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Evaluation of a human in vitro hepatocyte-NPC co-culture model for the prediction of idiosyncratic drug-induced liver injury: A pilot study

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Interactions between hepatocytes and immune cells as well as inflammatory episodes are frequently discussed to play a critical role in the alteration of the individual susceptibility to idiosyncratic drug-induced liver injury (iDILI). To evaluate this hypothesis and to face the urgent need for predictive *in vitro* models, we established two co-culture systems based on two human cell lines in presence or absence of pro-inflammatory factors (LPS, TNF), i.e. hepatoma HepG2 cells co-cultured with monocytic or macrophage-like THP-1 cells. HepG2 monocultures served as control scenario. Mono- or co-cultures were treated with iDILI reference substances (Troglitazone [TGZ], Trovafloxacin [TVX], Diclofenac [DcL], Ketoconazole [KC]) or their non-iDILI partner compounds (Rosiglitazone, Levofloxacin, Acetylsalicylic Acid, Fluconazole). The liver cell viability was subsequently determined via WST-Assay. An enhanced cytotoxicity (synergy) or a hormetic response compared to the drug effect in the HepG2 monoculture was considered as iDILI positive. TGZ synergized in co-cultures with monocytes without an additional pro-inflammatory stimulus, while DcL and KC showed a hormetic response. All iDILI drugs synergized with TNF in the simple HepG2 monoculture, indicating its relevance as an initiator of iDILI. KC showed a synergy when co-exposed to both, monocytes and LPS, while TVX and DcL showed a synergy under the same conditions with macrophages. All described iDILI responses were not observed with the corresponding non-iDILI partner compounds. Our first results confirm that an inflammatory environment increases the sensitivity of liver cells towards iDILI compounds and point to an involvement of pro-inflammatory factors, especially TNF, in the development of iDILI.

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1. Introduction

Idiosyncratic drug-induced liver injury (iDILI), which accounts for up to 17% of all cases of acute liver failure [1,2], remains a serious problem for public health due to the inability to predict those rare but severe adverse drug reactions. The prognosis for patients suffering from iDILI is very poor, ranging from 60 to 70% mortality without liver transplantation [3]. In addition, iDILI is the most frequent cause for the non-approval of a drug or its withdrawal from the market [4–6] and therefore represents a major issue for the drug development process and the marketing of drugs that are safe for the broad population. Consequently, the US Food and Drug Administration (FDA) demanded the majority of post-marketing restrictions (e.g. black box warnings) for drugs that induce idiosyncratic reactions [7]. What makes idiosyncratic reactions so difficult to predict, are their elusive characteristics [8]. A very low incidence of about 19 cases per 100,000 per year [9] makes it nearly impossible to recognize iDILI during pre-marketing trials, which typically cover only 1000–3000 subjects for a new drug application. Only the most evident hepatotoxicants can be expected to show cases of such severe outcomes as iDILI in those small subject numbers.

Abbreviations: CD, cluster of differentiation; DAMP, damage-associated molecular pattern; EC, effective concentration; EpCAM, epithelial cellular adhesion molecule; HSP, heat shock protein; iDILI, idiosyncratic drug-induced liver injury; JNK, c-Jun N-terminal kinase; LPS, bacterial lipopolysaccharide; NF-κB, nuclear factor kappa B; NPC, non-parenchymal cell; NSAID, nonsteriodal anti-inflammatory drug; PAMP, pathogen-associated molecular pattern; SD, standard deviation; TNF, tumor necrosis factor.

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Therefore, it is also understandable that iDILI cannot be predicted from regulatory animal toxicity studies. At present, iDILI can only be identified when a lot more patients are exposed to a certain drug post-marketing. Moreover, idiosyncratic reactions occur sporadically and show a variable onset relative to the start of exposure, this meaning that some patients develop toxicities soon after the start of exposure and some not until after a longer exposure period [10]. Drug concentrations that are able to induce iDILI are in the therapeutic dose range and are generally safe to the majority of the population. In addition, iDILI reactions are reported to be of a more dose-independent nature and unrelated to the pharmacologic action of the drug, but appear to reflect host factors and individual susceptibility [11,4,12]. To date it is not clear which factor(s) cause(s) this individual susceptibility and why some individuals develop iDILI and some do not. It seems possible that the susceptibility of a patient is triggered by an erratically occurring event that appears during the running drug therapy. In this case, the low incidence as well as the sporadic occurrence of iDILI would be explained. Whether the susceptibility factors are based on genetic differences or environmental factors is not yet understood, but it is most likely that the underlying mechanisms are multifactorial [8]. Amongst the variety of hypotheses that aim to explain the origin of iDILI, the inflammatory stress hypothesis has become the most studied and most promising approach. Roth et al. [13] suggested, that a mild inflammatory stress might render an individual susceptible to develop hepatotoxicity at an otherwise safe dose of the drug. Because inflammation often occurs in humans, but erratically and to a varying degree throughout the whole lifespan of an individual, it fulfills the requirements for a susceptibility factor that might account for iDILI. Therefore, a promising interplay to be studied in connection with inflammation-associated iDILI is that of liver parenchymal cells (PCs) and non-parenchymal immune cells (NPCs), such as liver resident macrophages (Kupffer cells) and infiltrating monocytes. It is well known that in particular Kupffer cells have a central function in hepatotoxicity by initiating and assembling local and systemic responses to liver injury and, together with recruited monocytes, rule the complex process of inflammation [14,15]. Xenobiotics can activate Kupffer cells directly or indirectly upon an initial hepatic insult, thereby resulting in the release of a variety of inflammatory mediators such as cytokines (e.g. TNF and interleukins), which can trigger a secondary response that appears to exacerbate the initial hepatocyte damage [14,11,16]. Moreover, an existing moderate inflammation due to the activation of Kupffer cells via e.g. bacterial lipopolysaccharide (LPS) appears to sensitize hepatocytes to toxic substances and can lower the threshold for hepatotoxicity [17,11]. An increasing number of recently developed *in vivo* and *in vitro* models for the prediction of (i)DILI incorporate immune cells and/or pro-inflammatory factors such as LPS and TNF, thus attempting to provide evidence for the inflammatory stress hypothesis. Most *in vivo* studies are based on rodents which are co-exposed to idiosyncratic drugs and LPS to induce a mild inflammatory background during drug exposure [18–20,17,21]. *In vitro* models are either based on the parenchymal cell itself and a co-exposure to pro-inflammatory factors [22–24] or a co-culture of hepatocytes and macrophages or monocytes including pro-inflammatory factors in most but not all cases [25–27]. All these studies confirm the suggestion that inflammation and the involved immune cells play a role in the development of iDILI. Unfortunately, most published studies are limited to one drug or one exposure scenario and are therefore not suitable for the establishment of a general iDILI testing approach that is applicable to structurally and mechanistically diverse iDILI compounds. *In vivo* animal studies in general lack predictability for hepatotoxicity in humans [28], mainly due to interspecies variations, and do not allow a reasonably high throughput for the screening of drugs in the preclinical development process. A simple well-controlled *in vitro* system, which saves time, money and animals, would strongly improve the early screening process for iDILI. In addition, a system that combines parenchymal with non-parenchymal cells and thereby allows intercellular communication is required to reflect multicellular phenomena like drug-induced toxicity and to understand how these interactions contribute to hepatotoxicity. Only a co-culture model can help to determine whether the communication to immune cells is necessary to predict iDILI or if (single) secreted pro-inflammatory factors might suffice to mirror iDILI in single PC cultures *in vitro*. Importantly, co-cultures represent a closer approximation to the *in vivo* situation and therefore have a higher relevance than models that are only based on the PC itself [29].

To this end, we developed an inflammatory *in vitro* liver co-culture model combining the human hepatoma cell line HepG2 with monocytic or macrophage-like THP-1 cells separated by a porous membrane. Monocytes were added to mimic the infiltration of immune cells during liver injury and inflammation and the macrophage-like cells as a surrogate for Kupffer cells. For the validation of this liver model we tested a panel of four drug pairs (Troglitazone – Rosiglitazone; Trovafloxacin – Levofloxacin; Diclofenac – Acetylsalicylic acid and Ketoconazole – Fluconazole), each consisting of a drug that is known to induce iDILI or a non-iDILI partner compound from the same substance class that has no potential to induce iDILI as a control [30,4,8,31,32]. Drugs were tested in mono- or co-culture and in the presence or absence of a pro-inflammatory background (induced by LPS or TNF) for comparison, resulting in nine different exposure scenarios per examined drug. Based on these tests, we aimed to identify whether the addition of immune cells and/or an additional pro-inflammatory environment to liver cell cultures could improve the detection of iDILI drugs and therefore represent a more sensitive liver model for the prediction of iDILI.

2. Materials and methods

2.1. Materials

All drugs except Diclofenac sodium salt were purchased from Sigma (Taufenkirchen, Germany). Diclofenac sodium salt (DcL) was obtained from Cayman Chemical (Ann Arbor, MI, USA). Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4 and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma and dimethyl sulfoxide (DMSO) from Carl Roth GmbH + Co. KG (Karlsruhe, Germany). DMEM (low glucose), fetal bovine serum (FBS), trypsin/EDTA (0.25%/0.02%) and phosphate buffered saline (PBS) were obtained from Biochrom GmbH (Berlin, Germany). Gentamycin (50 mg/mL) and UltraPure^{TM} 0.5 M EDTA solution were bought from Thermo Fisher Scientific (Waltham, MA, USA). Accutase solution was purchased from PromoCell GmbH (Heidelberg, Germany). Human TNF-α, premium grade, was obtained from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany). Cell Proliferation Reagent WST-1 was purchased from Roche Deutschland Holding GmbH (Penzberg, Germany) and the DuoSet® ELISA for human CXCL8/IL-8 from R&D Systems (Minneapolis, MN, USA). The antibodies (mouse anti-human CD32-PE; mouse anti-human CD11b-APC; mouse anti-human CD14-APC) and the fixative for FACS measurements were obtained from Beckman Coulter (Brea, CA, USA), while the mouse anti-human EpCAM-FITC (CD326) and mouse anti-human CD45-BV510 were from BD Biosciences (Franklin Lakes, NJ, US). Isotype controls (EpCAM-FITC: mouse IgG1 FITC; CD14-BV510: mouse IgG1 BV510; CD11b-APC: mouse IgG1 APC; CD14-APC: mouse IgG2a APC) were purchased from BD Biosciences (Franklin Lakes, NJ, US). Mouse IgG2a PE, the isotype control for CD32-PE, was bought from eBioscience (San Diego, CA,

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