Toxicology and Applied Pharmacology 332 (2017) 100–108

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/taap

Omics-based identification of the combined effects of idiosyncratic drugs and inflammatory cytokines on the development of drug-induced liver injury

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ARTICLE INFO

Article history:
Received 9 May 2017
Revised 8 July 2017
Accepted 17 July 2017
Available online 18 July 2017

Keywords:
Drug-induced liver injury
Microarray
Toxicogenomics
Ceramide
ER stress
Apoptosis

ABSTRACT

The mechanisms of idiosyncratic drug-induced hepatotoxicity remain largely unclear. It has demonstrated that the drug idiosyncrasy is potentiated in the context of inflammation and intracellular ceramides may play a role in this process.

To study the mechanisms, HepG2 cells were co-treated with high and low doses of three idiosyncratic (I) and three non-idiosyncratic (N) compounds, with (I+ and N+) or without (I− and N−) a cytokine mix. Microarray, lipidomics and flow cytometry were performed to investigate the genome-wide expression patterns, the intracellular ceramide levels and the induction of apoptosis.

We found that all I+ treatments significantly influenced the immune response- and response to stimulus-associated gene ontology (GO) terms, but the induction of apoptotic pathways, which was confirmed by flow cytometry, only appeared to be induced after the high-dose treatment. The ceramide signaling-, ER stress-, NF-kB activation- and mitochondrial activity-related pathways were biologically involved in apoptosis induced by the high-dose I+. Additionally, genes participating in ceramide metabolism were significantly altered resulting in a measurable increase in ceramide levels. The increases in ceramide concentrations may induce ER stress and activate the JNK pathway by affecting the expression of the related genes, and eventually trigger the mitochondria-independent apoptosis in hepatocytes. Overall, our study provides a potential mechanism to explain the role of inflammation in idiosyncratic drug reactions.

1. Introduction

Acute drug-induced liver injury (DILI) accounts for >50% of all cases of acute liver failure and is a common reason for drug withdrawal from the market (Kaplowitz, 2005). Since idiosyncratic drug hepatotoxicity occurs only in a small fraction of human patients (<1%) and is unrelated to the pharmacological action of the drug (Cosgrove et al., 2010), it can hardly be predicted by standard preclinical tests (cell culture and animal models, clinical trials) and is often not evident until after approval for human use (Kaplowitz, 2005). These idiosyncratic drug reactions, which represent a wide spectrum of hepatic manifestations, often result in patient morbidity and mortality, and, consequently, require regulatory actions including drug withdrawal or specific warnings on medication leaflets.

Recent studies have identified a close relationship between inflammatory stress and drug idiosyncrasy (Shaw et al., 2010). For certain drugs, increased idiosyncratic hepatotoxicity has been observed in rodent models with enhanced inflammatory background induced by bacterial lipopolysaccharide (LPS) (Deng et al., 2009). Besides, it has been indicated that the idiosyncratic drug-induced hepatocellular stresses synergize with LPS-triggered inflammatory cytokine signaling to produce acute hepatotoxicity (Cosgrove et al., 2009). Although the exact mechanism remains unclear, several hypotheses have been proposed for the causation of this immune- and inflammatory-mediated adverse

Abbreviations: BP, biological process; CPDB, ConsensusPathDB; DEG, differentially expressed gene; DILI, drug-induced liver injury; FDR, false discovery rate; GO, gene ontology; I, idiosyncratic compound; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; N, non-idiosyncratic compound; SPH, sphingosine; S1P, sphingosine 1-phosphate.

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http://dx.doi.org/10.1016/j.taap.2017.07.014
0041-008X/

drug reaction (Shaw et al., 2010). According to the immune idiosyncrasy hypothesis, the adaptive immune response induced by drug-protein adducts and/or metabolite-protein interactions play an essential role in idiosyncratic drug reactions. After covalent binding of a chemically reactive drug or its metabolite to a protein, the resulting hapten is processed by antigen-presenting cells and presented to T-cells. The immune system recognizes this adduct as a foreign protein and subsequently initiates an immune response. Under normal conditions, a drug-protein adduct is not sufficient to cause a full immune response and an additional signal is required to act as an adjuvant. The so-called danger hypothesis suggests that when a drug or its metabolite binds to a protein, it induces stress responses, including cell death or cytokine release, and produces a "danger" signal. Alternatively, mild hepatic injury, concomitant infection and/or inflammations also act as the "danger" signal and further augment the immune response. As a consequence, the activation of the immune response triggers the production and recruitment of effector lymphocytes to the liver and stimulates the release of reactive oxygen species (ROS), proteases, cytokines or other mediators that can cause tissue damage (Deng et al., 2009).

The stimulation of stress factors, such as inflammatory cytokines, has been linked to the increase in the production and the accumulation of intracellular ceramides in cells (Bikman and Summers, 2011). Ceramides, together with sphingosine (SPH) and sphingosine 1-phosphate (S1P), are known as sphingolipids, which not only are essential structural components of cellular membranes but also participate in signal transduction pathways and regulate a variety of biological functions including cell survival, proliferation, and apoptosis (Nojima et al., 2015). Ceramides can be endogenously generated by various metabolic pathways, including *de novo* synthesis and sphingomyelin hydrolysis (Brann et al., 2002). In addition, it can also be produced through sphingolipids catabolism and re-usage of sphingosine, which has been referred to as sphingolipid salvage/recycling pathway (Kitatani et al., 2008). Studies have shown that pro-inflammatory cytokines activate the formation of ceramides *via* regulating the expression of genes encoding the rate-limiting enzymes along different ceramide metabolism pathways (Kaipia et al., 1996; Davis et al., 2000). Opposite to S1P which acts as an anti-apoptotic molecule enhancing cell growth and survival, ceramides and SPH have received much attention as apoptotic second messengers that mediate growth arrest and cell death (Taha et al., 2006). The shift in the dynamic balance between S1P and sphingosine/ceramides, which is also referred to as the 'sphingolipid rheostat', could lead to apoptosis (Mandala et al., 2000; Taha et al., 2006). Therefore, the alteration in ceramide metabolism may have a functional role in the development of inflammatory-mediated adverse drug reactions.

To test this hypothesis, we have advanced an *in vitro* cytokine synergy model developed in HepG2 cells (Cosgrove et al., 2009) for the purpose of unravelling underlying mechanisms of inflammation-related idiosyncratic drug toxicity by integrating metabolomics and transcriptomics. We co-exposed HepG2 cells to six compounds, including three idiosyncratic (I) and three non-idiosyncratic (N) drugs, and a cytokine mix to emulate the drug-related hepatotoxicity under the inflammatory condition. The several ceramide concentrations were analyzed by UPLC–MS/MS and cell apoptosis representing the relevant endpoint of toxicity was determined by flow cytometric analysis. We hypothesized that the underlying mechanisms of inflammatory-mediated DILI may be revealed by integrating whole-genome expression responses and metabolomic profiling of ceramides in HepG2 cells after exposure to idiosyncratic and non-idiosyncratic compounds, in the absence and presence of the cytokine mix. Furthermore, the gene expression changes in response to the co-administration of I compounds and cytokines may lead to the increased formation of ceramides and the induction of apoptosis.

2. Materials and methods

2.1. Compound selection and cytotoxicity tests

Three pairs of known idiosyncratic drugs with their non-idiosyncratic "comparison" drugs (*i.e.* showing similar molecular target and clinical indication and, if possible, similar chemical structure) were selected based on the publication of Cosgrove et al., 2009. The main criterion for selecting these compounds was the difference in cytotoxicity between incubations with and without the addition of cytokines, assuming the strongest idiosyncratic effects in those compounds showing the largest difference. We selected Nimesulide, Nefazodone, and Trovafloxacin as model idiosyncratic compounds and Aspirin, Buspirone, and Levofloxacin as their respectively non-idiosyncratic equivalent (Cosgrove et al., 2009).

Incubation concentrations were determined using a combination of the results of LDH and MTT testing. The LDH test was used to select doses based on the largest difference in cell damage induced by various drugs with and without the addition of cytokines (at 24 h) (Fig. 1). The MTT test was used to select non-cytotoxic doses (viability of ≥80% at 24 h). The doses that meet these conditions were selected for the high-dose exposures and the half of these doses were used for the low-dose exposures to investigate dose-dependent effects (Table 1).

2.2. Cell culture and treatment

HepG2 cells were cultured in T25 flasks in the presence of minimal essential medium supplemented with 1% nonessential amino acids, 1% sodium pyruvate, 2% penicillin/streptomycin, and 10% fetal bovine serum (all from Gibco BRL, Breda, The Netherlands) at 37 °C in a humidified chamber with 95%/5% air/CO_2. After reaching 80% confluence, the culture medium was replaced with fresh medium containing one of the selected idiosyncratic (I) and non-idiosyncratic (N) compounds, or vehicle control (see Table 1) with (+) or without (−) the co-treatment of a cytokine mix as described previously (Cosgrove et al., 2009) (TNF 100 ng/mL + IFNγ 100 ng/mL + IL-1α 20 ng/mL + IL-6 20 ng/mL + LPS 10 μg/mL). Cells were incubated for 6, 12 or 24 h before being used for gene expression analysis. Levels of apoptosis induced by each treatment were measured at 24 h using flow cytometry. Three independent biological experiments were conducted for each treatment condition.

2.3. RNA isolation

Total RNA was isolated from the cells using QIAzol reagent with the RNeasy kit according to the manufacturer's protocol. RNA concentrations were measured on a nanodrop spectrophotometer and the quality of each RNA preparation was determined with a bio-analyzer (Agilent Technologies, The Netherlands). Only samples with a good quality (clear 18S and 28S peaks and RNA integrity number > 6) were used for hybridization. Extracted RNA was stored at −80 °C until it was used as the template for cDNA synthesis.

2.4. Target preparation and hybridization

Labeling and hybridization of RNA samples were done according to Agilent's manual for microarrays (Agilent Technologies, Breda, The Netherlands). Samples were labelled with Cyanine 3 (Cy3) following the Agilent one-color Quick-Amp labeling protocol (Agilent Technologies, Amstelveen, The Netherlands) and applied on the Agilent 4x44K Whole Human Genome microarray, and hybridized and washed according to Agilent's manual. Slides were scanned using an Agilent Microarray Scanner (Agilent Technologies, Amstelveen, The Netherlands). Raw data on pixel intensities were extracted from the scan images using Agilent Feature Extraction Software (Agilent).