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BOOK of ABSTRACTS

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Use of lung cell models to assess the toxicity of nanomaterials: Bioavailability and gene expression analysis to establish toxicity profiles

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Nanomaterials are widely distributed in the environment and at workplaces. However, to support the manifold potentials of nanotechnology, it is crucial to perform a sound risk assessment. One principal question to be answered is whether or not there are modes of action unique for nanomaterials as compared to particles in the microscale range, or in comparison to water-soluble compounds of the same composition. Since it is not feasible to perform extensive toxicological testing with every single material, it is essential to establish basic strategies to investigate and evaluate materials and to elucidate general toxic mechanisms for groups of materials. Within our studies, we focussed on metal-based nanomaterials, which are applied increasingly as nanoparticles, but also as so-called nanowires. To establish models for inhalative expsoure, we used adenocarcinoma A549 and BEAS-2B lung cells as well as differentiated macrophage cells in mono- and co-culture systems exposed either submersed or at the air-liquid interface (ALI) and investigated the impact of different metal-based nanomaterials on genomic stability. As one main research tool to establish toxicity profiles, we applied a high-throughput RT-qPCR system, which enables the parallel assessment of the impact of 96 samples on 95 sected genes, coding for stress response, DNA damage response, specific DNA repair factors, cell cycle control, apoptosis and inflammation. Also, we established a procedure to quantify intracellular metal ion levels in soluble cytoplasmic and nuclear fractions. We observed that potential toxic effects of metal-based nanomaterials are diverse and depend on a multitude of different factors, among which, in addition to physico-chemical properties, intracellular bioavailability of metal ions appears to play an outstanding role. For example, the modulation of cellular signaling was most pronounced by CuO NP, due to an increase in ROS, DNA damage and the interaction with redox-sensitive transcription factors. Most decisive appeared to be highly elevated levels of copper in the nucleus caused by CuO NP but not by CuO MP or by $CuCl_2$. In contrast, largely insoluble nanomaterials like TiO_2 and CeO_2 revealed no toxicity in our test systems. Interestingly, in case of silver-based nanomaterials appeared to be largely insoluble in model fluids, but exerted high bioavailability in cellular test systems, accompanied by pronounced changes in gene expression and genotoxicity. Altogether, the applied test systems will create a comprehensive data basis for metal-based nanomaterials which will contribute to a more refined risk assessment.

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Integration of NAMs into the European regulatory assessment of Endocrine Disruption (ED) – Experiences and challenges when addressing Thyroid ED

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In the EU pesticides, biocides and chemicals require an ED assessment, including the thyroid pathway. When this was implemented in 2018, no accepted methodologies for thyroid activity MIEs nor for the human relevance assessment of potential thyroid ED related adverse effects in animals were in place. While this lack gave room for maneuver to introduce and apply NAMs into the regulatory process, at the same time experimental and regulatory challenges had to be addressed and overcome to allow a reliable and comparable assessment.
Consequently, efforts were initiated by CROs, regulatory bodies and industry to develop, establish, validate and compare suggested NAMs (e.g. TPO, NIS, comparative enzyme activity in vitro) and to come up with accompanying or alternate approach to enable a better assessment strategy. The current proceedings are presented which allow some initial understandings of the opportunities, limitations and challenges of these approaches.
Up to now none of the NAMs mentioned has reached the step to be regulatory fit for purpose i.e. being fully validated and integrated into internationally accepted guidelines, IATAs or defined approaches. For the activity endpoint related assays like TPO or NIS several methodologies are applied whose read-outs vary and are not necessarily comparable. Clear definition criteria to judge and discriminate a positive from a negative or equivocal outcome need to be defined. Currently, the data quality is too limited to allow a qualified judgement on robustness, transferability and reproducibility of any of the methods across laboratories. To characterize the capabilities but also limitations of the methodologies it is key to define and assess relevant reference compounds including also weak positive effectors. Cell-based assays like the NIS require a proper understanding of the cytotoxicity threshold as transporter activity may be unspecific impaired without evident fatal cell-damage. IVIVE approaches are needed to translate relevance of these NAM read-outs to the human.
The induction levels for thyroid hormone glucuronidation in hepatocytes are way less pronounced than CYP-activities, requiring alternate assessment strategies. An extended 7-day incubation provides more robust results then shorter duration. Moreover, the basal activity levels of T4-glucuronidation between rat and human varies by a factor of about 10, which implies that subtle treatment related changes have a more relevant impact on the hormone concentrations in the rat as compared to human. A novel assessment strategy to account for these differences is presented
Alternate approaches may allow a more holistic way to assess the thyroid axis and its potential impairment in men but require further development and validation.
NAMs can provide relevant information for or along the AOPs if properly designed and understood. However, one should be careful deducing adverse outcome predictions using single NAM derived information, only.

The chicken chorioallantoic membrane assay: A short-term *in vivo* model for cancer research

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In vivo tumor models are essential for studying the biology of cancer. Considering the request for the minimisation of animal experiments and following the "3R"-rule (replacement, refinement, reduction), it has become crucial to develop alternative experimental models in cancer biology. The chorioallantoic membrane (CAM) of the chick serves as a short-term model that overcomes many limitations to studying the biology of cancer *in vivo*. Since the CAM is a well vascularized extra-embryonic tissue and the chick is naturally immunodeficient, the CAM readily supports the engraftment of cells and tissues. Wide ranges of tumours have been studied using CAM and several studies have already described the CAM model as an alternative to rodents, suitable to investigate growth, progression, angiogenesis and metastasis of various types of cancer.

In our laboratory, we have established the *ex ovo* CAM assay and use this method for various applications in cancer research. We find that carcinoma cell lines form vascularized CAM-tumors exhibiting phenotypic characteristics of primary tumors. Here we report on some applications and describe the advantages but also the disadvantages of the CAM assay.

Toxicity triggered by modulation of mass and structural integrity of non-cytoskeletal organelles: a new perspective for organ-on-a-chip experiments?

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In the last decades, toxicological research was strongly shaped by the need to reduce, refine and replaced animal-based studies. Among the different possibilities, promising strategies imply the use of complex in vitro model systems. These try to reproduce as close as possible cell physiological function in the body, including combination of different cell types in co-culture, development of 3D models and the inclusion of biomechanical stimulation reproducing the motility of cells and body fluids. As a matter of fact, recent advances in the comprehension of cell pathophysiology clearly demonstrate that physical cues orchestrate essential cellular functions, including metabolic competence, antioxidant response capacity and proliferation [1]. This obviously reflects on the complex mechanosensory apparatus that enable the cells to transduce the physical stimuli into biochemical pathways and, from the toxicological perspective, if and how this can be targeted by noxious stimuli. Taking this as a starting point we started to investigate if intracellular organelles other than the cytoskeletal elements could contribute to the biomechanical compliance of cells. Along this line we focused on the idea that structures like mitochondria and endoplasmic reticulum could contribute not only to essential cell functions, but with their mass and intracellular distribution also to the mechanotransducive apparatus of the cells [2, 3]. To test this hypothesis, we used T24 bladder cancer cells and systematically applied several stimuli modulating mitochondrial intracellular distribution and turnover or the appearance of the endoplasmic reticulum-ER (e.g. rapamycin 1-100 nM; bafilomycin 10 nM or thapsigargin 0.1-100 nM, brefeldin A 10-40 nM). With viability maintained between 80 and 90 % in comparison to solvent controls, the treatments were effective in rearranging the mitochondrial networks and/or ER distribution. In parallel to the redistribution of the organelles in the intracellular compartment, migration capacity was altered with marginal or rather secondary involvement of the cytoskeletal network. Additionally, the capacity of the cells to respond to biomechanical cues, as those typically applied in microfluidics systems or experienced by the cells in vivo, was also modified. In conclusion, these findings offer an intriguing perspective in the comprehension and interpretation of data generated in complex organ-on-chip model systems.

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Environmental ligands of the aryl hydrocarbon receptor associated with airborne particles and their interference with nuclear receptors

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Activation of the aryl hydrocarbon receptor (AhR) represents a key toxic event elicited by numerous persistent organic pollutants, as well as other environmental contaminants, including as polycyclic aromatic hydrocarbons (PAHs). The activation of the AhR is associated not only with the regulation of the metabolism of PAHs and related compounds, but it impacts further intracellular signaling modules including nuclear receptors, in particular steroid receptors. In first part of the talk, the mechanisms underlying the impact of various types of polycyclic aromatic compounds on steroid signaling will be outlined, as our recent data indicate that the AhR plays a dual role in the impact of PAHs (and their complex mixtures) on steroid signaling. In second part of the presentation, the AhR-mediated activities of various PAHs, substituted PAHs and other polyaromatic compounds will be discussed. Importantly, these contaminants are often neglected, when toxicities of complex mixtures of environmental pollutants are being evaluated. Our past work has revealed that such compounds can indeed contribute significantly to overall AhR-mediated toxicities of mixtures of organic pollutants, including airborne particles or direct products of combustion engines, such as diesel exhaust particles. Overall, the current presentation is intended to provide an overview of principle groups of polycyclic aromatic contaminants, their AhR-mediated activities and strategies necessary to fill the gaps in our current knowledge of their toxicity, including their potential roles in endocrine or metabolic disruption.

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Pitfalls and problems when evaluating combinatory effects – a case study on the mycoestrogen zearalenone and isoflavones

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Daily, humans and animals are exposed to multiple known and unknown substances which might have a negative health impact alone or in combination. For example, in case of food and feed, secondary plant metabolites, such as the soy isoflavones (ISF) genistein (GEN) and daidzein (GEN) occur naturally and are associated with both beneficial and negative effects. The same food and feed might be co-contaminated with the secondary fungal metabolite zearalenone (ZEN) which is known to exhibit estrogenic effects in humans and animals. So far, risk assessment is usually based on toxic effects of single compounds, but the interest in combinatorial studies is increasing.
In the present study, we investigated in vitro the estrogenic potential of the mycoestrogen ZEN and some of its metabolites alone or in combination with the ISF GEN, DAI, and the DAI-metabolite equol (EQ). In this presentation we want to focus on the difficulties of evaluating combinations and the problematic that guidelines, like OECD, might not be always applicable. For the evaluation of the estrogenic potential two different cell lines were used to determine the mode of action. First, the activity of the alkaline phosphatase (ALP) was assessed in the human endometrial cancer cell line "Ishikawa" expressing both estrogen receptors α (ERα) and β (ERβ). Second, following the OECD-guideline No 455 the luciferase reporter gene assay was performed using the stably transfected hERα-HeLa-9903 expressing only ERα. In the Ishikawa cell model, the tested concentration range included 0.001 to 10 nM with multiplication steps of 10 in between for the mycoestrogens, while for the ISF 1000 times higher concentrations were investigated. For the hERα-HeLa-9903 only selected combinations were evaluated.
For the individual substances the expected order of estrogenicity was obtained in the Ishikawa cells and in the combinatorial studies an influence on the estrogenic response was observed. Especially lower concentrations of the mycoestrogens (0.001 - 0.01 nM) in combination with low ISF concentrations (0.001 - 0.1 µM) strongly increased the estrogenic response compared to the single substances. ZEN and its metabolites have a relative high binding affinity to both ERs, while ISF preferably interact with ERβ. Therefore, no enhanced estrogenic effects in the hERα-HeLa-9903 cell line were expected. Indeed, no synergistic effects were induced when compared to the effect of the single substances. However, at concentrations above 1 µM of the ISF a superinduction of luciferase was observed that surpassed the effect induced by the positive control (1 nM 17-ß-estradiol) and showed a limitation of the OECD method.

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Innovative Approach to Control Bladder Infections Using MANNylation Strategies for Mesoporous Silica Nanoparticles

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D-mannose is a simple sugar (a monosaccharide isomer of glucose), which is known to stop the adhesion of bacteria to the urothelium. D-mannose can naturally be found in small amounts in various fruits such as cranberries, apples, and mangos as well as foods like egg white, soybeans, kidney beans and peanuts. Mannose is absorbed in the upper gastrointestinal tract and excreted in the urine. [1] Mannose was found to be a affective supplement to treat acute urinary tract infections in women.[2] Even though the human urinary tract is consists of strong barriers made of urothelial cells, about 150 million people are affected worldwide by urinary tract infections (UTIs).[3] Urinary tract infections (UTIs) are the most common type of infections for outpatients, according to the healthcare system.[4] Furthermore, indwelling urinary catheterization for hospitalized patients is a significant risk to urinary tract infection[5]. With estimates as high as 25% of hospitalized patients receiving a short-term indwelling urinary catheter, the risk for UTIs is increased. Indwelling urethral catheter usage is the reason of 70–80% of urinary tract infection within hospitalized patients.[6] Prevention of infections caused by these devices is an important goal of health-care system.
To come closer to that goal, different MANNOSylation methods were developed for the modification of mesoporous silica nanoparticles (MSNs) and were loaded with an antibiotic, commonly used for bladder infections. A human urinary bladder cancer cells line (T24) was used to study the impact of the cytotoxicity and the localization of a crucial component of the inflammatory cascade like the Toll-like receptor 4 (TLR4). Experiments were performed in presence or absence of bacterial lipopolysaccharide (LPS) stimulation. With this experimental layout, we observed that non functionalized MSNs, in the same mass concentration than MANNOSylated particles, showed severe cytotoxicity, compared to the mannose modified MSNs. In addition to reduced cytotoxic potential, the different chemical linkers used for the binding of mannose showed a strong impact on TLR4 localization. These results support that MANNOSylation could be a promising strategy for possible catheter coatings to support reduction of urinary tract infections in the future.

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The assessment of the risks to vultures and other necrophagous birds in the EU from the use of veterinary medicinal products

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In the European Union diclofenac, a non-steroidal anti-inflammatory substance has been authorised for veterinary use since 1993. The dramatic decline in vulture populations, which was estimated to be more than 95%, led in 2006 to the prohibition of the sale of VMPs containing diclofenac in India, Nepal, Pakistan and Bangladesh. In 2014, the European Commission presented to the European Medicines Agency (EMA) a request for an opinion from the Committee for Medicinal Products for Veterinary Use (CVMP) regarding the risk that the use of VMPs containing the diclofenac may represent to vultures and to other necrophagous birds in the Union. In addition, the opinion should address any actions or mitigation measures that could manage the risk. The assessment of the risks to vultures and other necrophagous birds in the EU from the use of veterinary medicinal products (VMPs) is not a standard environmental risk assessment (ERA) prescribed by the CVMP in Guidelines on ERA for VMPs. Therefore, an ad-hoc approach was agreed upon. The most suitable specie to be used as a model organism for the assessment was identified, as well as the most adequate inter-and intraspecies extrapolation factors based on the expert judgement were determined. The calculation of exposure concentrations was based on the social, foraging and feeding behaviour. Assessment of risk to specific populations considered the reproduction strategy of species. In the study of the toxicity of diclofenac on vultures were calculated values LD50 of 0.225 mg/kg bw and LD10 of 0.074 mg/kg bw. The maximum concentration of residues of diclofenac in tissue to ensure the safety of vultures would result in a value of 3 µg/kg. The reasonable worst-case amount of diclofenac in the injection sites available for consumption by birds would be approximately 37 mg in cattle and 0.9 mg in pigs. For residues in cattle, data show that at the injection site and in the liver, levels are higher than the safe value of 3 µg/kg until 10 days after the last administration of diclofenac. For other tissues, residues after 8 days are below 3 µg/kg. For pigs, 9 days after the final injection onwards, the residue concentrations of diclofenac were below 3 µg/kg in all tissues. Uncertainty related to the risk remains when animals die shortly after the treatment and are disposed to necrophagous birds. The vulture population could suffer great losses due to feeding frenzy behaviour when numerous birds are attracted by the carcass of a treated animal.

Interaction of A-, B-, and C-type prymnesins with sterols

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Prymnesium parvum is a haptophyte alga associated with harmful algal blooms. Their production of ichthyotoxic allelochemicals called prymnesins (PRMs) is likely to be the cause of massive fish kills. A distinguishing feature of PRMs is their large diversity for which they have been grouped into A-, B-, and C-type PRMs. Differences in the toxic potential between the groups have been observed previously, where A-type PRMs showed the strongest cytotoxicity towards fish gill cells. PRMs cause fish mortality by damaging fish gills through cell lysis, apparently via direct interaction with the cell membrane.
We examined the differences in hemolytic activity between A-, B-, and C-type PRMs. Furthermore, we looked into interactions of the three PRM-groups with cholesterol, epicholesterol, and ergosterol to better understand their membrane interactions. Comprehending sterol affinities and interactions can provide valuable insights into the pore formation and distinction in subsequent toxic potential.
The hemolytic potential was assessed by measuring the absorbance of released hemoglobin from a 1.25%-red blood cell solution in Tris-buffer, using blood from Atlantic salmon (Salmo salar). Subsequently, the half maximal effective concentration of each sample was established and used for co-incubation experiments. Different concentrations of cholesterol and epicholesterol were tested for their potential to inhibit lysis caused by PRMs. Interaction kinetics of A- and B-type PRMs with the three sterols were investigated using surface plasmon resonance (SPR). Sterols were coupled to an alkanethiol surface, and A- and B-type PRMs were tested for binding affinity using a single concentration of 1 µM.
The hemolytic potential of PRMs mirrored that of their cytotoxicity, with A-type being the most and B-type the least potent. Higher concentrations than those for cytotoxicity in fish gills were needed to reach a 50% effect, which could be a hint at the physiological susceptibility of fish gills. Co-incubation with cholesterol clearly lowered hemolysis of RBCs, as was observed in earlier studies. Strikingly, cholesterol seemed to have a stronger effect on C-type PRM compared to A-type. Moreover, as in previous findings for the structurally similar karlotoxin, cholesterol appeared to have a stronger affinity for PRMs than epicholesterol, which differs only in its α-position of the C3 hydroxy-group. This further indicates a direct interaction between membrane sterols and PRMs and the high specificity PRMs have for cholesterol. This affinity was also shown in the dissociation constants Kd (M) obtained from SPR testing for A-type PRM. B-type PRM had the lowest Kd (M) for cholesterol, and the highest for epicholesterol. Pore formation and/or membrane disruption mechanics may be subject to the PRM group.

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Vorstellung und Tätigkeiten der Vergiftungsinformationszentrale

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Die **Vergiftungsinformationszentrale (VIZ)** bietet unter der **Telefonnummer 01/4064343** bei Vergiftungsverdacht rund um die Uhr Beratung für Laien und medizinisches Fachpersonal an. Laien werden angeleitet welche Maßnahmen im Vergiftungsverdachtsfall zu setzen sind. Ärztinnen und Ärzte werden hinsichtlich einer Risikoabschätzung und Prognose sowie Entscheidungshilfe für therapeutische bzw. diagnostische Maßnahmen unterstützt, insbesondere auch spezifische Behandlungsmöglichkeiten (zB Antidota) unter Berücksichtigung der jeweiligen fallbezogenen Indikationen und Kontraindikationen betreffend. Zudem unterstützt die VIZ Ärztinnen und Ärzte bei diagnostischen und differenzial-diagnostischen Überlegungen in unklaren Fällen.

Zur individuellen Beratung sind **folgende Informationen wichtig:**

- **Was:** möglichst genaue Bezeichnung der Substanz bzw. des Produkts, etc.
- **Wie viel:** möglichst genaue Mengenangabe
- **Wer:** Alter, Gewicht, Geschlecht und Zustand der betroffenen Person(en)
- **Wann:** Zeitpunkt des Geschehens
- **Wo:** Ort des Geschehens
- **Wie:** Verschlucken, Einatmen, Hautkontakt, etc.
- **Warum:** versehentlich oder absichtlich

Alternatives to vertebrate testing

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The competent authorities responsible for the regulation and risk assessment of pesticides and plant protection products have to ensure the protection of human health and the environment. Therefore, health hazards resulting from substances such as pesticides have to be identified. Currently, many accepted test methods to assess such adverse effects are based on the use of laboratory animals. However, animal-based testing has a number of recognized disadvantages including high costs, moral and ethical issues and for some endpoints there are even difficulties to assess the human relevance of findings from different animal test species. Alternative new approach methodologies (NAMs) are promising alternatives to conventional toxicological in vivo testing strategies by combining human relevant in vitro methods and in silico models. This talk will give an overview on the integration of NAMs in the regulatory assessment of human health hazards of pesticides today and on the future perspective for a new paradigm shift towards animal-free safety testing as proposed by the latest external scientific report published by European Food Safety Authority [1] and the European Chemicals Agency [2].

[1] Development of a Roadmap for Action on New Approach Methodologies in Risk Assessment. EFSA Journal 2022;19(6):EN-7341
[2] Non-animal approaches - Current status of regulatory applicability under the REACH, CLP and Biocidal Products regulation. ECHA-17/R/24/EN

Next Generation Risk Assessment - Adopting a probabilistic perspective on the required scope of in vitro testing.

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A central aspect of animal-free hazard assessment is the development of integrated approaches to testing and assessment (IATA) based on new approach methods (NAMs). However, the scope of investigation in a fully NAM-based approach remains an open question. This case study explores the applicability of the EU-ToxRisk in vitro toolbox (EUT-TB) to derive a human equivalent reference concentration that can be used as a point of departure (PoD) for risk assessment (RA).
It is well known, that six main target organs including liver and kidney are found more frequently than others at the lowest observed effect level (LOEL) determined in preclinical rodent oral dosing studies. With these six targets/organs examined, the LOEL can be predicted with a probability of about 90%. Apical findings in other target organs are often observed together with toxicological effects in the six main target organs. This case study investigates whether the same principle can be applied to the development of an in vitro test battery.
For this purpose, the EUT-TB was used to assess the hazard of three tin chlorides, two imidazoles, two thioureas, and butanone oxime. The compounds were selected because they induce adverse effects like immunotoxicity or anemia in preclinical in vivo rodent studies. These adverse toxicological effects are not directly covered by dedicated assays in the EUT-TB, but the EUT-TB may still display responses at concentrations, which translate to human protective PoDs.
The EUT-TB comprises five human in vitro models, namely RPTEC/TERT1 (kidney), PHH and HepG2 (liver), PBEC (lung), and LUHMES cells (neuronal system). Readouts include transcriptome (human S1500+ gene panel of TempO-Seq®; 3,565 genes), cytotoxicity and high content imaging (HCI) data for analysis of mitochondrial dysfunction in (metabolically competent) HepG2 cells. Further, 32 CALUX®- and 10 HepG2 BAC-GFP reporter assays provide data on a broad range of known perturbation mechanisms.
Benchmark concentrations were derived from nominal media concentrations per gene/pathway/functional read-out. These were extrapolated to unbound intracellular concentrations. Finally, in vitro to in vivo extrapolation yielded human equivalent plasma concentrations for use as in vitro-based PoDs for RA. Forward dosimetry was applied to the LOEL values of the in vivo rodent studies to derive human equivalent plasma concentrations, too.
For the majority of test compounds, the PoD values derived from in vitro models were about 1 to 4 orders of magnitude lower compared to the corresponding values from the in vivo studies, indicating that such PoDs would have been protective. However, the number and type of active in vitro assays differed remarkably between the tested compounds/compound classes, a fact that needs to be considered in the ongoing assessment of the uncertainties of this approach.

This study is part of the EUToxRisk project which received funding from the EU (Horizon 2020; grant agreement No 681002).

An analysis of the limitations and uncertainties of animal based toxicity assessments to identify the potential for non-animal approaches in regulatory toxicology

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Change towards regulatory reliance on non-animal-methods (NAMs) appears essential in the light of the current green policy advocating challenging goals like safe and sustainable chemicals and a comprehensive (eco)toxicological knowledge base on chemicals allowing to work towards the zero-pollution goal. A critical challenge for the greater reliance on NAMs is the difficulty of deciding on acceptable uncertainty from their use. Transparency on the limitations and uncertainties of current animal testing based approaches may provide a useful, objective benchmark that may support such decision making.
For a set of different regulatory endpoints, i.e. developmental neurotoxicity, carcinogenicity and acute aquatic toxicity, a systematic description of the limitations and uncertainties of animal testing based approaches was carried out in qualitative and quantitative terms. This was accomplished by re-purposing an OECD format originally developed to characterize NAMs. The tabulated information was compared with the conceptual uncertainties of the respective NAM based approaches. Similarities between the three different endpoints are discussed.
The following conclusions appear valid for the three analyzed regulatory fields and may therefore be equally relevant for all fields of regulatory toxicology:

- The relative practical advantages of NAM based approaches are the abolishment of the 3Rs conflict, the higher throughput, the lower costs and the deeper mechanistic information.
- Relative to animal tests, NAMs are usually standardized to a higher degree and validated for experimental variability. Moreover, for NAMs the basic study design is much easier to improve in terms of replicates, number of concentrations, and inclusion of study-internal positive controls and the development of a larger historical negative and positive control database.
- The extrapolation from experimental animal data to real life, be it human or multi-species environments, is necessarily uncertain. Pragmatism and data-based extrapolation models are needed for the regulatory use of experimental animal data. Mode of action coverage is limited and uncertain. Similar considerations apply to the use of NAMs.

NAMs allow (eco)toxicological data to be generated for many more chemicals, provide mechanistic information for extrapolation needs and provide lower uncertainty for data variability. This can increase the availability, reliability and chemical-to-chemical comparability of (eco)toxicological data for globally effective safety regulation. Assessing the significant uncertainties for extrapolation from animal test data to real human or multi-species environments, it appears realistic that with the use of NAMs at least similar protection levels can be achieved than with animal test data. This work on systematic uncertainty analysis of animal tests was already carried out for eye irritation, skin sensitization, acute rodent toxicity, in vivo point of departure derivation and is currently being followed up more broadly by the US National Academy of Science. This information shall be used for the development of NAM based IATAs for developmental neurotoxicity, non-genotoxic carcinogenicity and acute aquatic toxicity which is ongoing at OECD level. However, ultimately *out of the cage thinking* will be necessary to fully rely on NAMs in regulatory toxicology.

Alternative assays in the frame of DART testing programs- experience with the latest revision of ICH S5

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Alternative methods can be useful to improve current reproductive toxicity testing strategies as they have the potential to provide a better understanding of adverse developmental outcome pathways. Although alternative assays - i.e. in vitro, ex vivo and non-mammalian in vivo assays - are typically used for screening purposes these studies are rarely part of regulatory submission packages as their regulatory acceptance remains challenging. In contrast, the ´gold standard´ - a package of studies evaluating effects on fertility, embryo-fetal development and postnatal development in rodents and usually rabbits as second species in embryo-fetal development studies - remains unchanged since decades.
The third revision of the ICH S5 guideline on detection of toxicity to reproduction for human pharmaceuticals provided the chance to incorporate experience gained with the testing of pharmaceuticals using current and novel testing paradigms enhancing human risk assessment. Above that it describes qualification of alternative assays, potential scenarios of use, and provides options for deferral of developmental toxicity studies – for the first time in a guidance on a true global level.
Furthermore a maintenance procedure for the guideline was introduced, allowing a swift update on a global level in case new data or new scenarios of use become available.
The presentation will also elaborate on the efforts undertaken on a european level to advance the development of alternative assays.

Non-Clinical Development of ATMPs - Challenges and Regulatory Specifics

Alexandra Günzl

Advanced therapy medicinal products (ATMPs) are innovative therapies that hold great promise for patients suffering from mostly severe medical conditions with high unmet medical need. These opportunities come with great challenges for both regulators and those developing such highly complex products.

Overall, the same basic principles apply to ATMPs as to all other medicinal products for human use, i.e., the need to demonstrate efficacy and safety to not pose unreasonable risks to healthy volunteers or patients. However, 'standard' non-clinical programs are mostly not suitable to account for the specific nature and risks of therapies within this diverse product class and product-tailored development plans need to be designed case-by-case.

Already the classification of a product candidate can pose a first hurdle, as the term 'ATMP' encompasses four different product types: (i) somatic cell therapy medicinal products (sCTMPs), (ii) tissues engineered products (TEPs), (iii) gene therapy medicinal products (GTMPs) and (iv) combined ATMPs. A complex legal and regulatory framework exists for ATMPs, including a set of product type-specific guidelines issued by the EMA that define requirements and aid at tackling the challenging development of ATMPs. The 'risk-based approach' guideline outlines a highly recommended strategy, which helps designing the non-clinical program based on a very structured determination of specific risks and identification of contributing risk factors that need to be covered. Detailed expertise is needed to adequately address the product-specific risks by designing an appropriate non-clinical development program, as there are numerous challenges to be overcome, such as: selecting adequate animal models for efficacy and toxicity studies, finding an experienced contract research organization that is capable of conducting the high-quality studies needed (in a GLP environment), choosing an adequate test item to be used in non-clinical studies, dealing with limitations based on the fact that repeated dose toxicity studies might not be feasible due to immunogenic reactions, tackling complex bioanalytics, defining the starting dose for first-in-human studies, and many more.

As a result of the product-specific risk factors, risks and aforementioned challenges, a non-clinical program for an ATMP may deviate considerably from a 'standard' program of e.g., small molecules, as outlined in ICH M3(R2), in terms of type, extent and design of (safety) studies.

Respective regulatory guidance documents for ATMPs acknowledge that conventional requirements may not always apply to ATMPs, but there is a need to document and scientifically justify any deviations from these 'standard' requirements. In any case, engaging with competent Authorities early on in the development of ATMPs to seek advice and to agree upon an adequate non-clinical testing strategy in a Scientific Advice Meeting is of importance and highly recommended.

Establishing a human starting dose: a specific challenge for cell and gene therapies

Anika Schröter
Dr. Anika Schroeter e.U.

Determination of the human starting dose is key for a successful transition from the preclinical development phase into the clinical phase.

For small molecules and biotechnology-derived products, defined and well-established approaches are available, which help to guide developers in finding a starting dose that meets the requirements for the first-in-human study; i.e. being safe for healthy volunteers or being reasonable safe with a chance for a benefit for a patient. Common strategies include for example i) estimating the maximum safe starting dose based on the “no observed adverse effect level” determined in non-clinical toxicology studies or ii) defining the “minimally biological effect level” as basis for starting dose selection. Both strategies are also described in regulatory guidelines as FDA "Guidance for Industry - Estimating the Maximum Safe Starting Dose in Initial Clinical trials for Therapeutics in Adult Healthy Volunteers" (2005) or EMA "Guideline on Strategies to identify and mitigate Risks for First-in-human and early Clinical trials with Investigational Medicinal Products" (2017), which thus provide further detailed support around establishment of the human starting dose.

In contrast to small molecules or biologics, cell and gene therapies represent a diverse class of products and no general approach can be defined. Consequently, there is no specific guidance, providing clear recommendations on how to determine a human starting dose for cell and gene therapies.
Additional challenges arise from peculiarities of this product class, which include limitations in the non-clinical program as the absence of relevant animal models for pharmacology or toxicology testing. As a result, product-specific risks cannot be adequately addressed pre-clinically, but are transferred into the clinical testing phase - highlighting the importance of selecting the appropriate starting dose to not put healthy volunteers or patients at risk.
Thus, individual, product-tailored and innovative approaches are needed, which require a thorough understanding of the indication, mode of action and potential risks related to the product.

Posters:

P1 - Benchmarking the potential toxicity of dietary fatty acids on the mechano-transductory apparatus of the intestinal cells Janice Bergen
P2 - The Alternaria mycotoxin alternariol suppresses the DNA damage-induced phosphorylation of the histone H2AX Francesco Crudo
P3 - Atomic force microscopy: integration of an ultra-high resolution imaging technique in toxicological research Livia Gruber
P4 - Non-clinical Cardiovascular Risk Assessment of Lefamulin Michael Hafner
P5 - Exploring toxicological implications for Krüppel-Like Factors Maximilian Jobst
P6 - Austrian Children's Biomonitoring Survey 2020 Part B: Mycotoxins Olga Lanaridi
P7- Discovery, mode of action & biosynthesis of marine biotoxins Magdalena Pöchhacker
P8 - Comparison of the metabolite profile in bovine rumen fluid, plasma, saliva and feces by anion exchange chromatography-high resolution mass spectrometry Heidi Schwartz-Zimmermann

Benchmarking the potential toxicity of dietary fatty acids on the mechanotransductory apparatus of the intestinal cells

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Piezo1 are Ca2+-permeable non-selective cation channels, which opens in response to mechanical triggers. Being sensitive to variation of cell morphology, novel findings suggest that Piezo channels might play a role in pathophysiological processes including gut inflammation, cancers and cardiovascular mechanobiology (PMID 26402601) However, data describing their involvement of the regulation of intestinal response to xenobiotics are limited. Recent studies have shown that margaric acid as well as polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid (EPA) can have a profound effect on the activation and inactivation of Piezo channels (PMID 30867417). Moreover, manipulation of membrane structure via cholesterol load or with cholesterol-lowering agent lovastatin, significantly affect the distribution of the Piezo channels, with functional effects on the capability of intestinal cells to cope with biomechanical stimulation (PMID 35461954). Among dietary lipids, palmitic acid (PA) and oleic acid (OA) are among the most prevalent fatty acids. Despite their abundance, awareness is rising about potential detrimental effects related to excessive consumption, hence cancer cells seem to be able to benefit from their uptake and foster in this way metastasis formation (PMID 34759321). Building on this, we investigated the effects of PA and OA (25-500µM) on non- transformed human intestinal epithelial cells (HCEC-1CT) in comparison to colon carcinoma cells HCT 116. The response of cerulenin (5-100µM) – a fatty acid synthase (FAS) inhibitor – was compared to the response PA and OA had on both cell lines. With this experimental layout we started to explore the hypothesis that the two fatty acids could affect intestinal motility. Cytotoxicity (WST-1 assay) was observed for PA starting from the concentration of 250 µM and for OA only at 500µM. Significant and concentration dependent reduction of cell membrane fluidity was triggered by PA and OA in the cancer cell line (≥ 25µM). This effect was not seen in the HCEC-1CT cells, which displayed rather an increase in membrane fluidity when incubated with 500µM PA or OA. With the exception of an increase of Piezo1 in the HCT 116 cell line incubated with 100 µM PA, there was no significant difference noticeable in the expression of Piezo1 after treatment with PA and OA. However, a clear rearrangement in actin cytoskeleton was visible in both cell lines (PA and OA; 25 and 100 µM). Taken together our data suggest that PA and OA can modify membrane and cytoskeleton of intestinal cells. These effects appear more pronounced in the cancer cells in comparison to the non-tumorigenic cell line and, occurring in a concentration range which is not cytotoxic, retain in this way the potential to modulate the biomechanical compliance of intestinal cells.

The Alternaria mycotoxin alternariol suppresses the DNA damage-induced phosphorylation of the histone H2AX

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Molds of the genus Alternaria can contaminate a wide variety of crops and raw materials and produce several mycotoxins showing a broad spectrum of adverse effects. Among the most recurrent mycotoxins, alternariol (AOH) has been reported to induce several types of DNA damage: theses include oxidative DNA damage, single strand breaks and double strand breaks (DSBs).

Cellular response to DNA damage consists in the activation of complex repair mechanisms involving several factors. In this context, DSBs are known to trigger the phosphorylation of the histone H2AX, a key event necessary for the assembly of DNA repair proteins at the site of damage and for the control of the cell cycle progression. Quantification of the phosphorylated histone γH2AX is, therefore, often used for the indirect detection of DSBs.

In the present study we investigated the DNA damage potential of AOH on the HepG2 cell line by applying the In-cell Western assay of γH2AX and COMET assay. The mechanisms underlying the effects observed on the formation of γH2AX were further investigated through an in vitro/in silico approach. To assess the impact on cell viability, the CellTiter-Blue® (CTB) assay was performed.

Results from the γH2AX and CTB assays clearly indicated the ability of AOH to induce the formation of γH2AX and decrease cell viability in the concentration range 10-100 µM. Of note, the increase in γH2AX fluorescence signal triggered by AOH was moderate and rapidly reached a plateau. Based on these results, the hypothesis of a possible inhibition by AOH of kinases involved in the formation of γH2AX was formulated. To verify this hypothesis, 5µM and 50 µM AOH were co-incubated with 2.5 µM of the chemotherapeutic drug doxorubicin, a known inducer of DSBs. Surprisingly, co-incubation of doxorubicin with both concentrations of AOH resulted in a decreased γH2AX expression compared to doxorubicin tested alone. To confirm that the reduced γH2AX fluorescence signal was not a consequence of a reduced genotoxic effect, COMET assays were performed in order to better clarify the degree of DNA damage occurring in cells exposed to the compounds in combination. Results of the assay showed an increased DNA damage in cells co-incubated with Doxorubicin and AOH compared to the single treatments. To further confirm the ability of AOH to interact with kinases involved in the H2AX phosphorylation, an in silico docking study was performed. In silico results confirmed the ability of AOH to interact with the ATP pocket of these enzymes, thus providing further data to support the inhibitory potential of the mycotoxin.

To conclude, results obtained in the present study suggest the ability of AOH to inhibit the DSB-induced formation of the phosphorylated histone γH2AX, thus triggering an event of crucial importance for the activation of DNA repair mechanisms. Further studies are needed to better clarify the mechanisms of inhibition.

Atomic force microscopy: integration of an ultra-high resolution imaging technique in toxicological research

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Our body, cells constantly sense and respond to a diverse set of mechanical signals such as tensional forces due to the presence of neighbouring cells and movement of body fluids. This process of translating physical forces into biochemical information is called mechanotransduction. It is involved in several fundamental processes including adhesion, spreading, migration, gene expression and cell-cell interactions. However, dysregulation of mechanical responses contributes to major diseases like atherosclerosis, hypertension, osteoporosis, muscular dystrophy, myopathies and cancer. Similarly, it is known from laboratory practice that cytotoxicity events can be accompanied by a loss of cell shape. Since there is a clear relationship between pathological conditions and cell biomechanical compliance, it is of increasing interest to develop novel technologies that allow incorporation of biomechanical stimulation to experimental layouts. This applies to several biomedical sciences, including toxicology, and supports the development of novel animal-free test strategies [1-4].
The study of how mechanical properties influence cell behaviour in relation to their surrounding microenvironment requires a profound knowledge of extracellular and/or intracellular forces, stiffness values and mechanical stresses from micro- to nano-scales [5]. Atomic force microscopy (AFM) is a non-optical, ultra-high resolution imaging technique, which can be used to observe the surface ultrastructure of living cells under near-physiological conditions and to quantitatively measure their physicochemical parameters. The principle of AFM imaging and mechanical property detection is based on the attraction and repulsive forces between the atoms at the tip of the cantilever probe and those on the surface of the sample [6]. Recording of force–distance curves allows contouring of the surface of biological samples with simultaneous mapping of their physical properties including height, adhesion, contact force, sample deformation and stiffness (Young´s modulus), at nanometre resolution [7]. From a toxicological perspective, it is intriguing to hypothesize how many chemicals could actually modify cell biomechanics in addition to well-known endpoints like for instance metabolism, DNA integrity or genes/proteins expression. Indeed, AFM microscopy enables the visualization and the measurement of cell structure including information on mechanical and functional heterogeneity of complex biological systems and promise to be a precious tool for novel pharmaco-toxicological profiling.

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Non-clinical Cardiovascular Risk Assessment of Lefamulin

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Safety pharmacology studies are defined in guidelines ICH S7A/S7B and focused on identifying adverse effects on physiological functions including the cardiovascular system.
Following these guidelines, the effect of the pleuromutilin antibiotic lefamulin on cardiovascular functions was investigated in in vitro IKr assays, conscious cynomolgus monkeys, and rabbit Purkinje fibers.
The effect of lefamulin on hERG tail current was assessed using the whole-cell patch clamp technique on Chinese Hamster Ovary (CHO) cells stably transfected with hERG-1 cDNA. The IC50 was 14 µg/mL and 40 µg/mL in another study. In a similar study using Human Embryonic Kidney 293 cells, the IC50 for lefamulin was 24 µg/mL.
IV administration (30-minute infusion) of lefamulin at 7.5, 15, or 40 mg/kg to 4 conscious, chronically telemetered male cynomolgus monkeys caused no significant test article-related changes in clinical observations, body weights, and food consumption. There were no changes in qualitative (Lead II configuration) ECG assessment, quantitative ECG parameters (PR, QRS, RR intervals, QRS-duration), arterial blood pressure parameters (SBP, DBP, MAP), or heart rate (HR).
At 40 mg/kg, a mean maximal QT interval prolongation of 31 ms above baseline was noted. Dosing at 15 mg/kg and 40 mg/kg caused a QTcF interval prolongation of 21 ms and 37 ms above baseline, respectively. The control-adjusted mean maximal QT/QTcF interval prolongation were 33 and 40 ms, respectively, at 40 mg/kg lefamulin. This delay in cardiac repolarization was considered test article related as peak QT and QTcF interval values were observed between 0.25 and 1.5 hours after the start of infusion, consistent with maximal concentration of lefamulin at the end of infusion. This prolongation was transient and reversible with baseline values restored within 4 to 6 hours following dosing. There was no occurrence of “Torsades de Pointes” ventricular arrhythmias at any dose level for the duration of the recording (which was at least 24 hours postdose).
These results of the rabbit Purkinje fiber study suggested no significant effects on the inward rectifier potassium and sodium currents at ≤10 µg/mL. In contrast, block of the delayed rectifier potassium channel was observed from the lowest concentration tested (0.5 µg/mL) with a slight action potential triangulation at 10 µg/mL. At the concentration tested there was no occurrence of EADs at low pacing rates (20 and 12 pulses/minute). Overall, treatment with lefamulin demonstrated QT/QTc interval prolongation, with a pro-arrhythmic potential at 10 µg/mL, but not at 0.5 µg/mL and 3 µg/mL.
Together, these data indicate a potential for effects on the QT interval. Based on the results of the safety pharmacology studies a comprehensive ECG monitoring was performed during clinical development of lefamulin. A thorough clinical QT/QTc study as described in ICH E14 was not requested.

Exploring toxicological implications for Krüppel-Like Factors

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Krüppel-like Factors (KLF) are part of the SP/KLF family of transcription factors, which are defined by their DNA binding zinc finger motifs. KLF are emerging players in the orchestration of the cellular response to xenobiotics and in pathological conditions. For example KLF2 is involved in antioxidant and anti-inflammatory action, by modulating the expression of NRF2. [1] KLF4 is one of the most researched members of the family because of its role in the induction of pluripotent stem cells, but its functions are much more diverse, ranging from the regulation of skin barrier function, mitochondrial biogenesis, to recruiting p53 and p21 in response to DNA damage. [2] In line, we previously described that the mycotoxin deoxynivalenol (DON) hampered the lipid biosynthesis machinery in epidermal cells, as well as the mitochondrial network and these effects were all connected to KLF4 as a single common denominator. [3] This led us to postulate a role for KLF2 and KLF4 in the capacity of cells to cope with metabolic and environmental stressors, but also opened the question about factors potentially influencing the localization of transcription factors. Here we applied a previously described laser scanning microscopy protocol [3] and established a novel high-throughput immunofluorescence method for the screening of Krüppel-like factors translocation potential in T24 bladder cancer cells. Importantly, we could establish a dependency of the localization of the transcription factors based on chemical intervention, as for instance in the selective modulation of cytoskeletal elements. In addition, we could observe a concentration dependent translocation potential upon incubation with YODA1 (0.1-5µM), which is an agonist of mechanogated PIEZO1-channels. Crucially, we could also measure an altered localization pattern in presence of biomechanical stimulation, which would be relevant for the performance of experiments in presence of a microfluidics system or organ-on-chip setup. In conclusion, we could demonstrate that crucial regulators of cell proliferation and metabolism like Krüppel-like factors can be modulated by chemical and physical intervention, opening in this way novel perspectives for the interpretation of the results obtained in complex cell culture models.

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Austrian Children's Biomonitoring Survey 2020 Part B: Mycotoxins

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Mycotoxins are toxic secondary metabolites of filamentous fungi that can adversely affect human health. Young children are particularly vulnerable to mycotoxins owing to their lower detoxification capacity and body weight. Thus, continuous monitoring of levels of mycotoxins in human biofluids is important, in view of their acute and chronic health effects. In this study, the levels of mycotoxins in urine of Austrian school children, aged six to ten, were quantified. In total, 85 first-morning urine samples were assessed for the presence of mycotoxins using an ultra-sensitive LC-MS/MS-based assay. Eight mycotoxins were quantified in the urine samples, with zearalenone (100%; range: 0.03-0.59 ng/mL), deoxynivalenol (DON) (99%; range: 0.95-165 ng/mL) and alternariol monomethyl ether (41%; range: 0.01-0.66 ng/mL) being most frequently detected. Estimated tolerable daily intake and margin of exposure for DON and ochratoxin A, respectively, indicate possible health risks for a few children. This study revealed exposure to multiple mycotoxins in the cohort and warrants further exposome-scale biomonitoring in vulnerable populations.

Discovery, mode of action & biosynthesis of marine biotoxins

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A harmful algal bloom (HAB), is a rapid expansions of phytoplankton populations and is a major threat to the health of diverse coastal and freshwater aquatic ecosystems. Particularly problematic are HABs caused by toxin-producing microalgae as the species *Karlodinium armiger* and *Karlodinium veneficum*. Those species, besides many others, are able to produce so-called ichthyotoxins which are responsible for a large number of fish killings due to their gill cell-damaging properties. Two groups of toxins are already known in these species, karlotoxins in *K. veneficum* and so-called karmitoxin in *K. armiger*. Both have a very similar hairpin-like structure with the main difference of a primary amino group in karmitoxin. Nowadays a few karlotoxins have been identified but in total little is known yet, especially regarding the species *K. armiger*.

Within our project we aim to discover new toxins in the previously mentioned species and investigate their mode of action.

The first step is the cultivation of the algal strains in our lab and taking the supernatant of the cultural medium containing the putative toxic compounds. Different assays for the assessment of their cytotoxic and apoptotic properties were performed, comprising the in vitro use of fish gill cells on the one hand and the algal species *Rhodomonas salina* on the other hand. Toxic supernatants will further be fractionated using preparative liquid chromatography followed by another toxicity evaluation of the fractions. Active fractions will be analyzed using liquid chromatography coupled to high resolution mass spectrometry.

First toxicity assays showed a higher potential of *K. armiger* supernatants compared to *K. veneficum* but also a variation within strains of the same species. We now need to investigate if these variations come from different growth behavior of the algae in culture or have their origin in a deviating constellation of synthesized compounds.

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Comparison of the metabolite profile in bovine rumen fluid, plasma, saliva and feces by anion exchange chromatography-high resolution mass spectrometry

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It is a common practice to feed grain rich diets to cattle in order to increase their productivity. Such feeding practices lead to an accumulation of short chain fatty acids (SCFAs) and lactate in the rumen, which disrupts the rumen's homeostatic acid-base balance regulation [1]. This results in a number of severe dysfunctions, commonly known as the subacute ruminal acidosis metabolic complex, which has become a prevalent health disorder in dairy and feedlot cattle [2]. In this work, we used metabolomics approaches to determine biomarkers associated with metabolic disorders, and to provide a deeper understanding of bovine rumen-gut health. For this purpose, nine rumen-cannulated non-lactating cows were first fed a forage diet and then gradually transitioned to a grain-rich diet [3, 4]. During this experiment, samples of several different matrices were taken once a week. Four of these - rumen fluid, plasma, saliva and feces - were analyzed by a targeted metabolomics approach utilizing anion exchange chromatography coupled to high resolution mass spectrometry. This method is capable of quantifying 89 compounds (mainly carboxylic acids, nucleotides, sugars and sugar phosphates). In feces, concentrations of carboxylic acids, SCFAs and sugars increased noticeably after the switch from forage-rich diet to grain rich diet. In plasma, the change in feed composition led to a slight decline in some analytes, while others increased immediately after the change in feed composition, in part followed by a decline to the original concentration before grain rich diet was fed. In rumen fluid, the concentrations of all sugars increased noticeably, whereas concentrations of some SCFAs and carboxylic acids increased, while no discernible change occurred in others. In saliva, several large outliers containing very high SCFA concentrations were found, likely because these cows ruminated shortly before the saliva sample was taken, thus leading to a contamination of the samples with rumen fluid. Despite different metabolite profiles in the individual matrices, certain common metabolites were identified in every investigated matrix.

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