Annual Meeting 2021 – Poster Session

Poster 01

UroKin: Linking gastrointestinal metabolism with pharmacokinetics of dietary bioactives.

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As the gut microbiome is emerging as an additional site of xenobiotic metabolism, the impact of its bacterial composition on the chemical transformation of bioactives – and thus its contribution to interindividual differences in the host's response to ingested chemicals - is an important research question. Pursuing a quantitative approach, the UroKin project aims to link the microbial bioactivation of food constituents with physiologically-based pharmacokinetic (PBPK) modeling for urolithins, the beneficial gastrointestinal metabolites of dietary ellagitannins. Both, the type and the quantity of urolithin metabolites depends on the availability of yet unknown bacteria in the colon, so an additional goal is to identify the involved enzymes and microbes in order to facilitate quantitative in vitro to in vivo extrapolation (qIVIVE), taking into account interindividual differences in the composition of the gut microbiome.

To incorporate the colon as a dedicated compartment of metabolism in a PBPK model, we determined gut metabolism kinetics using anaerobic fecal fermentation techniques coupled with liquid chromatography. Liver metabolism was assessed by measuring glucuronidation in S9 fraction incubations. Further, using a combined in silico / in vitro biofermentation approach, we identified an enzyme class that performs the dehydroxylation of upstream metabolites to urolithin A, one of the final transformation products of interest.

These results are relevant for functional nutrition, and are expected to enable the prediction of population groups possessing a high average capability to utilize urolithins as nutraceuticals. Furthermore, the experimental methodology provides a blueprint for further research on the quantitative assessment of the impact of gut metabolism on pharmaco/toxicokinetics of chemicals.

Keywords: microbial metabolism, pharmacokinetics, PBPK modeling, biotransformation

Poster 02

Genome-wide mapping of O6-methylguanine

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The widely used chemotherapeutic drug temozolomide induces DNA alkylation such as O6-methylguanine (O6-MeG). O6-MeG causes mismatches upon replication leading to apoptosis by mismatch repair overload. However, O6-MeG can be recognized and directly repaired by O6-methylguanine-DNA methyltransferase (MGMT). Patients with high MGMT expression tend to be resistant towards temozolomide.

To study this resistance, identifying genomic patterns of temozolomide-induced DNA alkylation is crucial. Until now this has not been possible for O6-MeG at single nucleotide resolution. To address this limitation, we developed a new method for precisely locating O6-MeG in the whole genome. Our approach involves immunoaffinity enrichment with an anti-O6-MeG antibody and subsequent stalling of a high-fidelity polymerase at the damaged site. Sequencing data from DNA exposed to temozolomide revealed preferential formation of O6-MeG in certain trinucleotide sequence contexts. Moreover, this DNA damage signature was highly similar to the mutational signature found in patients treated with temozolomide.

In our current work, we aim to uncover how the MGMT expression level affects damage distribution, which will lay ground for understanding resistance to chemotherapeutic drugs.

Keywords: DNA damage, whole genome sequencing, temozolomide, MGMT

Poster 03

Reactivity of Benzo[a]pyrene towards RNA

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Benzo[a]pyrene (B[a]P) is a ubiquitous environmental carcinogen generated by incomplete combustion of organic material. Main sources of daily exposure are high-temperature processed food and cigarette smoke. Upon metabolic activation, B[a]P forms the highly reactive metabolite B[a]P-7,8-dihydrodiol-9,10-epoxide (BPDE) that reacts with genomic DNA to form mutagenic BPDE-deoxyguanosine (BPDE-dG) adducts. Early literature has shown that BPDE reactivity is not limited to DNA but also targets RNA. However, the abundance, stability and biological role of these BPDE-RNA adducts remain elusive.

The aim of this study was to get a better understanding of the BPDE reactivity towards RNA and structurally characterize the resulting adducts. We exposed total RNA to various concentrations of (±)-anti BPDE and could thereby demonstrate the formation of RNA-BPDE adducts by LC-MS/MS. While BPDE-guanosine (BPDE-G) was the major adduct formed, we could also detect traces of BPDE-cytidine (BPDE-C) and BPDE-adenosine (BPDE-A). Furthermore, BPDE-G adducts formed instantly after carcinogen addition and increased linearly with increasing concentration of (±)-anti BPDE.

Our study is one of the first to demonstrate the formation and relative levels of BPDE-RNA adducts and thus sets the basis for further investigations aimed at demonstrating their existence and biological function in human cells. Thus, RNA adducts may be promising biomarkers for diseases and powerful alternatives to currently used genotoxic markers.

Keywords: RNA adducts, Benzo[a]pyrene, LC-MS/MS

Poster 04

Improving chemotherapeutic drugs by targeting protein-protein interaction of DNA polymerase ζ

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Platinum-based chemotherapeutic agents are used to treat a diverse range of cancer types. An adverse outcome of this therapy is the development of drug resistance. One strategy to combat this resistance is inhibiting enzymes that have a proclivity to replicate past DNA adducts formed from cells exposed to these drugs. Human DNA polymerase ζ (hPol ζ) is one such enzyme that can bypass platinum-based DNA adducts. It has been found in clinical studies that human Pol ζ expression increases following platinum drug therapy and, therefore, contributes to drug resistance. Human Pol ζ is a tetrameric protein composed of a catalytic subunit (Rev3), a non-catalytic subunit (Rev7) and two accessory subunits (PolD2 and PolD3). The binding of Rev3 to the "safety belt" region of Rev7 is essential for the activity of the enzyme and induces a conformational change in Rev7, from an "open" to "close" state. Due to the lack of enzymatic activity of Rev7, and the difficulty in expressing and purifying human Rev3 because of its large size (353 kDa), our current understanding about the Rev3-Rev7 interaction is limited. This gap in knowledge restricts our ability to target hPol ζ and learn more about its biochemical function.

The goal of this work is to structurally evaluate the Rev3-Rev7 interface to develop inhibition strategies to combat resistance. This work will present (1) the rational design of small molecules to inhibit the Rev7-Rev3 interaction (2) the chemical synthesis of small molecules and (3) the development of microscale thermophoresis and Ni2+ pull-down assays to study the structure-activity relationship of these small molecules on their ability to disrupt the safety belt interaction.

Overall, this work will provide structural insight into human TLS Pol ζ and contribute to the development of more effective chemotherapeutic strategies aimed at combating drug resistance.

Keywords: Drug resistance, DNA polymerase, translesion DNA synthesis, platinum-based drugs, drug discovery.

Poster 05 (selected for Short Presentation)

Indirect embryo-fetal risks of nanoparticles: Impact on human placental function, the release of placental signaling factors and subsequent alterations on angiogenic and neurodevelopmental processes

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Prenatal exposure to several environmental and engineered nanoparticles (NPs) has been associated with severe adverse effects on fetal development and health later in life. Concurrently, mechanisms underlying the developmental toxicity of NPs remain largely unknown. Since maternal to fetal transfer of NPs is often low or even absent while particles tend to accumulate in the placental tissue, we speculate that indirect placenta-mediated effects are involved in or responsible for the observed developmental toxicity of NPs. Specifically, NPs could interfere with placental signaling pathways essential to successful pregnancy outcomes. Our aim is to investigate the impact of selected NPs (titanium dioxide (TiO2), nanosilica (SiO2) NPs and diesel exhaust particles (DEPs); developmental toxicity previously described), on human placental tissue viability and functionality with a focus on the secretion of placenta-specific vascular, endocrine and inflammatory factors.

Non-lethal concentrations of the NPs were defined in BeWo trophoblast cells for subsequent functional studies. We then successfully established human placental explant cultures from early and late pregnancy and evaluated their viability and functionality. In human placental explants, TiO2 and SiO2 NPs did not induce major cell death. However, they altered hCG secretion levels in placental explants from early pregnancy. Furthermore, secretion profiling (multiplex arrays) indicate effects of tested NPs (TiO2 NPs, SiO2 NPs, and DEPs) with inflammatory (eg. G-CSF), vascular (eg. Angiopoietin-2) and endocrine (eg. leptin) signaling factors.

In conclusion, results revealed significant interference of TiO2 and SiO2 NPs with key pregnancy hormone secretion, hCG, as well as potential interference of TiO2 NPs, SiO2 NPs and DEPs with placental signaling processes. Further studies are ongoing to 1) perform an unbiased secretomics profiling and 2) understand whether conditioned media from NP-treated explants affect angiogenic and neurodevelopmental processes relevant to fetal wellbeing. Our mechanistic insights on NP-induced toxicity at the placenta will be indispensable for the safe use of NPs and the protection of pregnant women and their unborn children.

Keywords: Nanoparticles, placenta, developmental toxicity, cell-signaling

Poster 06

Coupling H295R with U2OS ERα and AR CALUX bioassays increases steroidogenesis test sensitivity

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Over the last years, increased regulatory and public attention has been put on endocrine active substances, including those with effects on steroid hormone synthesis. To screen and test chemicals that potentially affect steroidogenesis, the in vitro H295R assay (OECD TG 456) utilizes a cell line to detect interference with 17β-estradiol and testosterone production. However, the detection methods currently in use (immunoassay or mass spectrometry) lack the required sensitivity for the detection of minimal changes in hormone levels. This can be a major drawback for the analysis of mixtures originating from food or food contact materials, which may only influence hormone levels to a small extent.

The present study trialed the use of the U2OS ERα and AR CALUX receptor activity as a detection system for the original H295R assay, as CALUX tests exhibit a substantial change in reporter gene activity in response to a small change in hormone levels. To accommodate the CALUX detection system, the following refinements were made: i. perfluorooctanesulfonic acid and forskolin were used as positive controls for induction and ii. parallel CALUX assays were performed on the same plate without prior exposure to H295R cells to control for any direct effects of the chemicals on the androgen and estrogen receptors. The performance of the assay was evaluated in a proof of concept study with chemicals from the original OECD validation study. The respective lowest observed effect concentrations (LOECs) were at least equivalent to, and often lower than, those described by the OECD. For some substances, direct estrogenic and anti-androgenic activities were identified, complicating H295R assay interpretation and suggesting that some steroidogenesis-mediated endocrine activities could be masked by direct receptor (ant)agonism of test substances. Nonetheless, these modalities are also relevant for the characterization of endocrine active substances and can provide additional valuable mechanistic information.

Taken together, the results from this proof of concept study indicate a solid overall performance with significant additional gains in the detection of small changes in steroidogenesis, suggesting that the linked H295R-CALUX assay might have promise for the analysis of mixtures originating from food or food contact materials.

Keywords: steroidogenesis, H295R, CALUX, endocrine activity, endocrine disruption

Poster 07

Investigation of the interplay between oxidative stress and antioxidant defense in 3-dimensional primary human liver microtissues

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Poster presented by **Fabrice Mueller**

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Reactive oxygen species (ROS) have been implicated in the pathogenesis of numerous diseases as well as hepatotoxicity. Menadione, a synthetic analog of vitamin K (K3), is a redox cycling quinone known for causing oxidative stress. N-acetyl cysteine (NAC), a precursor for glutathione (GSH) synthesis, plays a pivotal role in protecting cells from ROS. Here, we investigated the relationship between menadione-induced toxicity and antioxidant response in 3D primary human liver microtissues (MTs).

3D InSight™ Human Liver Microtissues (Insphero AG, Schlieren, Switzerland) consisting of primary hepatocytes and non-parenchymal cells were used as a reliable model for hepatotoxicity studies. The MTs were pre-incubated with medium (control) or 250 µM NAC for 4 h to boost antioxidant response. MTs were subsequently treated for 1.5 h, 4 h or 20 h with 12.5 µM menadione (with or without NAC co-incubation). The response to the different treatments was evaluated using viability assay (ATP content), GSH content, expression of genes involved in antioxidant response (qPCR), and superoxide formation (MitoSOX™ staining).

Menadione induced toxicity in MTs with an EC50 of approximately 11 µM; cytotoxicity was completely prevented by NAC. Time-course experiments showed that menadione alone induced a significant and time-dependent GSH depletion after 4 and 20 h while cytotoxic effects shown by ATP depletion and superoxide formation were only detectable after 20 h of incubation. A significant upregulation of Nrf2-regulated genes (HMOX1, NQO1, SOD1, and TXN) was observed after 20 h incubation with menadione alone, but not in presence of NAC. Moreover, heme oxygenase 1 (HMOX1) was transcriptionally induced in a time-dependent manner.

Oxidative stress caused by menadione led to immediate cellular GSH depletion followed by superoxide formation and cell death. Effects on GSH-content were observed already at 1.5 h, whereas increased superoxide concentration was seen only after 20 h incubation. Consistent with the early onset response, menadione treatment triggered the Nrf2 antioxidant defense pathway, with first gene expression changes detectable as early as 1.5 h after treatment. Our experiments demonstrate for the first time in primary human liver MTs that menadione-induced

toxicity can be completely prevented by boosting GSH content with NAC. Overall, our results show that primary human liver MTs are a suitable model to study ROS-related hepatotoxicity and to elucidate the kinetic of antioxidative responses in complex, multicellular systems.

Poster 08

Biological evaluation of steroid 11β-hydroxylase inhibitors identified by virtual screening

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Humans are exposed to a multitude of xenobiotics that can potentially interfere with the endocrine system, including steroidogenesis. Recent reports documented cases of severe hypertension and hypokalemia as a result of an inhibition of the steroidogenic enzyme steroid 11β-hydroxylase (CYP11B1). The enzyme catalyzes the formation of cortisol and corticosterone from deoxycortisol and deoxycorticosterone, respectively. Its inhibition leads to pseudohyperaldosteronism, causing an excessive activation of the mineralocorticoid receptor.

This study applied in silico screening of the DrugBank database to identify drugs potentially inhibiting CYP11B1 and causing secondary hypertension. Hits were screened in a cell-free mitochondrial assay prior to evaluation in a cell-based model overexpressing human CYP11B1. Three inhibitors were identified, the most potent being liarozole with an IC50 of 4.2 nM. Tipifarnib and talarozol were less potent with IC50 values of 195 nM and 1.77 µM, respectively.

This project describes a strategy to identify potential endocrine disruptors leading to mineralocorticoid excess thus increasing the risk of secondary hypertension.

Keywords: CYP11B1, hypertension, endocrine disruptor, screening, inhibitor

Poster 09

A quantitative map of DNA damage in the human genome caused by benzo[a]pyrene

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Benzo[a]pyrene (BaP) is a known human carcinogen (IARC group 1) found in food, coal tar, cigarette smoke, and industrial smoke. Its diol-epoxide metabolites (BPDE) can react with DNA, predominantly forming N2-BPDE-deoxyguanine (N2-BPDE-dG). While the ability of BPDE to alkylate DNA and induce mutations is well described, little is known about how genomic features influence its reactivity profile and how repair mechanisms shape the resulting mutational landscape in the human genome.

To bridge this gap, we adapted a recently published damage-sequencing method to enable genomic localization of N2-BPDE-dG. We then combined this technique with liquid chromatography with tandem mass spectrometry (LC–MS/MS) to generate a single-nucleotide resolution quantitative map of N2-BPDE-dG in the human genome.

We observed a dose-dependent increase in damage formation, with the damaged DNA being enriched in the same manner. Furthermore, we performed mRNA sequencing and bisulfite sequencing and observed that the global distribution of N2-BPDE-dG appears to be correlated with the guanine–cytosine content, and its local distribution is possibly influenced by chromatin state, transcriptional activity, and DNA methylation levels. Finally, we resolved the preferred local sequence contexts for N2-BPDE-dG formation, which revealed a high cosine similarity with relevant mutational signatures extracted from cancer patient samples.

These findings show that single-nucleotide-resolution damage sequencing is a powerful tool for identifying factors that lead to preferential DNA damage and repair and deepens our understanding of damage-induced mutagenesis/carcinogenesis.

Keywords: Benzo[a]pyrene, BPDE-dG adduct, DNA damage sequencing, single-nucleotide resolution, mutational signature

Poster 10

Activation of retinoic acid-related orphan receptor γ(t) by parabens and UV-filters

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Retinoic acid-related orphan receptor γt (RORγt) regulates the expression of inflammatory mediators and its impaired function contributes to inflammatory and autoimmune diseases. To treat such pathologies, several synthetic inverse agonists have been developed to suppress RORγt-dependent inflammatory target genes, including interleukin-17A (IL-17A). Less is known on exogenous chemicals from environmental sources that may exert adverse effects by activating RORγt. Parabens and UV-filters are frequently used as additives in cosmetics and body care products and intensively inspected for endocrine disrupting properties.

This study assessed whether parabens and UV-filters can interfere with RORγ activity in CHO-RORγ-Tet-on cells. Transactivation experiments revealed hexylparaben, benzylparaben and benzophenone-10 as RORγ activators, with estimated EC50 values of 144 ± 97 nM, 3.39 ± 1.74 μM and 1.67 ± 1.04 μM, respectively. These compounds also were able to restore RORγ activity after suppression by an inverse agonist. Furthermore, they enhanced RORγt-dependent IL-17A and/or IL-22 expression in EL4 mouse T lymphocytes. Virtual screening of a cosmetics database for structurally similar chemicals and in vitro testing of the most promising hits revealed benzylbenzoate, benzylsalicylate and 4-methylphenylbenzoate as additional RORγ agonists, with lower micromolar EC50 values.

Importantly, the newly identified compounds showed additive agonistic effects towards RORγ. By activating RORγt the identified parabens and UV-filters may potentially aggravate pathophysiological conditions, especially skin diseases where highest exposure of such chemicals can be expected. Follow-on studies need to assess whether the identified RORγt agonistic parabens and benzophenones can reach relevant concentrations, either alone or as mixtures, in tissues and target cells to affect RORγt regulation in vivo.

Keywords: Retinoic acid-related orphan receptor gamma; paraben; UV-filter; Th17 cell; autoimmune disease.

Poster 11

Larval zebrafish recover rapidly from insecticide-induced developmental and behavioral toxicity

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Insecticides are inevitably released into the environment and may cause harm to non-target organisms. It is therefore particularly important to assess potential effects on non-target organisms and to estimate the risk posed by insecticides before they are authorized and released. In order to determine the concentrations at which an adverse effect can be observed, standardized toxicity tests are applied. However, these methods do not include the potential recovery or reversibility of effects. Neglecting this parameter in risk assessment may lead to an overestimation of risk.

We therefore assessed whether zebrafish larvae are able to recover from the effects caused by exposure to different insecticides. From 2 hpf to 144 hpf, zebrafish were exposed to a single concentration of the respective insecticide. At 144 hpf, larvae were transferred to chemical-free embryo medium (depuration phase) and where kept until 216 hpf (9 days). To monitor the recovery process, swimming activity was measured every 24 hours of the depuration phase, while body length and heart rate were measured only on day 9. For dimethoate, methomyl, imidacloprid, and thiacloprid a full recovery of the swimming activity was observed within the first 24 hours of depuration.

For larvae exposed to diazinon, recovery took a little longer as they showed full recovery of locomotion after three days. Larvae exposed to pirimicarb, however, did not recover. The heart rate measurements showed that the heart rate of diazinon-exposed fish did not recover within three days, while a recovery was observed for fish exposed to dimethoate, methomyl, and thiacloprid. With regard to body length, three days of depuration from diazinon and thiacloprid led to a recovery to control level. However, body length did not recover when larvae were exposed to pirimicarb and methomyl. Generally, we found that zebrafish larvae were able to recover from insecticide exposure within a short time. This indicates that developmental exposure to the tested insecticides did not alter the neuronal structure but rather induced changes in neurophysiology.

Differences in recovery after exposure to insecticides with same mode of action is most likely due to differences in absorption, distribution, metabolism, and excretion of the respective chemical.

Poster 12

Benzoyl-CoA conjugate accumulation as an initiating event for male reprotoxic effects in the rat: Structure-activity analysis, species specificity and *in vivo* relevance

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A number of para substituted benzoic acids (p-BA) and fragrance aldehydes such as 3-(4-tert-butylphenyl)-2-methylpropanal (BMHCA) metabolized to p-BA have been found to confer adverse effects in male rats on sperm viability, motility and morphology following oral gavage dose of >25 mg/kg body weight. These effects are putatively associated with the metabolism of p-BA to toxic intermediates. We had shown that p-BA lead to accumulation of high levels of benzoyl-CoA conjugates in plated rat hepatocytes.

Here we further investigated relevance of this metabolic pathway for the reprotoxic effects in rats and rabbits. Intracellular formation of CoA conjugates from either p-alkyl-phenyl-propanals directly or from their benzoic acid metabolites was assessed for 19 chemicals in plated primary rat hepatocytes using high-resolution LC-MS. The nine reprotoxic compounds metabolizing to p-alkyl-benzoic acids led to accumulation of high and stable levels of p-alkyl-benzoyl-CoA conjugates in plated rat hepatocytes, whereas benzoyl-CoA conjugates of ten non-reprotoxic compounds were only transiently formed in this in vitro system which confirms the very strong correlation between benzoyl-CoA-accumulation in rat hepatocytes and the toxic outcome. Species specificity was probed by comparing rat, rabbit and human hepatocytes. Benzoyl-CoA accumulation was found to be specific to the rat hepatocytes, not occurring in human hepatocytes. There was also very limited accumulation in hepatocytes from rabbits that are a non-responder species in in vivo studies. Tissues of rats orally treated with 3-(4-isopropylphenyl)-2-methylpropanal were analyzed and p-isopropyl-benzoyl-CoA conjugates were detected in the liver and in the testes in animals at toxic doses indicating that the metabolism observed in vitro is relevant to the in vivo situation and the critical metabolite does also occur in the reproductive tissue.

These multiple lines of evidence further support benzoyl-CoA accumulation as a key initiating event for a specific group of male reproductive toxicants, and indicate a species specific effect in the rat.

Keyword: Male reproductive toxicity, metabolism, acyl-CoA, in vitro, fragrance aldehyde

Poster 13 (selected for Short Presentation)

Estrogen activity characterization coupling bioassays to chemical identification

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New chemical analysis and *in vitro* assays have been developed for identification of estrogen activity (EA) in food and food-related items. However, consistency between the chemical analysis and the measured biological activity has not yet been addressed. A multidisciplinary approach combines targeted chemical analytics and effect-based *in vitro* bioassays to facilitate characterization of EA present in food. Cell culture-based bioassays are available for the detection of endocrine receptor activation of pure compounds or mixtures. However, in the case of mixtures, this approach gives no insight about the molecules responsible for the activity. The coupling of the bioassay to a prior High Performance Thin-Layer Chromatography (HPTLC) is proposed as a solution to facilitate identification of responsible substances. HPTLC includes sample separation and coupling to cell-based assay to highlight targeted bioactive bands. In addition, it allows to recover the bioactive bands for chemical identification of the substance(s) responsible for the biological activity.

Case study was applied using soy protein isolates. Soy isolate samples were analyzed for estrogen receptor (ERα) mediated activity by both the CALUX assay and the HPTLC coupled to an estrogen activation yeast-based assay (p-YES). Identified active bands were then analyzed using targeted LC-MS/MS.

Extracts of soy isolates induced dose-dependent estrogen receptor activation using the CALUX and the pYES assays. Concordance between the quantified molecules identified with LC-MS/MS targeted analysis and both *in vitro* assays was analysed. The molecules identified in pYES bioactive bands were consistent with the chemical analysis. New bioactive signals corresponding to two molecules were found and identified by LC-MS/MS untargeted screening. The performance of the HPTLC coupled to bioassays overcomes some limitations of the multi-well plates approaches with good detection limit, reliability, and versatility in its application. Overall, using detection tools like the pYES assay has demonstrated the advantages of the approach to characterize qualitatively and quantitatively estrogenic activity. The approach may be extended to other effect-based *in vitro* toxicological endpoints for prioritization and decision-making.

Keywords: Food safety, endocrine activity, in vitro toxicology, HPTLC coupled to bioassays

Poster 14

Application of HPTLC-AChE inhibition for assessment of food contact materials in the context of the TTC

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The threshold of toxicological concern (TTC) is widely used in risk assessment of substances with known structure for which toxicological data are absent or limited. The assessment starts with questioning on the presence of structural alerts for genotoxicity. A second level consists in identifying chemicals structurally similar to organophosphates (OPs) and carbamates (CBs), which exert neurotoxicity by inhibiting the enzymatic activity of acetylcholinesterase (AChE). Some chemicals used in food contact materials (e.g. phosphites used as antioxidants that might be oxidized into their phosphate forms) may also inhibit AChE, although they do not exhibit the structural alert and the TTC for OP/CBs would not apply. Such activity would interfere with the interpretation of a potential packaging contamination with CBs and/or OPs. Their identification in packaging migrates is relevant for safety assessment.

In this context, we applied the HPTLC-S9-AChE inhibition assay on two migrates and one extract of a can. Initial testing of concentrated samples (5 µL, 40X) showed two inhibition bands (in absence and presence of S9 metabolic fraction). HRMS analysis indicated the presence of cyclic oligomers and linear alkylbenzene sulfonates (LAS). Once tested individually in a concentration-range, LAS inhibited the AChE activity at high level while oligomers were inactive. No inhibition bands were observed after the direct application (200 µL) of non-concentrated extract and migrates. The additional concentration step resulted in the generation of false positive results. As our model is sensitive enough, it is recommended to work with non-concentrated samples. A risk assessment was performed on the presence of these substances in the packaging material.

Keywords: Acetylcholinesterase inhibition, HPTLC, food contact materials, TTC

Poster 15

Reducing the obstacles for the use of *in vitro* toxicity data of nanomaterials in Life Cycle Assessment and Human Risk Assessment

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The choice of doses used in in vitro toxicity testing of nanomaterials is often not scientifically justified, which makes it questionable whether the observed effects are relevant for humans or whether they are caused by cells being overloaded (Gangwal et al. 2011). This is a challenge for those methodologies such as Human Health Risk Assessment and Life Cycle Assessment that would benefit from using *in vitro* toxicity data as a replacement for animal data, which is becoming more and more scarce.

To help with the selection and evaluation of in vitro doses we developed a Combined Dosimetry model (CoDo) that calculates the exposure levels corresponding to the concentrations used in *in vitro* submerged systems (thus focusing on the inhalation exposure route). The model is based on the combination of *in vitro* dosimetry, which estimates the deposited dose *in vitro*, and lung dosimetry, which simulates the deposition of particles in the human lung. Multiple scenarios can be considered to account for different conditions in the *in vitro* system and in the human exposure scenario.

To demonstrate the usefulness of the model we tested it on a systematically-collected data set of titanium dioxide toxicity data. First, we compared the doses used in vitro with the Occupational Exposure Limit for titanium dioxide in Switzerland, confirming that the doses used *in vitro* are generally representative of longer exposure times on the workplace. Then, we calculated the Benchmark Doses for both the *in vitro* data set and *in vivo* data for Titanium dioxide, and observed in vitro effects such as cytotoxicity and cytokine release to occur at higher doses than inflammation in rodents, despite both data displayed great variability.

Overall, we demonstrated that combined dosimetry can be an effective way for the selection and analysis of in *vitro* doses, and the comparison of *in vitro* and in *vivo* effects.

Keywords: Nanoparticles, Dosimetry, Occupation Exposure Level, Inhalation

Poster 16

Differential contributions of UVA and UVB in the formation of the common deletion in human skin fibroblasts

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The common deletion (CD) is a pathological mutation found in mitochondrial DNA (mtDNA) associated with physiological aging, neuromuscular disorders, and certain types of cancer. While perturbations in mtDNA replication and repair have been connected to the generation of the CD, mechanisms underlying its formation remain largely unexplored.

Given the described accumulation of the CD in UV-exposed fibroblasts, in this study we aimed to dissect the differential contributions of UVA and UVB in the generation of this deletion. We irradiated human skin fibroblasts with UVA and UVB and performed assays to monitor the CD content and mitochondrial function. We found that UVA and UVB induce CD formation, albeit by different mechanisms. UVA treatments resulted in increased reactive oxygen species (ROS), oxidation of mtDNA bases, and elevated CD content. By treating fibroblasts with antioxidants, we found that the UVA-dependent increase in CD content is inhibited, suggesting oxidative stress as a determinant in the generation of this mutation. Conversely, UVB irradiation leads to the formation of mtDNA cyclobutane pyrimidine dimers and, unlike UVA, suggests a ROS-independent pathway for the CD formation.

We also monitored changes in the expression of genes encoding mtDNA maintenance factors and found deregulated expression in several genes involved in mtDNA replication and repair. We overexpressed a panel of these factors in a cybrid cell line derived from a patient with a pathology typified by high CD levels. We found that overexpression of these genes decreases the CD, directly connecting these genes to mechanisms controlling its maintenance.

Taken together, these findings contribute new mechanistic insight underlying the accumulation of the CD and could help in developing treatments targeting the CD in pathological conditions.

Keywords: Mitochondria, common deletion, UV light, DNA repair, DNA replication

Poster 17

Nanopore detection of carboxymethylated DNA bases

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Carboxymethyl DNA adducts are chemical modifications associated with high red and processed meat consumption. They can impede DNA replication machinery and induce mutations potentially leading to the development of colorectal cancer. Studies in polymerase knock-out cells indicate that carboxymethyl DNA adducts, such as O6-CMG, can be bypassed by human DNA polymerases and induce mutations. However, the repair and distribution of DNA carboxymethylation and how it affects mutational distribution in the human genome is not well characterized, in part due to a lack of methods for single-base resolution detection of carboxymethyl adducts.

In this work, we developed a nanopore-based approach for the detection of the carboxymethyl adduct O6-CMG in DNA. With nanopore sequencing, nucleotides can be identified by measuring current changes as DNA passes through a nanopore. Thus, any structural modifications to DNA could be detected, making it an ideal but underutilized platform for mapping DNA lesions. We synthesized DNA oligonucleotides containing a methylated and carboxymethylated guanine and incorporated them into a plasmid. Characteristic signals for specific modified bases were observed with high coverage.

Application of this approach to biological samples is expected to lead to better characterization of the chemical and molecular basis of carboxymethyl DNA adduct mutagenicity and elucidation of how the mechanisms of adduct formation and repair affect mutation distribution.

Poster 18 (selected for Short Presentation)

Fibrosis-specific extracellular miRNAs promote stellate cell activation

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In this work, we sought to identify extracellular miRNAs that are potential specific biomarkers for liver fibrosis and may be considered a diagnostic alternative to liver biopsies, which are a currently applied clinical method. miRNAs are single-stranded, non-coding RNAs that can be detected in tissues, body fluids, cells and cell culture supernatants. In patients, several miRNAs have been linked to tissue damage and disease, including liver failure and liver fibrosis. The exact role that released miRNAs may play in the progression of the cellular damage is often unclear.

We set out to identify and investigate miRNAs released from a well-characterized 3D-multicellular hepatic culture system undergoing fibrosis. Such biomarkers may be translational and applicable for *in vitro* investigations of liver fibrosis and potentially also for clinical diagnosis. Moreover, we sought to elucidate the potential role of the released miRNAs as actuators promoting stellate cell activation and fibrosis.

We used a human liver microtissues (MTs) model comprising HepaRG, hTERT-HSC and THP-1 cells and compared the effects of the profibrotic methotrexate (MTX) and the acute hepatotoxicant acetaminophen (APAP). We corroborated clinically relevant compound-specific responses, as MTX induced a fibrotic response and APAP did not. Using miRNA-seq of cell culture supernatants from MTX and APAP treated MTs, we identified potential miRNA biomarkers (miR-199a-5p, miR-214-3p, miR-125a-5p and miR-99b-5p) that were associated with a fibrotic phenotype induced by MTX. Furthermore, we demonstrated that miR-199a-5p, miR-214-3p and miR-99b-5p play a role in HSC activation. Transfection of hTERT-HSCs with miR-199a-5p, miR-214-5p and miR-99b-5p mimics led to increased α-SMA expression; while miR-199a-5p and miR-214-3p also promoted hTERT-HSC migration.

In conclusion, we suggest that extracellular miR-125a-5p and more specifically miR-214-3p, miR-99b-5p and miR-199a-5p, which promote HSC activation, could contribute towards a liver fibrosis-specific panel of miRNAs.

Poster 19

Black Box: Toxicology of Oligomers from Food Contact Materials

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The global production and use of food contact materials (FCM) is steadily increasing. More than 30% of packaging materials are made from plastics. During the polymerization reaction or degradation of such polymeric materials, linear and cyclic oligomeric molecules can be formed. Depending on the type of polymer, the contact conditions and the physicochemical properties of the oligomer, it can migrate into food when it comes into contact with it. Despite the dietary intake and high concentration of oligomers in FCMs, they have been poorly characterized toxicologically. This is mainly due to their large number and variety, lack of analytical methods, and very limited commercial availability, making toxicological testing in accordance with regulatory standards challenging. Due to the diversity and complex mixtures, it is difficult to determine their concentrations in food and estimate exposure, resulting in large knowledge gaps. The development of a systematic approach should allow chemical grouping and read-across to perform a reasonable risk assessment for all oligomers within a specific group.

Therefore, a multidisciplinary approach is developed using polyethylene terephthalate (PET) oligomers as a proof-of-concept. The aim is to better understand biological activity ranges of a group of oligomers in the context of other disciplines, using chemical, migration, exposure, toxicokinetic, and toxicological data.

This approach may enable hazard profiling and facilitate risk assessment of all PET oligomers, which can in a next step be extended to other materials and their respective oligomers.

Keywords: Polyethylene terephthalate, oligomers, risk assessment

Poster 20

Fluorescence-based Quantitation of Common Alterations in Genomic DNA for Accurate Damage Sequencing

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The integrity of DNA is constantly challenged by exposure to chemicals or radiation. To better understand adverse outcomes initiated by DNA damage formation, new DNA damage sequencing methods are rapidly emerging. A common strategy for single-nucleotide resolution damage sequencing relies on producing a 3′-hydroxyl group at the damage position of interest by using repair enzymes. However, these enzymes also generate interim apurinic sites and single strand breaks, which are by nature abundant in genomic DNA, making it impossible to uncover low abundance damage patterns.

Therefore, we used a ligation-mediated fluorescent probe-based damage quantitation approach to develop a strategy for minimizing background damage in genomic DNA. By this approach, we could detect elevated levels of 3′-hydroxyl groups based on increased fluorescence intensity between enzymatically treated vs. native DNA. Then, to mask pre-existing damage, we optimized enzymatic blocking of apurinic sites, gaps and single strand breaks in genomic DNA samples and validated the strategy based on decreases in fluorescence signal compared to native genomic DNA.

In conclusion, we developed an effective and general method for the quantitation and removal of adventitious common DNA damage products needed for genome-wide characterization of genotoxicants.

Poster 21

Human proximal tubule kidney barrier for toxicity testing under static and perfusion conditions

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The proximal tubule of the kidney consists of epithelial cells (RPTEC) that are involved in secretion and reabsorption of molecules, drugs, and metabolites via active transporters. These cells are common targets for toxicity. RPTECs form a tight barrier and are ciliated and constantly exposed to flow. Vitrofluid is a multicompartmental microphysiological system for perfused cultures which can accommodate one or several tissues. Here, we set off to characterize RPTECs maintained under static or dynamic culture conditions in terms of cell behavior and response to toxicants.

Primary human RPTECs were cultured on collagen-coated polycarbonate membranes and maintained over 14 days under static (transwell) or flow (Vitrofluid, Philip Morris International, Neuchâtel, Switzerland; flow rate of 150 µL/min) conditions. The formation of a leak-tight barrier was demonstrated by immunostaining (zonula occludens 1: ZO-1) and the apparent permeability of Lucifer Yellow. Gene expression of specific transporters (SLC22a2, SLC22a6, LRP2, hOAT-1, CUBN, and AQP1) and albumin uptake were also determined. The morphology and orientation of cilia were investigated by immunostaining (acetylated tubulin). The effect of several nephrotoxicants (colistin, tenofovir, benzo[α]pyrene, cadmium chloride, ochratoxin A, and methylmercury II chloride) on RPTECs exposed for 48 h was evaluated by viability assays (ATP content) and cell proliferation (Ki-67 immunostaining).

The human kidney barrier model developed with human primary RPTECs under both static and dynamic conditions was viable and functional for 14 days. The cells formed an integral barrier characterized by tight junctions and low permeability. The RPTEC barrier retained the ability to reabsorb albumin and expressed several proximal tubule transporters (SLC22a2, SLC22a6, CUBN, and AQP1) but did not express LRP2 or hOAT1. Shear stress did not have any damaging effects, as assessed by barrier integrity and expression of transporters. However, continuous medium flow led to a marked change in the morphology of the cilia, which became significantly longer and aligned with the flow. RPTECs in static conditions responded to most test compounds with an expected decrease in cell viability. As an exception, tenofovir did not cause cytotoxicity, as it requires uptake via hOAT-1. Furthermore, studies with colistin under static or flow conditions showed that RPTECs grown in both systems exhibited a similar dose–response behavior to the compound.

Our results demonstrate the suitability of a human RPTEC model for transport and nephrotoxicity studies. Physiological flow conditions changed the morphology of the cilia without affecting barrier function or the sensitivity of the cells to colistin. Further studies should evaluate the sensitivity of the cells under flow conditions to other compounds. In addition, the exposure of proximal tubular cells to more physiological conditions (such as artificial urine) on their basolateral/luminal side could help restore LRP2 and hOAT1 expression.

Poster 22

NanoAmes™: An Ultra-miniaturized Format of Ames Test

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The assessment of mutagenicity potential of complex mixtures containing unidentified substances is challenging. Today, the most widely used test to identify mutagenic substances is the Ames test, which is associated with the lowest calculated limit of biological detection (LOBD) values. Miniaturized formats of the Ames test were reported to be more sensitive than the standard regulatory Ames assay but the LOBDs are still inadequate to cover regulatory and safety requirements for complex mixtures, which possibly contain mutagenic agents at very low, but health-relevant concentrations.

An ultra-miniaturized agar-based Ames method, the NanoAmes™, with promisingly improved detection capacity, was developed and evaluated with a set of 11 mutagenic compounds, selected to compare the dose response curves on five bacterial strains (in presence and absence of metabolic activation) to those of MicroAmes assays run in parallel. Mutagenicity effect was consistently detected at a significant lower sample requirement and with more favorable lowest effective concentration values. Aiming to address the determination of the LOBD, a new statistical approach is proposed to analyze Ames dose response curves to extract the minimum detectable value (MDV). According to the current study, the NanoAmes™ performed better in terms of lower detectability capacity (in average 68× compared to the MicroAmes).

Further analyses applying complex mixtures samples with spiking analysis at low dose of mutagenic compounds will confirm the application of this promising tool for mutagenicity assessment of unknown compounds potentially present in mixtures.

Keywords: Mutagenicity, miniaturized assay, food contact material, consumer products, consumer safety, Ames test, genotoxicity, 3R initiative

Poster 23

Herbicide-induced proteome modulation of Human Brain Microvascular Endothelial Cells and associated Extracellular Vesicles

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Paraquat is an herbicide known to induce damaging effect on several human brain cell types by generating reactive oxygen species. However, very little is known about PQ-induced effect on human brain microvascular endothelial cells (HBMECs). They are the most represented cells in the blood-brain barrier, whose main function is to protect the brain against toxic molecules. Additionally, extracellular vesicles (EVs), a nanosized particle released from the cells into the circulation, might be a cargo reflecting the PQ-induced toxic effect on HBMECs. The purpose of this research was to characterize the proteome content of EVs and whole cells from HBMECs after PQ exposure.

EVs were isolated using EVtrap beads (Tymora Analytical Operations) and characterized by their size distribution (NTA), their concentration (NTA) and the presence of well-known EV markers (western blots). High-throughput mass spectrometry-based quantitative proteomics by Data Independent Acquisition was then applied on whole HBMECs and HBMECs-derived EVs. Signature pathways of PQ-exposed HBMECs and EVs were analyzed by gene ontology terms and pathway enrichment (MetaCoreTM). Cellular assays were performed to verify proteomics results.

The main results highlighted that HBMECs and HBMECs-derived EVs exposed to PQ have common modulated pathways, namely the ubiquinone metabolism and the transcription HIF-1 targets. Both biological pathways are associated to the oxidative stress process confirming a damaging well-known mechanism of PQ. Interestingly, omics data also underlined that cholesterol metabolism was altered in HBMECs exposed to PQ.

In conclusion, our data confirmed that PQ has a detrimental effect on HBMECs by modulating proteins involved in the oxidative stress process. This effect is also observable in HBMECs-derived EVs. As EVs mirror their cell of origin, their common biological pathways may potentially be molecular signatures of the PQ-induced toxicity in the circulation.

Keywords Brain endothelial cells, extracellular vesicles; paraquat; proteomics; oxidative stress

Poster 24

Use of preclinical toxicity and pharmacokinetic studies in the Environmental Risk Assessment (ERA) of human pharmaceuticals

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During the development of human medicines, preclinical toxicity (PT) and pharmacokinetic (PK) studies are conducted before the environmental impact is considered. When the environmental risk assessment (ERA) is due, they are completed with no samples remaining for further investigations. This can be problematic, as an unfavorable result of the ERA will lead to a leaflet warning. After the launch of the medicinal product, competitors are likely to use this situation to their advantage, placing blame upon the new medicine. This may even be possible if the competing products are worse for the environment, as the evaluation standards became stricter with time and a re-evaluation of products already on the market is not foreseen. Planning ahead for the ERA during the PT and PK studies can open possibilities for additional measurements, which can help avoid apparently bad environmental properties such as consideration as PBT (persistent, bioaccumulative and toxic or very persistent and toxic) or other type of substance of very high concern (SVHC). The poster shows examples and gives some hints for a smarter approach.

Keywords: preclinical toxicity (PT) studies, pharmacokinetic (PK) studies, environmental risk assessment (ERA), leaflet warning, substance of very high concern (SVHC), persistent, bioaccumulative and toxic (PBT)

Poster 25

Simultaneous profiling of gut microbiota xenobiotic transformations

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The gut microbiota modulates host health not only through alterations in the levels of chemical-signaling metabolites, but also through xenobiotic transformation of chemicals. These xenobiotic transformations can be uniquely catalyzed by gut microbes and may lead to the formation of unexpected transformation products which can have altered toxicokinetic potential compared to the parent chemical. In recent years, many gut microbe-catalyzed xenobiotic transformations have been identified and found to be catalyzed by specific classes of gut bacteria. However, the impact of gut microbiota composition changes on the ability of the gut microbiota to perform xenobiotic transformation has not been comprehensively studied.

Here, we present an ex vivo method to assess the potential of a fecal microbiota to catalyze known and unknown gut microbe-mediated xenobiotic transformations. A mixture of chemicals known to be reduced or hydrolyzed by gut bacteria was used to assess the potential of the fecal microbiota to perform these transformations. The time-dependent change in amounts of chemicals and transformation products was tracked using targeted and untargeted LC-HRMS/MS, respectively. Furthermore, to investigate the effect of the addition of the chemical mixture on the fermentation of the fecal microbiota, we performed an untargeted metabolomic analysis.

We were able to successfully observe differences in several of the xenobiotic transformation potential of fecal microbiota. After 24 hours incubation with the chemical mixture, we could observe up to 100% loss of some of the starting chemicals.

Future research will aim to use this approach to profile the xenobiotic transformation potential of gut microbiota perturbed by chemical exposures as a basis of adverse health effects.

Poster 26 (Late submission)

The biotransformation and bioaccumulation of ionizable organic compounds in rainbow trout cell lines

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The assessment of chemicals for their bioaccumulative potential requires in vivo testing with fish. These tests are resource intense, costly, time consuming and of ethical concern due to the sacrifice of animals. Therefore, alternative in vitro models are being sought to replace these tests.

Recently, it has been demonstrated that in vitro bioassays with rainbow trout (Oncorhynchus mykiss) cell lines from liver (RTL-W1), gill (RTgill-W1) and gut (RTgutGC) can be used to determine biotransformation rate constants and, by means of in vitro-to-in-vivo extrapolation, predict bioconcentration of a neutral organic compound (benzo(a)pyrene) in rainbow trout. However, the usability of these cell lines to assess the biotransformation and bioaccumulation of ionizable organic compounds (IOCs) has not yet been investigated. IOCs comprise a large proportion of the chemicals in commerce and are ubiquitously detected in the environment and biota.

Therefore, we have started to explore how well fish cell lines represent the uptake of IOCs into fish. Based on the availability of high quality in vivo data and the substance's charge state at physiological pH, four anionic substances were selected. Among the selected IOCs are pesticides, pharmaceuticals and industrial chemicals. In a first step, non-toxic chemical concentrations were determined and chemical analytical procedures established such that chemical starting concentrations can safely be measured above quantification limits. Cell exposure is then performed over 48h, during which cells and exposure medium are sampled to derive chemical specific uptake and elimination rates. Two of the test substances showed bioconcentration in cell cultures while two other test compounds showed no bioconcentration. The conceptual approach, experimental results and further leading questions are presented.

Keywords: Biotransformation, bioaccumulation, ionizable organic compounds, fish cell lines, rainbow trout.

Poster 27 (Late submission)

RAINBOWFLOW CHIP: An impedance-based biosensor for chemical hazard assessment with fish cell lines at its core

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Fish form an integral part of aquatic ecosystems, and as such comprise a compulsory constituent of toxicity testing for chemical risk assessment and effluent testing; millions of fish are sacrificed annually in this context. One *in vitro* alternative is the use of fish cells. Cell lines of different species and organs exist, and have shown good correlation with *in vivo* results. In the RAINBOWFLOW CHIP project, we use an intestinal cell line of the rainbow trout (Oncorhynchus mykiss), RTgutGC, for impedance sensing: cells are seeded on a microfluidic electrode chip and their adherence creates a resistance to the electric current flow, which reflects the health status of the cells. A decrease in resistance is an indicator for loss of cell viability as can be elicited, for example, by exposure to chemicals5. Impedance sensing is non-invasive and can be measured in real-time, allowing for time-resolved analysis of the cells' sensitivity to different chemical concentrations. In addition, a flow-through system allows for constant replenishment of the test substance. This is especially important for difficult-to-test (i.e., volatile and hydrophobic) chemicals, which are prone to losses by sorption and evaporation, thus potentially leading to underestimation of toxicity. Our first results show that stable exposure concentrations could be maintained for three model substances with different physico-chemical properties for at least 24 hours. The next step is thus the toxicity testing with these model substances. The RAINBOWFLOW CHIP biosensor will allow the investigation of toxicity of chemicals with stable or purposefully fluctuating concentrations over time without the need to conduct animal experiments. Moreover, extensions of this setup – specifically for applications to on-line effluent monitoring in the field – are also currently being implemented.

Keywords: Aquatic ecotoxicology, alternatives to animal testing, fish cell lines, impedance spectroscopy, lab-on-chip