Gaining mechanistic insights into mitochondrial complex I inhibitors as potential anti-cancer drugs by using a preclinical toxicological *in vivo* test battery

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1. Introduction

1.1 The role of energy metabolism for cancer therapy

Cancer is among the most common causes of death in western civilizations. Five of the 17 most common death reasons were a kind of cancer with lung cancer even ranking as number three of death reasons in the EU (Eurostat 2019). Therefore, a continuous need exists to develop innovative strategies in oncology drug research to treat cancer. Besides e.g. classical chemical agents for chemotherapy, newer types of drug treatments like targeted therapies are currently available exploiting differences in metabolism of cancer vs. normal cells (Baudino 2015; NIH – National Cancer Institute at the National Institutes of Health 2020; American Cancer Society 2020). About 100 years ago, Otto Warburg described first observations of a difference in energy metabolism of malignant cancer cells compared to benign normal cells (Warburg et al. 1927; Warburg et al. 1924). This finding still provides a foundation for current oncology research (Kim 2019; Shahruzaman et al. 2018; Vander Heiden 2011). The altered metabolic pattern has become a promising target in cancer therapy, since it allows a targeted approach. Nevertheless, energy metabolism of non-cancer cells might still be affected by that kind of treatment, which may lead to high toxic side effects. To find the right balance between hitting cancer cells via their energy metabolism but maintaining that of non-cancer cells, a sophisticated battery of toxicological tests has to be applied to find drug candidates with an acceptable risk–benefit balance. That kind of test battery should cover necessary parameters to identify the toxicological profile of a substance and to determine its influence on the different metabolic pathways. Gene expression analysis in combination with hematological and clinical-chemical examinations could be one such avenue. To ensure a tailor made and appropriate study design, it is necessary to take a closer look at the two major metabolic pathways glycolysis and oxidative phosphorylation in cancer cells.

1.1.1 Glycolysis under normoxic and hypoxic conditions in cancer cells

In general, every cell, whether differentiated or not, relies strongly on glycolysis to generate energy. During this metabolic pathway, glucose is converted anaerobically into pyruvate via a series of enzymatic reactions to produce two molecules of adenosine triphosphate (ATP), a high-energy phosphate compound. Further cellular processing of pyruvate is usually determined by oxygen availability. In the presence of oxygen, pyruvate is converted to acetyl coenzyme A (acetyl CoA), which enters the tricarboxylic acid (TCA) cycle and subsequently oxidative phosphorylation (OXPHOS) in mitochondria for further oxidization steps into CO_2 and H_2O, leading to energy production with a typical yield of 36 molecules of ATP per unit of glucose. Under hypoxic conditions (lack of oxygen), pyruvate is reduced to lactate. This

process, so called fermentation, which is a generic term for anaerobic digestion of glucose (or other organic nutrients), delivers at least the small amount of energy via glycolysis (anaerobic glycolysis) in form of two molecules ATP (Nelson et al. 2010). Most cancer cells use this process even in the presence of oxygen (Warburg 1956; Warburg et al. 1927; Warburg et al. 1926). This peculiarity of cancer cells is considered as a hallmark of cancer and is called aerobic glycolysis or the Warburg effect (Koppenol et al. 2011; Warburg et al. 1927; Warburg et al. 1926; Warburg et al. 1924). Otto Warburg observed an increased glucose uptake and lactate production in tumor slices and cancer cells with an approximately tenfold higher metabolization rate in a given time compared to normal tissues (Koppenol et al. 2011; Warburg et al. 1927; Warburg et al. 1924). This process converting glucose to lactate is far less efficient than the metabolization via OXPHOS, by generating fewer ATP molecules per unit of glucose. Therefore, a high rate of glucose uptake is required to cover the enhanced energy demand to support the rapid proliferation of cancer cells. This increased glucose uptake in cancer cells is used clinically for diagnostic purposes to visualize tumors by 2-deoxy-2(18F)-fluoro-D-glucose in positron emission tomography (FDG-PET) scans (Gambhir 2002). In addition to providing energy, the enhanced glycolytic rate is also required to maintain the level of glycolytic intermediates needed for increased biosynthetic processes including synthesis of lipids, amino acids, nucleotides and NADPH, which are essential for tumor growth (Lunt and Vander Heiden 2011). Moreover, the elevated glycolytic rate generates high amounts of lactate and H^+ ions intracellularly, which subsequently are excreted into the extracellular space, leading to acidification of the tumorigenic microenvironment (Gillies et al. 1994; Griffiths 1991; van Sluis et al. 1999; Wike-Hooley et al. 1984). This low pH provides a specialized niche, which is highly beneficial for cancer cells by promoting e.g. increased invasion and metastasis (Bhujwalla et al. 2002; Martinez-Zaguilan et al. 1996; Rozhin et al. 1994; Schlappack et al. 1991), enhanced resistance to apoptosis or autophagy induction, increased angiogenesis and obscured immune surveillance (Peppicelli et al. 2017). However, for normal, healthy cells this acidic tumor microenvironment is intolerable and rather toxic by inducing apoptosis and necrosis whereas cancer cells have acquired resistance mechanisms to survive (Estrella et al. 2013; Park et al. 1999; Williams et al. 1999). In addition, it has been shown that the acidic extracellular environment of cancer cells reduces the efficacy of or even causes resistance to chemotherapeutic drugs (Stubbs et al. 2000). Otto Warburg hypothesized that the altered metabolism of cancer cells reflects a damage to mitochondrial OXPHOS suggesting that normal respiration is not possible to obtain sufficient ATP (Warburg 1956; Warburg et al. 1927) (see Figure 1A). However, around two decades later, several studies revealed functional mitochondria in many cancer cells with the capability to generate ATP via OXPHOS (Weinhouse 1976). Moreover, some cancer cells are even able to switch reversibly between glycolytic and oxidative metabolism. This fast switching is referred to as "Crabtree effect" and

may help cancer cells to adapt to their rather heterogeneous microenvironments, especially in malignant solid tumors with hypoxic conditions (Crabtree 1929; Diaz-Ruiz et al. 2009). Interestingly, another model proposed a cancer cell symbiosis theory by which cancer cells relying on aerobic glycolysis provide their secreted lactate to neighbor cancer cells consuming this lactate to generate ATP via TCA cycle and OXPHOS (Faubert et al. 2017; Sonveaux et al. 2008) (see Figure 1B). Therefore, the studies regarding cancer metabolism in the last years showed that the Warburg effect is more closely related to alterations in signaling pathways rather than to mitochondrial defects. During the past decade, several investigations revealed that aerobic glycolysis in many cancer cells is a combined result of different factors and adverse events like activated oncogenes or inactivated tumor suppressors (Bayley and Devilee 2012; Elstrom et al. 2004; Levine and Puzio-Kuter 2010), a hypoxic microenvironment (Jiang 2017), mutations in mitochondrial DNA (mtDNA) (Chandra and Singh 2011; Srinivasan et al. 2016) and the tissue of origin (Gaude and Frezza 2016). Thereby, altered levels of some transcription factors are very common in human cancers, which are responsible for the glycolytic phenotype, like increased levels of hypoxia-inducible factor HIF1 and the oncogene c-Myc, as well as inactivation of the tumor suppressor p53 (Dang et al. 2008; Koppenol et al. 2011; Yeung et al. 2008). Hif1 is a heterodimeric transcription factor consisting of the two subunits Hif1-α and Hif1-β. HIF1-α is usually stabilized by hypoxia followed by dimerization with Hif1-β. The dimer can induce the transcription of its target genes, including genes coding for pyruvate-dehydrogenase-kinase 1 (PDK1), glucose transporter 1 and 3 (GLUT-1; GLUT-3), lactate dehydrogenase A (LDHA) and other glycolytic enzymes (Bell and Chandel 2007; Chowdhury et al. 2008; Ivan et al. 2001; Jaakkola et al. 2001; Ratcliffe et al. 1998; Schofield and Ratcliffe 2004; Semenza 2004; Simon 2006). However, HIF1-α could even be induced under normoxic conditions by activated oncogenes like Ras, Akt and HER2 (Denko 2008; Duvel et al. 2010; Hu et al. 2012; Levine and Puzio-Kuter 2010; Li et al. 2005; Majmundar et al. 2010; Pylayeva-Gupta et al. 2011; Yecies and Manning 2011) or inactivated tumor suppressors like p53, pVHL and PTEN (Amelio et al. 2018; Elstrom et al. 2004; Kapitsinou and Haase 2008). Furthermore, HIF1 also enhances *Myc* expression, by binding to a DNA motif in the promotor of *Myc* and interacts with c-Myc to promote aerobic glycolysis. This includes the induction of hexokinase 2 (HK2), the first enzyme in glycolysis, which converts glucose to glucose-6-phosphate and PDK1, which is a negative regulator of pyruvate dehydrogenase (PDH) catalyzing usually pyruvate to acetyl-CoA in mitochondria (Dang et al. 2008). In addition, c-Myc supports the Warburg effect in cancer cells by upregulating GLUT-1 and lactate dehydrogenase A (LDHA), the enzyme that converts pyruvate to lactate and concomitant converts NADH to NAD^+ (Osthus et al. 2000; Shim et al. 1997). The tumor suppressor and transcription factor p53 regulates several biological functions including cellular energy metabolism with a central role in balancing between glycolysis and OXPHOS (Ma et al. 2007;

Matoba et al. 2006). Mutations of the *p53* gene are the most common gene mutations in human cancer (Perri et al. 2016). The resulting inactivation of p53 induces aerobic glycolysis in many aspects. This includes e.g. enhanced glucose uptake and an increase of HIF-1α as well as promoting the activity of the glycolytic enzymes HK2 and phosphoglycerate mutase (PGM), which catalyzes step 8 in glycolysis converting 3-phosphoglycerate (3PG) to 2-phosphoglycerate (2PG) (Kondoh et al. 2005; Ma et al. 2007; Wang et al. 2012a). Understanding these molecular pathways, which regulate aerobic glycolysis in the altered cancer metabolism, is very important for improved cancer therapy. Investigations in the past century regarding aerobic glycolysis have revealed many targets for therapeutic drugs including several key enzymes involved in glycolysis. This includes e.g. GLUT1, LDHA, hexokinases, pyruvate kinase isoform M2 (PKM2), an isoform which is highly expressed and active in tumor tissue (Christofk et al. 2008; Mazurek et al. 2005) and monocarboxylate transporter 1 and 4 (MCT1 and MCT4), which are plasma membrane transporters that catalyze the proton-linked transport of monocarboxylates such as lactate and pyruvate (Dimmer et al. 2000; Draoui and Feron 2011). Specific inhibitors described in literature are for example silibinin for GLUT1 (Deep and Agarwal 2013), GNE-140 for LDHA (Rani and Kumar 2016), lonidamine for hexokinases (Hamanaka and Chandel 2012), TLN-232 for PKM2 (Vander Heiden et al. 2010) and AZ3965 and AZ93 for MCT1 and MCT4, respectively (Morais-Santos et al. 2015). However, even if the enhanced dependency on glycolysis of most cancer cells serves as an appealing target for cancer therapy, several clinical trials have shown, that the use of glycolytic inhibitors may not be suitable for certain types of tumors rather relying on OYPHOS (Moreno-Sanchez et al. 2007). Therefore, further investigations focusing on the OXPHOS metabolic pathway are still important for developing appropriate anti-cancer drugs.

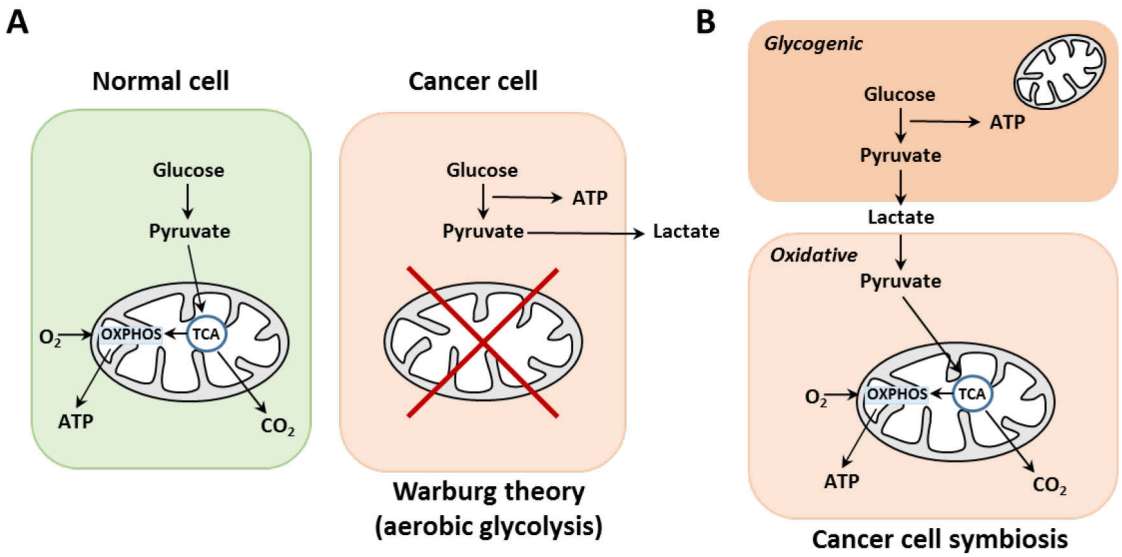

Figure 1: Simplified illustration of cancer energy metabolism. (A) Comparison of a normal cell and a cancer cell: In contrast to the normal cell generating ATP via TCA cycle and OXPHOS, the cancer cell shows the classical Warburg effect by relying on aerobic glycolysis and having impaired mitochondria, thus producing excessive lactate. (B) Cancer cell symbiosis: Secreted lactate from cancer cells relying on aerobic glycolysis is used by neighboring cancer cells to generate ATP via TCA cycle and OXPHOS. TCA: tricarboxylic acid cycle (Krebs cycle); OXPHOS: oxidative phosphorylation (adapted from Kim 2018).

1.1.2 Oxidative phosphorylation in cancer cells

The mitochondrial OXPHOS metabolic pathway is the last step of aerobic metabolism in normal cells. All oxidation steps of the breakdown of carbohydrates, fats and proteins lead to this final phase of cellular respiration, in which the oxidation energy triggers ATP synthesis. Energy is generated by the transport of electrons through a series of transmembrane protein complexes I-IV and the freely mobile electron transfer carrier ubiquinone (Coenzyme Q, CoQ) and cytochrome c in the mitochondrial inner membrane, also known as electron transport chain (ETC) or mitochondrial respiratory chain, coupled to a translocation of protons across the inner mitochondrial membrane. This generates an electrochemical gradient with proton accumulation in the intermembrane space, which is finally used by the ATP synthase (complex V) to generate ATP by providing a proton flux back into the mitochondrial matrix. In this process, NADH, FADH2 and succinate act as important electron carrier with oxygen as terminal electron acceptor in the chain (Nelson et al. 2010).

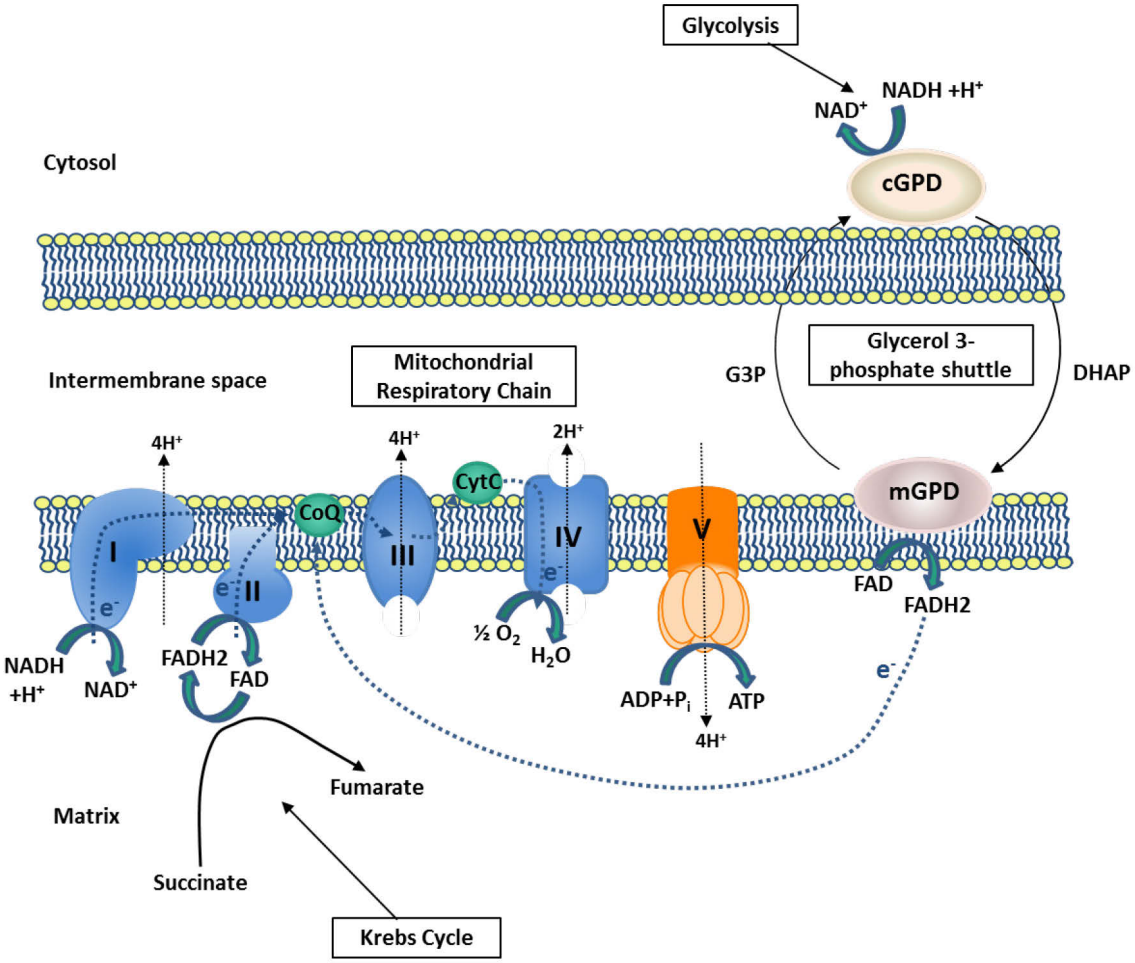

Figure 2: Schematic illustration of the mitochondrial respiratory chain (OXPHOS). I: complex I/NADH dehydrogenase, II: complex II/Succinate dehydrogenase, III: complex III/ CoQ:CytC-oxidoreductase, IV: complex IV/CytC-oxidase, V: complex V/ATP synthase, CoQ: Coenzyme Q/Ubiquinone, CytC: Cytochrome C, cGPD: cytosolic glycerophosphate dehydrogenase, mGPD: mitochondrial glycerophosphate dehydrogenase, G3P: glycerol-3-phosphate, DHAP: dihydroxyacetone phosphate, FAD: flavin adenine dinucleotide, NAD^+: nicotinamide adenine dinucleotide, ATP: adenosine triphosphate, ADP: adenosine diphosphate, Pi: inorganic phosphate (adapted from Nelson et al. 2010).

Beside producing energy in form of ATP, the mitochondrial OXPHOS appears to be an important source of reactive oxygen species (ROS) (Zhao et al. 2019). Even under physiological conditions it could be shown, that 0.15-2% of the ETC electrons do not pursue the normal transfer, thus leaking out of the ETC and interact with oxygen to form ROS like superoxide or hydrogen peroxide (Aguilaniu et al. 2003; Chance et al. 1979; Hansford et al. 1997; St-Pierre et al. 2002; Staniek and Nohl 2000). The main sites of ROS generation include several mitochondrial respiratory chain components like complex I (Esterhazy et al. 2008; Murphy 2009; Turrens and Boveris 1980; Vinogradov and Grivennikova 2016; Walker 1992), complex II (McLennan and Degli Esposti 2000; Quinlan et al. 2012; Zhang et al. 1998), complex III (Brand 2010; Drose and Brandt 2012; Murphy 2009) and the mitochondrial

glycerophosphate dehydrogenase (mGPD) (Drahota et al. 2002; Miwa and Brand 2005; Miwa et al. 2003; Mracek et al. 2013; Mracek et al. 2014; Vrbacky et al. 2007). The latter is a further integral component of the respiratory chain, which connects glycolysis with OXPHOS via the glycerol-3-phosphate shuttle and shows important interactions with the ETC by providing electrons to CoQ (Mracek et al. 2013) (see Figure 2). Moreover, OXPHOS can also be involved in mitochondrial-mediated programmed apoptosis, either through its overall functionality (Kwong et al. 2007), or through single enzyme complexes (Grimm 2013; Ricci et al. 2004). Especially, the generation of ROS by the ETC can directly trigger apoptosis (Redza-Dutordoir and Averill-Bates 2016). One hallmark of cancer is the ability to avoid apoptosis (Fernald and Kurokawa 2013). This would be in line with Otto Warburg's original proposal that all cancer cells have impaired mitochondria with a non-functional OXPHOS (Warburg 1956). However, as already mentioned in 1.1.1, it has been shown, that functional mitochondria can be present in cancer cells and can even play a major role in energy supply. Several studies have revealed that certain cancer types rely on OXPHOS like breast cancer (Whitaker-Menezes et al. 2011), ovarian cancer (Pasto et al. 2014), lung carcinoma (Hensley et al. 2016), melanoma (Fischer et al. 2017), or Hodgkin lymphoma (Birkenmeier et al. 2016). Moreover, even cancer stem cells show upregulated OXPHOS (Sancho et al. 2016), such as chronic myeloid leukemia stem cells (Kuntz et al. 2017) or pancreatic cancer stem cells (Sancho et al. 2015). Whether cancer cells utilize OXPHOS for energy generation and the degree of OXPHOS usage is dependent on different factors. Gene mutations can be a reason for OXPHOS upregulation in cancer cells. For example, mutational inactivation of the tumor suppressor liver kinase B1 (LKB1) can be found in several cancer types with upregulated OXPHOS like breast cancer, ovarian cancer and melanoma, but also in approximately 30% of lung adenocarcinoma (Gill et al. 2011; Guldberg et al. 1999; Sengupta et al. 2017; Shen et al. 2002; Tanwar et al. 2014). In addition, the proliferation rate of cancer cells is strictly interconnected with the use or non-use of OXPHOS. Fast growing cells often rely on aerobic glycolysis (Fernandez-de-Cossio-Diaz and Vazquez 2017), whereas dormant/slow-cycling or metastatic cells mainly rely on OXPHOS (LeBleu et al. 2014; Tan et al. 2015; Viale et al. 2015). Thus, OXPHOS seems to be a major factor in the determination, whether a tumor is metastatic or not. Viale and colleagues concluded that this might be the reason why the OXPHOS metabolism in cancer cells remained undiscovered for such a long time, since mainly the solid bulk was investigated (Viale et al. 2015). Moreover, a tumor can also be heterogeneous and contain both types of cells, the ones utilizing OXPHOS and the ones using only the glycolysis pathway (Hensley et al. 2016; Quintana et al. 2010). In this case, it might also depend on the availability of oxygen. While cells in a solid tumor might have to deal with a low oxygen availability, metastatic cells have a better oxygen supply. Furthermore, even in symbiotic tumors, as already mentioned in 1.1.1, cells that utilize aerobic glycolysis coexist with cancer cells exhibiting active OXPHOS. Here

OXPHOS plays a key role, enabling particular cells to participate in the symbiosis (Faubert et al. 2017; Sonveaux et al. 2008). Interestingly, several studies have shown that various cancers that have adapted to rely more on mitochondrial OXPHOS for survival and progression are more resistant to radiotherapy or chemotherapy (Bosc et al. 2017; Farge et al. 2017; Kalainayakan et al. 2018) and even to therapy targeting the glycolytic pathway (Hirpara et al. 2019). Therefore, candidate compounds targeting the ETC of these special cancer subtypes gained increasing interest in oncology research in recent years, especially, inhibitors of the different enzyme complexes of the OXPHOS (Ashton et al. 2018; Sica et al. 2020). Numerous compounds of therapeutic potential inhibiting OXPHOS, described in the literature are for example metformin (Koritzinsky 2015; Wheaton et al. 2014; Zannella et al. 2013), phenformin (Appleyard et al. 2012; Shackelford et al. 2013), BAY84-2243 (Chang et al. 2015; Ellinghaus et al. 2013) and fenofibrate (Brunmair et al. 2004a; Wilk et al. 2015) for complex I, the vitamin E analogue α-tocopheryl succinate (Dong et al. 2009) and lonidamine (Guo et al. 2016) for complex II, atovaquone (Ashton et al. 2016; Fiorillo et al. 2016; Xiang et al. 2016) for complex III, arsenic trioxide (Diepart et al. 2012) for complex IV and giboxin for complex V (ATP synthase) (Shi et al. 2019). In recent years, complex I has become a major target, which is also in the main focus of this thesis. Complex I, also known as NADH dehydrogenase, is the largest multi-subunit enzyme, with a molecular mass of about 1000 kDa, of the mitochondrial respiratory chain. It catalyzes one of the first steps of OXPHOS, by transferring electrons from NADH to CoQ with a simultaneous transmembrane proton pumping contributing to the electrochemical gradient (Nelson et al. 2010). It could be demonstrated, that mutations in nuclear as well as mitochondrial genes encoding complex I subunits are a contributing factor in various pathological conditions, like e.g. diabetes (Elango et al. 2014; Sethumadhavan et al. 2012), neurodegenerative diseases (Iommarini et al. 2013; Marin et al. 2013; Rodenburg 2016) and even cancer (Iommarini et al. 2013; Kurelac et al. 2013). Regarding cancer, it could be shown, that mutations in complex I genes can promote the progression of different cancer types like breast (Yu et al. 2015), prostate (Philley et al. 2016), lung (Su et al. 2016), renal (Horton et al. 1996; Kim et al. 2016), colorectal (Akouchekian et al. 2011), thyroid (Evangelisti et al. 2015; Gasparre et al. 2007), and head and neck cancers (Allegra et al. 2006). In addition, mutations in genes coding for complex I subunits have been linked to enhanced ROS-dependent metastatic potential in Lewis lung carcinoma and breast cancer cells (He et al. 2013; Ishikawa et al. 2008), as complex I is one of the major sides of ROS generation of the ETC. Moreover, a slight elevation of ROS level has been shown to support cancer cell proliferation and migration and to induce several signaling pathways associated with cell survival, which contribute to cancer growth and malignant transformation (Kumari et al. 2018). Considering the contributing role of mitochondrial complex I to metastasis, proliferation and survival of cancer cells, inhibition of its activity reveals to be a promising target for anticancer

therapy. Ongoing investigations to find new efficient therapeutic compounds targeting the mitochondrial complex I demonstrate the importance of this compound class in oncology research (Hirpara et al. 2019; Molina et al. 2018; Naguib et al. 2018).

1.2 Complex I inhibitors

In recent years, anticancer effects by complex I inhibition could be demonstrated in various cancer cell lines or animal models for several compounds (Appleyard et al. 2012; Brunmair et al. 2004a; Chang et al. 2015; Ellinghaus et al. 2013; Koritzinsky 2015; Shackelford et al. 2013; Wheaton et al. 2014; Wilk et al. 2015; Zannella et al. 2013). These include amongst others the two biguanides phenformin and metformin. Moreover, one classical, very strong and well known complex I inhibitor is the pesticide and piscicide rotenone, which could also demonstrate an anticancer potential in some *in vitro* and *in vivo* studies (Abdo et al. 1988; Armstrong et al. 2001; Chung et al. 2007; Cunningham et al. 1995; Deng et al. 2010; Isenberg et al. 1997; Shi et al. 2014; Tada-Oikawa et al. 2003; Tanaka et al. 2002; Yoshitani et al. 2001). In the following two sections, these substances are described in more detail.

1.2.1 Rotenone

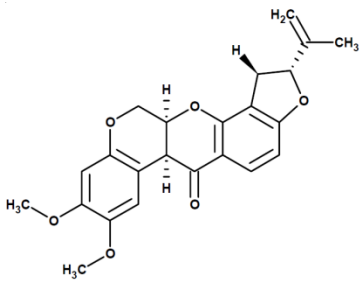

Figure 3: Chemical structure of rotenone. Rotenone's basic structure consists of a chromano-chromanone ring with a Dihydro-furan ring (Ling 2003; Fukami et al. 1959)

Rotenone is a highly lipophilic compound consisting of a basic structure of a chromano-chromanone ring with a Dihydro-furan ring (see Figure 3). It is a naturally occurring substance, which can be isolated from the roots and stems of *Lonchocarpus* and *Derris* species. Rotenone had been used worldwide as a broad spectrum pesticide and piscicide, but was then withdrawn from the market in many countries (OJEU 2008c) due to its toxicity. Its mechanism of action (MOA) concerning complex I comprises inhibition of electron transfer from the iron-sulfur centers in complex I to CoQ, leading to a blockade of OXPHOS with limited synthesis of ATP (Palmer et al. 1968) (see Figure 4). As a side effect of complex I inhibition, ROS can be formed due to incomplete electron transfer. A major impact of rotenone-induced ROS is the induction of apoptosis by damaging mitochondrial components, including mitochondrial DNA (Fato et al.

2009; Li et al. 2003). In addition to the effects on the OXPHOS, various studies have shown "that rotenone inhibits microtubule assembly independently of a specific energy-requiring step through tubulin binding, resulting in mitotic arrest and inhibition of cell proliferation (Brinkley et al. 1974; Marshall and Himes 1978; Srivastava and Panda 2007)" (manuscript I, Heinz et al. 2017). Due to these mechanisms and its high lipophilicity, enabling rotenone to readily cross biological membranes including the blood–brain barrier (Higgins and Greenamyre 1996), rotenone appeared to be neurotoxic (manuscript I, Heinz et al. 2017). In addition to this effect, it has been suggested that rotenone has anti-cancer activity (manuscript I, Heinz et al. 2017). Rotenone has been shown to induce apoptosis and inhibit cell proliferation *in vitro* of several human cancer cell lines (Armstrong et al. 2001; Chung et al. 2007; Deng et al. 2010; Shi et al. 2014; Tada-Oikawa et al. 2003). Moreover, it could be demonstrated, that rotenone can inhibit spontaneously and chemically induced liver tumors in mice (Abdo et al. 1988; Cunningham et al. 1995; Isenberg et al. 1997), and chemically induced colon tumors (Yoshitani et al. 2001) and tongue tumors in rats (Tanaka et al. 2002).

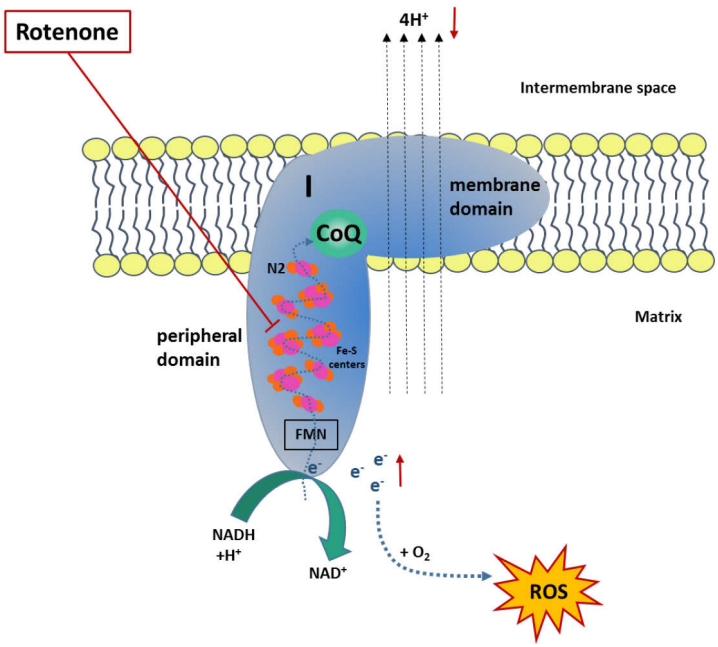

Figure 4: Schematic illustration of complex I inhibition by rotenone. Rotenone inhibits the transfer of electrons from the iron-sulfur clusters in complex I to CoQ. This leads to an accumulation of electrons within the mitochondrial matrix. Less protons can be pumped in the intermembrane space, resulting in a lower electrochemical gradient. Moreover, the surplus electrons can interact with cellular oxygen to form reactive oxygen species (ROS). NAD^+: nicotinamide adenine dinucleotide, CoQ: Coenzyme Q/Ubiquinone, $CoQH_2$: Ubiquinol, FMN: flavin mononucleotide, N2: final Fe-S cluster (adapted from Nelson et al. 2010).

1.2.2 Biguanides – Phenformin and Metformin

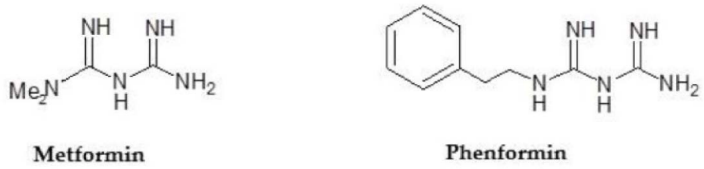

Figure 5: Chemical structures of Metformin an Phenformin. Metformin and Phenformin are both member of the class of biguanides with the same basic chemical structure. While metformin carries two methyl substituents at one of the terminal nitogen atoms, phenformin has a 2-phenylethyl group (Garcia Rubino et al. 2019)

Metformin and phenformin are plant derived biguanides and thus derivatives of guanidine which were discovered in the 1920s in extracts of *Galega officinalis* (French lilac) (see Figure 5). Due to their antihyperglycaemic effect, they have been widely used as antidiabetic drugs since 1957 (Schäfer 1983). While metformin has an excellent safety profile and is the first-line oral antidiabetic drug for Type II diabetes, phenformin has been withdrawn from many markets in the 1970s for causing fatal lactic acidosis (Williams and Palmer 1975). Reduction of the blood glucose level is the main effect that makes biguanides valuable for diabetes treatment. Although the complete MOA remains to be elucidated, “the blood glucose-lowering effect associated with increased cellular glucose uptake, suppression of gluconeogenesis, stimulation of glycolysis and fatty acid oxidation as well as improved insulin sensitivity, is generally thought to be induced by an impact on mitochondrial function (Owen et al. 2000)” (manuscript II, Heinz et al. 2019). Already in the early 1950s guanidines and biguanides have been demonstrated to have a specific effect on mitochondrial energy metabolism, including blockage of NADH-linked respiration of isolated mitochondria (Chance and Hollunger 1963; Davidoff 1971; Hollunger 1955; Schaefer 1964; Schafer 1969; Schafer 1980). In this context, as described in manuscript II (Heinz et al. 2019), several MOAs are currently under discussion, including “(1) inhibition of mitochondrial complex I activity (Owen et al. 2000), (2) direct or indirect activation of AMP-activated protein kinase (AMPK) (Shaw et al. 2005; Zhang et al. 2016; Zhang et al. 2007; Zhou et al. 2001), or (3) inhibition of the redox-shuttle enzyme mitochondrial glycerophosphate dehydrogenase (mGPD) (Madiraju et al. 2014; Madiraju et al. 2018). With respect to (1), inhibition of complex I activity, metformin is described as rather mild and transient inhibitor, whereas phenformin shows a more powerful inhibitory effect (Zhou et al. 2001; Bridges et al. 2014; Cameron et al. 2018). In general, this inhibition compromises the mitochondrial respiratory chain leading to an increase of the AMP-to-ATP ratio. This in turn leads to (2) activation of AMPK, considered to be responsible for many metformin and phenformin effects, due to its key role in fatty acid metabolism by suppressing fatty acid

synthesis and enhancing β-oxidation (Collier et al. 2006; Hawley et al. 2003; Zhang et al. 2007; Zhou et al. 2001), its involvement in enhancing glucose transport and insulin receptor function (Gunton et al. 2003) and its inhibitory effect on gluconeogenesis (Jeon 2016). The metformin- and phenformin-induced increase in AMP concentration in hepatocytes is also described to inhibit glucagon-induced elevation of cyclic adenosine monophosphate (cAMP) thus reducing activation of protein kinase A (PKA) (Gunton et al. 2003). Therefore, apart from AMPK activation, inhibition of PKA signaling may be partly responsible for biguanide-mediated stimulation of glycolysis and suppression of gluconeogenesis (Gunton et al. 2003; Herzig et al. 2001; Miller et al. 2013; Wang et al. 2012b; Yoon et al. 2001)" (manuscript II, Heinz et al. 2019). "Recently, it has been demonstrated, that a metformin-mediated AMP increase can also cause inhibition of fructose 1-6-bisphosphatase in the liver of mice, leading to reduced liver glucose production, suggesting a further mechanism for the blood glucose-lowering effect of metformin (Hunter et al. 2018). Besides indirect AMPK activation by an increased AMP-to-ATP ratio, more direct AMPK activation mechanisms are discussed for metformin (Meng et al. 2015; Shaw et al. 2005; Zhang et al. 2016)" (manuscript II, Heinz et al. 2019), but have not been demonstrated for phenformin. As the third (3) possible major MOA of biguanides, "Madiraju and colleagues (2014) demonstrated that inhibition of mGPD could also explain the decreased gluconeogenesis after metformin application" (manuscript II, Heinz et al. 2019). Whether phenformin acts similarly on mGPD is still elusive. "Considering all mentioned possible MOAs, the inhibition of complex I activity appears questionable, since it is observed only at high concentrations especially in the case of metformin (Drahota et al. 2014; Dykens et al. 2008; El-Mir et al. 2000; Owen et al. 2000). Hence, the other mechanism may be more likely to function *in vivo"* (manuscript II, Heinz et al. 2019). For an illustration of all possible MOAs of biguanides please see Figure 6.

"In addition to their antidiabetic activity, metformin and phenformin are under investigation as potential anti-cancer drugs, especially after an epidemiological study described a link between metformin treatment and a reduced cancer risk in diabetic patients (Evans et al. 2005). Since then, multiple laboratory-based studies reported that biguanides can inhibit proliferation and stimulate apoptosis in tumor cell lines *in vitro* (Ashinuma et al. 2012; Caraci et al. 2003; Geoghegan et al. 2017; Jackson et al. 2017; Jin et al. 2017; Kheder et al. 2017; Ko et al. 2016; Lu et al. 2018; Mogavero et al. 2017; Orecchioni et al. 2015; Rastegar et al. 2018; Wheaton et al. 2014; Xie et al. 2017; Yousef and Tsiani 2017), and can prevent spontaneously and chemically induced tumorigenesis *in vivo* (Anisimov et al. 2005a; Anisimov et al. 2011; Anisimov et al. 2005b; Appleyard et al. 2012; Bojkova et al. 2009; Dilman and Anisimov 1980; Dilman et al. 1978; Jackson et al. 2017; Jia et al. 2015; Ko et al. 2016; Yousef and Tsiani 2017). Furthermore, more than 100 clinical studies are currently ongoing or upcoming for metformin, in order to assess its role in the therapy of cancer (Chae et al. 2016; Kasznicki et al. 2014; MacKay et al. 2017; Zi et al. 2018; ClinicalTrials.gov)" (manuscript II, Heinz et al.

2019). In contrast, the effect of phenformin on cancer in clinical trials has rarely been studied and only one single clinical trial is ongoing (NCT03026517; ClinicalTrials.gov). For this potential anti-cancer activity of metformin and phenformin the apparent ability to inhibit complex I was suggested as MOA (Birsoy et al. 2014; Owen et al. 2000; Pollak 2014; Wheaton et al. 2014). Apart from ensuing indirect activation of AMPK via complex I inhibition, direct AMPK activation, leading to the inhibition of mTOR (mammalian target of rapamycin), a key regulator of cell proliferation, and its downstream pathways, thus blocking cell growth, is also under discussion for metformin and phenformin (Gong et al. 2016; Jackson et al. 2017; Jalving et al. 2010; Josse et al. 2016; Veiga et al. 2018) (see Figure 6). In addition, the ability to induce apoptosis via ROS production at the inhibited complex I, may be a further anti-cancer effect of biguanides (see Figure 6). Still for metformin the reported results in this context are ambiguous, showing ROS generation in some but not all experiments (Algire et al. 2012; Araujo et al. 2017; Batandier et al. 2006; Gao et al. 2016; Veiga et al. 2018).

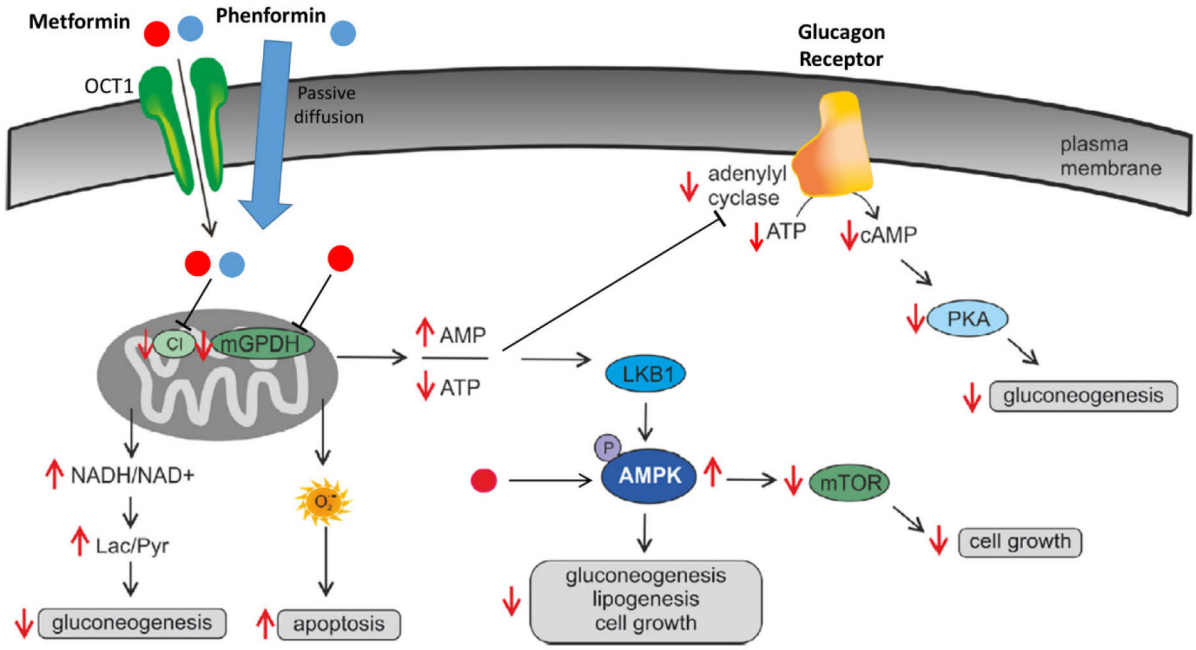

Figure 6: Simplified illustration of possible mechanisms of action of biguanides. Phenformin is either transported via organic cation transporter 1 (OCT1) or via passive diffusion, whereas metformin can be only transported via OCT1. Inside cell, metformin and phenformin may inhibit complex I, whereas metformin can also inhibit mitochondrial glycerophosphate dehydrogenase (mGPDH). Mitochondrial impairment can lead to (1) increased lactate/pyruvate (Lac/Pyr) ratio and decreased gluconeogenesis, (2) apoptosis due higher reactive oxygen species generation, (3) increased AMP/ATP ratio and subsequent inhibition of (a) adenylate cyclase, reduction of cAMP and protein kinase A (PKA) resulting in decreased gluconeogenesis and (b) activation of AMP-activated protein kinase (AMPK) leading to reduced gluconeogenesis, lipogenesis and cell growth. Metformin may also activate AMPK directly. (adapted from Pecinová et al. 2019)

1.3 Differences in toxic side effects of rotenone vs. biguanides

By targeting mitochondrial complex I of the ETC and thus cellular respiration, high toxicity could be expected, as the hazard of suffocation could be assumed. In this context, steep dose-response-curves for cell viability of mitochondrial ETC inhibitors have been demonstrated in cell culture (Marroquin et al. 2007). Therefore, a narrow therapeutic window for these kinds of compounds is likely. In this regard, the toxic effects seen for rotenone may exclude its use as an anti-cancer drug, even though the results in cancer cell lines and animal models suggest a possible anti-tumor activity. Besides rotenone's known high acute toxicity (MAK, 2003; Lightbody and Mathews, 1936), a subchronic 13-week feeding study, in which rats received rotenone at concentrations of 75, 150, 300, 600 and 1200 ppm (corresponding to 7.5, 15, 30, 60 and 120 mg/kg body weight/day) revealed no observed effect levels (NOELs) of 7.5 mg/kg body weight/day and of 15 mg/kg body weight/day for female and male rats, respectively. Animals in the higher dose groups showed atrophy of the bone marrow as well as inflammation and hyperplasia of the forestomach (≥ 30 mg/kg body weight/day for males and ≥ 15 mg/kg body weight/day for females) (Abdo 1988). Interestingly, several genotoxicity tests revealed that rotenone is not mutagenic, does not interact with DNA and induces no structural chromosomal aberrations (MAK, 2003). However, it was shown that rotenone causes numerical chromosome aberrations and increases the incidence of micronuclei through inhibition of microtubule assembly (MAK, 2003). Yet as mentioned above, this mechanism has been demonstrated to occur independently of a specific energy-requiring step through tubulin binding, leading to a mitotic arrest and inhibition of cell proliferation, which is associated with a no adverse effect level (Brinkley et al. 1974; Marshall and Himes 1978; Srivastava and Panda 2007). Moreover, as already mentioned in section 1.2.1, the combination of rotenone's ability to inhibit OXPHOS and mitotic chromosome distribution together with its ability to easily traverse biological membranes including the blood–brain barrier (Higgins and Greenamyre 1996), renders rotenone a neurotoxic compound. Chronic administration of rotenone correspondingly induced Parkinson's disease (PD)-like pathology in Lewis rats, including selective degeneration of nigral dopaminergic neurons and PD-like locomotor symptoms (Betarbet et al. 2000). Furthermore, rotenone exposure has been demonstrated to correlate with the occurrence of several PD-like symptoms in humans (Tanner et al. 2011). Compared to rotenone, biguanides show a much better toxicological profile. In a 6-month repeated dose toxicity study, in which rats received phenformin in the diet at concentrations of 2100 ppm, 6720 ppm and 21000 ppm (corresponding to 42, 150 and 275 mg/kg body weight/day), no toxic effects were detected in organs and no reduction of body weights were seen in the lowest and middle dose group. In the high dose group, only unspecific effects, such as a reduction of food consumption and body weight gain were observed. Therefore, a NOEL of 150 mg/kg body weight/day was derived. However, in another subchronic 45-days study, in which rats received

phenformin in the diet at concentrations of 300, 800, 1500, 3000, and 6000 ppm (corresponding to 27, 72, 135, 270 and 540 mg/kg body weight/day, using EFSA default converting factor (EFSA, 2012)) a delayed body weight gain was already observed in the lowest dose. No other parameters were tested (NCI, 1977). For metformin, a 6-month oral repeated dose toxicity study in rats is reported with doses of 120, 300, 600 and 900 mg/kg body weight/day administered via the diet (ECHA database, Registration Numbers.: 01-2119931228-39-0000/01-2119931228-39-0002). Minimal body weight suppression was already observed in the 300 mg/kg body weight/day dose group. Besides a highly reduced body weight gain in the two highest dose groups, slightly more frequent cytoplasmic vacuolation in renal tubuli were detected histopathologically only in female rats at the highest dose (900 mg/kg body weight/day). Therefore, a NOEL of 120 mg/kg body weight/day was derived for metformin in this study. The difference between the reported NOELs for rotenone and the two biguanides is therefore approximately a factor of 16-20. Moreover, neither a mutagenic or clastogenic potential could be shown for phenformin as well as for metformin in several genotoxic studies (NTP, 1973-1982; ECHA database Registration Numbers.: 01-2119931228-39-0000/01-2119931228-39-0002; Sant'Anna et al. 2013) nor has neurotoxicity been reported in humans for these biguanides. Despite the cited data may indicate that these two biguanides could have similar toxicological profiles, human epidemiological data reveal an important difference. As already mentioned in section 1.2.2, phenformin was withdrawn from many markets in the late 1970s due to an increased risk of lactic acidosis and associated mortality at its therapeutic use in diabetic patients (Williams and Palmer 1975). The reported incidence rate of phenformin-associated lactic acidosis ranged from 40 to 64 cases per 100000 person-years (Aguilar et al. 1992; Bailey 1992), whereas for metformin a much lower incidence was shown with approximately 0–9 cases per 100000 person-years (Bailey and Turner 1996; Berger 1985; Defronzo et al. 1995; Misbin et al. 1998; Stang et al. 1999). In this regard, Bando and colleagues investigated the potential risks of lactic acidosis induction by phenformin in comparison to metformin in rats by using equivalent doses of each biguanide. By that, comparable exposure levels relative to the therapeutic dose exposure levels in humans were guaranteed (Bando et al. 2010). It could be shown that blood lactic acid levels were increased significantly only in rats treated with phenformin, whereas no change was observed in metformin-treated rats. The authors considered that the repeated administration of the drug for 28 days may resulted in accumulation of phenformin since remarkably higher systemic exposure values of phenformin were measured after repeated treatment compared to those after a single-dose. It was concluded that this accumulation is the result of a saturated metabolic and/or elimination capacity for phenformin, because the metabolism of phenformin plays a major role in its excretion (Bando et al. 2010). Unlike metformin, which is mainly excreted as non-metabolized drug (Marchetti et al. 1991), phenformin is primarily hydroxylated to 4-hydroxy-phenformin by the cytochrome P450 2D6 (CYP2D6) enzyme in the human liver

(Shah et al. 1985). Interestingly, it could be demonstrated that 4-hydroxy-phenformin had no effect on blood lactate or glucose concentrations in rats, which were directly treated with this metabolite (Guest et al. 1980). Moreover, several investigations showed, that the well-known genetic polymorphism in the *CYP2D6* gene, coding for the cytochrome P450 2D6 hydroxylation enzyme, can be involved in an accumulation of non-metabolized phenformin in human (Bosisio et al. 1981; Shah et al. 1980, Oates et al. 1982). In this context, it was observed that poor metabolizers had higher plasma concentrations of phenformin and drastically increased blood lactate concentrations after a single therapeutic dose of phenformin, whereas extensive metabolizers had significantly lower concentrations (Oates et al. 1983). Nowadays, in pharmaceutical drug development, a potential drug candidate would be discontinued if it showed this polymorphism in preclinical studies.

Besides its potentially systemic accumulation, phenformin´s higher potency for inhibition of complex I activity compared to metformin (Zhou et al. 2001; Bridges et al. 2014; Cameron et al. 2018) may also contribute to a greater risk of lactic acidosis. As mentioned above the overall incidence of lactic acidosis is much lower for metformin, which in most cases could even be attributed to inappropriate high doses (Barrueto et al. 2002; Chang et al. 2002; Galea et al. 2007; Gjedde et al. 2003; Harvey et al. 2005; Heaney et al. 1997; Lacher et al. 2005; Nisse et al. 2003; Spiller and Sawyer 2006; Teale et al. 1998; von Mach et al. 2004). In addition, metformin is only unsafe for a subset of patients with risk factors for lactic acidosis, such as those with liver, kidney or heart dysfunctions (Crowley et al. 2017; Lalau 2010). Moreover, since metformin is mainly excreted as unchanged parent compound, the influence of a genetic polymorphism on its mode of action and potency is unlikely. Therefore, metformin shows an excellent toxicological profile, compared to phenformin and especially to rotenone.

1.4 Aim of this thesis

After the publication of an epidemiological study, which demonstrated a link between metformin treatment and reduced cancer risk in diabetic patients (Evans et al. 2005), oncology research started to investigate biguanides. It was originally suggested that biguanides exert their anti-cancer activity due to the inhibition of mitochondrial complex I (Birsoy et al. 2014; Owen et al. 2000; Pollak 2014; Wheaton et al. 2014). As introduced above, rotenone, a well-known strong complex I inhibitor, has also shown anti-cancer activity in several studies (Abdo et al. 1988; Cunningham et al. 1995; Isenberg et al. 1997; Yoshitani et al. 2001). However, other toxic effects like its neurotoxicity with a suggested induction of Parkinson's-like disease (Betarbet et al. 2000; Tanner et al. 2011), made it to a questionable candidate for an anti-cancer drug. In this context, the overall aim of this thesis was to investigate and to compare the strong complex I inhibitor rotenone with the suggested complex I inhibitors metformin and phenformin to elucidate potential mechanisms, which render biguanides, especially metformin, apparently less toxic than rotenone. With these investigations, the screening of new compounds in drug development could be supported by defining the required characteristics of an inhibitor of mitochondrial function with a still tolerable safety profile like metformin, but with potentially increased efficacy.

Therefore, the following points and questions were addressed:

1. Compilation of a suitable early preclinical toxicological *in vivo* test battery, which includes all important endpoints to identify MOA-based biomarkers and provide enhanced mechanistic insights into the action of complex I inhibitors
2. Mechanistic investigations of the strong complex I inhibitor rotenone
3. Mechanistic investigations of the apparently weak complex I inhibitors metformin and phenformin
4. Direct comparison of the different complex I inhibitors
5. Elucidation of potential mechanisms, which endows biguanides and future candidates with a potentially tolerable-risk-benefit balance than rotenone

2. Manuscripts

The publications, which emerged from this thesis, are attached below.

In the first publication ‘Mechanistic Investigations of the Mitochondrial Complex I Inhibitor Rotenone in the Context of Pharmacological and Safety Evaluation’ (manuscript I, Heinz et al. 2017) the classical, well known strong mitochondrial complex I inhibitor rotenone was evaluated in a comprehensive test battery. Based on recent indications that the mitochondrial complex I may serve as a site of action for anti-cancer drugs, especially for tumors relying on oxidative metabolism, rotenone was employed as tool compound to increase insight into the mechanism of action of this class of inhibitors with respect to their pharmacological action which at the same time may implicate safety issues. Within this publication a test battery was selected including parameters which have been measured before in rotenone studies by others. These were used to obtain quantitative results in our setting for direct comparison with those measured for the first time in our study. One of the novel test methods for rotenone was gene expression analysis. New mechanistic details for characterization of a toxic profile of a complex I inhibitor were revealed and discussed to find future candidates with a better safety profile.

The second publication ‘Energy metabolism modulation by biguanides in comparison with rotenone in rat liver and heart’ (manuscript II, Heinz et al. 2019) investigated potential MOAs of the biguanides metformin and phenformin, which were compared to the toxic complex I inhibitor rotenone, previously examined in manuscript I (Heinz et al. 2017). Within this publication an adapted test battery based on the experimental design in manuscript I (Heinz et al. 2017) was used. The different profiles of the compounds were critically discussed and potential mechanisms rendering biguanides apparently less toxic than rotenone allowing to define the required properties of anti-cancer drug candidates targeting the inhibition of mitochondrial function, yet with a better risk-benefit balance, were addressed.

2.1 Manuscript I: Mechanistic Investigations of the Mitochondrial Complex I Inhibitor Rotenone in the Context of Pharmacological and Safety Evaluation

Sabrina Heinz[1], Alexius Freyberger[1], Bettina Lawrenz[1], Ludwig Schladt[1], Gabriele Schmuck[1,*], and Heidrun Ellinger-Ziegelbauer[1,*]

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Gene expression data: Available from the Gene Expression Omnibus (GEO) database (accession number GSE86353), https ://www.ncbi.nlm.nih.gov/geo/

Contribution to the manuscript:

I desgined the studies and performed all the experiments with the study samples, except for the processing of the samples for the histopathological examination (pathology department) and the measurement of the clinical chemistry and haematology parameters and liver glycogen (Alexius Freyberger's team). Treatment of animals and daily observations took place with the help of Ludwig Schladt's team. Animal necropsy and sample collection was helped by Elke Hartmann's and Heidrun Ellinger-Ziegelbauer's team. I analyzed and evaluated the data and prepared the manuscript draft. Evaluation of the histopathological samples took place with the help of Bettina Lawrenz.

Paragraphs and/or sentences (in whole or in part) from this manuscript have been reused in this thesis, which is indicated by manuscript I (Heinz et al. 2017).

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Mechanistic Investigations of the Mitochondrial Complex I Inhibitor Rotenone in the Context of Pharmacological and Safety Evaluation

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Inhibitors of the mitochondrial respiratory chain complex I are suggested to exert anti-tumor activity on those tumors relying on oxidative metabolism and are therefore of interest to oncology research. Nevertheless, the safety profile of these inhibitors should be thoroughly assessed. Rotenone, a proven complex I inhibitor, has shown anti-carcinogenic activity in several studies. In this context rotenone was used in this study as a tool compound with the aim to identify suitable biomarker candidates and provide enhanced mechanistic insights into the molecular and cellular effects of complex I inhibitors. Rats were treated with 400 ppm rotenone daily for 1, 3 or 14 consecutive days followed by necropsy. Classical clinical endpoints, including hematology, clinical chemistry and histopathology with supporting investigations (FACS-analysis, enzymatic activity assays) were examined as well as gene expression analysis. Through these investigations, we identified liver, bone marrow and bone as target organs amongst approx. 40 organs evaluated at least histopathologically. Our results suggest blood analysis, bone marrow parameters, assessment of lactate in serum and glycogen in liver, and especially gene expression analysis in liver as useful parameters for an experimental model to help to characterize the profile of complex I inhibitors with respect to a tolerable risk-benefit balance.

Rotenone is a lipophilic, naturally occurring compound, mainly derived from the roots and stems of *Lonchocarpus* and *Derris* species. It had been widely used as pesticide and piscicide, however was then withdrawn from the market in many countries[1] due to its toxicity. Rotenone acts as a strong inhibitor of complex I of the mitochondrial respiratory chain (MRC). The mechanism of action (MOA) comprises inhibition of electron transfer from the iron-sulfur centers in complex I to ubiquinone, leading to a blockade of oxidative phosphorylation with limited synthesis of ATP[2]. Furthermore, incomplete electron transfer to oxygen could lead to the formation of reactive oxygen species (ROS). This rotenone-induced ROS production, with an assumed damage of mitochondria components, including mitochondrial DNA, can eventually lead to apoptosis[3,4]. In addition to the effects on the MRC, several studies have demonstrated that rotenone inhibits microtubule assembly independently of a specific energy-requiring step through tubulin binding, resulting in mitotic arrest and inhibition of cell proliferation[5–7]. Due to these MOAs and the high lipophilicity, enabling rotenone to easily cross biological membranes including the blood–brain barrier[8], rotenone became interesting for Parkinson's disease (PD) research. Defective mitochondrial function, especially decreased complex I activity and increased oxidative stress, has been demonstrated in a subset of patients with PD[9,10]. Due to their important role in neuronal polarity, axonal transport and synaptic plasticity, microtubule dysfunction may also play a role in PD progression[11,12]. Moreover, rotenone exposure has been shown to correlate with the occurrence of several PD-like symptoms in humans[13]. Chronic administration of rotenone has caused selective degeneration of nigral dopaminergic neurons with histopathological hallmarks of PD and PD-like locomotor symptoms in animal models[14]. Therefore, rotenone-based PD models have

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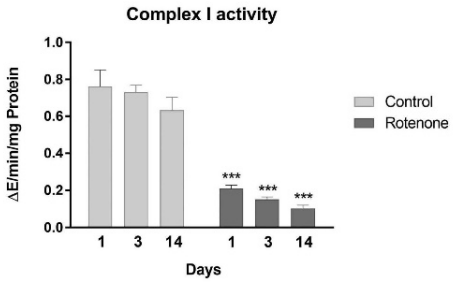


Figure 1. Complex I inhibition. Complex I activity in isolated mitochondria from the livers of control and rotenone treated animals (400 ppm) is shown as mean with SD (n = 5) after the three treatment durations. Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by ***$P < 0.001$ compared to time-matched control groups.

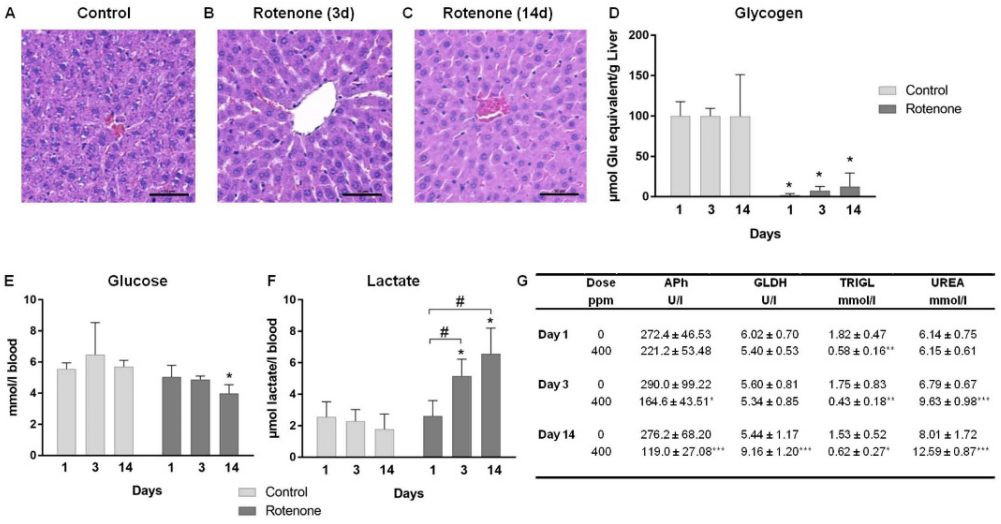
been developed for investigating behavioral syndromes and molecular mechanisms as well as screening novel anti-parkinsonian drugs and diagnostic methods[15]. In addition to rotenone´s neurotoxic effect, it has been suggested that rotenone has anti-carcinogenic activity. Rotenone has been known to induce apoptosis and inhibit cell proliferation of various human cancer cell lines[16–20], to inhibit spontaneously and chemically induced liver tumors in mice[21–23], and chemically induced colon tumors[24] and tongue tumors in rats[25]. Despite these results suggesting a possible anti-tumorigenic activity of rotenone, its neurotoxic effects may exclude its use as an anti-carcinogenic compound. However, other complex I inhibitors such as metformin, an antidiabetic drug, have also been suggested to exert anti-tumor activity on those tumors relying on oxidative metabolism[26]. Accordingly, complex I inhibitors are of interest in oncology research. In this context we performed a systemic study in rats with rotenone as a tool compound measuring classical clinical endpoints, including hematology, clinical chemistry and histopathology, with additional supporting investigations, like FACS-analysis and enzymatic activity assays. Several of these parameters have been measured before in rotenone studies by others. We still included these to obtain quantitative results in our setting for direct comparison with those measured for the first time in our study. The focus was mainly on organs and tissues with high proliferative activity, e.g. the hematopoietic system, and with high metabolic activity, e.g. the liver. Furthermore we performed gene expression analysis to examine additional pathways and functions affected by rotenone at the molecular level. In addition to the liver, the heart and brain stem were chosen for gene expression profiling due to their high energy demand and the known neurotoxic effect of rotenone. By using this experimental design we intended to identify MOA-based biomarkers and provide enhanced mechanistic insights into the action of complex I inhibitors to improve the assessment of compounds in drug development.

Results

General toxicological findings. The treatment of male rats with 400 ppm rotenone through the diet (resulting in a daily intake of 52.5 mg/kg body weight) led to a reduced body weight compared to the control group. After a decrease of around 10% within the first 2 days of treatment, body weight remained constant over the 2 week study. This resulted in a 33% lower weight at terminal necropsy (14d) compared to the weight increasing control group. Correspondingly, absolute organ weights of rotenone exposed animals were significantly reduced compared to control animals, which was especially the case for liver and kidneys. For a complete overview of body and organ weights see Supplementary Fig. S1 and Table S1. Histopathological investigations indicated no changes in kidneys, heart and brain, amongst many other organs evaluated. However, liver, hematopoietic tissue and bone were identified as primary target organs by histopathological and other supporting investigations like gene expression profiling, hematology and FACS-analysis. Detailed findings are described below.

Complex I inhibition. To determine the potency of complex I inhibition by rotenone after the different treatment durations, complex I activity was measured enzymatically in isolated liver mitochondria. Rotenone exposure induced a strong and significant decrease in complex I activity (Fig. 1), which was around 70–80% at every time point compared to the time matched control group (see Supplementary Fig. S2), indicating that the inhibition by rotenone was maintained even after isolation of the mitochondria.

Changes in liver after rotenone treatment. Histopathological findings in the liver (summarized in Table 1) revealed a distinct loss of glycogen in hepatocytes and condensation of the cytoplasm, most noticeably after 1 and 3 days of rotenone treatment (Fig. 2B). In addition, decreased granulation of the rough endoplasmic reticulum was observed, accompanied by substantial alterations in cytoplasmic morphology. These changes were also found in the 14-day treatment group. The histopathologically observed glycogen loss was consistent with an enzymatically determined significant decrease of the glycogen content in the livers of rotenone treated relative to control animals at every time point (Fig. 2D). In addition, triglycerides (TRIGL) were significantly decreased on all measurement days (Fig. 2G), and blood glucose was decreased after two weeks (Fig. 2E), whereas blood lactate concentration was significantly increased in a time-dependent manner (Fig. 2F). Overall, this suggests a hypocaloric status induced by rotenone exposure. Together, the liver-associated alterations summarized above,



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	Dose ppm	APh U/l	GLDH U/l	TRIGL mmol/l	UREA mmol/l
Day 1	0	272.4 ± 46.53	6.02 ± 0.70	1.82 ± 0.47	6.14 ± 0.75
	400	221.2 ± 53.48	5.40 ± 0.53	0.58 ± 0.16**	6.15 ± 0.61
Day 3	0	290.0 ± 99.22	5.60 ± 0.81	1.75 ± 0.83	6.79 ± 0.67
	400	164.6 ± 43.51*	5.34 ± 0.85	0.43 ± 0.18**	9.63 ± 0.98***
Day 14	0	276.2 ± 68.20	5.44 ± 1.17	1.53 ± 0.52	8.01 ± 1.72
	400	119.0 ± 27.08***	9.16 ± 1.20***	0.62 ± 0.27*	12.59 ± 0.87***

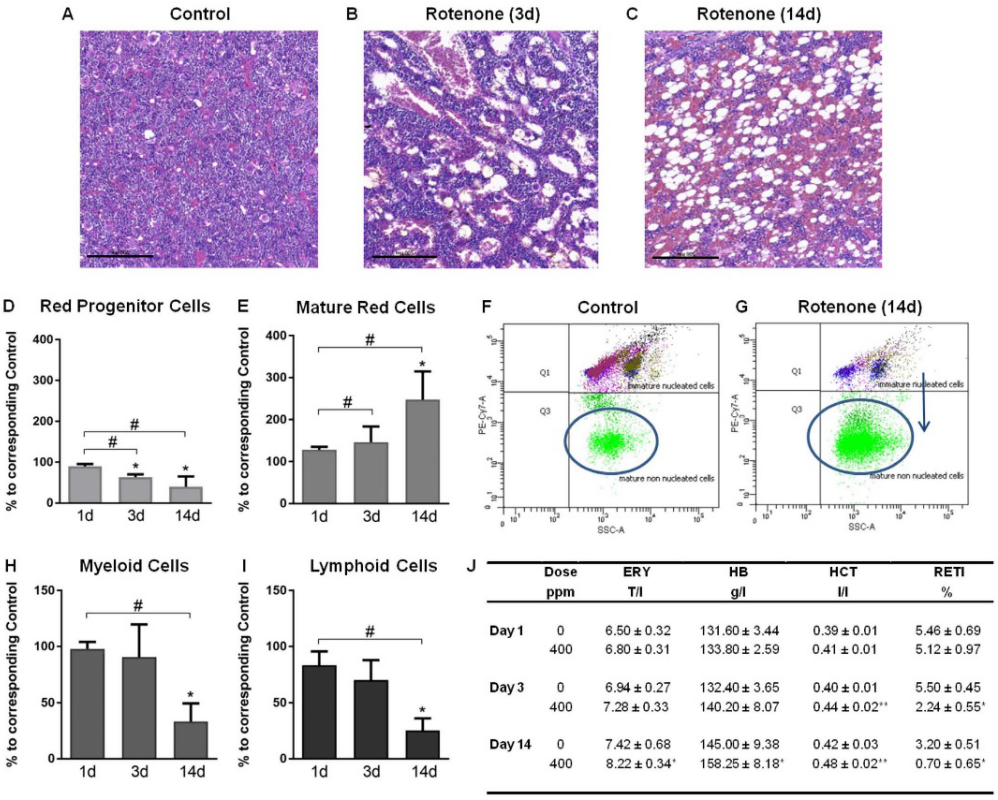
Figure 2. Changes in the liver after rotenone treatment. (**A–C**) Histopathological changes in the liver of rats treated with 400 ppm rotenone for 3 (**B**) and 14 days (**C**) compared to control (**A**). Shown are representative hematoxylin and eosin stained liver sections with a scale bar of 50 µm. (**D**) The enzymatically determined decrease of glycogen content in livers is shown as mean with SD (n = 5) at the three different time-points. (**E**) Decreased blood glucose, (**F**) time dependent increased blood lactate concentrations and (**G**) other significant blood parameters (alkaline phosphatase (APh), glutamate dehydrogenase (GLDH), triglyceride (TRIGL), urea (UREA)) are also presented as mean with SD (n = 5) at the three different time-points. Statistical analysis was performed with Two-Way ANOVA with Sidak multiple comparison test. Statistical significance is indicated by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with time-matched control groups or by $^{\#}P < 0.05$ for the factor "time" of one treatment group.

and increased urea and glutamate dehydrogenase (GLDH) and decreased alkaline phosphatase (APh) levels (Fig. 2G), indicate that rotenone predominantly affects metabolic processes in the liver. For a complete overview of clinical chemistry parameters see Supplementary Table S2.

Effects of rotenone on hematopoietic tissue. Histopathological investigations of the bone marrow in the femur and sternum showed subtle dilation and hyperemia of blood sinuses merely after 1 day of treatment. Bone marrow adipocytes started to increase after 3 days of dosing, indicating bone marrow depletion (Fig. 3B), which further increased by two weeks leading to a spongy atrophy of the marrow (Table 1 and Fig. 3C). One primary target of rotenone seemed to be erythropoiesis. In the spleen, extramedullary erythropoiesis was reduced in all animals after 3 days of treatment (Table 1). After 14 days, it was observed in all treated animals again with moderate severity, but also in three of five control animals with minimal severity. The latter may be related to the age of the animals used here, since extramedullary hematopoiesis declines with increasing age in rats[27]. FACS-analysis of femoral bone marrow indicated a significant increase in the erythroid cell lineage with a shift towards more mature forms over time (Fig. 3D–G). In addition, red progenitor cells, myeloid and lymphoid cells were significantly diminished notably after 14 days of rotenone treatment, confirming general bone marrow depletion (Fig. 3H+I).

The effects of rotenone on hematopoietic cells overall were investigated through the examination of red and white blood parameters and thrombocytes in blood. Significant increases in erythrocyte count, hemoglobin concentration and hematocrit were observed after 14 days in animals treated with rotenone compared to the control group (Fig. 3J). Moreover the number of reticulocytes was significantly decreased after 3 days in rotenone treated rats (Fig. 3H). These findings confirm the histopathological and FACS observations, revealing an effect of rotenone on erythropoiesis. For a complete overview of hematology parameters see Supplementary Table S3.

Effects of rotenone on bone. In the femur and tibia, minor changes in the growth plate were already encountered after 3 days of rotenone treatment. Dilation of subchondral blood sinuses, a slightly reduced height of the physis and reduction of spongiosa formation in the subchondral plate indicated the beginning of growth arrest and atrophy of the growth plate. After 14 days of treatment, a distinct suppression of the proliferative and hypertrophic zone of the cartilage was visible leading to a thin growth plate, cessation of primary enchondral ossification in the subchondral plate and a decrease in subsequent secondary ossification. No regressive changes were noted, and the growth zones rather resembled those in older rats, but with less trabecular stability in the subchondral plate (Fig. 4B). These histopathological findings are summarized in Table 1.



J

	Dose ppm	ERY T/l	HB g/l	HCT l/l	RETI %
Day 1	0	6.50 ± 0.32	131.60 ± 3.44	0.39 ± 0.01	5.46 ± 0.69
	400	6.80 ± 0.31	133.80 ± 2.59	0.41 ± 0.01	5.12 ± 0.97
Day 3	0	6.94 ± 0.27	132.40 ± 3.65	0.40 ± 0.01	5.50 ± 0.45
	400	7.28 ± 0.33	140.20 ± 8.07	0.44 ± 0.02**	2.24 ± 0.55*
Day 14	0	7.42 ± 0.68	145.00 ± 9.38	0.42 ± 0.03	3.20 ± 0.51
	400	8.22 ± 0.34*	158.25 ± 8.18*	0.48 ± 0.02**	0.70 ± 0.65*

Figure 3. Rotenone effects on hematopoietic tissue. (**A–C**) Histopathological changes in the bone marrow of rats treated with 400 ppm rotenone for 3 (**B**) and 14 days (**C**) compared to control (**A**), indicating decreased bone marrow cellularity and an increase in adipocytes after 3 days of treatment (**B**), with increased severity after 14 days (**C**). Shown are representative hematoxylin and eosin stained bone marrow sections with a scale bar of 200 μm. (**D–G**) FACS results of femoral bone marrow, including (**D**) red progenitor cells, (**E**) mature red blood cells, (**H**) myeloid cells and (**I**) lymphoid cells are shown as percent change (mean with SD, n = 5) at the three different time-points relative to the corresponding time-matched control. (**F**,**G**) Representative FACS plots of immature nucleated cells and mature non nucleated cells (blue circle) of a control animal (**F**) and a rotenone treated animal after 14 days (**G**). The blue arrow indicates a shift towards more mature erythroid forms. (**J**) Significantly affected blood cell parameters are presented as mean with SD (n = 5) at the three different time-points. Statistical analysis was performed with Two-Way ANOVA with Sidak multiple comparison test.

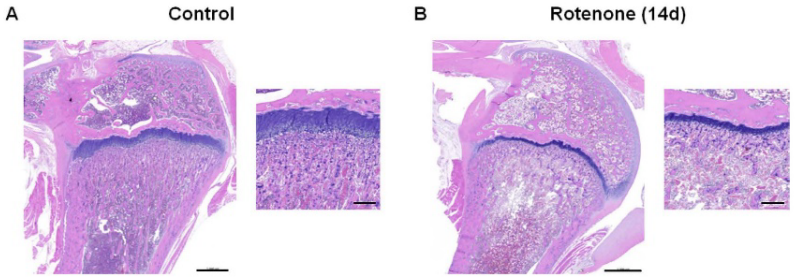


Figure 4. The effect of rotenone on bone. (**A+B**) Histopathological changes in the bone of rats administered with 400 ppm rotenone for 14 days (**B**) compared to control (**A**). Shown are representative hematoxylin and eosin stained bone sections of the femur with a higher magnification of the growth plates (scale bar of 1000 μm and 200 μm, respectively).

Gene expression analysis. Gene Expression Analysis was performed in several organs to examine pathways and functions affected by rotenone at the molecular level. The liver and heart were chosen due to the high metabolic activity and the high energy demand, respectively. For the brain, brainstem was selected as subregion, since it has been shown that Lewy bodies, the hallmark lesions of degenerating neurons in the brains of patients with Parkinson's disease (PD)[28], first appear in the olfactory bulb, medulla oblongata and pontine tegmentum, the letter two belonging to the brain stem[29]. Accordingly, in our short term study we expected gene expression changes rather in the brain stem as compared to other brain regions. After the analysis of the whole transcriptome, the strongest rotenone-induced effects on gene expression were observed in liver (1444 deregulated genes) compared to heart (650 deregulated genes) and brain stem (52 deregulated genes) (Fig. 5A). This finding supports the results reported above, proposing the liver as a primary target organ of rotenone. Only a small number of genes were deregulated in the brain stem after rotenone treatment compared to the liver and heart (Fig. 5A). For interpretation the deregulated genes were assigned to different biochemical categories and subcategories in the context of the main biological functions (a complete overview of genes assigned to such categories is given in Supplementary Table S4). The major functions (Fig. 5B–M) represented by the deregulated genes indicate, in line with the pharmacological action, increased expression levels of genes associated with mitochondrial genesis and the mitochondrial electron transport chain. Particularly genes encoding mitochondrial complex I subunits were up-regulated primarily in the liver (Fig. 5B). An increased expression of genes associated with fatty acid oxidation (Fig. 5C) and decreased expression of genes involved in fatty acid synthesis (Fig. 5D) and cell cycle/proliferation (Fig. 5E) were observed in liver and heart. Concerning the latter, genes encoding mitotic spindle components were down-regulated. Genes encoding cholesterol biosynthesis enzymes were initially down-regulated after 1 and 3 days of rotenone treatment, but were subsequently up-regulated after 14 days (Fig. 5F). However, several genes associated with bile acid synthesis were up-regulated throughout in the liver tissue (Fig. 5G). Genes known to be induced in response to oxidative stress were up-regulated in the liver, heart and brain stem (Fig. 5H). Interestingly, increased mRNA levels of the gene encoding *Sat2* (*spermidine/spermine N1-acetyltransferase family member 2*), involved in degradation of the hypoxia-inducible factor 1-alpha (Hif1α) were observed in the liver at all time points. However, Hif1 target genes and *Hyou1* (*Hypoxia up-regulated 1*), usually induced by hypoxia, were down-regulated (Fig. 5I). A rather unexpected down-regulation of genes encoding glycolysis enzymes in the liver and heart (Fig. 5J) and genes encoding glycogenolysis enzymes and regulators in liver were detected (Fig. 5K). In addition, the expression level of the gene encoding *GAA* (Lysosomal alpha-glucosidase), which is essential for breakdown of glycogen to glucose in lysosomes, was increased in the liver. Furthermore, genes belonging to the insulin pathway were down-regulated in the liver and heart (Fig. 5L). Interestingly genes associated with hematopoiesis and the oxygen carrier hemoglobin were down-regulated after 3 and 14 days of treatment in the liver and heart (Fig. 5M). Another interesting finding was the up-regulation of *Igfbp2* (*insulin-like growth factor binding protein 2*) mRNA after all durations of treatment and the down-regulation of *Igf1* (*insulin-like growth factor 1*) mRNA after 3 and 14 days. These two genes play a role in bone formation. Further implications of the gene deregulations described above will be discussed below.

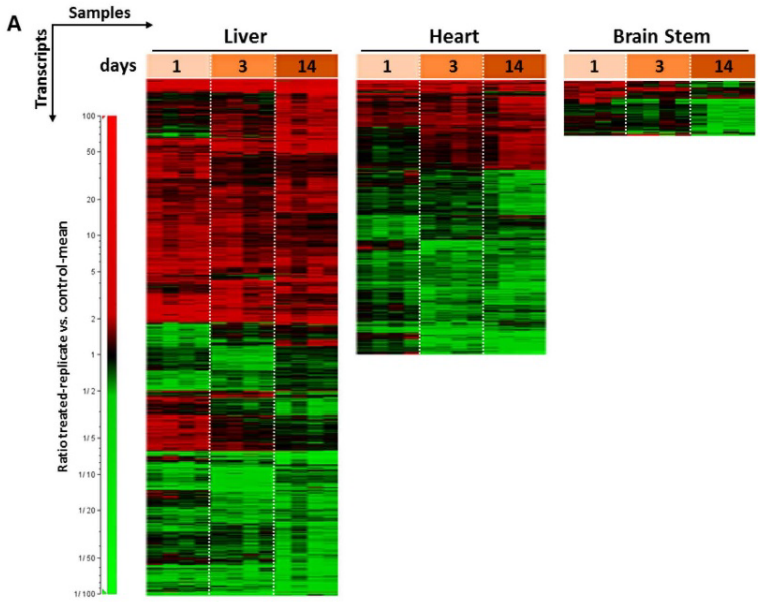
Discussion

Rotenone acts as a strong inhibitor of the mitochondrial complex I. The resulting incomplete electron transfer within the MRC leads to ATP depletion and in turn promotes the formation of ROS and thereby induces oxidative stress and apoptosis in cells[2,3]. Moreover, rotenone can inhibit microtubule assembly through binding to tubulin leading to the inhibition of cell proliferation[7].

In oncology drug research mitochondrial metabolism has recently evolved as a target for cancer therapy, especially for tumors relying on oxidative metabolism[26], with complex I suggested as one possible site of action. Since rotenone has also shown anti-carcinogenic activity in several studies[21–24], the aim of the present study was to identify biomarker candidates and provide enhanced mechanistic insights into the molecular and cellular effects of complex I inhibitors after *in vivo* treatment, using rotenone as a tool compound. Therefore rats were exposed to 0 or 400 ppm rotenone through their diet up to 14 days and various parameters including gene expression profiling were examined. Three target organs, liver, bone marrow and bone, were identified through these investigations.

Rotenone triggers a hypocaloric status. Complex I inhibition by rotenone was confirmed in the liver through measurement of complex I activity in isolated mitochondria after 1, 3 and 14 days of treatment. Gene expression analysis in liver revealed increased expression of mRNAs encoding proteins associated with mitochondrial genesis and the mitochondrial electron transport chain, especially of those encoding complex I subunits, after rotenone treatment. This could be a feedback reaction to complex I inhibition by rotenone, likely leading to reduced mitochondrial energy supply. It is proposed that this reduced supply was partially responsible for the weight loss within the first 2 days and the subsequent constant but not increasing body weight development observed during the 14 day treatment period.

Additionally, in the liver, heart and brain stem a number of genes involved in oxidative stress responses were up-regulated. This was potentially caused by an increase in the formation of ROS upon incomplete electron transfer due to the inhibition of complex I activity by rotenone, as previously described[4]. In parallel, increased expression of the gene encoding *Sat2* (*spermidine/spermine N1-acetyltransferase family member 2*), which plays a role in the degradation of hypoxia-inducible factor 1-alpha (Hif1α), was observed in the liver of rotenone treated rats. In addition, expression of Hif1 target genes e.g. *Lox* (*lysyl oxidase*) and *Loxl2* (*lysyl oxidase-like 2*) and the gene *Hyou1* (*Hypoxia up-regulated 1*), usually induced by hypoxia, was decreased. Hif1 is a heterodimeric transcription factor consisting of two subunits Hif1-α and Hif1-β and regulates cellular responses to low oxygen. Under normoxic conditions, Hif1-α is hydroxylated by oxygen-dependent prolyl hydroxylases (PHD) leading to its degradation via ubiquitination. Under hypoxic conditions, PHDs are inactive, thus Hif1-α becomes stabilized, dimerizes with Hif1-β and can induce transcription of its target genes[30–33]. Although activation of Hif1 in the



B

Electron transport chain - Mitochondrial genesis	Liver	Heart	Brain stem
Day 1	⇧	NDEG	NDEG
Day 3	⇧	NDEG	NDEG
Day 14	⇧	NDEG	NDEG

C

Fatty acid oxidation	Liver	Heart	Brain stem
Day 1	⇧	⇧	NDEG
Day 3	⇧	⇧	NDEG
Day 14	⇧	⇧	NDEG

D

Fatty acid synthesis	Liver	Heart	Brain stem
Day 1	⇩	⇩	NDEG
Day 3	⇩	⇩	NDEG
Day 14	⇩	⇩	NDEG

E

Cell cycle/proliferation - Mitotic spindle/cyclins	Liver	Heart	Brain stem
Day 1	⇩	NDEG	NDEG
Day 3	⇩	⇩	NDEG
Day 14	⇩	⇩	NDEG

F

Cholesterol biosynthesis	Liver	Heart	Brain stem
Day 1	⇩	NDEG	NDEG
Day 3	⇩	NDEG	NDEG
Day 14	⇧	NDEG	NDEG

G

Bile acid synthesis	Liver	Heart	Brain stem
Day 1	⇧	NDEG	NDEG
Day 3	⇧	NDEG	NDEG
Day 14	⇧	NDEG	NDEG

H

Oxidative stress response	Liver	Heart	Brain stem
Day 1	⇧	⇧	⇧
Day 3	⇧	⇧	⇧
Day 14	⇧	⇧	NDEG

I

Hif-pathway	Liver	Heart	Brain stem
Day 1	⇧⇩	NDEG	NDEG
Day 3	⇧⇩	NDEG	NDEG
Day 14	⇧⇩	NDEG	NDEG

J

Glycolysis	Liver	Heart	Brain stem
Day 1	⇩	⇩	NDEG
Day 3	⇩	⇩	NDEG
Day 14	⇩	⇩	NDEG

K

Glycogenolysis	Liver	Heart	Brain stem
Day 1	⇩	NDEG	NDEG
Day 3	⇩	NDEG	NDEG
Day 14	⇩	NDEG	NDEG

L

Insulin-pathway	Liver	Heart	Brain stem
Day 1	⇩	⇩	NDEG
Day 3	⇩	⇩	NDEG
Day 14	⇩	⇩	NDEG

M

Hematopoiesis - Oxygen carrier	Liver	Heart	Brain stem
Day 1	NDEG	NDEG	NDEG
Day 3	⇩	⇩	NDEG
Day 14	⇩	⇩	NDEG

Figure 5. Gene expression analysis. (**A**) Heatmaps represent expression profiles of transcripts significantly affected by rotenone treatment in the liver, heart (left ventricle apex) and brain stem over time. Each column represents a dataset from one organ sample (n = 4), and each row represents a transcript. Changes in gene expression are demonstrated by the color bar to the left of the diagram. Red represents increased and green represents decreased expression levels, indicated as ratios relative to the mean of the time-matched control group. (**B**–**M**) Tables show different affected pathways. Arrows represent the direction of deregulation of genes associated with the respective pathway. NDEG (not found as deregulated by our selected cutoffs) indicates that genes belonging to this pathway were not significantly deregulated in the specific organ and time point indicated.

context of hypoxia is suggested to be induced by mitochondrial ROS formation[34,35], complex I inhibitors, including rotenone, have been known to inhibit the Hif1-pathway even under hypoxic conditions[26,36,37]. This might be explained by reduced O_2 consumption in mitochondria with an increase in cytosolic oxygen levels due to complex I inhibition and subsequent reduced electron transport chain activity. This could result in greater availability of oxygen for PHDs, in turn leading to Hif-1α degradation.

Organ: finding	Treatment duration	Score	Control	Rotenone
Liver: cytoplasmic change/atrophy	1 day	incidence	0	5
		severity	—	3.2
	3 days	incidence	0	5
		severity	—	2.8
	2 weeks	incidence	0	5
		severity	—	3.6
Bone marrow femur: hyperemia/ sinus dilation	1 day	incidence	0	2
		severity	—	0.8
	3 days	incidence	0	5
		severity	—	2.0
	2 weeks	incidence	0	5
		severity	—	3.2
Bone marrow femur: depletion/ increased adipocytes	1 day	incidence	0	1
		severity	—	0.4
	3 days	incidence	0	2
		severity	—	0.6
	2 weeks	incidence	0	5
		severity	—	3.4
Bone marrow femur: atrophy	1 day	incidence	0	0
		severity	—	—
	3 days	incidence	0	0
		severity	—	—
	2 weeks	incidence	0	5
		severity	—	3.0
Spleen: reduced extramedullary hemopoiesis (erythropoiesis)	1 day	incidence	0	0
		severity	—	—
	3 days	incidence	0	5
		severity	—	3.0
	2 weeks	incidence	3	5
		severity	1.2	3.0
Bone, femur: atrophy/arrest growth plate	1 day	incidence	0	0
		severity	—	—
	3 days	incidence	0	4
		severity	—	1.6
	2 weeks	incidence	0	5
		severity	—	3.0

Table 1. Major histopathological findings. Incidence (number of animals; n = 5 per treatment group) of rotenone-caused histopathological changes in rats, after the three different treatment periods. The "severity" score indicates the mean severity from grading 1–5, representing minimal-slight-moderate-marked-massive, respectively.

This apparent reduced energy supply may also be responsible for other findings in liver. Histopathological investigations and direct glycogen measurements indicated early loss of liver glycogen within the first 3 days. Rotenone-treatment induced liver glycogen depletion was observed previously by others[38]. From these observations one may expect increased glycogenolysis leading to the release of glucose into the bloodstream, allowing energy generation through the glycolysis pathway. However, gene expression analysis showed mostly decreased expression of genes encoding proteins participating in glycogenolysis. This could potentially be explained by a negative feedback reaction resulting from the earlier increased degradation of glycogen. An exception to the mostly decreased expression of genes encoding glycogenolysis-associated proteins was an increased hepatic expression of the gene for *GAA* (Lysosomal alpha-glucosidase). *GAA* is essential for the breakdown of glycogen to glucose in lysosomes, suggesting all available resources of glycogen were utilized. Lactate, which is a component in the glycogen-glucose metabolic network, was increased in the blood after rotenone administration. Gene expression deregulation in this context indicated decreased expression of genes coding for proteins involved in the insulin pathway and for glycolytic enzymes in the liver and heart. Downregulation of glycolysis may be explained by feedback regulation to high blood lactate levels to avoid severe lactic acidosis. Blood glucose levels were only slightly reduced after two weeks of treatment presumably due to a tight regulation by the rat to maintain stable blood glucose levels. Further, the rat is able to avoid hypoglycemia through the modulation of metabolic pathways that generate glucose from other resources including the breakdown of glycogen, proteins, and lipids. This suggests the metabolic regulation network is supported by results from other blood parameters including

increased urea and decreased triglyceride levels potentially resulting from increased protein and lipid/fatty acid degradation, respectively. The latter is supported by the increased expression of genes encoding fatty acid oxidation enzymes in the liver and heart. The decreased expression levels of genes coding for proteins involved in cell proliferation and fatty acid synthesis at all time points in liver and heart, may potentially represent a response to reduced mitochondrial energy supply overall, to avoid not acutely required energy consuming processes. This includes e.g. the mRNA for *Srebf1* (*Sterol regulatory element binding transcription factor 1*), which is the major regulator of fatty acid enzyme gene transcription.

With respect to cholesterol biosynthesis, the expression of genes encoding the corresponding enzymes were decreased after 1 and 3 days, but then increased after 14 days of rotenone treatment. However, genes encoding bile acid synthesis enzymes were increased at all time points analyzed. This may be explained by stimulated increased synthesis of bile acids required as emulsifiers for dietary lipids in the intestine, leading to decreased cholesterol levels, in turn activating cholesterol synthesis at the later time point. Supporting evidence for this mRNA levels encoding *Srebf2* (*Sterol regulatory element binding transcription factor 2*), the major regulator of cholesterol synthesis gene transcription, were increased at the 14 day time point. In addition, increased mRNA levels were also seen for the gene encoding the key enzyme of cholesterol biosynthesis *Hmgcr* (*3-hydroxy-3-methylglutaryl-CoA reductase*), and others of this biosynthetic pathway.

Rotenone causes bone marrow atrophy and influences erythropoiesis. Depletion of the bone marrow as observed by histopathological analysis of the femur and sternum, started already after the first day of treatment, leading to marked atrophy after 2 weeks. Furthermore, FACS investigations showed a decrease of red progenitor cells, myeloid and lymphoid cells especially after 14 days of treatment, confirming general bone marrow depletion. Bone marrow atrophy was also observed after 13 weeks of oral rotenone administration of 300 ppm up to 1200 ppm through the diet in the NTP technical report (1988)[39].

Further, rotenone treatment had distinct effects on erythropoiesis, leading to decreased extramedullary erythropoiesis in the spleen, as detected histopathologically. FACS-analysis indicated increased numbers of erythroid cells in femoral bone marrow with a shift towards more mature forms over time. This shift to more mature erythroid stages in the bone marrow was paralleled in peripheral blood by an increase in red blood parameters (Ery, Hb, Hct) at the later time point, and a decrease of the number of reticulocytes, i.e. early immature erythroid cells, already on day 3 after rotenone treatment, in line with and as an indicator for the developing bone marrow depletion. In parallel to reduced numbers of reticulocytes, gene expression analysis showed decreased expression of genes encoding isoforms of the oxygen carrier hemoglobin in the heart and liver tissue. Further, decreased expression of genes involved in hematopoiesis mainly in the heart after 3 and 14 days, was observed. This likely reflects rotenone-induced processes in blood circulating through these organs.

Together, these results suggest a significantly impaired generation of hematopoietic cells. In general, hematopoietic tissue has a strong energy demand due to its high proliferative activity. Therefore mitochondrial complex I inhibition by rotenone leading to decreased energy supply could be a likely reason for bone marrow depletion. The additional inhibitory effect of rotenone on microtubule assembly[5–7] may also be involved in some of the observed effects, leading to mitotic arrest and inhibition of cell proliferation of these rapidly dividing cells. Previously, diminished pyrimidine synthesis was discussed as a potential reason for ineffective hemopoiesis after rotenone treatment[40] with the resulting depletion of nucleotides leading to reduced cell division and proliferation. In this context we observed increased mRNA levels of the gene encoding uridine phosphorylase 2 (*Upp2*) in the liver, which is involved in the salvation pathway of pyrimidine bases for nucleotide synthesis. Although not analyzed by gene expression, such genes may also be deregulated in hematopoietic tissue and may contribute to ineffective hemopoiesis. Furthermore, the assumed inhibition of the Hif1-pathway by rotenone, as described above, could be involved in bone marrow atrophy, as Hif1 is required for maintenance of the hematopoietic stem cell pool. Thus, deletion of Hif-1α promotes the differentiation and exhaustion of hematopoietic stem cells[41].

The acute transient increase in erythrocytes may furthermore be explained as a feedback reaction to eryptosis. This particular form of apoptosis seen only with erythrocytes can be induced by oxidative stress and energy depletion[42], both known effects of rotenone. In addition, treatment of erythrocytes with rotenone *in vitro* leads to eryptosis[43]. However, no increase in bilirubin, a breakdown product of eryptosis, was observed in the blood in the present study. Thus, the significance of eryptosis for our observations remains unclear. Although this potential mechanism has been insufficiently characterized and does not fit completely with all observations made in this study, it can be summarized, that after the onset of treatment, an acute mobilization of red blood cells occurs, followed by the development of severe bone marrow depletion. These events can be explained by both known MOAs of rotenone, i.e. inhibition of complex I activity and of microtubule assembly.

Rotenone treatment leads to bone growth suppression. In the femur and tibia, suppression of bone growth could be observed starting after 3 days of dosing and increasingly over the 2-week study period. The growth arrest became evident in the proliferation and hypertrophic zones of the epiphyseal growth plate. This could be caused by the observed bone marrow atrophy due to the fact that osteoblasts and osteoclasts, both required for bone remodeling, are differentiated from bone marrow cells. Osteoblasts, which are essential for bone reassembly, develop from bone marrow mesenchymal stem cells[44,45]. Additionally, osteoclasts, crucial for bone disassembly, are differentiated from macrophage precursors in the hematopoietic lineage in response to the cytokine Receptor Activator of NF-κB Ligand (RANKL)[46]. In this context, direct inhibition of osteoclast differentiation through down-regulation of RANKL is described for rotenone *in vitro*[47].

Another potential reason for the rotenone-induced bone growth suppression in our study could be an induction of *Igfbp2* (*insulin-like growth factor binding protein 2*), which was detectable in our gene expression data at all three time points, with highest levels after 14 days of treatment., Moreover, *Igf1* (*insulin-like growth factor*

1)-mRNA was down-regulated after 3 and 14 days. It has been shown, that overexpression of *Igfbp2* reduces bone mass in growth hormone transgenic mice[48]. In addition, Jehle *et al.*[49] observed a positive correlation between high serum levels of IGFBP-2 and bone loss in women and men. Furthermore, Amin and colleagues (2004)[50] reported that serum IGFBP-2 levels were inversely associated with bone mass in humans. Conversely, proliferation and differentiation of growth plate osteoblasts and chondrocytes could be stimulated by IGF-I[51–53]. Hence, a reduced bone density could be shown in IGF-I-knockout-mice[54]. Moreover, it has been considered that IGFBP-2 is a negative regulator for IGF-I-induced bone formation[55]. Thus, our observations, i.e. induction of *Igfbp2* mRNA and reduction of *Igf1* mRNA, suggest involvement of these gene deregulations in bone growth suppression. Other than this, the growth arrest in the proliferation zone in our study could also simply be explained with an overall growth reduction as a response to reduced energy storage, e.g. fat and glycogen.

The fibroblast growth factor 21 (FGF21)-pathway may represent a further mechanisms for negative regulation of bone mass, as it has previously been involved in the inhibition of osteoblastogenesis and enhancement of marrow adipogenesis[56]. *FGF21* expression is induced in the liver through PPARα activation[57,58] and in white adipose tissue through PPARγ activation[59–61]. Further, Kim *et al.*[62] demonstrated the ability of rotenone to induce *FGF21 in vitro* due to the stress response associated with ROS production[62]. However, our gene expression analysis in the liver, although exhibiting up-regulation of *PPARα* mRNA after one day of rotenone treatment, did not reveal increased expression of *FGF21* mRNA. The idea that *PPARγ* mediated *FGF21* expression is induced by rotenone within adipose tissue can be neither assumed nor ruled out and would warrant further investigation.

Administration of rotenone through the diet did not lead to obvious neurotoxicity. The high lipophilicity of rotenone enables it to cross all biological membranes easily, including the blood-brain barrier[63]. Nevertheless, histopathological investigations of the whole brain, which was sampled and processed according the STP Position Paper by Bolon *et al.*[64] to identify the different brain regions, including substatia nigra[64], could not detect rotenone-induced neurotoxicity in our study, as previously described for other rotenone studies. Further, gene expression analysis in brain stem identified a small number of genes with increased expression levels. A subset of these genes are known to be involved in an oxidative stress response which may be related to neurotoxicity. This rather weak effect in the brain in our study may be explained by the administration method used. Rotenone has limited bioavailability[65]. It is only incompletely absorbed in the stomach and intestine and is efficiently metabolized in the liver. Thus, high brain concentrations can only be achieved by parenteral administrations and presumably not by the oral route. The absence of extensive neurotoxicity after oral exposure is also consistent with the fact, that rats in the chronic two years study from Marking (1988)[66] did not develop any behavioral disorders or neuropathological effects at doses 30 times greater than in the rotenone-based PD model with a systemic infusion performed by Betarbet and colleagues (2000)[14]. In addition, no adverse effects in the brain were reported in the NTP technical report (1988)[39] after 13 weeks of oral rotenone administration up to 1200 ppm and after two years up to 75 ppm in male F344/N rats. Therefore the administration route reported for rotenone-based PD models is mainly parenteral[15], whereas administrations via the oral route as used in our study usually do not induce clear neurotoxic effects.

General considerations of toxicological compared to potential pharmacologic effects in cancer. In two-year rat NTP studies with up to 75 ppm rotenone survival was not affected, yet 14 day studies with higher doses indicated dose-dependent reduced body weight gain (50–600ppm) and body weight loss starting at 1200 ppm in 14-days studies. Our 14-day rat study with 400ppm suggested metabolic effects which may be related to positive effects in certain cancers (e.g. inhibition of fatty acid synthesis and cell cycle/proliferation), yet were accompanied by toxicological phenotypes including bone marrow atrophy and the overall hypocaloric status of the rats. However, although concentration ranges up to 75 ppm may be tolerated for a life time of the rat, it is unclear whether these doses are sufficient to induce anti-cancer responses. This is supported by observations of usually steep dose-response-curves of mitochondrial electron transport chain inhibitors[67] (and unpublished observations), suggesting that lowering the dose to some extent may already reduce toxicity, but in parallel also decrease potential anti-cancer activity.

Conclusions

In summary, the treatment of rats with rotenone administered through the diet induced an overall hypocaloric status, which was quite obvious in the liver, and characterized by e.g. glycogen depletion. Although this finding in general was already reported for rotenone in previous studies, our gene expression analysis, especially in the liver, adds new mechanistic details for the characterization of this status. In addition, our investigations provide new insights into the effect of rotenone on hematopoietic tissue and bone. Moreover, the lack of neurotoxicity despite the ability of rotenone to easily pass the blood-brain barrier indicates that the hematopoietic tissue and the bone are more sensitive to rotenone than the brain, in case of oral applications. In case complex I inhibitors were administered by routes allowing efficient brain exposure, the brain may be a preferential target, and absence of changes in the biomarkers suggested here would not exclude more sensitive effects in the brain. In these cases potential toxicity to dopaminergic neurons would need to be investigated in more details as described for Parkinson's disease models[68,69]. Although previously reported investigations suggest possible anti-carcinogenic effects of rotenone, the toxic effects described by others and in this study, including bone marrow depletion and bone atrophy, exclude its use as a safe anti-cancer compound. However, other complex I inhibitors such as metformin, a drug developed originally as an antidiabetic, have been suggested to exert anti-tumor activity on tumors relying on oxidative metabolism[26]. The safety profile of such compounds must still be rigorously assessed. Our experimental model with multiple time points, including bone marrow parameters, blood analysis, glycogen

assessment in liver, lactate assessment in serum, and in particular gene expression analysis in liver, in comparison to rotenone and potentially other reference compounds, should help to characterize this profile.

Methods

Animal studies. All animal experiments were performed according to the German guidelines for care and use of laboratory animals and were approved by the State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia in Germany (LANUV). Male RccHan Wistar rats aged 7–8 weeks were randomly assigned to the vehicle or treatment group (n = 5). All animals were housed under controlled standard conditions (12 h light and 12 h dark cycle, at 22 ± 2 °C) and received food and water *ad libitum*. 0 or 400 ppm rotenone (Sigma Aldrich, Steinheim, Germany) was administered through the diet for 1, 3 or 14 consecutive days, followed by necropsy. The diet was prepared by mixing 400 ppm of pure rotenone powder into the chow (V1534–0 ssniff R/M-H) using a mixing granulator (Loedige, Paderborn, Germany). Based on the food intake and body weight data, the rotenone intake was calculated as 52.5 mg/kg body weight per day (Pristima®). The individual animal body weights were determined daily and the food consumption was measured weekly. Clinical examinations of all animals were performed once and inspections on mortality and morbidity twice daily. On the day of necropsy, animals were sacrificed by exsanguination under isoflurane anesthesia. Blood was collected for clinical chemistry and hematology and several organs were removed, weighed, aliquoted, fixed in formalin or flash frozen in liquid nitrogen and stored at −80 °C, described further below.

Preparation of mitochondria. Mitochondria were isolated as described by Fernández-Vizarra and colleagues (2006)[70] with slight modifications. Frozen liver (500 mg) was disrupted in homogenization medium (0.075 M sucrose; 0.225 M mannitol; 1 mM EGTA; 0.01% BSA; adjusted to pH 7.4) using a loosely-fitting potter homogenizer (Potter S, B. Braun Biotech International) at 1500 rpm with 20 strokes up and down. The homogenate was centrifuged at 1000 × g for 10 min at 4 °C. The supernatant was transferred into a fresh tube and centrifuged at 12000 × g for 6 min at 4 °C. The resulting pellet was washed in homogenization medium and re-centrifuged at 12000 × g for 6 min at 4 °C. This pellet containing the mitochondrial fraction was solubilized in MAITE buffer (25 mM sucrose; 75 mM sorbitol; 100 mM KCl; 0.05 mM EDTA; 5 mM $MgCl_2$; 10 mM Tris-HCl; 10 mM H_3PO_4; adjusted to pH 7.4) and centrifuged once more at 12000 × g for 6 min at 4 °C. The final pellet was resuspended in 500 ml MAITE buffer. All steps above were performed on ice. Mitochondrial protein concentration was measured using the Pierce™ BCA Protein Assay Kit (Thermo Scientific, Rockford, USA) with bovine serum albumin as standard. The mitochondria were subsequently snap frozen in liquid nitrogen and stored at −80 °C until further use. All chemicals were purchased from Sigma Aldrich, Steinheim, Germany.

Measurement of complex I activity. Complex I (NADH ubiquinone reductase) activity was measured according to Birch-Machin and colleagues (1989)[71] by recording NADH oxidation by complex I using the coenzyme Q10 analogue decylubiquinone (dUb) as an electron acceptor. 300 µg of mitochondrial protein was mixed with 320 µl reaction-solution (20 mM KH_2PO_4 (pH 7.2), 5 mM $MgCl_2$, 0.65 mg/ml KCN, 25 mg/ml defatted BSA, 0.5 mg/ml antimycin), 4.19 mg/ml dUb and either DMSO or 2.5 mM rotenone in a final volume of 950 µl. After incubation for 2 min the reaction was started by addition of 1.92 mg/ml NADH. Oxidation of NADH was determined by following the decrease in absorbance of NADH at 340 nm for 2 min with a spectrophotometer. Complex I activity was calculated using the difference between the measured rates in the absence (DMSO only) vs. presence of rotenone. All chemicals were purchased from Sigma Aldrich, Steinheim, Germany.

Histopathological analysis. On the day of necropsy several organs and tissues (including liver, heart, kidneys, brain, spleen, femur and sternum with bone marrow) were fixed in 10% buffered formalin, embedded in paraffin (Paraplast®, Carl Roth GmbH & Co. Kg, Karlsruhe, Germany), sectioned (approximately 3 µm) and stained with hematoxylin and eosin (HE). The brain was sampled and processed according the STP Position Paper by Bolon *et al.*[64] and the different brain regions were identified by using the brain atlas of Paxinos and Watson (1997)[72]. All sections were examined by light microscopy and histopathological findings were graded by a semi-quantitative severity scoring system (grade 1 - minimal; grade 2 - slight; grade 3 - moderate; grade 4 - marked; grade 5 - severe). Histopathological findings were entered online into the PathData® software version 6.2c2 (Pathology Data Systems, Inc., Mt. Arlington, NJ, USA).

FACS-analysis. Flow cytometric analysis (FACS) of rat bone marrow was performed as described by Saad *et al.*[73] with slight modifications. Briefly, during necropsy the left femur was removed and trimmed, distal epiphyses were cut off, bone marrow tissue was flushed out with HBSS 1x +Ca/Mg (Hank´s Balanced Salt Solution; Thermo Fisher Scientific, Waltham, USA), suspended and filtered. The adjusted bone marrow cell suspension (1×10^6 cells/tube) was washed with ice-cold phosphate buffered saline (PBS, Alfa Aesar, Ward Hill, USA) containing 0.5% bovine serum albumin (BSA, Sigma Aldrich, Steinheim, Germany)(PBS/BSA) and centrifuged at 1200 rpm for 10 min at 4 °C. Cells were first incubated for 20 min in the dark at 4 °C with 5 µl of fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45 (leucocyte common antigen) and 10 µl of phycoerythrin (PE)-conjugated mouse anti-rat CD71 (transferrin receptor antigen) monoclonal antibodies (Becton Dickinson, Franklin Lakes, USA) and subsequently washed with ice-cold PBS/BSA and centrifuged at 1200 rpm for 5 min at 4 °C. The resulting cell pellet was resuspended in 400 µl ice-cold PBS/BSA. 20 µl of LDS-751 staining solution (a cell-permeate nucleic acid stain; Life Technologies, Carlsbad, USA) was added and tubes were incubated for 30 min in the dark at 4 °C. Cells were subsequently washed with ice-cold PBS/BSA, centrifuged at 1200 rpm for 5 min at 4 °C and resuspended in 400 µl ice-cold PBS/BSA. FACS-analysis was performed with a FACSCanto II flow cytometer and FACSDiva Software (Becton Dickinson, Franklin Lakes, USA) with data collection from 10000 cells. The following bone marrow cell populations were determined: lymphoid, myeloid and nucleated

erythroid cells. In addition to the workflow by Saad *et al.*[73] another parameter was added to discriminate between reticulocytes (CD71-positive cells) and mature erythroid cells (CD71-negative cells).

Glycogen determination in liver. Frozen liver (approximately 1 g) was used for enzymatic determination of the glycogen content using the amyloglucosidase-method described by Keppler and Decker (1974)[74].

Hematology and clinical chemistry analysis. On the day of necropsy blood samples were taken from the Vena jugularis of non-anesthetized animals for glucose and lactate determination. Blood samples for determination of all other parameters were collected during necropsy from the retro-bulbar venous plexus.

Determination of all major hematological parameters was conducted using the Hematology System ADVIA 2120i (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Serum clinical chemistry parameters including alanine aminotransferase (ALAT), aspartate aminotransferase (ASAT), alkaline phosphatase (APh), gamma-glutamyltransferase (gamma-GT), lactate dehydrogenase (LDH), creatine kinase NAC (CK), cholesterol (CHOL), triglycerides (TRIGL), creatinine (CREA), urea, total bilirubin (Bili-t), protein, albumin and glucose from deproteinized whole blood were measured with the ADVIA 2400 Analyzer (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany). Serum glutamate dehydrogenase (GLDH) and lactate from deproteinized whole blood were measured with the Cobas c501 Analyzer (Roche Diagnostics GmbH, Heiligenhaus, Germany).

RNA extraction. Total RNA was isolated from liver (70 mg) using the RNeasy Mini Kit; from heart (left ventricle apex, 30 mg), using the RNeasy Fibrous Tissue Mini Kit and from brain stem (70 mg) using the RNeasy Lipid Tissue Mini Kit, all according to the manufacturer's instructions (Qiagen, Hilden, Germany). RNA quality was determined using the Bioanalyzer® 2100 and RNA 6000 Nano Kits (Agilent, SantaClara, CA) and quantity was assessed using a Nanodrop® 1000 Spectrophotometer.

Gene expression profiling. Starting with 300 ng high quality total RNA from the liver, heart and brain stem of 4 replicate animals per treatment group, biotin labelled cDNA fragments for hybridization on the GeneChip® Rat Transcriptome Array (RTA) 1.0. (Affymetrix; Santa Clara, USA) were prepared according to the manufacturer's instructions. Using the Affymetrix GeneChip Scanner 3000, fluorescent images of the GeneChips were generated. The resulting CEL files were pre-processed by applying the Guanine Cytosine Count Normalization (GCCN) correction and the Signal Space Transformation (SST) algorithm developed by Affymetrix. Subsequently the Robust Multi-array Average (RMA) condensing method was used to obtain a single expression value for each transcript. The RTA 1.0 array covers 194,000 transcripts. Significantly deregulated genes were identified with Genedata® Analyst by using N-Way-ANOVA based on the factors treatment (liver: $P < 0.000001$; heart: $P < 0.00001$; brain stem: $P < 0.001$), time (liver: $P < 0.0001$; heart: $P < 0.0001$; brain stem: $P < 0.001$) and treatment + time (liver: $P < 0.0001$; heart: $P < 0.001$; brain stem: $P < 0.001$), and with a Kruskal-Wallis-test ($P < 0.05$ for all organs) between treated and control groups combined with a 1.8-fold deregulation cut-off.

The significance levels for N-Way-ANOVA were adjusted differently between organs to identify the most dominant changes in gene expression yet to obtain a reasonable number of genes for a detailed analysis. Ingenuity Pathway Analysis (IPA; Qiagen; Hilden, Germany) was used for further investigations.

Statistics. Statistical calculations for data shown in bar diagrams and tables were performed using Graphpad Prism (Vers.6). Statistical significance for the factors time and treatment were determined using a Two-Way ANOVA with the Sidak multiple comparison test. For each time point the data are represented as mean + SD from five replicate animals per treatment group (control; rotenone). Statistics for microarray analysis were performed as described in the '*Gene expression profiling*' section.

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Author Contributions

Experimental design: S.H., L.S., G.S., H.E.-Z.; experiment performance: S.H., A.F., B.L., L.S.; figure design: S.H., B.L., G.S., H.E.-Z.; manuscript writing: S.H., B.L., G.S., H.E.-Z.

Additional Information

Supplementary information accompanies this paper at http://www.nature.com/srep

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SUPPLEMENTARY MATERIAL

Mechanistic Investigations of the Mitochondrial Complex I Inhibitor Rotenone in the Context of Pharmacological and Safety Evaluation

Sabrina Heinz, Alexius Freyberger, Bettina Lawrenz, Ludwig Schladt, Gabriele Schmuck and Heidrun Ellinger-Ziegelbauer

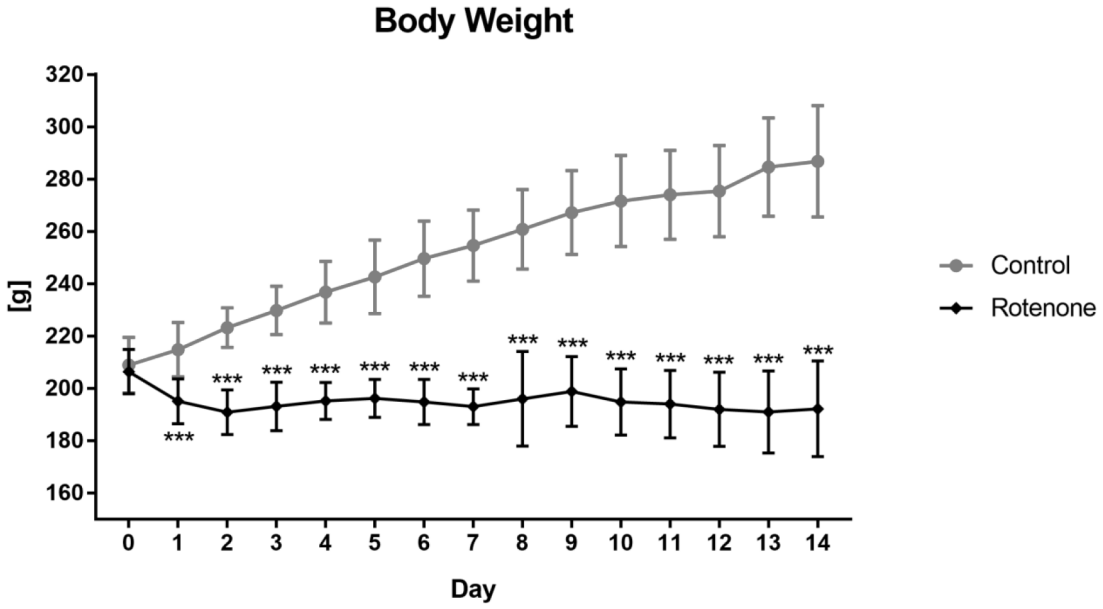

Fig. S1: Body weight after rotenone treatment. Development of body weights of control and rotenone treated animals (400 ppm) is shown as mean with SD (day 0 and 1: n=15; day 2 and 3: n=10; day 4-14: n=5). Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by ***P<.001 compared to time-matched control groups.

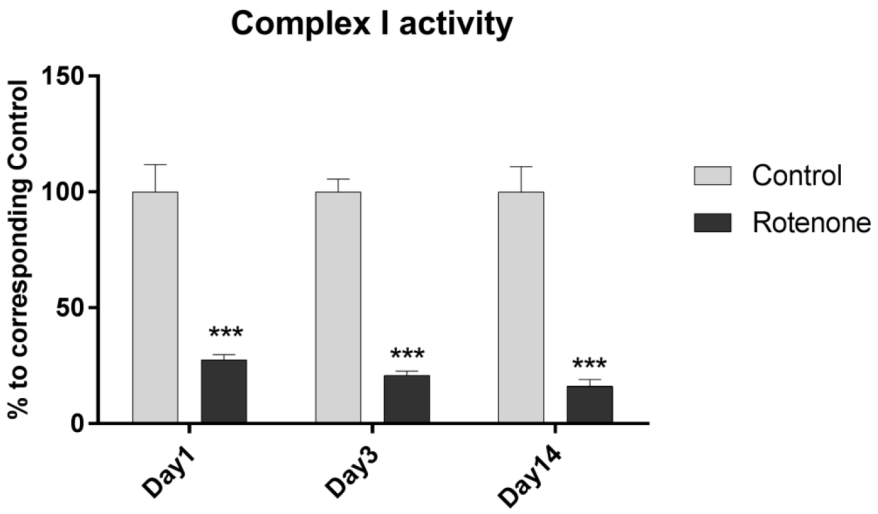

Fig. S2: Complex I inhibition. Complex I activity in isolated mitochondria from livers of control and rotenone treated animals (400 ppm) is shown in % to the corresponding control group with SD (n=5) after the three treatment durations. Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by ***P<.001 compared to time-matched control groups.

Table S1: Absolute and relative organ weights. Mean values with SD (n=5) of absolute and relative organ weights of control and rotenone treated animals after the three treatment durations. Body weight changes in treated animals are shown in % compared to time-matched control groups. Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by ***P<.001 compared to time-matched control groups.

Day	**Day 1**		**Day3**		**Day14**	
Dose [ppm]	**0**	**400**	**0**	**400**	**0**	**400**
Organ		**Rotenone**		**Rotenone**		**Rotenone**
Body weight [g]	211.6±14.5	197.2±1.9 (-7%)	229.2±6.8	190±7.7 (-17%**)	286.8±21.3	192.2±18.3 (-33%**)
Brain						
-absolute [mg]	1771.8±97.5	1818.4±49.5	1796.0±66.5	1805.0±51.0	1903.8±119.3	1827.6±82.7
-relative [g/0.1 kg BW]	0.8406±0.076	0.9222±0.019	0.7837±0.022	0.9504±0.015**	0.6654±0.045	0.9566±0.088**
Adrenal Glands						
-absolute [mg]	56.2±21.2	59.4±13.3	49.6±5.6	51.2±4.7	69.0±6.0	56.6±11.6
-relative [g/0.1 kg BW]	0.0262±0.008	0.0302±0.007	0.0216±0.002	0.0268±0.002*	0.0242±0.001	0.0294±0.004*
Heart						
-absolute [g]	930.0±126.0	1015.8±219.3	1067.4±234.4	956.6±178.6	1156.2±249.1	891.4±131.2
-relative [g/0.1 kg BW]	0.4404±0.062	0.5144±0.106	0.4666±0.106	0.506±0.109	0.4040±0.086	0.4692±0.1
Liver						
-absolute [mg]	9849.6±888.8	7227.2±671.9**	10269.8±728.0	7155.2±527.0**	11432.0±2073.6	7265.6±1074.3**
-relative [g/0.1 kg BW]	4.6521±0.195	3.663±0.314**	4.4792±0.256	3.7682±0.274**	3.9670±0.472	3.77±0.277
Kidneys						
-absolute [mg]	1729.4±156.8	1644.8±110.3	1784.0±69.6	1474.8±31.8**	2070.2±147.9	1457.0±74.9**
-relative [g/0.1 kg BW]	0.8172±0.047	0.834±0.048	0.7784±0.023	0.7772±0.031	0.7228±0.044	0.7632±0.081

Table S2: Overview of clinical chemistry parameters (Alanine Aminotransferase (ALAT); Aspartate Aminotransferase (ASAT); Alkaline Phosphatase (APh); Glutamate Dehydrogenase (GLDH); gamma-Glutamyl Transferase (gamma-GT); Lactate Dehydrogenase (LDH); Creatine Kinase (CK); Glucose; Cholesterol (CHOL); Triglycerides (TRIGL); Creatinine (CREA); Urea; Bilirubin (Bili-t); Lactate; Protein; Albumin). Mean values with SD (n=5) of clinical chemistry parameters of control and rotenone treated animals after the three treatment durations. Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by **P<.01 and ***P<.001 compared to time-matched control groups.

	Dose	ALAT	ASAT	APh	GLDH	gamma-GT	LDH	CK	GLUCOSE
	ppm	U/l	U/l	U/l	U/l	U/l	U/l	U/l	mmol/l
Day1	0	43.40±7.10	108.36±18.17	272.4±46.53	6.02±0.70	0.14±0.22	884.6±219.13	966.8±249.17	5.56±0.38
	400	30.56±2.99**	98.02±14.48	221.2±53.48	5.40±0.53	0.02±0.04	492.6±196.04*	694.6±457.08	5.05±0.74
Day3	0	44.62±3.70	100.98±12.47	290.0±99.22	5.60±0.81	0.52±0.04	547.4±182.79	775.4±232.58	6.48±2.06
	400	29.92±7.38**	105.12±31.81	164.6±43.51	5.34±0.85	0.54±0.19	756.4±382.28	735.8±289.81	4.88±0.22
Day14	0	46.74±5.43	96.44±11.72	276.2±68.20	5.44±1.17	0.56±0.09	766.6±359.58	678.4±226.15	5.71±0.40
	400	39.10±5.05	115.84±17.45	119.0±27.08***	9.16±1.20***	0.52±0.15	1083.2±601.98	1024.8±529.25	3.98±0.57**

Table S2 continued

	Dose	CHOL	TRIGL	CREA	UREA	Bili-t	Lactate	Protein	Albumin
	ppm	mmol/l	mmol/l	mmol/l	mmol/l	µmol/l	mmol/l	mmol/l	µmol/l
Day1	0	1.86±0.32	1.83±0.47	35.8±1.92	6.14±0.75	0.20±0.21	2.57±0.95	54.90±1.66	35.50±1.01
	400	2.23±0.47	0.58±0.16**	35.8±1.30	6.15±0.61	0.30±0.33	2.62±0.97	53.82±1.06	33.76±1.06*
Day3	0	1.84±0.24	1.75±0.83	34.6±1.52	6.79±0.67	0.22±0.11	2.31±0.72	53.22±2.56	34.72±1.51
	400	1.78±0.19	0.43±0.18**	38.0±2.55	9.63±0.98***	0.12±0.08	5.15±1.08**	53.96±0.74	36.02±0.36
Day14	0	1.73±0.33	1.53±0.52	36.4±0.89	8.01±1.72	0.32±0.24	1.78±0.97	56.96±1.31	37.40±0.60
	400	1.38±0.27	0.62±0.27*	37.8±3.11	12.59±0.87***	0.38±0.38	6.57±1.62**	53.64±3.60	36.30±2.32

2.2 Manuscript II: Energy metabolism modulation by biguanides in comparison with rotenone in rat liver and heart

Sabrina Heinz[1,a,*], Alexius Freyberger[1], Bettina Lawrenz[1], Ludwig Schladt[1], Gabriele Schmuck[1,‡], and Heidrun Ellinger-Ziegelbauer[1,‡,*]

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Contribution to the manuscript:

I desgined the studies and performed all the experiments with the study samples, except for the processing of the samples for the histopathological examination (pathology department) and the measurement of the clinical chemistry and haematology parameters (Alexius Freyberger's team). Treatment of animals and daily observations took place with the help of Ludwig Schladt's team. Animal necropsy and sample collection was helped by Elke Hartmann's and Heidrun Ellinger-Ziegelbauer's team. I analyzed and evaluated the data and prepared the manuscript draft. Evaluation of the histopathological samples took place with the help of Bettina Lawrenz.

Paragraphs and/or sentences (in whole or in part) from this manuscript have been reused in this thesis, which is indicated by manuscript II (Heinz et al. 2019).

Energy Metabolism Modulation by Biguanides in Comparison with Rotenone in Rat Liver and Heart

Sabrina Heinz[1,a,*], Alexius Freyberger[1], Bettina Lawrenz[1], Ludwig Schladt[1], Gabriele Schmuck[1,‡], and Heidrun Ellinger-Ziegelbauer[1,‡,*]

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Abstract

The biguanide metformin, a widely used antidiabetic drug, has received great interest in oncology research in recent years after an epidemiological study showed a link between metformin treatment and a reduced cancer risk in diabetic patients. Since mitochondrial metabolism has become a target for possible cancer therapeutic approaches, especially for tumors relying on oxidative metabolism, mitochondrial complex I inhibition is under discussion to be responsible for the anti-cancer effect of metformin. Rotenone, a well-known strong mitochondrial complex I inhibitor, yet associated with toxic effects, has also shown anti-cancer activity. Thus, we compared metformin and phenformin, another biguanide previously on the market as antidiabetic, with rotenone, to elucidate potential mechanisms rendering biguanides apparently less toxic than rotenone. Therefore, we conducted *in vivo* rat studies with metformin and phenformin, based on an experimental design previously described for mechanistic investigations of the effects of rotenone, including blood and tissue analysis, histopathology and gene expression profiling. These investigations show that the mechanistic profile of phenformin appears similar to that of rotenone, yet at a quantitatively reduced level, whereas metformin displays only transient similarities after one day of treatment. A potential reason may be that metformin, but not rotenone or phenformin, self-limits its entry into mitochondria due to its molecular properties. Thus, our detailed molecular characterization of these compounds suggests that inhibition of mitochondrial functions can serve as target for an anti-cancer mode of action, but should be self-limited or balanced to some extent to avoid exhaustion of all energy stores.

Keywords: biguanides; rotenone; mitochondrial metabolism; gene expression; liver; heart

Introduction

The biguanides metformin and phenformin are derivatives of guanidine, which were discovered in the 1920s in extracts of the plant *Galega officinalis* (French lilac). Due to their antihyperglycaemic effect, they have been used as antidiabetic drugs since 1957 (Schäfer 1983). While metformin has an excellent safety profile and is the most commonly prescribed antidiabetic drug for Type II diabetes, phenformin has been withdrawn from many markets in the 1970s for safety reasons, especially for the risk of fatal lactic acidosis with a mortality rate of 30-40% (Williams and Palmer 1975). Although the complete mechanism of action (MOA) of biguanides still remains elusive, the blood glucose-lowering effect associated with increased cellular glucose uptake, suppression of gluconeogenesis, stimulation of glycolysis and fatty acid oxidation as well as improved insulin sensitivity, is generally thought to be induced by an impact on mitochondrial function (Owen et al. 2000). Under discussion are (1) inhibition of mitochondrial complex I activity (Owen et al. 2000), (2) direct or indirect activation of AMP-activated protein kinase (AMPK) (Shaw et al. 2005; Zhang et al. 2016; Zhang et al. 2007; Zhou et al. 2001), or (3) inhibition of the redox-shuttle enzyme mitochondrial glycerophosphate dehydrogenase (mGPD) (Madiraju et al. 2014; Madiraju et al. 2018). With respect to (1), inhibition of complex I activity, metformin is described as a rather mild and transient inhibitor, whereas phenformin shows a more powerful inhibitory effect (Zhou et al. 2001). In general, this inhibition compromises the mitochondrial respiratory chain leading to an increase of the AMP-to-ATP ratio. This in turn leads to (2) activation of AMPK, considered to be responsible for many metformin and phenformin effects, due to its key role in fatty acid metabolism by suppressing fatty acid synthesis and enhancing β-oxidation (Collier et al. 2006; Hawley et al. 2003; Zhang et al. 2007; Zhou et al. 2001), its involvement in enhancing glucose transport and insulin receptor function (Gunton et al. 2003) and its inhibitory effect on gluconeogenesis (Jeon 2016). The metformin- and phenformin-induced increase in AMP concentration in hepatocytes is also described to

inhibit glucagon-induced elevation of cyclic adenosine monophosphate (cAMP) thus reducing activation of protein kinase A (PKA) (Gunton et al. 2003). Therefore, apart from AMPK activation, inhibition of PKA signaling may be partly responsible for biguanide-mediated stimulation of glycolysis and suppression of gluconeogenesis (Gunton et al. 2003; Herzig et al. 2001; Miller et al. 2013; Wang et al. 2012; Yoon et al. 2001). For an illustration of metformin´s influence on these pathways please see Fig. 3 in the review by Pernicova and Korbonits (2014). Recently, it has been demonstrated, that a metformin-mediated AMP increase can also cause inhibition of fructose 1-6-bisphosphatase in the liver of mice, leading to reduced liver glucose production, suggesting a further mechanism for the blood glucose-lowering effect of metformin (Hunter et al. 2018). Besides indirect AMPK activation by an increased AMP-to-ATP ratio, more direct AMPK activation mechanisms are discussed for metformin (Meng et al. 2015; Shaw et al. 2005; Zhang et al. 2016).

As a third possible major MOA of biguanide action, Madiraju and colleagues (2014) demonstrated that inhibition of mGPD could also explain the decreased gluconeogenesis after metformin application. Considering all mentioned possible MOAs, the inhibition of complex I activity appears questionable, since it is observed only at high concentrations especially in the case of metformin (Drahota et al. 2014; Dykens et al. 2008; El-Mir et al. 2000; Owen et al. 2000). Hence, the other mechanism may be more likely to function *in vivo*.

In addition to their antidiabetic activity, metformin and phenformin are under investigation as potential anti-cancer drugs, especially after an epidemiological study described a link between metformin treatment and a reduced cancer risk in diabetic patients (Evans et al. 2005). Since then, multiple laboratory-based studies reported that biguanides can inhibit proliferation and stimulate apoptosis in tumor cell lines *in vitro* (Ashinuma et al. 2012; Caraci et al. 2003; Geoghegan et al. 2017; Jackson et al. 2017; Jin et al. 2017; Kheder et al. 2017; Ko et al. 2016; Lu et al. 2018; Mogavero et al. 2017; Orecchioni et

al. 2015; Rastegar et al. 2018; Wheaton et al. 2014; Xie et al. 2017; Yousef and Tsiani 2017), and can prevent spontaneously and chemically induced tumorigenesis *in vivo* (Anisimov et al. 2005a; Anisimov et al. 2011; Anisimov et al. 2005b; Appleyard et al. 2012; Bojkova et al. 2009; Dilman and Anisimov 1980; Dilman et al. 1978; Jackson et al. 2017; Jia et al. 2015; Ko et al. 2016; Yousef and Tsiani 2017). Furthermore, more than 100 clinical studies are currently ongoing or upcoming for metformin, in order to assess its role in the therapy of cancer (Chae et al. 2016; Kasznicki et al. 2014; MacKay et al. 2017; Zi et al. 2018). Since it was suggested that metformin and phenformin exert their anti-cancer activity due to complex I inhibition (Birsoy et al. 2014; Owen et al. 2000; Pollak 2014; Wheaton et al. 2014), complex I inhibitors became of interest in oncology research, mainly for tumors relying on oxidative metabolism (Schockel et al. 2015). Rotenone, a withdrawn pesticide and piscicide, which is a known strong complex I inhibitor, has also shown anti-cancer activity in several studies (Abdo et al. 1988; Cunningham et al. 1995; Isenberg et al. 1997; Yoshitani et al. 2001). However, the reported neurotoxic effect of rotenone, including the suggested induction of Parkinson's disease (Betarbet et al. 2000; Tanner et al. 2011), makes it to an inappropriate candidate for an anti-cancer drug. In this context we compared metformin, suggested to act anti-tumorigenic via complex I inhibition, with phenformin and rotenone, which are known to inhibit complex I yet with different magnitudes. Our aim was to elucidate potential mechanisms, which render biguanides, especially metformin, apparently less toxic than rotenone. For this purpose, we performed *in vivo* studies in rats with metformin and phenformin, based on an experimental design we previously employed for investigations of the pathway-level effects of rotenone in selected organs with high proliferative (bone marrow) or metabolic activity (liver, heart) (Heinz et al. 2017). This included the analysis of various blood and tissue parameters, histopathology and in particular gene expression profiling in the liver and heart. Based on these investigations, we intended to characterize and compare the profiles of metformin, phenformin and rotenone, with respect to their risk-benefit balance. Our main goal was to define the required characteristic of an

inhibitor of mitochondrial function, or of energy generation via oxidative phosphorylation in mitochondria in general, with a still tolerable safety profile like metformin, but with potentially increased efficacy.

Materials and methods

Animal studies

All animal experiments were performed according to the German guidelines for care and use of laboratory animals and were approved by the State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia in Germany (LANUV, AZ 84-02.04.2012.A345). Male Rcc:Han Wistar rats at the age of 7-8 weeks were randomly assigned to the vehicle or treatment group (n=5). All animals were housed in groups (2 or 3 animals per cage) under controlled standard conditions (12 h light and 12 h dark cycle, at 22 ± 2°C, approx. 55% relative humidity, air change ≥ 10 per hour) and received food (V1534-0 ssniff R/M-H, 10 mm (pellet) by ssniff Spezialdiäten GmbH, D-59494 Soest, Germany) and tap water *ad libitum*. In one study, rats were treated orally once a day by gavage with 0 or 800 mg/kg body weight metformin (Sigma Aldrich, Steinheim, Germany) in Ethanol/Kolliphor HS 15®/Tap Water (10/40/50) with an administration volume of 10 ml/kg body weight, for 1, 3 or 14 consecutive days, followed by necropsy 24 hours after the last administration. In another study, 0 or 3000 ppm phenformin (Sigma Aldrich, Steinheim, Germany) was administered in the diet for 3 or 14 consecutive days, followed by necropsy. The diet was prepared by mixing 3000 ppm of pure phenformin powder into the chow (V1534-0 ssniff R/M-H, 10 mm (pellet) by ssniff Spezialdiäten GmbH, D-59494 Soest, Germany) using a mixing granulator (Loedige, Paderborn, Germany). The phenformin intake (mg/kg body weight) was then calculated based on the food intake and body weight data, delivering about 318.5 mg phenformin/kg body weight per day. In the following, this value is used for a better comparison of both studies. The body weight of every animal was determined daily. Clinical examinations of

all animals were performed once and inspections on mortality and morbidity twice daily. On the day of necropsy, animals were sacrificed by exsanguination under isoflurane anesthesia. Blood was collected for clinical chemistry and hematology and several organs were removed, aliquoted, fixed in formalin or flash frozen in liquid nitrogen and stored at −80 °C, as described further below.

RNA isolation

Total RNA from liver (70 mg) and heart (left ventricle apex, 30 mg) were extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany) and RNeasy Fibrous Tissue Mini Kit (Qiagen, Hilden, Germany), respectively, according to the manufacturer's instructions. RNA quantity was determined by using a Nanodrop® 1000 Spectrophotometer and qualitative RNA analysis was performed with the Bioanalyzer® 2100 and RNA 6000 Nano Kits (Agilent, SantaClara, CA).

Gene expression profiling

300 ng high quality total RNA from the liver and heart of 4 replicate animals per treatment group were used for the preparation of biotin labelled cDNA fragments, which were subsequently hybridized on the GeneChip® Rat Transcriptome Array (RTA) 1.0. (Affymetrix; Santa Clara, USA). All steps were conducted according to the manufacturer's instructions. Further handling, including fluorescent imaging and pre-processing to obtain a single expression value for each transcript, was performed as previously described in Heinz et al. (2017). Using Genedata® Analyst, significantly deregulated genes were identified with N-Way-ANOVA based on the factors treatment (metformin: liver $p \leq 0.0001$ and heart $p \leq 0.001$; phenformin: liver $p \leq 0.0001$ and heart $p \leq 0.001$), time (metformin: liver $p \leq 0.00001$ and heart $p \leq 0.001$; phenformin: liver $p \leq 0.0001$ and heart $p \leq 0.001$) and treatment+time (metformin: liver $p \leq 0.0001$ and heart $p \leq 0.001$; phenformin: liver $p \leq 0.0001$ and heart $p \leq 0.001$), and with a Kruskal-Wallis-test ($p \leq 0.05$) between treated and control groups, combined with a 1.8-fold deregulation

cut-off. The differently adjusted significance levels for the N-Way-ANOVA were used to identify the most dominant changes in gene expression per compound as well as to obtain a reasonable number of genes for a detailed analysis. These data were compared with the rotenone gene expression data from our previous study (Heinz et al., 2017). The gene expression data are available from the Gene Expression Omnibus (GEO) database (accession numbers GSE86353 and GSE122609), https://www.ncbi.nlm.nih.gov/geo/.

Histopathological analysis

Several organs and tissues (including liver, heart, spleen, femur and sternum with bone marrow) were fixed in 10% buffered formalin on the day of necropsy. Tissue samples were embedded in paraffin (Paraplast®, Carl Roth GmbH & Co. Kg, Karlsruhe, Germany), cut into 3 µm sections and stained with hematoxylin and eosin (HE). Sections were examined by light microscopy and histopathological findings were graded by a semi-quantitative severity scoring system (grade 1 - minimal; grade 2 - slight; grade 3 - moderate; grade 4 - marked; grade 5 - severe). All histopathological findings were entered online into the PathData® software version 6.2c2 (PathData is a registered trademark of Pathology Data Systems, Inc., Mt. Arlington, NJ, USA).

FACS-analysis

Flow cytometric analysis (FACS) of rat bone marrow was performed as described by Saad et al. (2000) with slight modifications as previously described in Heinz et al. (2017). In brief, cells from bone marrow of the left femur were examined after staining with fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD45 (leucocyte common antigen, Clone OX-1, Catalog# 554877, 0.5 mg/ml, BD Pharmingen™) and phycoerythrin (PE)-conjugated mouse anti-rat CD71 (transferrin receptor antigen, Clone OX-26, Catalog# 554891, 0.2 mg/ml, BD Pharmingen™) monoclonal antibodies (Becton Dickinson, Franklin Lakes, USA) to determine lymphoid, myeloid and erythroid cell populations. Nuclei were stained with LDS-751 (a cell-permeate nucleic acid stain; Life

Technologies, Carlsbad, USA). FACS-analysis was conducted with a FACSCanto II flow cytometer and FACSDiva Software (Becton Dickinson, Franklin Lakes, USA), collecting data from 10000 cells.

Hematology and clinical chemistry analysis

On the day of necropsy blood samples were either collected from the Vena jugularis of non-anesthetized animals (for lactate measurement) or from the retro-bulbar venous plexus during necropsy (for all other parameters). Serum lactate from deproteinized whole blood was determined with the Cobas c501 Analyzer (Roche Diagnostics GmbH, Heiligenh aus, Germany). All major hematological parameters were measured using the Hematology System ADVIA 2120i (Siemens Healthcare Diagnostics GmbH, Eschborn, Germany).

Statistics

Statistical analyses for the data shown in bar diagrams and tables were conducted using Graphpad Prism (Vers.6). Significance testing was based on a Two-Way ANOVA with the Sidak multiple comparison test for the factors time and treatment. All data are shown as mean +SD from five replicate animals per treatment group (control, metformin, and phenformin, respectively) and for each time point. Statistic calculations for gene expression analysis were performed as depicted in the '*Microarray* g*ene expression profiling'* section.

Results

General study findings

After treatment of male rats with 318.5 mg/kg/day phenformin, an impact on the body weight was seen compared to the control group. Weight loss of around 10% was observed within the first 2 days of treatment. Thereafter the animals gained body weight, but with a retarded development over the 2-week study compared to the control group,

resulting in an 11.4% lower body weight at terminal necropsy (14d). Treatment with 800 mg/kg metformin did not affect body weight development. For a complete overview of body weights, see Supplementary Fig. S1. Histopathological investigations of a standard set of approx. 40 organs suggested liver and hematopoietic tissue as target organs for phenformin, whereas metformin only affected liver, the latter in a different manner compared to phenformin. This profile was supported by investigations of several additional parameters evaluating the functional status of these organs. Furthermore, gene expression profiling suggested potential molecular phenotypes for these differences, also in comparison to rotenone (see below).

Effects on the hematopoetic tissue after phenformin and metformin treatment

In general, no changes in hematopoietic tissue were observed after metformin treatment, whereas the treatment with phenformin led to slight bone marrow depletion in the femur of single rats with a slight sinus hyperemia and adipocyte increase, especially after 14 days of treatment. In addition, in comparison to control animals, splenic extramedullary erythropoiesis was distinctly reduced in all animals already after 3 days of phenformin treatment, likely indicating a compound-related depression (Table 1). After 14 days, splenic extramedullary erythropoiesis was observed in all phenformin-treated animals again with moderate severity, but also in three of five control animals with minimal severity, in comparison to control animals at day 3. The latter could be related to the age of the animals used in our study. It is known, that with increasing age of rats extramedullary hematopoiesis decreases (Losco 1992). To further examine the observed effects of phenformin, we performed FACS analysis and measured red and white blood parameters and thrombocytes as previously reported in Heinz et al. (2017) for rotenone. FACS analysis of bone marrow cells did not reveal any significant alterations in cell numbers (see Supplementary Fig. S2) whereas hematological analysis of blood showed a significant reduction in the number of reticulocytes after 3 days and a

slight decrease after 14 days in phenformin-treated rats (Table 2). Additionally a slight yet significant increase in leucocytes and lymphocytes was observed after 14 days in animals treated with phenformin compared to the control group (Table 2). These blood parameters confirm the histopathological findings, showing a slight effect of phenformin on hematopoiesis. For a complete overview of hematology parameters, see Supplementary Table S1.

Table 1. Major histopathological findings on hematopoietic tissue after phenformin treatment

Organ: finding	Treatment duration	Score	Control	Phenformin
Bone marrow femur: hyperemia/sinus dilation	3 days	incidence	0	2
		severity	-	0.8
	2 weeks	incidence	0	3
		severity	-	1.2
Bone marrow femur: depletion/increased adipocytes	3 days	incidence	0	1
		severity	-	0.4
	2 weeks	incidence	0	4
		severity	-	1.6
Spleen: reduced extramedullary hemopoiesis (erythropoiesis)	3 days	incidence	0	5
		severity	-	3.0
	2 weeks	incidence	3	5
		severity	1.2	3.0

Incidence (total number of animals per treatment group: n=5) of histopathological changes in phenformin-treated and corresponding control rats, after the two different treatment durations. The “severity” score indicates the mean severity from 5 animals each for the following grades: grade 1 – minimal, grade 2 – slight, grade 3 – moderate, grade 4 – marked and grade 5 – severe

Table 2. Significantly affected hematology parameters after phenformin treatment

	Dose ppm	RETI %	LEUCO G/l	LYM G/l
Day3	0	5.50±0.45	6.18±1.62	5.05±1.57
	3000	3.18±0.42***	8.50±1.94	6.91±1.36
Day14	0	3.20±0.51	6.27±1.20	5.15±0.92
	3000	2.70±0.21	8.77±1.08*	7.30±1.08*

Mean values with SD (n=5) for control and phenformin-treated animals after the two treatment durations. The hematology parameters include % of reticulocytes among mature red blood cells (RETI), and the number ($G=10^9$) of leucocytes (LEUCO) and lymphocytes (LYM) per volume blood. Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by *P<0.05 and ***P<0.001 compared to time-matched control groups

Phenformin- and metformin-induced alterations in the liver

After 3 and 14 days of treatment, phenformin induced slight glycogen loss and cell condensation in the liver compared to the control group (Fig. 1B). In contrast, metformin led to increased glycogen storage in hepatocytes up to a moderate level from 3 days of treatment onwards (Fig. 1C), which is compatible with an enhanced glycogen synthesis due to metformin treatment. Blood investigations showed a slight but non-significant increase of blood lactate concentration after 14 days of phenformin treatment, (Fig. 1D), hinting at a potentially developing lactic acidosis, which is a well-known side-effect of phenformin (Williams and Palmer 1975), whereas metformin had no effect. Blood lactate concentrations measured in control animals were constant over time (see Supplementary Fig. S3). Thus glycogen depletion together with indication of increased blood lactate concentrations relative to time-matched controls after phenformin treatment suggest weakly increased anaerobic glycolysis which may be due to systemic inhibition of mitochondrial respiration.

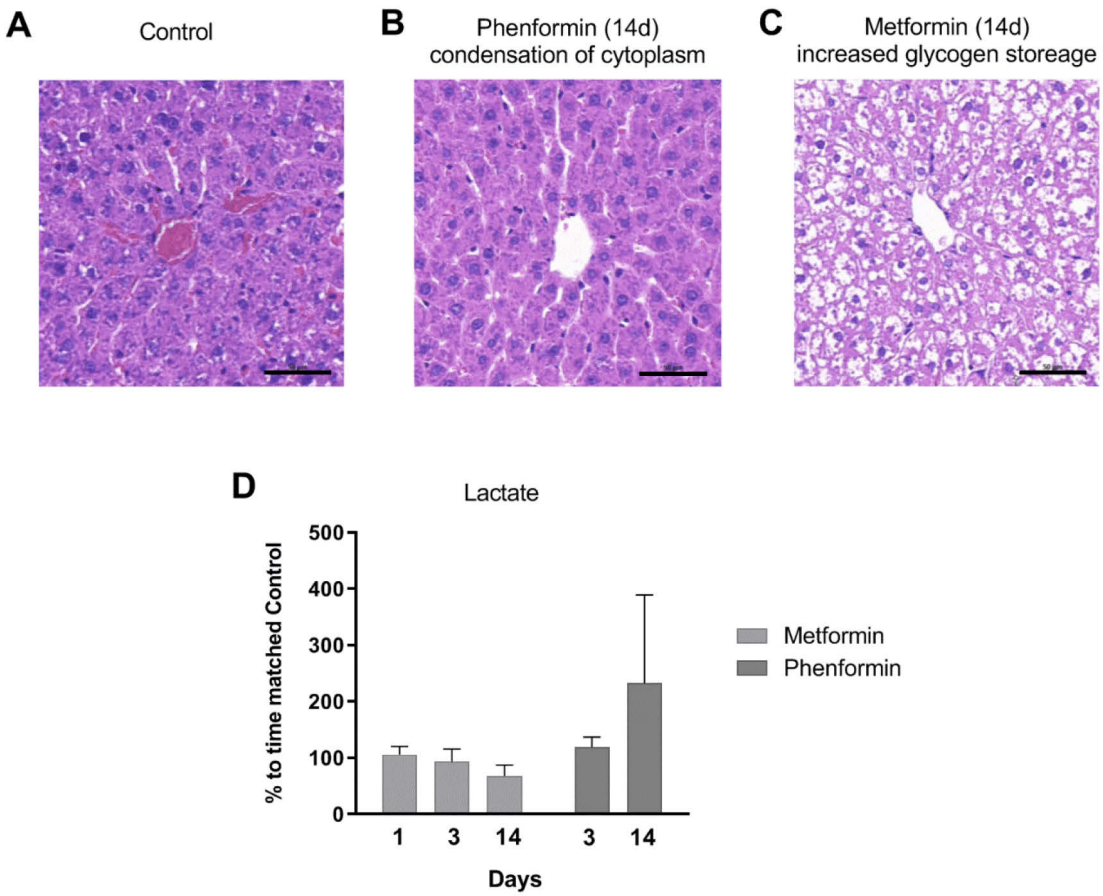

Fig. 1 Liver changes after metformin and phenformin treatment. (A-C) Histopathological changes in the liver of rats treated with (B) phenformin (318.5 mg/kg) or (C) metformin (800 mg/kg) for 14 days compared to (A) control. Shown are representative HE- stained liver sections with a scale bar of 50 µm. (D) Blood lactate concentrations of metformin (800 mg/kg)- and phenformin (318.5 mg/kg)-treated animals are shown as percent of the corresponding time-matched controls (mean with SD, n=5) at the different time-points. Thus 100% equates to no change as compared to the control. A Two-Way ANOVA with Sidak multiple comparison test was performed for the statistical analysis

Gene expression analysis after treatment with biguanides in comparison to rotenone

In order to examine different pathways and functions affected by metformin and phenformin, compared to rotenone at the molecular level, we performed gene expression analysis in liver. This organ was chosen due to its high metabolic activity, but also because it appears to be the main site of action of biguanides. The heatmap (Fig. 2A)

296 represents the expression profile of 1630 transcripts deregulated by rotenone, metformin
297 and/or phenformin. The gene expression profile associated with phenformin appeared
298 qualitatively similar, yet quantitatively weaker compared to rotenone whereas the gene
299 expression deregulations induced by metformin were partly similar to rotenone only on
300 day 1, and then appeared to even change the direction of deregulation for some genes
301 at the later time points. Accordingly, genes associated with fatty acid synthesis and with
302 glycolysis were down-regulated at all time points after treatment with rotenone and
303 phenformin, whereas after metformin treatment the genes were down-regulated only on
304 day 1, but up-regulated on day 3 and 14 (Fig. 2E+G). One known antihyperglycaemic
305 effect of metformin, namely reducing liver glucose release via inhibition of
306 gluconeogenesis (Hundal et al. 2000) could be supported by down-regulation of genes
307 associated with gluconeogenesis after metformin treatment. However, after phenformin
308 treatment genes related to this pathway were up-regulated (Fig. 2B). Furthermore,
309 metformin decreased the expression of genes associated with an oxidative stress
310 response at the latest time point only, whereas after phenformin and rotenone such
311 genes were up-regulated in liver already at earlier time points (Fig. 2D), possibly as
312 reaction to increased reactive oxygen species (ROS) levels upon complex I inhibition by
313 rotenone and phenformin. Potentially as a response to reduced mitochondrial energy
314 supply, increased expression of genes associated with fatty acid oxidation (Fig. 2H) and
315 decreased expression of genes associated with cell cycle/proliferation (Fig. 2I) was
316 observed after metformin, phenformin and rotenone treatment. Increased expression
317 levels of genes associated with the mitochondrial electron transport chain, especially
318 genes encoding complex I subunits (Fig. 2F) were apparent only in rotenone-treated rats.
319 This may be explained as part of a feedback reaction due to the strong direct complex I
320 inhibition by rotenone.

321 We similarly analyzed gene expression profiles in heart as an organ with higher energy
322 needs. The heatmap (Fig. 2B) represents the expression profile of 777 transcripts

323 deregulated by rotenone, metformin and/or phenformin, showing that the heart is less
324 affected by these compounds. As in the liver phenformin led to qualitatively overall similar
325 gene expression deregulations as rotenone, yet at a much lower level with respect to the
326 extent of in- or decreased expression relative to the controls. Especially the pathways
327 fatty acid oxidation (Fig. 2K) and synthesis (Fig. 2L) were similarly affected by rotenone
328 and phenformin (albeit indicating only a trend), with an up and downregulation,
329 respectively, of genes associated to these pathways. In contrast, metformin deregulated
330 the expression of only very few genes in heart with no similarity compared to phenformin
331 or rotenone.

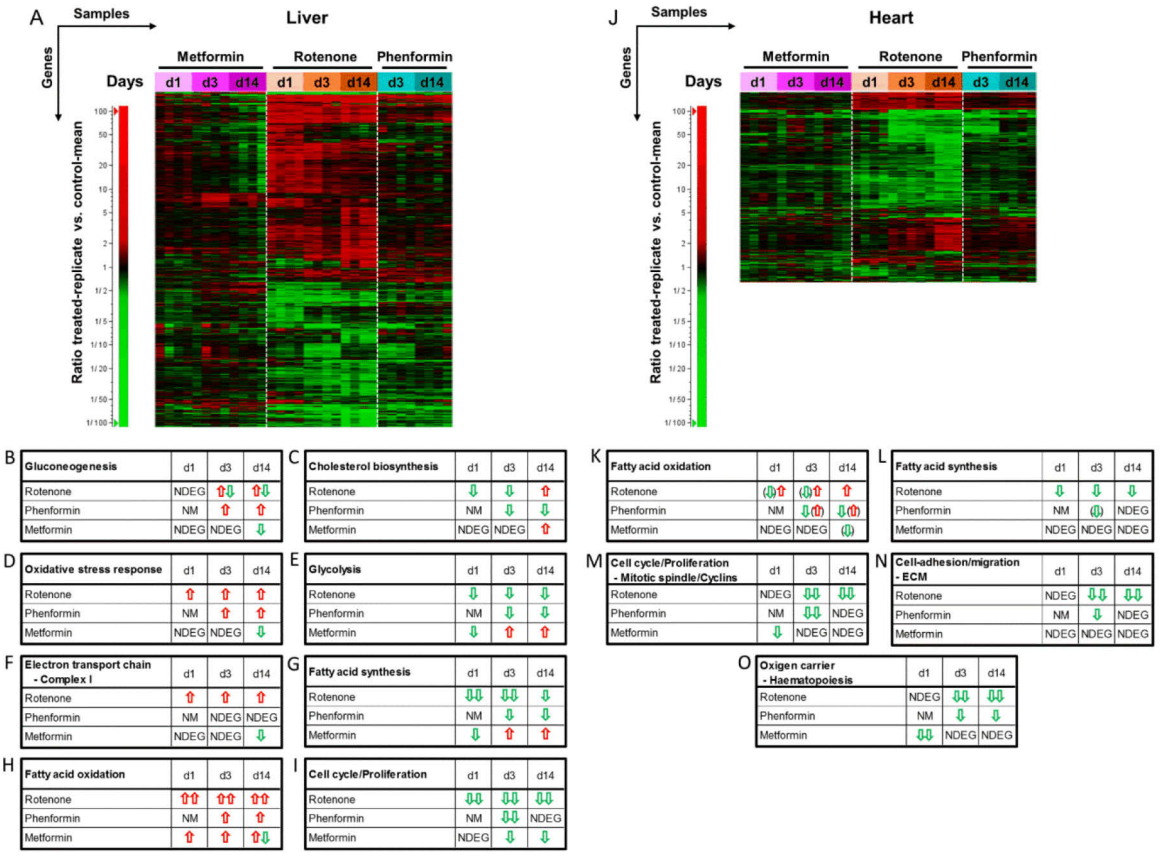

Fig. 2 Gene expression analysis. (A+J) Heatmaps, representing the expression profiles of transcripts significantly affected by metformin, phenformin and/or rotenone treatment in the liver and heart (left ventricle apex) over time. Each row represents a transcript, whereas each column represents a dataset from one organ sample. Samples from 4 different animals per time point and treatment were subjected separately for expression analysis. The color bar to the left of the diagram specifies the gene expression ratios, indicated as ratios relative to the mean of the time-matched control group. Red represents increased and green decreased expression levels. (B-I; K-O) Tables of different pathways affected by the treatment, with arrows demonstrating the direction of deregulation of genes associated with the respective pathway. Arrows in brackets demonstrate genes associated with this pathway, which are not significantly deregulated but showing a tendency by almost reaching the threshold. NDEG (not found as deregulated by our selected cutoffs) indicates that genes belonging to this pathway were clearly not significantly deregulated after the different treatment, in the specific organ and time point indicated. NM (not measured) shows time points, which were not analyzed

Discussion

In recent years, mitochondrial metabolism has evolved as a target for possible cancer therapeutic approaches, especially for tumors relying on oxidative metabolism (Fogal et al. 2010; Weinberg et al. 2010; Wheaton et al. 2014). In this context, the antidiabetic biguanides metformin and phenformin gained great interest after the publication of an epidemiological study, which indicated a link between metformin treatment and a reduced cancer risk in diabetic patients (Evans et al. 2005). Since then, multiple laboratory-based studies demonstrated that biguanides can inhibit tumorigenesis *in vitro* and *in vivo* (Anisimov et al. 2005a; Anisimov et al. 2011; Anisimov et al. 2005b; Appleyard et al. 2012; Ashinuma et al. 2012; Bojkova et al. 2009; Caraci et al. 2003; Dilman and Anisimov 1980; Dilman et al. 1978; Geoghegan et al. 2017; Jackson et al. 2017; Jia et al. 2015; Jin et al. 2017; Kheder et al. 2017; Ko et al. 2016; Lu et al. 2018; Mogavero et al. 2017; Orecchioni et al. 2015; Rastegar et al. 2018; Wheaton et al. 2014; Xie et al. 2017; Yousef and Tsiani 2017). Besides indirect impacts like their general blood glucose lowering effect with a consecutive reduction of insulin levels (Ish-Shalom et al. 1997; Kheirandish et al. 2018; Luengo et al. 2014), inhibition of mitochondrial complex I is discussed as a mechanism by which biguanides may act as anti-cancer agent (Birsoy et al. 2014; Owen et al. 2000; Pollak 2014; Wheaton et al. 2014). Since rotenone, a strong mitochondrial complex I inhibitor yet associated with toxic effects, has also shown anti-cancer activity in several studies (Abdo et al. 1988; Cunningham et al. 1995; Isenberg et al. 1997; Yoshitani et al. 2001), the aim of the present study was to compare the biguanides metformin and phenformin with rotenone, to elucidate potential mechanisms, which render biguanides apparently less toxic than rotenone. These investigations then may reveal approaches to identify drug candidates with a similar safety profile as metformin, but with increased efficacy with respect to anti-cancer activity.

Several previously reported studies confirmed mitochondrial complex I inhibition for both biguanides, whereas others questioned this MOA especially for metformin. Specific

inhibition of complex I by metformin and phenformin was reported especially with *in vitro* studies (Bridges et al. 2014; Brunmair et al. 2004; Detaille et al. 2005; El-Mir et al. 2000; Feldmann et al. 2000; Janzer et al. 2014; Owen et al. 2000; Scotland et al. 2013; Stephenne et al. 2011). Due to these investigations, metformin was described as a mild and transient inhibitor, whereas phenformin was found to exhibit stronger and much clearer inhibitory effects (Bridges et al. 2014; Cameron et al. 2018). In contrast, Algire and colleagues (2015) demonstrated, that neither metformin nor phenformin had an effect on complex I at concentrations up to 300 µM. We investigated potential complex I inhibition in liver mitochondria of rats, isolated after treatment in the studies described here and previously (Heinz et al. 2017). Whereas rotenone (52.5 mg/kg) showed clear effects confirming it as an effective complex I inhibitor and indicating that the assay in principle worked (Heinz et al. 2017), no clear inhibition could be detected with liver mitochondria isolated from metformin (800 mg/kg)- or phenformin (318.5 mg/kg)-treated rats (see Supplementary Fig. S4, method described by Heinz et al. (2017). These findings are in line with the previously reported irreversible inhibition of complex I by rotenone but reversible inhibition by metformin and phenformin (Bridges et al. 2014; Owen et al. 2000; Wheaton et al. 2014). Furthermore, Owen and colleagues (2000) assumed that a significant amount of accumulated metformin in their *in vivo* study got lost during mitochondrial preparation, which could also explain our results. A potential explanation for the positive outcome of several *in vitro* investigations, indicating direct complex I inhibition by metformin and phenformin, could be the used supra-pharmacological extra-cellular or extra-mitochondrial concentrations (Drahota et al. 2014; Dykens et al. 2008; El-Mir et al. 2000; Owen et al. 2000). Such concentrations are unachievable *in vivo* within the therapeutic dose range. Plasma metformin and phenformin levels in patients were reported to be in the micromolar (~10 µM) (Wheaton et al. 2014) and nanomolar range (~200 nM) (Schulz and Schmoldt 2003). However, it is also known that the positive charge of metformin and phenformin at physiological pH leads to accumulation in the mitochondrial matrix to concentrations up to 1000-times

403 greater than in the extracellular environment (Bridges et al. 2014; Owen et al. 2000;
404 Wheaton et al. 2014). This might explain the necessity of apparent supra-
405 pharmacological concentration to cause direct complex I inhibition *in vitro*.
406 Several other findings in the present study support mitochondrial impairment as a
407 possible MOA by which metformin and phenformin exert their anti-diabetic and
408 suggested anti-cancer actions. With regard to the hematopoietic system, phenformin
409 treatment indicated slightly impaired hematopoiesis particularly after 14 days of
410 treatment, due to bone marrow cell substitution by adipocytes as revealed by
411 histopathological analysis of the femur, resulting in decreased reticulocytes and
412 increased leucocytes and lymphocytes counts measured in blood. In comparison, in rats
413 treated with rotenone (400 ppm), depletion of the femur bone marrow started already
414 after the first day of treatment with much greater impact, leading to marked atrophy after
415 2 weeks (Heinz et al. 2017). Bone marrow atrophy was also described after 13 weeks of
416 oral rotenone administration (300 - 1200 ppm) (Abdo 1988). These results suggest that
417 phenformin and rotenone lead to impaired generation of hematopoietic cells, with much
418 stronger effects by rotenone, likely due to inhibition of mitochondrial activity leading to a
419 reduced energy supply to the highly proliferative and thus energy-consuming
420 hematopoietic system. In contrast, no changes in hematopoietic tissue were observed
421 after metformin treatment in this study, potentially contributing to its better tolerance
422 overall.
423 The histopathological findings in the liver in our study are supported by our gene
424 expression results. In general, phenformin showed a qualitatively similar albeit
425 quantitatively weaker gene expression profile after 3 and even 14 days of treatment,
426 compared to the strong complex I inhibitor rotenone. Unlike phenformin, the gene
427 expression pattern induced by metformin showed qualitative, but quantitatively much
428 weaker similarity to that induced by rotenone only after one day of treatment, but then
429 mostly differed after 3 and 14 days of treatment. This suggests a weak mitochondrial
430 impairment after one day of metformin exposure, which was then potentially self-limited

431 due to accumulation in mitochondria as mentioned above. In literature, it is described
432 that this accumulation depends on the plasma and mitochondrial membrane potential
433 and transport processes (Bridges et al. 2014; Shitara et al. 2013; Wang et al. 2003).
434 Metformin is highly hydrophilic and needs transporters to pass plasma or mitochondrial
435 membranes (He and Wondisford 2015). Inside the cell, the positive charge of metformin
436 may lead to mitochondrial membrane potential-dependent accumulation in the
437 mitochondrial matrix (Bridges et al. 2014; Owen et al. 2000; Wheaton et al. 2014). Since
438 metformin requires a robust mitochondrial membrane potential to accumulate inside
439 mitochondria (Wheaton et al. 2014), it is suggested that its accumulation is self-limited,
440 because inhibition of the mitochondrial complex I should simultaneously reduce the
441 mitochondrial membrane potential due to the interdependence of proton pumping and
442 electron transfer (Owen et al. 2000). The subsequent different gene-expression pattern
443 after 3 and 14 days of metformin treatment may be due to the effects of metformin on its
444 other mentioned targets, or indicate an adaption of the rats to a still existing weak
445 inhibition of complex I by metformin, which is not comparable to the effects and
446 associated gene expression changes induced by the stronger complex I inhibition
447 achieved with rotenone or phenformin. Further clarification of the exact MOA would need
448 detailed analyses of the amounts of metformin within mitochondria, e.g. with closely
449 spaced points after metformin addition to energized mitochondria *in vitro*, in conjunction
450 with complex I activity measurements. In case of rotenone, which due to its lipophilicity
451 easily crosses biological membranes (Talpade et al. 2000), a membrane potential-
452 dependent and ensuing self-limited accumulation has not been described. For
453 phenformin, both passive diffusion and transporter-mediated cell entry are suggested
454 (Hawley et al. 2010; Owen et al. 2000; Pollak 2013; Shackelford et al. 2013), thus
455 mitochondrial accumulation may be less self-limited than for metformin.
456 Consistent with the histopathological findings in the liver, reduced release of liver glucose
457 by inhibition of gluconeogenesis, a known effect of metformin (Hundal et al. 2000), is
458 supported by decreased expression of genes associated with gluconeogenesis after

459 metformin treatment. Especially the decreased expression of the gene encoding
460 *phosphoenolpyruvate carboxykinase*, as also shown by Yuan and colleagues (2002), is
461 in line with this assumption. In contrast to the reported suppression of gluconeogenesis
462 by phenformin (Medina et al. 1971; Ogata et al. 1974), our data showed an up-regulation
463 of genes associated with gluconeogenesis in the liver of phenformin- or rotenone-treated
464 rats. This may rather indicate increased glucose synthesis and could represent a
465 feedback response to decreased energy supply. The slight glycogen depletion in the
466 livers of phenformin-treated rats along with the tendency to increased blood lactate
467 concentration suggests a weakly increased anaerobic glycolysis. However, gene
468 expression analysis rather showed decreased expression of genes associated with
469 glycolysis. This may be explained by a feedback regulation to avoid severe lactic acidosis
470 which eventually will set in after continued treatment with phenformin, leading to its well-
471 known side-effect (Williams and Palmer 1975). Qualitatively similar, but even more
472 pronounced biochemical (glycogen, lactate) and gene expression responses were
473 observed with rotenone (Heinz et al. 2017). In comparison, metformin treatment only led
474 to transient down-regulation of genes associated with glycolysis on day 1, followed by
475 sustained up-regulation on day 3 and 14, in line with the antihyperglycaemic effect of
476 metformin. In addition to increased glycolysis, increased conversion of glucose into
477 glycogen has been reported to contribute to blood glucose lowering by metformin
478 (Wiernsperger and Bailey 1999). We similarly observed increased glycogen storage
479 detected by histopathological analysis, starting on day 3 of metformin treatment. In
480 contrast, phenformin rather induced a slight loss of glycogen after 3 and 14 days, as
481 previously described by Tutwiler and Fawthrop (1983). Rotenone (52.5 mg/kg body
482 weight) also led to glycogen loss in livers of rats, already after 1 day of treatment, and
483 with more extensive effects (Heinz et al. 2017). Since glycogen is used in case of
484 increased energy requirement, the decreased glycogen storage is likely a result of
485 mitochondrial impairment induced by phenformin and rotenone.

Increased expression of genes associated with fatty acid oxidation in the liver of metformin-, phenformin- as well as rotenone-treated rats may be related to an enhanced β-oxidation through activation of AMPK more directly by biguanides (Collier et al. 2006; Hawley et al. 2003; Zhang et al. 2007; Zhou et al. 2001), or indirectly by reduced energy supply in case of rotenone.

Our gene expression analysis also suggests differences between metformin compared to rotenone and phenformin with respect to ROS levels. Metformin treatment decreased the expression of genes associated with an oxidative stress response, in line with the previously reported suppression of ROS generation by metformin (Algire et al. 2012; Araujo et al. 2017; Batandier et al. 2006; Wheaton et al. 2014). In contrast, after phenformin and rotenone treatment, genes known to be induced in response to oxidative stress were up-regulated in the liver. This could be explained by increased ROS formation due to complex I inhibition by rotenone (Li et al. 2003) and likely also by phenformin leading to incomplete electron transfer to molecular oxygen (Fato et al. 2009; Miskimins et al. 2014). Besides mitochondrial complex I, several other respiratory chain components are also known to generate ROS. Especially mGPD, which is prone to leak electrons during the transfer of electrons to ubiquinone, appears to be a significant ROS producer. Therefore, the recently reported ability of metformin to inhibit mGPD (Madiraju et al. 2014), may also play a role in the reduction of mitochondrial ROS production. This assumption is supported by Rauchová and colleagues, who show strongly decreased ROS generation in mitochondria by direct inhibition of mGPD (Rauchova et al. 2014). In addition, the resulting limited electron-flow from ubiquinone to complex III, via inhibition of mGPD, may also contribute to reduced ROS formation.

In comparison to liver, gene expression deregulations in heart tissue of animals treated with metformin, phenformin and rotenone were less pronounced. However, as in the liver, phenformin led to qualitatively overall similar gene expression deregulations, yet quantitatively weaker compared to rotenone. Metformin did not lead to clear gene expression deregulations in the heart, and showed no similarity to those induced by

phenformin and rotenone. Thus in case of metformin, the liver appears to be main target organ.

In summary, for phenformin the gene expression alterations and the depletion of glycogen, together with the weakly enhanced blood lactate concentrations indicate a hypocaloric status, yet at a reduced level compared to the strong complex I inhibitor rotenone. This effect could be explained by systemic inhibition of mitochondrial activity due to complex I inhibition based on the overall qualitatively similar, although quantitatively weaker profile compared to rotenone. Nevertheless, other possible MOAs should be taken into account, including an inhibitory effect on mGPD, as already shown for metformin (Madiraju et al. 2014). For a schematic summary, see Supplementary Fig. S5. For metformin our results suggest only a weak impact on mitochondrial function and only after one day of treatment, followed by apparently qualitatively different effects at later time points. Thus, apart from potential complex I inhibition, the anti-cancer effects of metformin may be mediated by suppression of hepatic gluconeogenesis, inhibition of mGPD (Thakur et al. 2018), and/or even other MOAs like the non-mitochondrial activation of AMPK. The latter could explain the reported anti-diabetic effects of metformin and phenformin, but could also play a role in their anti-cancer effects via the LKB1 – AMPK – mTOR pathway (Zhao and Xu 2014). However, despite of whether and how metformin disrupts mitochondrial respiration via any of the targets discussed above, and potentially being self-limited in duration and/or strength, our gene expression analysis reveals molecular details of pathways likely contributing to the anti-cancer effect of metformin. For instance, reducing glucose in the blood by inhibition of gluconeogenesis, as evident from our gene expression analysis, with a simultaneous decrease of insulin, which has mitogenic and prosurvival effects on tumor cells (Ish-Shalom et al. 1997; Kheirandish et al. 2018; Luengo et al. 2014), is one of the known mechanisms for the anti-cancer properties of metformin.

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Compliance with Ethical Standards

Conflict of Interest: The authors declare that they have no conflict of interest.

Ethical approval: All animal experiments were performed according to the German guidelines for care and use of laboratory animals and were approved by the State Agency for Nature, Environment and Consumer Protection North Rhine-Westphalia in Germany (LANUV).

Data availability statement: The gene expression data are available from the Gene Expression Omnibus (GEO) database (accession numbers GSE86353 and GSE122609), https://www.ncbi.nlm.nih.gov/geo/. Further datasets generated or analysed during this study are included in the manuscript and its Supplementary material. Any other raw data generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Supplementary Data

Energy Metabolism Modulation by Biguanides in Comparison with Rotenone in Rat Liver and Heart

Archives of Toxicology

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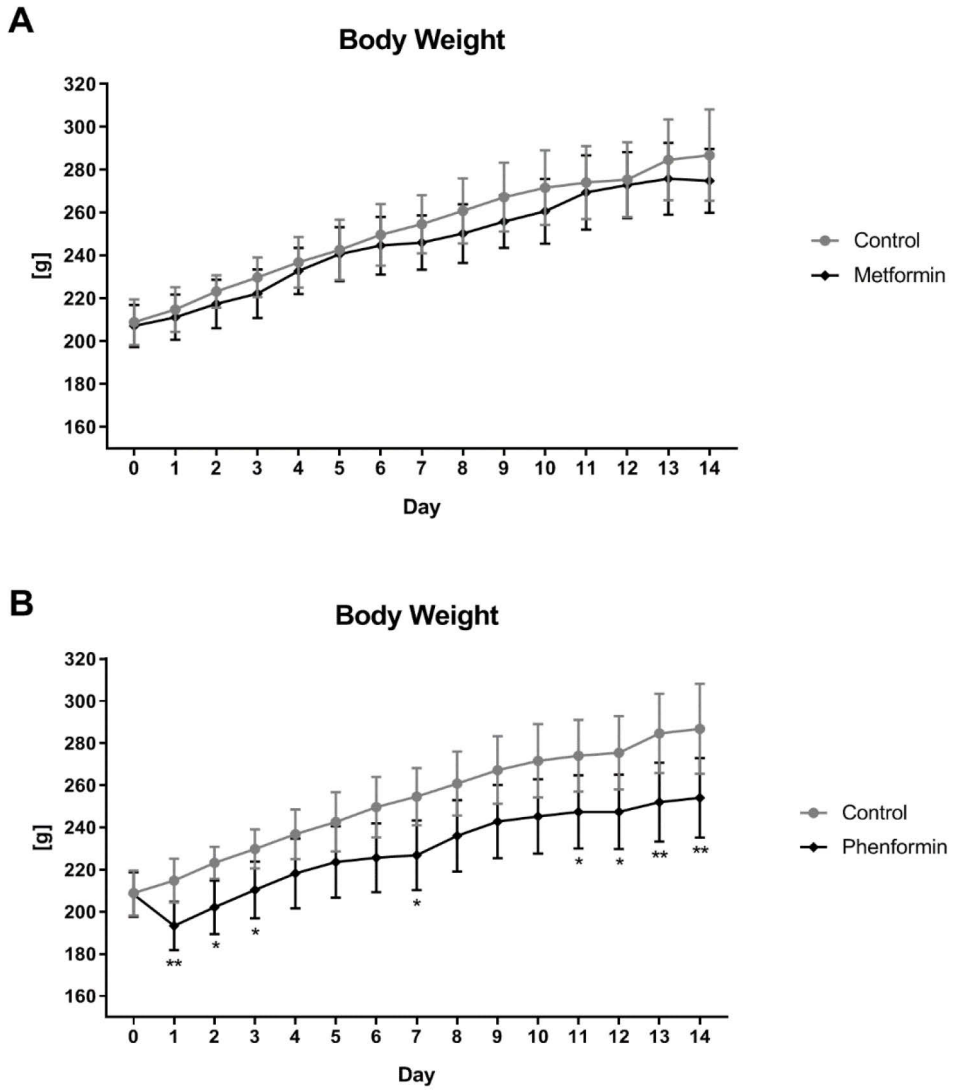

Fig. S1 Body weighst after metformin and phenformin treatment. The development of body weights of control and (A) metformin (800mg/kg) or (B) phenformin (3000 ppm) treated animals are shown as mean with SD (day 0 and 1: n=15 (metformin)/ n=10 (phenformin); day 2 and 3: n=10; day 4-14: n=5). Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by *P<.05 and **P<.01 compared to time-matched control groups

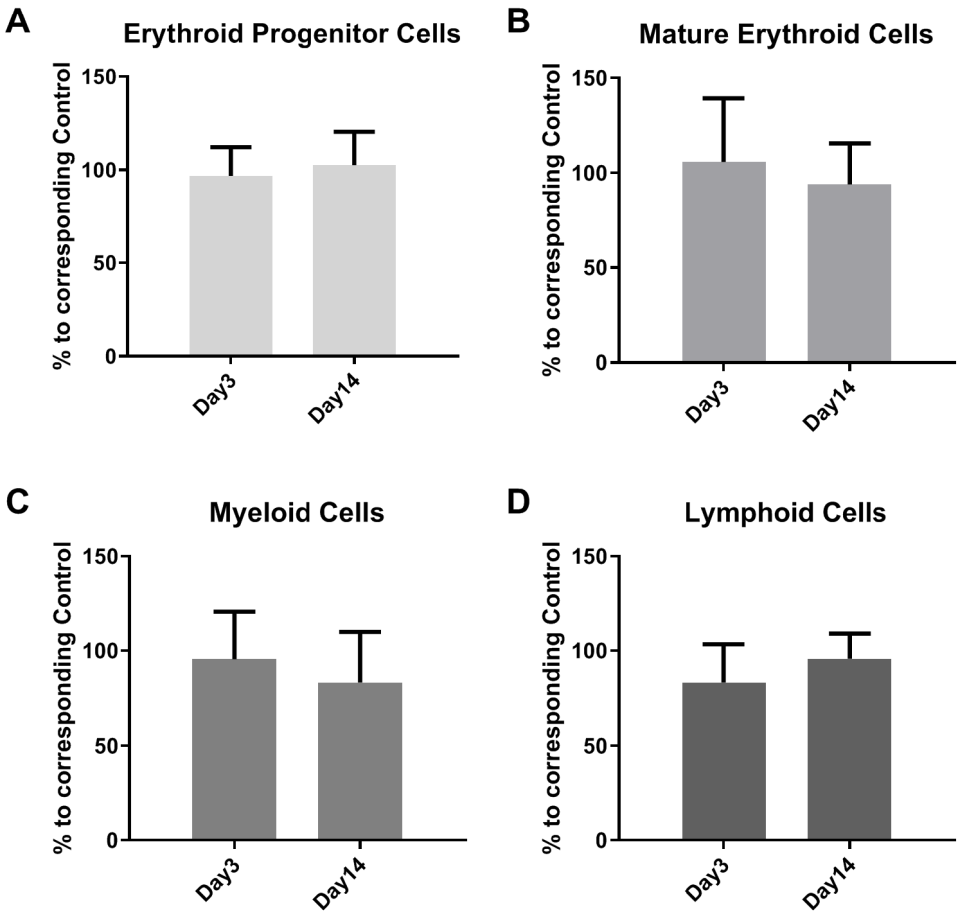

Fig. S2 FACS analysis of femoral bone marrow of phenformin treated rats. (A) erythroid progenitor cells, (B) mature erythroid cells, (C) myeloid cells and (D) lymphoid cells are shown as percent change (mean with SD, n = 5) at the two different time-points relative to the corresponding time-matched control

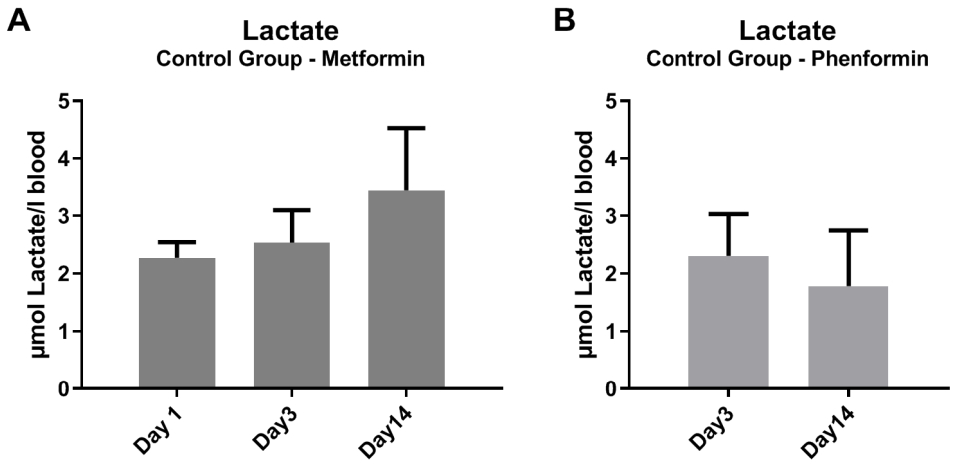

Fig. S3 Blood lactate concentrations of control rats. Blood lactate concentrations are shown as µmol lactate /l blood over time in control animals of (A) metformin study and (B) phenformin study

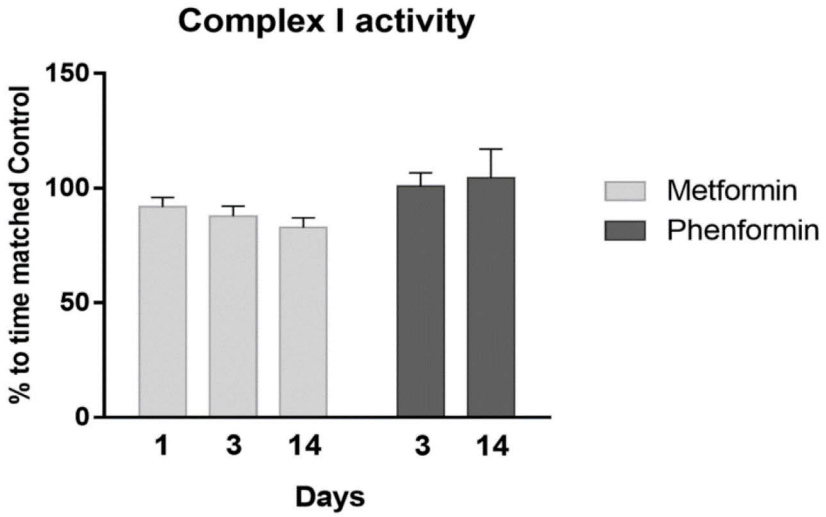

Fig. S4 *In vivo* complex I inhibition. Complex I activity in isolated mitochondria from livers of metformin (800 mg/kg) and phenformin (3000 ppm) treated animals is shown as percent change (mean with SD, n=5) after the different treatment durations relative to the corresponding time-matched control

Table S1 Overview of hematology parameters of phenformin treated rats

	Dose ppm	Ery T/l	Hb g/l	HCT l/l	MCH pg	MCHC g/l Ery	MCV fl	RETI %	THRO G/l
Day 3	0	6.94±0.27	132.4±3.65	0.40±0.01	19.08±0.59	330.6±3.21	57.74±1.31	5.50±0.45	1156.0±41.10
	3000	6.96±0.29	136.5±4.66	0.41±0.01	19.63±0.39	335.3±2.87	58.50±1.68	3.18±0.42***	1093.3±249.77
Day 14	0	7.42±0.68	145.0±9.38	0.42±0.03	19.56±1.01	343.0±6.12	57.02±2.16	3.20±0.51	868.8±266.01
	3000	7.67±0.14	144.6±3.78	0.43±0.01	18.84±0.45	333.6±4.41	56.54±0.56	2.70±0.21	921.2±138.21

	Dose ppm	LEUCO G/l	LYM G/l	NEUTRO G/l	Basophils G/l	EOS %	Mono %	Atypical G/l
Day 3	0	6.18±1.62	5.05±1.57	0.88±0.24	0.02±0.01	0.05±0.02	0.14±0.03	0.05±0.03
	3000	8.50±1.94	6.91±1.36	1.27±0.58	0.03±0.01	0.05±0.01	0.18±0.05	0.06±0.02
Day 14	0	6.27±1.20	5.15±0.92	0.86±0.31	0.02±0.01	0.07±0.05	0.12±0.09	0.04±0.03
	3000	8.77±1.08*	7.30±1.08*	1.15±0.18	0.02±0.01	0.08±0.02	0.15±0.04	0.07±0.02

Mean values with SD (n=5) of hematology parameters of control and phenformin treated animals after the two treatment durations (Erythrocytes (ERY); Hemoglobin (HB); Hematocrit (HCT); Mean Corpuscular Hemoglobin (MCH); Mean Corpuscular Hemoglobin Concentration (MCHC); Mean Cell Volume (MCV); Reticulocytes (RETI); Thrombocytes (THRO); Leucocytes (LEUCO); Lymphocytes (LYM); Neutrophils (NEUTRO); Basophils; Eosinophils (EOS); Monocytes (MONO); Atypical). Statistical significance (Two-Way ANOVA with Sidak multiple comparison test) is indicated by *P<0.05 and ***P<0.001 compared to time-matched control groups

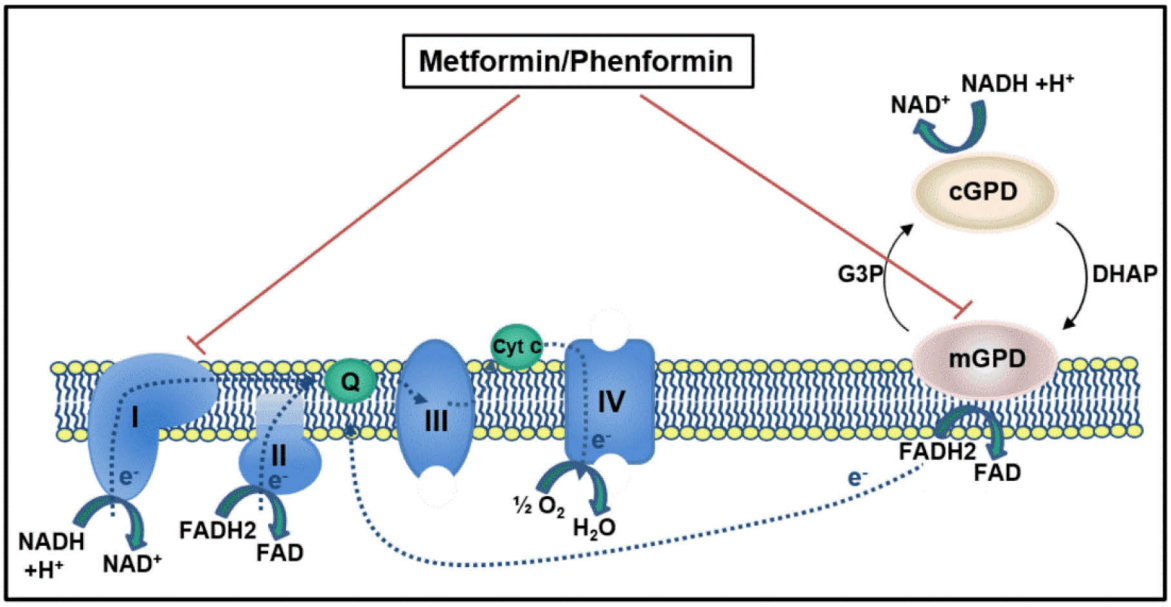

Fig. S5 Schematic illustration of potential inhibition sites of biguanides in mitochondria. Metformin and phenformin are suggested to inhibit mitochondrial complex I of the respiratory chain, leading to impaired oxidation of NADH+H+ to NAD+ (nicotinamide adenine dinucleotide) (Owen et al., 2000). Furthermore, metformin inhibits mitochondrial glycerophosphate dehydrogenase (mGPD) (Madiraju et al., 2014) (not shown for phenformin yet), which prevents the oxidation of FADH2 to FAD (flavin adenine dinucleotide), and thereby the reduction of ubiquinone (Q) and the electron transfer to complex III. In addition, inhibition of mGPD impairs the conversion of glycerol-3-phosphate (G3P) to dihydroxyacetone phosphate (DHAP) and in turn impedes the oxidation of NADH+H+ to NAD+ at the cytosolic glycerophosphate dehydrogenase (cGPD)

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Additional Data

Preparation of heart mitochondria

Collected rat hearts of untreated animals were first washed three times in ice-cold sodium chloride solution, freed from the large vessels and cut into small pieces. The heart muscle tissue was then washed with buffer 1A (25 mM K-PIPES; 0.3 M sucrose; 0.2 M KCl; 0.1 mM EGTA; 0.5 M EDTA and protease inhibitors (Aprotinin, Leupeptin, Pefabloc, Pepstatin A, Antipain); adjusted to pH 7.4) and afterwards homogenized in 6 ml buffer 1A per g heart muscle tissue using an Ultraturax at 1000 rpm with approximately 20 strokes up and down. The homogenate was centrifuged at 2800 rpm for 10 minutes at 4 °C. The supernatant was filtered through 5 layers of mull and centrifuged at 12750xg (10267 rpm) for 20 minutes at 4 °C. The resulting pellet containing the mitochondrial fraction was resuspended in 0.4 ml buffer 2 (200 mM mannit; 50 mM saccharose; 10 mM HEPES; 1 mM EDTA; adjusted to pH 7.3) per g heart muscle tissue. The mitochondrial suspension was sonicated using the UP100H sonifier (Hielscher Ultrasonics GmbH, Germany; cycle: 1, amplitude 90%) for 4 intervals (30 seconds, with a 3 minutes rest period between the intervals). Every step was performed on ice. Mitochondrial protein concentration was measured according to the Bradford protein assay (Bradford 1976). The mitochondrial suspension was then diluted with buffer 2 to 10 mg/ml and subsequently snap frozen in liquid nitrogen and stored at -80 °C until further use. All chemicals were purchased from Sigma Aldrich, Steinheim, Germany.

Measurement of complex I activity in heart mitochondria

For a comparison of the complex I inhibition of the different compounds, the complex I activity in heart mitochondria was measured after the treatment with methanol or substance (rotenone, metformin or phenformin diluted in methanol) with a dilution series from 10^{-11} mol/l to 10^{-5} mol/l. 10 µl of methanol or substance were mixed with 35 µl of cytochrome C solution (100 mg cytochrome C; 15 ml phosphate buffer pH 7.2; 52.6 µl sodium cyanide solution (100 mg sodium cyanide; 1000 µl phosphate buffer pH 7.2)) and 130 µl of NADH solution (1.962 mg NADH; 45 ml phosphate buffer pH 7.2)) per well in a 96-well plate (Nuclon 96 flat-bottom-transparent; Thermo Fisher Scientific, USA) and incubated for 5 minutes at 37°C. Subsequently, 0.85 µg of heart mitochondria diluted in CHAPS/KCl solution (62.5 mg CHAPS; 466 mg potassium chloride (KCl); 25 ml phosphate buffer pH 7.2; 37°C) were added per well. The 96-well plate were than shaken briefly and the complex I activity was measured by recording the NADH oxidation at 550 nm and 37°C in the TECAN Infinite microplate reader (Switzerland). All chemicals were purchased from Sigma Aldrich, Steinheim, Germany, except of CHAPS (Thermo Fisher Scientific, USA) and potassium chloride (Merck, Germany).

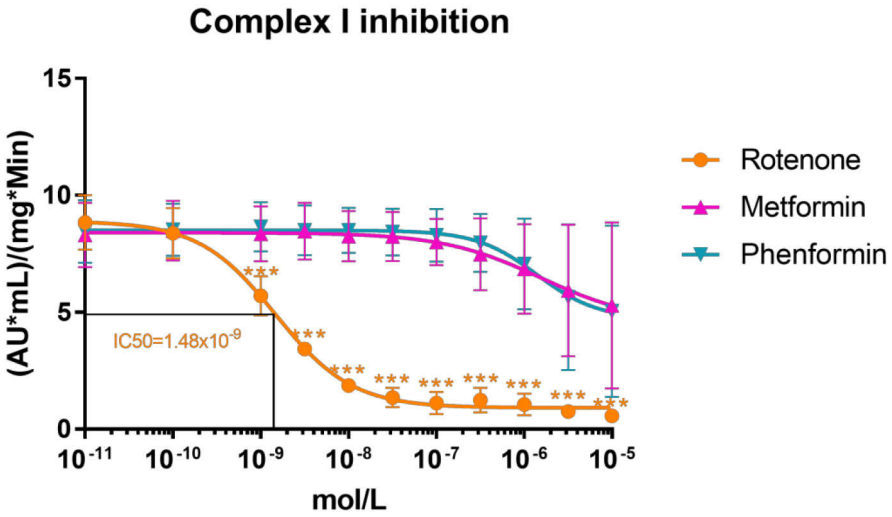

Figure. A *In vitro* complex I inhibition. Complex I activity in isolated rat heart mitochondria, treated with increasing concentrations of rotenone, metformin or phenformin (10^{-11}-10^{-5} mol/L) is shown as mean with SD (n=3). A sigmoidal curve fit was applied and for rotenone an IC_{50} value was calculated. As expected, rotenone induced a strong significant inhibition with an IC_{50} of $1.48x10^{-9}$ mol/L, whereas metformin and also phenformin caused no significant complex I inhibition. The 95% confidence interval for the IC_{50} value is $1x10^{-9}$-$2x10^{-9}$ mol/L. Significant differences (OneWay ANOVA with Sidak multiple comparison test) from the lowest concentration (10^{-11} mol/L) are indicated by ***P<.001.

3. Discussion

The treatment of cancer remains a major challenge due to the complexity of this disease. Therefore, the development of innovative approaches in oncology drug research is essential to overcome current limitations in cancer therapy. Discovering new anticancer drugs with less severe side effects as those chemical agents which were used for chemotherapy in the past, remains as important task. In recent years, targeted therapies gained great interest in oncology research due to their promising potential to treat mainly cancer cells and to minimize the damage to normal, healthy cells. However, there is still a strong demand to gain a better understanding of the cell physiology in cancer and, moreover, enhanced insight on the impact of novel anti-cancer candidates on normal cell physiology. The latter could help to discover or minimize unwanted side effects to allocate new drugs with an improved risk-benefit profile. Thus, investigations on the altered energy metabolism in cancer cells, which has been discovered by Otto Warburg in the early 20th century, became important to detect promising targets in cancer therapy. Despite Otto Warburg's theory that cancer cells only rely on aerobic glycolysis and contain impaired mitochondria, mitochondrial metabolism has evolved as a possible target for cancer therapy, especially for tumors relying on oxidative metabolism (Fogal et al. 2010; Weinberg et al. 2010; Wheaton et al. 2014). In this context, the mitochondrial complex I gained great interest as a possible site of action for anti-cancer drugs (Owen et al. 2000; Pollak 2014; Schockel et al. 2015; Wheaton et al. 2014). Based on an epidemiological study, which demonstrated a link between the treatment with the biguanide metformin and reduced cancer risk in diabetic patients, biguanides came into focus in oncology research (Evans et al. 2005). The effect was attributed to the alleged ability of biguanides to inhibit mitochondrial complex I.

In this thesis the biguanides metformin and phenformin, as well as the classical, rather toxic mitochondrial complex I inhibitor rotenone were molecularly characterized in detail (manuscript I, Heinz et al. 2017 and manuscript II, Heinz et al. 2019). Moreover, these molecular profiles were compared with each other to examine why biguanides are apparently less toxic than rotenone (manuscript II, Heinz et al. 2019). Overall, these investigations may allow to define required properties of anti-cancer drug candidates targeting the inhibition of mitochondrial function, yet with a better risk-benefit balance.

3.1 Compilation of suitable parameters to evaluate complex I inhibitors in preclinical studies

With the strong mitochondrial complex I inhibitor rotenone as a reference compound a suitable test battery was compiled (see Figure 7). In this context, a systemic oral study in rats was conducted with rotenone as well as with the biguanides metformin and phenformin with a

treatment duration of up to two weeks (manuscript I, Heinz et al. 2017 and manuscript II, Heinz et al. 2019). The suggested test battery consists of classical clinical endpoints, like hematology, clinical chemistry and histopathology, with additional supporting investigations, including FACS-analysis and enzymatic activity assays. Since mitochondrial complex I is important to generate energy as a main component in the ETC, the focus was mainly on organs and tissues, which have a high energy demand like e.g. the hematopoietic system needing energy for proliferation, or the liver requiring a considerable amount of energy due to its high metabolic activity (manuscript I, Heinz et al. 2017). To gain more insights into the mechanism of action of complex I inhibitors, gene expression analysis was performed in addition to examine further pathways and functions affected by these compounds at the molecular level. In addition to the liver, the heart was chosen for gene expression profiling, which has also a high energy demand (manuscript I, Heinz et al. 2017 and manuscript II, Heinz et al. 2019). To complete the picture for rotenone, also brain stem was analyzed for this compound by gene expression investigations due to its known neurotoxic effect (manuscript I, Heinz et al. 2017). The whole test battery delivered a comprehensive profile for each compound, enabling a comparative analysis. Especially gene expression analysis revealed interesting new insights into the mechanism of action of the three compounds (manuscript I, Heinz et al. 2017 and manuscript II, Heinz et al. 2019). Based on the results, the study design is suggested to provide relevant information for the characterization of an appropriate drug candidate with a tolerable risk-benefit balance. Results are discussed in detail in the following sections.

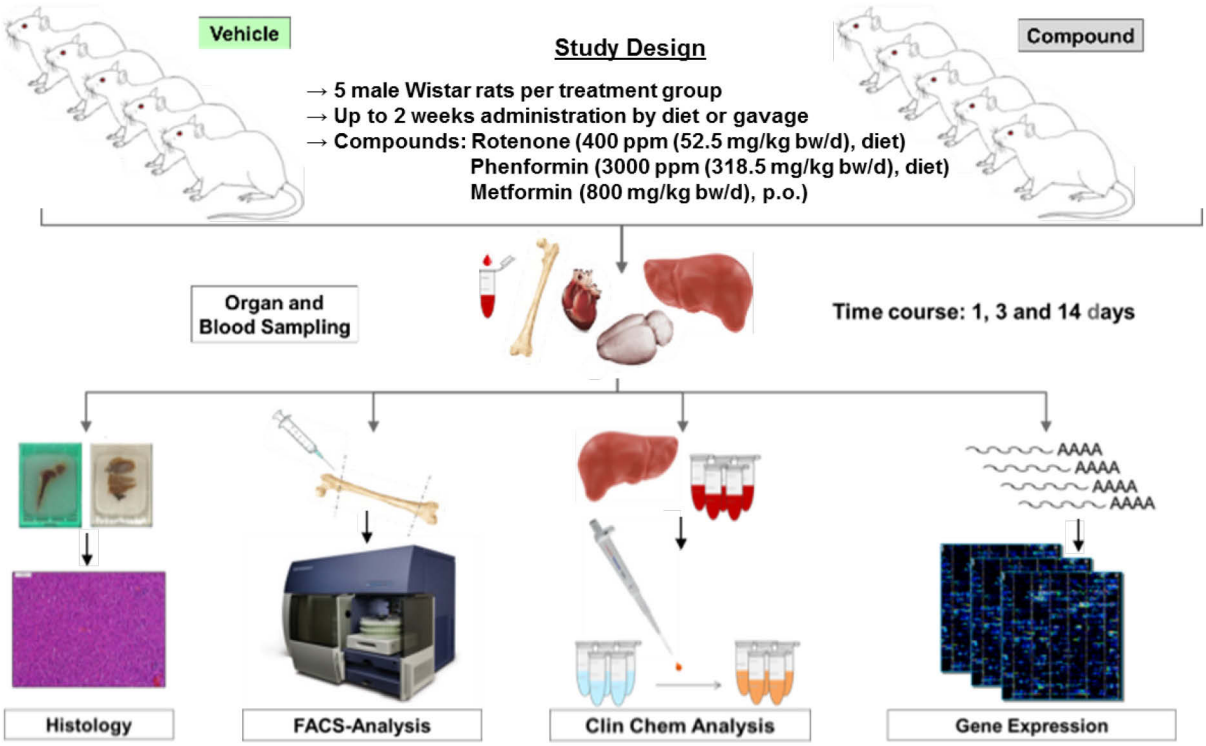

Figure 7: Schematic diagram of the study design and test battery used within this thesis. Male rats (5/group) were treated up to 2 weeks with the three different compounds. Several organs were investigated in a test battery using classical clinical endpoints, like histology and clinical chemistry (Clin Chem) analysis, including hematology and enzymatic activity assays, with additional supporting investigations, including FACS-analysis and gene expression profiling (FACS instrument (FACSCanto II) from Becton Dickinson (Franklin Lakes, USA) shown as example (BD Biosciences 2012))

3.2 Mitochondrial impairment by biguanides vs. rotenone

Rotenone is well known as strong inhibitor of the mitochondrial complex I and was therefore, due to resulting blockage of the respiration, widely used as pesticide and piscicide in many countries. On the other hand, mitochondrial complex I inhibition is under discussion since 2000, to be responsible for the anti-hyperglycemic as well as anti-tumor effects of metformin and phenformin (El-Mir et al. 2000; Owen et al. 2000). As already discussed in manuscript II (Heinz et al. 2019), several studies have apparently confirmed this MOA for both compounds, whereas others questioned such an inhibitory ability especially for metformin. “Specific inhibition of complex I by metformin and phenformin was reported especially with *in vitro* studies (Bridges et al. 2014; Brunmair et al. 2004b; Detaille et al. 2005; El-Mir et al. 2000; Feldmann et al. 2000; Janzer et al. 2014; Owen et al. 2000; Scotland et al. 2013; Stephenne et al. 2011)” (manuscript II, Heinz et al. 2019). Within these investigations, “metformin was described as a mild and transient inhibitor, whereas phenformin was found to exhibit stronger and much clearer inhibitory effects (Zhou et al. 2001; Bridges et al. 2014; Cameron et al. 2018)” (manuscript II, Heinz et al. 2019). In contrast, a study by Algire and colleagues (2015) (Algire et al. 2015)

demonstrated with a succinate-rescue assay in H1299 cells for determination of complex I inhibition, that neither metformin nor phenformin had an effect at concentrations up to 300 µM (manuscript II, Heinz et al. 2019). Moreover, a biochemical bovine complex I assay confirmed an apparent absence of direct Complex I inhibition. To compare the potency of complex I inhibition by metformin and phenformin with rotenone, complex I activity was also directly measured in isolated rat heart mitochondria as part of this thesis. As expected, rotenone caused a strong and significant inhibition with an IC_{50} of 1.48x10-9 mol/l, whereas metformin and phenformin induced no significant complex I inhibition up to a concentration of 10-5 mol/l (10 µM) (see Figure. A). However, these concentrations were even lower as the concentrations tested by Algire and colleagues (2015) (Algire et al. 2015). Potential complex I inhibition was also investigated in liver mitochondria of rats, which were isolated after treatment with the different compounds in the *in vivo* studies described in manuscript I and II (Heinz et al. 2017; Heinz et al. 2019). “Whereas rotenone (52.5 mg/kg) showed clear effects confirming it as an effective complex I inhibitor and indicating that the assay in principle worked (Heinz et al. 2017), no obvious inhibition could be detected with liver mitochondria isolated from metformin (800 mg/kg)- or phenformin (318.5 mg/kg)-treated rats. These findings are in line with the previously reported irreversible inhibition of complex I by rotenone but reversible inhibition by metformin and phenformin (Bridges et al. 2014; Owen et al. 2000; Wheaton et al. 2014)” (manuscript II, Heinz et al. 2019). It might also be the case that a significant amount of metformin got lost during the preparation of mitochondria (manuscript II, Heinz et al. 2019). Owen and colleagues (2000) already mentioned this in their own study. This assumption would also support our data (manuscript II, Heinz et al. 2019).

In the liver, gene expression analysis “revealed increased expression of mRNAs encoding proteins associated with mitochondrial genesis and the mitochondrial electron transport chain, especially of those encoding complex I subunits, after rotenone treatment” (manuscript I, Heinz et al. 2017). This could be explained by “a feedback reaction to complex I inhibition by rotenone, likely leading to reduced mitochondrial energy supply” (manuscript I, Heinz et al. 2017). For phenformin the expression of some of these genes encoding complex I subunits were also weakly increased, yet not statistically significant (1.4 to 1.7-fold deregulation compared to control), whereas for metformin no response was observed (manuscript II, Heinz et al. 2019). These results are in line with the in literature shown stronger inhibitory effects of phenformin compared to metformin (Zhou et al. 2001; Bridges et al. 2014; Cameron et al. 2018). The reduced energy supply, which can be inferred from the complex I activity measurement and the gene expression data, could be partially responsible for the weight loss of the rats within the first 2 days and the subsequent constant but not increasing body weight development observed during the 14-day treatment period for rotenone as well as for phenformin (manuscript I, Heinz et al. 2017 and manuscript II, Heinz et al. 2019). Metformin

treatment did not affect body weight development in rats, which is in line with the gene expression and enzyme activity outcome (manuscript II, Heinz et al. 2019).

As discussed in manuscript II (Heinz et al. 2019), a potential reason for the positive outcome of several previously reported *in vitro* studies, which indicated direct complex I inhibition by metformin and phenformin, could be the use of supra-pharmacological extra-cellular or extra-mitochondrial concentrations (Drahota et al. 2014; Dykens et al. 2008; El-Mir et al. 2000; Owen et al. 2000). “Such concentrations are unachievable *in vivo* within the therapeutic dose range. Plasma metformin and phenformin levels in patients were reported to be in the micromolar (~10 µM) (Wheaton et al. 2014) and nanomolar range (~200 nM) (Schulz and Schmoldt 2003)” (manuscript II, Heinz et al. 2019), respectively. Explanations for the necessity of the apparent supra-pharmacological concentrations *in vitro* for detection of direct complex I inhibition, are based on a requirement for accumulation of biguanides in the mitochondrial matrix (manuscript II, Heinz et al. 2019). Metformin and phenformin can accumulate up to 1000-times higher in the mitochondrial matrix compared to the extracellular environment. This can be explained by the positive charge of both components at physiological pH (Bridges et al. 2014; Owen et al. 2000; Wheaton et al. 2014; manuscript II, Heinz et al. 2019). According to this, the plasma concentrations mentioned above would correspond to mitochondrial concentrations of metformin and phenformin of 10 mM and 200 µM, respectively (Bridges et al. 2014; Owen et al. 2000). This accumulation depends on the plasma and mitochondrial membrane potential and transport processes (Bridges et al. 2014; Shitara et al. 2013; Wang et al. 2003; manuscript II, Heinz et al. 2019). While rotenone is able to cross all biological membranes easily due to its high lipophilicity (Talpade et al. 2000), metformin is highly hydrophilic and thus cannot pass the plasma or mitochondrial membrane by passive diffusion (manuscript II, Heinz et al. 2019). The organic cation transporters OCT1, 2 and 3 as well as the multidrug and toxin extrusion proteins (MATE1–2) are described as the main transporters of metformin in the plasma membrane, (He and Wondisford 2015). “For phenformin, both passive diffusion and transporter-mediated cell entry are suggested (Hawley et al. 2010; Owen et al. 2000; Pollak 2013; Shackelford et al. 2013)” (manuscript II, Heinz et al. 2019). Inside the cell, metformin´s positive charge may lead to mitochondrial membrane potential-dependent accumulation inside mitochondria (Bridges et al. 2014; Owen et al. 2000; Wheaton et al. 2014; manuscript II, Heinz et al. 2019)). Since metformin requires a robust mitochondrial membrane potential to accumulate in the mitochondrial matrix (Wheaton et al. 2014), it is suggested that this accumulation is self-limited, because inhibition of the mitochondrial complex I by metformin should simultaneously reduce the mitochondrial membrane potential due to the interdependence of proton pumping and electron transfer (Owen et al. 2000, manuscript II, Heinz et al. 2019)). For rotenone, which easily crosses biological membranes due to its lipophilicity, a membrane potential-dependent and ensuing self-limited accumulation has not

been described (manuscript II, Heinz et al. 2019). Moreover, for phenformin with similarity to both metformin and rotenone, mitochondrial accumulation may be less self-limited than for metformin (manuscript II, Heinz et al. 2019). Taken together, the possible ability of metformin to self-restrict its accumulation in mitochondria compared to phenformin and rotenone may partly be responsible for the better safety-profile of metformin (manuscript II, Heinz et al. 2019).

3.3 Oxidative stress response as indication of complex I inhibition?

The cellular oxidative stress response may be an additional indicator for an impaired ETC by inhibition of mitochondrial complex I. The main hallmark of oxidative stress is an increased generation of ROS and in this regard, the ETC appears to be an important source of ROS (Zhao et al. 2019). The gene expression analysis reported in manuscript II (Heinz et al. 2019) suggests differences between metformin compared to rotenone and phenformin with respect to ROS levels. Metformin treatment decreased the expression of genes associated with an oxidative stress response in the liver (manuscript II, Heinz et al. 2019). Interestingly, the expression of genes encoding for metallothioneins, for which an antioxidant function has been described (Ruttkay-Nedecky et al., 2013) were transiently up-regulated on day 1 of metformin treatment, followed by sustained down-regulation on day 3 and 14 (manuscript II, Heinz et al. 2019), which is in line with the possible ability of self-limitation of metformin as already discussed in section 3.2. These results also correspond to the previously reported suppression of ROS generation by metformin (Algire et al. 2012; Araujo et al. 2017; Batandier et al. 2006; Wheaton et al. 2014; manuscript II, Heinz et al. 2019). “In contrast, after phenformin and rotenone treatment, genes known to be induced in response to oxidative stress were up-regulated in the liver. This could be explained by increased ROS formation due to complex I inhibition by rotenone (Li et al. 2003) and likely also by phenformin leading to incomplete electron transfer to molecular oxygen (Fato et al. 2009; Miskimins et al. 2014). Besides mitochondrial complex I, several other mitochondrial respiratory chain components are known to generate ROS” (manuscript II, Heinz et al. 2019), including mGPD (Madiraju et al. 2014). Therefore, the most recently suggested mechanism of metformin, namely inhibition of mGPD (Madiraju et al. 2014) may also play a role in the reduction of mitochondrial ROS production (manuscript II, Heinz et al. 2019). “This assumption is supported by Rauchova and colleagues, who show strongly decreased ROS generation in mitochondria by direct inhibition of mGPD” (manuscript II, Heinz et al. 2019) with α-tocopheryl succinate in their study (Rauchova et al. 2014). Furthermore, the resulting limited electron-flow from CoQ to complex III, via inhibition of mGPD, may also contribute to reduced ROS formation in metformin treated rats (manuscript II, Heinz et al. 2019). In parallel to the deregulated genes involved in oxidative stress responses, deregulated genes involved in the hypoxia signaling pathway were detected. In this context, increased expression of the gene encoding *Sat2 (spermidine/spermine N1-*

acetyltransferase family member 2), which plays a role in the degradation of Hif1-α, was observed in the liver of rotenone treated rats (manuscript I, Heinz et al. 2017). Moreover, expression of Hif1 target genes e.g. *Lox (lysyl oxidase) and Loxl2 (lysyl oxidase-like 2)* and the gene *Hyou1 (Hypoxia up-regulated 1)*, usually induced by hypoxia, was decreased (manuscript I, Heinz et al. 2017). "Although activation of Hif1 in the context of hypoxia is suggested to be induced by mitochondrial ROS formation (Simon 2006, Bell and Chandel 2007), complex I inhibitors, including rotenone, have been known to inhibit the Hif1-pathway even under hypoxic conditions (Wheaton et al. 2014; Agani et al. 2002; Ellinghaus et al. 2013). This might be explained by reduced O_2 consumption in mitochondria with an increase in cytosolic oxygen levels due to complex I inhibition and subsequent reduced electron transport chain activity. This could result in greater availability of oxygen for PHDs, in turn leading to Hif-1α degradation" (manuscript I, Heinz et al. 2017). In comparison to rotenone, the expression of genes involved in the Hif1-pathway were mainly not affected after metformin or phenformin treatment (manuscript II, Heinz et al. 2019), which is in line with the much stronger complex I inhibition by rotenone compared to the biguanides. Nevertheless, in contrast to the observed down-regulation of the expression of the Hif1 target gene *Hyou1* in the liver of rotenone treated rats, metformin treatment lead to up-regulation of the expression of this gene on day 3 and day 14 (manuscript II, Heinz et al. 2019), which at first glance could be an indication of ROS formation activating the Hif1 pathway. However, this up-regulation might rather be due to the blood glucose lowering effect of metformin, since it was shown, that the protein Hyou1 encoded by the gene *Hyou1*, also known as 170kDa Glucose-Regulated Protein, can be upregulated at low glucose conditions (Krętowski et al. 2013; Catrina et al. 2004).

3.4 Possible other pathways playing a role in the anti-cancer effects of biguanides

Although the findings and interpretations discussed above could in principle explain the different strength of effects on mitochondria, they do not prove that direct complex I inhibition by biguanides is the primary MOA *in vivo* as it is obvious for rotenone. As briefly mentioned in manuscript II (Heinz et al. 2019), alternative primary MOAs are clearly possible. In this context, and as alluded to before, Madiraju and colleagues (2014) demonstrated that metformin inhibits the redox-shuttle enzyme mGPD resulting in impaired oxidation of FADH2 to FAD, which is reported to impede reduction of CoQ in the inner mitochondrial membrane and thereby electron transfer to complex III (see Figure 2). In addition, inhibition of mGPD inhibits conversion of glycerol-3-phosphate to dihydroxyacetone phosphate during gluconeogenesis from glycerol, one likely MOA for the anti-hyperglycemic effects of metformin. Furthermore, mGPD inhibition decreases the levels of oxidized NAD^+ in the cytosol which impairs conversion of lactate to pyruvate by lactate dehydrogenase, resulting in decreased gluconeogenesis and accumulation

of lactate. The latter could be the cause of lactic acidosis, a well-known side-effect of some biguanides in humans (Brown et al. 1998; Williams and Palmer 1975), even if not suggested for metformin (Aharaz et al. 2018). These findings are further supported by Madiraju and colleagues (2018), who report redox-mediated suppression of hepatic gluconeogenesis by metformin from lactate and glycerol. Moreover, first investigations show a relation between this inhibitory MOA on mGPD by metformin and its anti-cancer effects (Thakur et al. 2018). Altogether, the potential target sites of biguanides in mitochondria include inhibition of the respiratory chain complex I and suppression of mGPD.

Besides the inhibitory MOAs of biguanides in mitochondria, another MOA, namely activation of AMPK must be taken into account. AMPK, a heterotrimeric protein complex of a catalytic α subunit and regulatory β and γ subunits, is a master regulator of several metabolic pathways maintaining energy homeostasis (Hardie et al. 2012; Lin and Hardie 2018). These pathways are considered responsible for many metformin and phenformin anti-diabetic effects, including suppression of fatty acid synthesis, enhancement of β-oxidation (Collier et al. 2006; Hawley et al. 2003; Zhang et al. 2007; Zhou et al. 2001), inhibition of gluconeogenesis (Jeon 2016), and increased glucose transport and insulin receptor function (Gunton et al. 2003). It was suggested that metformin and phenformin treatment can activate AMPK indirectly by increasing cellular AMP levels due to mitochondrial complex I inhibition, leading to an intracellular increase in the AMP-to-ATP ratio (Foretz et al. 2014; Zhang et al. 2007). However, studies in cells treated with metformin showed an activation without alteration of the AMP-to-ATP ratio (Cao et al. 2014; Hawley et al. 2002; Zhou et al. 2001). Since, as summarized above, complex I inhibition by metformin per se is questionable, possible mechanisms of direct AMPK activation were also investigated, suggesting the involvement of its upstream kinase LKB1 (Shaw et al. 2005) direct binding (Meng et al. 2015) and/or formation of protein complexes (Zhang et al. 2016).

Activation of AMPK by such non-mitochondrial MOAs could equally explain reported anti-diabetic effects of metformin and phenformin, but could also play a role in their anti-cancer effects. For instance, LKB1, the upstream kinase of AMPK, is a known tumor suppressor by suppressing the mTOR pathway via AMPK activation (Zhao and Xu 2014). mTOR is a catalytic subunit of the multiprotein complexes mTORC1 and mTORC2 (Wullschleger et al. 2006). Especially mTORC1 is involved in the regulation of cellular growth as it functions as a nutrient/energy sensor and controls protein synthesis (Ben-Sahra and Manning 2017). Inhibition of mTOR thereby can impair tumor cell proliferation. In this context, Zhang and colleagues (2016) demonstrated that metformin inactivates mTORC1 by the AXIN/LKB1-v-ATPase-Ragulator pathway. In addition, other studies reported that metformin shows a suppressing effect on the NF-κB and mTOR signaling pathways via AMPK activation (Kim et

al. 2011; Chaudhary et al. 2012). Inhibition of mTORC1 signaling via activation of AMPK has also been reported for phenformin (Gwinn et al. 2008). Interestingly, one study also indicated a direct influence of metformin on mTOR through an AMPK-independent mechanism via the p53/REDD1 axis. It could be shown, that metformin exerts its anti-proliferative effects in prostate cancer cells in a p53-dependent manner, boosting the level of REDD1 (regulated in development and DNA damage 1), which is a negative regulator of mTOR (Sahra et al., 2011).

Despite these possible direct anti-cancer mechanisms of metformin, another indirect effect has to be taken into account. Metformin´s anti-diabetic effect, reducing glucose in the blood by inhibition of gluconeogenesis, which was also evident from the gene expression analysis shown in manuscript II (Heinz et al. 2019), with a simultaneous decrease of insulin, also plays a role in tumor impairment. It could be shown, that increased insulin levels have mitogenic and prosurvival effects on tumor cells (Ish-Shalom et al. 1997; Kheirandish et al. 2018; Luengo et al. 2014). In this regard, it was demonstrated that insulin activates the PI3K (phosphoinositide 3-kinase)-mTOR signaling pathway, which in turn favors mRNA translation, cell survival and proliferation (Bertuzzi et al. 2016; Du et al. 2014). Therefore, since metformin is known to lower insulin levels and hence may reduce the activation of the PI3K-mTOR pathway, malignant growth could be indirectly impaired without requiring the proposed mitochondrial MOAs of metformin.

4. Conclusion

Treatment of rats with rotenone and phenformin, the latter at a quantitatively reduced level, seems to lead to the use of every metabolic source, which can deliver energy for maintenance of the most important cellular and organismal functions. This includes supply and thus some synthesis of glucose to sustain the glucose requirements of the brain, which was quite obvious in the liver of the treated rats, and characterized by e.g. glycogen depletion. Especially the treatment of rats with rotenone tended to result in an overall borderline hypocaloric status, with additional toxic effects, including bone marrow depletion and bone atrophy, excluding its use as a safe anti-cancer compound. Metformin, on the other hand, appears to induce catabolism of major energy sources only transiently, which became obvious by differential gene expression patterns. The gene expression deregulations induced by metformin were partly similar to rotenone on day 1, but then appeared to change the direction of deregulation for some genes at the later time points. This effect, may be sufficient to keep tumor growth in check, but may avoid the complete depletion of energy resources. Overall, this may be responsible for the much better safety profile of metformin, despite some disruption of mitochondrial respiration. These differences are likely due to differential molecular characteristics of these compounds, with metformin, in contrast to rotenone, requiring a certain mitochondrial potential to allow accumulation in this organelle, which may self-limit its inhibitory effects there. Furthermore, other target functions differ between these compounds. For example, rotenone also has the ability to act as a microtubule inhibitor, whereas metformin and phenformin directly or indirectly inhibit AMPK and thus AMPK pathway-associated metabolic functions, which may also play a role for the different anti-cancer properties of these two compounds. Overall, the more detailed molecular characterization of these compounds suggests that inhibition of mitochondrial functions can serve as target for an anti-cancer mode of action, yet should be self-limited or balanced to some extent to avoid exhaustion of all energy stores. The suggested preclinical experimental model with multiple time points and different toxicological endpoints, including bone marrow parameters, blood analysis, glycogen assessment in liver, lactate assessment in serum, and in particular gene expression analysis in liver, in comparison to rotenone, phenformin and metformin, could help to characterize this profile and may support to find drug candidates with an acceptable risk–benefit balance.

5. Summary

In recent years, mitochondrial metabolism evolved as a target for cancer therapy, especially for tumors relying on oxidative metabolism. Complex I is suggested as one possible site of action for anti-cancer drugs. In this context, various studies have shown that the biguanides and anti-diabetic agents metformin and phenformin can inhibit tumorigenesis *in vitro* and *in vivo*, for which mitochondrial complex I inhibition is discussed as a mechanism of action. Furthermore, rotenone, a well-known and strong complex I inhibitor, has shown anti-cancer activity in several studies. Yet rotenone is also associated with severe systemic toxic effects, like its neurotoxicity, disqualifying it as an anti-cancer drug. Therefore, the aim of this thesis was to compile a suitable early preclinical toxicological *in vivo* test battery to analyze and compare the strong complex I inhibitor rotenone with the suggested complex I inhibitors metformin and phenformin. These investigations shall help to elucidate potential mechanisms, which render biguanides, especially metformin, apparently less toxic than rotenone. Moreover, they are suggested to support oncology research for screening of new drug candidates by defining the required characteristics of such an inhibitor, yet with an improved risk-benefit balance. To reach this goal, rats were orally treated with the three compounds daily for 1, 3 or 14 consecutive days. The test battery included classical clinical endpoints, such as histopathology, clinical chemistry and hematology, as well as supportive investigations, like FACS-analysis and enzymatic activity assays in combination with gene expression analysis. These investigations demonstrated that the mechanistic profile of phenformin appears similar to that of rotenone, however, at a quantitatively reduced level. It seemed that the treatment of rats with these compounds lead to utilization of all available metabolic sources to maintain the most vital functions. In contrast, metformin showed only transient similarities after one day of treatment compared to rotenone and phenformin. This was especially obvious for the gene expression changes induced by metformin, which were qualitatively similar to rotenone on day 1, but then changed the direction of deregulation for some genes at the later time points. This might be explained by the different molecular properties of metformin, compared to rotenone or phenformin, which self-limits its entry into mitochondria and thus its inhibitory effects there, avoiding the exhaustion of all energy stores.

In conclusion, the detailed molecular characterization of these compounds using the proposed preclinical experimental model with multiple time points and different toxicological endpoints could serve to support the identification of new drug candidates with a still tolerable safety profile like metformin, but with potentially increased efficacy.

6. Zusammenfassung

In den letzten Jahren rückte der mitochondriale Stoffwechsel für eine gezielte Krebstherapie in den Fokus, insbesondere für die Behandlung von Tumoren, die auf oxidativen Stoffwechsel angewiesen sind. Hier wird Komplex I als ein möglicher Wirkort für ein Anti-Krebsmittel beschrieben. Die Biguanide und Antidiabetika Metformin und Phenformin zeigten in verschiedenen Studien, dass sie die Tumorbildung hemmen können, wobei die mitochondriale Komplex-I-Hemmung als Wirkmechanismus diskutiert wird. Rotenon, ein bekannter starker Komplex I-Inhibitor, hat in mehreren Studien ebenfalls eine krebshemmende Wirkung gezeigt, wurde aber durch seine schwere, systemische Toxizität als Krebsmedikament ausgeschlossen. Ziel dieser Arbeit war es daher, eine geeignete frühe präklinische toxikologische *in vivo*-Testbatterie zusammenzustellen, um den starken Komplex I-Inhibitor Rotenon mit den mutmaßlichen Komplex I-Inhibitoren Metformin und Phenformin zu vergleichen. Diese Untersuchungen sollten dabei helfen, mögliche Mechanismen aufzudecken, die die geringere Toxizität der Biguanide, insbesondere Metformin, im Vergleich zu Rotenon erklären. Darüber hinaus könnte die onkologische Forschung beim Screening neuer Medikamentenkandidaten unterstützt werden, indem die erforderlichen Eigenschaften eines solchen Inhibitors mit einem besseren Nutzen-Risiko-Verhältnis definiert werden. Um dieses Ziel zu erreichen, wurden Ratten täglich bis zu 14 Tage oral mit den drei Substanzen behandelt. Die Testbatterie umfasste klassische klinische Endpunkte, wie Histopathologie, klinische Chemie und Hämatologie, mit unterstützenden Untersuchungen, wie FACS-Analysen und enzymatischen Aktivitäts-Assays, in Kombination mit Genexpressionsanalysen. Diese Untersuchungen zeigten für Phenformin ein ähnliches mechanistisches Profil im Vergleich zu Rotenon, wenn auch auf einem quantitativ reduzierten Level. Es schien, dass die Behandlung der Ratten mit diesen Substanzen zu einer Nutzung aller verfügbaren Stoffwechselressourcen zur Aufrechterhaltung der lebenswichtigen Funktionen führte. Im Gegensatz dazu zeigte das Profil von Metformin nur am ersten Behandlungstag vorübergehende Ähnlichkeiten zu Rotenon und Phenformin. Dies wurde insbesondere im Genexpressionsmuster deutlich. An Tag 1 wies dieses qualitativ ähnlich induzierte Genexpressionsänderungen wie bei Rotenon auf, zeigte aber zu späteren Zeitpunkten eine veränderte Richtung der Deregulation einiger Gene. Erklärt werden könnte dies durch die unterschiedlichen molekularen Eigenschaften von Metformin im Vergleich zu Rotenon oder Phenformin, wodurch es seinen Eintritt in die Mitochondrien und damit seine hemmenden Effekte selbst limitiert, sodass eine Ausschöpfung aller Energiespeicher vermieden wird.

Folglich könnte die detaillierte molekulare Charakterisierung dieser Substanzen anhand der vorgeschlagenen präklinischen Testbatterie als Unterstützung bei der Suche nach neuen Wirkstoffkandidaten dienen, die ein noch tolerierbares toxikologisches Profil wie Metformin aufweisen, aber potenziell wirksamer wären.

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Abbreviations

2PG	2-phosphoglycerate
3PG	3-phosphoglycerate
ADP	adenosine diphosphate
Akt	protein kinase B
ALAT	alanine aminotransferase
AMP	adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	analysis of variance
APh	alkaline phosphatase
ASAT	aspartate aminotransferase
ATP	adenosine triphosphate
Bili-t	bilirubin
BSA	bovine serum albumin
Ca	calcium
cAMP	cyclic adenosine monophosphate
CD	cluster of differentiation (surface antigen)
cGPD	cytosolic glycerophosphate dehydrogenase
CHOL	cholesterol
CK	creatine kinase
CO_2	carbon dioxide
CoA	coenzyme A
CoQ	coenzyme Q/ubiquinone
$CoQH_2$	ubiquinol
CREA	creatinine
CYP2D6	cytochrome P450 2D6
CytC	cytochrome C
d	day
DHAP	dihydroxyacetone phosphate
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid

dUb	decylubiquinone
ECHA	European Chemicals Agency
EDTA	ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
EOS	eosinophils
ERY	erythrocytes
ETC	electron transport chain
EU	European Union
FACS	Fluorescence-activated cell scanning
FAD	flavin adenine dinucleotide
FADH2	flavin adenine dinucleotide (hydroquinone form)
FDG-PET	2-deoxy-2(18F)-fluoro-D-glucose in positron emission tomography
Fe-S	iron-sulfur
FGF21	fibroblast growth factor 21
FGF21	gene encoding fibroblast growth factor 21
Fig.	figure
FITC	fluorescein isothiocyanate
FMN	flavin mononucleotide
G3P	glycerol-3-phosphate
GAA	gene encoding Lysosomal alpha-glucosidase
gamma-GT	gamma-Glutamyl Transferase
GCCN	Guanine Cytosine Count Normalization
GLDH	glutamate dehydrogenase
GLUT-1/-3	glucose transporter 1 / 3
H_3PO_4	phosphoric acid
HB	hemoglobin
HBSS	Hank´s Balanced Salt Solution
HCT	hematocrit
HE	hematoxylin and eosin
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

HER2	human epidermal growth factor receptor 2
Hif1	hypoxia-inducible factor 1
Hif1-α/-β	hypoxia-inducible factor 1-alpha / -beta
HK2	hexokinase 2
Hmgcr	gene encoding 3-hydroxy-3-methylglutaryl-CoA reductase
Hyou1	gene encoding Hypoxia up-regulated 1
IC_{50}	half maximal inhibitory concentration
Igf1	gene encoding insulin-like growth factor 1
Igfbp2	gene encoding insulin-like growth factor binding protein 2
IPA	Ingenuity Pathway Analysis
KCl	potassium chloride
KCN	potassium cyanide
KH2PO4	potassium dihydrogenphosphate
K-PIPES	potassium-piperazine-N,N′-bis(2-ethanesulfonic acid)
Lac	lactate
LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen
LDH	lactate dehydrogenase
LDHA	lactate dehydrogenase A
LDS	lithium dodecyl sulfate
LEUCO	leucocytes
LEUCO	leucocytes
LKB1	liver kinase B1
Lox	gene encoding lysyl oxidase
Loxl2	gene encoding lysyl oxidase-like 2
LYM	lymphocytes
LYM	lymphocytes
MATE1–2	multidrug and toxin extrusion protein 1-2
MCH	mean corpuscular hemoglobin
MCHC	mean corpuscular hemoglobin concentration
MCT1/MCT4	monocarboxylat-transporter 1/4
MCV	mean cell volume

Mg	magnesium
MgCl2	magnesium chloride
mGPD	mitochondrial glycerophosphate dehydrogenase
MOA	mechanism of action
MONO	monocytes
MRC	mitochondrial respiratory chain
mRNA	messenger RNA
mtDNA	mitochondrial DNA
mTOR	mammalian target of rapamycin
NAD^+	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide hydrogen
NEUTRO	neutrophils
NOEL	no observed effect level
NTP	National Toxicology Program
O_2	oxygen
OCT	organic cation transporter
OXPHOS	oxidative phosphorylation
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD	parkinson’s disease
PDH	pyruvate dehydrogenase
PDK1	pyruvate-dehydrogenase-kinase 1
PE	phycoerythrin
PGM	phosphoglycerate mutase
PHD	prolyl hydroxylases
Pi	inorganic phosphate
PI3K	phosphoinositide 3-kinase (phosphoinositide 3-kinase
PKA	protein kinase A
PKM2	pyruvate kinase isoform M2
PPARα	peroxisome proliferator-activated receptor *α*
PPARα	gene encoding peroxisome proliferator-activated receptor *α*

PPARγ	gene encoding peroxisome proliferator-activated receptor *γ*
PTEN	phosphatase and tensin homolog
pVHL	Von Hippel–Lindau tumor suppressor
Pyr	pyruvate
qRT-PCR	quantitative real-time polymerase chain reaction
RANKL	Receptor Activator of NF-κ B Ligand
Ras	rat sarcoma
REDD1	regulated in development and DNA damage 1
RETI	reticulocytes
RMA	Robust Multi-array Average
RNA	ribonucleic acid
ROS	reactive oxygen species
RTA	Rat Transcriptome Array
Sat2	gene encoding spermidine/spermine N1-acetyltransferase family member 2
SD	standard deviation
Srebf1	gene encoding Sterol regulatory element binding transcription factor 1
Srebf2	gene encoding Sterol regulatory element binding transcription factor 2
SST	Signal Space Transformation
TCA	tricarboxylic acid
THRO	thrombocytes
TRIGL	triglycerides
Tris-HCl	tris(hydroxymethyl)aminomethane - hydrogen chloride
Upp2	gene encoding uridine phosphorylase 2
USA	United States of America

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Eidesstattliche Erklärung/Declaration

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Dissertation selbstständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität“ erstellt habe.

Ich versichere außerdem, dass ich die vorliegende Dissertation nur in diesem und keinem anderen Promotionsverfahren eingereicht habe und, dass diesem Promotionsverfahren keine endgültig gescheiterten Promotionsverfahren vorausgegangen sind.

Sabrina Anna-Maria Fritz,

Düsseldorf, April 2021