123

Assessing reproducibility, robustness and predictivity of an *in vitro* method to assess DIO1 inhibition in human liver microsomes

A. Weber[1], B. Birk[1], C. Herrmann[1], H. A. Huener[1], K. Renko[2], S. Coecke[3], S. Schneider[1], D. Funk-Weyer[1], R. Landsiedel[1]
[1]BASF SE, BASF SE, RB/TB, Ludwigshafen, Germany
[2]Bundesinstitut für Risikobewertung (BfR), German Centre for the Protection of Laboratory Animals (Bf3R), Berlin, Germany
[3]European Commission, Joint Research Centre (JRC), Ispra, Italy

Impairment of thyroid hormone homeostasis has been associated with several adverse effects. Regulatory requirements are increasing to identify different mode of actions (MoA) impacting thyroid hormone (TH) signaling pathways. The European Union Reference Laboratory for alternatives to animal testing (EURL ECVAM) is coordinating the validation of multiple *in vitro* methods focusing on different thyroid MoA by cooperating with a network of EU laboratories (EU-NETVAL).

Deiodinases (DIO) are important, local regulators of TH action by enzymatically activating or inactivating TH via deiodination. DIO1, one of the three isoforms and mainly expressed in thyroid, liver, and kidney tissue, serves as one main source for circulating T3 via deiodination of T4 and plays a role in recycling of iodide via deiodination of inactive TH metabolites.

A non-radioactive approach to determine DIO1 inhibition based on enzymatic activity in human liver microsomes was transferred to our laboratory [1] and further developed following the GIVIMP concept [2]. The released iodide was quantified via the Sandell-Kolthoff (SK) reaction. The reproducibility assessment (Part 1) testing of six known DIO1 inhibitors has been finalized and resulting acceptance criteria were used to test 40 blinded items (Part 2) assessing predictivity of the method. 22 test items were de-blinded by ECVAM and compared to available *in vitro* data. Additional testing strategies were implemented to show specificity of the observed DIO1 inhibition.

Reproducibility is given for all six test items with IC50 values in range of literature (e.g., IC_{50}: 6-Propyl-2-thiouracil: 3.8 μM in this study, compared to [3]: 5.4 μM). High concordance with *in vitro* data obtained in recombinant enzyme [3] is given for 22 unblinded substances. Silychristin, a natural substance from milk thistle and described thyroid hormone transport inhibitor, interfered with DIO1-SK assay and was not applicable.

The DIO1 inhibition assay using human liver microsomes is a robust and reproducible *in vitro* assay to assess potential DIO1 inhibition of chemicals with high concordance with known *in vitro* data. Additional tests on specificity improves the assessment of the data. Finally, integrative testing strategies considering other thyroid related MoA are needed to assess the biological relevance of the assay.

[1] https://doi.org/10.1210/en.2011-1863

[2] https://doi.org/10.1787/9789264304796-en

[3] https://doi.org/10.1093/toxsci/kfy302

167

SEMO-1 (Y37A1B.5), a novel selenium-binding protein ortholog and hydrogen sulfide source, mediates selective stress resistance in the model organism *C. elegans*

V. A. Ridolfi[1], *T. M. Philipp*[1], *W. Gong*[1], *J. Priebs*[1], *H. Steinbrenner*[1], *L. O. Klotz*[1]
[1]Friedrich-Schiller-Universität Jena, Nutrigenomik, Jena, Germany

Question: The *Caenorhabditis elegans* ortholog of human selenium-binding protein 1 (SELENBP1), Y37A1B.5 (Y37), is a pro-aging factor. Knock-down of Y37 resulted in elevated lifespan and better resistance against oxidative stress [1]. SELENBP1 catalyzes the conversion of methanethiol to hydrogen sulfide (H_2S), hydrogen peroxide (H_2O_2) and formaldehyde, thus acting as methanethiol oxidase (MTO). Here, we tested whether Y37 has MTO activity, whether it is involved in selenium homeostasis, and whether energy metabolism is involved in the observed modulation of lifespan by Y37.

Methods: MTO activity was measured using a coupled assay based on *in situ*-generation of methanethiol as catalyzed by a bacterial recombinant L-methionine gamma-lyase, followed by detection of MTO-generated H_2S and H_2O_2 [2]. Lifespan analyses were performed using standard *C. elegans* culture. For stress resistance analyses, nematodes were exposed to toxic concentrations of selenite or paraquat. Worms employed included N2 wildtype and mutant strains deficient in Y37 or in the AMPK ortholog, AAK-1/-2.

Results: Like SELENBP1, isolated recombinant Y37 has MTO activity. While MTO activity was detected in lysates from wild-type nematodes, the Y37-deficient strain was devoid of it. A Y37-deficient mutant strain generated through CRISPR/Cas technology exhibited an extended lifespan, similar to the previously reported worms exposed to Y37-specific RNAi. Moreover, resistance against the redox-cycler paraquat was also improved in the Y37-deficient strain. In contrast to paraquat, selenite was more toxic in the Y37-deficient strain, as compared to wild-type worms. Lifespan extension following Y37 depletion was abrogated in a mutant strain deficient in both isoforms of the catalytic AAK subunit, while Y37 depletion through RNAi appeared to enhance AAK phosphorylation in wild-type worms.

Conclusions: (1) Y37 acts as MTO in *C. elegans*; thus, we named it SEMO-1 (SELENBP1 ortholog with MTO activity). (2) SEMO-1 mediates selective stress resistance. It renders worms susceptible to oxidative stress but also serves as selenium buffer, protecting against high doses of selenite. (3) SEMO-1 is involved in the AAK-mediated regulation of energy metabolism, thereby affecting organismal lifespan and stress resistance.

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[1] Köhnlein *et al.*, Redox Biol. 28:101323 (2020), [2] Philipp *et al.*, Redox Biol. 43:101972 (2021).

151

Potency ranking of PAs in human liver cells using an *in vitro* genotoxicity battery and impact of OCT1-mediated uptake

M. Haas[1], K. Wirachowski[1], J. H. Küpper[2], D. Schrenk[1], J. Fahrer[1]
[1]Technical University of Kaiserslautern, Food Chemistry and Toxicology, Kaiserslautern, Germany
[2]Brandenburg University of Technology Cottbus-Senftenberg, Molecular Cell Biology, Brandenburg, Germany

Introduction: Pyrrolizidine alkaloids (PAs) are known contaminants in numerous plant-based foods like herbal teas and dietary supplements. CYP450 enzymes are necessary for metabolic activation of PAs and formation of DNA reactive metabolites. It is well known that PAs are genotoxic, hepatoxic and can cause liver cancer. Recent data provide evidence that the genotoxic and cytotoxic potential of PAs is largely structure-dependent.

Objectives: The aim of the study was to analyse the relative genotoxic potential of eleven structurally different PAs in metabolically competent human liver cells using an *in vitro* genotoxicity battery and to detail the impact of OCT1-mediated uptake.

Material & Methods: HepG2 cells with CYP3A4 overexpression were used as cell model, which were incubated with eleven different PAs for up to 24 h. The genotoxic potential was investigated using western-blot analysis of the DNA damage markers γH2AX and p53 as well as the alkaline comet assay. Data were subject to BMD modelling via PROAST software to derive BMDL values for assessing the relative genotoxicity. The impact of OCT1 on PA uptake was studied using pan-OCT and OCT1-specific inhibitors. As endpoints, cell viability and genotoxicity markers were included.

Results: Overall, monoesters such as lycopsamine show the lowest and cyclic di-esters including retrorsine the strongest genotoxic effects within our test battery. Furthermore, the results of all genotoxic assays demonstrate a structure-dependent genotoxicity of the PAs and the same correlation regarding the potency ranking based on BMDL values. The lowest BMDL values ranging from 0.1 – 0.8 μM were obtained for cyclic and open di-ester such as retrorsine and lasiocarpine, whereas monocrotaline and lycopsamine showed the highest BMDL values. After OCT1 inhibition, a strong reduction in both genotoxicity and cytotoxicity induced by lasiocarpin was determined.

Conclusion: Our findings show a concentration- and structure-dependent toxicity based on the degree of esterification. The alkaline comet assay results are perfectly in line with the western blot experiments. Our data strongly supports the notion that PAs can be ranked according to their relative genotoxic and cytotoxic potencies. Furthermore, the OCT inhibition studies displayed that heliotrine, lasiocarpine and riddelliine are generally transported by OCT1, which is currently validated by genetic knockdown studies.

258

Machine Learning Prediction of Cyanobacterial Toxin (Microcystin) Toxicodynamics in Humans

R. Fotler[1], S. Altaner[1], S. Jäger-Honz[2], I. Zemskov[3], V. Wittmann[3], F. Schreiber[2], D. Dietrich[1]

[1]Universität Konstanz, Biologie / Human & Ökotoxikologie, Konstanz, Germany

[2]Universität Konstanz, Bioinformatik, Konstanz, Germany

[3]Universität Konstanz, Bioorganische Chemie, Konstanz, Germany

Microcystins (MC) represent a family of cyclic peptides with approx. 250 congeners, some of which were demonstrated to be toxic to humans. The toxicological profile of MC is characterized by the active cellular uptake of MC via organic anion transporting polypeptides (OATPs), and the subsequent irreversible inhibition of primarily ser/thr protein phosphatases (PPP) amongst a number of cellular proteins. Although a comparison between rodents and humans demonstrated that rodents are poor surrogates for humans with regard to the i) type of OATP expressed in the various tissues, ii) the affinity and iii) capacity of expressed OATPs for specific MC congener transport, risk assessment is still based on a single MC congener and a 90-day toxicity study in mice. The observation that humans demonstrate major differences in OATP expression and thus susceptibility to MC only compounded the fact that current risk assessment premises could severely underestimate the potential toxicities of MC due to their congener-specific kinetics. In view of the ever-increasing number of identified MC congeners, yet lacking the ability to synthetize these in sufficient purity and amounts for *in vitro* or *in vivo* testing, an *in silico* approach using toxicodynamic data could provide a first step towards a better toxicity assessment of uncharacterized MCs with relevance for humans. Accordingly, the aim of this study was to develop a comprehensive dataset of toxicodynamics, i.e., the PPP inhibitory capacities of a limited number of MC congeners. These *in vitro* data were then used as a comparative basis driving an *in silico* approach using machine learning (ML). The inhibition of PPP1, PPP2A and PPP5 by 18 structurally different MC was determined and demonstrated MC congener-dependent inhibition activity and a lower susceptibility of PPP5 to inhibition than PPP1 and PPP2A. The data were employed to train a ML algorithm that allows prediction of PPP inhibition (toxicity) based on 2D chemical structure of MC. IC50 values were classified into three toxicity classes, and three ML models were used to predict the toxicity class, resulting in 80-90% correct toxicity predictions, thereby providing an initial step towards in silico hazard predictions for MC and thus a basis for improved risk assessment.

26

Toxicological evaluation of carbon fibre dusts after air-liquid interface exposure of lung cell cultures

A. Friesen[1], S. Fritsch-Decker[2], S. Mülhopt[3], C. Weiss[2], D. Stapf[3], A. Hartwig[1]

[1]Karlsruhe Institute of Technology, Institute of Applied Biosciences, Department of Food Chemistry and Toxicology, Karlsruhe, Germany

[2]Karlsruhe Institute of Technology, Institute of Biological and Chemical Systems - Biological Information Processing, Eggenstein-Leopoldshafen, Germany

[3]Karlsruhe Institute of Technology, Institute of Technical Chemistry, Eggenstein-Leopoldshafen, Germany

The role of carbon fibres (CF) as an advanced material is growing in various sectors of the industry, for example in the automotive, aerospace or wind power industries. However, there are only few studies addressing the release of CF dusts and their toxicological impact after inhalation. To fill this gap, within the CarbonFibreCycle project air-liquid interface (ALI) cultures composed of bronchial epithelial cells (BEAS-2B) and macrophage-like cells (THP-1) were established and exposed to a single dose of differently processed CFs via the Vitrocell® Automated Exposure Station. A high modulus CF was either milled in a ball mill or heated in a tube furnace at 800°C and then milled. The CF fragments were aerosolized and fed into the exposure station. The deposition within the exposure chamber and the resulting doses were measured by digital microscopy as well as by quartz crystal microbalance. After different post-incubation periods, the cultures were assessed with regard to viability (LDH, cell count), genotoxic effects (alkaline unwinding), gene expression (high-throughput RT-qPCR) and cytokine release (IL-8).

Exposure towards mechanically processed CFs did not generate any cytotoxic response in mono- (BEAS-2B) or cocultures (BEAS-2B/dTHP-1). In monocultures, gene expression profiles revealed a time dependent response in the inflammation and apoptosis clusters, which peaked at 1 hour of exposure and decreased with increasing post-incubation time. The initial increase in pro-inflammatory gene expression was mirrored in the IL-8 ELISA; however, cytokine release further increased with longer post-incubation times. While differences between mono- and cocultured cells were minor at the level of gene expression, IL-8 release was higher in cocultures.

Exposure towards thermally-mechanically processed CFs also did not induce cytotoxic effects as assessed with the LDH assay. However, this treatment resulted in a reduced cell count. The underlying mechanisms are still to be investigated.

In conclusion, we generated ALI cultures that are stable, easy to use and suitable for the assessment of fibre toxicity. Although the fibres were not highly toxic in the applied cultures, a specific response was detected on the gene expression and protein level, especially with respect to inflammation. The distinct responses induced by mechanically and thermally-mechanically processed fibres show that toxicological effects are highly dependent on fibre treatment and properties.

225

Laser ablation-ICP-MS as an alternative method to determine spatially resolved and quantitatively the organ burden directly in histological sections

S. B. Seiffert[1], I. Nordhorn[2], L. Ma-Hock[3], S. Gröters[3], O. Hachmöller[1], M. Wiemann[4], U. Karst[2], S. Kröger[1]

[1]BASF SE, Elemental Analysis, Ludwigshafen, Germany

[2]University of Münster, Institute of Inorganic and Analytical Chemistry, Münster, Germany

[3]BASF SE, Experimental Toxicology and Ecology, Ludwigshafen, Germany

[4]IBE R&D Institute for Lung Health gGmbH, Münster, Germany

Nowadays nanomaterials (NMs) are essential for a variety of applications and therefore their production volume along with their pollution into the environment is rapidly increasing. To access potential risks to organism, animal testings still remain a necessity in regulatory toxicology. However, the development of novel analytical tools and strategies can help to reduce the number of animals.

Whenever adverse reactions are found in the organs of NM-treated animals, additional animals are required to determine their organ burden. For this purpose, inductively coupled plasma-mass spectrometry (ICP-MS) or optical emission spectroscopy (OES) after digestion protocols are utilized. But these results are limited to the bulk concentration of NMs within the respective organ whereas spatially resolved information are lost. In contrast, laser ablation (LA)-ICP-MS provides spatially resolved and quantitative information of elements directly in histological sections without the need of additional animals.

In this study, we used LA-ICP-MS with matrix-matched gelatine standards to quantify various elemental distributions in different organs derived from animal testings on NMs. The developed method was validated by an interlaboratory comparison of the quantitative iron distribution in spleens from different aged rats, showing that it is independent of the sample thickness and sample preparation procedure. Furthermore, the quantitative NM distribution of samples after a short-term inhalation of ZnO NPs and samples after a long-term inhalation of CeO_2 are in good agreement to organ burden calculations by organ digestion of additional animals. Our data indicate that LA-ICP-MS is a promising method to quantify NMs in histological sections without the need of additional animals.