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Minimizing DILI risk in drug discovery — A screening tool for drug candidates

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ABSTRACT

Drug-induced liver injury (DILI) is a leading cause of acute hepatic failure and a major reason for market withdrawal of drugs. Idiosyncratic DILI is multifactorial, with unclear dose-dependency and poor predictability since the underlying patient-related susceptibilities are not sufficiently understood. Because of these limitations, a pharmaceutical research option would be to reduce the compound-related risk factors in the drug-discovery process.
Here we describe the development and validation of a methodology for the assessment of DILI risk of drug candidates. As a training set, 81 marketed or withdrawn compounds with differing DILI rates – according to the FDA categorization – were tested in a combination of assays covering different mechanisms and endpoints contributing to human DILI. These include the generation of reactive metabolites (CYP3A4 time-dependent inhibition and glutathione adduct formation), inhibition of the human bile salt export pump (BSEP), mitochondrial toxicity and cytotoxicity (fibroblasts and human hepatocytes). Different approaches for dose- and exposure-based calibrations were assessed and the same parameters applied to a test set of 39 different compounds. We achieved a similar performance to the training set with an overall accuracy of 79% correctly predicted, a sensitivity of 76% and a specificity of 82%. This test system may be applied in a prospective manner to reduce the risk of idiosyncratic DILI of drug candidates.

1. Introduction

Drug-induced liver injury (DILI) is a leading cause of acute hepatic failure and a major reason for market withdrawal of drugs. While drugs that cause direct DILI with a clear dose dependency and high incidence are often recognized in animal testing or during early clinical development phases, idiosyncratic DILI usually occurs with low incidence and with no direct correlation to the dose. In many cases there are strong hints for the involvement of the adaptive immune system (Kaplowitz, 2013). Often idiosyncratic DILI occurs first after a latency of days to months, while showing a rapid onset upon re-challenge (Peters et al., 2014). Common features include systemic hypersensitivity, fever, rash, eosinophilia, autoimmune hepatitis and human leukocyte antigen (HLA) association. Most idiosyncratic hepatotoxins can be linked with HLA if investigated, with some exceptions such as isoniazid and diclofenac (Kaplowitz, 2013). Strong evidence suggests that an individual subject's genetic predisposition combined with drug hapten/autoantigen formation leading to adaptive immune response causes idiosyncratic DILI (Kaplowitz, 2013).

Because patient-related susceptibilities of idiosyncratic DILI are not sufficiently understood, the current approach in the pharmaceutical industry is to minimize the major, drug-related, risk factors. To support the selection of drug candidates with low idiosyncratic DILI risk, it is important to address these liabilities early in the drug discovery phase when chemical optimization is still possible. Therefore, the test systems used for this optimization process need to be suitable for profiling numerous compounds with rapid turnaround cycles.

Several approaches to assess the DILI potential of compounds in pharmaceutical discovery and development have recently been published. Since animal studies typically do not predict idiosyncratic DILI in man (Olson et al., 2000), different in vitro assays mostly involving human cells or enzymes are used. A major mechanism presumably contributing to DILI is the formation of reactive metabolites, which can be assessed by investigating glutathione (GSH) adduct formation (Sakatis

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et al., 2012), covalent binding (CVB) to hepatic proteins (Nakayama et al., 2009), or time-dependent enzyme inhibition (TDI) (Zimmerlin et al., 2011). The inhibition of the hepatobiliary bile salt export pump, BSEP, leading to intrahepatic cholestasis was also proposed to contribute to DILI (Fattinger et al., 2001; Funk et al., 2001; Pedersen et al., 2013). Mitochondrial (Nadanaciva and Will, 2011), lysosomal (Nadanaciva et al., 2011) and inflammatory effects (Cosgrove et al., 2009) as well as different methods and cell systems to assess general cytotoxicity have been suggested. Thompson proposed a combination of assays (Thompson et al., 2012). They suggest the use of a hazard matrix, based on covalent binding burden combined with an in vitro panel of five assays, addressing cytotoxicity in different cell lines and inhibition of canalicular transporter proteins BSEP and MRP2 with individual cut-off values for each assay. Compound exposure and dose were identified as additional important risk factors (Warner et al., 2012) that can be further modulated by active hepatic uptake processes impacting the local intracellular concentration (Huang et al., 2012). Systemic drug exposure was also used in combination with the inhibition of BSEP (Dawson et al., 2012; Morgan et al., 2013). Sakatis et al. (2012) base their risk assessment on in vitro assays associated with reactive metabolite generation (TDI, GSH trapping, CVB), in combination with a dose cutoff of ≥100 mg/day. Aleo et al. (2014) investigated an approach combining mitochondrial and BSEP inhibition.

Here, we describe a refined approach to address human DILI based on calibration of the assays with dose or exposure. Eighty-one marketed or withdrawn compounds were categorized for DILI based on FDA-approved drug labeling (Chen et al., 2011). The assays applied covered various mechanisms and endpoints associated with human DILI. They included the generation of reactive metabolites, namely TDI of Cytochrome P450 3A4 and GSH adduct formation, inhibition of the human bile salt export pump (BSEP), mitochondrial toxicity and cytotoxicity. In a second step, different approaches to calibrate the assay data with the daily dose and/or systemic exposure at clinically relevant doses were assessed.

2. Material and methods

All chemicals and reagents were used at the highest quality available and were obtained from standard commercial suppliers. The pharmaceutical test compounds were obtained from Sigma Aldrich (Buchs, Switzerland), Toronto Research Chemicals (Toronto, Canada), ArkPharm (Libertyville, USA) or from the Roche Compound Depository. The categorization of compounds for human DILI concern was based on the currently approved label or according to Chen et al. (2011). Clinical dose and exposure information was obtained from the literature as listed in the supplemental material.

Pooled mixed gender human liver microsomes (HLM) from 150 individuals (UltraPoolTM HLM 150) were purchased from BD Biosciences (Woburn, Massachusetts). Vesicles prepared from Sf9 cells expressing recombinant human BSEP were obtained from SOLVO Biotechnology (Budaörs, Hungary). Cryopreserved plateable primary human hepatocytes were obtained from Life Technologies (today Thermo Fisher, Frederick, USA) or Celsis In Vitro (today BioreclamationIVT, Brussels, Belgium). Compounds were tested in hepatocytes from two to three different donors. 384-well collagen I coated cell culture plates were purchased from Becton Dickinson Biocoat, Cat Nr 354664 (today Corning, Tewksbury, USA). Cryopreserved Hepatocyte Recovery Medium (CHRM®; CatNr. CM7000) was obtained from Invitrogen (now Thermo Fisher, Columbia, USA). CellTiter-Glo Luminescent Cell Viability Assay (Cat Nr G7571) was purchased from Promega (Madison, USA). Hepatocyte Plating Medium (HPM) consisted of In Vitro GRO CP Medium (Z99029; Celsis (now BioreclamationIVT, Brussels, Belgium)) supplemented with 2.2% Torpedo Antibiotic Mix™ (Z99000; Celsis (now BioreclamationIVT, Brussels, Belgium)). Hepatocyte Incubation Medium (HIM) consisted of glutamine-free and phenol-free William's medium E (W1878; Sigma) supplemented with 1% Pen/Strep/L-Glutamine Solution (10378-016; Invitrogen (now Thermo Fisher, Paisley, UK)), 10 µg/ml insulin (I9278; Sigma) and 50 nM hydrocortisone (H6909; Sigma).

Detailed assay descriptions are given in the Supplementary material.

2.1. GSH adduct assay

The GSH adduct assay was performed as described by Brink et al. (2014). Briefly, compounds were incubated at 20 µM with 5 mM GSH and 1 mM NADPH in 0.1 M sodium phosphate buffer at pH 7.4 containing human liver microsomes (HLM, 1 mg/ml). After 60 min, the reaction was quenched and analyzed by LC–MS. The resulting MS^E data files were processed in batch mode using Mass-MetaSite. Troglitazone, Diclofenac, Nefazodone and a proprietary Roche compound were included as experimental positive controls with each batch of compounds.

2.2. BSEP inhibition assay

The assay was performed according by Glavinas et al. (2008) and Herédi-Szabó et al. (2012): Test compounds were incubated at 37 °C with BSEP-transfected membrane vesicles (25 µg protein/ well) in assay buffer containing the probe substrate Na taurocholate (NaTC; 0.2 µM, partially ^{3}H-labeled) and either ATP or AMP (diffusion control). Afterwards the vesicles were thoroughly washed, dried and the intravesicular amount of ^{3}H-NaTC was quantified via liquid scintillation counting. IC_{50} values were generated by testing a serial dilution of seven concentrations. Vehicle control wells (blank DMSO) were used as negative control, cyclosporine A (CsA) at 10 µM was used as positive control marking 100% relative inhibition.

2.3. CYP3A4 TDI assay

Experiments were performed using the conventional two-step dilution method (Hyland et al., 2010) with test compounds at 10 and 50 µM. Inhibition of 1-OH Midazolam formation was measured by LC–MS/MS. As the cutoff for flagging time-dependent inhibition, >33% apparent enzyme inactivation was defined, corresponding to approximately three fold background rate of enzyme inactivation. As positive controls, verapamil and ethinyl estradiol were included in all experiments.

2.4. Cytotoxicity in human hepatocytes

Experiments were performed in a 384-well plate format using cryopreserved primary human hepatocytes (10,000 cells/well) in monolayer culture. For treatment, test compounds were serially diluted in serum free WME (0.1% DMSO final). Highest test concentration was 200 µM. Viability was assessed upon 48 h test compound exposure by determination of intracellular ATP. 2 to 3 independent experiments using cells from different donors were performed for each test compound and obtained TC_{50} values averaged. Two reference compounds, troglitazone and flutamide, were included in all experiments to check for assay performance. An assay run was accepted, if the TC_{50} for troglitazone and flutamide were below 100 µM and 200 µM respectively and TC_{50} for troglitazone was lower compared to the TC_{50} for flutamide.

2.5. Mitochondrial toxicity and cytotoxicity in NIH 3T3 fibroblasts

The mitochondrial and cellular toxicity assay was performed as described by Marroquin et al. (2007) using mouse NIH 3T3 fibroblasts pre-adapted to glucose or galactose medium for at least 2 weeks. A ratio of IC_{50} glucose/IC_{50} galactose of ≥3 was considered a mitochondrial toxicity flag. Three reference compounds were included in all experiments to check for assay performance. Rotenon and antimycin A served as positive controls for mitochondrial toxicity and chlorpromazine for cellular toxicity.