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The enhanced value of combining conventional and "omics" analyses in early assessment of drug-induced hepatobiliary injury

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ABSTRACT

The InnoMed PredTox consortium was formed to evaluate whether conventional preclinical safety assessment can be significantly enhanced by incorporation of molecular profiling ("omics") technologies. In short-term toxicological studies in rats, transcriptomics, proteomics and metabolomics data were collected and analyzed in relation to routine clinical chemistry and histopathology. Four of the sixteen hepato- and/or nephrotoxicants given to rats for 1, 3, or 14 days at two dose levels induced similar histopathological effects. These were characterized by bile duct necrosis and hyperplasia and/or increased bilirubin and cholestasis, in addition to hepatocyte necrosis and regeneration, hepatocyte hypertrophy, and hepatic inflammation. Combined analysis of liver transcriptomics data from these studies revealed common gene expression changes which allowed the development of a potential sequence of events on a mechanistic level in accordance with classical endpoint observations. This included genes implicated in early stress responses, regenerative processes, inflammation with inflammatory cell immigration, fibrotic processes, and cholestasis encompassing deregulation of certain membrane transporters. Furthermore, a preliminary classification analysis using transcriptomics data suggested that prediction of cholestasis may be possible based on gene expression changes seen at earlier time-points. Targeted bile acid analysis, based on LC-MS metabonomics data demonstrating increased levels of conjugated or unconjugated bile acids in response to individual compounds, did not provide earlier detection of toxicity as compared to conventional parameters, but may allow distinction of different types of hepatobiliary toxicity. Overall, liver transcriptomics data delivered mechanistic and molecular details in addition to the classical endpoint observations which were further enhanced by targeted bile acid analysis using LC/MS metabonomics.

Introduction

Within recent years, several large-scale collaborative projects involving academic and industrial institutions as well as regulatory agencies have been initiated with the aim of delivering tools for improved preclinical safety assessment and prediction of toxicity. While the Predictive Safety Testing Consortium (http://www.c-path.org/pstc.cfm), a public–private partnership led by the non-profit Critical Path Institute (C-Path), focuses on qualifying pre-clinical

Abbreviations: Tx, Transcriptomics; Mx, Metabolomics; Px, Proteomics; LC/MS, Liquid chromatography followed by mass spectrometry; PP, peroxisome proliferation; XME, Xenobiotic metabolism; SVM, Support vector machine; HD, high dose; LD, low dose; LOQ, limit of quantification; CA, cholate; GCA, glycocholate; TCA, taurocholate; TβMCA, tauro-β-muricholate; ΔTβMCA, unsaturated tauro-β-muricholate; ALT, alanine aminotransferase; AST, asparate aminotransferase; ALP, alkaline phosphatase.

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safety biomarkers of key problem toxicities, i.e. carcinogenicity, and kidney, liver, muscle and vascular injury, the Innomed-PredTox consortium (http://www.innomed-predtox.com) set out to assess the value of combining results from "omics" technologies together with results from conventional toxicology methods for more informed decision making in preclinical safety evaluation. "Omics" responses to 14 drug candidates, which previously failed during non-clinical development in part due to hepatotoxic and/or nephrotoxic effects, were extensively characterized and integrated with conventional endpoints to obtain better understanding of key mechanisms of toxicity and identify more sensitive safety biomarkers. This comprised systemic toxicity studies in rats treated with hepatotoxic or nephrotoxic compounds at two dose levels for 1, 3, or 14 days by oral gavage, with the high dose having previously been associated with organ toxicity after 14 days of exposure. Organ samples and body fluids were then analyzed both for conventional endpoints and by using "omics" technologies, including transcriptomics, proteomics and metabolomics.

Different principles may be used to group item expression profiles and select significantly deregulated items, with items representing either genes, metabolites or proteins in the case of transcriptomics (Tx), metabolomics (Mx) or proteomics (Px), respectively. An often used principle in toxicogenomics is grouping of item profiles according to classical toxicological endpoints (Mattes et al., 2004; Ganter et al., 2006), which may allow investigations of the molecular mechanisms associated with different phenotypes of toxicity, and the extraction of diagnostic or predictive item signatures after having conducted studies with a reasonable number of compounds.

To investigate molecular functions and pathways leading to or associated with a particular phenotype, studies conducted within the frame of the Innomed-PredTox project were grouped according to common liver or kidney pathologies (Suter et al., 2011). Four of the compounds, FP004BA, FP005ME, FP007SE and FP014SC, abbreviated in the following as 4BA, 5ME, 7SE, and 14SC, induced similar liver phenotypes involving both the liver parenchyma and the bile duct compartment (4BA, 5ME, 7SE) or at least bile homeostasis (14SC). In the present study, deregulated genes in the target organ liver were determined in relation to histopathological scores associated with samples showing hepatobiliary injury. These genes were then mapped to pathways or functions in the context of hepatotoxicity.

In addition to providing mechanistic insight, Tx profiles may be used to classify compounds with respect to the pathological phenotype they induce upon treatment (Ganter et al., 2006; Blomme et al., 2009). It can be expected that compounds eliciting similar pathologies affect the same or related mechanisms and are therefore likely to deregulate similar sets of genes in a comparable manner. These gene signatures may then be employed in a diagnostic or predictive manner. For example, expression profiles generated from rat liver after short term treatment would be used to predict the potential for a specific toxicity earlier than a clearly detectable histopathological lesion and aid the interpretation of classical parameter measurements. Since a major objective of Innomed-PredTox was more informed decision making earlier in preclinical safety evaluation, we evaluated the potential of Tx data obtained from studies showing hepatobiliary toxicity for early prediction of cholestasis. Similarly, to test the value of individual bile acids as sensitive markers of hepatobiliary injury and to shed further light on potential mechanisms associated with the cholestatic phenotypes seen in this study group, metabonomics LC/MS data was used to determine the effect of individual compounds on a range of conjugated and unconjugated bile acids in urine and serum.

Methods

In addition to the methods described here, further details on the methods and technologies used are given in the accompanying overview paper by Suter et al. (2011).

Animal treatment. All animal studies were conducted in accordance to European or national animal welfare regulations using a harmonized study protocol as previously described (Mulrane et al., 2008). Briefly, male Wistar rats (8–10 weeks old, weighing 170–200 g) were distributed into three dose groups ($n=5$ per group and time-point) and dosed with (+)-(1*R*)-1-[4-(4-fluorophenyl)-2,6-diisopropyl-5-propyl-pyridin-3-yl]ethanol (4BA) at 0, 20 and 100 mg/kg/day, (1-(2-trifluoromethoxyphenyl)-2-nitroethanone (5ME) at 0, 15 and 350 mg/kg/day, 3-pyrrolidineacetic acid, 5-[[[4′-[imino[(methoxycarbonyl) amino]methyl] [1,1′-biphenyl]-4-yl]oxy]methyl]-2-oxo-,methyl ester, (3S-*trans*) (7SE) at 0, 100 and 1000 mg/kg/day or tetraethyl[(3-hydroxy-2-pyridyl)amino]methanediphosphonate (14SC) at 0, 280 and 1120 mg/kg/day by oral gavage for 1, 3 or 14 days, followed by necropsy after an overnight fasting period. After 1, 3 and 12 days of dosing, 16 h urine samples were collected from those animals treated for a total of 14 days, aliquoted and stored at −80 °C until use. At necropsy, blood and serum was collected for clinical chemistry, hematology, metabonomics and blood transcriptomics as indicated below. Organs (liver, kidney) were removed, aliquoted, fixed in formalin or flash frozen in liquid nitrogen and stored at −80 °C. Sections (3–4 mm) were fixed in 10% phosphate buffered formalin and subsequently embedded in paraffin blocks, sectioned, stained with hematoxylin and eosin and examined by light microscopy by the respective study pathologist.

Immunohistochemistry. Immunohistochemical staining of proliferating cell nuclear antigen (PCNA), neutrophile gelatinase associated lipocalin (NGAL/lipocalin-2) and tissue inhibitor of metalloproteinases 1 (TIMP1) was performed on tissue microarrays as previously described (Adler et al., 2010; Hoffmann et al., 2010). Antibodies used were a monoclonal anti-mouse PCNA (sc-56, Santa Cruz, 1:400), a goat anti-rat lipocalin-2/NGAL (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany), and a rabbit anti-human TIMP1 (Millipore, Schwalbach, Germany, 1:400).

Transcriptomics. Approximately 100 mg of frozen tissue was homogenized for total RNA extraction using RNeasy columns (Qiagen). Purified total RNA was submitted to DNase digestion using RNase-Free DNase (Qiagen) before 5 μg purified total RNA were used for cDNA synthesis, in vitro transcription, and fragmentation using the GeneChip Expression 3′ Amplification One-Cycle Target Labeling kit and Control Reagents (Affymetrix). Fragmented in vitro transcripts (cRNAs) were hybridized onto RAE 230_2.0 microarrays (Affymetrix), scanned and used for further analysis. Microarray quality was evaluated using the Refiner software (Genedata). Statistical gene expression analysis was performed using Expressionist (Genedata).

Selection of deregulated genes by statistical methods. Expression values for all probe sets were obtained by applying the Robust Multi-array Average (RMA) condensing method to batches of arrays corresponding to the same compound. In order to normalize the data and to make them comparable across all studies, all condensed probe set expression values of the treated animal samples of a given study were divided by the median of the respective time-matched vehicle group. Subsequently, the data from individual vehicle samples were normalized in this way.

Positive and negative groups of samples were defined based on the histopathology scores as follows. The histopathology terms "hepatocyte cell death", "bile duct epithelial cell mitosis/hyperplasia", and "bile duct inflammation" were considered as summary terms. In addition, a "combined histopathology score" representing the three individual pathological summary terms was employed in the same way as the individual summary terms for deregulated gene selection. The maximum score of the three individual summary terms, "hepatocyte cell death", "bile duct epithelial cell mitosis/hyperplasia" and "bile duct inflammation", was set as the "combined histopathology