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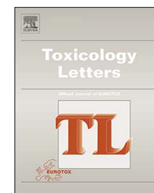

Metabolomics profiling of steatosis progression in HepaRG® cells using sodium valproate

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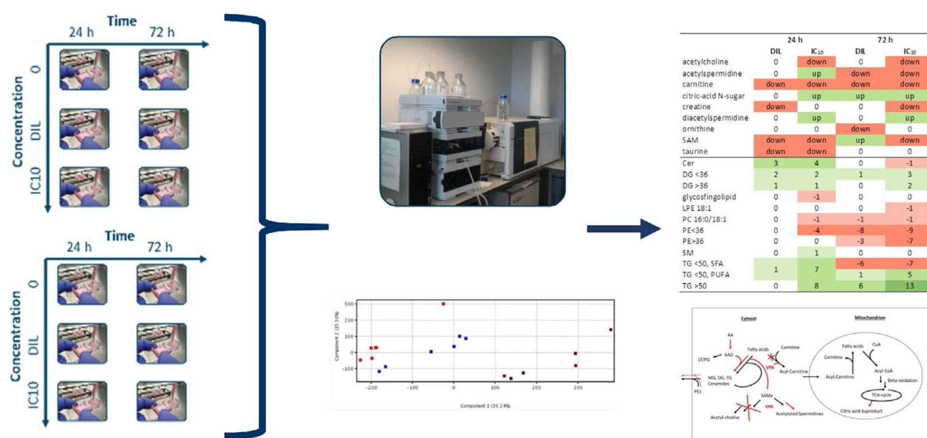
GRAPHICAL ABSTRACT



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ABSTRACT

Non-alcoholic Fatty Liver Disease (NAFLD) is a frequently encountered Drug-Induced Liver Injury (DILI). Although this stage of the disease is reversible, it can lead to irreversible damage provoked by non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis. Therefore, the assessment of NAFLD is a paramount objective in toxicological screenings of new drug candidates. In this study, a metabolomic fingerprint of NAFLD induced in HepaRG® cells at four dosing schemes by a reference toxicant, sodium valproate (NaVPA), was obtained using liquid-liquid extraction followed by liquid chromatography and accurate mass–mass spectrometry (LC–AM/MS).

Abbreviations: ACN, acetonitrile; ADP, adenosyl-diphosphate; AM/MS, accurate mass/mass spectrometry; ATP, adenosyl-triphosphate; BHT, butyl-hydroxytoluene; CAWG, chemical analysis working group (metabolomics society); Cer, ceramide; $CHCl_3$, chloroform; DG, diacylglycerol; DIL, concentration 1/10 of IC10; DILI, drug-induced liver injury; EDTA, ethylenediaminetetra-acetic acid; FA, formic acid; FDR, false discovery rate; HAc, acetic acid; HILIC, hydrophilic interaction liquid chromatography; HMDB, human metabolome database; IC_{10}, inhibitory concentration 10%; IPA, isopropanol; LC, liquid chromatography; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MeOH, methanol; MFE, molecular feature extractor algorithm; MFG, molecular formula generator algorithm; mRSD, median relative standard deviation; MS, mass spectrometry; MSI, metabolomics standards initiative; MS/MS, tandem mass spectrometry; MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steato-hepatitis; NH_4Ac, ammonium acetate; $(NH_4)_2CO_3$, ammonium carbonate; NH_4F, ammonium formate; NR, neutral red; NRU, neutral red uptake; PBS, phosphate buffer saline; PC, phosphatidylcholine; PCA, principal component analysis; PE, phosphatidylethanolamine; PLS-DA, partial least squares discriminant analysis; QC, quality control; QTOF, quadrupole time of flight mass spectrometer; RP-LC, reversed phase liquid chromatography; RSD, relative standard deviation; SAMe, *S*-adenosylmethionine; SM, sfingomyelin; TCA, tri-carboxylic acid cycle; TG, triglyceride; VIP, variable importance projection; VLDL, very-light density lipoprotein; NaVPA, sodium valproate

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The combination of a strict design of experiment with a robust detection method, applied on sodium valproate, validated the possibilities of untargeted metabolomics in hepatic toxicological research.

Distinctive patterns between exposed and control cells were consistently observed, multivariate analyses selected up to 200 features of interest, revealing hallmark NAFLD-biomarkers, such as diacylglycerol and triglyceride accumulation and carnitine deficiency. Initial toxic responses show increased levels of *S*-adenosylmethionine and mono-acetylspermidine in combination with only a moderate increase in triglycerides. New specific markers of toxicity have been observed, such as spermidines, creatine, and acetylcholine. The described design of experiment provides a valuable metabolomics platform for mechanistic research of toxicological hazards and identified new markers for steatotic progression.

1. Introduction

Adverse drug reactions form an important risk when patients are exposed to pharmaceuticals. Liver toxicity is one of the most frequent targets of toxicity since the liver is the central organ involved in the biotransformation and excretion of xenobiotics. Its high metabolic capacity makes it a strong, yet vulnerable organ; therefore the detection of liver toxicity is a priority in pre-clinical testing (Rang et al., 2012; Hodgson, 2010).

A key aspect in toxicology is the determination of safe concentration levels for acute and chronic exposures (ECHA, 2012). However, conventional tests do not provide additional insight in the mechanism of toxicity (Ramirez et al., 2013; van Ravenzwaay et al., 2007, 2014). Modern toxicology aims at mechanistic interpretations of adverse drug reactions using alternative tools to accurately assess the hazards associated with xenobiotics (Ramirez et al., 2013). The fingerprinting of toxicological events allows the accurate determination of mechanisms of the toxicological outcome. Metabolomics, the study of small organic molecules, provides the most downstream information of biochemical pathways, providing a summary of the eventual outcome after exposure to xenobiotics (Dunn et al., 2012; Madji Hounoum et al., 2016; Leon et al., 2013). Since metabolomics do not measure proteins or genes, the phenotypical toxicological response on metabolite level can be investigated as an alternative to gene or protein alterations on a (sub) histological level (van Ravenzwaay et al., 2007, 2014). Furthermore, in the context of the Adverse Outcome Pathway approach, metabolomics may provide novel markers of toxicity that can be related to the molecular initiating events of the adverse effect under study (Vinken, 2013).

This study investigated the intracellular endogenous metabolic profiles of HepaRG cells, a human metabolically competent hepatic cell line (Gripon et al., 2002; Guillouzo et al., 2007), exposed to sodium valproate (NaVPA) at different time points and dosage schemes to observe acute and sub-chronic effects on two different dosing levels. NaVPA is an anti-epileptic drug which is frequently prescribed to reduce the number of epileptic seizures (Rang et al., 2012; Informatie and voor, 2014). Due to its structural similarity with fatty acids, it competes with the endogenous lipid metabolism, provoking a hazard to induce non-alcoholic fatty liver disease (NAFLD) or steatosis (Silva et al., 2008).

The metabolomic fingerprint of NaVPA as a reference toxicant for steatosis validates the set-up of a designed experimental platform including the *in vitro* exposure model, cell extraction and metabolomic analysis of the cell extracts. Multivariate analyses are used to select the signals related to exposure; further identification using physicochemical characteristics such as the accurate mass, MSMS spectra and retention times to generate a list of endogenous metabolites (Kind and Fiehn, 2007, 2006). With the obtained information, a biochemical interpretation of the observed results can be inferred, allowing an accurate determination of pathways involved in the potential toxicological hazards associated with the xenobiotic of interest.

2. Materials and methods

2.1. Standards and chemicals

Cryopreserved differentiated HepaRG® cells (HPRGC10) and recommended culture media and supplements (Basal Hepatic Cell Medium (MIL600), Thawing/Plating/General Purpose Medium Supplement with antibiotics (ADD670) and Additives for Maintenance/Metabolism with antibiotics (ADD620)) were obtained from Biopredic International (Rennes, France). Collagen type 1 was obtained from Corning (Wiesbaden, Germany). Dry ice was purchased from Strombeek IJsfabriek (Strombeek, Belgium). Ultrapure (milliQ) water was obtained by the use of a PURELAB device from Elga LabWater (Tienen, Belgium). Phosphate-buffered saline (PBS) was prepared on site at the research group *In Vitro* Toxicology and Dermato-Cosmetology (IVTD, VUB, Belgium).

From Thermo Scientific (Rochester New York, VS) the Lab-Tek Chamber Slide w/Cover Permanox Slide Sterile 2 well were purchased. Sterile cell scrapers with sharp edge (type GBO 541070) were purchased from Greiner Bio-One (Vilvoorde, Belgium). The equipment for the use of the TC10 Automated Cell Counter (Cell Counting Slides for TC10™/TC20™ Cell Counter, Dual-Chamber en Trypan Blue Dye, 0,40% solution) were purchased from BioRAD Laboratories (Temse, Belgium).

Ammonium acetate (for analysis, > 98%) (NH_4Ac), formic acid (for analysis, > 98%) (FA), acetic acid (glacial, anhydric for analysis, 100%) (HAc), chloroform (for analysis) ($CHCl_3$), ammonium carbonate (extra pure), 2-propanol (for analysis) (isopropanol, IPA) and neutral red (NR) were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile (HPLC grade) (ACN) and methanol (LC–MS grade) (MeOH) were obtained from Fisher (Loughborough, VK). Sodium Valproate (NaVPA), ammonium formiate (97%) (NH_4F), L-ascorbic acid (BioXtra, > 99%), butylhydroxytoluene (> 99%) (BHT) and ethylenediaminetetraacetic acid (trace metal basis, 99,995%) (EDTA) were purchased from Sigma-Aldrich (Steinheim, Germany).

The isotope labeled standards D-tryptophane-2′,4′,5′,6′,7′-d5 (98%), laurylic acid-12,12,12-d3 (99%) and cholesterol-25,26,26,26-d4 (99%) were obtained from CDN Isotopes (Pointe-Claire, Quebec, Canada). The standards 1,2-diheptadecanoyl-sn-*glycero*-3-phosphocholine (PC-17:0), 1,2-diheptadecanoyl-sn-*glycero*-3-fosfaat (PA-17:0), N-heptadecanoyl-sphing-4-enine (Cer-17:0) and 1-heptadecanoyl-2-hydroxy-sn-*glycero*-3-phosphocholine (LPC-17:0) were purchased from Avanti Lipids (Alabaster, Alabama, VS). ATP-$^{13}C_{10}$, TG-($^{12}C_{15}$-^{13}C:0)$_3$, lysine-$^{13}C_6$-$^{15}N_2$, glucose-$^{13}C_6$, ADP, ATP, stearic acid, folic acid, mono-, di- and trioleylglycerol, misoprostol, cholic acid-d4, phosphoenolpyruvate, ornithine, glutamate-d4, leucine-d3, adenine, glucosephosphate, citric acid, caffeine, N-acetylglucosamine, pyridoxal-d3, dopamine-d4, palmitoylcarnitine, cholesterylpalmitate, succinic acid-d4 and a standardised amino acid mix (AAS18) were purchased from Sigma-Aldrich (St. Louis, Missouri, VS).

2.2. Determination of testing concentrations

Cryopreserved differentiated HepaRG® cells were thawed and seeded in chamber slides at a density of 0.089×10^6 cells/well using

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