Mitochondria in chemical-induced toxicity

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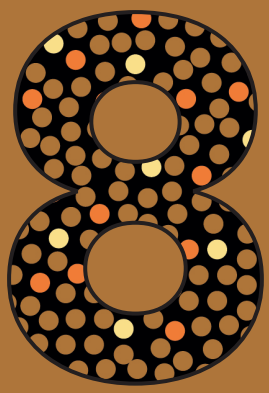

Discussion and future perspective

Perturbation of cellular homeostasis is a major cause of organ toxicity occurring upon chemical exposure. Malfunctioning of one of the most important cellular organelles, the mitochondria, is observed upon exposure to 15% of 10,000 environmental chemicals (TOX library) [Attene-Ramos 2015, Xia 2018]. Testing for chemical-induced mitochondrial malfunctioning is becoming more prominent during the development of chemicals and is largely based on proper functioning of vital mitochondrial processes, like respiration and metabolism [Dykens 2007, Dykens 2014]. Despite the current testing strategy, mitochondrial perturbations are still observed upon exposure to existing environmental chemicals, signifying the need for improvements in risk assessment. We hypothesized that the use of a more mechanism-driven testing approach that also monitors the overall cellular responses, besides functioning of the vital mitochondrial processes, will strengthen the prediction of potential mitochondrial toxicity during chemical risk assessment.

Mechanism-based research links mitochondrial perturbation to cellular adversity

Organ toxicity is the result of both perturbation of the mitochondrial proteins and enzymes followed by a (partial) loss of their function and the incapability of the cell/tissue to overcome this. Assessment of changes in cellular behavior upon perturbation of mitochondrial processes will improve the identification of conditions leading to cellular adaptation or toxicity. To enable proper classification of generic, chemical class- and substance-specific effects, a comprehensive characterization of vital mitochondrial processes, cellular behavior and toxicity is required.

Vital mitochondrial process can generally be subdivided in 6 processes, as reviewed in **chapter 2**: metabolism, respiration, protein homeostasis, morphology dynamics, calcium homeostasis and mitophagy. The assays monitoring proper functioning of these mitochondrial processes should allow us to discriminate between normal fluctuations (non-toxic conditions) and a perturbed state (toxic conditions), and ideally provide scaled results, matching the potency of chemicals. This information supports flagging of chemicals that require further testing. In addition, the ability to separate mitochondrial toxicants with different modes-of-action will support the use of more tailored risk assessment pipelines, with specific testing steps only relevant for subgroups of chemicals. In **chapter 3**, we focused on the evaluation of well-known biochemical assays for vital mitochondrial functions, which monitor (complex-specific) respiration, mitochondrial integrity, glycolytic capacity and cell viability for mitochondrial transport inhibitors. These assays were all capable of discriminating inactive (e.g., complex II inhibitors) and active chemicals (e.g., complex I and III inhibitors) and could be used to determine potency differences within the group of

active chemicals. ToxCast and Tox21 have used similar approaches, confirming that these assays can identify different classes of mitochondrial electron transport chain inhibitors [Xia 2018, Willw 2015, Hallinger 2020]. Others have demonstrated that mitochondrial membrane potential readouts for a large set of chemicals allowed the identification of chemical structure clusters with increased risk for mitochondrial-related toxicity [Attene-Ramos 2015]. Nonetheless, this data did not differentiate classes of mitochondrial toxicants and likely identified only the most potent structures. Identification of inhibitors that have low potency or that demonstrate only partial inhibition remains important in case of prolonged exposure (i.e., a low dose of agro-chemicals) or upon exposure to mixtures (i.e., a combination of drugs and food additives), which could result in accumulative or even synergistic effects [Nandipati 2016, Vasan 2020].

Cellular behavior upon perturbation of vital mitochondrial processes can be monitored based on cellular signaling indicating, among others, the initiation of adaptation responses and induction of cell death. The emerging opportunities that come with the omics technologies allow us to study cellular signaling based on information gathered: at gene, protein and metabolite level, from any cell system, at any moment in time and for any chemical concentration [Brockheimer 2017, Heijne 2005]. We examined the relationship between mitochondrial perturbation and cellular signaling using targeted transcriptomics (**chapter 4**). The overall concentration-dependent change in expression of the set of toxicology relevant transcripts illustrated that we can distinguish chemical potencies based on the transcriptome profiles. The study of the up- and down-regulated transcripts did not allow separation of the chemicals targeting the different protein complexes responsible for mitochondrial respiration, which indicates a shared cellular response upon exposure to this class of chemicals [Pearson 2016, Simon 2019]. The incorporation of chemicals with other mode-of-actions (mitochondrial metabolism and morphology), multiple time points (covering primary and secondary responses) and the use of the whole transcriptome is required to study the clustering behavior of different classes of mitochondrial toxicants and will support studies that aim to distinguish between the primary targets of all chemical classes.

Based on our work and that from others, we envision that the ultimate assay design for testing the relationship between mitochondrial events and cellular toxicity should incorporate multiple readouts in one run such that their relative influence can be studied. A set of consecutive readouts could be an early drop in ATP levels monitored using ATP-FRET (used in **chapter 4 and 5**), followed by the up- or downregulation of relevant downstream fluorophore-labeled proteins and eventually the induction of cell death markers at a later stage. Alternatively, a set of concurrent readouts addressing multiple features of one cellular response, or several features of different cellular

responses will strengthen the identification of toxic conditions. These combined high-content imaging approaches are only limited by the number of available lasers, compatibility of the used fluorophores and the resolution required to capture the studied biological process. The use of these single cell measurements will allow the identification of exact thresholds amongst the various subsequent events occurring between mitochondrial perturbation and toxicity, which will help to understand why one cell survives and another cell will not.

Single cell concurrent measurements are already commonly used in a sequencing context [Hwang 2018]. The sequencing captures both nuclear and mitochondrial genome-driven gene expression and the co-expression patterns of both have been used to understand occurrence and development of mitochondrial diseases [Lareau 2020]. These patterns could also be used to understand possible adaptive or adverse responses upon exposure to chemicals targeting mitochondrial genes and/or proteins. In the last decades, single cell sequencing and imaging-based readouts are integrated in one assessment pipeline to link phenotypic readouts, like protein expression and morphology, to concurrent transcriptomic changes. This approach relies on sequencing of single cells that were selected based on interesting phenotypes or were stained for interesting markers after collection [Binan 2016, Liu 2020]. In this way, imaging-based selection of cells with specific mitochondrial phenotypes and concurrent single cell sequencing enables linking of different mitochondrial appearances to the expression of relevant proteins (**chapter 5**).

Altogether, this work demonstrated that using a tiered testing strategy that monitors mitochondrial processes followed by detailed assessment of cellular signaling will provide valuable information to unravel the relationship between mitochondrial perturbation and cellular adversity, which will support identification of both potent and less pronounced mitochondrial toxicants.

Mechanism-based studies enable the use of a variety of in vitro cell models

Nowadays toxicology and chemical risk assessment is focused on reducing the animal burden in all phases of chemical development and evaluation. Since 2013, the use of products tested on animals is completely banned in the cosmetic field and the use of alternative testing methods is preferred and promoted in the context of chemical assessment under REACH [European Committee 2009, European Committee 2006]. The choice of cell system to use in alternative testing methods influences the throughput, relevance and complexity of the outcome. The outcome of especially mitochondria-related readouts can vary between cell systems, because of large differences in mitochondrial mass and need for mitochondrial respiration

[Pagliarini 2008, Wang 2010]. Nevertheless, the use of a tiered mechanism-driven testing strategy allows the use of all available models; this requires proper selection for readouts relevant for the used cell type and its complexity.

The first tier in the assessment of mitochondrial toxicity includes the study of chemical-target interaction. The molecular structures of mitochondrial targets are assumed to be comparable between different human cell types. This implies that any cell type, independent of its origin or need for mitochondrial respiration, could be used to assess the potency of chemicals for interaction with mitochondrial targets in high-throughput formats assessing large sets of chemicals. We demonstrated that HepG2 cells do support the assessment of complex-specific inhibition of respiration, loss of mitochondrial integrity and decreased ATP production (**chapter 3, 4, 6 and 7**). Moreover, related changes in downstream signaling processes could be picked-up with HepG2 cells that do not exhibit toxicity, indicating HepG2 is "fit-for-purpose" to analyze these early events.

These "simple" models, like HepG2 cells, can be forced to rely more on mitochondrial respiration. We achieved this via inhibition of glycolysis, which resulted in an increase in the observed cellular toxicity. This new phenotype better resembled the effects observed in cell lines that already rely more on mitochondrial respiration, like RPTEC-TERT1 (**chapter 3 and 4**, Delp 2019). Another option is to increase the complexity of model systems by changing the culture environment via the introduction of an extracellular matrix. In **chapter 3**, we demonstrated that HepG2 cells cultured in 3D protein matrices establish a different phenotype including the reduction of glycolysis-related factors. Furthermore, exposure to electron transport chain inhibitors, specifically complex I inhibitors, led to toxicity already in response to a single dose.

Nevertheless, certain human tissues are more susceptible towards mitochondrial perturbation upon chemical exposure and therefore require extra attention in follow-up research. Differences between tissues arise from their reliance on mitochondrial respiration or high energy demand as is observed for respectively neurons and muscles. To enable proper assessment of mitochondrial toxicity, it is necessary to incorporate *in vitro* assays monitoring readouts specific for proper functioning of these susceptible tissues. In **chapter 7** and related work [Delp 2020], we additionally monitored the outgrowth and degradation of neurons to capture central nervous system specific effects. It is clearly demonstrated that chemicals known to induce neuronal disorders, like rotenone, also display a more pronounced effect in the susceptible tissues. Assessment of specific muscle function can be achieved *in vitro* by assessing myotube formation via morphological features, immunofluorescence staining and their adhesion to each other and cell culture materials [Ishikawa 2019, Murphy 2016].

More advanced model systems for mitochondrial, or any type of toxicity studies, are models that capture a form of higher tissue complexity. This higher complexity can be achieved by using multiple tissues (organ-on-a-chip), multiple cell types (micro tissues) or multiple cell lines derived from one genetic background (IPSCs technology). Organ-on-a-chip combines multiple tissues to enable monitoring of metabolism and signaling in the development of toxicity [Bavli 2016]. This combination will create a more realistic homeostasis between different substrates and metabolites involved in mitochondrial respiration and regulation of glycolysis. Micro tissues will support the study of interactions within one tissue, to understand why particular cell types are more vulnerable and how intercellular signaling is involved in the development of toxicity. This concept combined with IPSCs was explored in the form of a "mini brain" and could provide valuable information into the relationship between mitochondrial perturbation and neurological disorders [Govindan 2021]. Moreover, IPSCs differentiated into multiple cell lineages enables more in-depth studies when creating tissues with different mitochondrial mass (kidney, cardio, liver, and neuro) from the same genetic background [Shi 2017].

To summarize, the integration of specific *in vitro* models into the different steps of a tiered testing strategy enables flagging of chemicals in early stages using simple cell models followed by testing for specific toxicity in susceptible tissues or upon prolonged exposure using tissue relevant and more complex systems. The proper use of these *in vitro* methodologies will help to move from complete *in vivo* testing strategies to more hybrid forms in which *in vitro* work will reduce the required number of test animals, and ultimately to a chemical risk assessment without the need of animals.

Integration of in vitro and in silico strengthens mitochondrial perturbation prediction

The use of quantitative rather than qualitative mechanistic information facilitates the development of toxicity prediction models assessing the probability that chemical exposure will result in cellular adversity. *In silico* simulations enable the generation of detailed mathematical descriptions of experimental data allowing intra- or extrapolation to untested conditions, which will indicate if a chemical is safe or not and inform about required further testing.

Understanding the exact relationship between the degree of mitochondrial perturbation and the balance of cellular adaptation or adversity is required to enable the identification of mitochondrial toxicants in general, but specifically for chemicals that have a less pronounced effect.

To facilitate the identification of these adversity thresholds, it is essential to describe the observed changes in mitochondrial functioning. In **chapter 4 and 6**, we monitored mitochondrial perturbation using temporal high content imaging of mitochondrial membrane potential fluctuations in detail. We described the time-dynamics using *in silico* approaches based on a phenomenological model and an ordinary differential equation (ODE) model. Others employed ODE models to describe the relationship between mitochondrial DNA and mitochondrial morphology [Kornick 2019], the interaction between Ca^{2+} homeostasis and mitochondrial swelling [Moshkforoush 2019, Efendiev 2020], and the balance between all mitochondrial metabolites [Bazil 2010]. The obtained parameters from both phenomological and ODE models could serve as a classification of hazardous and non-hazardous chemicals. Nevertheless, ODE descriptors fit best in a biology-driven assessment.

Moreover, the use of machine learning approaches, supervised or unsupervised, help to establish tools in which information obtained from *in vitro* and *in silico* approaches together support chemical clustering and as a result improved classification. In **chapter 5**, we used unsupervised machine learning algorithms to classify the mitochondrial morphology phenotypes into two sub-groups. The unbiased algorithm enables the assessment of the change in distribution between the two mitochondrial types of mitochondrial morphology upon chemical exposure. A combination of processes, including MMP and ATP, should be perturbed before mitochondrial fragmentation occurred, rather than only a decrease in MMP [Jones 2017]. Machine learning approaches to classify mitochondria morphologies are also exploited by others using both supervised and unsupervised assessment to classify mitochondrial phenotypes [Zahedi 2018, Fisher 2020]. Using high resolution images, they achieved the characterization of fragmented and tubular objects, though when integrating morphology features into a high-content analysis pipeline, the use of lower resolution pictures is standard practice. Therefore, in **chapter 5** we focused on the discrimination of clear differences rather than identification of detailed structures. The next step in understanding the implications of mitochondrial morphology change for the development of toxicity is to link the phenotypes besides to mitochondrial processes also to the dynamics of morphology machinery proteins. Others already demonstrated that changed expressions of these machinery proteins can be both protective and disruptive upon exposure to several known mitochondrial inhibitors [Hwang 2014, Alaimo 2014, Patten 2014, Civiletto 2015, Grohm 2012, Tian 2014, Jones 2017].

The incorporation of these *in silico* approaches into *in vitro* testing platforms allow detailed quantification of chemical-induced temporal, spatial and concentration-dependent change in mitochondrial parameters, including ATP, MMP and mitochondrial morphology. We envision that combining this approach with the

quantitative description of cellular behavior upon chemical exposure will support prediction of toxicity upon a broad variety of exposure scenarios.

Chemical risk assessment revisited?

Incorporation of *in vitro*-based mechanistic studies into chemical risk assessment requires broad acceptance amongst regulatory agencies. To achieve this, new methodologies need to gain trust and successful examples are vital in this process. Integration of new methodologies into the identification of the risk a chemical poses for human health can be done at various levels of the assessment process, including hazard and exposure assessments.

Chemical risk assessment attempts to estimate the hazard of a particular chemical by its identification and characterization. Hazard identification for mitochondrial toxicants can be performed both bottom-up or top-down. A bottom-up approach is based on biochemical assays focusing on known mitochondrial targets, such as respiration, metabolism, or morphology. This approach can be used to identify chemicals that completely perturb particular targets or to flag chemicals that demonstrate less pronounced effects but could be of risk in combined or prolonged exposures. A top-down approach is built around the cellular response, for instance transcriptomic profiles, observed upon mitochondrial target perturbation and could flag chemicals that induce responses known to be linked to levels of mitochondrial perturbation above the cells capacity to adapt.

Hazard characterization takes it a step further and focuses on understanding the temporal, concentration, and spatial relationship among the mode-of-actions and the occurrence of adversities. It assesses the risk of a chemical upon acute vs prolonged exposure, the concentration at which adaptation turns into adversity and the effects of an accumulated dose in specific tissues. In this case more complex *in vitro* model systems, for instance, the 3D spheroids or IPSC based cell systems can support the study of different dosing regimens. In addition, systems specific for mitochondrial susceptive tissues, like neurons and muscles, help to identify chemicals which both interfere with the mitochondrial targets and perturb susceptible tissue-specific features.

The complete risk characterization also requires a likelihood estimation if a person or a specific group of people will be exposed to a dose that would pose a threat to their health. This is based on expected exposure levels based on intake and exposure levels that will induce toxicity. The hazardous exposure levels are generally determined based on *in vivo* toxicity assessments combined with a range of safety factors assessing inter- and intra-species variations, duration of exposure and the output used to define an effect. Reduction of uncertainty or even only identification

of the existing uncertainty in the safety factors will create more confidence in the obtained information and potentially reduces the large numbers of animal studies [EFSA 2018, Bokkers 2017].

The *in vitro* approaches discussed in this thesis are not capable of replacing all exposure related data obtained from *in vivo* tests. Especially the assessment of various exposure scenarios and tissue interactions are challenging to mimic *in vitro*. Nevertheless, mechanistic studies could support in reducing the safety factors concerning the identification of the effect levels (point of departure = PoD). The PoD is based on an observed first effective dose (lowest/no observed effect level = LOAEL/NOAEL) or modelled based on the complete tested dose range (benchmark dose = BMD) both obtained from *in vivo* studies. *In vitro* assays, in particular will help to identify relevant concentration ranges, which will focus and reduce follow-up experiments *in vivo*. *In vivo* relevant doses can be extrapolated from *in vitro* concentration ranges for any desired (mitochondrial-related) readout when using bioavailability studies to determine the *in vitro* available concentration [Fisher 2019] and PBPK modeling to translate this into an *in vivo* available dose [Shebley 2018] (as we performed in **chapter 7** and related work [OECD 2020a, OECD 2020b, OECD 2019, Van der Stel 2021 submitted, Escher 2021 submitted]).

Finally, the detailed mitochondrial toxicity data obtained with *in vitro* mechanistic studies, especially with the broad omics assessments, also support the identification of the expected type(s) of adversity in humans. This informs us about the possible susceptive users and the features which should be monitored to timely identify possible mitochondrial perturbation during development phases and possibly thereafter.

Concluding remarks

Altogether, this thesis contributes to the integration of *in vitro* mitochondrial-related toxicity assessment into chemical risk assessment. We evaluated existing and new mitochondrial toxicity biomarkers for their potential application in high-content imaging approaches and established a transcriptomic analysis pipeline that supports more mechanism-driven assessment of the relationship among mitochondrial perturbation, cellular adaptation and adversity. Ultimately, our *in vitro* mitochondrial toxicity strategy will support safer use of chemicals and reduce the animal burden to achieve this goal.