Predicting long-term toxic effects using computer models based on systems characterization of organotypic cultures

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Final publishable summary report

EXECUTIVE SUMMARY

NOTOX developed *in vitro* cultivation systems amenable to systems biology *omics* analyses specifically serum free long term cultivation. In order to mimic the *in vivo* situation as close as possible, human organotypic 3D cultivation of its various cell systems comprising of HepaRG cells, PHH, co-cultures with non-parenchymal cells, human stem cell derived cardiomyocytes were developed. Modern microscopic methods were used to derive structural and activity data at different levels of magnification and resolution. NOTOX established a unique approach of deriving diverse and dynamic *omics* data from the same experiment with close link with computational modelling. The omics endpoints provided rich insights into the mechanisms of action of the tested compounds. These data were used to build up large scale computational models of varying complexities including metabolic network models, PBPK models, and tissue organization and regeneration models. The systems biology toolbox developed in NOTOX will have a significant impact in the scientific and industrial communities. The NOTOX approach is the step forward towards animal fee repeated dose toxicity assessment using human *in vitro* models.

SUMMARY DESCRIPTION OF PROJECT CONTEXT AND OBJECTIVES

NOTOX is the cornerstone project of the SEURAT-1 initiative towards the replacement of repeated dose systemic toxicity testing in human safety assessment. Legislative pressure in the form of the cosmetics directive (Directive 76/768/EEC) and the scientific need for animal free models for risk assessment led to this jointly funded initiative by Cosmetics Europe and the European Commission.

There is a lack of validated alternative assessment methods for long-term systemic toxicity specifically for repeated exposures. Animal models do not adequately predict human toxicity and at the same time incur huge costs to the industry in the safety assessment of their products. It is expected that a combination of *in vitro* and *in silico* approaches would lead the way towards animal free human relevant and reliable safety predictions (Figure 1). *In vitro* test systems based on human cells could provide dose response data for the estimation of toxicity threshold. *In silico* tools and models taking into account population stratification could be used for the prediction of concentration-time profiles and the safe exposure limits. In addition, various omics data is set to

provide mechanistic details on the adverse effects for a better understanding of the pathways involved in the biological response.

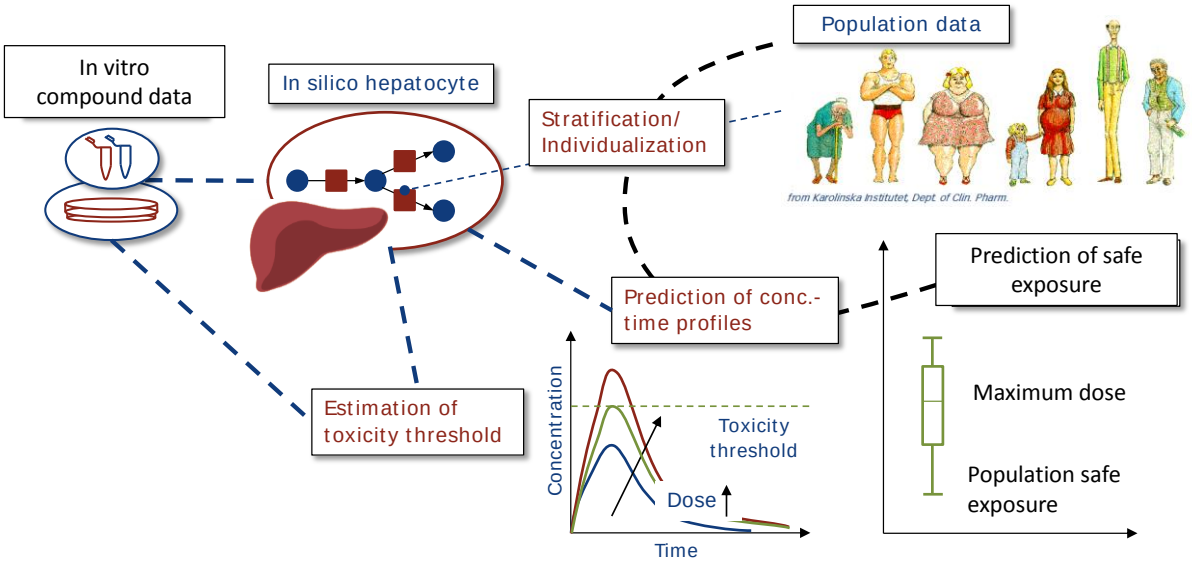

Figure 1. Replacement of cost- and time-intensive animal or preclinical studies by *in vitro* and computational approaches. Detailed *in silico* hepatocyte models which are validated by experiments permit the prediction of dose-dependent concentration-time profiles of compounds. The knowledge of appropriate toxicity thresholds and the implementation of population data into predictive models enable an individualized or stratified estimation of maximum serum concentrations of test compounds and their metabolites as well as an individualized risk assessment.

NOTOX used an integrated multifaceted experimental and computational platform using a systems biology approach for long term toxicity prediction on the molecular and cellular levels. The experimental work focused on the application of cellular systems that come closest to the human *in vivo* situation while at the same time allowing their transfer into applicable and easy to handle test systems. In these test systems viability and physiological toxicity response parameters (*omics*) were monitored together with epigenetic and structural characteristics in parallel. Large-scale network models of regulatory and metabolic pathways and cellular systems together with bioinformatics integration of human and across species literature data was used for toxicity prediction (Figure 2).

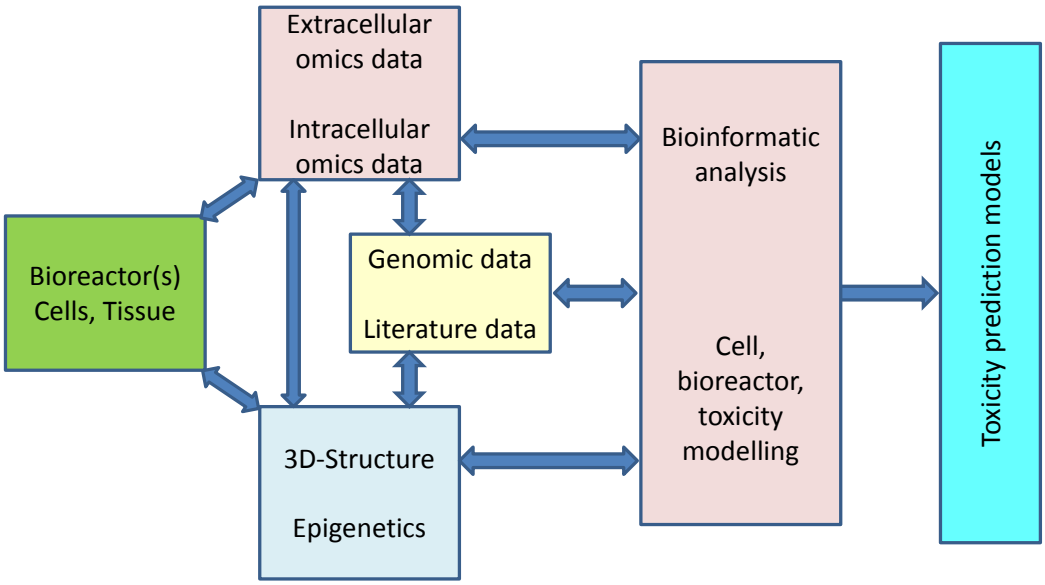

Figure 2. The NOTOX approach combining human based cell systems including 3D culture with computational modelling for the prediction of toxicity.

The major objectives of **NOTOX** given in the proposal were achieved as described below:

Supplying a versatile methodology for systems-based analysis and prediction of long-term toxicity of test compounds on organotypic 3D cultures

Since testing on the target organisms (humans) is hardly possible, human organotypic cultures were developed and applied to permit reproducible and transferrable testing of the highest possible relevance. The obtained experimental data was used to predict human long-term toxicity in a multi scale modelling approach. Since the liver plays a central role in metabolism, in both its inherent and xenobiotic conversion functions, we selected hepatic cultures for the NOTOX project. As human hepatic cells derived from stem cells are not yet readily available with sufficient functionality, we selected HepaRG, a hepatocarcinoma cell line, and primary human hepatocytes (PHH) for NOTOX. The HepaRG cell line has been shown to be closest to primary human cells in terms of the metabolism of xenobiotics, expressing important CYPs at high levels (Kanebratt and Andersson, 2008a; 2008b). At the beginning of the project the suitability of the HepaRG cell line **for toxicity assessment** was not known. NOTOX contributed significantly to introduce this cell line to the scientific community and to industry. For validation purposes and for the development of new techniques, we also use PHH.

The organotypic cultivation was extended not only to co-cultures models of hepatocytes (Leite et al., 2016) but also to heart model based on stem cell derived cardiomyocytes.

Development and application of experimental and computational methods for continuous, non-invasive and comprehensive physiological monitoring (respiration, metabolomics, fluxomics, proteomics and peptidomics, epigenomics, transcriptomics, viability and toxicity reporters, cellular toxicity models) of organotypic test systems upon exposure to selected test compounds

In NOTOX developed cell systems, viability and physiological toxicity-response parameters (*omics*) were monitored together with epigenetic and structural characterisation. Large-scale network models of regulatory and metabolic pathways and cellular systems, together with bioinformatics integration of human and across species literature data was used for prediction. The organotypic model systems are exposed to repeated low doses of selected test compounds over long timescales. The selected test compounds were of industrial relevance and have known mode-of-action (MoA) relevant to toxicity. These compounds were chosen from the gold compound list harmonized by ToxBank. The physiological effects of test compounds on the test systems were monitored by determining *omics* data (epigenomics, transcriptomics, proteomics, metabolomics, fluxomics) at various time points. The design of experiments incorporated toxicophysiolog**ical** data curated from literature and databanks as well as from ***in silico*** simulations.

NOTOX carried out two important case studies using acetaminophen and valproic acid as model compounds. Large scale *omics* data was derived and used to understand the pathways involved in the modes of action of these compounds. Mechanistic computational models were set up for prediction.

Development and application of experimental and computational methods for the comprehensive characterisation of 3D organotypic cultures after long-term repeated dose exposure to selected test compounds (individual epigenetic chromosomal profiling, 3D electron tomography, 3D-topographic analysis and modelling, bioinformatics characterisation)

3D spatial organisation of tissue structures, cell-cell contacts and intracellular structural features were characterised by 3D cryo-electron tomography and light/confocal microscopy. We also use a newly established multi-scale mathematical modelling approach, where toxic effects on 3D organotypic cultures, including tissue microarchitecture as well as tissue function, can be simulated in a dose-dependent manner.

Development of causal and predictive large-scale computer models based on the integration of the experimental data with available data (from various databases) and high-performance grid computing for identification of predictive endpoints

The effects of long-term exposure to test compounds were quantified and analysed using bioinformatics methods. Data from databases, literature, experiments and simulations was integrated through bioinformatics tools to create a knowledge base for quantitative understanding of adverse outcome pathways and regulatory networks at the molecular level (NOTOX Wiki). These data provide the bases for prediction models. Large-scale modelling of regulatory and metabolic pathways provided information on the key events in toxic responses. Since such large-scale computational systems biology models often comprise a large set of equations and may include millions of data points, strategies were developed using state-of-the-art computing for analysis and exploration of these models.

*Providing cheaper, more ethical, scientifically based testing strategies for repeated dose toxicity in order to meet the European legislative demands. For this purpose we illustrate how computer models calibrated with **in vitro** experiments could be used in combination with human parameters to predict the possible toxicity in humans*

NOTOX developed and established a spectrum of systems biology tools including experimental and computational methods for: i) organotypic human cell and tissue cultures suitable for long-term toxicity testing with focus on the mode-of-action (MoA); and ii) the identification and analysis of adverse outcome pathways (AOP). The overall goal was the prediction of long-term toxicity (repeated dose) on the basis of these models and well-designed experiments using an iterative systems approach. Furthermore, predictive endpoints for repeated dose toxicity are identified. NOTOX provided information on the pathways such as energy metabolism, amino acid metabolism, glucose and bile acids homeostasis which are involved in toxicity responses. This information can be used in broader context in testing strategies for repeated dose toxicity in various industrial sectors.

Equally important, NOTOX used reverse dosimetry to predict oral equivalent doses in human for bosentan and valproic acid from the long-term repeated exposure data. NOTOX shows how a combination of *in vitro* study and simple PBPK modelling could help in the assessment of safe dose in human.

DESCRIPTION OF MAIN S & T RESULTS/FOREGROUNDS

NOTOX used a systems biology approach based on data rich *omics* technologies and modelling. Traditional cell culture is usually carried out in the presence of foetal bovine serum which leaves a xenogenic signature in the various omics readouts. NOTOX developed serum free long term cultivation of its cell systems which are compatible with the systems toxicology methods (Klein et al., 2014). In long-term repeated-dose experiments the long term-effects of a series of compounds including valproic acid, bosentan and chlorpromazine were characterized. In parallel, NOTOX developed organotypic cultivation of its human cell systems in order to mimic as close as possible the *in vivo* situation. 3D HepaRG spheroid cultures were characterized in detail (Gunness et al., 2013). These 3D HepaRG cultures were used in acute but also repeated-dose toxicity studies (Mueller et al., 2014). HepaRG spheroid cultures were successfully used for the identification and study of compounds with cholestatic liability. 3D cultivation techniques developed with HepaRG spheroid cultures were further extended to primary human (PHH) as well as to co-cultures with non-parenchymal cells for fibrotic (Leite et al., 2016) and inflammatory responses. Additionally, 3D cardiomyocyte cultures derived from stem cells were also applied for long-term studies.

Applying new methods of two-photon microscopy, dynamics of transport of fluorescent compounds into hepatocytes and secretion into biliary structures *in vivo* and *in vitro* provided data for modelling of liver tissue. A simple work-flow in cryo-electron tomography (CET) and sub-volume averaging (SVA) was introduced that allows to obtain improved high-resolution, reliable results with little expert-supervision. This technology can be used as a tool to link cell biology to structural biology aiming towards a more complete understanding of physiological processes particularly related to the interaction of compounds with macromolecular and supramolecular structures in the cell.

Long-term toxicity was characterized with *omics* analyses. A major NOTOX case study focused on valproic acid long-term repeated-dose toxicity (14 days) in 2D in a joint large-scale experiment. A similar setup was used on a smaller scale with 3D HepaRG spheroids. Various omics methods were improved (e.g., epigenetics using ChiP-Seq, expression using Affymetrix GeneChip HTA, proteomics with improved sensitivity for spheroids). A reference proteome of HepaRG was created (4000 extracellular and 240 extracellular proteins) to support studies.

For simulations aiming at *in vitro* to *in vivo* extrapolation (IVIVE), models of different levels of complexity are developed and applied for long-term toxicity. In all cases, biokinetics were considered for modelling. A simple model based on PBPK principles was used to estimate oral equivalent dose (OED). Drug degradation and time-dependent response was measured *in vitro* and a virtual

population was applied to capture potential variability. Dynamic EC_{10} determinations over two weeks permitted safety predictions very close to *in vivo* data (Klein et al., 2015).

For both joint large scale case studies using acetaminophen and valproic acid, detailed mode of action based kinetic models were developed comprising gene expression, metabolism and transport and calibrated with the rich experimental *omics* data. The acetaminophen MOA model focuses on processes relate to oxidative stress, e.g. formation of radicals (NAPQI) and glutathione metabolism. Expression analyses of PHH in sandwich culture exposed to APAP showed time dependent exposure effects clearly distinguished from culture effects. The acetaminophen MOA model was incorporated into a liver lobule module resulting in a multi-scale model describing APAP damage, including apoptosis pathways, cell death, regeneration, flow and transport. The model of VPA metabolism includes glucuronidation, beta-oxidation and CYP oxidation. The VPA-MOA model couples kinetics in the signalling and gene regulation of fatty acid synthesis and metabolic enzymes. These models were incorporated into PBPK models and used for IVIVE. These support the identification and validation of key events in the adverse outcome pathways (AOP).

A whole toolbox for agent-based modelling of toxicological responses was developed: (i) TiQuant for image processing and analysis, (ii) TiSim for simulation of a liver lobule including cell death, regeneration, flow and transport and (iii) CellSys II for toxicity testing simulations. Models of spheroids were developed to describe *in vitro* experiments for the identification of relevant parameters for IVIVE using *in vivo* liver models. This model has been used to simulate APAP toxicity in 3D spheroids

Major results of the NOTOX project are listed below followed by a more detailed description:

1. Long-term *in vitro* cultivation of human cells for biological studies
2. 3D-hepatic model using HepaRG cells for acute and repeated dose toxicity assessment
3. A 3D *in vitro* HepaRG model for the identification and study of compounds with cholestatic liability
4. Functional imaging on hepatocyte *in vitro* systems for toxicological testing
5. Supramocular EM-imaging for compound interaction.
6. *In vitro* based prediction of human hepatotoxicity using expression analysis on human primary hepatocytes (PHH
7. Multi omics analyses for long term toxicity characterization (APAP, VPA)
8. Toxicity assessment by combining multi-*omics* analysis and database search
9. Quantitative proteomics for toxicity assessment in 2D and 3D cultures of hepatocytes

10. *In silico* modelling for the prediction of dose and pathway related adverse effects in humans from *in vitro* repeated-dose studies
11. Combined PBPK and metabolic models for toxicity prediction
12. Agent based modelling for *in vitro* to *in vivo* toxicity extrapolation

1. Long-term *in vitro* cultivation of human cells for biological studies

A system for long-term cultivation of liver cells has to maintain high liver specific functions as well as viability over time. In an effort to find ideal conditions for long-term repeated-dose exposure, serum-free conditions for long-term cultivation of HepaRG cells were established in 2D (Klein et al., 2014) as well as 3D (spheroids) environment. HepaRG cells were maintained for 30 days without the loss of viability and only minor loss of liver specific functions. Four different conditions without foetal bovine serum (FBS) were compared with standard long-term medium with FBS. SFM1, 2 and 3 were supplemented with growth factors (GF) making up for withdrawal of FBS. SFM4 was used for cultivation without FBS and GF. SFM1, 2 and 3 differed in respect to their DMSO concentrations (no DMSO, 0.5% DMSO and 1.8% DMSO respectively). Investigation of viability of 2D HepaRG cultures over time showed that HepaRG cells can be cultivated without addition of FBS for at least 30 days, while maintaining cells viable (SFM1 and SFM2).

At the same time, the cytochrome P450 (CYP) activity of HepaRG cells during long-term cultivation in different media was monitored. Results are depicted in Figure 3. While activities for CYP1A2, CYP2C9 and CYP2D6 only differ slightly between the tested conditions (SFM2 and serum supplemented medium (SSM)), we found significantly higher activities for CYP2B6 and CYP2D6 when cells were cultured in SSM with 1.8% DMSO. For both, SFM2 and SSM, CYP activities generally decreased from day 0 to day 30, however were still on a high level (around 65% on day 30 for both conditions relative to original activity on day 0). This finding is very important since CYPs are key enzymes for the metabolization of xenobiotics.

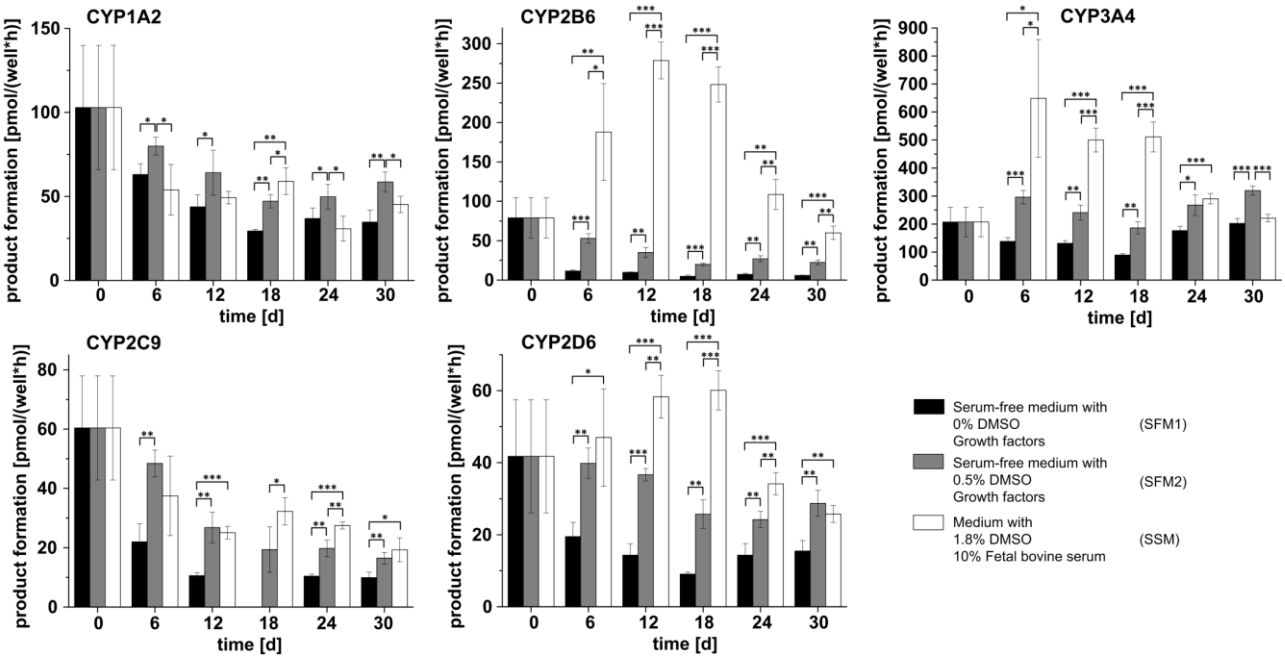

Figure 3. Activities of CYP1A2, CYP2B6, CYP3A4, CYP2C9 and CYP2D6 enzymes in HepaRG cells during long-term cultivation with daily medium renewal. Error bars indicate standard deviation (n = 3). *, **, *** indicate $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively (Klein et al., 2014).

Under these conditions, we analysed the extracellular metabolome (amino acids, glucose, pyruvate and lactate) of HepaRG cells. The uptake/production of various amino acids is depicted in Figure 4. The lactate/glucose ratios on days 6 and 30 were identical for SSM, SFM1 and SFM2.

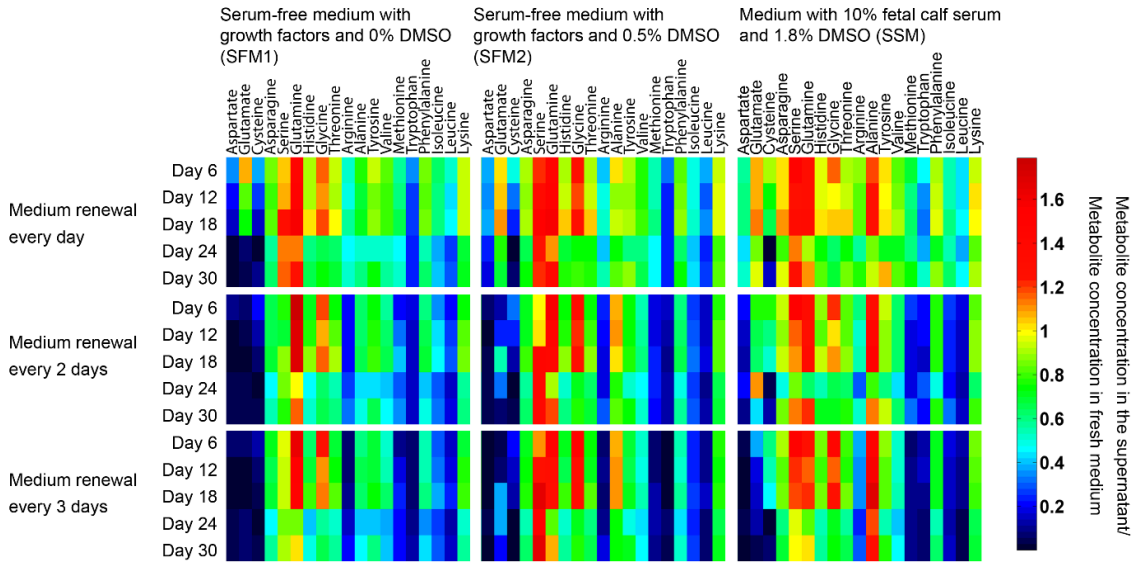

Figure 4. A heat map showing the ratio of amino acid concentrations in the supernatants upon medium renewal every day, second or third day versus amino acid concentrations in the fresh medium, given for each

investigated time point. An orange to red colour indicates production of amino acids, a yellow colour indicates that the amino acid was neither consumed nor produced. Green to blue colour indicates increasing consumption (Klein et al., 2013).

Moreover, metabolic flux analysis was applied to analyse metabolic changes over time and to investigate differences in metabolism between the different cultivation conditions. Metabolic flux analysis was shown to be a valuable tool for understanding the effects of experimental conditions and substrates as well as toxic compounds on cells. Several reaction and transportation rates are depicted in a flux distribution map (Figure 5). Reaction rates in the glycolytic pathway of HepaRG cells were significantly lower in serum-supplemented medium (SSM) as compared to serum-free medium (SFM). In accordance, lactate secretion was lower for cells maintained in SSM. Generally, glycolytic activity increased for all conditions over time. For cells cultivated in medium without DMSO (SFM1), approximately 40% of the glycolytic pyruvate was converted to lactate; for cells kept in 0.5% and 1.8% DMSO respectively (SFM2 and SSM), about 50% to 55% of glycolytic pyruvate was metabolized to lactate. Increased uptake of nutrients and consequential increased TCA cycle fluxes at later time points of cultivation for all conditions may be associated with increased energy demand for general maintenance, e.g. repair of DNA damages. An increased uptake of the branched-chain amino acids (BCAAs) isoleucine and leucine in SSM cultivation was observed. These amino acids are mostly metabolized to acetyl-CoA.

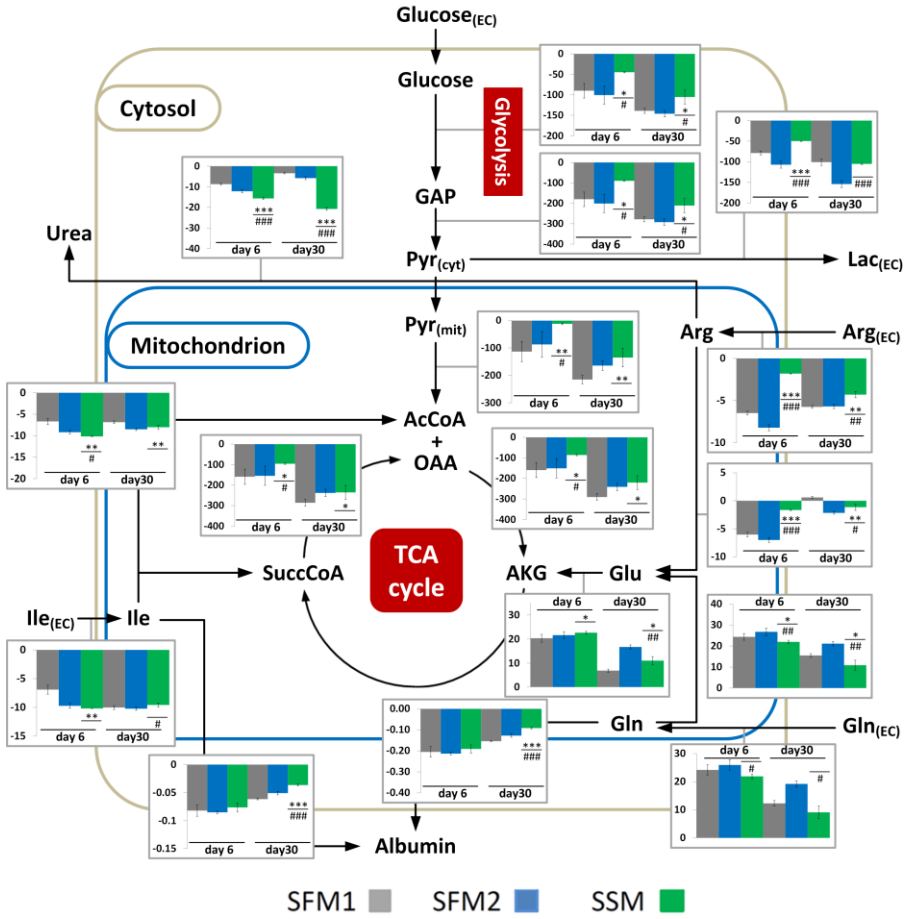

Figure 5. A flux distribution map of HepaRG cells upon long-term cultivation for days 6 and 30 for SFM1 / 2 (serum-free medium with growth factors and 0% or 0.5% DMSO, respectively) and SSM (serum-supplemented medium with 1.8% DMSO). Negative values indicate fluxes into the direction of the arrow and positive values in reversed direction. Error bars indicate standard deviations (n = 3). *, **, *** (comparison of SSM to SFM1) / #, ##, ### (comparison of SSM to SFM2) indicate significance at $p<0.05$, $p<0.01$ and $p<0.001$, respectively. GAP, glyceraldehyde 3-phosphate; Pyr, pyruvate; AcCoA, acetyl coenzyme A; OAA, oxaloacetate; AKG, α-ketoglutarate; SuccCoA, succinyl coenzyme A; Lac, lactate; Glu, glutamate; Gln, glutamine; Arg, arginine; Ile, isoleucine; cyt, cytosolic; mit, mitochondrial; EC, extracellular; TCA, tricarboxylic acid.

2. 3D-hepatic model using HepaRG cells for acute and repeated dose toxicity assessment

Due to lack of adequate *in vitro* models or methods of limited application, repeated-dose toxicity is still heavily dependent on animal studies in the assessment of long-term safety of compounds such as drugs and chemicals. This is mainly due to the fact that liver cultures rapidly lose their functions in 2D cultures and cannot be maintained viable and functional longer than a few days. Additionally, many transport reactions depend on cell-cell contants and transporter activities often not sufficiently present in 2D cultures. Therefore, NOTOX established 3D cultures of liver cells (HepG2, primary human hepatocytes and HepaRG cells) and maintained them in culture for several weeks. The viability was constant for 4 weeks and longer. These hepatic cultures were functional during the

tested period of 3 weeks as was shown by constant albumin production, transporter and CYP 450 activity.

The established system allows accurate adjustment of spheroid size, medium refreshment and spheroid harvesting and can therefore be used for the analysis of intra- and extracellular parameters. HepaRG cells formed compact spheroids between days 2 – 3 after initial seeding which did not grow in size since differentiated HepaRG cells do not further proliferate under used conditions (Figure 6). Cells containing up to 2000 cells per spheroid were found not exhibiting any visible transport limitations of e.g. oxygen, amino acids and other nutrients.

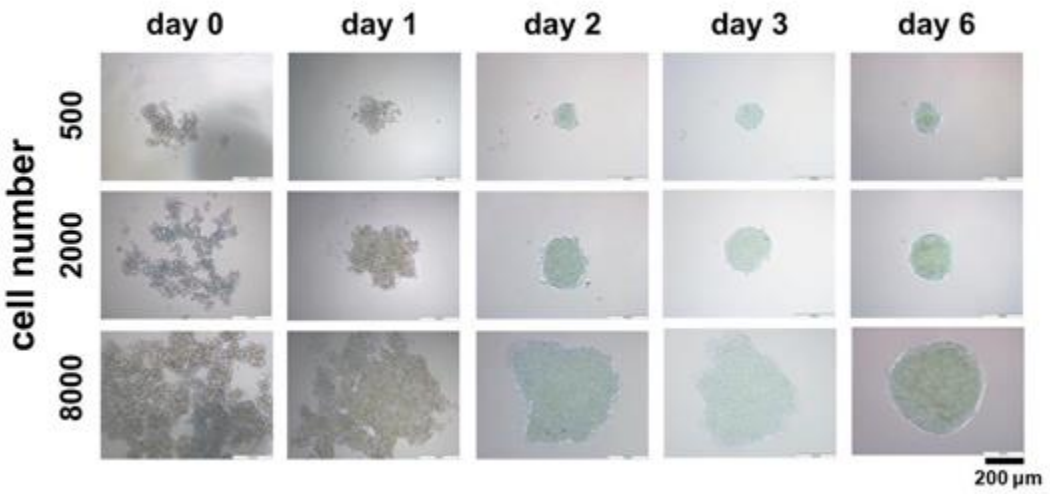

Figure 6. Formation of HepaRG spheroids (initial cell numbers 500 – 8000) during 6 days of cultivation (d0 = seeding). Scale bars = 200 µm.

Morphological analysis was performed using light microscopy and transmission electron microscopy. In Figure 7 electron microscopy pictures of HepaRG spheroid cross sections are depicted. Typical structures found *in vivo* were consistently found in 3D HepaRG spheroids (Figure 7A). This includes typical liver structures such as bile canaliculi and microvilli (Figure 7B &C) as well as transport vesicles found inside the bile canaliculi, indicating that HepaRG cells have an intact transport within these channels

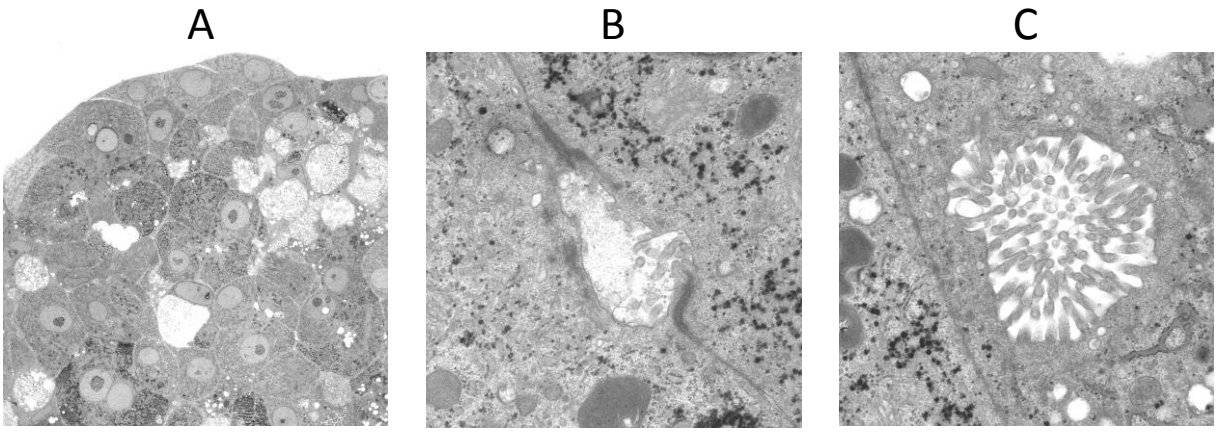

Figure 7. TEM picture showing a HepaRG spheroid. A) Healthy HepaRG cells are showing the same characteristics as hepatocytes in normal liver, such as the variable quantity of glycogen (the dark stained material). B) A bile canaliculus is formed as an extracellular space, limited by two plasma membranes, sealed by tight junctions. In the cytoplasm next to the canaliculus, small irregular tubulo-vesicular structures of the smooth endoplasmic reticulum (SER) are seen. Dark granules are glycogen particles. C) Bile canaliculi with microvilli.

The spheroid cultures were characterized for transporter activities and show e.g. functional MRP-2 transporters (Figure 8). In spheroids the CMFDA dye is exported into the bile canaliculi like structures as seen by the green spots.

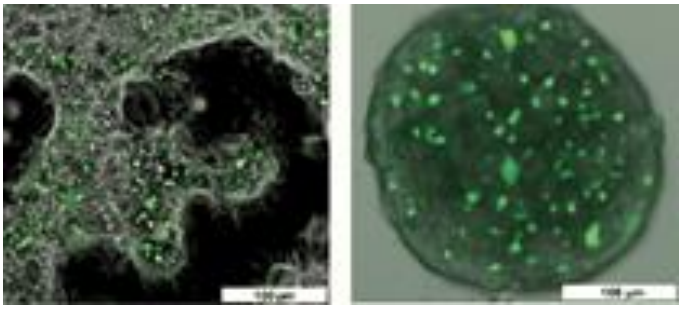

Figure 8. Multidrug resistance transporter (MRP2) activity in 2D (left) and 3D (right) HepaRG cultures. CMFDA was used as a substrate.

Several compounds were tested in repeated dose toxicity studies applying 3D HepaRG cultures (Gunness et al., 2013). Compared to the 2D cultures, the 3D cultures were significantly more sensitive to acetaminophen exposure (Figure 9 a & b). The results are supported by those from the CYP2E1 enzyme activity assay where consistently higher enzyme activity was observed in the 3D

versus the 2D cultures over the experimental period. The toxic metabolite of acetaminophen, N-acetyl-p-benzoquinone imine (NAPQI) is produced *via* metabolism by CYP2E1 (McGill et al., 2011).

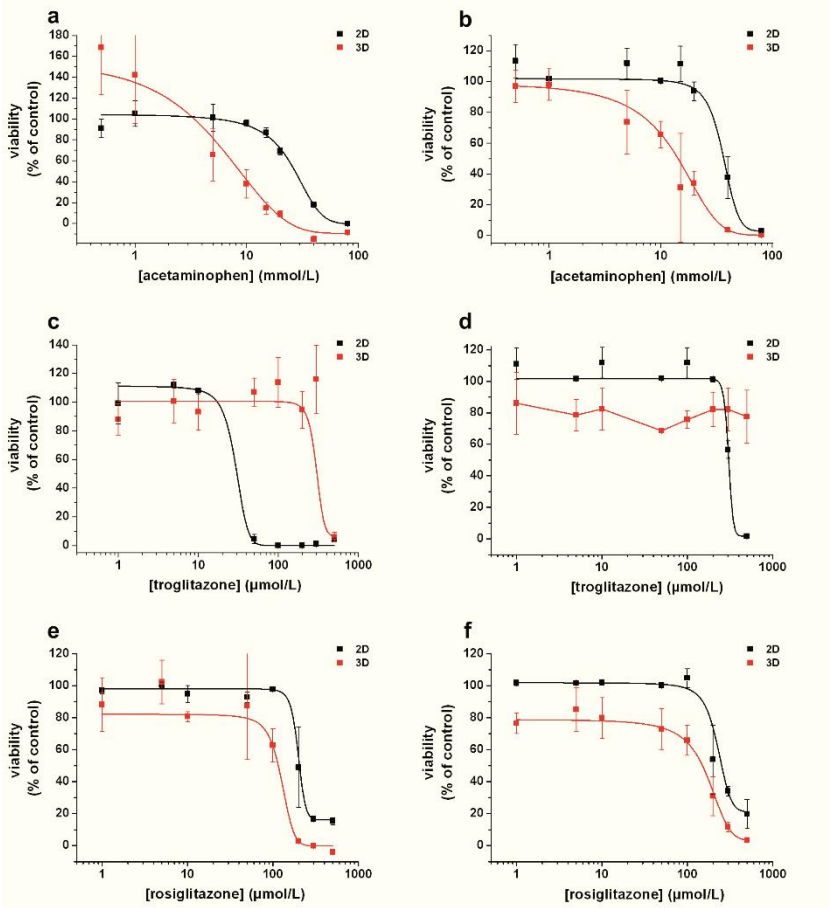

Figure 9. Acute toxicity in 2D and 3D HepaRG cultures. The 2D and 3D HepaRG cultures were exposed to acetaminophen (a & b), troglitazone (c & d) and rosiglitazone (e & f) for 24 hours on culture days 4 or 21, respectively. Cell viability was assessed by measuring the intracellular ATP content.

In contrast to the 2D cultures, troglitazone was not toxic to the 3D cultures at both assessment time points (Figure 9 c & d). The mechanism of troglitazone – induced hepatotoxicity is not fully understood and it is uncertain whether troglitazone causes direct or idiosyncratic toxicity (Isley, 2003). A plethora of mechanism has been suggested including the formation of reactive metabolites, mitochondrial damage, apoptosis, inhibition of the bile salt export pump (BSEP) and activation of inflammatory responses (Chojkier, 2005). The 3D cultures were more sensitive to rosiglitazone exposure at both exposure time points (Figure 9 e & f). Rosiglitazone is not hepatotoxic *in vivo*, however, rosiglitazone was found to be toxic *in vitro* in some donors of primary human hepatocytes (Lloyd et al., 2002).

In another study, 2D and 3D HepaRG cultures were tested upon repeated exposures. 3D cultures of HepaRG are more sensitive to toxic response upon long term repeated exposure as seen by the shift in the dose-response curve (Figure 10 b).

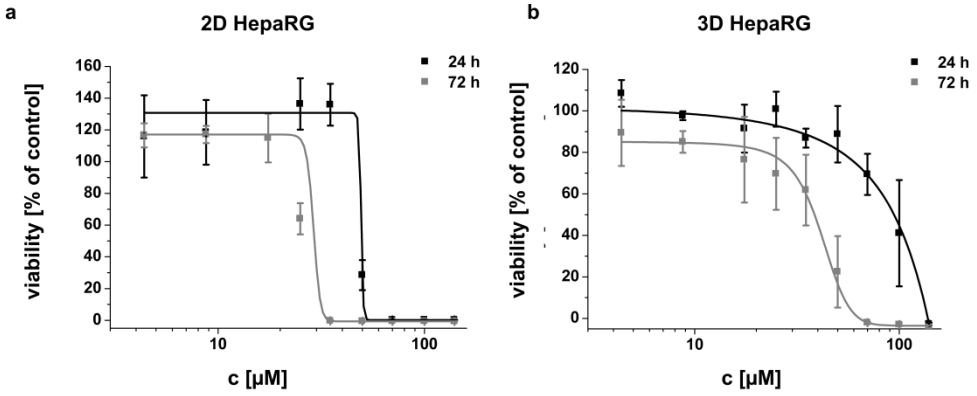

Figure 10. Repeated dose toxicity of chlorpromazine in 2D and 3D HepaRG cultures

Taken all together, the results suggest that the 3D HepaRG cultures are suitable for metabolism mediated toxicity and can be extensively applied since these are amenable to high throughput screening of compounds. In conclusion, 3D organotypic HepaRG cultures are a promising preclinical tool in the study of human relevant long-term repeated effects and in the assessment of chronic drug-induced hepatotoxicity.

3. A 3D *in vitro* HepaRG model for the identification and study of compounds with cholestatic liability

Drug-induced cholestasis (DIC) is one of the leading causes of drug-induced liver injury and often only manifests weeks/months after the start of drug treatment. Preclinical detection of DIC is still often limited to measuring the compound's potential to inhibit the bile salt export pump (BSEP). Yet, recent studies emphasize the importance to consider other mechanisms by which drugs can induce cholestasis and it is clear that there is a need for novel *in vitro* models which allow for a comprehensive analysis of the cholestatic risk of compounds. HepaRG cells and spheroids have previously been described as appropriate for long-term toxicity testing and here we show that the spheroids accurately express two main bile acid (BA) transporters, bile salt export pump (BSEP) and multidrug resistance-associated protein 2 (MRP2) at the site of the bile canaliculi (Figure 11A); this makes them suitable for long-term toxicity testing of cholestatic drugs. By co-exposing the HepaRG spheroids to a non-toxic dose of a bile acid mix and compounds known to cause cholestasis

(bosentan, chlorpromazine and troglitazone) we show that the bile acid co-exposure led to enhanced toxicity after 14 days of repeated dosing (Figure 11B). Importantly, this effect was not observed with the hepatocellular toxicant paracetamol or steatosis-inducing tetracycline (Figure 11B). Furthermore, in accordance with the definition of cholestasis, chlorpromazine induced accumulation of bile acids after 8 days of repeated dosing in the spheroids, is accompanied by decreased BSEP mRNA expression (Figure 11C). Finally, when further investigating the mechanism behind the synergistic toxicity between bile acids and chlorpromazine, we found a selective increase in oxidative stress dependent sulfiredoxin 1 (SRXN1) expression as well as an increase in death receptor 5 (DR5) RNA (Figure 11D). In summary the use of HepaRG spheroids allows for distinction of compounds with cholestatic liability, as well as in depth mechanistic studies of cholestatic liver injury.

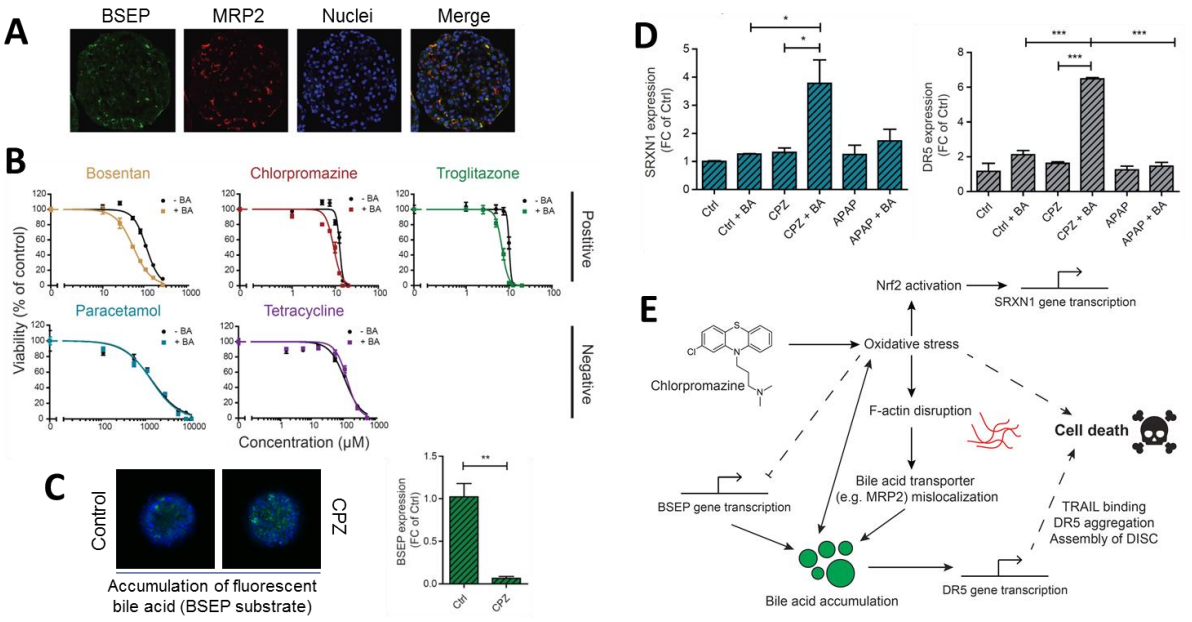

Figure 11. HepaRG spheroids as a model for the detection of compounds with cholestatic liability and for mechanistic studies. A – Immunofluorescent staining of fixated and cryosectioned HepaRG spheroids; B – The viability (ATP levels) after repeated dosing of compounds for 14 days with and without the presence of bile acids (BA); C – Live confocal imaging of fluorescent bile acid and RNA expression of BSEP in spheroids exposed to chlorpromazine (CPZ) for 8 days; D – CPZ+BA selectively induces the oxidative stress Nrf2 target gene SRXN1 as well as the death receptor 5 (DR5) RNA; E – Proposed mechanism of chlorpromazine induced bile acid accumulation (cholestasis) and their combined toxicity.

4. Functional imaging on hepatocyte *in vitro* systems for toxicological testing

The spheroid cultivation system displays a distinct cellular differentiation compared to classical hepatocytes monolayer cultures. The formation of bile canalicular structures at hepatocyte interfaces is a sign of marked differentiation (Figure 12). In collaboration with other projects an automated

quantification system for bile canalicular dynamics was developed. This system offers the possibility to systematically study disturbed bile canalicular dynamics which is a major reason for intrahepatic cholestasis (Reif et al., 2015).

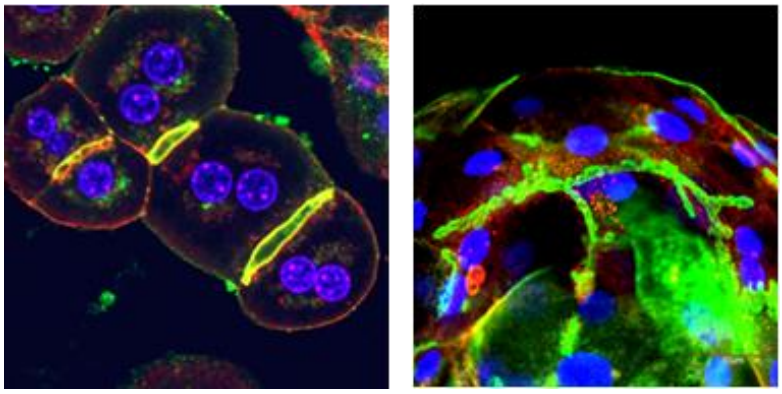

Figure 12. Immunostaining for bile canaliculi in sandwich culture (cells embedded in a collagen matrix) and spheroids: Green fluorescence shows the bile canalicular structures visualised with an antibody against the marker protein DPPIV. Both cultivation systems, left sandwich culture - right spheroid, display distinct cell polarity with bile canaliculi formed at the apical cell membrane. Nuclei in blue and plasma membrane in red.

The compound uptake efficiency in different hepatocyte cultivations systems was compared in order to estimate the applicability for compound testing in toxicological studies. For this purpose, passively diffusing Hoechst and actively taken up CMFDA were chosen as model compounds. Live cell imaging of these compounds into the spheroids revealed a distinct delay for the inner hepatocytes compared to the outer cell layer. The functionality of the formed bile canalicular system was shown by the biliary elimination of fluorescent metabolite of CMFDA (Figure 13).

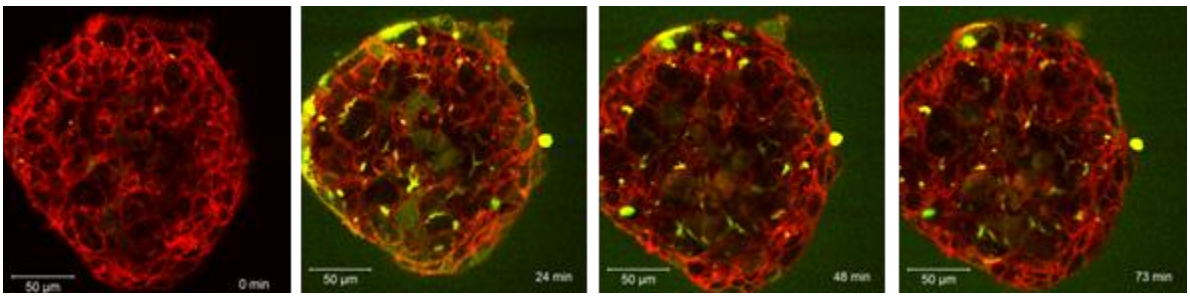

Figure 13. Uptake of CMFDA into spheroids: Spheroids of primary mouse hepatocytes (td-tomato) were fixed after four days in cultivation on a collagen layer and were exposed to 3.2 µM CMFDA (metabolite in green). Compound activation and elimination was measured in a time dependent manner using two-photon microscopy.

In order to compare the uptake efficiency between different culture systems, live cell imaging was also carried out for hepatocytes in sandwich cultures (Figure 14).

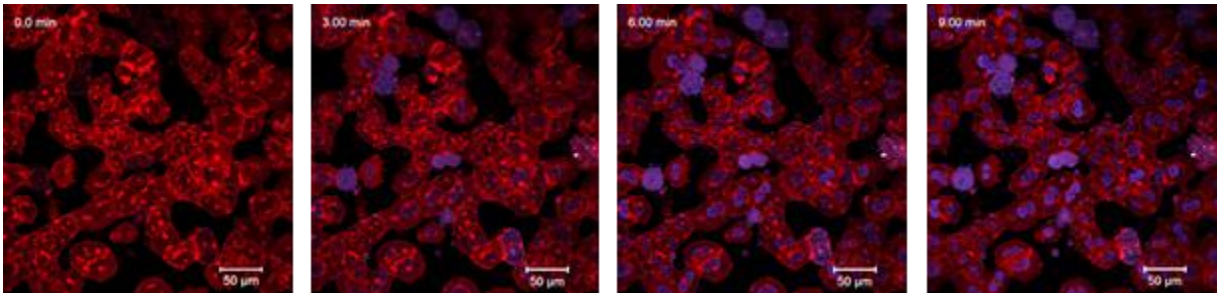

Figure 14. Passive diffusion of Hoechst in sandwich cultures: Primary mouse hepatocytes (td-tomato) were cultured for 4 d in collagen sandwiches and exposed to 0.05 mM Hoechst (nuclear marker). Compound uptake was measured in a time dependent manner using two-photon microscopy.

In sandwich cultures the uptake of the model compounds was fast and homogeneous at the tested concentrations. To compare uptake efficiency, kinetic parameters are determined for both cultivation systems using various concentrations of the fluorescent compounds. Finally these kinetic parameters are compared to the uptake measurements *in vivo*. This method contributes to transport related toxic effects in liver. In conclusion, both *in vitro* systems, hepatocyte sandwich cultures and spheroids can be used to study bile canalicular transport. CMFDA is taken up by hepatocytes, converted into the corresponding acid that exhibits green fluorescence and is transported to bile channels *via* the MRP2 transporter. This has a great potential for monitoring of cholestatic compounds.

5. Supramocular EM-imaging for compound interaction

New methods of high resolution EM tomography allows near *in vivo* adequate imaging of macromolecules and supramolecular complexes. To detect and interpret biologically and toxicologically relevant drug-induced macromolecular changes, high-resolution structures with sufficient details have to be extracted from the tomograms. One of the issues that limit obtaining such structures with resolution sufficient for detection of drug-induced changes is modulation of the measured projections by so-called contrast transfer function (CTF), which is caused by microscope aberrations and defocusing. CTF causes a periodic inversion of contrast in high frequencies of the measured projections, making their direct interpretation impossible. The correction of CTF is performed by first creating a mathematical model of the effective CTF, i.e. the CTF that has actually

distorted the acquired projections and then correcting the projections by de-convoluting them with this model CTF. To model the CTF, its key parameter - the microscope defocus for each projection - has to be estimated directly from those projections.

NOTOX developed a technique to estimate this defocus for each projection in the tilt series, drawing on *a priori* information about the series acquisition process and noise properties of the estimates. This estimation is incorporated into the whole pipeline as follows. Tilt-series is acquired and then aligned using the IMOD software package. A final result of this procedure is an electron scattering potential model of a representative of each structurally unique class. Figure 15 details the structure of these representatives. Panel A) in Figure 15 represents a 70S ribosome locked by EF-G, panel B) is a 70S without the EF-G, and panel C) highlights the EF-G alone. Finally panel D) shows that we were able to identify also standalone 50S subunits, which are expected to be in the sample, since not all of the ribosomes in a cell are fully assembled. What should be noted is that neither of those classes has been specifically searched for. They have been revealed automatically by the classification procedures. This shows the potential of the method to reveal unexpected structural events on the macromolecules upon exposure to a drug. It allows to semi-quantitatively characterizing the biochemical processes, such as effectiveness of the antibiotics binding, since we could count the total number of EF-G bound and EF-G free ribosomes, giving an approximation of the drug binding efficiency.

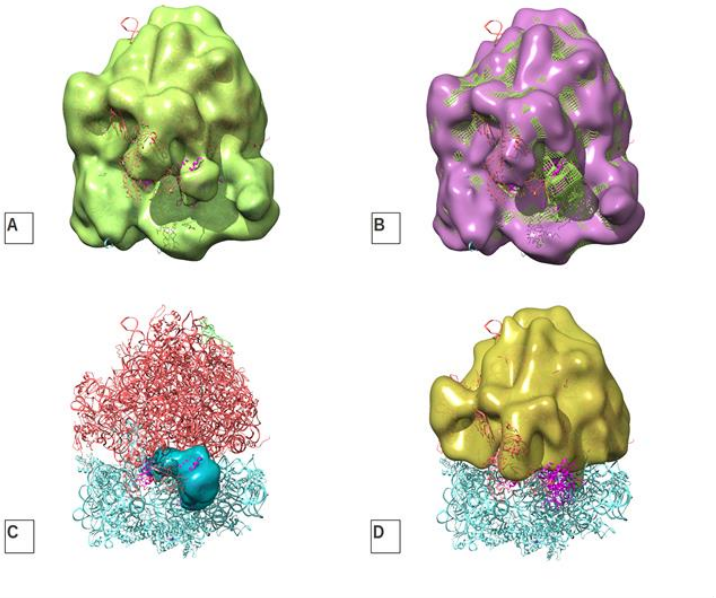

Figure 15. cryo-ET Structures of *ribosomes*. Structures of E. coli ribosomes with docked inside the X-ray structures of Thermus thermophilis 70S. In green is shown the EM-map of the 70S+EF-G (surface panel A and green mesh panel B), in magenta the density relative to the 70S without EF (surface panel B), where we have

coloured in yellow (panel D) the density of the 50S only. The small density in turquoise (panel C) corresponds to the EF-G only. The X-ray structures docked in the EM map shows ribbons of the 50S in green, the 30S in cyan, and the EF-G in pink. A) 70S+EF-G. Most of the density of the cryo-ET map, including the one relative to the EF-G, is occupied by the pdb model, as expected. B) Overlap of the 70S+EF-G and 70S only. The density relative to the EF-G is present only in the map 70S+EF-G (mesh). C) Subtraction, made with Chimera in between the 70S+EF-G (green map) and 70Sonly (magenta map). D) Map of the 50S only.

6. In vitro based prediction of human hepatotoxicity using transcriptomic data of human hepatocytes

In vitro based prediction of hepatotoxicity is challenging, because it requires an *in vitro* system, which reflects critical mechanisms of *in vivo* toxicity. In this project we evaluated transcriptomic readouts to identify predictive biomarkers and established an *in vitro* based model to predict human hepatotoxic blood concentrations. We used publically available, genome wide expression data from 150 compounds tested in primary human hepatocytes (PHH) at a slightly cytotoxic concentration. The following strategy was applied to identify potential biomarker candidate genes: (i) Identification of genes that are altered by many compounds. (ii) Identification of genes, which are as well altered in human liver diseases such as cirrhosis, hepatocellular carcinoma and non-alcoholic steatohepatitis. (iii) Exclusion of unstable baseline genes, which are altered just by the hepatocyte isolation and cultivation procedure. (iv) Selection of genes belonging to various biological motifs to cover the most relevant toxic mechanisms. From the top genes with the highest fold changes among all compounds, 7 genes were selected as biomarkers: CYP 1B1, CYP 3A7, SULT 1C2, G6PD, TUBB2B, RGCC and FBXO32 (Table 1). These genes cover the biological motifs metabolism of xenobiotics, energy and lipid metabolism, cell cycle and cytoskeleton as well as protein degradation.

Table 1. Identified biomarkers for further evaluation

Symbol	Liver disease	SV Up (FC3)	Gene Function	Category
CYP1B1	UP	18	metabolic enzyme in the ER (phase I enzyme)	Metabolism xenobiotics
CYP3A7	UP	39		
SULT1C2	UP	22	cytosolic enzye; catalyzes sulfonation (phase II enzyme)	
G6PD	UP	7	Enzyme in pentose phosphate pathway → fatty acid synthesis	Energy and lipid metabolism
TUBB2B	UP	5	major constituent of microtubules; functions in mitosis and intracellular transport	Cytoskeleton Cell cycle
RGCC	UP	25	cytosolic protein; induced by p53 modulates the activity o fcell cycle specific kinase in response to DNA damage	
FBXO32	UP	29	cytosolic protein; ubiquitination and proteasomal degradation	Protein degradation

A set of hepatotoxic as well as non-hepatotoxic compounds was defined and literature search was performed to identify plasma peak concentrations at therapeutic doses. HepG2 cells as well as PHH were exposed for 24 h and each compound was tested in a concentration range covering the plasma peak concentration but also ranging up to slightly cytotoxic concentrations. Two readouts were used to evaluate the hepatotoxic potential of the compounds: (i) the expression of the selected biomarker genes was analysed. The *in vitro* alert concentration was defined as the lowest concentration that causes a significant increase of at least 2.5 fold induction of at least one biomarker. (ii) Cytotoxicity tests were performed to identify the lowest cytotoxic concentration, corresponding to 20% loss of viability. Both readouts were considered to identify the lowest observed effect concentration *in vitro*, which was finally compared to the plasma peak concentration of a therapeutic dose *in vivo*. Already in HepG2 cells, the prediction model separates hepatotoxic from non-hepatotoxic compounds. The majority of hepatotoxic compounds show alerts at concentrations *in vitro* which correspond to therapeutic doses *in vivo* (Figure 16). In PHH, the prediction sensitivity improves and hepatotoxic effects are observed at even lower concentrations. Preliminary results indicate that both systems are suitable to predict human hepatotoxic blood concentrations, at least within a certain error range.

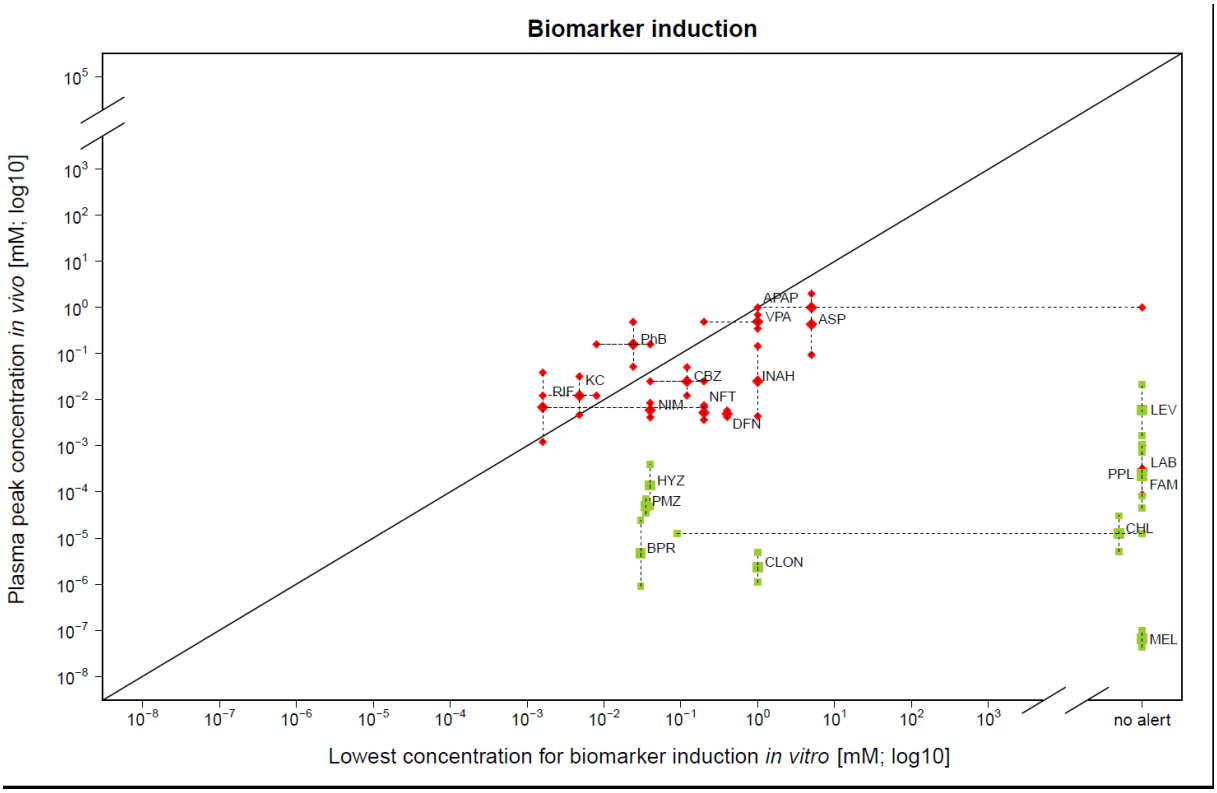

Figure 16. Prediction of hepatotoxic blood concentrations in primary human hepatocytes. The plot shows the lowest observed effect concentration vs the plasma peak concentration in humans of 9 different non-hepatotoxic (Buspirone, Famotidine, Propranolol, Hydroxyzine, Promethazine, Chlorpheniramine, Clonidin, Melatonin, Levofloxacine - green) and 12 hepatotoxic (Rifampicin, Acetamiophen, Carbamazepine, Diclofenac, Ketoconazole, Valproic acid, Nitrofurantoin, Nimesulide, Phenylbutazone, Labetalol, Isoniazide, Aspirin – red) compounds. The closer the *in vitro* determined concentration to the diagonal is, the more accurate the hepatotoxicity prediction was.

7. Multi-*omics* analyses for long term toxicity characterization

In an early step towards ***omics* analyses on NOTOX cell systems**, the expression profiles in different cell types, namely HepG2, HepaRG and PHH were compared (Figure 17). Clusters of coherent gene expression kinetics were identified and the potential regulatory circuitry underlying them was studied. This coarse-grained description of the transcriptional network in HepaRG serves as the basis for characterising more subtle responses during long-terms exposure and low dose experiments.

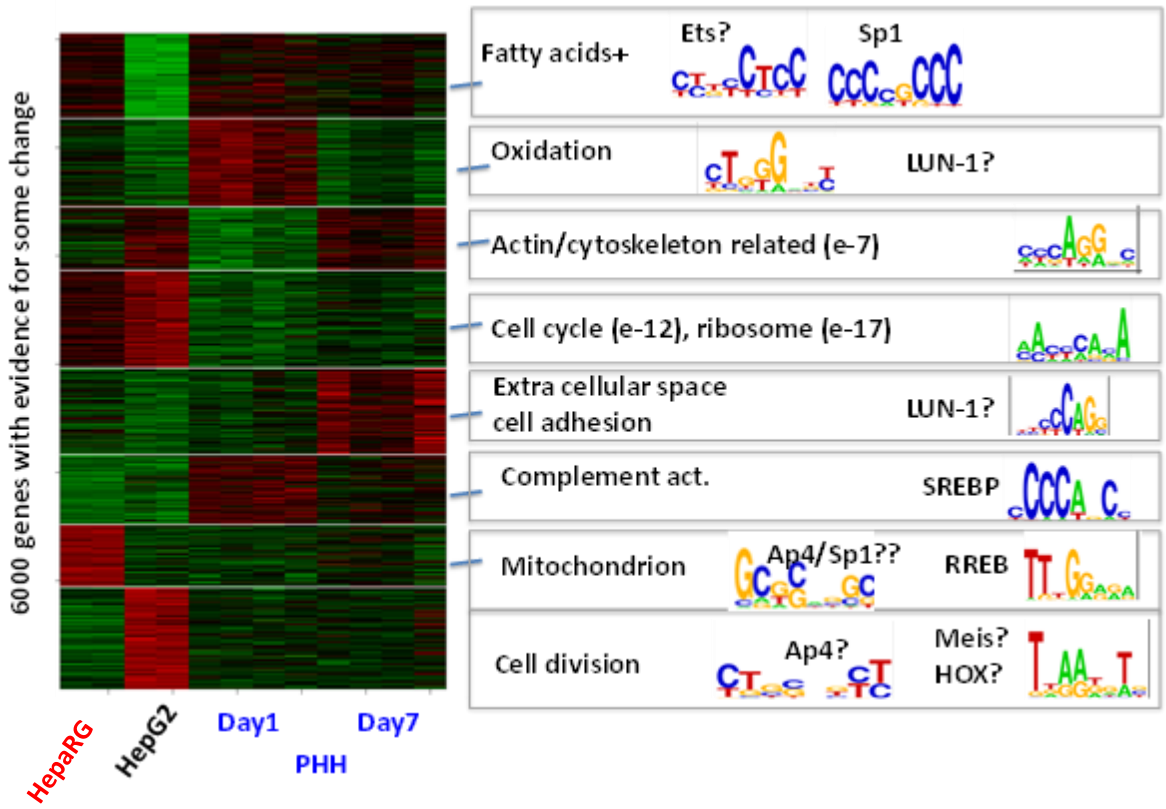

Figure 17. Gene expression of HepaRG, HepG2 and exposed PHH cells was clustered using a variety of methods (K-means is shown here), as depicted by the heat map on the left (green – low, red – high). Encode-derived enhancer and promoter functional sequences were then associated with genes according to spatial proximity, generating a set of potentially regulatory sequences associated with each responding gene. Functional enrichment using GO annotation was associating putative function with the gene in each cluster (Fisher exact p-value is shown). De-novo motif finding was performed using the inferno algorithm developed at 'Weizmann Institute', which is capable of fully controlling for background distribution heterogeneity in enhancer sequences. PWM logos of identified motifs are shown.

The multi-*omics* studies were extended in a joint experiment for the assessment of **long term effects of valproic acid (VPA)**. VPA is among the gold compounds of the TOXBANK and is used in the treatment of several diseases like epilepsy, bipolar disorders and migraine headaches. Molecular mechanisms of VPA effects, however, are still poorly understood. Within NOTOX effects of VPA were studied using HepaRG cells in 2D and 3D cultures. Viability of HepaRG cells during two-week VPA treatment decreased in a dose- and time dependent manner. Treatment with concentrations below the c_{max} (0.29 mM), resulted in a decreased viability after 8 and 14 days.

Treatment of HepaRG cells with VPA resulted in decreased uptake of glucose, followed by a decrease in secretion of lactate (Figure 18). Likewise, rates in the tricarboxylic acid (TCA) cycle and those of branched-chain amino acid (BCAA) degradation decreased. These observations are consistent with the literature *in vivo* effects of VPA.

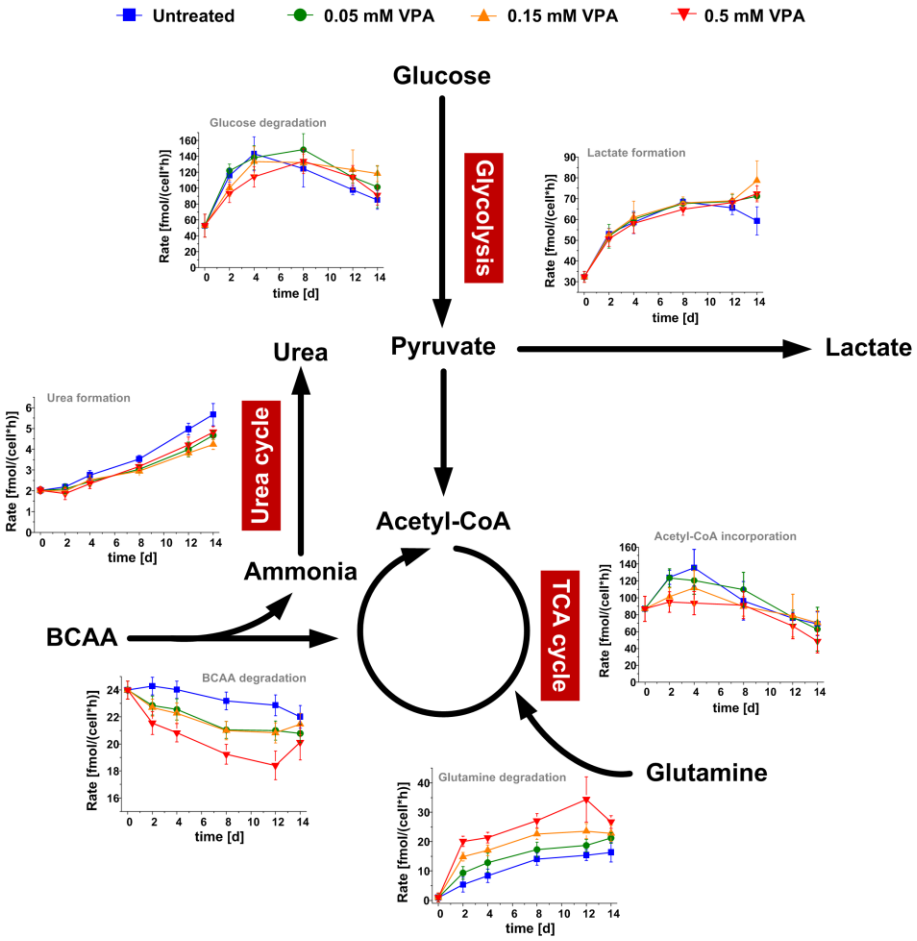

Figure 18. Selected conversion rates (metabolic fluxes) in the central carbon metabolism of HepaRG cells upon repeated dose treatment with VPA. A reaction occurring in the direction of the arrow is indicated by a positive sign. Error bars indicate standard deviation (n=3). BCAA = branched-chain amino acids.

Observed changes in the glucose metabolism and urea production may have a pronounced impact in susceptible patients such as those with compromised liver function and urea cycle deficiency leading to idiosyncratic toxicity. The combination of modelling based on long-term *in vitro* repeated-dose data and metabolic changes allows the prediction of human relevant *in vivo* toxicity with mechanistic insights.

On the gene expression level, approximately 300 genes were found to be up- or down-regulated more than 1.5 folds (Figure 19). Based on a cluster analysis, the genes can be divided into 4 different groups. In one of the groups of genes in Figure 19, one can observe a considerable induction as a late response after 14 days of VPA treatment. Among these genes we identified are several stress response genes, e.g. FOS, JUN, ATF3, which indicate a more severe toxic response after long-term VPA treatment of the HepaRG cells.

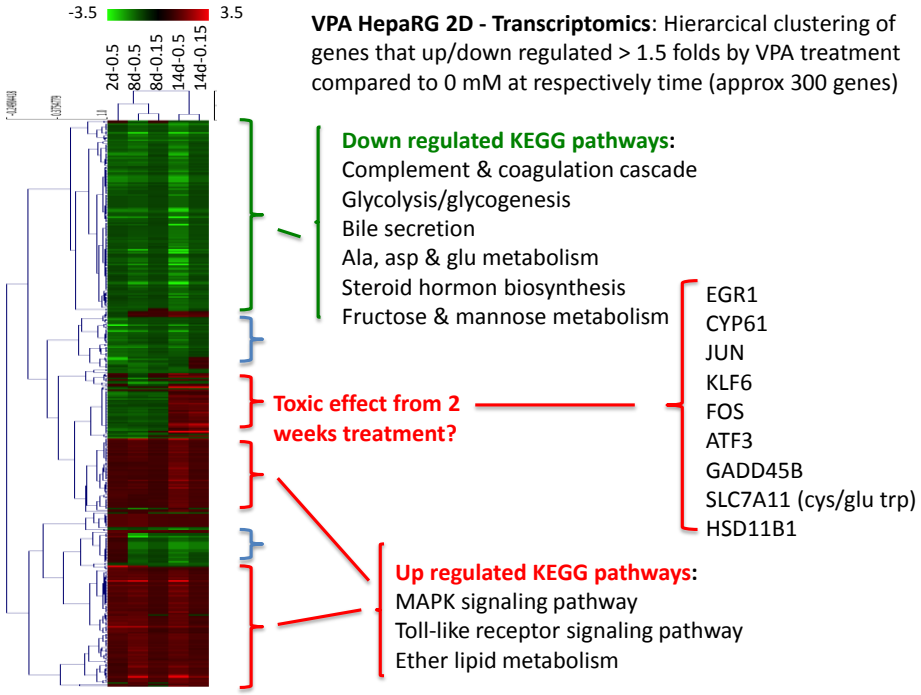

Figure 19. Hierarchical clustering of FC at each time points compared to the corresponding control.

Among the down-regulated genes the bile secretion pathway can be exemplified with the BSEP transporter, which is down regulated already after 2 days treatment with 0.15 mM VPA *(*Figure 20 left).

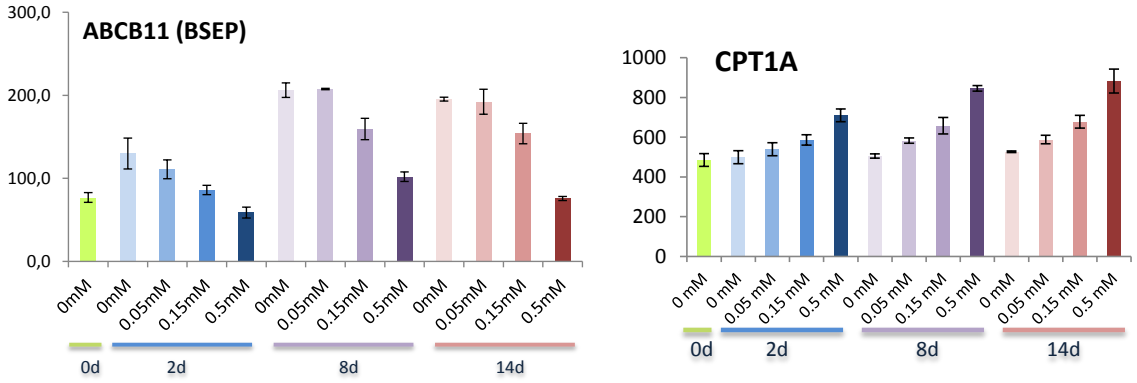

Figure 20. Signal intensity of gene expression analysis of BSEP mRNA (left) carnitine palmitoyl transferase 1 mRNA (right).

VPA enters the liver mitochondria with the help of carnitine and carnitine palmitoyltransferase I (CPT1A). Long-term treatment with VPA will deplete carnitine and inhibit the CPT1A enzyme, resulting in a compensatory up-regulation of the CPT1A gene expression (Figure 20 right).

On the proteomics level, using a label-free proteomics approach including manual validation of more than 4000 peptide signals, 1300 proteins could be quantified across all groups analysed with an intragroup coefficient of variation of less than 20%. Abundance of around 200 proteins changed significantly between all samples in a dose- and time-dependent manner, i.e. the longer and the more VPA was applied, the more proteins turned out to be significantly altered. Pathway analysis of the differential proteins indicated that especially lipid metabolism was affected by VPA-treatment, which is in accordance with the expected steatotic effects of VPA. Furthermore, the effects on the proteome were visible already when commonly applied endpoint assays like e.g. cell viability or ATP content did not yet indicate toxicity.

VPA is known to induce epigenetic changes. Therefore, genome-wide DNA methylation (Illumina 450K BeadChip array) and histone modification analysis (ChIP-seq) at different time points were applied. Epigenetic data were also correlated with global gene expression data (Affymetrix). As VPA is known to inhibit histone deacetylases, HepaRG cells cultivated for 14 and 21 days were analysed for the histone modifications H3K9ac and H3K4me3 using ChIP-seq. Preliminary bioinformatic analysis showed an overall decrease of normalised H3K9ac peaks during cultivation (Figure 21A), while H3K4me3 appears to be more stable (Figure 21B). This points to VPA blocking partially the loss of H3K9ac. Interestingly, also new H3K9ac and H3K4me3 peaks (Figure 21A and B) were detected at d14 and d21, among them sites that coincide with changes in gene expression.

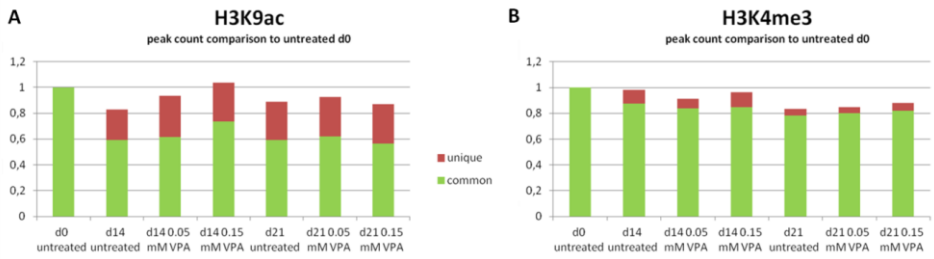

Figure 21. Effects of long-term VPA treatment on histone modifications H3K4me3 and H3K9ac in HepaRG. A: Normalised H3K9ac peak comparison to d0 untreated control (peaks that occur in d0 and remain in all samples analysed are displayed in green, sample-specific peaks in red); B: Normalised H3K4me3 peak comparison to d0 untreated control.

In brief, clear dose and time dependent trends were observed at all *omics* levels. These omics endpoints are now assessed in detail to develop a pathway of VPA effects. An overarching analysis of all the *omics* is underway.

8. Toxicity assessment by combining multi-*omics* analysis and database search

In order to develop computational models of biological pathways for long term toxicity assessment, comprehensive NOTOX Knowledge Base - a database for long term toxicity data, was established, to enable experimental partners obtain information on all selected gold compounds together with their therapeutically relevant targets (proteins/genes), for each compound, a list of all metabolizing enzymes, nuclear hormone receptors, other affected proteins and deregulated genes in the context of long term toxicity and all the literature references for each relation in the knowledge base. We performed systematic knowledge extraction (text mining and expert curation of the relevant scientific literature and publicly available knowledge bases, Figure 22A), in order to populate the NOTOX database and this prior knowledge (the information published in the scientific literature from its beginnings until now), specifically knowledge of protein targets of compounds known previously to act on them in the context of long term toxicity, contributes greatly to experimental studies within the NOTOX consortium and the analysis of *omics*- data. To be able to analyse and visualize imported *omics* data in context of their mode of action on the molecular level, we developed a module for importing different *omics* data (proteins and genes). Visualisation of pathways provides relevant information about molecular interactions, including up- and down regulated genes' effects and allows to make biologically informed decisions on further actions. Moreover, by using pre-processed experimental data from partners, we designed and completed the module for analysing *omics* data in context of most strongly indicated toxicological pathways related to exposure to test compounds and associated with genes/proteins from the *omics* data (Figure 22B). Toxicity assessment of compounds used for NOTOX pilot studies (acetaminophen and valproic acid) considers curated existing knowledge, experimental *omics* data and data derived from bioinformatics analysis in order to enable super-imposition of characterized *omics* data onto maps of affected biological pathways, in order to create hypothesis-based models of mechanism of action.

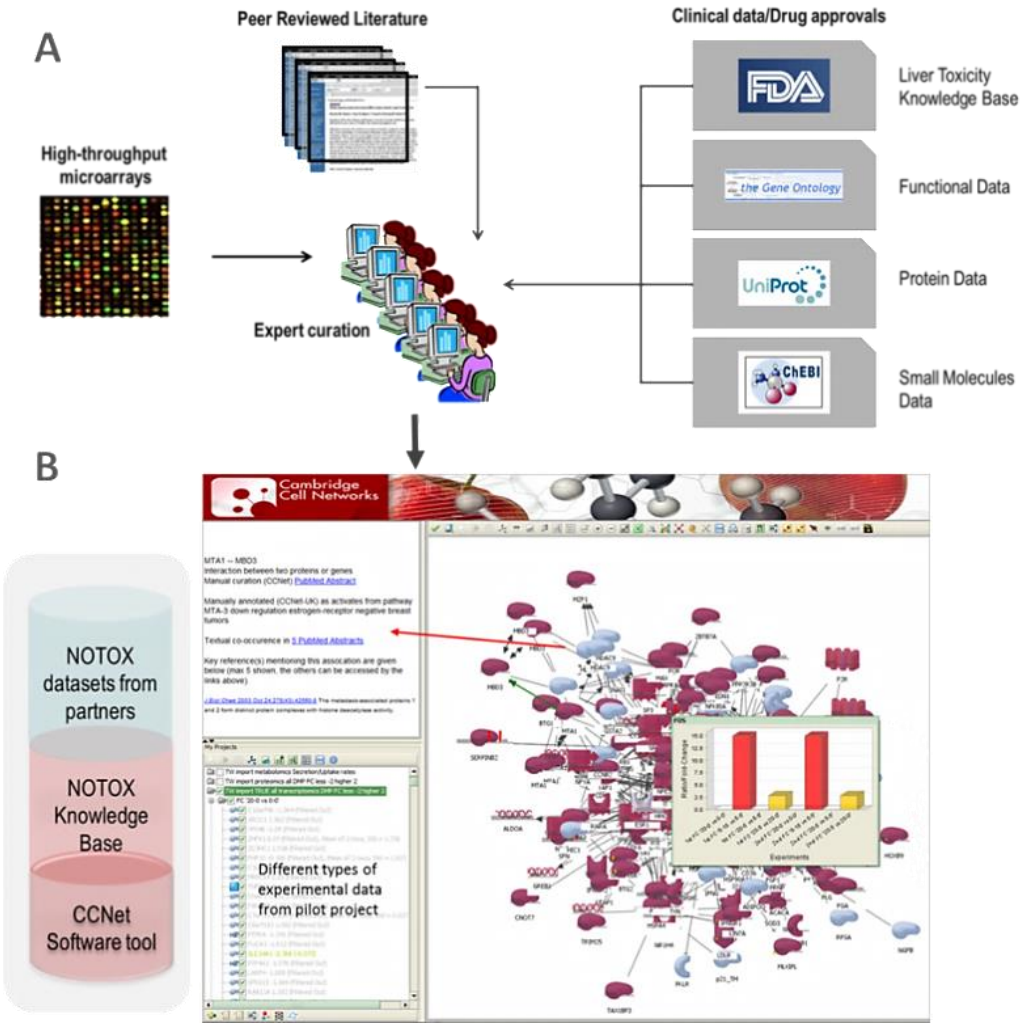

Figure 22. The manually curated database (Liver Toxicity). The database was developed by manual annotation of 30 000 scientific publications related to drug-induced hepatotoxicity. It contains 5000 compounds, nearly 10000 individual expression values and more than 2500 articles (A) as well as proteomics, transcriptomics and metabolomics data from NOTOX partners (CNRS, KI, USAAR-EH). These data are accessible for analysis and visualization *via* project workspace in NOTOX Knowledge Base.

9. Quantitative proteomics for toxicity assessment in 2D and 3D cultures of hepatocytes

The emerging field of toxicoproteomics has been boosted by recent advances in proteomic technologies and its increasing applications in toxicology testing/research. In the context of toxicants, studying cellular protein dynamics on a global scale is indispensable for identifying molecular initiating events as well as adverse outcome pathways, especially in connection with other systems biology approaches like transcriptomics and metabolomics. Therefore, in an attempt to develop *in vitro* toxicological approaches, *in vitro* screening tests for toxicity evaluation nowadays should take advantage of the wide diversity of proteomic platforms and *omics* approaches in general. NOTOX developed quantitative proteomics workflows for investigating both intracellular and extracellular proteomes of hepatocytes in both holistic and targeted approaches as well as bioinformatic methods

for *omics* data integration. The developed analytical methods provide good coverage of the different cellular compartments including the extracellular space (up to 4000 proteins analysed; Figure 23), bearing in mind that secreted proteins are a valuable source for biomarkers of drug-induced toxicity. Moreover, the methods allowed accurate and reproducible relative quantification of more than 1200 intracellular and 200 extracellular proteins in large-scale toxicological studies comprised of up to 13 groups including time series and different doses, a common experimental setup in toxicity studies. Using the quantitative data we differentiated treatment- as well as cultivation-dependent changes in the intracellular proteome upon treatment with acetaminophen and valproic acid (Figure 23 B-C). Proteomics results were integrated with other *omics* data using in-house developed bioinformatics tools (Carapito et al., 2014), as exemplified in Figure 23D.

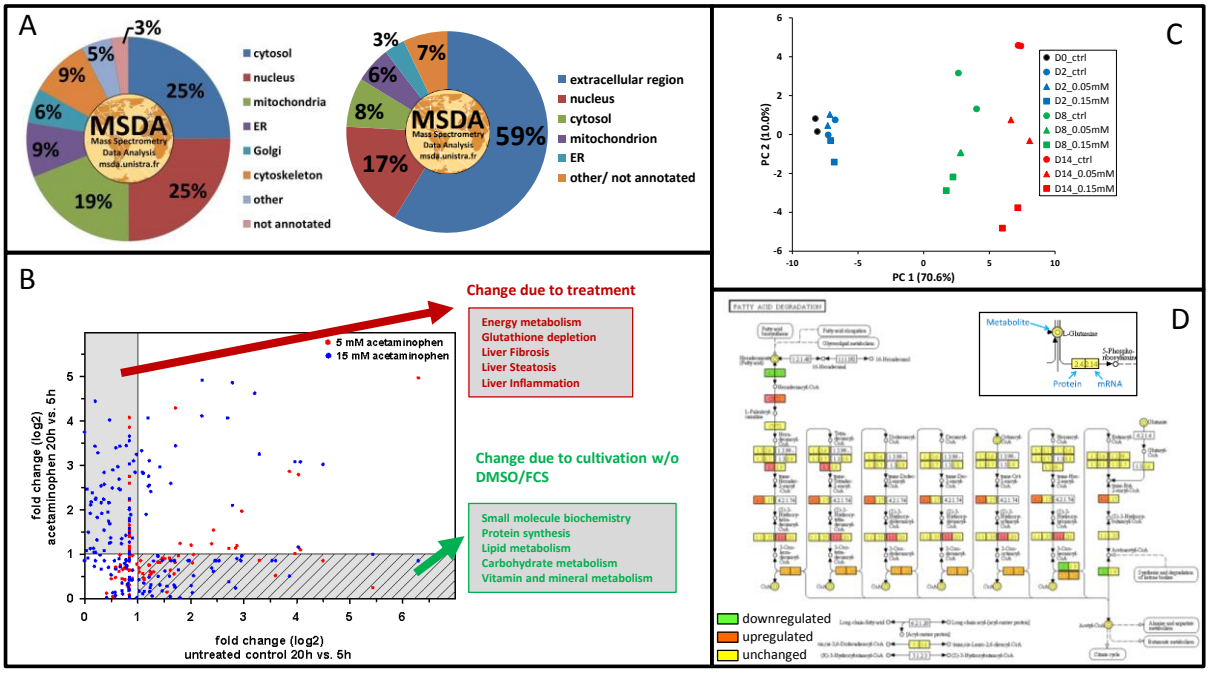

Figure 23. Global toxicoproteomics within NOTOX. Subcellular locations of identified intracellular (A, left) and extracellular/secreted (A, right) proteins were determined, as well as changes in protein abundances upon treatment with acetaminophen (B) and valproic acid (C). Simultaneous visualisation of significantly changing transcripts, proteins and metabolites in HepaRG cells data was created using in-house developed bioinformatics tools (msda.unistra.fr), as exemplified here for long-term effects of valproic acid exposure on fatty acid degradation (D).

10. In silico modelling for the prediction of dose and pathway related adverse effects in humans from *in vitro* repeated-dose studies

For the reduction and ultimately replacement of animal studies in preclinical long-term toxicity assessment, a combined strategy of using advanced *in vitro* cell culture methods based on functional

human cell cultures and computational modelling is expected to play an essential role. HepaRG cultures remain viable and functional for long periods and have been applied in toxicity studies. NOTOX established conditions for HepaRG cells in which they retain viability, transporter and metabolic activity. These cultures were used to assess long term toxicity of valproic acid and bosentan over a period of 28 days. *In vitro* biokinetics were assessed for the consideration of plastic binding and compound degradation (Figure 24A). The long-term dose response data was used to calculate oral equivalent doses for both valproic acid (Figure 24C) and bosentan. Using a simple PBPK model (Figure 24B) with a virtual population of 100, *in vitro* to *in vivo* extrapolation (IVIVE) is possible as shown for valproic acid (Figure 24D). The model predicts that valproic acid is hepatotoxic in 4 and 47% of the virtual population at the maximum daily recommended dose after 3 and 4 weeks of exposure, respectively (Klein et al., 2015).

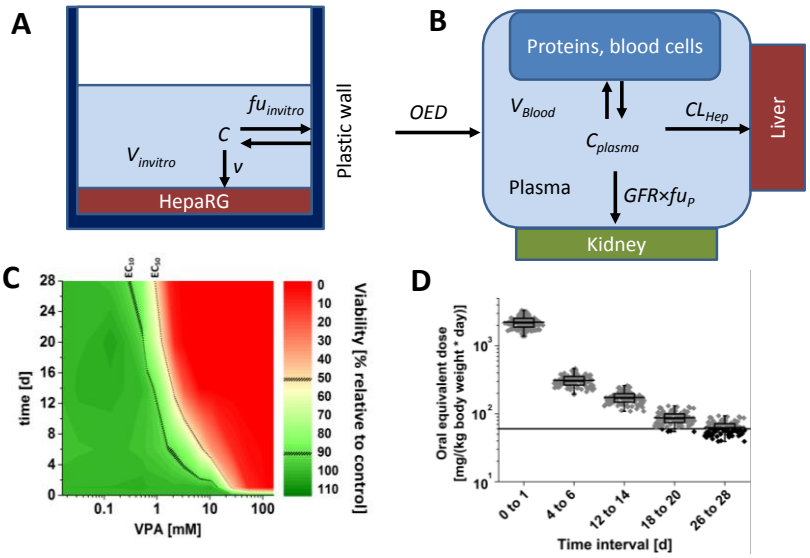

Figure 24. Estimation of oral equivalent doses (OEDs) from *in vitro* data using a simple physiological model. A – model scheme for *in vitro*; B – human physiological model; C - Viability data from 2D *in vitro* HepaRG culture during VPA exposure; D – estimated OEDs using a population 100 individuals.

11. Combined PBPK and metabolic models for toxicity prediction

Cellular metabolic and toxicity-related mechanism of action models coupled to pharmacokinetic or physiologically-based pharmacokinetic (PBPK) models provide insight into the relation of route and dose of administration to local effective concentrations. NOTOX provides a multi-scale whole body model describing the molecular toxic mechanisms of valproic acid (VPA). For this purpose, a PBPK model of valproic acid (Figure 25A) is combined with a model of the hepatic VPA metabolism (Figure 25B), based on a set of coupled ordinary differential equations, as well as toxic mechanisms/mode of

action (MOA) steps which attenuate cell viability due to toxic metabolites and disturbance of lipid metabolism. The coupled PBPK-VPA-MOA-model was pre-set with *a priori* parameters, to fulfil following demands: (i) The coupled PBPK-VPA-MOA-model was adapted to reflect *in vivo* plasma concentration profiles (Figure 25C). (ii) Further, the PBPK-VPA-MOA-model was enabled to reflect potential VPA-triggered dynamic response in key players in fatty acid synthesis and oxidation (Figure 25D). The hepatic VPA-model was further identified with experimental metabolite, transcript and protein data from the VPA experiment on HepaRG culture. The combined modelling approach introduced supports the dose-dependent simulation of cellular VPA dynamics and toxic effects and can be adapted and extended to a large range of compounds.

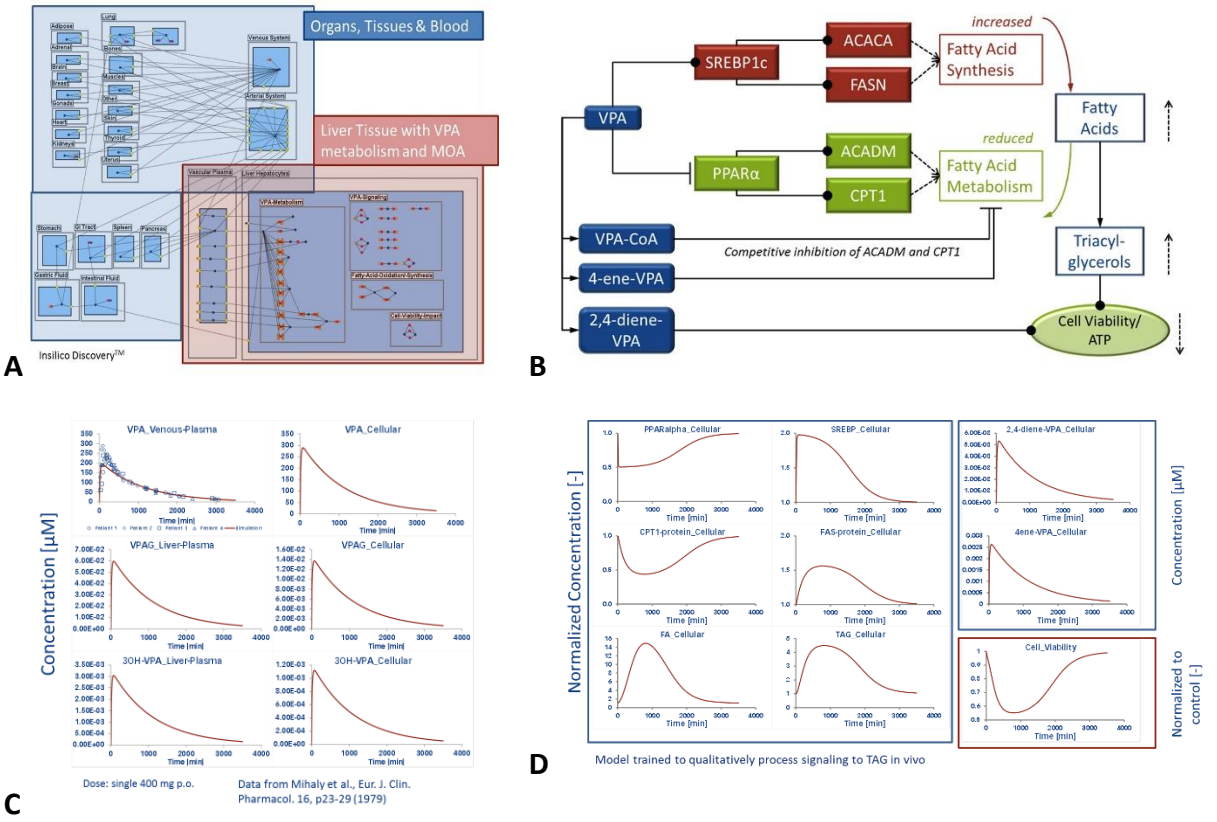

Figure 25. (A) The VPA-MOA-model is integrated in the liver compartment of Insilico's PBPK model. (B) VPA toxic mechanism/mode of action model reflecting FA (Fatty Acid) metabolism disturbance and cell viability impact. (C) Plasma and Cellular VPA and –metabolites' profiles (D) Potential mode of action response profiles in fatty acid regulation and metabolism and in cell viability.

12. Agent based modelling for *in vitro* to *in vivo* toxicity extrapolation

Agent-based models display every individual cell and hence permit representing the precise experimental setting of *in vitro* experiments as well as the precise architecture of liver lobules which constitute the smallest anatomical and functional unit of liver. Figure 26 shows how the detailed

mechanistic model on the molecular level is incorporated into an agent-based representation of a liver lobule that is then incorporated into a whole body PBPK model.

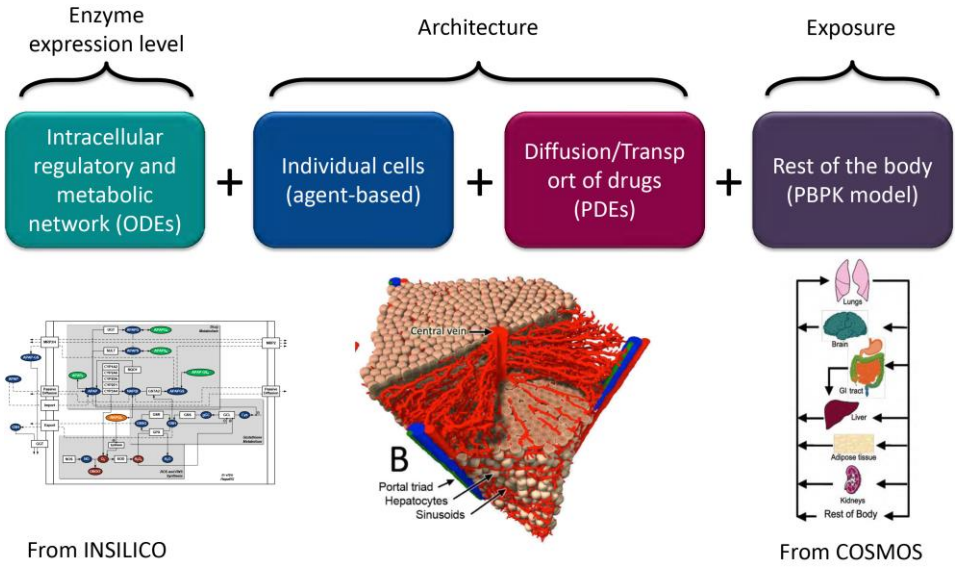

Figure 26. Concept of application of agent-based multi-scale modelling for IVIVE. A detailed regulatory and metabolic model is plugged into individual cell constituting an organ or the elementary unit of an organ, e.g. a liver lobule. This organ model is then part of a classical PBPK model primarily describing the distribution of compounds in the body as well as their removal, e.g. by the kidney.

Zonated ammonia detoxification. We investigated how liver function and in particular ammonia detoxification is impaired during drug-induced liver damage and regeneration. We showed that the classical reaction scheme (Figure 27A) for ammonia detoxification in a compartment model was sufficient to reproduce the measured concentrations of ammonia and glutamine at the liver outlet in the healthy case but not in the case of a drug-damaged liver (Schliess et al., 2014). In an integrated metabolic spatio-temporal model, it was shown that adding an *ad hoc* ammonia sink during the damage could resolve the mismatch. The reversible reaction catalysed by GDH is a good candidate that could act as an ammonia sink during the damage, whereas producing ammonia in the healthy situation. We developed and implemented an alternative model taking into account the GDH reaction (Figure 27B). This model was now able to describe cell damage by ammonia as well as the recovery of the cells (Figure 28). As could be shown in an animal model, the identified mechanism can be used therapeutically to prevent hyperammonemia.

A B

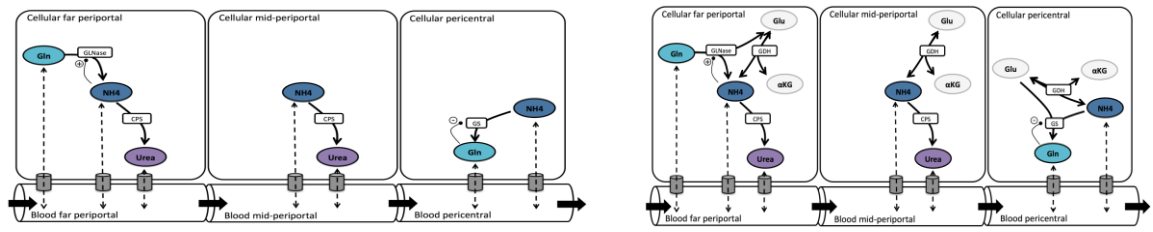

Figure 27. Left: Classical ammonia detoxification model without GDH composed of three zones. Right: Ammonia detoxification model extended by GDH.

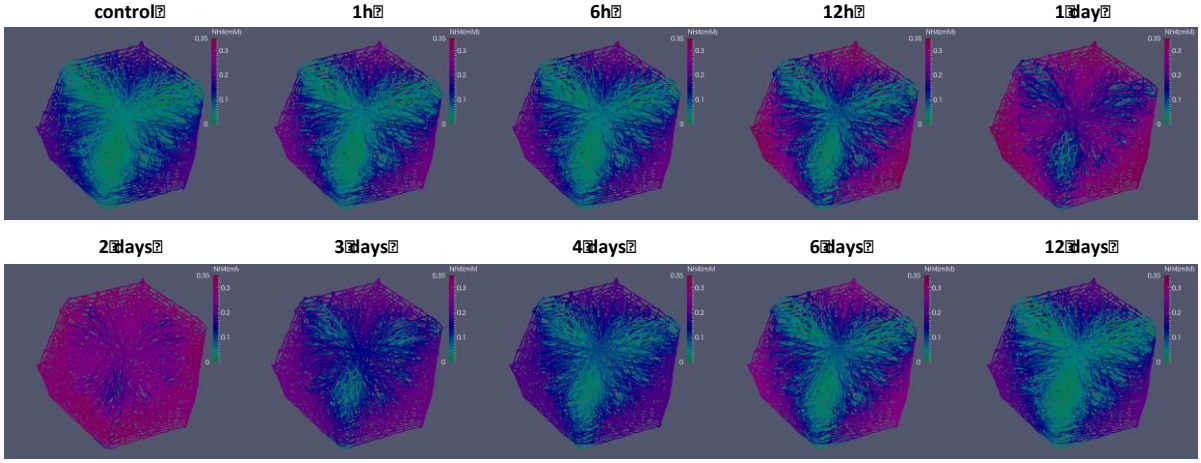

Figure 28. Simulation snapshots of ammonia concentration in lobule (green: low, violet: high). 3 portal veins per lobule were implemented in the model together with a septum-like connection of the sinusoidal network in the portal field with the portal veins. Ammonia detoxification is better along the porto-central axis.

Acetaminophen (APAP) overdosing causes necrosis in hepatocytes expressing CYP2E1 and CYP1A2 (about 50% of all hepatocytes) resulting in a necrotic lesion in the central region of liver, similar to ammonia. Necrosis most severely affects hepatocytes expressing the enzyme glutamine synthetase, localized in the lobule centre. These most efficiently detoxify the blood from ammonia. The integrated mathematical model described above (Figure 27) recently predicted the existence of a hypothetical ammonia sink (Schliess et al., 2014) that could later be identified experimentally (Ghallab et al., 2015). NOTOX used a similar modelling strategy to infer the *in vivo* APAP toxicity from *in vitro* experiments by stepwise extending a mathematical model calibrated with *in vitro* data to extrapolate *in vivo* toxicity. In its final version, the model is multi-scale and spatial temporal, representing liver lobule architecture, including APAP detoxifying pathway in each hepatocyte, and the flow of blood and transport of APAP with the blood. First a population of hepatocytes exposed to different concentrations of APAP has been modelled to reproduce the measured *in vitro* toxicity curves. Cell-to-cell variability has been taken into account by permitting 30% variability for enzymes involved. Next differences of the *in vivo* to *in vitro* situation were systematically explored: (i) the

difference in the temporal profile of exposure, (ii) the higher expression of metabolic enzymes *in vivo* than *in vitro*, (iii) the lobule architecture. While including (i) generates dose-independent cell survival (Figure 29 a), including in addition (ii), all CYP positive cells died (Figure 29 b). Integrating the APAP toxic pathway into each CYP2E1/CYP1A2 positive hepatocyte of a spatial-temporal model resolving each hepatocyte as a single agent within realistic lobule architecture, first simulations indicate that still all cells are dying (Figure 29 c). We conclude that our modelling tool permits systematic *in silico* testing of death mechanisms guiding experimentation.

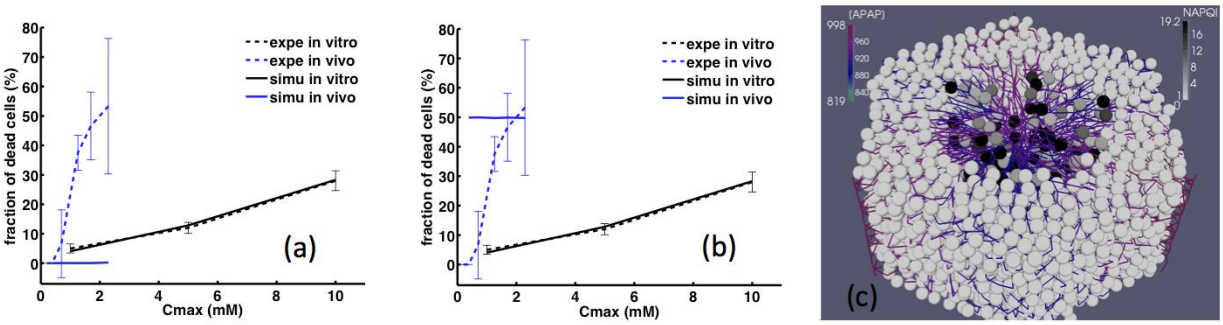

Figure 29. (a)-(c) Modelling steps to infer *in vivo* from *in vitro* toxicity (full lines: model, dashed: experiments). (a) temporal exposure profile included, (b) higher CYP levels *in vivo* are included in addition, (c) both in spatial temporal model (black: dead cells), snapshot before all cells have died.

Various simulations of a series models showed that a detailed agent-based model is especially important when tissue regeneration or tissue formation plays an important role as was shown for ammonia, CCl_4 and also acetaminophen. A simplified model involving several compartments, e.g. mimicking the different cellular characteristics along the sinusoid, may be necessary and sufficient for compounds that are metabolised with relatively high rate and for taking into account the different sensitivities of these cells to the toxic compound they are exposed to. In those case where the first pass through the liver would not create significant gradients, a simple one compartment model may be sufficient.

POTENTIAL IMPACT AND MAIN DISSEMINATION ACTIVITIES AND EXPLOITATION RESULTS

1. Strategic Impact of NOTOX

Huge efforts have already been committed for the development of alternative, non-animal safety tests in the European Union but also in other highly industrialized countries like the USA and Japan. These have created considerable success but still lack a complete coverage of all relevant areas of safety assessment. Some *in vitro* tests, e.g. for genetic toxicity tests, skin and eye irritation and corrosion, phototoxicity or skin penetration are now applied routinely. However, for pivotal toxicological endpoints e.g. repeated dose toxicity, target organ toxicity, toxicokinetics, reproductive toxicity and carcinogenicity, no alternative tests are currently available. This leaves a huge gap for the consumer and pharmaceutical industry to ensure the safety of their products especially for long term toxicity. This is not only a scientific problem but is an area affected by regional regulatory scrutiny and control as well as issues like ethics of animal testing.

The NOTOX project directly addressed some of these problems and delivers new methods based on a systems biology approach. The NOTOX approach created examples of *in silico* predictive models at various levels of details that will be beneficial to all stakeholders, consumers, regulatory bodies and producers and paves the way how new alternative testing methods may be developed in the future. Particularly the development of alternative methods in long term repeated dose toxicities will have a tremendous impact on all these communities and will also bring about harmonization, transparency and better understanding on various levels. The potential impact of NOTOX may be seen in different directions: strategically, with respect to the application in safety assessment, to innovation and to the support of European industries.

Successful results of the project, if these are adopted by the concerned European industries will have an impact in:

- Ensuring safety of consumer products as defined by the European Scientific Committee on Consumer Products (SCCP) directives
- Providing *in vitro* alternative methods for long term toxicity testing based on predictive computer models that have potential to enter pre-validation and finally validation
- Significantly decreasing animal testing
- Reducing the workload during product development and testing
- Improving predictability of safety of substances of importance for cosmetics and other industries (chemical and pharmaceutical)
- Allowing new systems-oriented approach for novel cost-efficient and time-efficient product testing
- Reducing time by using a new multi-sector approach to market as well as time to intellectual property

- Strengthening competitive edge of cosmetic and pharmaceutical industries by providing knowledge-based solutions
- Accelerating systems-oriented toxicity testing in product development

2. Impact on safety assessment

The potential impact can be best explained by briefly looking at some major results of NOTOX that promise integration into safety assessment procedures in the foreseeable future. We are fully convinced that future testing will very much rely on methods based on systems biology approach that is characterized by a broad understanding of mechanisms of toxicity often called mode of action. Mode of action of a compound can be most thoroughly studied using *omics* methods, comprising gen, epigenomics, proteomics, metabolomics and fluxomics combined with powerful new imaging methods using fluorescence techniques and high resolution 2D/3D electron microscopy. Indispensably, this requires reliable cellular systems mimicking the human *in vivo* situation as closely as possible.

NOTOX paved the way to using only human cells, particularly cell lines (HepaRG, stem cell derived hepatocytes and cardiomyocytes) for long term toxicity assessment instead of animals. At the beginning of NOTOX, HepaRG cells were occasionally used for toxicity testing, almost exclusively by the groups of Christiane Guguen-Guillouzo and Andre Guillouzo in Rennes, France, but not for long term effects. However, during the course of time, the NOTOX results have shown the suitability of HepaRG cells in long term applications. Many other SEURAT projects started using the HepaRG model. Moreover, NOTOX realised early the value of emerging new methods for 3D cultures as was indicated already in the proposal. NOTOX started to extensively use such techniques in toxicity studies which were mainly based on 2D cultures or animals in the near past. In addition, NOTOX extended high content screening methods to the 3D cultures, i.e. spheroids and sandwich cultures. More recent developments are further stimulating the use of human cells, particularly the increasing availability thanks to the breath taking progress in the isolation, propagation and differentiation of embryonic and induced pluripotent stem cells (hESC and hiPSC). NOTOX presents examples for the cultivation of such cells including micro tissues of multi-organ mimicking systems (hepatocytes and cardiomyocytes). Activities of cellular systems were characterized with a range of *omics* methods and with optical and electron microscopy methods. Bioinformatic methods for the evaluation of the huge amount of data created were applied and further developed. Computational modelling is still a tedious and challenging effort, however, in NOTOX, mechanistic and predictive mathematical models with much higher resolution and degree of detailing were created in its last phase.

a. Application of NOTOX methods for safety assessment

A crucial question is how such new developments can contribute to the safety assessments of compounds in the cosmetics, pharmaceutical and other industries. Figure 30 shows schematically some important levels of testing that contribute to safety assessment. A first and nowadays quick computational method uses chemical knowledge combined with a priori knowledge on structurally related chemicals for read across, as has been successfully further developed within the SEURAT-1 initiative by the project COSMOS.

NOTOX contributes significantly to methods in the other three areas indicated in Figure 1 by providing a whole toolbox of methods for testing compounds using human cell *in vitro* and *in silico* methods.

b. Human in vitro cell culture application for the estimation of oral equivalent doses (OED)

This first method capitalizes on the developed organotypic cultures of the HepaRG cell line that has been extensively studied in NOTOX and combines it with simple PBPK type of modelling (Klein et al., 2015). It was stimulated by American activities like ToxCast (Rotroff et al., 2010; Wetmore et al., 2012). Our newly developed 2D and 3D cultivation methods are not only applicable for short-term toxicity testing but allow also long-term and repeated-dose testing.

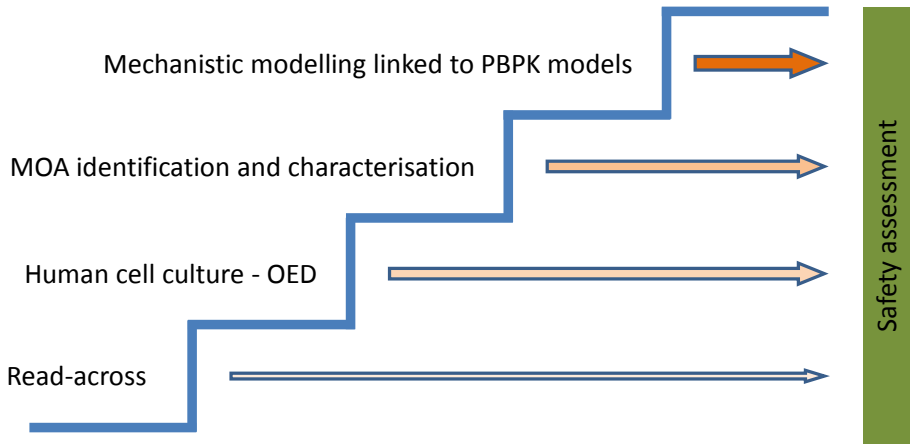

Figure 30. The NOTOX approach combining human based cell systems including 3D culture with computational modelling for the prediction of toxicity. OED- oral equivalent dose, PBPK - physiologically based pharmacokinetic modelling.

The principle of oral equivalent dose (OED) determination is again highlighted in Figure 31 (see also S & T results described above) to elaborate more on questions of its practical applicability. Generally, a simple model of the *in vitro* systems of Figure 31A allows the estimation of parameters needed for the *in vivo* estimations. The pharmacokinetic parameters liver clearance and plastic binding are

determined using modern analytical techniques, usually involving mass spectrometry. For reliable determination of liver clearance, cell cultures with *in vivo* like activities of liver specific enzymes and transporters are essential that are now available by the 2D and 3D culture methods using HepaRG (Gunness et al., 2013; Mueller et al., 2014) or other human cells. The serum free media (Klein et al., 2014) developed in NOTOX improves such studies by practically eliminating protein binding effects *in vitro*. Protein binding is determined in separate *in vitro* experiments or estimated by computational methods.

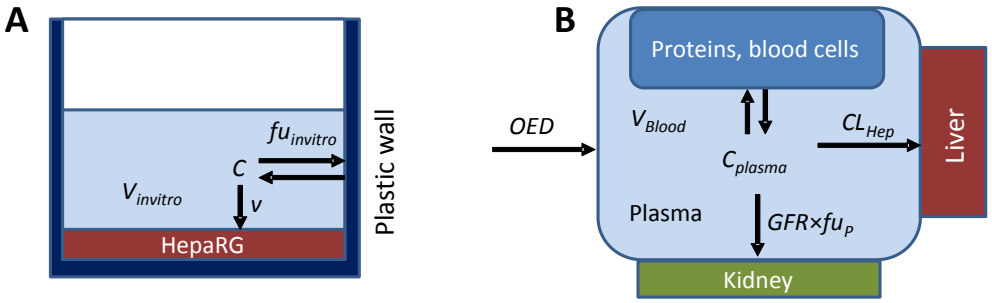

Figure 31. Estimation of oral equivalent doses (OEDs) from *in vitro* data using a simple physiological model. A – model scheme for *in vitro* study; B – human physiological model.

In wells as shown in Figure 2A, also dose-response curves for liver cells can be determined reliably using our HepaRG cultures. All these studies can be made in a high-throughput manner using e.g. 96-well plates. Having the glomerular filtration rate (GFR) hepatic clearance can be estimated (Figure 31B). Taking known data of liver weight, serum protein concentration and blood volume allows a direct estimate of OED. In combination with a useful exposure scenario the assessment of the risk of liver toxicity of possible also for a population (Wetmore et al., 2015). This method is also straight forward applicable for new liver cell lines that will be developed, e.g. derived from human embryonic or human of induced pluripotent stem cells (hESC or hiPSC). It has potential to be applied for liver co-culture systems, e.g. with Kupffer or stellate cells. Such systems have been explored within NOTOX with Kupffer cells and with stellate cells in collaboration with HeMiBio (Leite et al., 2016).

Additionally HepaRG cells can be combined with cells of another organ of interest for the determination of metabolism mediated toxicity. We recently used cardiomyocytes derived from hESC and hiPSC cells (by Scr&Tox project of the SEURAT-1 initiative) for long term toxicity testing. These cultures can be kept in a common medium to test for toxic compounds created by liver metabolism. This shows the huge future potential of methods developed within NOTOX.

c. Identification and characterisation of mode of action for safety assessment

The NOTOX approach with its multi-*omics* and high content imaging characterisation combined with informatics analysis of the data permits the identification and characterisation of pathways leading to toxic effects. Specific data, e.g. expression of certain genes, changes in the proteome, metabolic changes or changes related to the generation of reactive oxygen species, can later serve as markers for the quantification of effects. This can then be used for quantitative IVIVE. Examples from NOTOX are:

- **Steatosis** (e.g. changes in expression of CPT1 and BSEP, changes in metabolic fluxes, changes in histone modification and various protein levels, etc. see S & T results, chapter 6)
- **Cholestasis** (modified susceptibility of HepaRG detects compounds with cholestatic liability; functional imaging in sandwich and spheroid cultures allows determination of bile flux, see S & T results, chapters 3 and 4)
- **Oxidative stress** (formation of reactive oxygen species in response to treatment with paracetamol, see S & T results, chapter 11)
- **High resolution EM-tomography** established for future exploration of MIEs (see S & T results, chapter 5)

d. Detailed mechanistic signalling and metabolic models incorporated into PBPK model

Two detailed case studies using paracetamol (acetaminophen, APAP) and valproic acid (VPA) permitted the generation of detailed gene regulatory and metabolic models that could be incorporated into agent-based models and further into PBPK models that allow a quantitative description of the metabolism and distribution of compounds in the human body. Using such models various scenarios of exposure can be studied in detail, e.g. uptake via different routes, oral or via the skin.

- Case study APAP (mechanistic model for APAP metabolism and metabolism of reactive oxygen species incorporated in agent based model, see S & T results, chapter 11)
- Case study VPA (mechanistic model of valproic acid VPA metabolism, related gene expression set up and incorporated into PBPK model, see S & T results, chapter 10)

3. **General innovative impact**

NOTOX particularly extended the spectrum of "*omics*" techniques as epigen*omics*, transcriptomics, proteomics, metabolomics and fluxomics by systems oriented multi scale structural analysis using cryo-3D electron microscopy. This provides besides epigenomics a second extremely powerful method for the detection of integrative effects that seem most important in characterizing long term toxic effects. NOTOX established and improved high-content screening methods, e.g. for the characterisation of bile flux in 2D, sandwich and 3D spheroid cultures (Reif et al., 2015). NOTOX

established and applied large scale mathematical and bioinformatic modelling integrating the spectrum of experimental data for long term toxicity assessment. NOTOX is still working on improved methods for a comprehensive, overarching bioinformatic analysis of multi-*omics* (transcriptome, proteome, fluxome) analysis of long-term exposure data.

The scientific results of the NOTOX project show:

- Improved *in vitro* alternative (non-animal) methods based on human cell culture for human long term repeated dose toxicity. NOTOX delivered protocols for the long term toxicity assessments for liver and cardiac toxicity.
- Exploitation of the potential of organotypic systems with their close resemblance to *in vivo* physiological conditions and human relevance with 3D microtissue cultures. These 3D cultures were established and tested for both liver and stem cell derived cardiac microtissues for application in long term toxicity study setups.
- Novel combination of functional and structural systems biology characterization using an integrative multi-scale approach (see part on description of main S & T results and foreground). High resolution EM-tomography was established for future exploration of molecular initiating events (MIEs).
- Computer *in silico* large scale models based on "*omics*" for long term toxicity prediction (see part on description of main S & T results and foreground).
- New predictive solutions will stimulate research towards personalized health-care and personalised medicine.

4. **Strengthening the community industry**

The NOTOX project by its diverse activities focusing on improved prediction of long term toxicity has potential impact on the community industry. This can be elaborated as follows:

- The SMEs which are partners in the project namely; Biopredic International, Insilico Biotechnology and Cambridge Cell Networks, have access to the results and prototypes for own business development.
- The dissemination of results such as the developed predictive models for long term toxicity according to the exploitation plan of the project during the project can be used by new or existing SMEs in the bioinformatic and bioinstrumentation business sector (see dissemination and exploitation results).
- New methods for cosmetics, pharmaceutical and chemical industries were developed and disseminated.

5. **Integration in community research**

The importance of the area addressed in the proposal call, requires actively engaging the scientific community. Especially a systems biology approach necessitates a joint effort by a multidisciplinary

team composed of biologists, bioinformatics, biophysicists, toxicologists, engineers, mathematicians and modellers.

The NOTOX project integrated the expertise of diverse community research groups as follows:

- By a cross disciplinary research effort between frontline researchers in the bioinformatics and biomedicine sciences, in particular with the German “Virtual Liver Initiative”
- NOTOX collaborated with the other SEURAT-1 cluster projects by exchange of people, of methods and material. These collaborations resulted in joint publications either already published or under preparation for wider scientific community.
- Stimulating innovative and integrative research with high quality scientific publications.
- Mobilizing front researchers and users by thorough dissemination activities in scientific community by organizing satellite meeting/workshops at the ESTIV and EUROTOX meetings. In addition, general public was also reached by media communications and press releases (See also dissemination and exploitation results).
- NOTOX was actively involved in SEURAT case studies
- NOTOX was actively supporting SEURAT workshops

6. Animal welfare policy and 3R principle

The complete ban on animal testing for cosmetics and cosmetic ingredients from March, 2013 (Cosmetics directive 76/768/EEC) necessitates the development of non-animal test systems. To date no alternative validated long term repeated dose toxicity methods exist. The NOTOX project considered the situation of experimental animal testing as stated in the Community Directive 86/609/EEC (OJ L 358, 18.12.1986, p.1) and is in agreement with the protocol annexed to the Treaty of Amsterdam on Animal Protection and Welfare regarding the formulation and implementation of Community Policies including research. In addition, this project is in accordance with the 3Rs concept (reduce, refine or replace the use of laboratory animals) of Russell & Burch (Russell, W.M.S. & Burch, R.L., 1959, The principles of humane Experimental Technique, 238 p.p., Methuen, London).

NOTOX supports this policy:

- By reducing the need for animals during compound screening by using organotypic human cell cultures in 2D and 3D (HepaRG cells, primary human hepatocytes, human stem cell derived hepatocytes and cardiomyocytes) for long term repeated dose toxicity studies.
- By replacing animal tissue material with human cell and tissues systems in addition to replacement of foetal bovine serum with other components allowing serum free cultivation.
- By providing powerful *in vitro* bioinformatic and mathematical models of human cell systems. Such causal computer models can be used for long term toxicity studies that are

also more predictive for responses in humans than in animals. An example close to application is the OED calculation using *in vitro* long term data and PBPK modelling.

7. NOTOX dissemination to scientific community and general public

During the life of the NOTOX project, two important extended dissemination activities were organized at the auspicious of Europe's international conferences. One of the workshops was organized as satellite to the European Society of Toxicology *In vitro* (ESTIV) in Egmond aan Zee, Netherlands in 2014. Two guest lecturers were invited: Richard Judson from the National Center for Computational Toxicology of the U.S. Environmental Protection Agency and Mathieu Vinken of Vrije Universiteit Brussel, Belgium. The NOTOX project was represented by lectures of Magnus Ingelman-Sundberg (Karolinska Institutet, Sweden), Fozia Noor (Saarland University, Germany), Géraldine Cellière (INRIA, France) and Elmar Heinzle (Saarland University, Germany)

The other public NOTOX symposium was organized for the dissemination of the NOTOX results at the EUROTOX meeting in Porto in 2015. The following topics were featured

- Improved *in vitro* systems for prediction of hepatotoxicity, Magnus Ingelman-Sundberg (Karolinska Institutet, Stockholm, Sweden)
- Toxicoprote*omics* applied to *in vitro* investigation of liver toxicity using HepaRG cells, Fabrice Bertile (CNRS, Strasbourg, France)
- Model and *in vitro* based prediction of human hepatotoxicity , Jan G. Hengstler (IfADo, Dortmund, Germany)
- Prediction of long term toxic effects by genome based network models, Lothar Terfloth (INSIL, Stuttgart, Germany)

Figure 32. NOTOX Satellite Meeting at ESTIV (Egmond aan Zee, The Netherlands, 2014) and NOTOX Symposium at EUROTOX 2015 (Porto, Portugal, 2015).

In addition, NOTOX participated in SEURAT meetings and workshops and contributed with posters, lectures and hands on trainings. NOTOX also participated in the summer schools organized by the COACH project of the SEURAT.

NOTOX contributed to the scientific community by numerous (44) publications in peer reviewed journals. NOTOX scientists also presented project results and achievements in the form of posters and lectures in several national and international conferences and workshops.

For general public a movie showing NOTOX approach and methods was released in 2013 (http://www.notox-sb.eu/film). Recently, NOTOX also actively participated in the SEURAT movie for dissemination of results released by COACH (www.seurat-1.eu).

From the beginning of the NOTOX project a professional webpage was created for wider scientific and public interest and information (www.notox-sb.eu).

NOTOX also targeted general public and stakeholders through numerous press and releases and local TV interviews (German ARD channel). NOTOX also informed of its activities and results in EU magazines (research*eu results magazine (10/2015), Horizon - the EU research and innovation magazine (12/2015)).

8. Exploitation results

A number of software packages were developed in the NOTOX project (some available freely) and are ready for exploitation. Examples of these software packages are

- Cell Sys and TiQuant are software packages of INRIA for agent based modelling
- Ettention software framework for tomographic reconstructions by DFKI is publicly available also after the end of the project.

The company EYEN SE was founded by former NOTOX researcher Lukas Marsalek as a Spin-Off from DFKI GmbH. The company aims to provide electron tomography, sub-tomogram averaging and macromolecular structural analysis as a commercially available service and thus is a large step to transfer some part of the techniques and pipelines developed during NOTOX to a persistent state usable after the end of the project.

The companies participating in NOTOX benefitted in various ways

- INSIL – was able to extend its business to a new field of activity
- Biopredic – learned more about the suitability of their HepaRG cell line for toxicity testing. The application of this cell line in this area has certainly grown considerably both in academia as well as in industry.

- CCNet – got more exposure to the community. CCNet created new structures for integrating and evaluating multi-*omics* data. It also helped expand their business network to the cosmetics industry

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