NOTOX EXECUTIVE SUMMARY
3rd project period

CONTEXT & OBJECTIVES

NOTOX will develop and establish a spectrum of systems biological tools including experimental and computational methods for (i) organotypic human cell cultures suitable for long-term toxicity testing and (ii) the identification and analysis of pathways of toxicological relevance. NOTOX will initially use available human HepaRG and primary liver cells cultures especially in 3D systems to generate own experimental data to develop and validate predictive mathematical and bioinformatic models characterizing long-term toxicity responses. Cellular activities will be monitored continuously by comprehensive analysis of metabolome, proteome and by estimation of metabolic fluxes using ^{13}C labelling techniques (fluxome). At selected time points a part of the cells will be removed for in-depth structural (3D-optical and electron microscopy tomography), transcriptomic, epigenomic, metabolomic, proteomic and fluxomic characterizations. When applicable, cells derived from human stem cells (hESC or iPS) and available human organ simulating systems or even a multi-organ platform developed in SCR&TOX and HEMIBIO will be investigated using developed methods. Together with curated literature and genomic data these toxicological data will be organised in a toxicological database (cooperation with DETECTIVE, COSMOS and TOXBANK). Physiological data including metabolism of test compounds will be incorporated into large-scale computer models that are based on material balancing and kinetics. Various "-omics" data and 3D structural information from organotypic cultures will be integrated using correlative bioinformatic tools. These data also serve as a basis for large scale mathematical models. The overall objectives are to identify cellular and molecular signatures allowing prediction of long-term toxicity, to design experimental systems for the identification of predictive endpoints and to integrate these into causal computer models.

Specific objectives of NOTOX:

O1: Supplying a versatile methodology for systems based analysis and prediction of long-term toxicity of test compounds on organotypic 3D cultures.

O2: 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.

O3: Development and application of experimental and computational methods for the comprehensive characterization of 3D organotypic cultures after long-term repeated dose exposure to selected test compounds (epigenetic chromosomal profiling, 3D-EM-tomography, 3D-topographic analysis and modelling, bioinformatic characterization).

O4: Development of causal and predictive large scale computer models based on the integration of the experimental data with available data (various databases) and high performance grid computing for identification of predictive endpoints.

O5: Development of predictive causal computer models aimed at entering pre-validation as guided by the integrative project (TOXBANK) and as defined by ECVAM.

O6: Finally the ultimate objective is to provide cheaper, more ethical, scientifically based testing strategies for repeated dose toxicity in order to meet the European legislative demands. For this purpose we will illustrate how computer models calibrated with *in vitro* experiments could be used in combination with human parameters to predict the possible toxicity in humans.

SCIENTIFIC RESULTS

3D HepaRG spheroid cultures were characterized in detail and further extended to primary human (PHH) as well as to co-cultures with non-parenchymal cells for fibrotic (with HeMiBio) and inflammatory responses. A first series of EM images of 2D and 3D HepaRG cultures showed healthy-looking hepatocytes exhibiting typical hepatocyte structures. Screening for compounds with cholestatic potential was established in 3D spheroid cultures in the presence of bile acids. Additionally 3D cardiomyocyte cultures derived from hiPS and hES cells by SCR&TOX are applied for long-term studies. Biokinetics of test compounds (plastic binding, degradation) were studied by LCMS, partly in collaboration with Toxbank. 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.

Two large-scale joint consortium wide studies on acetaminophen (APAP) and valproic acid (VPA) focused on adverse outcome pathways. For VPA DNA methylation was not changed and histone modifications are under investigation. Transcriptomic analysis of the VPA experiment provided in-depth information about mode-of-action, e.g. CPT-1, CYP4X1A and SPP1. Bioinformatic analysis of expression and proteome data allowed distinguishing culture effects from VPA exposure effects and delivered clusters of dose-dependent changes in genes/proteins, e.g. CYPs and metabolic enzymes, even at very low dose. In accordance metabolic flux analysis showed changes in energy and branched-chain amino acid metabolism. 13C metabolic flux analysis was carried out using acetaminophen and diclofenac.

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). Dynamic EC10 determinations over two weeks permitted safety predictions very close to in vivo data. 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 (Virtual Liver project). 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.

EXPECTED FINAL RESULTS AND IMPACTS

NOTOX project's main objective is to go beyond the state-of-the-art technology by using the most modern techniques and developing them for the purpose of prediction of long-term toxicity. NOTOX is particularly extending the spectrum of -omics techniques as epigenomics, transcriptomics, proteomics, metabolomics and fluxomics by systems oriented multi-scale structural analysis using cryo-3D electron microscopy and 3D light microscopy applied on modern *in vitro* human cell assays. NOTOX develops bioinformatic tools to analyse, interpret and integrate various omics data. NOTOX delivers a human based 3D cultivation model using HepaRG cells which mimics the *in vivo* liver. The methods developed by NOTOX are successfully applied to other tissue types including heart tissue, i.e. cardiomyocytes derived from human stem cells, as well as co-cultures of liver cells.

NOTOX is extending prediction of toxic effects on humans based on human cell *in vitro* assays significantly by applying various types of *in silico* models to predict long-term toxicity. These range from simple to large-scale mathematical models integrating the spectrum of experimental data. These models will be very useful in preclinical setting as well as for *in vitro* to *in vivo* extrapolation (IVIVE) for cosmetic ingredients and for pharmaceuticals.

We already show a proof of concept and the feasibility of these systems for long-term repeated dose toxicity testing. NOTOX extended prediction of oral equivalent dose (OED) for long-term *in vitro* assays. The cases investigated showed very good prediction for OED. More complex models are enriching the adverse outcome pathways (AOP) within a global effort to change the paradigm of toxicity testing putting it on a mechanistic basis.

The various strict regulations by the European regulatory bodies for consumer products and pharmaceuticals require the community industries to prove the safety of their products. At the same time, testing on animals is highly discouraged and stringently controlled. While many validated alternative methods for testing are available, the community industry is at a loss where long-term repeated dose toxicity is concerned. Any serious effort in this direction will strengthen the community industry and will be welcome. The NOTOX project is, by its diverse activities, focused on improved prediction of long-term toxicity having an important impact on the community industry.

To our knowledge, no validated alternative method yet exists for long-term repeated dose toxicity. The methods developed by NOTOX are expected to support successfully the intensive endeavours of the cosmetics industry to provide safe products without any animal testing. Delivering such a human-based alternative method will have a huge impact on not only the cosmetics industry but also on the chemical and pharmaceutical industry. This will promote the principle of 3Rs (reduce, refine, replace). It will further decrease the costs incurred in animal testing and will thus have an overall economic benefit.

In this way the project supports EU interests that are expressed in corresponding EU Directives, at the same time supporting the 3Rs principles of animal testing (reduce, refine or replace the use of laboratory animals).

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