# Untargeted *in vitro* metabolomics to identify hepatotoxic modes of action

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## Introduction

Liver toxicity is a major concern in toxicological safety assessment and many drug candidates are revoked because they induce hepatotoxicity. The implementation of "omics" technologies in toxicological research resulted in a paradigm shift towards mechanistic interpretations to complement the dose-related safety assessment.
Metabolomics play a vital role in the investigation of Adverse Outcome Pathway models since it is most downstream of the cellular organisation. Combining metabolomics with *in vitro* techniques creates an untargeted platform which can differentiate between different modes of action (MOA).

## Methods

For *in vitro* metabolomics experiments, the HepaRG® cell-line was selected based on its characteristics to differentiate into hepatocellular clusters and biliary-like cells, while maintaining important hepatic functions, such as CYP-related biotransformation capacity and bile efflux. Cells were cultured in collagen coated chamber slides and exposed to IC10 and a $1/10^{th}$ of IC10 of the hepatotoxicants sodium valproate and bosentan during a 24 h and 72 h (repeated dose) exposure in duplicate.
Cells were quenched in liquid nitrogen and intracellular metabolites were extracted using 80 % (*v/v*) MeOH. A polar and non-polar fraction were retrieved during a Bligh and Dyer based Liquid/liquid extraction using water, methanol and chloroform and using anti-oxidants and chelating agents to prevent analytical degradation. Polar and non-polar fractions were analysed using tailored HILIC- and RP-UPLC-QTOF methods.
Untargeted data analysis was performed with vendor software and XCMS, extracted features were blank-subtracted, filtered for sparsity and variance. The statistical dataset was normalised and analysed using principal component analysis (PCA), partial-least-squares-discriminant analysis (PLS-DA) and random forest classifiers (RF).

Markers of toxicity were selected based on redundancy between two experimental batches and identified using accurate mass, isotope pattern and MS/MS fragmentation, which were matched against the Metlin and LipidMaps public databases.

## Results & discussion

PCA-based interpretations of both toxicants revealed the extensive impact of the IC10 exposure on the metabolome of HepaRG cells. The subcytotoxic concentration does show a shift in the metabolome and lipidome, but this shift is more distinct for sodium valproate than for bosentan. All exposure groups were successfully discriminated from the negative control group through a linear PLS-DA.

Markers of toxicity for steatosis included hallmark metabolites, such as diacylglycerol and triacylglycerol accumulation and carnitine deficiency. Non-typical markers included a downregulation of creatine and acetylcholine, and an upregulation of acetyl-spermidines. Markers for cholestasis comprised of a general upregulation of the phosphorylation state, downregulations of nucleotides and changes within the aminergic amino acid and peptides were observed. alterations in lipid metabolism were mainly related to a moderate accumulation of triglycerides and a conversion of phosphatidylethanolamines (PE) to lysoPEs.
Comparison of both metabolomes revealed similarities, such as the ceramide upregulation, which can be applied as a non-specific marker of toxicity. Certain metabolites were altered in both MOAs but were changed in an opposite way upon exposure. Furthermore, many metabolites were only altered specifically for one MOA. Therefore, qualitative discrimination of the mode of action can be performed based on the metabolomics fingerprints.

## Conclusion

The combination of *in vitro* HepaRG® cultures and untargeted UPLC-AM/MS metabolomics was successfully applied to determine *in vitro* markers for steatosis and cholestasis.
Besides non-specific markers of toxicity, differentiation between different modes of action can be annotated based on both the lipidomic and metabolomic intracellular profiles. The identified markers of toxicity are linked to the MOA of the toxicants, allowing a mechanistic interpretation of the toxicological insults.