Mitochondria in chemical-induced toxicity

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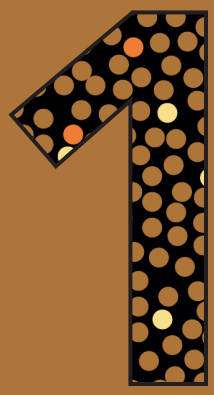

General introduction, aim and scope of thesis

Chemical-induced organ toxicity is a major concern in the development and societal application of chemicals, including pharmaceutical drugs, pesticides, industrial chemicals and cosmetics. In the last decades, the onset and progression of chemical-induced organ-toxicity have been linked to the perturbation of various cellular targets and processes, one of which is mitochondrial functioning. This is illustrated by a study involving 10,000 tested environmental chemicals and drugs (the TOX21 library) from which 15% perturbs mitochondrial respiration upon exposure [Attene-Ramos 2015, Xia 2018]. Mitochondria appear to be a major target after exposure to higher chemical concentrations in the case of liver, heart or kidney toxicity [Rana 2019]. Unraveling the mechanisms relating mitochondrial perturbations to organ-toxicity is essential to improve hazard identification and risk assessment for new and existing chemicals. This thesis elaborates on the development of quantitative markers for the assessment of chemically-induced mitochondrial perturbation. In this first chapter I will introduce the biological role of mitochondria, their susceptibility to perturbations and the role of perturbed mitochondria in development of organ failure. Furthermore, I will discuss how biomarkers of mitochondrial functioning could improve the mechanistic understanding of the progression of chemical-induced organ-toxicity and thereby support early stage risk assessment of chemicals.

Mitochondria

Mitochondria are organelles essential for the energy production in eukaryotic cells. They are thought to originate from aerobic prokaryotes which established an endosymbiosis with the ancestors of today's eukaryotes. Although its precise trigger remains unknown, it is clear that this endosymbiosis provided the eukaryotic cell the opportunity to survive in more oxygen-rich atmospheres, develop into more complex architectures and support multi-cellular collaborations [Martin 2015]. In eukaryotic cells the mitochondrion consists of a double membrane enclosing the mitochondrial matrix with its unique circular DNA. This mitochondrial DNA (mtDNA) encodes for the majority of proteins and associated ribosomal RNAs involved in the oxygen-dependent production of energy via the electron transport chain (ETC). The double membranes provide the opportunity to create a proton gradient used to drive the production of energy carriers and the enclosed matrix is used to store ions like calcium. An energy carrier like ATP is of major importance for the effective functioning of active transport, signal transduction pathways and RNA/protein synthesis in the cell [Alberts 2002, Lynch 2015][1,2]. Active transport is a form of transport which consumes ATP to be able to transport molecules, like for example glucose, over membranes against a concentration or electrochemical gradient. ATP itself or the energy stored in ATP is also used to transport information concerning extracellular conditions from the cellular membranes into the cell, for instance signal transduction supported by

ATP-driven phosphorylation or the use of cAMP which is created from ATP. Not only transport and signaling requires the energy, also both RNA transcription and protein translation require the input of energy to allow the cell to assemble all proteins in the cell.

Furthermore, mitochondrial specific enzymes present in the mitochondrial matrix provide the necessary equipment to produce crucial building blocks, including amino acids, nucleotides, lipids, and Fe-S clusters, needed for protein translation, RNA transcription, membrane construction and enzyme functioning [Pfanner 2019, Friedman 2014]. All together this prominent role of the mitochondria in energy, amino acid, nucleotide and lipid production, makes the organelle the perfect sensor for the detection of abnormalities in the cellular homeostasis.

Energy production in the cell

Energy required for cellular functioning is obtained from three sources: carbohydrates, proteins and fatty acids originating from our diet [Nelson 2017]. They are metabolized via an interplay between enzymatic reactions in the cytoplasm and mitochondria. Which of these three substrates is used depends on the availability in our food, the requirements of the cell itself, the presence of enzymes/substrates needed for all intermediate steps and the presence of oxygen.

Carbohydrate metabolism and especially glucose metabolism occurs in three successive metabolic steps: glycolysis, the citric acid cycle and mitochondrial respiration (figure 1). Glycolysis, an anaerobic process occurring in the cytoplasm of the cell, is the conversion of imported glucose via multiple enzymatic steps into pyruvate with a net yield of 2 ATP molecules. Pyruvate is subsequently transported into mitochondria by the mitochondrial pyruvate carriers 1 and 2 (MPC 1 and MPC2) [Bricker 2012], converted into 2-carbon acetyl-CoenzymeA (acetyl-CoA) and processed by the citric acid cycle: a series of metabolic steps resulting in the creation of NADH, $FADH_2$ and CO_2. The produced energy rich NADH and $FADH_2$ molecules are the fuel for the mitochondrial respiration. In an oxygen-poor environment cells rely completely on the less efficient ATP production via the glycolysis and do not shuttle pyruvate through the citric acid cycle, but instead convert pyruvate to lactate by fermentation.

Fatty acids are imported from the cytoplasm into the mitochondria where they are further metabolized. The import is through active transport for chain length longer than 14 carbons (carnitine carrier) [Indiveri 2011]. The complete metabolism of the fatty acid carbon chains is termed beta-oxidation, which is the gradual removal of all carbon pairs and coupling them to CoA resulting in acetyl-CoA. This process yields both NADH and $FADH_2$ molecules. Acetyl-CoA enters the citric acid cycle together

with the acetyl-CoA obtained from the glycolysis. In case of fasting, the intermediates of the citric acid cycle are depleted and the mitochondria start to produce ketone bodies from acetyl-CoA (ketogenesis). This form of energy production allows the beta-oxidation and mitochondrial respiration to continue without the need of the citric acid cycle. However, prolonged production of ketone bodies will result in an undesired drop in the pH of body fluids, eventually resulting in toxicity.

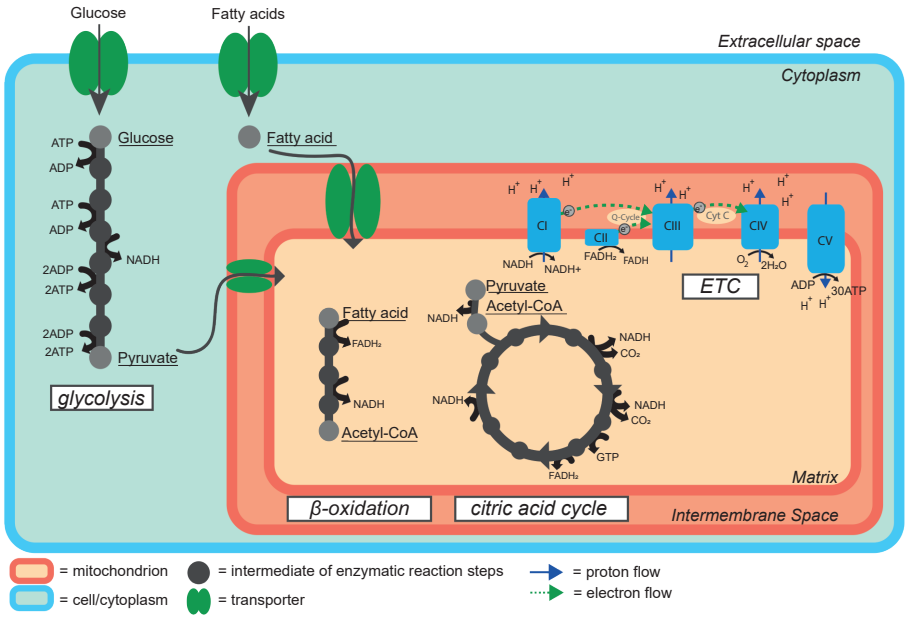

Figure 1: Energy production in the cell from glucose and fatty acids. Glucose and fatty acids are converted in the cytoplasm and mitochondria to ATP, NADH and $FADH_2$ via an interplay of glycolysis, β-oxidation, citric acid cycle and subsequently the electron transport chain (ETC). The intermediate steps in the glycolysis, β-oxidation and citric acid cycle are omitted for clarity and the protein complexes involved in the ETC are mitochondrial complex I, II, III, IV (respectively CI, CII, CIII, CIV and CV) .

The amino acids extracted from various proteins enter the citric acid cycle at various steps. In humans, there are two subgroups of amino acids: ketogenic amino acids, which can be converted to ketone bodies, and glucogenic amino acids, which can be used to create glucose and glycogen. The ketogenic amino acids are converted to acetyl-CoA and enter the citric acid cycle together with the acetyl-CoA created from carbohydrates and fat, while the glucogenic amino acids are pre-cursors for various intermediate substrates in the citric acid cycle or can be converted to pyruvate.

Mitochondrial respiration (Electron transport chain)

The NADH and $FADH_2$ energy carriers that are created in the beta-oxidation and citric acid cycles are used in the mitochondrial respiration chain. To free the energy stored in

the electrons from NADH and $FADH_2$, they are passed over 4 protein complexes called the electron transport chain (ETC): complexes I (NADH dehydrogenase), II (Succinate dehydrogenase), III (Cytochrome bc1 complex) and IV (Cytochrome c oxidase), each transferring electrons from electron donors to electron acceptors. The energy that is released in these redox reactions is used to shuttle protons over complex I, III and IV from the mitochondrial matrix into the intermembrane space. Finally, low energy electrons are used by complex IV to reduce O_2 into H_2O, making the electron transport chain a form of aerobic energy production. The produced proton gradient drives the conversion of 30 ADP molecules to ATP in mitochondrial respiration chain complex V (ATPsynthase), making the mitochondrial respiration the major contributor to ATP production in the cell.

Mitochondrial susceptibility towards damage

The central role of mitochondria in energy production and amino acid metabolism makes perturbation of these organelles a major risk factor in the cellular response to toxic chemicals. Furthermore, various features of the organelle itself, including the double membrane, the charged inner compartment and the presence of detoxifying enzymes and DNA, create vulnerability towards chemical toxicity [Meyer 2013].

Mitochondria are sensitive to the accumulation of chemical structures with various physicochemical properties within their matrix [Ross 2006, Battogtokh 2018]. Lipophilic compounds are attracted because of the lipid rich mitochondrial membranes, while cationic metals accumulate in the mitochondria because of the presence of a negative charged and a slightly alkaline matrix. Furthermore, the combination of lipophilic membranes and a charged/alkaline matrix attracts chemicals with amphiphilic properties. Besides accumulation of the parent chemical also the accumulation of metabolites bears the risk to disrupt mitochondrial integrity. The presence of metabolic enzymes in the mitochondria and the closely connected endoplasmic reticulum (ER) results in increased levels of reactive metabolites in the mitochondrial matrix [Sangar 2010].

Disruption of the mitochondrial membranes can also have secondary consequences. Under normal conditions the mitochondrial DNA is safely stored within the mitochondrial compartment. However, mitochondrial DNA leakage into the cytosol upon loss of mitochondrial integrity can trigger an undesired immune reaction targeted against free-nucleotides, causing or aggravating toxicity [Maekawa 2019].

Cells contain hundreds of mitochondria and therefore severe damage to a small fraction of these mitochondria will not immediately result in cytotoxicity and, consequently, tissue or organ malfunctioning [Alberts 2002]. The occurrence of

cytotoxicity upon chemical exposure largely depends on accumulation of damaged mitochondria and the speed by which the mitochondrial pool can restore itself. Single mitochondria possess an arsenal of options to restore homeostasis, including the activity of molecular chaperones to restore misfolded proteins [Munch 2018] and detoxifying enzymes to neutralize reactive parent chemicals and metabolites [Forred 2017, Holley 2011]. Furthermore, the pool of mitochondria in a cell provides the opportunity to cope with low amounts of damage via organelle interaction. The mitochondrial interaction is a balance between fusion – fusion of two or more organelles – and fission – splitting of one organelle into two or more – [Westermann 2010, Gaicomell 2020]. Together they provide the opportunity to exchange DNA and proteins, but also split off unrepairable components, which can be degraded specifically via the process termed mitophagy [Youle 2011, Hamacher-Brady 2016]. All processes combined make that the cell system can handle stress internally until a certain threshold is reached. Beyond this tipping point, the system will decide to sacrifice individual cells to save the organ and with that the species.

Mitochondria as player in chemical-induced organ toxicity

As mentioned above, the cell and its mitochondria possess various adaptive mechanisms which can help to cope with limited amount of damage. However, acute high levels of perturbation or prolonged presence of damage can lead to organ toxicity. Tissues and organs which demonstrate high susceptibility towards toxicity include those involved in chemical metabolism and excretion. Disturbing either of these processes can result in accumulation of parent chemicals or its metabolites, as observed in liver and kidney [Doull's 2008]. Organs, like heart, kidney and muscle, with a high energy demand rely more heavily on mitochondrial respiration and are therefore more susceptible to mitochondrial specific organ toxicity [Pagliarini 2008, Wang 2010]. Finally, tissues, like neurons, with limited capacity to counteract the impaired mitochondrial respiration, via the upregulation of glycolysis, also demonstrate a higher occurrence of toxicity [Fernandez-Fernandez 2012, Zhao 2019].

Mitochondrial perturbation has been observed as off-target effect upon intake of various drug classes [Dykens 2007a, Dykens 2007b, Will 2019]. These chemicals can target various components of the mitochondria, including electron transport chain complexes, enzymes/ substrates, mitochondrial DNA, structural proteins or the mitochondrial permeability transition pores. Another subset of the chemicals affecting the mitochondria are designed to target mitochondria of others species (e.g. pesticides and piscicides like rotenone and antimycin), but demonstrate undesired affinity for human mitochondrial proteins.

The class of chemicals designed to interfere with mitochondrial respiration include pesticides manufactured to inhibit mitochondrial complex I of the ETC. Various retrospective studies speculate about an association between the hazard of developing Parkinson's-like diseases and prolonged exposure to complex I inhibitors, like rotenone [Tanner 2009, Tanner 2011, Nandipati 2016]. *In vivo* assessment of the toxicity of rotenone indicated the development of neuronal defects, which correlates with the possible development of neurological disorders [Betarbet 2000]. The occurrence of the undesired toxicity across species has led to environmental use restriction regulations in various countries [Gonzalez-Coloma 2013].

The undesired occurrence of mitochondrial perturbation as off-target effect upon exposure to chemicals has also led to usage restrictions (box warnings) or even withdrawal of chemicals and especially drugs from the market. The observed adversities are mostly occurring in the liver and heart, but malfunctioning of muscles, intestine or even systemic failure (development of lactic acidosis) have also been reported [Boelsterli 2007, Dykens 2017a, Dykens 2017b, Will 2019]. Examples of drugs withdrawn from the market include the liver injury inducing drugs nefazodone (anti-depressant), troglitazone (anti-diabetic) and trovafloxicin (antibiotic). An overrepresented class of drugs which have a box warning are commonly used pain medication drugs, including acetaminophen and NSAIDs (non-steroidal anti-inflammatory drugs) like, acetylsalicyl acid, celecoxib, diclofenac, ibuprofen, indomethacin, mefenamic acid, meloxicam, nabumetone, naproxen, nimusulide, piroxicam and sulindac.

Mitochondria as biomarker for toxicity

In the past, a number of mitochondria perturbing agents have entered the market undetected. To reduce these incidences, industry introduced mitochondrial toxicity testing in their current chemical development pipelines [Dykens 2014]. However, to keep improving the mitochondrial risk assessment, it is important to validate existing biomarkers, introduce new candidates and to integrate them into structured risk assessment strategies.

To optimize the use of existing biomarkers and assays, it is important to select and validate their biological relevance. Currently, the assessment of the chemical impact on mitochondrial health is mostly based on biochemical readouts for the different mitochondrial processes, including oxidative phosphorylation (OXPHOS), metabolism, organelle dynamics, protein homeostasis, ion homeostasis and organelle degradation. These assays are specifically relevant for mitochondrial functioning and are therefore very suitable for the identification of direct and efficient inhibitors of mitochondrial targets. The assessed process can be relevant for mitochondrial functioning, however it is also important to consider the various *in vitro* parameters and their relationship

to the *in vivo/in human* situation. This includes among others the selection of the cell system, the environmental conditions and the moment of measurements. As mentioned above, tissues and their cell types vary greatly in their number of mitochondria and reliance on mitochondrial respiration. Moreover, the selected *in vitro* culture conditions affect mitochondrial respiration status and activity [Pagliarini 2008, Bogert 1992]. While assessing effects upon mitochondrial functioning *in vitro*, it is also important to keep in mind that *in vivo/in human* observed perturbation can be direct and indirect and one should adjust the assay and moment of measurement accordingly.

The introduction of new biomarkers is required to improve the quality and predictivity of mitochondrial toxicant detection. One of the main limitations of existing biochemical assays, used in the past, is that they cannot completely capture the interconnectivity between the mitochondria and the various organelles in the cell. Cytotoxicity occurring upon partial target interaction and prolonged or repeated exposures can be overlooked when assessing only mitochondrial targets. Incorporation of the mitochondrial interconnectivity via a combined strategy of assessing mitochondrial functioning and the correlated cellular responses could improve the identification of all mitochondrial perturbing agents. Opportunities to support biomarker identification are, among others, live confocal imaging and omics approaches.

Live confocal imaging using, among others, fluorescent tagged proteins provides the opportunity to follow multiple processes simultaneously over time in the same cell. The use of living intact cells enables tracking of signal transduction and protein expression, which includes valuable information allowing us to follow the development of adversity in a time-dependent manner [Wink 2017, Wink 2018].

Omics techniques can help bridge the gap between mitochondrial and cellular signaling present in single endpoint biochemical assays [Heijne 2005]. The assessment of the nuclear transcriptome gives information concerning regulation of essential mitochondrial components, and possible related cellular signaling [Joseph 2017 Cui 2010]. The mitochondrion-nucleus communication is a close connection exchanging information concerning the status of both organelles; this dynamic interaction could potentially produce an early biomarker for mitochondrial perturbation.

To support efficient and large-scale assessments of chemicals based on both existing and new biomarkers, the introduction of high throughput toxicity identification is required. Automated high throughput confocal imaging offers the opportunity to follow in a concentration- and time-dependent manner the effect of dozens of chemicals in all types of model systems using a variety of culture conditions. Furthermore, the establishment of cheaper alternatives for transcriptome analyses,

like the targeted transcriptome assessment of crucial genes in chemical-toxicity, also supports the assessment of chemical-induced time- and concentration-dependent alterations of the cellular response [Waldmann 2014, Limonciel 2018].

The addition of *in silico* assessments in any assay setup can support large scale pattern recognition, unbiased selection of predictive parameters or replace *in vitro* work. *In silico* set-ups can for example be used to pre-screen existing chemical libraries for the identification of pharmacophores susceptible for mitochondrial toxicity or chemical structures likely to bind to specific mitochondrial targets [Naven 2013, Troger 2020, Hemmerick 2020].

To be able to integrate the obtained high dimensional information into risk assessment and regulations, it is important to condense and structure information into documentation accepted by regulatory agencies (like an Integrated Approaches to Testing and Assessment (IATA) approach). One option to condense toxicological information is the use of the adverse outcome pathway (AOP) framework. AOPs are descriptions of adversity pathways divided in subsequent and essential events ranging from the interaction of a chemical with the cellular system to occurrence of adversity in tissue, organ or individual [Ankley 2010]. When integrating these mostly retrospective qualitative descriptions with all types of preferably *in vitro* data, it will be possible to create a quantitative description of adversity and provide a structured roadmap to perform targeted assessments for all types of toxicity [Oki 2019].

Aim and scope of this thesis

This thesis aims to provide insights into the mechanisms underlying the occurrence of mitochondria-related organ toxicity. In **chapter 2**, we summarized the current knowledge concerning mitochondrial biomarker measurements and their use in a quantitative adverse outcome pathway approach. In **chapter 3**, we assembled an *in vitro* mitochondrial toxicity screening platform to systematically assess chemical-induced mitochondrial perturbation and applied it to assess the effects of chemical exposure upon two cell models: i) HepG2 cells, a liver carcinoma cell line; and ii) RPTEC-TERT1, an immortalized human renal proximal tubular epithelial cell line. Using a set of 23 prototypical ETC inhibitors we evaluated our testing platform for its performance. The introduction of more liver-like 3D cultured HepG2 cells improved the adversity predictions compared to 2D cultured HepG2 and made the outcomes comparable to results from RPTEC-TERT cells. In **chapter 4**, we systematically evaluated the effects of ETC inhibitors upon mitochondrial integrity using high content confocal imaging and targeted transcriptomic studies. High content imaging techniques revealed clear concentration- and time-dependent effects of mitochondrial complex I and III inhibitors exposure upon mitochondrial membrane potential and unfolded protein

response components. The targeted transcriptomic studies provided information for pathway and transcription factor enrichment studies and resulted in a gene profile specific for mitochondrial toxicants that was further evaluated for application in the assessment of drug-induced mitochondrial toxicity liability. In **chapter 5**, we evaluated changes in mitochondrial morphology as an early sign of mitochondrial toxicity. We studied the causal relationship between mitochondrial membrane potential with associated ATP production and mitochondrial morphology. These studies allowed us to get insight into the predictivity of the mitochondrial morphology for toxicity. In **chapter 6**, we used a combination of *in vitro* and *in silico* modeling (based on ordinary differential equations) to study the dynamics of mitochondrial membrane potential upon electron transport chain inhibitors exposures. We revealed that pharmacokinetics and proton leakage were essential parameters for the adjustment of the model to the different chemicals tested. In **chapter 7**, we addressed the possibilities of using chemical read across based on biological similarities for chemical risk assessment in the case of mitochondrial liabilities. Lastly, **Chapter 8**, provides a summary of all findings discussed in this thesis and outlines the implications and future perspectives of these studies.