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Untargeted liquid chromatography-mass spectrometry metabolomics to assess drug-induced cholestatic features in HepaRG® cells

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Abstract

Cholestasis is a liver disease associated with retention of bile in the liver, which leads to local hepatic inflammation and severe liver damage. In order to investigate the mode of action of drug-induced cholestasis, in vitro models have shown to be able to recapitulate important elements of this disease. In this study, we applied untargeted metabolomics to investigate the metabolic perturbances in HepaRG® cells exposed for 24 h and 72 h to bosentan, a cholestatic reference toxicant. Intracellular profiles were extracted and analysed with liquid chromatography and accurate-mass spectrometry. Metabolites of interest were selected using partial least-squares discriminant analysis and random forest classifier models. The observed metabolic patterns associated with cholestasis in vitro were complex. Acute (24 h)
exposure revealed metabolites related to apoptosis, such as ceramide and triglyceride accumulation, in combination with phosphatidylethanolamine, choline and carnitine depletion. Metabolomic alterations during exposure to lower dosages and a prolonged exposure (72 h) included carnitine upregulation and changes in the polyamine metabolism. These metabolites were linked to changes in phospholipid metabolism, mitochondrial pathways and energy homeostasis. The metabolic changes confirmed the mitotoxic effects of bosentan and revealed the potential involvement of phospholipid metabolism as part of the mode of action of drug-induced cholestasis.

Graphical abstract

**Keywords:** Bosentan, drug-induced cholestasis, HepaRG, Liquid Chromatography-Mass spectrometry, metabolomics, *in vitro*

**Highlights:**

- Drug-induced cholestasis was investigated *in vitro* using LC-MS metabolomics.
- HepaRG® cells reduce bile acid synthesis during exposure to bosentan.
- Bosentan causes mitochondrial impairment and a lower phosphorylation state.
- Adaptive responses include phospholipid production and polyamine activation.
- Cytotoxic effects include ceramide production and phospholipid depletion.

**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full name</th>
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<tbody>
<tr>
<td>BSEP</td>
<td>Bile salt efflux pump</td>
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<tr>
<td>CAWG</td>
<td>Chemical analysis working group</td>
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<tr>
<td>Cer</td>
<td>Ceramide</td>
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<tr>
<td>DG</td>
<td>Diacylglycerol</td>
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<tr>
<td>DIC</td>
<td>Drug-induced cholestasis</td>
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<tr>
<td>DILI</td>
<td>Drug induced liver injury</td>
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<td>HMBD</td>
<td>Human metabolome database</td>
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<tr>
<td>IC</td>
<td>Inhibitory concentration</td>
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<tr>
<td>LC</td>
<td>Liquid chromatography</td>
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<tr>
<td>MeOH</td>
<td>Methanol</td>
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Liver injuries are frequent adverse effects of both drugs and phytotherapeutic medicines and can be considered to be the main reason for drug attrition in preclinical research. Among the different forms of drug-induced liver injuries (DILI), drug-induced cholestasis (DIC) is defined as the accumulation and/or reduced excretion of bile due to drug intake. The reduced bile flow results in hepatic damage, causing the recognisable symptoms of jaundice and pruritus. This damage is reflected by increased serum values for liver enzymes, such as alkaline phosphatase, gamma-glutamyltranspeptidase and, to a limited extent, aspartate and alanine amino transferases, in combination with a hyperbilirubinemia. According to the adverse outcome pathway, DIC is linked to Bile Salt Efflux Pump (BSEP) inhibition as a molecular initiating event (MIE). Downstream effects include oxidative stress, mitochondrial damage, liver failure and accumulation of bile acids and bilirubin, which ultimately give the specific jaundice symptoms.

Metabolomics, the study of small endogenous molecules, has proven to be a valuable tool in in vivo toxicological studies investigating DIC. Bosentan is known to induce cholestatic liver injury through BSEP inhibition and can therefore serve as a reference toxicant for DIC. Metabolomics applications for in vitro hepatotoxicity studies are increasing, but articles reporting DIC are limited.

The combination of HepaRG® cultures and metabolomics has already been used to study non-alcoholic fatty liver disease. These cells have also been proven valuable for in vitro cholestatic research.
The hepatocyte-like cells form clusters with functioning bile ducts, which forms a reliable platform to study bile metabolism\(^\text{14}\). Rodrigues et al. performed a pilot NMR metabonomics study to support transcriptomic data acquired from HepaRG\(^\circ\) under cholestatic stress\(^\text{9}\). The current article focuses on a more extensive ultra-pressure liquid chromatography and accurate mass spectrometry (UPLC-AM/MS) metabolomics analysis of both the lipidome and metabolome of HepaRG\(^\circ\) cells exposed to bosentan in different exposure regimes, allowing the monitoring of alterations at the most downstream level of the cellular organisation\(^\text{15-17}\).

2. Materials and methods

Exposure and acquisition methods have been performed as described previously\(^\text{11}\). A brief description highlighting the principles is mentioned in-text and full details concerning the protocol are provided in the supplementary information SI-1 to SI-5.

2.1 Determination of testing concentrations

Seven days after initial cell seeding, the wells were divided in two negative control groups and eight groups that were exposed to bosentan at different concentrations ranging from 8 µg/mL to 2 800 µg/mL for a period of 24 h and 0.45 µg/mL to 1 000 µg/mL for a period of 72 h in a repeated dose exposure with a medium refreshment every 24 h (Table SI-2.1). Viability was assessed using the neutral red uptake (NRU) assay\(^\text{18,19}\). Full details are available in the Supplementary Information SI-2.

2.2 Metabolomics experiments

2.2.1 Seeding of the HepaRG\(^\circ\) cells and exposure to bosentan

Cryopreserved differentiated HepaRG\(^\circ\) cells were thawed and seeded in collagen-coated 2-well Lab-Tek chamber slides at a density of 1.03 x 10\(^6\) cells/well. After seven days cell cultures were visually checked for hepatocyte/biliary cell ratio to prevent any differentiation bias and randomly assigned to a negative control group or to any of the two exposure groups: (1) the high dose (230 µg/mL and 95 µg/mL for 24 h and 72 h exposure, respectively) and (2) a 1/10 dilution of the high dose (low dose: 23 µg/mL and 9.5
µg/mL for 24 h and 72 exposure, respectively) (Figure 1). Two non-cultured chamber slides were used as blank samples in each experiment and all exposure experiments were performed twice to reduce the number of false positive results, i.e. features that do not show similar alterations across independent experiments.

2.2.2 Sample preparation

The cell cultures were harvested and processed by the protocol of Wu et al. and adapted by Cuykx and Mortelé et al., which is fully explained in SI-320,21. The cells were washed twice with phosphate buffer saline (PBS) (37°C), flash frozen on liquid nitrogen and scraped from the surface with three times 200 µL of a cooled (-80°C) 80 % (v/v) methanol (MEOH)/milliQ water solution. Two fractions were obtained through Liquid/Liquid extraction using ultrapure water, methanol, and chloroform. Quality Control (QC) pools were generated through the collection of aliquots of all samples for the polar and non-polar phases.15 The extracts were evaporated to dryness and reconstituted in LC-MS compatible solvents.

2.2.3 LC-MS analysis

LC-MS analysis was performed according to previously established protocols; details of the four analytical runs are available in SI-411,22. The non-polar fraction was analysed in two runs (one positive, one negative mode) on a Kinetex XB-C18 (150 x 2.1 mm; 1.7 µm particle size, Phenomenex, Utrecht, the Netherlands). Mobile phase composition consisted of mixtures of methanol, isopropanol (IPA) and water with ammonium acetate (pH 6.7) and mixtures of acetonitrile (ACN), IPA and water with an acetate buffer (pH 4.2) for negative and positive mode, respectively. The polar fraction was analysed in positive mode with a HILIC system using an iHILIC column (100 x 2.1 mm; 1.8 µm particle size, HILICON, Umeå, Sweden) using ACN, MeOH and water with an ammonium formate buffer (pH 3.15). In negative ionisation mode, a polymeric iHILIC Fusion Column (100x 2.1 mm, 5 µm particle size, HILICON) was used in combination with ACN, MeOH and water with an ammonium carbonate ((NH₄)₂CO₃) buffer (pH 9.0). LC-separation was performed on an Agilent Infinity 1290 UPLC (Agilent Technologies, Santa Clara, USA), connected to an Agilent 6530 QTOF with Agilent Jet Stream nebuliser (Agilent Technologies). Injections were block-
randomised and the LC-MS system was equilibrated with 15 QC-injections, 1 QC injection was performed after every 4 sample injections to monitor instrumental drifts.

2.2.4 Data analysis

2.2.4.1 Data-quality control

Internal and external standards were controlled to evaluate the precision and repeatability of the analytical method regarding retention time and m/z-accuracy within and between the experimental batches. The raw data were searched for the internal standards using the Find by Formula algorithm (Agilent Technologies) with the following parameters: formula matching ± 10 ppm, expected variation 2 mDa ± 8 ppm.

2.2.4.2 Data-pretreatment

Acquired data were imported on the MassHunter Qualitative software (Agilent Technologies, v 2.06.00) and converted to centroid mzdata. The generic data files were processed using the XCMS package in the R workspace\textsuperscript{23,24}. Features representing the ions of the extracted metabolites were searched using the centWave algorithm. Features were aligned with the ObiWarp algorithm\textsuperscript{25} and grouped by density. Missing peaks were re-extracted using the fillPeaks algorithm\textsuperscript{24}. All parameters during feature extraction are available in SI-5.

The dataset was cleaned up by blank subtraction, a frequency filter and a variability filter using the MetaboMeeseeks package\textsuperscript{26}. A principal component analysis (PCA) was performed and outliers were removed for further analysis (SI-7). After outlier removal, the filter process was re-iterated and samples were normalised using probabilistic quotient normalisation\textsuperscript{27}. Missing values in extracted features were checked with a Fisher-test between the different exposure groups and missing values with a non-significant missing value distribution (p>0.05) were imputed through a k-nearest neighbour algorithm with 5 neighbours\textsuperscript{27,28}. The final dataset was once more evaluated using a PCA to assess important trends and their potential impact on the subsequent multivariate analysis.

2.2.4.3 Statistical analysis
Univariate statistical analysis was performed through the non-parametric Mann-Whitney U test with a Benjamini-hochberg correction for multiple testing using the multtest package in R\textsuperscript{29}. In addition, a partial least squares discriminant analysis (PLS-DA) and a random forest classification were performed as multivariate analyses\textsuperscript{26,30}. Performances were checked using leave-one-out cross-validation. Metabolites of interest were obtained from significant features with a high importance in the multivariate models based on the latent values of the first component of the PLS-DA and on the variable importance measure (VIM) of the random forest classifier model. The raw signals of the selected metabolites were manually checked before being selected as metabolites of interest.

2.2.4.4 Identification

In MassHunter, the signals corresponding to potential metabolites of interest were selected and potential formulas were calculated with the molecular formula generator algorithm. The identification was based on the m/z-value, the isotope pattern, the measured retention time and the fragmentation spectra acquired during the equilibration runs supported by reference spectra from the metabolite databases METLIN, LIPID MAPS, the Human Metabolome Database (HMDB) and/or ChemSpider\textsuperscript{31–36}. The resulting levels of identification are based on the standardised reporting rules published by the Chemical Analysis Working Group (CAWG) and the Metabolomics Standards Initiative (MSI), and an example has been given in SI-6\textsuperscript{37–39}.

2.2.4.5 Biotransformation products

To compare the biotransformation activity of HepaRG cells, the data were actively searched for biotransformation products mentioned in literature\textsuperscript{40}. Full details concerning the biotransformation products are available at supplementary information SI-16. Briefly, m/z values were extracted and integrated. The values were qualitatively compared between the different exposure groups using boxplots.

3 Results

3.1 Experimental observations
The dose-response curves of the viability assay showed a clear sigmoid curve correlating the viability with the dose during the 72 h repeated dose exposure (SI-2, Figure 2). 24 h exposure data showed higher viability at concentration levels between 44 µg/mL and 230 µg/mL. The IC_{10} was calculated based on 4-parametric analysis of the response curves of three plates using Masterplex® QT curve-fitting software and resulted in IC_{10} values of 510 ± 10 µg/mL and 96 ± 3 µg/mL for the 24 h and 72 h exposure, respectively. The best-fit curves in the program do not follow this initial higher expression of neutral red uptake during the 24 h exposure, which biases the calculation of the IC_{10} values towards the higher dosages of 500 µg/mL. A transition towards cytotoxicity was observed from 230 µg/mL, which was set as the high-dose concentration for the 24 h exposure experiments.

Evaluations of the cell cultures prior to extraction (Figure 3), showed no morphological differences between the treated cultures and the negative controls, except for the presence of cell debris in the medium of the cells exposed to the high dose.

The analysis of biotransformation products in SI-16 confirmed the active CYP2C9 and CYP3A4 biotransformation capacity of the HepaRG cultures. This highlights the relevance of the culture considering potential bioactivation and toxicokinetics.

### 3.2 Data Quality

A limited number of samples (± 1 %, Table SI-7) did not meet the quality assurance standards and were consecutively removed. The calculated mRSDs (Table SI-8) were below 20% for all QC samples in the non-polar fraction, while in the polar fraction in positive ionisation mode mRSDs did not exceed 25%. The mRSDs of the polar fraction in negative ionisation mode were higher, reaching up to 35%. The high values of these mRSDs could be related to the co-extraction of noisy features by the algorithms, augmenting variation in the dataset. However, the within-group variations were lower than the variation between all exposure groups.

All PCAs are available in Figure SI-9, two examples are shown as examples in Figure 4 and Figure 5. Batch-related differences introduce high variation in the dataset, represented by the first principal component of all non-polar datasets and of the polar datasets during 72 h exposure and by the second principal
component in the polar datasets of the 24 h exposure. Clear differences between the high-dose exposure and the negative control group could be observed through PC 2, with exception for the polar datasets of the 72 h exposure. The samples exposed to the low-dose were not fully discriminated from the negative control group but intra-batch trends were noticeable. The observable trends in the PCA-plots supported the decision to conduct further multivariate analysis to select metabolites potentially associated with DIC.

As reported in Tables SI-10 to SI-13, all classifier models could accurately discriminate between the cytotoxic concentration and the negative control group. For the non-polar fraction, discriminatory power was stronger for the 72 h repeated dose exposure, although the Q² values of the 24 h exposure were still good (>0.6). The polar fraction had a more distinguishable metabolic profile for the 24 h exposure time point than at 72 h exposure, revealing a higher number of significant metabolites.

Modelling a classifier between the group exposed to the low dose and the negative control was not performant, with no discriminating power of the polar fraction between the groups for a 72 h exposure and limited discriminatory impact for the non-polar fraction for a 24 h exposure. The models overfit for exposure at sub-cytotoxic concentrations, resulting in low Q² values.

Regarding the selection of metabolites associated with DIC, random forest models concurred with the PLS and ± 50 % of the selected metabolites of interest were identified by both classifier models.

### 3.3 Selection of metabolites associated with DIC

A full list of identified metabolites provided with their VIM and LV for the multivariate modes is presented in Table SI-14 and a comprehensive table with qualitative alterations is given in Table 1. Boxplots of these features are also provided in SI-15.

In all conditions exposure of HepaRG cultures to bosentan induced higher signals related to creatine, phosphorylated metabolites, aminergic oligopeptides, ceramides, diacylglycerols, ether-triglycerides and poly-unsaturated triglycerides with heavy free fatty acids. Lower signals were observed for nucleotides, trimethylammonium butanoic acid, uridine diphosphate (UDP) glucuronic acid, phosphatidylethanolamines, triglycerides with a low m/z value and bile acids.
Carnitine was upregulated in the low-dose exposure but downregulated upon exposures to high dosages of bosentan. Phosphorylethanolamine was downregulated during acute exposures and upregulated during prolonged exposure.

Species which were selected in only one condition included isoputreanine, methylhydroxylysine, pantothenic acid, phosphocholine and a phosphatidylserine for the 24 h exposure and spermidine, ceramide derivatives, poly-unsaturated phosphatidylethanolamines and phosphatidylglycerols for the 72 h exposure regimen.

To gain an overview of pathways involved, the metabolites were grouped according to their presence in metabolic pathways in Figure 6 and 7.

4 Discussion

The differentiation of HepaRG cells into hepatocyte-like clusters with active bile ducts in combination with an active bile salt metabolism and functional transporters provides a reliable and in vivo-relevant in vitro platform to study DIC. An in vivo hallmark of DIC is the increased presence of bile acids in the hepatic tissue and serum. Observations in cholestatic tissue reveal strong bile acid leakage from the biliary ducts into the extracellular matrix, together with a strong infiltration of inflammatory cells. However, the in vitro model revealed significant downregulation of different bile acids. The identified bile acids were linked to the conjugated taurocholate, which is the most present bile acid in HepaRG cell cultures. Since the excretion of bile salts is prevented through the MIE of DIC induced by bosentan, lower levels are probably linked through an adaptive feedback mechanism, reducing bile acid synthesis. In addition, an important factor to consider when assessing cholestatic mechanisms in vitro is the limited presence of bile acids in standard cell culture medium, which is often compensated through the extra addition of bile acids to the medium, simulating a down-stream accumulation e.g. through bile duct ligation.

The decrease of phospholipid precursors, such as phosphorylethanolamine, choline and phosphocholine indicated either a consumption or a decreased production of these phospholipids, shifting the metabolism from these phospholipids to ceramides in order to induce apoptotic events.
Signals related to carnitine, an important amino-acid for lipid consumption and metabolism, were higher when HepaRG® cultures were exposed to the low dose concentrations, while they were lower during high-dose exposure. The observed decrease of its precursors (methylhydroxylysine and trimethylammonium butanoic acid) is possibly explained by an initial compensatory upregulation of carnitine production to sustain the energy homeostasis of the cell culture.

The observation of a consistent cofactor decrease might be related to mitochondrial toxicity as to a higher phosphorylation state of the cultures, shown as several phosphorylated metabolites. This effect can be an activation of different pathways in order to adapt to the toxicological insult. Other effects linked to the mitochondrial malfunction could be noticed in chain prolongation, resulting in the higher-weight TGs and DG increments (Table 1, SI-13 for all separate lipid species). The increased incorporation of highly unsaturated, long-chain TGs can be explained by activated pathways of arachidonic acid, potentially linked to a pro-inflammatory environment.

The conversion of UDP-hexoses to UDP glucuronic acid was inhibited. Since glucuronic acid is the main metabolite in matrix production, being a precursor of hyaluronic acid and chondroitin, this downregulation resulted in a negative impact on the matrix production. Glucuronic acid also functions as a ligand during phase-II biotransformation processes to increase excretion efficiency of xenobiotics. The downregulation of this pathway could be explained by a shift of metabolic priorities, converting all energy to cell survival rather than extracellular interactions.

Differences between an acute (24 h) and sub-acute (72 h, repeated dose exposure) can be related to dose-related effects. Higher concentrations of bosentan during a 24 h exposure might increase the impact of the MOA, shifting the pathway especially towards the cytotoxic events (Figure 7). Longer exposures to lower absolute dosages did not imply as many altered metabolites, which might suggest an adaptive response of the culture, reducing the short-term toxic outcome (Figure 6). This hypothesis explains why ceramide upregulation is less pronounced during a 72 h exposure than in a 24 h time-frame, combined with a higher decrease of PUFA-PEs and short-chain TGs. It also explains different alterations, such as a putrescine downregulation towards a spermidine production, a less pronounced creatine upregulation and an upregulation of phosphorylethanolamine to counteract PE depletion.
The identified metabolites associated with DIC and their role in the pathways of toxicity have been compared with available in vitro data. As such, Van den Hof et al reported similarities during cholestatic events on HepG2 cells using cytotoxic (IC20) dosages of cyclosporine regarding initial carnitine upregulation and cofactor downregulation, but reported mostly amino acids to be upregulated including alanine, leucine and glutamine. Differences might be related to both the cell type (HepG2 vs. HepaRG) as to the product and the dosage (Cyclosporine at IC20 vs Bosentan at IC10). The limited transporter function of HepG2 cells in comparison to HepaRG cells can be an important factor to explain the differences among the experimental outcomes. However, the observed similarities also revealed that next to BSEP inhibition other factors are involved, such as feedback mechanisms in bile acid synthesis and conjugation.

A possible explanation for the decreased levels of intrahepatic bile acids has been described for HepaRG cells in literature and is related to a decreased uptake through sodium dependent uptake transporters (NTCP) and organic anion transporter 1B (OATP1B). Other factors of the decreased presence include a downregulation of bile acid synthesis through active feedback mechanisms of the FXR and PXR receptors.

Changes in phospholipid metabolism, and especially the levels of phosphatidylethanolamines are often reported in in vitro metabolomics research and are known to have an impact on both membrane fluidity and biosignalling functions. The lower intrahepatic choline levels in concordance with altered PC-metabolims and hepatobiliary damage has also been reported by Kohjima et al. and Zhao et al.

Mitochondrial impairment was reported by Rodrigues et al. and Krahenbuhl et al. through the increase of leucine metabolites, such as methyl-oxovalerate, and lactate and carnitine distortion. The latter study reported lower levels of acylcarnitines and free carnitine in vivo, mainly due to the inhibition of carnitine production and carnitine-acyltransferases. The upregulation of free carnitine in vitro matches the observations by Van den Hof and Rodrigues for a 24 h exposure regimen.

Downstream effects of mitochondrial impairment, such as nucleotide downregulation concurs with Van den Hof, although the metabolite species are not identical. The upregulation of single amino acids has not been observed.
Other effects mentioned in literature are tumour necrosis factor and interleukin 6/8 production, suggesting oxidative stress and inflammation. This might be linked to upregulation of PUFA-esters as side effects of leukotriene production. Indeed, pro-inflammatory effects are proposed to be a key factor in cholestatic injuries in vivo.

Polyamine metabolism was investigated by Dayoub et al. in the context of regeneration processes, and this could be an explanation of putrescine conversion into spermidines. Different alterations were described in function of time and dose. This pattern was observed for 72 h exposure, adaptive responses were not observed upon 24 h exposure, potentially due to the high dose of bosentan.

5. Conclusions
Drug-induced cholestasis is a form of DILI related to reduced bile efflux. Untargeted metabolomics analysis using HepaRG® cell cultures revealed features related to different exposure experiments, representing the balance between adaptive and cytotoxic mechanisms.

The described pathways are linked to cytotoxic mechanisms related to mitochondrial impairment, carnitine depletion and ceramide production; but also include adaptive mechanisms to sustain cell viability, such as a decreased bile acid presence, polyamine production and reduced glucuronate metabolism. Untargeted metabolomics support the findings of complementary -omics techniques. The reported patterns underscore the complexity of in vitro cholestasis research and highlight that mechanistic interpretation is dependent on the design of the exposure experiment.

Acknowledgements
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Conflicts of interest

The authors declare no conflicts of interest

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Table 1: Summary of metabolites of interest identified in HepaRG cells after 24h and 72h exposure to bosentan with a confidence of identification of 3 and higher. The full list of identified metabolites and their confidence in identification is mentioned in Table SI-13. Colours represent the degree of up- (red) or down-regulation (green), the intensity of the colour is related to the number of metabolite species altered.

<table>
<thead>
<tr>
<th>Metabolites</th>
<th>24 h exposure</th>
<th>72 h repeated dose exposure</th>
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<tbody>
<tr>
<td></td>
<td>Low-dose 23 µg/mL</td>
<td>High-dose 230 µg/mL</td>
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<tr>
<td></td>
<td>Low-dose 9.5 µg/mL</td>
<td>High-dose 95 µg/mL</td>
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<td>Aminergic oligopeptides</td>
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<td>Carnitine</td>
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<td>TG(&gt;50, PUFA)</td>
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<tr>
<td>TG(&gt;50, non PUFA)</td>
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<tr>
<td>TG(&lt;50)</td>
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</tbody>
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Cer: ceramide, DG: diacylglycerol, LPE: lysophosphatidylethanolamine, PE: phosphatidylethanolamine, PG: phosphatidylglycerol, PS: phosphatidylserine, PUFA: species containing mostly unsaturated fatty acids,
TG: triglyceride with more (> or less than (<) 50 carbons divided over all acyl chains, UDP: uridyl diphosphate.

**Figure 1:** Graphical representation of the experimental design for the metabolomics experiments.  

**Figure 2:** Example of the NRU viability curves (SI-2) for the 24 h (left) and 72 h repeated dose (right) exposure.

**Figure 3:** Photos of the HepaRG cell cultures at a 10x10 amplification as an example of cell morphology at different stages. A-C: control, low dose (23 µg/mL) and high dose (230 µg/mL) during 24 h exposure, respectively and D-F: control, low dose (9.5 µg/mL) and high dose (95 µg/mL) exposure after 72h, respectively. Healthy hepatocellular clusters (A full circle) and biliary-like cells (B: dotted circle) show a clear organisation and the absence of debri in the cytoplasm. Exposure to bosentan disturbed the hepatocellular organisation (F: full circle) and bile-pocket formation (F: arrows). Debri in biliary-like cells (C: circle) and floating debri in cell culture medium (C: arrow) are observable.
Control | low dose | high dose
---|---|---
24 h single dose exposure

72 h repeated dose exposure
Figure 4: exemplary PCA plots of the non-polar fraction in positive mode during the 72 h exposure showing PC1 vs PC2. PC1 represent very strong batch effects, while PC2 is oriented according to a concentration-dependent exposure to the high dose and the low dose/negative control group. (Green = control group, red = group exposed to the low dose (9.5 µg/mL), black = group exposed to the high dose 95 µg/mL), blue = QC; circles = experimental replicate 1; triangles = experimental replicate 2).
Figure 5: exemplary PCA plot of the polar fraction in positive mode during the 24 h exposure showing PC1 vs PC2 (up) and PC3 vs PC4 (down). A higher variance in the method acquisition is observable through a broader distribution of the QC samples, but no distinctive batch effects dominate the first two components. Differentiating trends between the high exposure dose and the negative control group is observable through PC1 and PC2. Discriminatory trends between the low dose and the negative control group are limited. (Green = control group, red = group exposed to the low dose (9.5 μg/mL), black = group exposed to the high dose 95 μg/mL), blue = QC; circles = experimental replicate 1; triangles = experimental replicate 2).
Figure 6: metabolite classes altered during exposure to low concentrations of bosentan. Colours indicate higher (red) or lower (green) signals when exposed to bosentan.
Figure 7: metabolite classes altered during high-dose exposure to bosentan. Colours indicate higher (red) or lower (green) signals when exposed to bosentan.