatment of steatotic drugs may also lead to cirrhosis and finally to liver failure (Begriche et al., 2011).

Multidrug resistance-associated protein 2 (Mrp2) is an ATP-binding cassette (ABCC2) transporter located at the bile canalicular membrane. It is a major efflux transporter involved in biliary excretion, playing a crucial role in the biliary excretion of a wide variety of organic anions, including glutathione, glutathione conjugates, sulfated and glucuronidated bile acids (Borst et al., 2006). In addition, Mrp2 plays an important role for the biliary excretion of bilirubin: the absence of Mrp2, such as in patients affected by Dubin–Johnson Syndrome (DJS) or in transport deficient (TR^-) rats, has been associated with deregulation of bilirubin homeostasis resulting into hyperbilirubinemia (Kartenbeck et al., 1996; Paulusma et al., 1996). Inhibition of Mrp2-mediated biliary clearance may result in lipid homeostasis impairment and toxic accumulation of metabolites in the hepatocytes (Tang, 2007).

Phospholipidosis (PLD) is a lysosomal storage disorder characterized by excessive accumulation of phospholipids in several tissues, such as liver, kidney and lung. Cationic amphiphilic drugs

Abbreviations: DILI, drug-induced liver injury; Mrp2, multidrug resistant protein 2; HCI, high content imaging.

* Corresponding author. Address: Discovery and Investigative Safety, Preclinical Safety, WKL-135.2.86, Novartis Institutes for Biomedical Research, Klybeckstrasse 141, CH-4057 Basel, Switzerland. Tel.: +41 61 324 1735; fax: +41 61 324 1893.
E-mail address: armin.wolf@novartis.com (A. Wolf).

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(CADs) have been demonstrated to possess a high potential to induce PLD (Halliwell, 1997). The impaired degradation of phospholipids by lysosomal phospholipases following CADs administration seems to be the main mechanism (Reasor and Kacew, 2001). Despite the evidence that drug-induced PLD is often reversible and that toxicological implications remain uncertain, it is still considered an adverse side effect by regulatory authorities (Berridge et al., 2007) and some challenge for pharmaceutical companies to circumvent. Therefore, the use of characterized predictive models is highly recommended in order to identify toxicity potential in preclinical phases.

Primary hepatocytes are regarded as the gold standard for assessing drug transport and metabolism *in vitro*. However, following isolation and culture, primary hepatocytes may fail to maintain their typical oriented apical and basolateral morphology as well as hepatic functions. Without embedding in an extracellular matrix, the expression and activity of cytochrome P450 (CYP) enzymes in hepatocytes cultured on plastic remains stable only during a short period. Loss of polarity can be avoided by culturing primary hepatocytes in sandwich configuration allowing longer periods of culture (Dunn et al., 1991), maintenance of liver functions (LeCluyse et al., 1994; Tuschl et al., 2009) and characteristic gene expression (Kim et al., 2010). Despite these evidences, many studies are performed between 24 and 48 h, therefore exposing the cells to a range of acute high doses not comparable to physiological concentrations. Here, a long-term culture and treatment of primary rat hepatocytes in sandwich configuration (Collagen I-Matrigel™) was used; cells could be kept in culture for 15 days and were shown to maintain typical liver functions. Regular additions of Matrigel™ layers have been found to improve significantly the quality and functions of the hepatocytes. In order to mimic a chronic treatment and to investigate the effect of 10 drugs with known adverse side effects in rats and human, cells were exposed daily to subtoxic concentrations for two weeks. The drugs were selected by the Predict-IV (FP7) consortium, a collaborative large-scale project, whose aim is to support the development of better drug testing strategies, which can be used for safety profiling of the most frequently affected target organs of toxicity (liver, kidney and CNS) (Wolf et al., 2013). Multi-parametric morphological and functional cellular responses, such as intracellular accumulation of neutral lipids and lysosomal phospholipids together with inhibition of Mrp2-mediated canalicular transport, were analyzed with the use of HCI technology, which has been shown to be a valuable tool for investigating specific drug-induced hepatotoxic events (Donato et al., 2012; McDonough et al., 2009; Tolosa et al., 2012; van de Water et al., 2011; Xu et al., 2008).

2. Materials and methods

2.1. Isolation and purification of primary rat hepatocytes

Five to six weeks old male Wistar rats were purchased from CBP Harlan. The animals had free access to food and water. Hepatocytes were isolated according to a two-step collagenase perfusion method (Paine et al., 1979; Seglen, 1976) with modifications (Paine, 1990). Briefly, rats were anaesthetized with an intraperitoneal injection of sodium pentobarbitone (75 μg/g body weight). Liver perfusion was performed through the portal vein with Ca^{2+}/Mg^{2+}-free pre-perfusion buffer (0.5 mM EGTA (Fluka) supplemented with 10 mM HEPES-buffered Hanks' balanced salt solution (Invitrogen)) at a constant flow rate of 25 ml/min. After 7–8 min the pre-perfusion solution was replaced by the perfusion buffer (DMEM/F12 (Gibco) supplemented with 0.3 M $CaCl_2$ (Sigma), 1 M HEPES (Gibco), 1% Pen/Strep/Glut (Invitrogen) containing, and 100 U/ml collagenase A (Roche)) at decreasing flow rates from 25 to 12 ml/min. After additional 8–10 min perfusion, the liver was dissected and manually dissociated in 25 ml collagenase buffer. The resulting cell suspension was filtered through a 100 μm gauze into a 50 ml plastic tube and then filled with cold wash buffer (DMEM/F12 supplemented with 20% FCS (HyClone), 1 M HEPES, 1% Pen/Strep/Glut). Parenchymal cells were separated from the previous cell suspension by two cycles of low-speed centrifugation (50 g, 5 min, 4 °C). The supernatant was discarded and the cells were then re-suspended in 25 ml of attachment medium (Williams' Medium E supplemented with 10% FCS, 10 mM HEPES, 0.17 μM insulin (Sigma), 0.3 μM dexamethasone (Sigma), 1% Pen/Strep/Glut) and added to a Percoll gradient (Percoll™ Plus (GE Healthcare), HBSS 10X (Gibco), 1 M HCl). After 10 min spinning (50 g, 4 °C) the cells were re-suspended and washed in attachment medium before viability determination by Trypan Blue. Hepatocytes preparations with viability greater than 85% were used for cultivation.

2.2. Cell culture and long-term treatments

Hepatocytes were seeded (3.5×10^5 cells/well) on 24-well collagen I-coated plates (BD Biocoat). After 2–3 h, non-attached cells were removed and a top layer of Matrigel™ (250 μg/ml; BD #356237) diluted in serum-free medium (DMEM/F12 supplemented with sodium pyruvate (Gibco), 1X Insulin/Transferrin/Selenium (Gibco), 0.03 μM dexamethasone, 1% Pen/Strep, albumin solution from bovine serum (Sigma)) was applied with pre-cooled pipette. Medium was changed every 24 h. Hepatocyte morphology was monitored daily. Other layers of Matrigel™ were added at day 4 and 8 and 12 of culture. For selected experiments rat hepatocytes were cultured in presence of DMEM/F12 supplemented with Recombinant Human Epidermal Growth Factor (hEGF, Invitrogen) or 0.5% FCS and with HCM™ Bullet Kit (Hepatocyte Culture Medium, Lonza). The following compounds chosen from a training set used in the 7th EU Framework project Predict-IV were used for the long-term term treatment: Cyclosporin A, Metformin (Calbiochem, Switzerland); Rosiglitazone, Troglitazone (Cayman Chemicals, USA); Amiodarone, Chlorpromazine hydrochloride, Fenofibrate, Ibuprofen, Acetaminophen, Valproic Acid sodium salt (Sigma–Aldrich, Germany). Non-cytotoxic concentrations were chosen (Table 2) and rat hepatocytes were exposed 14 days to perform chronic treatment. The treatments started 24 h after cell seeding. All compounds were dissolved in DMSO and added to the medium with a final concentration of 0.1% vol/vol DMSO. Cells incubated in the presence of 0.1% vol/vol DMSO were used as control.

2.3. Biochemical assays

ATP assay: ATP was measured with CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA) according to manufacturer's instructions.

Lactate Dehydrogenase (LDH) release: LDH release was measured with Cytotoxicity Detection Kit^{Plus} (Roche, Germany) according to manufacturer's instructions.

Urea synthesis: Urea synthesis was measured with Biochain's Urea Assay Kit (Biochain, USA) according to manufacturer's instructions.

Albumin secretion: Albumin content was assessed with Rat Albumin ELISA Quantitation Set (Bethyl Laboratories (Montgomery, TX, USA) according to the manufacturer's instructions.

2.4. Microscopy imaging and analysis and functional assays

All fluorescence microscopy images of were taken with Thermo Scientific Cellomics™ Arrayscan® VTI, with XF93 Hoechst, FITC, TRITC excitation/emission filters and 10×/20× objective. An amount of at least 2000 cells per well were imaged (8–10