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New strategies to evaluate mitochondrial function

Mitochondria produce the majority of the cell’s energy. Any dysfunction in, or interference with mitochondrial function can have severe consequences. And yet, it was only within the last decades that screening for potential mitochondrial toxicity was included as a routine toxicity assay during early drug development. Despite improved recognition of drug-related side effects on mitochondrial function, and progress in method development, translation from the in vitro to the in vivo situation and from animal to human tissues still remain obstacles. Mitochondrial dysfunction can also have genetic origin, with similar consequences. Current treatment options for mitochondrial disease are limited. In the present thesis, human peripheral blood cells were evaluated as a model for investigation of drug-induced mitochondrial toxicity and for efficacy assessment of pharmacological treatment strategies to support mitochondrial function.
Mitochondrial medicine

New strategies to evaluate drug toxicity and develop pharmacological protection of the cell’s powerhouse

Sarah Piel

LUND UNIVERSITY

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Current treatment options for mitochondrial disease are limited. Succinate, a natural metabolite of the TCA cycle and a direct substrate of complex II of the respiratory chain that can increase mitochondrial function has shown to improve clinical symptoms in case studies. However, it has limited cell-permeability. We developed novel cell-permeable succinate prodrugs and characterized them using human peripheral blood cells. We demonstrated improved oxidative phosphorylation in different models of mitochondrial dysfunction. Methylene blue, which has previously been described to improve mitochondrial function in experimental models, failed to improve oxidative phosphorylation in human peripheral blood cells under the same conditions.

To investigate the bioenergetic response of human peripheral blood cells relative to other, more metabolically active tissues we further evaluated mitochondrial inhibitors and pharmacological treatment strategies in muscle fibres and human-derived primary cells and cell lines. Changes in the respiratory profiles of human peripheral blood cells reflected changes in the respiratory profiles of other, more metabolically active human cells.

In conclusion, we demonstrate that human peripheral blood cells are a suitable model for evaluation of potential drug-induced mitochondrial toxicity and pharmacological bypass strategies for the support of mitochondrial function. Human peripheral blood cells, in this context, reflect the metabolic responses of other, more metabolically active human cells.
Mitochondrial medicine

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Sarah Piel
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The following article is of relevance to this thesis, but is not formally included:

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<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADR</td>
<td>adverse drug response</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>APAP</td>
<td>acetaminophen</td>
</tr>
<tr>
<td>cyt c</td>
<td>cytochrome C</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>CI</td>
<td>complex I</td>
</tr>
<tr>
<td>CII</td>
<td>complex II</td>
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<tr>
<td>CIII</td>
<td>complex III</td>
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<tr>
<td>CIV</td>
<td>complex IV</td>
</tr>
<tr>
<td>CV</td>
<td>complex V</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
</tr>
<tr>
<td>ETS</td>
<td>electron transfer system</td>
</tr>
<tr>
<td>FA</td>
<td>fatty acid</td>
</tr>
<tr>
<td>FADH$_2$</td>
<td>1,5-dihydro-flavin adenine dinucleotide</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FCCP</td>
<td>carbonyl-cyanide p-(trifluoromethoxy) phenylhydrazone</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>MB</td>
<td>methylene blue</td>
</tr>
<tr>
<td>MEM</td>
<td>minimum essential medium</td>
</tr>
<tr>
<td>mGPD</td>
<td>mitochondrial glycerophosphate dehydrogenase</td>
</tr>
<tr>
<td>MILA</td>
<td>metformin-induced lactic acidosis</td>
</tr>
<tr>
<td>MMP</td>
<td>mitochondrial membrane potential</td>
</tr>
</tbody>
</table>
mtDNA mitochondrial DNA
NAC N-acetylcysteine
NAD⁺ nicotinamide adenine dinucleotide, oxidized form
NADH nicotinamide adenine dinucleotide, reduced form
NADPH nicotinamide adenine dinucleotide phosphate, reduced form
NAPQI N-acetyl-p-benzoquinone imine
nDNA nuclear DNA
NV241 cell-permeable succinate prodrug
NV118 cell-permeable succinate prodrug
NV189 cell-permeable succinate prodrug
NV161 cell-permeable malonate prodrug
OXPHOS oxidative phosphorylation
PBMC peripheral blood mononuclear cells
PRP platelet-rich plasma
PDH pyruvate dehydrogenase
RC respiratory chain
RET reversed electron transport
ROS reactive oxygen species
RT room temperature
SDH succinate dehydrogenase
SUIT substrate-uncoupler-inhibitor-titration
TBI traumatic brain injury
TCA tricarboxylic acid
TMPD N,N,N’-tetramethyl-p-phenylenediamine dihydrochloride
TMRM tetramethylrhodamine methyl ester perchlorate
Summary

Mitochondria produce the majority of the cell’s energy. Any dysfunction in, or interference with mitochondrial function can have severe consequences. And yet, it was only within the last decades that screening for potential mitochondrial toxicity was included as routine toxicity assay during early drug development. Despite improved recognition of drug-related side effects on mitochondrial function and progress in method development, translation from the \textit{in vitro} to the \textit{in vivo} situation and from animal to human tissues still remain obstacles. Mitochondrial dysfunction can also have genetic origin, with similar consequences.

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In conclusion, we demonstrate that human peripheral blood cells are a suitable model for evaluation of potential drug-induced mitochondrial toxicity and pharmacological bypass strategies for the support of mitochondrial function. Human peripheral blood cells, in this context, reflect the metabolic responses of other, more metabolically active human cells.
Background

The mitochondrion

The presence of mitochondria in the eukaryotic cell traces back to the merger of two cells. Billions of years ago the mitochondrion developed from an α-proteobacterium that was taken up by a host cell providing an evolutionary advantage which kept it alive. Different theories exist to whether the host cell was of eukaryotic origin or a prokaryote, with the latter theory being favoured (1; 2). Over time, most of the bacterial genome was transferred to the genome of the host but a small percentage still remained in the mitochondrion. As a result, the mitochondrial machinery is encoded by two genomes in separate compartments within the same cell, the mitochondrial DNA (mtDNA) and the nuclear DNA (nDNA) (1; 3-5).

The mitochondrion is formed by two phospholipid bilayers enclosing the mitochondrial matrix (6; 7). The outer mitochondrial membrane faces the cytosol and is permeable to metabolites required for mitochondrial function. The inner mitochondrial membrane, in contrast, tightly regulates the transport of even the smallest ions, the smallest being the hydrogen ion which eventually drives phosphorylation of adenosine diphosphate (ADP) to adenosine triphosphate (ATP) (8). The inner mitochondrial membrane is highly folded to further increase its surface area which forms cristae, the regions at which mitochondrial ATP is generated (7; 8).

The mitochondrion is highly versatile, dynamic and has multiple functions. It can buffer calcium and, in doing so, maintains cytosolic and mitochondrial calcium levels to regulate the diverse set of cellular enzymatic reactions (9). It also regulates metabolite levels to provide precursors for the synthesis of amino acids, nucleotides and fatty acids (FA) and is involved in the cellular response to stress (6; 10). The primary function of the mitochondrion, and the one most relevant for this thesis project, is its role in energy production: it produces energy in form of ATP through a process referred to as oxidative phosphorylation (OXPHOS). The energy stored in carbohydrates, fats and proteins is broken down to generate high energy electrons which are carried down a chain of protein complexes at the inner mitochondrial membrane, the respiratory chain (RC). This electron transport is coupled to proton pumping across the inner mitochondrial membrane, generating a proton gradient.
which is used by the ATP-synthase, also known as complex V (CV), to phosphorylate ADP (11; 12). The coupling of redox processes to phosphorylation of ADP was first described by Peter Mitchell in 1961 (13) and later awarded him the Nobel prize in chemistry.

The electron transfer potential harvested from the breakdown of carbohydrates, fats and proteins is delivered to the RC via the tricarboxylic acid cycle (TCA) cycle. The TCA cycle, also known as Krebs cycle or citric acid cycle, takes place in the mitochondrial matrix and consists of a chain of eight consecutive enzymatic reactions. Four out of eight of the enzymatic reactions of the TCA cycle generate the reducing equivalents nicotinamide adenine nucleotide (NADH) and 1,5-dihydro-flavin adenine dinucleotide (FADH2). With each turn of the cycle, the oxidation of isocitrate, α-ketoglutarate and malate yields one molecule NADH each and the oxidation of succinate results in one molecule of FADH2 (14; 15). In addition, either one molecule of GTP or ATP is generated at the TCA cycle via substrate level phosphorylation. The energy charges of both GTP and ATP are equivalent and either form is produced depending on which isozyme catalyses the reaction (16). The succinate dehydrogenase (SDH), which oxidizes succinate and generates FADH2, is a unique enzyme of the TCA cycle. It is not only one of the eight enzymes driving the TCA cycle but also one of the protein complexes of the RC and thus, physically links the TCA cycle and the RC together (14; 17; 18). Due to the importance of the TCA cycling, the enzymatic reactions are tightly regulated by the cell’s redox state and energy charge (14).

The RC couples electron flux to proton pumping and is found at the inner mitochondrial membrane. It consists of five multi-subunit protein complexes which are embedded into the membrane, and two smaller, lipid soluble proteins. Complex I (CI) is the main entry point for electrons to the RC (11; 12). The electron transport from CI to complex III (CIII) is facilitated by the lipid soluble protein ubiquinone which mediates the electron transfer between the two complexes. Between CIII and complex IV (CIV) electrons are transferred via the lipid soluble protein cytochrome C (cyt c) (12). The final step of the redox reactions driving phosphorylation of ADP is the reduction of molecular oxygen to water. The redox reactions at CI, CIII and CIV are coupled to proton pumping. The protons are pumped from the mitochondrial matrix to the intermembrane space, the space between the two mitochondrial membranes, creating an electrochemical gradient across the inner mitochondrial membrane. The primary pathway for protons back into the mitochondrial matrix is the ATP-synthase which finally couples the redox reactions to phosphorylation reactions and uses the electrochemical gradient to drive the phosphorylation of ADP to ATP (11; 12). Parallel to CI, electrons can enter the RC through complex II (CII), the SDH. CII oxidizes succinate and shuttles electrons into the RC via FADH2 to ubiquinone. The mitochondrial glycerophosphate
Dehydrogenase (mGPD) and the electron transfer flavoprotein both bypasses the TCA cycle entirely and also deliver electrons to the RC via FADH₂ to ubiquinone.

Closely linked to mitochondrial function is lactate metabolism. Lactate is formed from pyruvate in the cytosol by the enzyme lactate dehydrogenase to the expense of cytosolic NADH. By now, it is widely recognized that lactate is not just a by-product of glycolysis. It is a precursor for the synthesis of alanine, can enter the TCA cycle, partake in OXPHOS and be used as fuel for gluconeogenesis. Under normal circumstances lactate production and utilization are kept at a constant rate. When oxygen is depleted or mitochondrial function is impaired, lactate generation exceeds lactate utilization due to an upregulation of the glycolytic rate to compensate for the reduced mitochondrial ATP production. Under these circumstances, pyruvate metabolism is increasingly shifted towards lactate generation to meet the increased NAD⁺ demand, causing elevated lactate production and acidification (19-21).

**Studying mitochondrial function**

The most common aspect investigated for functional assessment of mitochondrial function is mitochondrial respiration. As oxygen is the final acceptor of electrons during OXPHOS, monitoring oxygen consumption of intact or permeabilized cells, homogenized tissues or isolated mitochondria allows evaluation of the integrated function of the RC. Currently, both clark-type electrodes, such as the high-resolution oxygraph (Oroboros-O2k, Oroboros Instruments, Innsbruck, Austria), and fluorescence-based techniques, such as the Seahorse XFe96 Analyzer (Agilent technologies, Massachusetts, USA) are commonly used in the community of mitochondrial research. Both types of respirometer allow respiration measurements of intact or permeabilized cells, homogenized tissues or isolated mitochondria (22). Next to measuring mitochondrial respiration, assessment of the MMP, mitochondrial oxidant production, mitochondrial ATP production and the capacity of calcium uptake are frequently evaluated (22).

Mitochondrial function can be assessed in the isolated organelle, tissue homogenates, permeabilized cells or in intact cells. Traditionally isolated mitochondria were used. Substrates and inhibitors gain full access to the mitochondrion and can be tightly controlled to investigate the site of mitochondrial dysfunction. The lack of cellular context can make interpretation of results easier. From a methodological point of view, the use of isolated mitochondria allows for an easier assessment of other mitochondrial aspects, such as the MMP, calcium uptake, ATP and ROS production, due to the lack of cellular components thereby reducing concomitant confounding factors (11; 22; 23). The use of isolate mitochondria, however, requires tissue from invasive biopsies which poses a
discomfort and risk for the study subject (22; 24-26) and which makes it less likely
to obtain human samples. The isolation procedure can potentially lead to selection
of different mitochondrial populations, is rather harsh and can thereby lead to
disruption of mitochondrial structure and function (22; 27; 28). Alternatively,
mitochondrial function can be analysed in tissue homogenates or permeabilized
cells that, like isolated mitochondria, allow full control of the analysis of respiratory
states. The advantages, compared to isolated mitochondria, are that the structure of
mitochondria and other cellular components together with their possible interactions
are kept intact. Also, no selection of different mitochondrial populations during the
preparation takes place (11; 22).

The loss of cellular context when working with isolated mitochondria,
permeabilized cells or tissue homogenates is advantageous in terms of substrate
control and easiness of interpretation but important information can be lost by
removing intracellular metabolism from the equation. Working with intact cells
gives restricted information on mitochondrial dysfunction due to the inaccessibility
of certain substrates and inhibitors (11). However, it is the most physiological
approach (11; 22; 29). It gives information on the cells energy demand at
physiological substrate and nucleotide concentrations and keeps compensatory
cellular mechanisms intact (11). Because the use of intact cells involves no
homogenization or permeabilization, there is a lesser risk to negatively affect the
mitochondrial membrane integrity and related coupling efficiency at the RC (11).

Separately analysing the activity of the different complexes of the RC presents
another alternative to evaluated mitochondrial function. This is usually done with
mitochondrial fractions where the mitochondrial membrane is permeabilized by
repetitive freeze and thaw cycles or with sub-mitochondrial particles where the
mitochondria are opened up by permeabilization with a detergent or by sonication.
In either case, the mitochondrial matrix content is lost so that the pure complex
activity can be evaluated (30; 31). Pyruvate dehydrogenase (PDH) deficiency, a
common inborn error of metabolism, would not be detected using mitochondrial
fractions or sub-mitochondrial particles because the enzyme is found in the
mitochondrial matrix (32-35). And yet, decreased activity of the PDH would result
in decreased TCA cycling and OXPHOS. This sample type has the advantage that
very small amounts of tissue are required (~10-20 mg) for measuring enzymatic
activity of the RC. In addition, samples can be stored up for 10 years (30; 36). For
diagnosis of inherited mitochondrial disease it is the standard method and hence,
offers the additional advantage that a large reference literature is available (36; 37).
However, because only the activities of the enzyme complexes of the RC can be
evaluated information on important aspects of mitochondrial function are missing
when working with mitochondrial fractions or sub-mitochondrial particles.
Drug-induced mitochondrial toxicity

Drug-induced mitochondrial toxicity has mainly been discovered retrospectively when the organ specific toxicity associated with a certain drug has been investigated more thoroughly. Two of most prominent examples are the antidiabetic drug troglitazone and lipid lowering drug cerivastatin. Both drugs were withdrawn from the marked due to safety concerns over the associated liver injury and rhabdomyolysis, respectively, which later was discovered to be potentially related to their effect on mitochondrial function. Other drugs, such as the antibiotic isoniazid, received black box warnings for hepatotoxicity and have also shown to interfere with mitochondrial function (38). Adverse drug responses (ADR) are among the leading causes of death in the US and EU and, with over 100 000 deaths annually, more people die due to ADRs as compared to diabetes, acquired immune deficiency syndrome or car accidents (39; 40). Although it is believed that mitochondrial toxicity only accounts for a small fraction of ADRs (39; 41), the numbers are likely underestimated considering the large numbers of drugs which are suspected to induce mitochondrial toxicity but where a consensus has not been found due to opposing results from the research community.

Drugs can have off-target effects on multiple aspects of mitochondrial function. They have shown to interfere with the electron transport system (ETS), impair the integrity of the mitochondrial membrane, induce mitochondrial production of ROS or impact mitochondrial maintenance. But also FA oxidation, glycolysis and protein metabolism or the mitochondrial antioxidant defence system were shown to be affected by drugs (39; 42; 43). Often, not only one aspect of mitochondrial function is impaired by a single drug (38; 43).

The liver, kidneys and the heart are predominantly affected by drug-induced toxicities (38; 39). What makes them more susceptible to drug-related side effects are the common routes of drug administration, distribution, metabolism and excretion which many drugs share. In addition, they are organs with high energy demands so that any interference with the mitochondrial ATP-production will become apparent rather quickly and, depending on the extend of toxicity, can result in organ failure. Mitochondrial toxicity is further related to the electrochemical properties and permeability of drugs as well as specific chemical motifs which exert the mitochondrial toxicity (39; 41; 42; 44; 45).
Figure 1: Possible off-target effects of drugs on mitochondrial function. Reproduced with the permission of the publishers (38).

Standard drug-toxicity tests were unable to pick up the toxicity of reported mitochondrial toxicants. Troglitazone and cerivastatin, for instance, passed the preclinical stage which included routine toxicity testing in cells and animal models. This indicates the need for specific screening assays to evaluate drug-related effects on mitochondrial function (38; 41). Drug-induced mitochondrial toxicity has been investigated in the academic environment for more than 50 years but only recently screening assays for mitochondrial toxicity were routinely included in the drug development process (38; 39; 41; 42; 44). This is partially due to the general lack of knowledge of mitochondrial function and dysfunction but also because the expertise on mitochondrial physiology has long staid within the academic environment (41).

Despite improved recognition of drug-related side effects on mitochondrial function and progress in method development, translation from the in vitro to in vivo situation and from animal to human tissues still remain obstacles, indicating the further need for methodological improvement (41; 42). Within the course of this thesis project, the following drugs and formulation excipients were investigated in depth for their effect on mitochondrial function to better understand drug-induced mitochondrial toxicity, eventually prevent it or find potential treatment strategies.

Metformin

The antidiabetic drug metformin is the first line treatment for type 2 diabetes. (46; 47). Although the exact mechanism of action of metformin’s therapeutic effect is still not entirely resolved it is now believed that its antidiabetic effect, at least partially, is mediated through the drugs effect on mitochondrial function (48-50). Madiraju et al. (48) demonstrated inhibition of human hepatic mGPD at therapeutic metformin concentrations. The mGPD is part of the glycerol-3-phosphate shuttle and its inhibition results in reduced hepatic gluconeogenesis (48). Inhibition of
hepatic gluconeogenesis has previously been reported by others and it is now well established that the liver is one of the main target organs of metformin’s antidiabetic effect (46; 51; 52). Metformin has further been shown to decrease glucose absorption into the blood through the gastrointestinal tract and increase glucose uptake in peripheral tissues and thus, lower blood glucose levels. However, less is known about the specific mechanism behind these findings (46). Metformin is considered safe but is been associated with several cases of lactic acidosis each year. Lactic acidosis is defined by increased blood lactate levels (> 5 mM) and decreased blood pH (< 7.35) (52; 53). Metformin-induced lactic acidosis (MILA) occurs primarily in patients with kidney failure when metformin’s concentration in the blood can exceed therapeutic levels (53-55). Pathological conditions affecting the cardiovascular system, respiratory system or the liver can further exacerbate the lactic acidosis as they affect the body’s capacity to control lactate metabolism and/or the acid-base balance (53-55). Like its therapeutic mechanism of action, the pathogenesis of MILA is not fully elucidated yet. Not surprisingly, metformin’s effect on mitochondrial function has also been implicated in the development of MILA (47; 49; 50; 56). The impairment of mitochondrial ATP-production would cause a shift towards glycolytic ATP production with lactate as by-product. Similar side-effects were seen with buformin and phenformin, two antidiabetic agents from the same drug class which were withdrawn from the market due to the high incidence of associated lactic acidosis (41). To date, treatment strategies consist of supportive measures, forced clearance to remove the drug and correction of the acidosis. However, with a mortality of around 20 % with MILA (57; 58) there is a need for complementary treatment strategies.

Acetaminophen

The analgesic and antipyretic drug acetaminophen (APAP), also known as paracetamol, is sold over-the-counter and available worldwide. It is safe at therapeutic dosing but has been associated with the development of liver toxicity at higher doses. APAP-induced liver toxicity is the most common cause of drug-induced liver injury and one of the leading causes for acute liver failure in the western world which is associated with a high mortality rate (59-63). The drug-induced toxicity of APAP is not thought to be related to APAP itself but its toxic metabolite N-acetyl-p-benzoquinone imine (NAPQI). At therapeutic doses only a small fraction of APAP is metabolized to generate NAPQI. At high doses, however, more NAPQI is formed which depletes glutathione, that is responsible for NAPQIs detoxification, so that free NAPQI can bind to cellular proteins, including mitochondrial proteins, and induce oxidative stress (60; 64; 65). Impaired mitochondrial function during APAP-induced hepatotoxicity has been reported but the exact mechanism of APAP-induced mitochondrial injury is still unclear (64; 66; 67). In addition, human data on the mechanism of APAP-induced liver toxicity are limited. Currently, the only treatment option for APAP intoxication is the
antioxidant N-acetylcysteine (NAC) which prevents further liver damage by increasing intracellular glutathione levels and improving the cells antioxidant defense. Consequently, with increasing time of APAP intoxication to NAC administration, NAC loses effectiveness (61; 68; 69). Alternative treatment strategies which are less time-dependent are needed to prevent APAP-induced liver failure.

*Kolliphor® EL*

Kolliphor® EL, previously branded Cremophor® EL, is an emulsifying agent frequently used in drug formulations (70). It is one of the formulation excipients of the branded intravenous ciclosporin formulation Sandimmune® Injection. Ciclosporin has immunosuppressive properties and is widely used for transplantations and autoimmune diseases due to its inhibitory action on calcineurin in T-cells (71). In the late 1980s, it was further demonstrated that ciclosporin inhibits mitochondrial permeability transition (72). Because of the inhibition of mitochondrial permeability transition it has been suggested as potential treatment for traumatic brain injury (TBI) (73; 74). In form of the branded intravenous ciclosporin formulation Sandimmune® Injection it has been associated with anaphylaxis, nephrotoxicity, cardiotoxicity and muscle toxicity, side effects that are thought to be at least partially related to the effect on mitochondrial function by one of the formulations excipients, Kolliphor® EL (75-78). If similar effects are observed in human tissues, alternative ciclosporin formulations will need to be developed to utilize the potential of ciclosporin for indications where intravenous formulations are needed.

**Inherited mitochondrial disease**

Mitochondrial dysfunction can also have genetic origin. Approximately 1 in more than 5000 people has an inherited mitochondrial disease. It can be caused by mutations of the mitochondrial or nuclear genome (10; 30; 43; 79; 80). Impaired translation, assembly, structure or maintenance of the OXPHOS machinery can be the result (18; 79). The most common defects are at CI, CIV or the PDH, which can appear isolated or in combination with other defects (30; 81; 82). One or more tissues, commonly highly dependent on OXPHOS, are usually affected in inherited mitochondrial disease, such as the nervous system, skeletal and cardiac muscles and endocrine organs (18; 30; 79; 80). Heteroplasmy can also further contribute to the heterogeneity in clinical presentations among patients. Mutations originating from the mtDNA are not necessarily expressed on all mtDNA copies within the same cell.
which leads to the presence of both healthy (wild-type) but also mutant mtDNA, which is referred to as heteroplasmy. Depending on the degree of heteroplasmy the disease phenotype can range from mild to severe. And to further complicate this, the degree of heteroplasmy can change over time and differ between and within individuals (10; 43; 79).

The result is a highly heterogeneous genetic, biochemical and clinical presentation, making a clear diagnosis difficult (26; 36). Once mitochondrial disease is suspected, family and clinical history together with different molecular tests aid in the diagnosis. However, the patients do not always meet all the criteria for a clear identification so that the diagnosis becomes challenging (26; 43). To date, assessment of the enzymatic activity of the complexes of the RC in tissue biopsies is the golden standard for a molecular diagnosis along with identification of genetic mutations (26; 43; 80). Advances in next generation sequencing, proteomics and metabolomics will make diagnosis of mitochondrial disease easier in the future (6; 43; 80; 83) and further advance the development of therapeutics for mitochondrial disease.

**Mitochondrial medicine**

Currently, the standard treatments for mitochondrial disease lack scientific evidence supporting their use but aim at upregulating mitochondrial function and mitochondrial content, reducing consequences of the mitochondrial disease, such as lactic acidosis and oxidative stress, and improving the energy storage of cells (84; 85). Supplementation with ubiquinone, also known as coenzyme Q10, or riboflavin is intended to upregulate mitochondrial activity by providing components of the RC. Ubiquinone is a natural component of the RC which mediates the electron transfer from CI, CII, the mGPD and the electron transfer protein down to CIII. Riboflavin is a precursor of the flavoprotein which is a component of CI and CII and additional serves as cofactor for TCA cycle reactions and FA oxidation (84). Other strategies increase the substrate supply to mitochondria or bypass parts of the RC. Thiamine and L-carnitine, for instance, increase the supply of acetyl-CoA which is required for the TCA cycle (84; 86). Succinate, a natural metabolite of the TCA cycle, increases OXPHOS by providing electrons to the RC downstream of, and thereby bypassing, CI. It has been shown to improve clinical symptoms in case studies (87; 88). However, exogenously given succinate has low permeability which limits its ability to reach the intracellular environment (89). Supplementation with antioxidants, such as vitamin C and E, lipoic acid or NAC, but also the redox agent ubiquinone intend to reduce oxidative stress (84; 85). A combination of the above mentioned supplements is common (79; 85). Despite some improvements on the
biochemical level and on isolated endpoints, such as muscle strength or post-exercise lactate levels, these treatment strategies, however, did not improve clinical outcome to warrant their use (79). This may partially be due to the heterogeneity of the disease and molecular background of patients which were included in these trials (85). Due to the lack of alternatives, they are, however, regularly employed (84). For more accurate evaluation of potential treatment strategies, future clinical trials will require the involvement of multiple centres to be able to include a sufficient number of patients with the same disease and molecular deficit (85). There is only one drug approved in the EU for mitochondrial disease, idebenone. Idebenone is a more soluble analogue of ubiquinone with improved pharmacokinetics. Like ubiquinone it facilitates electron transfer along the RC and acts as an antioxidant. However, it’s use is restricted to Leber’s hereditary optic neuropathy (85; 90). Dichloracetate, which keeps the PDH active to increase pyruvate flux down to the TCA cycle, is a synthetic compound that has shown success in some cases. However, long-term treatment with dichloracetate has been associated with the development of peripheral nerve toxicity (79; 91). With increased recognition of the role of mitochondrial dysfunction in disease more strategies have emerged and are currently in development for different indications (92; 93). One example is the redox agent methylene blue (MB) which has been extensively described in the context of neurodegenerative diseases (94-96). It is readily permeable and thought to shuttle electrons from NADH- and nicotinamide adenine dinucleotide phosphate (NADPH)-dependent dehydrogenases, such as CI, to cyt c at the RC, thereby recycling itself. Due to its redox activity it further has antioxidant properties and some studies have further reported upregulation of markers of mitochondrial biogenesis. Next to MB, mitochondria-targeted antioxidants (Mito-Tempo, MitoQ, EPI-743), inhibitors of the permeability transition (ciclosporin), pharmacological stimulators of mitochondrial biogenesis (dimethyl fumarate, resveratrol) and uncoupling agents (2,4-dinitrophenol, carbonyl-cyanide p-(trifluoromethoxy) phenylhydrazone), have been investigated for different indications with involvement of mitochondrial dysfunction (73; 84; 93; 97-101).
Objectives

The overall objective of this thesis was to develop and evaluate new strategies for assessment of drug-induced mitochondrial toxicity and pharmacological bypass strategies for the support of mitochondrial function. The specific aims were as follows:

1. To evaluate respirometry of freshly isolated human peripheral blood cells as a model for assessment of drug-induced mitochondrial toxicity (paper I and II).

2. To evaluate respirometry of freshly isolated human peripheral blood cells as a model for assessment of pharmacological bypass strategies for the support of mitochondrial function (paper III and IV).

3. To investigate if changes in mitochondrial respiration of human peripheral blood cells reflect changes in mitochondrial respiration of more metabolically active human cell types (paper II and III).

4. To investigate how changes in mitochondrial respiration correlate to phosphorylating capacity and affect glycolytic lactate metabolism (paper I, III and IV).

5. To evaluated the use of pharmacological bypass agents as a diagnostic tool to investigate mitochondrial function in intact cells (paper II and III).
Methods

Sample acquisition and preparation

Human peripheral blood cells

All blood cell experiments were performed with the approval of the regional ethics review committee of Lund, Sweden (permit no. 2013/181). Venous blood from healthy volunteers was collected after informed written consent was obtained. The blood was drawn in K$_2$EDTA tubes (Vacutainer®, BD, Plymouth, UK) by venous puncture and further processed within 1-3 h. Fresh blood was centrifuged at 500 g at room temperature (RT) for 10 min to obtain platelet-rich plasma (PRP). PRP was collected and further concentrated by centrifugation at 4600 g at RT for 5-8 min. The resulting platelet pellet was gently re-suspended in 1-2 ml platelet free plasma and was then ready for analysis (24). Peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep™ (Axis-Shield PoC AS, Oslo, Norway). PRP from whole blood was removed as described above. The remaining blood suspension was washed with physiological saline and gently layered over 3 ml Lymphoprep™. After centrifugation at 800 g at RT for 30 min the PBMC layer was collected and washed with physiological saline. Following a centrifugation at 250 g for 10 min the supernatant was removed and the cells were re-suspended in 50-100 µl of the donor’s own plasma and 200-400 µl of physiological saline. Cell count was performed using an automated haemocytometer (Swelab Alfa, Bouled Medical AB, Stockholm, Sweden) (102; 103).

Human cardiac muscle fibres

Ethical approval was granted by the regional ethics review committee of Lund, Sweden (permit no. 2013/701) and written informed consent was obtained from patients prior to surgery. Human cardiac muscle biopsies (up to 2 g), which otherwise would have been discarded, were obtained from scheduled open-heart surgeries. The sample was immediately transferred to ice-cold preservation medium (BIOPS) (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM dithiothreitol, 6.56 mM MgCl$_2$, 5.77 mM ATP and
15 mM phosphocreatine, pH 7.1). Under the microscope the tissue was dissected using forceps to remove fat and connective tissue and the muscle fibres were gently separated (104). The wet weight of each sample was obtained prior to respirometry.

Cell culture

Primary human fibroblasts were kindly provided by the Welcome Trust Centre for Mitochondrial Research at Newcastle University and experiments were approved by the Newcastle and North Tyneside 1 NRES Committee (REC reference 2002/205). Skin fibroblasts derived from a patient with Leigh syndrome due to nuclear-encoded CI deficiency and age-matched control fibroblasts were cultured under the same conditions. The culture medium consisted of minimum essential medium (MEM) (ThermoFisher Scientific, Bleiswijk, Netherlands) supplemented with 10 % fetal bovine serum (FBS) (Sigma Aldrich Chemie GmbH, Schnellendorf, Germany), 1 % MEM vitamins, 1 % MEM non-essential amino acids, 2 mM L-glutamine, 50 µg/ml streptomycin, 50 U/ml penicillin, 50 µg/ml uridine and 1 mM sodium pyruvate (all ThermoFisher Scientific, Bleiswijk, Netherlands). The immortalized human liver cell line HepG2 was purchased from Sigma-Aldrich Chemie GmbH (Schnelldorf, Germany). Cells were cultured in MEM supplemented with 10 % FBS, 1 % non-essential amino acids, 2 mM L-glutamine, 50 µg/ml streptomycin and 50 U/ml penicillin. The immortalized human proximal tubule kidney cell line HK2 was kindly provided by Redox Medicine, Division of Infection Medicine, Department of Clinical Sciences Lund and cultured in keratinocyte serum-free medium containing human recombinant epidermal growth factor 1-53 and bovine pituitary extract (ThermoFisher Scientific, Bleiswijk, Netherlands). The cells were kept at 37°C and 5 % CO₂ and collected for experiments at 70-80 % confluence using trypsin (0.05 %) (ThermoFisher Scientific, Bleiswijk, Netherlands). They were re-suspended in 1-3 ml culture medium, counted with an automate cell counter (TC20TM Automated Cell Counter, Bio-Rad laboratories, Solna Sweden) and prepared for respirometric analysis.

Primary human hepatocytes were obtained from ThermoFisher Scientific (Bleiswijk, Netherlands) and prepared the day before the experiments according to the supplier’s instructions. The cells were plated in William’s eagle Medium supplemented with 5 % FBS, 1 µM dimethyl sulfoxide (DMSO), 100 µg/ml streptomycin, 100 U/ml penicillin, 4 µg/ml human recombinant insulin, 2 mM GlutaMAXTM and 15 ml HEPES for 4 h at 37°C and 5 % CO₂. After plating, the medium was removed and exchanged for culture medium of the same composition as for HepG2 cells.
Respirometry

Two different respirometers were used throughout this project. Mitochondrial respiration of human blood cells and HepG2 cells was measured with a high-resolution oxygraph (Oroboros-O2k, Oroboros Instruments, Innsbruck, Austria). Respiration of primary human fibroblasts, primary human hepatocytes and HK2 cells was evaluated using the Seahorse XFe96 Analyzer (Agilent technologies, Massachusetts, USA).

**Oroboros O2k**

High-resolution respirometry with the Oroboros-O2k was performed at 37°C, with 2 ml active chamber volume, and a stirrer speed of 750 rpm. Settings, instrumental background corrections and air calibration were done according to the manufacturer’s instructions. Respiration was measured every 2 s either in the mitochondrial respiration medium (Mir05) (0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2PO4, 20 mM HEPES, 110 mM sucrose and 1 g/L bovine serum albumin) or phosphate-buffered saline (PBS) containing glucose (5 mM) and EGTA (5 mM) (24). Respiratory values were corrected for the oxygen solubility factor of the media (24; 104) and the chambers were re-oxygenated when the O2 concentration fell below 50 µM (24). Respiration measurements were performed at cell concentrations of 200 x 10^6 platelets per ml, 5 x 10^6 PBMCs per ml, 0.5 x 10^6 HepG2 cells per ml and ~ 10 mg cardiac muscle fibres per chamber.

**Seahorse XFe96 Analyzer**

Primary human fibroblasts and hepatocytes were prepared for respirometry the day before the experiments. Fibroblasts were seeded out in their regular culture medium at 25 000 cells per well on collagen-coated 96-well plates. Hepatocytes were plated at 20 000 cells per well on collagen-coated 96-well plates according to the supplier’s instructions as described above. After plating time, the plating medium was exchanged for culture medium of the same composition as for HepG2 cells. HK2 cells were seeded out on uncoated 96-well plates at 25 000 cells per well in their regular culture medium. After seeding, the cells were kept overnight at 37°C and 5 % CO2 until use. On the day of the experiment, the culture medium was replaced with XF-Base medium (Agilent technologies, Waghaeusel-Wiesental, Germany) supplemented with 10 mM glucose, 2 mM L-glutamine and 5 mM sodium pyruvate.
(pH 7.4) and the cells were left to equilibrate for 1.5 h at 37°C and atmospheric O₂ and CO₂ until the experiment was started.

**Evaluation of potential drug-induced mitochondrial toxicity**

Using high-resolution respirometry, the antidiabetic drugs metformin and phenformin (paper I and IV), the analgesic drug APAP (paper II), the branded intravenous ciclosporin formulation Sandimmune® Injection, its separate formulation ingredients and the alternative, novel ciclosporin formulation NeuroSTAT® (unpublished data) were evaluated for potential drug-induced mitochondrial toxicity. The exposure to the drugs was primarily done in the respective respirometer. As first step, increasing doses were titrated on intact cells to investigate whether they would decrease mitochondrial respiration instantly (105). If no immediate negative effect on mitochondrial respiration was observed mitochondrial respiration was evaluated after prolonged exposure to the test compounds. Doses for further characterization of mitochondrial toxicity were selected based on the dose response of each drug. Detailed characterization of mitochondrial toxicity was performed with intact and permeabilized cells by application of substrate-uncoupler-inhibitor-titration (SUIT) protocols following specified time of exposure. If drug exposure for more than 8 h was necessary, the exposure was performed outside of the respective respirometer prior to preparing the cell suspensions for respiration measurements. Additional protocols were performed in isolated cases where cells were washed after exposure to investigate the reversibility of the mitochondrial toxicity. Vehicle controls to each test compound were run in parallel.

*Permeabilized cells*

Application of protocols with permeabilized cells allowed detailed investigation of the origin of mitochondrial toxicity (29). Following exposure, the cell’s plasma membrane was permeabilized with digitonin to allow exogenous, otherwise impermeable substrates access. The optimal concentrations of digitonin required to permeabilize the plasma membrane without disrupting the mitochondrial membranes and affecting respiration has been determined for blood cells by Sjovall et al. (24) and was evaluated for HepG2 cells in the course of this thesis project. Substrates were given at saturating amounts to evaluate the maximal capacity of the OXPHOS system (24). After exposure to the drugs and subsequent permeabilization, CI-linked OXPHOS capacity was measured with the NADH-related substrates malate, pyruvate and glutamate and in the presence of ADP (OXPHOSCI). The following addition of the FADH₂-related substrate succinate additionally stimulated CII-linked OXPHOS capacity, allowing measurements of convergent OXPHOSCI+II capacity and calculation of isolated CII-linked OXPHOSII.
capacity (24). Non-phosphorylating respiration was measured after inhibition of the ATP-synthase by oligomycin. Because the respiration after oligomycin addition is due to compensation for the leakage of protons across the inner mitochondrial membrane this respiratory state is referred to as LEAK. It can indicate a compromised mitochondrial membrane integrity. The addition of oligomycin further allows the evaluation of coupling of mitochondrial respiration to phosphorylation, here referred to as coupled respiration. Maximal uncoupled respiration with convergent electron input to the electron transport system (ETS\textsubscript{CI+II}) was measured by careful titration of the protonophore carbonyl-cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP) which dissipates the MMP and activates the electron flux along the respiratory chain (29). ETS\textsubscript{CI} was subsequently evaluated by inhibition of CI with rotenone and non-mitochondrial respiration, also known as residual oxygen consumption, was measured after addition of the CIII inhibitor antimycin A. At last, CIV activity was evaluated by addition of the artificial substrate N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD) which reduces cyt c. Due to the high autooxidation of TMPD, CIV was subsequently inhibited with sodium azide to measure the remaining chemical background oxygen consumption which was subtracted from the cyt c-linked respiration for calculation of CIV activity (24; 29). Additional protocols were employed to evaluate the mitochondrial membrane integrity and possible cyt c loss (29).

**Intact cells**

The use of intact cells allows the evaluation of mitochondrial function under more physiological conditions. In addition to assessing the effect of the drugs on endogenous respiration, hereto referred as routine respiration (24), their effect on LEAK and ETS can be studied at their physiological concentrations of substrates and adenosine nucleosides (29). In some cases, the cell-permeable succinate prodrug NV241 was additionally used to evaluate CII-linked mitochondrial respiration in intact cells which was crucial for characterization of mitochondrial toxicity in primary hepatocytes and HK2 cells. For evaluation of potential mitochondrial toxicity using the Seahorse XFe96 Analyzer the number of additions per sample is limited to four. By combining the addition of the CI inhibitor rotenone and the cell-permeable succinate NV241 CII-linked respiration was measured.
Evaluation of pharmacological bypass strategies for the support of mitochondrial function

Cell-permeable succinate prodrugs

Primarily blood cell respirometry was used to evaluate the cell-permeable succinate prodrugs for their potential to support mitochondrial function. The application of blood cell respirometry as an adjunct diagnostic method for paediatric mitochondrial disease (26) lead to the foundation of the drug discovery program described in paper III. Since then, it has been used to evaluate more than 50 drug candidates. The above mentioned drug discovery program applied two different protocols for the evaluation of drug candidates (105). The effect of the drug candidates was first assessed in intact human platelets with rotenone-induced CI dysfunction with the aim to evaluate efficacy. Each drug candidate was titrated to give a 100 µM, 500 µM and 5 mM final, accumulative dose and the cells were subsequently permeabilized with digitonin. After stabilization of respiration, the prodrugs active payload succinate (reference) was given for comparison and the protocol was terminated by addition of antimycin A to inhibit CIII and measure non-mitochondrial respiration. A second protocol was performed with platelets where the effect of each drug candidate on convergent respiration was assessed in the presence of FCCP to accelerate electron flux along the ETS. After step-wise titration of each drug candidate, the remaining CII-linked respiration was measured by inhibition of CI with rotenone and the protocol was terminated by addition of antimycin A to inhibit CIII and measure non-mitochondrial inhibition. The highest dose tested in the second screening protocol was higher than the highest dose assessed in the first screening protocol to gain information on possible negative effects on mitochondrial respiration.

Both protocols were taken into account for the selection of drug candidates for further development. The drug candidates to be moved forward in the drug discovery program should possess the following properties (105):

1. It should stimulate CII-linked respiration to a larger extent than its reference, succinate (\(a > a'\)).
2. It should stimulate CII-linked respiration of intact cells to a similar degree as the reference, succinate, does in permeabilized cells (\(a \sim b \sim b'\)).
3. The maximal respiration after stimulation of respiration by the drug candidate and the reference, succinate, after permeabilization of the plasma membrane and should be similar to the maximal respiration reached in permeabilized cells by the reference alone (\(c \sim c'\)).
4. It should stimulate convergent CI+II-linked respiration while its vehicle, DMSO, leaves respiration unchanged (\(e > e'\)).
5. It should stimulate isolated CII-linked respiration to a larger extent than its vehicle, DMSO ($f > f'$).

6. The drug candidate should not stimulate non-mitochondrial respiration as compared to the reference, succinate, and its vehicle, DMSO ($d \sim d'$).

7. It should stimulate both convergent CI+II-linked and CII-linked respiration at low concentration.

Figure 2. Schematic illustration of two respirometry protocols for the evaluation of cell-permeable succinate prodrugs. A Drug candidates were evaluated in cells with rotenone-induced complex I dysfunction. Either drug or reference (succinate) was added at increasing doses. The cells were subsequently permeabilized and succinate was given two both treatment groups to assess maximal CII-linked mitochondrial respiration. B Drug candidates were evaluated in cells with partially uncoupled respiration by FCCP to accelerate electron flux at the respiratory chain. After titration of increasing doses of drug candidates or vehicle, complex I of the respiratory chain was inhibited by rotenone. Both protocols were terminated by inhibition of mitochondrial complex III with antimycin A to measure non-mitochondrial respiration.

Three drug candidates were moved forward for detailed characterization (paper III). Proof of the drug delivery concept was evaluated by comparing the stimulation of respiration by the prodrugs in intact and permeabilized cells and comparing the response to the reference, succinate. The drug candidates should stimulate respiration in intact cell but not in permeabilized cells as intracellular metabolism, which would release the succinate core, is reduced by permeabilization of the plasma membrane and dilution of the cytoplasmic contents. The mechanism of action of the cell-permeable succinate prodrugs was characterized in intact cells by inhibiting CI with rotenone, subsequently stimulating CII-linked respiration with the prodrugs followed by inhibition of CII with NV161, a specifically designed cell-permeable version of the otherwise impermeable malonate. Stimulation of CII-linked respiration with the drug candidates should be blocked again by addition of
NV161 as it blocks the electron input by the prodrugs at CII. To assess whether the increase in respiration with the cell-permeable succinate prodrugs translates to an increase in phosphorylation capacity, coupled respiration was assessed following the stimulation of respiration by the prodrugs and maximal uncoupled respiration was measured by titration with the protonophore FCCP in the presence of the prodrugs. Both coupled respiration and ETS capacity should be increased by the drug candidates due to the increased substrate supply. The prodrugs potential to stimulate mitochondrial respiration was evaluated in non-inhibited mitochondria (paper III), in different models of drug-induced mitochondrial CI inhibition (paper III and IV) and patient-derived primary cells with inherited, CI-related mitochondrial dysfunction (paper III). Stimulation of mitochondrial respiration by the cell-permeable succinate prodrugs was assessed in human platelets, PBMCs, cardiac muscle fibres, hepatocytes and fibroblasts as well as the immortalized liver and kidney cell line HepG2 and HK2, respectively, to evaluate its efficacy in different and more metabolically active tissues.

**Methylene blue**

The electron carrier MB was evaluated and characterized in human platelets with a more limited number of protocols as its effect on mitochondrial function has previously been described extensively by others (68; 94-96; 106-111). The effect of MB was first evaluated in intact human platelets with rotenone-induced CI inhibition. MB was titrated step-wise to stimulate CII-linked respiration until no further increase in response to the redox agent was observed. The experiment was terminated by subsequent addition of antimycin A and sodium azide to inhibit CIII and CIV, respectively, and measure non-mitochondrial respiration. MB’s potential to link the stimulation of respiration to phosphorylation pathways was then evaluated in two models of mitochondrial dysfunction, rotenone-induced CI inhibition and metformin-induced mitochondrial dysfunction. After inducing mitochondrial inhibition, MB was added to stimulate respiration followed by inhibition of the ATP-synthase by oligomycin to measure coupled respiration. Control experiments were performed without addition of oligomycin to account for background oxygen consumption. Subsequently, the respiratory chain was shut down by consecutive inhibition of CIII and CIV to measure non-mitochondrial inhibition.
Lactate production

When the cell’s powerhouse shuts down, the cell upregulates glycolysis to compensate for the reduced mitochondrial ATP production. Under these circumstances, pyruvate metabolism is increasingly shifted towards lactate generation to meet the increased NAD⁺, causing elevated lactate production and acidification. Increased lactate levels and decreased pH has been associated with many drugs which are suspected to induced mitochondrial toxicity (19-21) and is also considered as hallmark of mitochondrial disease (26). Increased lactate production due to upregulation of glycolytic lactate metabolism was used as concept to develop an additional assay for assessment of drug-induced mitochondrial toxicity and pharmacological bypass strategies for treatment of mitochondrial dysfunction.

Rotenone and different putative mitochondrial inhibitors were used to investigate how changes in mitochondrial respiration correlate to changes in glycolytic lactate metabolism. For this purpose, 400 x 10⁶ platelets were incubated in glucose-containing PBS (10 mM) at 37°C and under stirring conditions (750 rpm). Constant air calibration of the medium was allowed to avoid hypoxia. Lactate levels in the medium were measured over time using the Lactate Pro™ 2 blood lactate meter (Arkray, Alere AB, Lidingö, Sweden) (112; 113). After measuring starting lactate levels in the medium, platelets were exposed to a maximally inhibiting dose of the CI inhibitor rotenone or the putative inhibitors of mitochondrial function (phenformin, metformin and APAP) and lactate levels were measured over time. In selected experiments, the pH of the medium was measured at specified time points using a PHM210 Standard pH Meter (Radiometer, Copenhagen, Denmark). A limited set of experiments was performed for proof of concept in PBMCs.

After proof of concept (paper I), the assay was applied to evaluate the efficacy of the cell-permeable succinate prodrugs (paper III and IV) and MB (paper IV) to lower rotenone-induced and metformin-induced increases in lactate production. After establishing a difference in lactate production between controls (vehicle) and mitochondrial inhibitors over 60 min, co-treatment with either the cell-permeable succinate prodrugs or MB was started. One dose regimen was selected for the cell-permeable succinate prodrugs with dosing every 30 min. Two different treatment regimens were chosen for MB due to its attributed self-recycling property. It was given either as a single, higher dose at the start of intervention or as lower dose every 30 min. Controls and untreated mitochondrial inhibitors were run on each occasion. To explore whether both bypass strategies lower lactate production through mitochondrial effects treatment groups were included where the electron input of either drug was blocked by mitochondrial inhibitors (cell-permeable succinate prodrugs: antimycin A; MB: sodium azide). Lactate levels recorded from
the onset of intervention until the end of the assay (60-240 min) were used to calculation lactate production using a nonlinear regression model.

**Mitochondrial membrane potential**

The MMP, which is build up by the electrochemical gradient over the inner mitochondria membrane, is also frequently used to evaluate mitochondrial function (22; 114). Cell-permeable succinate prodrugs were further characterized by their effect on the MMP in human platelets (paper III). The MMP was measured using a flow cytometer (FACSAria III, BD, Franklin Lakes, USA) and the fluorescent probe tetramethylrhodamine methyl ester perchlorate (TMRM; ThermoFisher Scientific, Bleiswijk, Netherlands) in non-quench mode. TMRM was excited by 561 nM 40 mM laser and collected with 582/15 band pass filters. After 30 min equilibration with the fluorescent probe, CI was inhibited by rotenone and the platelets received either vehicle or the cell-permeable succinate prodrug. The protocol was terminated by consecutive additions of the internal controls oligomycin, FCCP and antimycin A. The change in MMP was measured at each respiratory state in the same sample.

**Metabolomics and isotope labelling**

To explore intracellular metabolism of the cell-permeable succinate prodrugs, metabolomics was performed. 116 metabolites were measured in PBMCs with or without rotenone and with or without co-treatment of the cell-permeable succinate prodrugs. Capillary electrophoresis time-of-flight mass spectrometry for cationic compounds and capillary electrophoresis tandem mass spectrometry for anionic compounds was used for quantification of metabolites. In a second set of experiments, metabolomics was performed in platelets where all four carbons of the succinate core of the succinate prodrug were isotopes. The [13C] metabolites were measured after 15, 30, 120 or 240 min. Metabolites of both experiments were extracted with methanol, filtered, dried up and analysed by Human Metabolome Technologies Inc., Tsuruoka Japan, based on their theoretical m/z value and migration time (115).
Data analysis

Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, California, USA). Respiratory values from high-resolution respirometry of human blood cells have previously been found to be normally distributed (24; 102) and hence, parametric tests were used for analysis of differences. Standard non-linear regression models were used to calculate half maximal inhibitory concentrations (IC_{50}) and lactate production slopes. A p value of 0.05 or less was considered to indicate significant difference. No blinding or randomization was performed.
Results

Evaluation of drug-induced mitochondrial toxicity using respirometry of human blood cells

Figure 3. Metformin, acetaminophen and Kolliphor® EL inhibit NADH-linked mitochondrial respiration in human blood cells. NADH-linked mitochondrial respiration of human blood cells was significantly reduced in response to the antidiabetic drug metformin, analgesic agent acetaminophen and the formulation ingredient Kolliphor® EL. Mitochondrial complex II (CII) linked respiration in the presence of succinate was unaffected by the pharmaceuticals, indicating that the site of drug-induced mitochondrial toxicity is upstream of or at complex I (CI).

Metformin dose-dependently inhibited mitochondrial respiration of platelets and PBMCs. Detailed respirometric protocols with permeabilized platelets and PBMCs demonstrated that metformin reduced CI-linked mitochondrial respiration. The integrity of the mitochondrial membrane, CII-linked respiration and CIV activity were unaffected by metformin. Metformin further decreased mitochondrial respiration of intact cells in a time-dependent manner at concentrations relevant for clinical metformin intoxications. The mitochondrial inhibition induced by metformin was not reversed upon extra- and intracellular removal of metformin. Phenformin, a structurally similar antidiabetic drug from the same drug class which was withdrawn from the market due to the high incidence of associated lactic acidosis, was simultaneously evaluated for structure-activity-relationship analysis and also demonstrated inhibition of CI-linked mitochondrial respiration. Like with metformin, CII-linked respiration, CIV activity and the mitochondrial membrane...
integrity were unaffected by phenformin. Compared to metformin, phenformin was a more potent inhibitor of CI-linked respiration.

The analgesic and antipyretic drug APAP dose-dependently decreased endogenous mitochondrial respiration of intact platelets. Detailed analysis with both intact and permeabilized cells demonstrated that CI-linked respiration was impaired by APAP. CII-linked respiration measured in permeabilized platelets in the presence of succinate or in intact cells in the presence of the cell-permeable succinate prodrug NV241 was unaffected by APAP, excluding CII or downstream complexes of the electron transport system as site of drug-induced mitochondrial toxicity. The inhibition of mitochondrial respiration observed with APAP did not increase further over time (data not shown). Further experiments need to be performed to distinguish whether CI or upstream processes of CI are inhibited by APAP.

Endogenous mitochondrial respiration and maximal uncoupled respiration was inhibited in the presence of the branded intravenous ciclosporin formulation Sandimmune® Injection and its formulation ingredient Kolliphor® EL (Figure 3A). Exposure to ethanol, ciclosporin and the novel ciclosporin lipid emulsion NeuroSTAT® resulted in similar levels of respiration than control. The integrity of the inner mitochondrial membrane and CII-linked mitochondrial respiration, which was evaluated in the presence of the cell-permeable succinate prodrug NV241, was similar between all groups (Figure 3B). These results indicate that the branded intravenous ciclosporin formulation Sandimmune® Injection induced mitochondrial toxicity through its formulation ingredient Kolliphor® EL. Likely CI or upstream processes of CI are inhibited by Kolliphor® EL. Further experiments need to be performed with permeabilized cells to confirm the results.
Figure 4. Effect of Kolliphor® EL on mitochondrial respiration of human platelets.

(A) Mitochondrial routine respiration of human platelets was impaired in the presence of the branded intravenous ciclosporin formulation Sandimmune® Injection (85 µM ciclosporin) and the corresponding dose of the formulation ingredient Kolliphor® EL. Ethanol, ciclosporin and the novel ciclosporin lipid emulsion NeuroSTAT® did not affect mitochondrial respiration.

(B) Kolliphor® EL did not affect the integrity of the inner mitochondrial membrane but reduced maximal uncoupled respiration. Complex II-linked respiration, assessed by addition of the cell-permeable succinate prodrug NV241, was not affected by Kolliphor® EL. Data are expressed as mean ± SD. n=4.
Evaluation of pharmacological bypass strategies for the support of mitochondrial function using respirometry of human blood cells

Cell-permeable succinate prodrugs

More than 50 drug candidates from a drug discovery program around cell-permeable succinate prodrugs were evaluated by high-resolution respirometry of human blood cells. The results are illustrated exemplary for the candidate NV118 below. The cell-permeable succinate prodrug NV118 increased respiration of CI-inhibited human platelets to a larger extent than the reference, succinate (a > a’) (Figure 5A). The CII-linked respiration of the group receiving NV118 before permeabilization of the plasma membrane was slightly lower but not significantly different to the respiration after permeabilization of the plasma membrane (a ~ b ~ b’) (Figure 5A). CII-linked respiration of both groups was similar after permeabilization of the plasma membrane and remained similar when the reference, succinate, was given to both groups (c ~ c’) (Figure 5A). When NV118 was given to non-inhibited cells in the presence of FCCP, which accelerates the electron flux along the respiratory chain, it stimulated convergent CI+II-linked mitochondrial respiration while the vehicle control left respiration unchanged (e > e’) (Figure 5B). After subsequent inhibition of CI by rotenone, CII-linked respiration was higher in the group treated with NV118 than in the control (f > f’) (Figure 5B). Titration of NV118 on convergent CI+II-linked respiration indicated a safe concentration range up to 1 mM (Figure 5B). In neither of the two screening protocols the cell-permeable succinate prodrug NV118 increased non-mitochondrial respiration (d ~ d’) (Figure 5). The drug candidates NV118, NV189 and NV241 were selected from the screening protocols for further characterization. All three candidates stimulated respiration of intact but not permeabilized human platelets, confirming the dependence on intracellular metabolism for release of the active payload succinate and providing proof of concept of the prodrug strategy. When respiration was inhibited with the cell-permeable malonate prodrug NV161 after stimulation of CII-linked respiration with either of the candidates, respiration immediately decreased in human platelets, demonstrating that the increase in respiration was due to electron supply to CII by the cell-permeable succinate prodrugs. Measurements of coupled respiration, ETS capacity and MMP in the presence of the cell-permeable succinate prodrugs provided evidence that the stimulation of mitochondrial respiration in human platelets is linked to increased substrate supply and phosphorylation potential. The cell-permeable succinate prodrugs were effective in healthy and rotenone-inhibited models with different cell types. In the metformin-model of mitochondrial dysfunction the cell-permeable succinate prodrug NV118 only showed a tendency for improved phosphorylation activity.
Figure 5: Effects of the cell-permeable succinate prodrug NV118 on mitochondrial respiration.

(A) NV118 was titrated on intact human platelets with rotenone-induced CI dysfunction (a) and the plasma membrane was subsequently permeabilized with digitonin (b), followed by addition of the reference, succinate (c). The protocol was terminated by addition of antimycin A to inhibit complex III of the respiratory chain and measure non-mitochondrial respiration (d). (B) The effect of NV118 on convergent respiration was assessed in the presence of FCCP to accelerate electron flux along the electron transport system. After step-wise titration of NV118 (e), the remaining CII-linked respiration was measured by inhibition of CI with rotenone (f) and the protocol was terminated by addition of antimycin A to inhibit CIII and measure non-mitochondrial inhibition (d). Data are expressed as mean ± SD. n=3 (vehicle, NV118), n=4 (reference).

**Methylene blue**

After initial step-wise titration of MB on human platelets with rotenone-induced mitochondrial CI dysfunction one dose was selected for further characterization. The potential treatment effect of MB was assessed both in a rotenone-model and metformin-model of mitochondrial dysfunction. Due to the high non-mitochondrial oxygen consumption introduced by MB an intermediate dose was selected to allow more accurate analysis of its effect on mitochondrial respiration. Consecutive shut down of the complexes of the ETS with rotenone (CI), antimycin A (CIII) and sodium azide (CIV) indicated stimulation of mitochondrial respiration through electron input downstream of CIII. The stimulation of endogenous routine respiration in both models of mitochondrial dysfunction was not linked to increased phosphorylation capacity.
Changes in mitochondrial respiration of human blood cells reflect changes in respiration of more metabolically active human cells

Because possible drug-induced liver dysfunction is a problem for metformin and APAP intoxication both drugs were evaluated for their effect on mitochondrial respiration in liver cells. Like in blood cells, metformin induced a dose-dependent and time-dependent inhibition of endogenous respiration of intact HepG2 cells which resulted in decreased coupled respiration (data not shown). The CI-linked mitochondrial inhibition by APAP was likewise confirmed in both the immortalized human liver cell line HepG2 and primary human hepatocytes (data not shown). The branded intravenous ciclosporin formulation Sandimmune® Injection has been associated with nephrotoxicity, among other side effects. Like in platelets, endogenous respiration of the immortalized proximal tubule kidney cell line HK2 was dose-dependently inhibited by Sandimmune® Injection and Kolliphor® EL but not with ciclosporin alone (the results for the corresponding dose of 5 µM ciclosporin are illustrated in Figure 6A). CII-linked respiration was similar between all groups at the lower dose investigated (dose corresponding to 2 µM ciclosporin) but was decreased in response to Sandimmune® Injection at a corresponding dose to 5 µM ciclosporin (the results for the corresponding dose of 5 µM ciclosporin are illustrated in Figure 6B). Further experiments with permeabilized cells need to be performed to confirm the results.

The evaluation of the cell-permeable succinate prodrugs described in paper III was primarily done in human blood cells. Key experiments were then repeated with human cardiac muscle fibres and primary human skin fibroblasts. Like in blood cells, the cell-permeable succinate prodrugs also increased CII-linked mitochondrial respiration in cardiac muscle fibres and primary human skin fibroblasts.
Figure 6. The formulation excipient Kolliphor® EL of the branded intravenous ciclosporin formulation Sandimmune® Injection induces mitochondrial inhibition in the immortalized human kidney cell line HK2. (A) Using the Seahorse XFe96 Analyzer mitochondrial respiration of intact HK2 cells was monitored in the presence of Sandimmune® Injection and its separate formulation ingredients Kolliphor® EL, and ciclosporin. After stable endogenous routine respiration was established, mitochondrial respiration was uncoupled from phosphorylation pathways by addition of FCCP to accelerate electron flux along the respiratory chain and increase the potential to detect toxicity towards the electron transport system. Subsequently, the branded intravenous ciclosporin formulation Sandimmune® Injection or the separate formulation ingredients Kolliphor® EL, and ciclosporin were injected at a corresponding dose to 5 µM ciclosporin, followed by assessment of CII-linked respiration by combinatorial injection of the complex I inhibitor rotenone and the cell-permeable succinate prodrug NV241. (B) Quantification of the effect of Sandimmune® Injection or the separate formulation ingredients on uncoupled respiration and CII-linked respiration in the presence of the cell-permeable succinate prodrug NV241. Data are expressed as percentage of endogenous routine respiration (first measurement point) with mean ±SD. n=3.
Changes in the respiration of human blood cells affect their glycolytic lactate metabolism

For proof of concept, we studied lactate production of human platelets which were exposed to rotenone and different putative mitochondrial inhibitors (paper I). Platelets were exposed to two doses of metformin, one dose phenformin or a maximal inhibitory dose of the CI inhibitor rotenone. Lactate levels increased in the medium over time and pH simultaneously decreased with all inhibitors of mitochondrial function. The increase in lactate production and decrease in pH correlated to an increasing degree of mitochondrial inhibition. Preliminary experiments further showed that a near half maximal inhibitory dose of APAP also increased lactate production of human platelet (data not shown). A limited set of experiments with human PBMCs and the immortalized liver cell line HepG2 demonstrated the potential use of this assay with other cells (data not shown).

After establishing the proof of concept with inhibitors of mitochondrial function, we applied this assay to evaluate two different pharmacological bypass strategies for their potential to lower lactate production caused by mitochondrial dysfunction. The cell-permeable succinate prodrugs reduced rotenone- (paper III) and metformin-induced (paper IV) lactate production in human platelets. Despite the failure to increase coupled respiration we hypothesized that the redox agent methylene blue could potentially lower lactate production by replenishing the cytosolic NAD+ pool by oxidizing cytosolic NADH independent of pyruvate-to-lactate conversion. However, methylene blue was unable to reduce metformin-induced lactate production in human platelets.

Intracellular delivery and metabolism of cell-permeable succinate prodrugs in human blood cells

To investigate the intracellular metabolism of the cell-permeable succinate prodrugs we performed metabolomics with and without isotope-labelled compound in human PBMCs. The lactate-to-pyruvate ratio was significantly increased by inhibition of CI with rotenone but restored to control levels with the cell-permeable succinate prodrug. TCA cycle metabolites were rapidly increased after treatment with the succinate prodrugs, indicating intracellular delivery of the cell-permeable succinate prodrugs and its mitochondrial metabolism by the TCA cycle was further demonstrated by the time-course evaluation using isotope labelled compound.
Discussion

Studying mitochondrial function

The application of intact cells was crucial for characterization of mitochondrial toxicity of APAP as its toxicity has been ascribed to its toxic metabolite NAPQI (60; 64). If APAP were given to permeabilized cells or isolated mitochondria, the mitochondrial toxicity likely would be underestimated. Beneficial effects of potential treatment strategies, such as the cell-permeable succinate prodrugs, would likewise be largely lost if drug candidates were evaluated in permeabilized cells, isolated mitochondria, mitochondrial fractions or sub-mitochondrial particles. Interestingly, the evaluation of prodrug strategies in permeabilized cells in comparison to intact cells can serve as proof of concept of the prodrug strategy, as described in paper III. Working with intact cells gives restricted information on mitochondrial dysfunction. But because the use of intact cells involves no homogenization or permeabilization, there is a lesser risk to negatively affect the mitochondrial membrane integrity (11), which is of utmost importance when evaluating the coupling of electron transfer and phosphorylation pathways in response to a potential treatment strategy, as it was done in paper III and IV. Mitochondrial toxicity due to uncoupling of electron transport and phosphorylation capacity by pharmacological agents would affect mitochondrial OXPHOS while leaving the respiratory complexes and their activities intact and would likely not be picked up using mitochondrial fractions or sub-mitochondrial particles. The use of this method can also lead to overestimation of potential treatment strategies. MB, for instance, has primarily been evaluated using mitochondrial fractions and sub-mitochondrial particles by measuring NADH or succinate oxidation with or without the presence of the drug (95; 106; 111). Although this tells you whether MB can oxidize substrates, and to some degree, if the accepted electrons are shuttled to other components of the RC, it does not give the information whether the electron transfer is put to good use, *i.e.* if the shuttling of electrons is linked to phosphorylation pathways.
Blood-based markers of bioenergetic health

Blood cells

The use of human blood cells as a model to evaluate mitochondrial function in disease has grown in interest in the last decade. Several neurodegenerative diseases and sepsis have been associated with altered mitochondrial function of human peripheral blood cells (70; 102; 116; 117). Human blood cells have further been proposed to be useful in the diagnosis of mitochondrial disease (26; 118-120). In a few cases they were further used to evaluate suspected drug-induced mitochondrial toxicity (47; 121-123). Measuring mtDNA levels in PBMCs of patients with human immunodeficiency virus taking antiretroviral therapy, which has been described to interfere with the mitochondrial specific DNA polymerase gamma (POLG), impair mtDNA replication and consequently cause mtDNA depletion, has been suggested as a potential clinical management tool and is probably the most well-established use of blood cells to evaluate drug-induced mitochondrial toxicity (39; 124; 125).

Except for erythrocytes all blood cells contain mitochondria. Because obtaining human blood is less invasive compared to biopsies they present an easily accessible source of human mitochondria. It will also allow for easier repeated sampling over time. Blood cells consist of several different cell types with different physiological and bioenergetics function. Platelets and PBMCs are the blood cells which depend the most on OXPHOS for energy production and they are the main blood cell types used for evaluation of mitochondrial function (126-128). Although human blood cells are not the primary tissue which is affected in the above described diseases and medical conditions they may reflect systemic metabolic responses and thus, could function as a model for mitochondrial research. Some attempts have been made to investigate the relationship of blood cell mitochondrial function and systemic mitochondrial function. In non-human primates, blood cell respirometric profiles have shown to correlate with those of skeletal and cardiac muscle as well as brain metabolism (129; 130) and in a porcine model of cardiac arrest changes in the respiratory profile of the brain were associated with alterations in mitochondrial respiration of platelets (131). Limited data exist on the relationship in humans. In older human adults, gait speed, a measure of general physical health and functionality, has shown to correlated with respiratory profiles of both blood cells and skeletal muscle. Due to the risks and invasive procedures associated with human biopsies the direct correlation of human blood cell bioenergetics with those of other, more metabolically active fresh human tissues is difficult to investigate. In paper II and III, blood cells were analysed along with cells from more metabolically active human cells and tissues. The qualitative changes we observed in human peripheral blood cells in response to mitochondrial toxicants and pharmacological treatment
strategies were also found in human-derived liver and kidney cells, fibroblasts and muscle fibres.

**Serum biomarkers**

In addition to blood cells, other blood based markers have been used to investigate systemic bioenergetic function. The lactate-to-pyruvate ratio would be elevated with upregulation of glycolysis to compensate for the loss of mitochondrial ATP production. An increased β-hydroxybuturate/acetoacetate ratio reflecting the cellular redox state has been associated with drug-induced mitochondrial toxicity and has also been reported to be elevated in patients with Leigh-syndrome (48; 83). In the same study, levels of TCA cycle metabolites, FA levels and intermediates of NAD⁺ synthesis were likewise altered, indicating their possible use to monitor systemic mitochondrial function (83). Increased levels of serum mtDNA and glutamate dehydrogenase, which is usually confined to the mitochondrial matrix, have also been linked to mitochondrial dysfunction in other organs. Their levels were increased in blood of animals and patients with APAP-induced liver injury (66). Increased serum mtDNA copy numbers were further described to correlate with alterations of cerebral mitochondrial function and were suggested as potential serum biomarker for TBI (132). The use of the above mentioned serum markers to monitor mitochondrial function seems promising but will need to be further established before systemic mitochondrial function can be judged solely on their profiles.

**Blood cells as model cells in drug development**

In drug development, there are additional aspects which need to be considered when using blood cells as model cells for drug evaluation. The use of blood cells from healthy donors provides the advantage of genetic diversity, gender differences and idiosyncratic responses, aspects that are lacking in animal studies and when using standard human cell lines (41; 133). In addition, healthy donors are not drug-naïve, cover a wider age range and are exposed to environmental factors and co-medications (41). In drug development, immortalized cancer cell lines are often used to screen and/or evaluate drug efficacy and toxicity. These cell lines are often more glycolytic, which could impact evaluation of drug-induced mitochondrial toxicity and treatment efficacy of drugs for the support of mitochondrial function. They also have shown different gene expression profiles and altered metabolic functions as compared to primary cells (134; 135). In contrast, primary cells do not grow indefinitely (135). Strategies were developed to grow cancer cell lines under conditions where they are more dependent on OXPHOS, such as the glucose-galactose assay (41). In this assay, cells are grown either in glucose or galactose and

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their mitochondrial function is assessed. Cells grown in galactose are more dependent on mitochondrial ATP production due to slower glycolytic metabolism of galactose which, as a consequence, makes them more sensitive to drug-induced mitochondrial toxicity (41). PBMCs have shown to express cytochrome P450 (CYPs) enzymes (136; 137) which are involved in the formation of the toxic metabolite of APAP (64). Our data suggest that these enzymes might also be expressed in platelets as the mitochondrial toxicity induced by APAP was also demonstrated in human platelets. In the search for translational markers of drug toxicity, gene expression profiles of PBMCs have been investigated in response to known nephrotoxicants and hepatotoxicants and shown to be specific for the corresponding organ toxicity (138; 139). Our results and those of others indicate human blood cells as a suitable tissue for evaluation of mitochondrial toxicity and pharmacological treatment strategies for the support of mitochondrial function. Their use, however, is limited to 24 h. Whole blood can be stored up to 24 h at RT without loss of mitochondrial function (24). This time window of 24 h limits the use of blood cells for evaluation of certain treatment strategies. As an example, markers of mitochondrial biogenesis have been upregulated with MB and β-lapachone treatment, a natural quinone evaluated in vitro as treatment for mitochondrial disease (108; 110; 140). However, these effects were seen only after 24 h treatment.

Ex vivo application of human blood cell respirometry could also be useful in drug development. Monitoring mitochondrial function in human blood cells during clinical trials and even post-marketing, e.g. by respirometry, could allow more accurate assessment of the incidence of drug-induced mitochondrial toxicity and could also guide safe dosing regimens (38; 105). This model could be further used to monitor drug efficacy. In paper III, we demonstrated that cell-permeable succinate prodrugs increased levels of TCA cycle metabolites, decreased the lactate-to-pyruvate ratio and increased mitochondrial respiration of intact human blood cells. Monitoring those parameters prior to initiation of and during treatment with cell-permeable succinate prodrugs in patients could potentially be used to evaluate treatment efficacy on a molecular and cellular basis.

Evaluation of mitochondrial toxicity

“Alle Dinge sind Gift, und nichts ist ohne Gift; allein die dosis machts, daß ein Ding kein Gift sei.” – Paracelsus, 1538

The concept of modern toxicology is not a new one. Already in the 16th century Paracelsus introduced the concept of dose response stating that only the dose distinguishes a poison from a therapeutic agent (141). And this is still true today. The antidiabetic agent metformin is considered safe at therapeutic dosing but has
been associated with cases of lactic acidosis under circumstances where the drug accumulates (52; 53). It is now believed that both its antidiabetic effect and toxic effect are, at least partially, mediated through the drug’s effect on mitochondrial function (48-50). In paper I, we demonstrated that metformin induces lactate production in peripheral blood cells through specific mitochondrial CI inhibition at concentrations that are relevant for clinical metformin intoxication. This inhibitory action of metformin on mitochondria was restricted to high metformin concentrations (> 1mM). We were unable to detect any specific inhibitory action of metformin on mGPD due to the nature of the SUIT protocol applied on permeabilized cells. The SUIT protocols used in paper I and II do only allow evaluation of NADH-linked and succinate-linked respiration. FA oxidation or oxidation of glycerol-3-phosphate was not evaluated in this protocol and is therefore a limitation in the methods used in paper I and II. However, in both studies the effect of the drugs was additionally evaluated in intact cells where such an effect eventually would contribute to the inhibition of mitochondrial function. In the same study, phenformin inhibited mitochondrial CI and increased lactate production in platelets and did so with a higher potency relative to clinical dosing. The difference in mitochondrial toxicity and increase in lactate production could potentially explain the difference in the incidence of associated lactic acidosis reported with either drug.

Like metformin the analgesic drug APAP is safe at therapeutic levels. It is only at drug levels reached during intoxication that APAP has been associated with hepatotoxicity and liver failure (60; 142; 143). In paper II, we demonstrated that APAP induces mitochondrial toxicity through CI in human platelets and hepatic human cells. CII or complexes downstream of CII were not affected by APAP. Others have reported mitochondrial permeability transition, increased mitochondrial oxidative stress and decreased mitochondrial ATP production with APAP (59; 64; 68). While we cannot exclude effects of APAP on mitochondrial permeability transition and oxidative stress as these aspects of mitochondrial function were not analysed in the present study, the limited set of experiments on APAP-induced lactate production indicate that the APAP-induced CI inhibition was associated with a switch from mitochondrial ATP production to glycolytic ATP production. Interestingly, in contrast to metformin, the inhibition of mitochondrial respiration induced by APAP did not increase further over time (data not shown), which potentially could explain that a longer time was required to see a difference in lactate production between controls and APAP-treated platelets. APAP intoxication has primarily been associated with the development of hepatotoxicity but nephrotoxicity has also been reported (60). Both liver and kidneys play an important role in drug metabolism and excretion. The liver is the major site of CYP expression (144), enzymes that are critical to the formation of APAP’s toxic metabolite NAPQI (64). Nevertheless, CYP enzymes are also expressed in the kidney and blood cells (137). This is in agreement with our data where
mitochondrial toxicity of APAP was not limited to hepatic tissue. The organ specific toxicity seen with APAP is likely not due to a difference in hepatic biotransformation and generation of NAPQI. Instead, it is more likely that the organ-specific toxicity is related to the generally higher exposure of liver and kidneys to drugs (145; 146).

Not only active pharmaceutical ingredients have the potential to induce mitochondrial toxicity. Kolliphor® EL, an emulsifying excipient frequently used in drug formulations, has also been implicated to inhibit mitochondrial respiration (70; 77; 78). This was confirmed in the present thesis project. Ciclosporin and ethanol, an additional formulation ingredient, left mitochondrial respiration unchanged whereas Sandimmune® Injection and Kolliphor® EL induced inhibition of respiration in human platelets. To investigate the relationship of Kolliphor® EL-induced mitochondrial toxicity and the reported nephrotoxicity for Sandimmune® Injection the effect of the complete formulation, Kolliphor® EL and ciclosporin were further investigated in the kidney cell line HK2 with qualitatively similar results and at doses similar to peak concentrations of ciclosporin after a bolus injection of Sandimmune® Injection during acute treatment (147). Similar results have also been reported by others (75-78). In human platelets, we further compared the effects of Sandimmune® Injection and Kolliphor® EL on mitochondrial respiration to the novel ciclosporin lipid emulsion NeuroSTAT® which is free of ethanol and Kolliphor® EL. No inhibition of mitochondrial respiration of human platelets was observed with NeuroSTAT® presenting it as a suitable alternative ciclosporin formulation. Our data further indicate that Kolliphor® EL likely inhibits the RC upstream of CII. This was demonstrated in intact platelets using the cell-permeable succinate prodrug NV241 and measuring CII-linked respiration in the presence and absence of Sandimmune® Injection, Kolliphor® EL, ciclosporin and ethanol (unpublished data). Further experiments with permeabilized cells would need to be performed to confirm the results. Even though intact cells are more physiological, evaluation in permeabilized cells allows measurements of maximal respiratory capacities which may unmask potential toxic effects at sites downstream of complex II. In line with this, others have reported that CIV was affected by Kolliphor® EL when analysed in permeabilized muscle fibres (77; 78).

As briefly mentioned in the introduction, the potential of a drug to induce mitochondrial toxicity is mostly related to its electrochemical properties and specific chemical motifs which exert the mitochondrial inhibition (41). Charge and lipophilicity are two properties that increase permeability of the plasma and/or mitochondrial membranes. Metformin, for instance, is positively charged and due to this property thought to accumulated in the highly negatively charged environment of the mitochondrial matrix (11; 49; 50; 148). But also the electrophilicity of compounds, such as the toxic metabolite NAPQI (149) can increase the risk of mitochondrial toxicity. Often, the potential to induce
mitochondrial toxicity is common among a drug class but the potency to do so is variable among the different drug candidates (41), as was demonstrated for metformin and phenformin in paper I and by Dykens et al. (148) for metformin, buformin and phenformin. Similar structure-activity-relationship was reported for the thiazolidinediones troglitazone, rosiglitazone and pioglitazone which have all been described to inhibit mitochondrial function and whose potency to do so correlated with the reported incidence of associated hepatotoxicity observed with these drugs (150). As these electrochemical properties and mitochondrial toxic chemical motifs are embedded in the chemical structure of drugs, mitochondrial toxicity could potentially be predicted similar to structure-activity-relationship during rational drug design.

Cell-permeable succinates as a pharmaceutical drug

Succinate has previously been used in individual non-controlled case studies for treatment of mitochondrial disease. In a patient with MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like symptoms), long-term treatment with succinate improved neurological symptoms. A patient with Kearns-Sayre/chronic external ophthalmoplegia plus syndrome with confirmed CI and partial CIV and V dysfunction showed improved respiratory function with combinational treatment with CoQ10 and succinate (87; 88). Succinate treatment has further been suggested as a potential treatment strategy for sepsis and TBI (89; 151-154). There is less data on the use of succinate for drug-induced mitochondrial dysfunction. Hinke et al. (155) previously proposed the bypass of metformin-induced CI toxicity with succinate as a potential treatment strategy. Methyl succinate improved the mitochondrial reduction potential of pancreatic β-cells during exposure to metformin and reduced metformin-induced cell death (155). However, regular succinate has limited permeability (89). Consequently, millimolar concentrations of succinate was added in the above study to achieve an effect (155).

In paper III and IV, we showed that cell-permeable succinate prodrugs improved mitochondrial respiration coupled to phosphorylation. The improved phosphorylation potential translated into a decrease in rotenone-induced lactate production (paper II). However, the phosphorylation potential after metformin exposure was not significantly improved with the cell-permeable succinate prodrug NV118 (paper IV) and yet, metformin-induced lactate production was reduced. This may partially be due to experimental variation. Another reason contributing to the discrepancy between the rotenone- and metformin model of mitochondrial dysfunction could be the different degree of inhibition of mitochondrial respiration induced by the different substances. Rotenone was used at a dose which maximally
inhibits mitochondrial respiration before treatment with NV118 was evaluated. Metformin, in contrast, did not inhibit mitochondrial respiration maximally at the start of intervention. It is a much milder inhibitor of mitochondrial function which is primarily due to its slow permeation and accumulation within the mitochondrial matrix. In the patient-derived human fibroblasts with known CI dysfunction used in paper III we did observe an increased response to the cell-permeable succinate prodrugs which could be due to the increased dependence on alternative substrates, such as NV118. Karlsson et al. (156) further reported decreased succinate levels in muscle biopsies in a pig model with acute rotenone-induced CI inhibition, further indicating a possible high dependence on CI-linked substrates in severe models of mitochondrial CI dysfunction. In contrast to the respiratory model of metformin-induced mitochondrial dysfunction, the lactate production in response to metformin was measured over more than 1 h which better accounts for the slow uptake of metformin. Like in the respiratory model, lactate levels measured in the assay medium were not different between metformin- and vehicle treated cells after 1 h. After 2 h exposure to metformin, however, we did observe a difference in lactate levels in the assay between the two treatment groups. Preliminary data indicate a better treatment effect with NV118 when the metformin-induced mitochondrial inhibition is more severe (data not shown). It is intriguing to speculate that the lactate assay is more sensitive for detection of beneficial effects by NV118.

Evaluation of coupled respiration in response to NV118 assesses only the drug’s effect at the RC but bypasses any effects the drug might have on substrate level phosphorylation through anaplerosis of the TCA cycle. TCA cycle intermediates were increased after treatment with the cell-permeable succinate prodrug NV189 and the time study with isotope labelled cell-permeable succinate prodrug indicated continuous TCA cycle metabolism which could potentially improve cellular energy charge by in increasing ATP or GTP by substrate level phosphorylation.

In paper III, we demonstrated that the supplementation with cell-permeable succinate prodrugs increased intracellular succinate levels. Elevated levels of succinate are believed to induce reversed electron transport (RET) to CI and increase mitochondrial ROS production during ischemia-reperfusion injury. ROS production is part both of physiological and pathophysiological process. Excess ROS production, however, can cause oxidative damage to lipids, nucleic acids and proteins (6). Increased ROS production via RET at CI due to increased succinate oxidation is thought to contribute to ischemia-reperfusion injury where oxidative stress after reperfusion is a hallmark (157). This view was, however, recently been challenged with the arguments that the redox state during early reperfusion is unfavourable for RET and the quick disappearance of succinate after reperfusion could also be due to succinate efflux instead of succinate oxidation (158). Inhibitors of CI, such as rotenone and metformin, have been described to inhibit RET under experimental conditions (159) and similar results were observed in a mouse model.
of Leber hereditary optic neuropathy with CI dysfunction (160), leaving the question open whether increased ROS from RET due to increased oxidation of succinate from the cell-permeable succinate prodrugs would be an issue for inherited CI-related mitochondrial disease or drug-induced CI-dysfunction. If increased ROS production by the cell-permeable succinate prodrugs would potentially be an obstacle remains to be evaluated.

Cell-permeable succinates as a diagnostic tool

In paper III, the cell-permeable succinate prodrugs were primarily evaluated as a potential treatment strategy for CI-related mitochondrial disease. Experiments with patient-derived fibroblasts with a known CI defect further demonstrated their usefulness for characterization of mitochondrial function in intact cells. In the patient-derived, CI deficient fibroblasts we identified a higher dependence on CII-linked substrate oxidation as compared to control cells. Their use as a diagnostic tool in intact cellular models was also demonstrated in cellular models of drug-induced mitochondrial toxicity (paper II, and unpublished data on the mitochondrial toxicity induced by Kolliphor® EL). One limitation of the use of cell-permeable succinate prodrugs as a diagnostic tool is that maximal capacity cannot be evaluated in intact cells due to the limitation of endogenous supply of nucleotide levels. However, the results can be used to guide further assessment of mitochondrial function. Regular succinate has limited permeability (89). Consequently, millimolar concentrations of succinate would need to be used to achieve an effect (151; 154; 155). The cell-permeable succinate prodrugs, in contrast, were highly permeable and effective at micromolar concentrations when succinate or methyl succinates did not improve mitochondrial function at identical doses.

Coupled or non-coupled, that is the question

As demonstrated in this study, an increase in mitochondrial respiration does not necessarily result in an increased coupled respiration. The increase in mitochondrial respiration with MB was due to increased uncoupling and did not improve OXPHOS. These data do not support the use of MB for acute treatment of MILA to correct for the mitochondrial metabolic defect. The apparent discrepancy between the data presented in paper IV and other studies which reported the bioenergetic bypass potential of MB might be explained by use of different methods. Most of these studies reported increased oxidation of NADH in isolated, sonicated mitochondria or sub-mitochondrial particles (95; 106; 111). Although it is possible
to evaluate the oxidation of NADH at CI with this approach it does not allow judgement whether the electron shuttling by MB is used to normalize the mitochondrial membrane potential and phosphorylation pathways. Others (94; 95) have studied MB’s potential to shuttle electrons and support mitochondrial respiration in intact cells which theoretically allows evaluation of its effect on the fully integrated mitochondrial function. Unfortunately, they did not calculate the coupled respiration in response to MB to investigate the quality of the increase in respiration. The lack of correlation of stimulation of mitochondrial respiration and phosphorylation potential could be related to the limited number of protons that are pumped at CIV as compared to CI and CIII, as MB donates electrons via cyt c to CIV. CI and III pump double the number of protons and thereby contribute to a larger degree to the build-up of a proton gradient that is required by the ATP-synthase to drive phosphorylation of ADP (12). MB has also been described to improve blood pressure through inhibition of the guanylate cyclase and has been used successfully in a small number of metformin-related lactic acidosis cases with this objective (109; 161; 162). This effect, however, is unrelated to mitochondrial function. MB has further been shown to increase markers of mitochondrial biogenesis (108; 110) which would be less relevant for acute critical care illnesses like MILA but could be of interested as long-term treatment for other indications.
Final conclusions

- High-resolution respirometry of freshly isolated human peripheral blood cells is a suitable model for evaluation of drug-induced mitochondrial toxicity and pharmacological bypass strategies for the support of mitochondrial function.

- Changes in the mitochondrial respiration of human peripheral blood cells qualitatively reflected changes in mitochondrial respiration of human muscle fibres, fibroblasts, hepatocytes, and kidney cells.

- Potential pharmacological bypass strategies have to be carefully investigated; stimulation of mitochondrial respiration has to be related to phosphorylation activity.

- Changes in mitochondrial respiration linked to phosphorylation pathways affect glycolytic lactate metabolism in human blood cells and the human-derived liver cell line HepG2.

- Cell-permeable succinate prodrugs are a useful tool to characterize mitochondrial function and dysfunction in intact cells but should be employed together with substrate-inhibitor-titration protocols in permeabilized cells to measure maximal respiratory capacities and confirm the results.
Future perspective

Cell-permeable succinates as a pharmaceutical drug

Cell-permeable succinates as potential treatment for acetaminophen-induced liver failure

APAP is the most common cause for drug-induced liver injury, the main cause for acute liver failure in the western world which not uncommonly ends fatally (59; 60; 62). Currently, the only treatment option for APAP overdose is NAC. NAC increases reduced glutathione levels and, thereby can prevent NAPQI from causing any further damage. This also means that the timing of its administration is crucial. However, patients often present several hours after APAP ingestions when NAPQI formation and liver damage have already occurred (60; 61; 163). Therefore, alternative and less time-dependent treatment strategies are needed. Because CII or downstream complexes of CII were unaffected by APAP-intoxication, cell-permeable succinate prodrugs may be beneficial as treatment for APAP-induced mitochondrial dysfunction and, potentially also hepatotoxicity. Succinate has previously shown to increase ATP content and reduce cell death in models of oxidative stress-induced and metformin-induced mitochondrial dysfunction (155; 164).

Proof of concept in vivo

Currently, the cell-permeable succinate prodrugs have not been evaluated in vivo. The next step in drug development of the cell-permeable succinate prodrugs would be the evaluation of their treatment potential in vivo. A suitable model for in vivo proof of concept would be the large animal model of rotenone-induced mitochondrial CI dysfunction described by Karlsson et al. (156). In this model, rotenone infusion caused a decrease in mitochondrial and whole body oxygen consumption due to rotenone-induced CI inhibition. Glycolytic metabolites were upregulated correspondingly due to upregulation of glycolysis to compensate for the loss of mitochondrial ATP production. The large animal model of metformin-
induced mitochondrial dysfunction by Protti et al. (165) presents an alternative in vivo model for efficacy assessment of this drug class.

**Mitochondrial dysfunction in Drug-Induced Lactic Acidosis-MIDDILA**

A study has been initiated with the aim to investigate the incidence of drug-induced lactic acidosis and elucidate the role of mitochondrial (dys-) function in its pathogenesis. This study was approved by the regional ethics committee of Lund, Sweden (permit no. 2017/518). Patients with suspicion of drug-induced metabolic acidosis admitted to the ICUs or emergency departments of Skåne University Hospital and Helsingborg hospital are included. Patients with lactate $\geq 4$ mmol/l and concurrent treatment with at least one of the following drugs are eligible for inclusion: metformin, paracetamol, linezolid, isoniazid, malarone, dudanosine, stavudine, lamivudine, zidovudine, abacavir, propofol, sevoflurane, desflurane and ketamine. Age below 18, pregnancy, known mitochondrial disease, haematological malignancy and thrombocytopenia $< 10^{9}$ /l are defined as exclusion criteria. Blood samples are taken from an existing arterial, central venous or peripheral line or by venous puncture. Two blood samples of 20 ml each are taken, the first within 24 h of admission and the second 48-72 h later. Blood from twenty healthy volunteers are taken by venous puncture at a volume of 60 ml. Written informed consent is acquired from the patient or next of kin and healthy volunteers. Human platelets and PBMCs will be isolated as previously described (24). In the second, explorative part of this study, the chemical laboratory data bank of Region Skåne will be reviewed for cases of serum lactate $\geq 4$ mM. From this list of patients, further clinical parameters will be extracted from the digital hospital charts.
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Eftersom mitokondrierna producerar majoriteten av cellens energi kan en störning av deras funktion ha allvarliga konsekvenser. Ändå är det bara under de senaste decennierna som eventuell mitokondriell toxicitet av läkemedel börjat undersökas under tidig läkemedelsutveckling. Trots att denna typ av läkemedelsbiverkan nu är känt och att det skett framsteg i metodutvecklingen för att undersöka mitokondrietroxicitet, är överföringen av fynd och kunskap från labbänken till klinisk användning av läkemedel fortfarande bristfällig.

Vi har utvecklat en diagnostisk metod för undersökning av mitokondriell funktion i friska mänskliga blodceller. Metoden bygger på konceptet att mitokondriell ATP-produktion är kopplad till syrgasförbrukning, och blodcellerna, vita blodkroppar och blodplättar, används som modell för frisk mänsklig vävnad. I denna avhandling har modellen utvärderats för att bedöma läkemedelsinducerad mitokondriell toxicitet och använts för utveckling av nya mitokondriestödjande farmakologiska strategier.


För att ytterligare undersöka om förändringar av mitokondriell funktion i vita blodkroppar och blodplättor reflekterar potentiella förändringar i andra, mer metaboliskt aktiva vävnader utvärderade vi både mitokondrie-toxiska läkemedel och cellpermeabla prodroger av succinat på humana fibroblaster, leverceller, njurceller och muskelfibrer. Vi visade att förändringar i syreförbrukningen i humana blodkroppar och blodplättor återspeglas i syreförbrukningen hos andra, mer metaboliskt aktiva mänskliga celler.
Sammanfattningsvis visade vi att vita blodkroppar och blodplättar är lämpliga modeller för utvärdering av läkemedelsrelaterad mitokondriell toxicitet och nya farmakologiska strategier för att stödja mitokondriell funktion. Förändringar i mitokondriefunktionen i vita blodkroppar och blodplättar på grund av läkemedelseffekter reflekterar förändringar hos andra, mer metaboliskt aktiva mänskliga vävnader.
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New strategies to evaluate mitochondrial function

Mitochondria produce the majority of the cell’s energy. Any dysfunction in, or interference with mitochondrial function can have severe consequences. And yet, it was only within the last decades that screening for potential mitochondrial toxicity was included as a routine toxicity assay during early drug development. Despite improved recognition of drug-related side effects on mitochondrial function, and progress in method development, translation from the in vitro to the in vivo situation and from animal to human tissues still remain obstacles. Mitochondrial dysfunction can also have genetic origin, with similar consequences. Current treatment options for mitochondrial disease are limited. In the present thesis, human peripheral blood cells were evaluated as a model for investigation of drug-induced mitochondrial toxicity and for efficacy assessment of pharmacological treatment strategies to support mitochondrial function.