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How useful are clinical liver function tests in *in vitro* human hepatotoxicity assays?

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ABSTRACT

In preclinical hepatotoxicity testing cell based assays are frequently employed. However, prediction of clinical drug induced liver injury (DILI) remains a major challenge. Here we examined the usefulness of frequently employed markers of hepatocellular injury in cultures of primary human hepatocytes (PHH) in response to treatment with either paracetamol, rifampicin, petadolex and/or amiodarone. The changes in the metabolic competency (urea and albumin) and cellular injury (AST, ALT, ALP, LDH, γGT and succinate dehydrogenase) were determined at therapeutic and above drug concentrations as to evaluate the utility of these markers in *in vitro* systems. Initially, treatment of PHH with any of the drugs caused a statistically significant reduction in enzyme activities to suggest a switch from basic amino acid metabolism towards induced detoxification. However, treatment for prolonged periods of time caused cytolysis, as evidenced by the significant rise in extracellular LDH and the concomitant increase in ALT and AST activity. Notably, amongst the various endpoints studied, urea was best to demonstrate dose dependent metabolic stress, while other markers of hepatocellular injury were highly variable. Taken collectively, urea measurement proofed to be robust in predicting hepatocellular stress; therefore it should be included in preclinical testing strategies for an improved prediction of DILI.

1. Introduction

Drug induced liver injury (DILI) is a major reason for drug failures in the drug development process and a primary cause for regulatory action to result in boxed warning and even removal from the market. Despite extensive safety testing DILI remains the most common cause of acute liver failure in the US (Ghabril et al., 2010) and owing to its complexity the need for the development of reliable testing strategies remains. Several programs have been initiated including the annual FDA hepatotoxicity meeting, the NIH LiverTox database, the LTKB project of the FDA (Chen et al., 2011) and the German funded "Virtual Liver Network" as to improve patient safety and to foster the development of predictive and cost-effective preclinical assays.

There is unmet need for the development of novel testing strategies for an early identification of drugs with risk for hepatotoxicity with high-content screening assays, "omics" and QSAR approaches being extensively evaluated; however, its unfailingly translation into the clinics and vice versa into the drug development process lacks behind (Chen et al., 2011). Moreover, the preclinical safety testing strategy relies on animal studies but next to ethics and the much needed advance in the 3R concept (Reduce, Replace and Refine) along with the time and cost involved, these studies fail to predict about 50% of clinically relevant DILI (Olson et al., 2000). Finally, species specific differences in ADME frequently hinder extrapolation of findings from animals to humans. The FDA therefore emphasized the importance of *in vitro* and *in silico* models to eventually replace the *in vivo* models in the regulatory science field (Hamburg, 2011).

Indeed, in a recent food for thought article the complexity faced in translational sciences was addressed with animal models failing to recapitulate the pathophysiology seen in patients. Thus, 95% of drugs entering clinical trials will not reach the market and the

Abbreviations: DILI, drug induced liver injury; FDA, Food and Drug Administration; NIH, National Institute of Health; LTKB, Liver Toxicity Knowledge Base; QSAR, quantitative structure activity relationship; ADME, absorption, distribution, metabolism and excretion; ALT, alanine aminotransferases; AST, aspartate aminotransferases; γGT, γ glutamyl transferases; ALP, alkaline phosphatase; MTT, 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; EGTA, ethylene glycol tetraacetic acid; HEPES, (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid); DMSO, dimethyl sulfoxide; h, hour; p.t., post treatment; ELISA, enzyme linked immuno sorbant assay; APAP, acetaminophen; OCT, ornithine carbomyl transferase; CYP, cytochrome P; GSH, glutathione S-transferase.

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reliance on animal models has dropped in the pharmaceutical industry (Hartung, 2013). In recent years, considerable efforts are being invested to restore hepatocyte functionality by use of complex culture systems for improved prediction of clinically relevant drug-induced hepatotoxicity and mechanisms underlying DILI. Notably, chip based microfluidic and other advanced 3D co-culture systems enable hepatocytes to maintain stable morphology and prolonged metabolic competence which in turn contributes towards better prediction of DILI (Bhushan et al., 2013; Kratschmar et al., 2013; Materne et al., 2013; Godoy et al., 2013). In this regard, Kostadinova et al. (2013) reported that 3D scaffolds are capable of preserving the functionality of hepatocytes and non parenchymal cells for up to 3 months and therefore would permit repeated dose studies to predict drug toxicity more reliably.

In the clinics DILI is usually diagnosed by the monitoring of so called "liver function tests", i.e. by determining serum enzyme activities of alanine aminotransferases (ALT), aspartate aminotransferases (AST), glutamyl transferases (γGT), alkaline phosphatase (ALP), the international normalized ratio for prothrombin time (INR) and bilirubin. However, markers of hepatocellular damage based on serum transaminase activity are not used for the prognostic evaluation of disease severity in end stage liver disease. Here, the so called MELD score (=model for end-stage liver disease) is determined based on the parameters bilirubin, INR and serum creatinine for the prioritization of allograft allocations. Nonetheless, markers of hepatocellular damage are also employed in cell based preclinical testing strategies (Corsini et al., 2012; Ozer et al., 2008).

To evaluate the sensitivity of different markers of hepatocellular damages, cultures of primary human hepatocytes were treated with either paracetamol, rifampicin, petadolex and/or amiodarone and the dose and time dependent activities of ALT, AST, ALP, γGT, LDH, MTT and the production of urea and albumin were assayed at therapeutic and above concentrations. The rationale of choosing these drugs is based on their different mechanisms in causing hepatotoxicity. Specifically, with paracetamol a frank dose dependent hepatotoxicity is observed leading to hepatocellular necrosis and the hepatocellular damage is primarily caused by the exaggerated production of NAPQI, a reactive metabolite. Paracetamol overdosing accounts for about 50% of all liver transplantation cases in the US and serum acute phase reactants may predict individuals at risk for liver injury (Borlak et al., 2013). We also investigated the hepatotoxicity of butterbur extracts which contain pyrrolizidine alkaloids; its excessive ingestion is associated with venoocclusive liver disease. Thus, Petadolex is prepared as a butterbur extract essentially free of most pyrrolizidine alkaloids but was shown to be a mitochondrial toxin and to inhibit bile acid transport at above therapeutic doses (Anderson et al., 2009). The potential to cause liver injury is well documented for Rifampicin, i.e. a macrocyclic antibiotic. It is extensively metabolized and induces CYP3A4 and MRP2. Its mechanism of toxicity is unclear and mostly of idiosyncratic nature. Possibly, the formation of some toxic metabolic products causes hepatic injury (Marschall et al., 2005). Finally, the antiarrhythmic agent amiodarone, has been used for decades and its metabolite N-desmethylamiodarone causes phospholipidosis in liver by inhibition of lysosomal phospholipase A1 and A2 (Hussain et al., 2013). This drug is also associated with non-alcoholic steatohepatitis seen in some patients after long term use.

2. Materials and methods

2.1. Isolation of primary human hepatocytes

Human hepatocytes were isolated from specimens obtained from patients undergoing hepatic resections as described by Borlak et al. (2003). Basic information on individual donors is provided in Supplementary Table S1.

Each patient gave written consent and the experiments were approved by the ethical committee of Hannover Medical School (Tr/L 040999/130309La) and were therefore performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments. The liver specimens were immediately transferred in ice cold physiologic saline solution to the laboratory. Vessels visible on the cut surface were cannulated and the perfusion was done with 200 ml of EGTA-containing HEPES buffer (Roche, Germany) at pH 7.4 and 37 °C. This was followed by perfusion with 200 ml HEPES buffer. Thereafter Liberase CI (Roche, Germany) perfusion was performed with 200 ml of HEPES buffer containing Liberase CI and calcium chloride dihydrate at 37 °C. The Liberase CI perfusate was recirculated. Following perfusion, the liver capsule was carefully removed and the cells were liberated by gentle shaking of the liver specimen in ice cold buffer containing Hanks buffered salt HEPES and bovine serum albumin. The resulting cell suspension was filtered through a nylon mesh and washed three times in buffer at 4 °C. Viability of the hepatocytes was assessed by the Trypan blue exclusion assay and cells were counted in the Neubauer chamber.

2.2. Collagen sandwich culture of isolated hepatocytes

Primary hepatocytes were cultured in a collagen sandwich as described previously (Dunn et al., 1991; Borlak et al., 2002). Rat tail collagen solution (BD Biosciences, Germany) was used for coating 6-well plates (Techno Plastic Products AG, Switzerland). The collagen was prepared according to the manufacturer's instructions. Approximately 1 million of hepatocytes were seeded per well. After attachment to the collagen, the medium along with nonadherent cells was aspirated and a second layer of collagen was pipetted on top of the cells. After gelation of this second layer, supplemented William's E culture medium (Lonza GmbH, Germany) was added and cell cultures were incubated at 37 °C with 100% humidity and 5% CO_2. The medium of the cells was changed at every 24 h and the cultures were observed daily for any morphological changes by phase contrast microscopy.

2.3. HepG2 cell culture

HepG2 cells were procured from American Type Culture Collection (ATCC). Cells were grown and maintained in Dulbecco's modified Eagle's medium (DMEM) with high glucose supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 10 mM HEPES buffer at 37 °C under 5% CO_2 (95% air). All the cell culture medium and reagents used were purchased from Gibco, USA.

2.4. Treatment of hepatocytes

Prior to drug exposure, cells were allowed to recover from the stress caused by the isolation procedure for 4 days. Following recovery, the cells were exposed to different doses and duration of paracetamol, *Petasites hybridus* extracts, rifampicin and amiodarone (Table 1). Test substances were diluted in DMSO. The final concentration of DMSO in cell cultures was 0.1% (V/V). The control values were set to 100% and the results of treated groups were expressed as percentage of control.

2.4.1. Paracetamol

Hepatocyte cultures were treated with 132 μM (D1), 662 μM (D2), 3300 μM (D3), 6600 μM (D4) and 19,800 μM (D5) of paracetamol (Sigma–Aldrich, Germany). The lowest concentration of 132 μM was chosen to mimic the effect of therapeutic plasma