Abstract—An increasing number of commonly prescribed drugs are known to interfere with mitochondrial function, which is associated with almost half of all Food and Drug Administration black box warnings, a variety of drug withdrawals, and attrition of drug candidates. This can mainly be attributed to a historic lack of sensitive and specific assays to identify the mechanisms underlying mitochondrial toxicity during drug development. In the last decade, a better understanding of drug-induced mitochondrial dysfunction has been achieved by network-based and structure-based systems pharmacological approaches. Here, we propose the implementation of a tiered systems pharmacology approach to detect adverse mitochondrial drug effects during preclinical drug development, which is based on a toolset developed to study inherited mitochondrial disease. This includes phenotypic characterization, profiling of key metabolic alterations, mechanistic studies, and functional in vitro and in vivo studies. Combined with binding pocket similarity comparisons and bottom-up as well as top-down metabolic network modeling, this tiered approach enables identification of mechanisms underlying drug-induced mitochondrial dysfunction. After validation of these off-target mechanisms, drug candidates can be adjusted to minimize mitochondrial activity. Implementing such a tiered systems pharmacology approach could lead to a more efficient drug development trajectory due to lower drug attrition rates and ultimately contribute to the development of safer drugs.

Significance Statement—Many commonly prescribed drugs adversely affect mitochondrial function, which can be detected using phenotypic assays. However, these methods provide only limited insight into the underlying mechanisms. In recent years, a better understanding of drug-induced mitochondrial dysfunction has been achieved by network-based and structure-based system pharmacological approaches. Their implementation in preclinical drug development could reduce the number of drug failures, contributing to safer drug design.

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This work has not (yet) been presented.

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I. Mitochondrial Dysfunction as a Major Determinant in Adverse Drug Reactions

Mitochondria are well known for their classic role in cellular energy production, as they harbor many central metabolic pathways, including the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation (OXPHOS; Fig. 1). Consequently, they generate the majority of cellular ATP (Galluzzi et al., 2012a, 2012b). When cellular energy demand is high, such as in renal proximal tubule and heart muscle cells, fatty acids (FA) are used as preferred substrate for ATP production via β-oxidation. In the cytosol, FAs are converted into acyl-CoA and transferred into the mitochondrial matrix by the carnitine/acylcarnitine carrier, driving β-oxidation and leading to the production of acetyl-CoA that fuels the TCA (Fig. 1). Although fatty acid β-oxidation is the most efficient ATP-producing mechanism, this pathway implies a high oxygen request and will therefore be limited to such conditions, whereas other substrates might be used when high oxygen requirement cannot be fulfilled.

The compartmentalized structure of mitochondria provides the required microenvironment for these and many other metabolic pathways located within the mitochondrial matrix, such as heme biosynthesis, iron-sulfur cluster assembly, part of gluconeogenesis, ketogenesis, part of amino acid metabolism, and calcium storage (Galluzzi et al., 2012b). Additionally, mitochondria play a pivotal role in cellular life, stress, and death and are more recently implicated in the initiation and propagation of inflammatory responses (Galluzzi et al., 2012b; Weinberg et al., 2015; Riley and Tait, 2020; Tiku et al., 2020). Combined with their metabolic roles, this led to the inevitable association with many common diseases, for instance neurodegenerative disorders (i.e., Alzheimer’s and Parkinson’s disease), type II diabetes, several cancers, and cardiovascular disease (Sivitz and Yorek, 2010; Walters et al., 2012; Galluzzi et al., 2013; Alam and Rahman, 2014; Rao et al., 2014; Weinberg and Chandel, 2015; Murphy and Hartley, 2018). Hence, mitochondria have gained much interest as therapeutic targets (Lanzillotta et al., 2019; Patel et al., 2019; Seo et al., 2019; Roth et al., 2020).

In addition, an increasing number of commonly prescribed drugs are known to interfere with mitochondrial function (e.g., cholesterol-lowering and antidiabetic drugs, antibiotics, chemotherapeutics, and immunosuppressants). Accordingly, these drugs often affect tissues with a high energy demand, including the central nervous system, skeletal muscle, heart, liver, and kidneys (Rolo et al., 2004; Amacher, 2005; Begriche et al., 2011; Montaigne et al., 2012; Pessayre et al., 2012; Schirris et al., 2015a; Wallace et al., 2020). The relevance of mitochondrial toxicity as targets for adverse drug effects is exemplified by the observation that approximately 50% of all U.S. Food and Drug Administration black box warnings are associated with mitochondrial dysfunction (Haughey et al., 2007; Lee et al., 2011; Montpellier et al., 2012; Pessayre et al., 2012). Therefore, understanding the mechanisms of mitochondrial dysfunction induced by drugs is crucial for the development of safer medications.

**ABBREVIATIONS:** AD, Alzheimer’s disease; AOP, adverse outcome pathway; ETC, electron transport chain; FA, fatty acid; KEGG, Kyoto Encyclopedia of Genes and Genomes; MFN, mitofusin; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; OPA, optic atrophy; OXPHOS, oxidative phosphorylation; ROS, reactive oxygen species; TCA, tricarboxylic acid.
with drug-induced mitochondrial dysfunction (a representative overview for cardiovascular, renal, and hepatic toxicity of drugs is shown in Table 1) (Dykens and Will, 2007; Nadanaciva and Will, 2009; Pereira et al., 2009). A screen of 676 unique compounds demonstrated that 73% negatively affected mitochondrial function (e.g., inhibition of the mitochondrial electron transport chain and mitochondrial uncoupling) in an in vitro assay using primary renal proximal tubule cells (Wills et al., 2015). Although drugs could interfere with the protein binding pocket and thereby the function of all approximately 1200 mitochondrial proteins (Calvo et al., 2016), off-target mechanisms are generally categorized as (1) inhibition of multisubunit OXPHOS complexes (Fosslien, 2001; Schirris et al., 2015a); (2) respiratory uncoupling (Madiraju et al., 2014); (3) permeability transition pore opening; (4) suppression of fatty acid β-oxidation and carnitine shuttling pathways for several drugs, including diloefacin, ibuprofen, and zidovudine (Massart et al., 2013; El-Gharbawy and Vockley, 2018; Console et al., 2020); (5) mitochondrial transporter inhibition (Dolce et al., 2001; Divakaruni et al., 2013; Kalghatgi et al., 2013); and (6) affected mitochondrial DNA replication, transcription, or translation (Brinkman et al., 1998; Chan et al., 2005; Dykens, 2008; Payne et al., 2011; McGill et al., 2012). Functionally, these mechanisms are often associated with a reduction of oxygen consumption, increased levels of reactive oxygen species (ROS) or changes in mitochondrial substrates [e.g., reduced nicotinamide adenine dinucleotide (NADH)], decreased ATP levels, or increased oxygen consumption with uncouplers, as well as disturbed calcium homeostasis. Although many compounds are “mito-active” in vitro (and thus have an intrinsic mitochondrial hazard), it is important to emphasize that not all result in mitochondrial toxicity in vivo (i.e., pose a mitochondrial toxicity risk), and in some cases the activity is central to a drug’s pharmacology. Translation of in vitro hazard to in vivo risk is determined by multiple factors but predominant are potency, exposure (including to specific sensitive tissues), and the target tissue’s ability to adapt to the metabolic challenge.

As alluded to, for some drugs the potential to perturb mitochondrial function contributes to their therapeutic efficacy (Lin et al., 2015). The antidiabetic effect of the commonly used drug metformin has, for example, been reported to act through inhibition of mitochondrial glycerol-3-phosphate dehydrogenase and mitochondrial complex I activity at micro- and millimolar concentrations, respectively. The consequent reduced pyruvate and increased adenosine monophosphate levels result in a decreased hepatic gluconeogenesis, via respective positive feedback signaling and adenosine monophosphate-activated protein kinase activation, explaining its use as first-line therapy in type II diabetes mellitus (Owen et al., 2000; Madiraju et al., 2014). The ability of metformin to inhibit mitochondrial function also led to the exploration of its potential use in several cancer types (Viollet et al., 2012; Kheirandish et al., 2018; Thakur et al., 2018). In addition, drug-induced mitochondrial dysfunction has been associated with the therapeutic efficacy of many other anticancer drugs, including etoposide (cell death induction via the mitochondrial-dependent p53 pathway), doxorubicin (inhibition of OXPHOS complexes, respiratory uncoupling, suppression of FA- and TCA- associated protein expression, inhibition of topoisomerase II, and reduction in mitochondrial DNA (mtDNA) content), taxol [opening of the mitochondrial permeability transition pore (mPTP)], thapsigargin (opening of the mPTP), and apicidin (apoptosis via mitochondrial-dependent caspase cascade) (Kwon et al., 2002; Swain et al., 2003; Dykens, 2008; Lebrecht et al., 2010; Quintanilla et al., 2013; Canta et al., 2015; Jamil et al., 2015; Yadav et al., 2015; Babaei et al., 2020).

The impact of drug-induced mitochondrial toxicity can be very significant as emphasized by the market withdrawal of a number of commonly prescribed drugs due to serious mitochondrial adverse effects, such as troglitazone-induced severe liver injury (respiratory uncoupling and opening of the mPTP), cerivastatin-induced rhabdomyolysis (respiratory uncoupling, inhibition of glutamate-driven respiration, and induction of ultrastructural changes), and fatal lactic acidosis by phenformin and buformin (Furberg and Pitt, 2001; Tirmenstein et al., 2002; Bova et al., 2005; Seachrist et al., 2005; Westwood et al., 2005; Kaufmann et al., 2006; Masubuchi et al., 2006; Dykens and Will, 2008; Bridges et al., 2014; Segawa et al., 2018; Totten et al., 2021). Although the withdrawals of phenformin and buformin date from the 1970s, it was only over the last two decades that mitochondrial activity of drugs gained more attention (Dykens and Will, 2008; Amoedo et al., 2017; Meyer et al., 2018; Rana et al., 2019; Bellance et al., 2020). A comprehensive overview of novel cases of mitochondrial toxicity, including enhanced insights into the underlying pathomechanisms, has been reviewed by others (Will et al., 2019; Wu et al., 2020; Leuthner and Meyer, 2021). To understand the physiologic effects of a drug or compound on mitochondrial function and correlate this mechanistic, biologic, and chemical information with clinically relevant toxicity, an extensive mitochondrial toxicity database (MitoTox) has recently been established (Lin et al., 2021). It combines pharmaceutical information with experimental data of over 1,400 small molecules and drugs and aims to integrate knowledge on mitochondrial-related toxicants and their targets. Molecules related to mitochondrial toxicity are classified according to their action on the target, including membrane potential (e.g., depolarization, hyperpolarization, uncoupling, and redox cycling), function of mitochondria (e.g., oxidative phosphorylation and glucose/lipid/amino acid metabolism), organization of mitochondria (e.g., morphology, mass, biogenesis, mitophagy,
<table>
<thead>
<tr>
<th>Drug class</th>
<th>Drug</th>
<th>Toxicity</th>
<th>Mitochondrial Toxic Effects</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Alkylating agents</td>
<td>Cisplatin</td>
<td>Renal</td>
<td>Complex I and IV inhibition, declined MMP, low mtDNA, lower FAO, inhibition of protein synthesis</td>
<td>Miller et al., 2010; Pereira et al., 2009; Santos et al., 2007; Zeengeller et al., 2012</td>
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<tr>
<td></td>
<td>Ifosfamide</td>
<td>Renal</td>
<td>Complex I inhibition, OCR reduction, mitochondrial swelling</td>
<td>Nissim et al., 2006</td>
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<td>Bupivacaine</td>
<td>Cardiovascular</td>
<td>Complex I inhibition, reduction in ATP, OXPHOS uncoupling, MMP dissipation</td>
<td>Hiller et al., 2013</td>
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<td>Anesthetic</td>
<td>Amiodarone</td>
<td>Cardiovascular</td>
<td>Complex I inhibition, reduction in ATP, OXPHOS uncoupling, MMP dissipation</td>
<td>Karkhanis et al., 2018</td>
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<td>Antiarrhythmic</td>
<td>Disopyramide</td>
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<td>Dofetilide</td>
<td>Cardiovascular</td>
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<td>Ibutilide</td>
<td>Cardiovascular</td>
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<td>Anthracyclines</td>
<td>Daunorubicin</td>
<td>Cardiovascular</td>
<td>MMP dissipation, ROS elevation, lipid peroxidation, inhibition of topoisomerase II (mtDNA)</td>
<td>Bloom et al., 2016; Luo et al., 2009; Wu et al., 2014</td>
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<td></td>
<td>Doxorubicin</td>
<td>Cardiovascular/Renal</td>
<td>Loss of cytochrome C, downregulation TCA protein expression, lipid peroxidation, decreased mtDNA content, oxidative stress</td>
<td>Benzer et al., 2018; Brando et al., 2021; Gnanapragasam et al., 2007; Lahoti et al., 2012; Lebrecht et al., 2010; Oz et al., 2006; Pereira et al., 2016</td>
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<td>Epirubicin</td>
<td>Cardiovascular</td>
<td>Nitrosative stress, inhibition of antioxidant enzymes, ROS elevation, lipid peroxidation, inhibition of topoisomerase II (mtDNA)</td>
<td>Guven et al., 2007</td>
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<td>Idarubicin</td>
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<td>Antibiotics</td>
<td>Gentamicin</td>
<td>Renal</td>
<td>Decreased MMP, reduced mtDNA, ROS elevation, complex II inhibition</td>
<td>Chen et al., 2017b; Gai et al., 2020</td>
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<td>Isoniazid</td>
<td>Hepatic</td>
<td>ROS elevation through complex I–III inhibition, increased lipid peroxidation, dissipation MMP, mitochondrial swelling, cytochrome C release</td>
<td>Ahadpour et al., 2016</td>
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<td>Ketoconazole</td>
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<td>Complex I–IV inhibition, ATP depletion, decreased mtDNA, decreased MMP, superoxide accumulation</td>
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<td>Anti-cancer</td>
<td>Arsenic trioxide</td>
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<td>Structural mitochondrial damage, abnormal mPTP opening, ROS elevation, downregulation mitochondrial biogenesis</td>
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<td>MMP dissipation, ATP depletion, complex I inhibition</td>
<td>Ball et al., 2016; Coe et al., 2007; Fau et al., 1994; Zhang et al., 2018b</td>
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<td>Gemtuzumab</td>
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<td>Mitoxantrone</td>
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<td>Methotrexate</td>
<td>Hepatic</td>
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<td>Pentostatin</td>
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<td>Tamoxifen</td>
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<td>MMP dissipation, ATP depletion, complex I inhibition</td>
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<td>Antivirals</td>
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<td>Hepatic</td>
<td>Inhibition of mtDNA polymerase gamma, mtDNA depletion and inhibition of mtDNA polymerase gamma</td>
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<td>Didanosine</td>
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<td>mtDNA depletion and inhibition of mtDNA polymerase gamma</td>
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<td>Toxicity</td>
<td>Mitochondrial Toxic Effects</td>
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fission, and fusion), movement of mitochondria (e.g., mitochondrial transport and motility), oxidative stress (e.g., ROS generation, antioxidants, and scavenger of mitochondrial superoxide), mtDNA (e.g., mtDNA replication, maintenance, and mutation), cell death (e.g., apoptosis, necroptosis, and autophagy), and signaling (e.g., mTOR, AMPK, and MAPK), as summarized in Fig. 2A (Lin et al., 2021). In this review, we focus on specific mitochondrial characteristics that explain why mitochondria are particularly prone to adverse drug effects (Fig. 2). These include the lipid abundance of both mitochondrial membranes that facilitates accumulation of lipophilic drugs. Second, the inner mitochondrial membrane contains high levels of the phospholipid cardiolipin, required for proper functioning of many proteins embedded in this membrane (e.g., OXPHOS complexes). Cardiolipin’s negative charge, however, enhances interactions with cationic drugs (de Wolf et al., 1993; Parker et al., 2001). Such interactions exacerbate membrane fluidity, which together with drug accumulation can eventually result in mitochondrial dysfunction (Unsay et al., 2013). Third, mitochondrial transport proteins and channels, such as the mitochondrial calcium uniporter, allow accumulation of drugs in the mitochondrial matrix and specifically metal ions (e.g., lithium) that interact with essential proteins or disturb the redox cycle (Salimi et al., 2017; Pathak and Trebak, 2018). Fourth, the highly negative electrochemical membrane potential (approximately 120–180 mV) (Griffiths, 2000) over the mitochondrial inner membrane facilitates a strong accumulation (approximately 300- to 500-fold) of lipophilic and amphiphilic cationic drugs (Boelsterli and Lim, 2007; Meyer et al., 2013). Fifth, mitochondrial DNA repair mechanisms are limited (Meyer et al., 2013), and mtDNA is more vulnerable to drug-induced damage compared with nuclear DNA, which is explained by the difference in DNA packing by protective histones, although recent studies have suggested that mtDNA is less “naked” than previously anticipated and packed in histone-like nucleoids (Bogenhagen, 2012; Campbell et al., 2012; Gilkerson et al., 2013; Meyer et al., 2013). Increased mtDNA vulnerability to drug exposure compared with nuclear DNA is especially relevant in the elderly. Both mitochondrial function and mtDNA content decline with age, while simultaneously an increase in age-related diseases and a consequent higher use of medication in the elderly is observed, which is expected to lead to an increase in drug-induced mitochondrial dysfunction (Will et al., 2019). Sixth, mtDNA is closely located to major cellular ROS generation sites, and the scarcity of noncoding sequences that are particularly involved in regulation of gene expression prevents this

### TABLE 1—Continued

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<thead>
<tr>
<th>Drug class</th>
<th>Drug</th>
<th>Toxicity</th>
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<td>Piroxicam</td>
<td>OxPHOS uncoupling</td>
<td>Reduction in ATP content, MMP dissipation</td>
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<td>Salsalate</td>
<td>Mitochondrial uncoupling, membrane dissipation, ATP depletion</td>
<td>Leite et al., 2006</td>
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<td>Tolmetin</td>
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ANT, adenine nucleotide translocase; CNS, central nervous system; FDA, Food and Drug Administration; FAO, fatty acid β-oxidation; MMP, mitochondrial membrane potential; mPTP, mitochondrial permeability transition pore; mtDNA, mitochondrial DNA; NSAID, nonsteroidal anti-inflammatory drug; NtRTI, nucleotide reverse transcriptase inhibitor; OCR, oxygen consumption rate.

Drugs for which the toxicity is related to mitochondrial liabilities are shown in bold. The reported toxic effects on mitochondrial function are indicated.
Fig. 2. Unique functional and structural characteristics render mitochondria particularly vulnerable to adverse drug effects. (A) Drug-induced mitochondrial liabilities are categorized in eight main groups according to their action on the different targets, as specified in Section I. (B) Mitochondria harbor various structural and functional characteristics that enhance their vulnerability for adverse drug effects. (I) Lipophilic drugs easily accumulate in the phospholipid-rich inner and outer mitochondrial membrane (Comte et al., 1976) and can interact with cardiolipin (CL). Especially, cationic drugs are as such trapped in the mitochondrial matrix due to cardiolipin’s negative charge. (II) Mitochondrial transport proteins and channels, such as the mitochondrial calcium uniporter, allow accumulation of drugs and metal ions in the mitochondrial matrix; the latter can interact with essential proteins or disturb the redox cycle. The additional highly negative electrochemical membrane potential over the mitochondrial inner membrane causes strong accumulation (approximately 300- to 500-fold) of lipophilic and amphiphilic cationic drugs. (III) Only a limited number of mechanisms exist that repair damaged mtDNA, of which base excision repair is the main and best understood DNA repair pathway in human mitochondria (Alexeyev et al., 2013; Zinovkina, 2018). (IV) mtDNA is packed in histone-like nucleoids, consisting of proteins including Twinkle, Taf, and mitochondrial single-strand DNA-binding protein (SSB). The noncoding displacement or D-Loop region acts as promoter in replication of mitochondrial DNA (Sharma et al., 2005). (V) OXPHOS complexes are main generation sites of radicals, such as reactive oxygen species (ROS). mtDNA is in close proximity to these sites. Moreover, mitochondria harbor several cytochrome P450 enzymes that facilitate the conversion of xenobiotics into toxic and reactive metabolites, which could accumulate in the matrix but also directly damage mitochondrial proteins and DNA and lipids.
control, thereby increasing vulnerability to potentially harmful substances, including drugs (Boelsterli and Lim, 2007; Meyer et al., 2013). The functioning of mtDNA is also influenced by other factors, as shown by recent developments in environmental exposure assessment linking environmental toxicants, including airborne pollutants, heavy metals, and therapeutic drugs, to impaired mitochondrial epigenetics (e.g., reduced mtDNA methylation), leading to altered expression patterns of mtDNA-coding proteins. Since the interaction between these and nuclear proteins is required for maintenance of cellular health and homeostasis, as well as mitochondrial metabolic pathways, epigenetic perturbations have been linked to a variety of conditions such as cancer, neurodegenerative disorders, disturbed cellular metabolism, and alterations in circadian rhythm (Meyer et al., 2017, 2018; Ramachandran et al., 2018; Zhou and Huang, 2018; Sharma et al., 2019; Zhou et al., 2020). Seventh, mitochondria harbor several cytochrome P450 enzymes that can convert certain drugs into toxic metabolites that could damage mitochondrial proteins, DNA, and lipids (Hartman et al., 2017; Orhan et al., 2021). Finally, the interplay of biogenesis, fission, fusion, and mitophagy makes mitochondrial morphology highly dynamic (Beerreiter-Hahn and Voth, 1994), which may further increase mitochondrial vulnerability to adverse drug effects. The dynamic character arises from sequentially switching between fusion of two separate mitochondria or budding off smaller structures from a single mitochondrion (fission). This enables the adequate coordination of mitochondrial metabolism in response to cellular demands (Tilokani et al., 2018; Ramachandran et al., 2021). Elongated mitochondria are generally associated with conditions in which ATP production is increased; therefore, mitochondrial fusion presumably stimulates the distribution of these high-energy molecules throughout the cell (Mitra et al., 2009; Mishra and Chan, 2016; Ramachandran et al., 2021). Stability and replication of mtDNA and tolerance to mtDNA mutations are also thought to be fusion-dependent, as it was found in skeletal muscle that these events appear to be linked to proteins that regulate the inner and outer mitochondrial membrane fusion (e.g., mitofusin (MFN) 1 and 2) (Chen et al., 2010; Silva Ramos et al., 2019; Sidarala et al., 2022). In cells undergoing stress, mitochondrial fission seems to be the predominant dynamic event, and it is suggested to occur as an adaptive mechanism and a necessary step for the induction of mitophagy, in which dysfunctional or severely damaged mitochondria are directed to Parkin-mediated lysosomal degradation, as has been reviewed in detail (Ni et al., 2015; Tilokani et al., 2018; Ramachandran et al., 2021).

It has been shown that after challenging cells to various toxic conditions, mitochondrial dynamics induce changes in organelle number and morphology to maintain cell viability (Karbowski and Youle, 2003). These changes are linked to the regulation of mitochondrial metabolism and have been shown to influence each other (e.g., for cardiac and muscle cell contraction) (Mishra and Chan, 2016; Wai and Langer, 2016; Abdelwahid, 2017). Consequently, mitochondrial biogenesis, typically occurring in response to loss of functional mitochondria, is fundamental to maintaining cellular homeostasis and regeneration. Especially after exposure to toxic compounds, controlled mitochondrial biogenesis, mediated by the upregulation of the transcription factor PGC1α, enables recovery of cellular function by maintaining respiration and other vital processes. This coordinated action is regulated between mitochondria on the one hand and nuclear transcription and translation on the other, to ensure proper functioning of newly synthesized mitochondria (Ramachandran et al., 2021).

An example involving drug interference with mitochondrial dynamics is cardiotoxicity induced by doxorubicin (Kuznetsov et al., 2011; Tang et al., 2017). In vitro exposure to doxorubicin has been shown to decrease the mitochondrial fusion proteins optic atrophy (OPA) 1 and MFN1/2 and to increase phosphorylation of dynamin-1-like protein 1, which is a fundamental component of mitochondrial fission, resulting in inhibition of fusion and promotion of fission (Li et al., 2014; Tang et al., 2017; Osataphan et al., 2020). In addition, etoposide (OXPHOS inhibition, dissipation of the mitochondrial membrane potential, and ROS elevation), zidovudine (nucleoside reverse transcriptase inhibitor—OXPHOS inhibition, opening of mPTP, dissipation of the mitochondrial membrane potential, inhibition of ATP/ADP carrier, antioxidant enzyme, and DNA polymerase, and remdesivir (antiviral—OXPHOS inhibition) have also been identified as disruptors of mitochondrial dynamics, thereby promoting their fragmentation (Nomura et al., 2017; Nemade et al., 2018; Kwok et al., 2022; Tang et al., 2022). Moreover, in liver injury it has been demonstrated that exposure to acetaminophen (analgesic—inhibition of OXPHOS complexes by toxic metabolite, opening of the mPTP; and respiratory uncoupling) to primary mouse hepatocytes resulted in spheroid-shaped mitochondria before progressing to pathologic irreversibly fragmented mitochondria (Kon et al., 2004; Hanawa et al., 2008; Hu et al., 2016; Umbaugh et al., 2021). On the other hand, liver regeneration after acetaminophen-associated toxicity could be induced by facilitating mitochondrial biogenesis, which is in line with the observation that impaired biogenesis contributes to age-dependent liver damage in experimental sepsis (Du et al., 2017). Since mitochondrial biogenesis restores oxidative metabolism in bacterial sepsis, it is considered an important and early pro-survival factor (Haden et al., 2007). Sustained cellular stress could also lead to mitochondrial remodeling, as alterations in morphology and biogenesis are thought to shift mitochondrial homeostasis to support cell survival. This is a
phenomenon observed in various processes associated with hepatic, cardiovascular, and metabolic diseases, for instance insulin resistance in non-alcoholic fatty liver disease (Gottlieb and Bernstein, 2016; Shannon et al., 2021). It is well established that mitochondrial dynamics underlie cellular homeostasis and that its dysregulation is inseparable from pathophysiological conditions.

Previous drug withdrawals highlight the historic lack of sensitive and specific assays to detect mitochondrial toxicity during drug development. The standard battery of in vivo toxicology studies mandated during drug development relies on healthy animals, which have a high metabolic reserve capacity and can easily adapt to moderate metabolic challenges without showing adverse signs or pathology. This contrasts with many patient groups who are subject to comorbidities, comedications, lifestyle choices, age, and genetic factors, which can all erode their metabolic reserve capacity. As part of an alternative approach, systems pharmacology has proven to be effective to pinpoint mitochondrial off-target effects (Bisson et al., 2007; Wagner et al., 2008; Fannin et al., 2010; Lee et al., 2013; Schirris et al., 2015b). This review aims to provide an overview of these strategies. We propose the implementation of a tiered systems pharmacology approach to aid the identification of mechanisms underlying mitochondrial dysfunction of existing and new drugs under development.

II. Current Methods to Identify Drug-Induced Mitochondrial Dysfunction

Regularly applied assays to evaluate drug-induced mitochondrial dysfunction include measurements of OXPHOS complex enzyme activities, mitochondrial membrane potential, lactate and cellular ATP generation, mtDNA, and calcium levels (Dykens and Will, 2018). Most of these parameters are determined as an endpoint observation. Screening of cellular oxygen consumption rates (i.e., using Seahorse extracellular flux analysis or a fluorescent oxygen sensor) has been introduced and is widely applied to detect mitochondrial activity (Hynes et al., 2006; Hynes et al., 2009; Beeson et al., 2010). The importance of measuring respiratory capacity of the mitochondrial energy generating system is based on the notion that virtually all mitochondrial bioenergetic pathways converge in the OXPHOS system. Moreover, OXPHOS complexes are often observed as important off-targets involved in adverse effects of drugs (Nadanaciva et al., 2007; Hargreaves et al., 2016), and their adequate function depends on the presence of an electrochemical membrane potential. Consequently, measuring respiratory rates instantly provides information about a variety of mitochondrial functional parameters. A reduced oxygen consumption rate and decreased OXPHOS function are associated with increased reductive stress. The resulting surplus of electrons may react with cellular oxygen to produce excessive ROS. A gamut of intracellular molecular probes to sense ROS (Forkink et al., 2010) or detect ROS-induced damage (i.e., lipid and protein peroxidation) are increasingly applied in the investigation of drug-induced mitochondrial damage (Belousov et al., 2006; Forkink et al., 2010; Kalyanaraman, 2011). These fluorescent compounds include small molecules such as hydroethidine, CM-H2DCFDA, dihydrorhodamine 123, and C11-BODIPY that require incubation to get into the cell. On the other hand, protein-based reporter molecules, which can be introduced into the cell by stable or transient transfection, can be used to detect cellular ROS levels, including circularly permuted yellow fluorescent protein, HyPer, and reduction-oxidation sensitive green fluorescent protein. It is important to note that each of these probes can be used to gain insight into the formation of ROS molecules, which are known to have different origins. For example, the primary mitochondrial ROS molecule O$_2^-$ results from electron reduction of O$_2$ and is generally detected by HEt.

The importance of these experimental approaches to distinguish between ROS types is emphasized by the notion that ROS molecules can also serve as cellular signaling molecules. Low levels of ROS and downstream products are key to cellular health and have beneficial effects, for example, in the defense against microbial agents (Valko et al., 2007). Consequently, distinguishing different ROS molecules is useful in separating oxidative stress-related toxic mechanisms from beneficial signaling events (Forkink et al., 2010).

Although phenotypic assays have proven to be very powerful in the detection of drug-induced mitochondrial dysfunction (Wills et al., 2015), these do not provide information about the exact mitochondrial off-target. Furthermore, whether a drug directly affects mitochondria or whether mitochondrial function is influenced secondary to other cellular mechanisms is difficult to distinguish.

The introduction of high-content imaging with mitochondria-selective fluorescent and phosphorescent dyes has facilitated the evaluation of mitochondrial function, morphology, and mitochondrial biogenesis using live-cell imaging (Ferrick et al., 2008; Wagner et al., 2008; Iannetti et al., 2016; Düssmann et al., 2017; Zhang et al., 2017), which enables monitoring of drug effects over prolonged time courses. Besides overcoming the limitation of phenotypic endpoint assays, it also allowed the simultaneous determination of multiple parameters using multiple probes. Recently, spectral unmixing (e.g., linear unmixing) methods have further advanced high-content imaging, as it allows scientists to analyze fluorescent probes with overlapping excitation and emission spectra (Valm et al., 2016; Meghiani et al., 2017). This application has increased the number of fluorescent labels up to 120 for live-cell imaging. Moreover, combining imaging techniques with machine learning made it particularly amenable to disentangle the effects of drugs on
mitochondrial function and morphology (Blanchet et al., 2015; Iannetti et al., 2016; Iannetti et al., 2019). These methods have enabled the successful, unbiased identification of beneficial drug effects on primary cells with a genetically encoded mitochondrial defect and of drug-induced mitochondrial dysfunction (Leonard et al., 2015; Zahedi et al., 2018). They have also significantly aided in the screening of large drug libraries for mitochondrial activity. Importantly, the sensitivity to detect drug-induced mitochondrial dysfunction has been shown to increase in such assays with multiple parameters (Wagner et al., 2008; Wills et al., 2015). The high costs of fluorescent live-cell imaging and rather low capacity, however, limit their use to late-phase compound characterization (Haney et al., 2006; Smith et al., 2012). Clearly, the identification of therapeutic targets and pharmaceutical drug development finally requires in situ complementation studies and even in vivo validation of lead compounds to exclude any potential compound-associated (mitochondrial) toxic hazard and verify safety in a physiologic system, as described in more detail in the section “A Tiered Approach to Implement Mitochondrial Systems Pharmacology in Drug Development.”

Even though these advanced methodologies have increased the capability to detect drug-induced mitochondrial dysfunction, they still predominantly measure the phenotypic consequences, rather than identifying the primary target being affected. In addition, the large number of possible pathways regulating mitochondrial function limits the use of traditional research techniques that are based on an a priori hypothesis about the mechanisms involved. Only a subset is represented, which is expected to hamper the detection of relevant off-target mechanisms. Consequently, there is great need for an unbiased systems analysis in which the complete network of cellular metabolic processes and pathways is considered. This, however, will depend on the availability of large data sets collected without an a priori hypothesis, to avoid inherent selection bias of known pathways (Go et al., 2018).

III. Application of Systems Pharmacology to Investigate Drug-Induced Mitochondrial Dysfunction

In contrast to hypothesis-driven strategies as described previously, systems biology integrates data on multiple levels, including experimental (e.g., mechanistic studies), omics, and predictive bioinformatics data sets. This enables an overall understanding of mechanisms underlying mitochondrial dysfunction on a systems level, which opens up opportunities for targeted investigations of adverse events.

Integrative and unbiased observations from big databases allow the examination of global fluctuations in cellular metabolism, instead of studying the effects on a smaller scale (e.g., single genes, proteins) (Fasano et al., 2016). However, to understand these metabolic effects, gene expression and metabolite concentrations need to be mapped on cellular metabolic networks to connect all individual reactions. The feedback inhibition of amino acid biosynthetic pathways was one of the first metabolic networks constructed more than 60 years ago. Despite this, a clear definition of systems biology is lacking, but it is generally considered to be an integrative approach at the level of full organism, tissue, or cell. It is aimed to understand the physiology and pathology using complex molecular response networks (Klipp et al., 2009). Systems biology is based on a holistic methodology combining all possible targets and pathways involved. The classic systems biology cycle is initiated by data acquisition at a patient, animal, or cell model level, as described in Fig. 3. Types of data include clinical phenotypes; cellular responses; omics-derived data (e.g., genomics, transcriptomics, proteomics, and metabolomics); biochemical reactions or pathways; and drug-related data on pharmacodynamics, pharmacokinetics, and toxicity. Clinical samples, for example, use patient-derived body fluids (e.g., blood or plasma) for RNA sequencing and mass spectrometry–based untargeted metabolomics (e.g., next-generation metabolic screening), as increasingly applied in diagnostic screening for inborn errors of metabolism and mitochondrial disease (Miller et al., 2015; Tebani et al., 2016a,b; Bonte Buzkova et al., 2018; Coene et al., 2018; Hoegen et al., 2021; Thistlethwaite et al., 2022).

By measuring as many metabolites as possible, a metabolic fingerprint can be generated that is representative of a biochemical phenotype, thereby offering novel opportunities for diagnostic screening (Hoegen et al., 2021). The next step is to integrate data by incorporating the obtained knowledge of biochemical pathways, molecular interactions, and omics-derived data into a computer database coupled to correct ontology terms, used for interpretation of a given pathway or process. A similar systematic approach has previously been applied to build the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, which collects, combines, and maintains data on genetics (KEGG GENES database), biochemistry (KEGG PATHWAY database), and molecular and cellular biology (KEGG LIGAND database) (Kanehisa, 1997; Ogata et al., 1999). Computational methods are then employed to model various experimental conditions, including gene functions in terms of gene networks and molecules, reconstruction of biochemical pathways, and prediction of biologic systems. The three modeling approaches that are characteristically applied are discussed in detail later and in Fig. 4. Computational models are typically validated with experimental data ranging from in vitro (cells), in vivo (animal) to clinical studies investigating mitochondrial function (e.g., OXPHOS enzyme activities, cellular ATP levels, and mtDNA, as described in the section “Current Methods to Identify Drug-Induced Mitochondrial Dysfunction”). If model refinement is needed, the cycle is reinitiated. Systems biology typically employs top-
down, bottom-up, or middle-out models (Fig. 4). The top-down approach applies a coarse-grained model of an entire system, which is often refined using large-scale omics data, including proteomic, interactomic (viz. all interactions between and among proteins and molecules within a cell and their consequences), transcriptomic, genomic, or metabolic data (Rolland et al., 2014; Wan et al., 2015; Bludau and Aebersold, 2020). The use of these “omics” data sets enable the construction of biologic networks that represent interactions between genes, transcripts, proteins, and metabolites and aids in the identification of novel pathophysiological mechanisms, as well as new biomarkers and therapeutic targets, as extensively discussed by Maldonado et al. (Suomalainen et al., 2011; Maldonado et al., 2019). These network models represent interacting molecules by nodes (e.g., genes or proteins) and edges (e.g., chemical transformations such as biochemical reactions or regulatory relationships) (Albert, 2007). Nodes that interact with several others are referred to as hubs that split the network into isolated clusters upon loss, whereas node disruption does not cause major loss of connectivity (Albert, 2007; Maldonado et al., 2019).

In the context of mitochondrial disease, these network-based approaches are powerful in studying mitochondrial (dys)function as numerous interactions can be explored, enabling the elucidation of integrative mitochondrial functions that may have been missed using traditional experimental techniques (Maldonado et al., 2019). These top-down network models are generally holistic by their nature as they involve an in-depth investigation of the whole system. As an example, a top-down workflow applied in mitochondrial research involves sample (e.g., patient-derived) collection, processing by high throughput methods (e.g., omics), and analysis by bioinformatics tools to gain a better understanding of function (Maldonado et al., 2019). Bottom-up models rely on mechanistic hypothesis-driven studies of molecular interactions. In contrast to the top-down strategy, they are typically based on (database or literature-driven) experimental data and described by a smaller number of interactions involved. Processes are studied individually and integrated into a model, such as certain metabolic pathways, including glycolysis and catabolism (Teusink et al., 2000; Klipp et al., 2009; Cortassa et al., 2019;
Marín-Hernández et al., 2020) mitochondrial malate-aspartate and citrate-pyruvate shuttles (Korla et al., 2015), mitochondrial messenger RNA translation (Korla and Mitra, 2014), ROS generation in the mitochondrial matrix (Korla, 2016), and more comprehensive mitochondrial (Wu et al., 2007) and cellular models (Grass et al., 2022).
Interestingly, such bottom-up dynamic metabolic models have recently been further refined with the inclusion of circadian cellular patterns, as time-dependent changes in metabolic activity (Rowland Adams and Stefanovska, 2021). The construction of genome-scale metabolic models using pre-existing databases combined with literature input is also powerful in modeling biologic systems, as they aim to fully encompass all interactions within a system. Especially in the context of mitochondrial disorders, generation of these metabolic models has contributed to the assessment of the functional consequences of genetic changes or to the identification of therapeutic targets facilitating the design or repurposing of drugs (O’Brien et al., 2015; Brunk et al., 2018; Maldonado et al., 2019). As described earlier for the use of a top-down strategy in mitochondrial research, the bottom-up workflow can be characterized by identification and collection of molecular data (e.g., database-driven data on glycolysis), formatting this into a model (e.g., genome-scale metabolic models), followed by prediction of solutions to gain a better understanding of the underlying mechanisms (Maldonado et al., 2019).

While bottom-up models are built on the individual kinetic equations describing biochemical reactions, such as the Michaelis-Menten kinetics for enzyme activity, the top-down model is designed to represent a good global fit of the in vivo behavior (Klipp et al., 2009). Nevertheless, it is clear that integration of different data types is key in creating a complete representation of biology, but although the available integrative tools are expanding, they are still scarce and complex to use (Maldonado et al., 2019). As previously reviewed, studying the mechanisms underlying mitochondrial diseases benefits from a multidisciplinary approach that combines clinical, molecular, and computational research to achieve better diagnostics and improve the development of therapeutic agents (Maldonado et al., 2019). Recent developments in multomics approaches have already been demonstrated to be a valuable tool in improving patient care (Maldonado et al., 2019). However, as the integration of large omics data sets can lead to modeling problems, methods such as similarity network fusion have been developed to aggregate and analyze multiple complex omics data sets on a genomic scale (Wang et al., 2014). xMWAS is an approach that identifies associations and correlations between molecules based on multomics data and allows integration of more than two data sets (Uppal et al., 2018; Hu et al., 2020). Expansion of these knowledge bases, including xMWAS and MitoCarta, is an essential next step toward more efficient integration of multomics data for providing deeper insights into specific mitochondrial network responses. (Hu et al., 2020).

In practice, the data types used are the ones that are sufficiently available from various experimental conditions and models, often applying a combination of bottom-up and top-down methodologies, known as the middle-out strategy. This method aims to integrate data from different levels of complexity using a dynamic network modeling approach. Here, the biologic network of interactions connects to the dynamic behavior of a system and has proven powerful in effectively integrating experimental and literature data to gain a holistic understanding of complex biologic systems (Albert, 2007; Sun et al., 2018) (Fig. 3). A similar systems biology approach has been applied successfully to identify Alzheimer’s disease (AD)-related genes and to discriminate molecular regulatory networks and pathways associated with healthy and diseased states in AD (Hu et al., 2017). Moreover, aberrant function of cellular metabolic pathways has been associated with phenotypic disease characteristics in AD using multiple high-throughput technologies, including genomics, transcriptomics, proteomics, and even interactomics (Soler-López et al., 2011; Kristensen et al., 2012; Zhang et al., 2014; Ng et al., 2017; Wang et al., 2019). Such integrative approaches benefit the construction of interpretable and predictive models.

Over the last 20 years, systems biology analyses were also applied in pharmacological and toxicological research (Kongsbak et al., 2014; Turner et al., 2015; Hartung et al., 2017; Yahya et al., 2021). To evaluate the dynamic interaction between drugs and biologic networks, physicochemical-based macromolecular structure modeling has been incorporated in experimental data-driven and mathematical-based pharmacokinetic and pharmacodynamic models (Ward et al., 2013; Xie et al., 2014). Combined with pharmacogenomic data, these models typically represent a systems pharmacology approach that allows deeper insight into mechanisms underlying both beneficial and adverse drug effects (Xie et al., 2014) and predicts personalized drug responses.

Systems pharmacology can methodologically be categorized in either pathway- and network-based approaches or proteome-wide exploration of drug targets using binding pocket similarity comparison. Successful identification of drug-induced metabolic network perturbations has been demonstrated using relatively simple pathway models. However, more extensive genome-scale metabolic networks combined with metabolomic or proteomic data have the potential to detect drug-induced mitochondrial dysfunction. The use of metabolomics as a comprehensive analysis strategy of metabolites and low molecular weight molecules in a biologic specimen goes beyond the scope of standard clinical laboratory techniques and allows precise analyses of hundreds to thousands of compounds. The application of techniques such as liquid/gas chromatography and mass spectrometry provides an objective lens to view the complex link between physiology and external conditions and measure responses to perturbations such as those associated with disease. In addition, as metabolomics allows detailed characterization of metabolic phenotypes, these techniques are valuable for discovering
new therapeutic targets and biomarkers used to diagnose disease or monitor effects of therapeutic compounds (Clish, 2015). Unraveling drug-induced alterations in biochemical pathways because of mitochondrial dysfunction has benefitted from using metabolomic approaches, as previously illustrated for acetaminophen and troglitazone (Fannin et al., 2010; Lee et al., 2013). Recently, systematic evaluation of the effects of electron transport chain (ETC) inhibitors on both mitochondrial and cellular signaling identified that the induction of the specific amino acid response is initiated by ETC inhibition (van der Stel et al., 2022). Combining experimental data with in silico methods, including pathway and gene network analysis, proved promising in unraveling mechanisms of mitochondrial toxicity. These studies also emphasize the importance of experimental data to inform mechanistic computational models, enabling the identification of drug-induced mitochondrial toxicity (van der Stel et al., 2020, 2022). In parallel, significant progress has been made in the development of a bottom-up description of mitochondrial metabolism. Comprehensive dynamic models of one or more mitochondrial metabolic pathways, such as the OXPHOS system, the TCA cycle, or metabolite transport, have been constructed (Wu et al., 2007; Heiske et al., 2017; Bolaji O, 2018). Recently, the application of mathematical modeling of time-dependent high-content imaging data has shown great promise in obtaining a quantitative understanding of mitochondrial adaptation upon exposure to a set of known ETC inhibitors (Yang et al., 2021). By modeling the dynamics of the mitochondrial membrane potential and integrating this with the pharmacokinetics of the studied compounds, it was concluded that data-driven computational modeling is a powerful tool to unravel experimental complexities, such as drug-induced mitochondrial toxicity (Yang et al., 2021). These types of dynamic models benefit from the combined application of system-level metabolic responses and flux stimulations, which is not possible with general metabolic pathway databases such as the KEGG and the BioPlanet database (Kanehisa, 1997; Huang et al., 2019). Over the years, more human metabolic network models have become available, such as Edinburgh Human Metabolic Network (Ma et al., 2007), Human Metabolic Reaction (Agren et al., 2012), and Recon1/2, the latter being a comprehensive consensus-based network (Thiele et al., 2013). A reconstruction of the human metabolic network has recently also been applied to predict drug-induced mitochondrial dysfunction of 18 statogenic drugs (AbdulHameed et al., 2019). Such molecular networks have also been applied to identify gene ontologies, as for example in the development of the Ingenuity Pathway Analysis software, which applies algorithms to infer omics networks based on functional similarity (Calvano et al., 2005). Recently, Recon3D was developed to functionally characterize disease-associated mutations and identify metabolic responses upon exposure to drugs using three-dimensional metabolite and protein structure data (Brunk et al., 2018).

In addition to network-based approaches, the use of structure-based off-target predictions has acquired a central position in the field of systems pharmacology and toxicology. These are based on the notion that virtually all drugs are promiscuous and bind multiple targets (i.e., polypharmacology). Drug-network studies estimated that the average number of drug targets can be as high as 6.3, if therapeutic and predicted drug targets are included (Mestres et al., 2008; Schenone et al., 2013). Hence, various in silico techniques were developed to explore similarity between structural features of primary drug-binding pocket and other binding pockets to reveal drug off-targets (Xie et al., 2011; Ferreira et al., 2015). Although all examine binding pocket similarity, different methods are applied, such as comparison of the protein binding pocket itself (e.g., ProBiS; Konc and Janezic, 2012), SMAP (Ren et al., 2010), comparison of binding pocket pharmacophores (e.g., KRIPO; Ritschel et al., 2014), or ligand comparison (e.g., SEA; Keiser et al., 2007). These algorithms were successfully applied to identify (mitochondrial) targets, including antimicrobial activity of several drugs (i.e., fosfomycin, sulfathiazole, and trimethoprim; Chang et al., 2013), mitochondrial complex III inhibition by statins (Schirris et al., 2015a), mitochondrial ADP/ATP exchange inhibition (Schirris et al., 2015b), inhibition of heat shock protein 27 (Heinrich et al., 2016), and β-secretase (i.e., BACE-1) by gefitinib (Niu et al., 2014). More recently, application of deep learning (i.e., DeepDrug3D and BionoiNet; Pu et al., 2019; Shi et al., 2020) and artificial intelligence has further advanced these techniques, which increased their accuracy by accommodating for specific binding characteristics, such as involvement of hydrogen-bond acceptor and donor sides, as well as aromatic and hydrophobic contacts.

Other strategies adapted from drug design methodology have been used to systematically search for off-targets based on drug promiscuity and target similarity, such as inverse virtual screening (Salentin et al., 2014). In parallel, several experimental techniques to search for protein-small molecule interactions have been described that have developed into proteome-wide target identification. A powerful example is provided by stable isotope labeling of amino acids in cell culture, combined with affinity chromatography and mass spectrometry (Ong et al., 2009; Xie et al., 2011). Although these are robust methods to identify drug off-targets, they can also be used in a more targeted manner to validate in silico structural off-target predictions described above.

Finally, efforts in the field of systems toxicology contributed to the development of a global toxicological network that spans various hierarchical levels of biologic organization and drug-induced perturbations.
of physiologic mechanisms (Bai and Abernethy, 2013). Using genomic, transcriptomic, and adverse phenotypic data, interrelated network models on drug-protein, protein-pathway, pathway-organ, and organ-phenotype interactions have been constructed. Data sources provided to these models can be experimental, literature-based, or adverse event reporting databases, which most optimally are organized according to ontology terms. In this respect, the recently developed, online-available, fully searchable database MitoTox integrates comprehensive information on mitochondrial toxicity-related molecules and their targets. Over 1,400 small-molecule compounds, 870 mitochondrial targets, and more than 4,000 mitochondrial toxic-target associations described in scientific journals and electronic databases are included (Lin et al., 2021). It correlates chemical, biologic, and mechanistic data on clinically relevant mitochondrial toxicity and provides applications that include toxicity classification, prediction, reference, and even education. Moreover, a recent study combined metabolic networking with pharmacokinetic models to construct whole-body physiologically based pharmacokinetic models, which demonstrated phenotype-specific cases of drug-induced metabolic perturbations (Cordes et al., 2018). Lastly, integration of data from experiments, modeling prediction, and exposure assessment in adverse outcome pathways (AOPs) has aided toxicological risk assessment and has shown to be promising in replacing animal studies for these purposes (Hecker and LaLone, 2019). The use of AOP-based testing strategies in exploring the opportunities to flag chemical and structurally related substances for potential mitochondrial respiratory chain-mediated neurotoxicity hazards was described by van der Stel et al. (2021). This shows that practical application of AOPs integrated with new approach methods, including in silico docking and in vitro assays, could be a promising strategy for drug safety assessment (Van der Stel et al., 2021).

In summary, various applications of systems pharmacology have demonstrated great potential to identify drug off-targets. Knowledge about off-target actions is potentially providing a rationale for novel interventions to attenuate drug adverse effects, for example by stimulation of metabolic compensatory pathways. Second, a newly identified off-target effect could indicate novel susceptibility factors, such as genetic variation of the off-target drug binding site. Lastly, expanding knowledge on off-target effects can be valuable in the construction of toxicological networks, in which combinations of drugs and targets are integrated with other relevant parameters of different levels of complexity.

IV. A Tiered Approach to Implement Mitochondrial Systems Pharmacology in Drug Development

Efforts in the field of systems pharmacology and toxicology have successfully contributed to elucidating the mechanisms underlying adverse drug effects, including drug-induced perturbation of mitochondrial function (Yang et al., 2015; Watkins, 2020). Consequently, these strategies hold great promise to detect drug-induced mitochondrial dysfunction in early stages of drug discovery. In view of the large number of drugs that have a mitochondrial liability (Wills et al., 2015) and the serious consequences if this translates into mitochondrial toxicity (Nadanaciva and Will, 2009; Pereira et al., 2009), the early understanding of drug actions on mitochondria is expected to help reduce drug attrition during late-stage drug development. Currently, systems pharmacology methods are still labor intensive, making their application most suitable when a smaller number of compounds (i.e., 5–10) is evaluated, like lead development stages. Here, we propose a tiered evaluation of drug-induced mitochondrial dysfunction in preclinical drug development, with a key position for systems pharmacology approaches during lead development. This approach is based on a toolset developed for the clinical investigation of inherited mitochondrial disease, as described in Fig. 5 and Table 2. It is important to note that the proposal here is set out in such a way that the resource required at each stage matches the stage of development of the compound(s). However, once the capability is built for each tier, there are a number of elements of the proposal that could be moved progressively earlier as the case knowledge and validation increase to the point where early chemistry decisions can be influenced to remove or significantly reduce the intrinsic hazard of mitochondrial activity.

A. Tier 1: Phenotypic Screening During Hit Identification

The first diagnostic phase for mitochondrial diseases is mainly focused on clinical chemistry abnormalities, which can be compared with a phenotypic toxicity screening during drug development, as both aim to identify most significant phenotypes. Clinically, a broad range of parameters is assessed to examine which is most relevant for disease state. Similarly, general measures of mitochondrial function could be used to initially flag compounds with a potential intrinsic mitochondrial toxic hazard. Clinical chemistry abnormalities leading to a high suspicion of mitochondrial defects include increased blood lactic acid concentrations. However, only 30% of mitochondrial-diseased children present with elevated venous lactate levels (Munich et al., 1996). Therefore, the suspicion of a mitochondrial defect depends on multiple signals, and the chance of such a disease increases with the number of phenotypic alterations observed. Low suspicion of mitochondrial disease often results from single-organ system effects (cardiomyopathy, impaired neurodevelopment, exercise intolerance) and reduced ATP production (Koopman et al., 2016). In the case of drug-induced mitochondrial dysfunction, reduced ATP levels are expected to have a lower predictive value, as cellular ATP levels are maintained by compensatory mechanisms (Dyken et al., 2007). For example, phosphagen pools and relevant kinases hold ATP at unity.
by maintaining adenine nucleotide pools. Phosphagens (e.g., phosphocreatine) are found in tissues that experience quickly changing energy demands, such as muscles and nerves, and function as immediate access reserve of high-energy phosphates needed to rapidly generate ATP from ADP (Dykens et al., 1996). Therefore, a reduction in cellular ATP levels mostly associates with severe and not mild mitochondrial activity (Will and Dykens, 2014). Phenotypic assays for mitochondrial activity assessment are not universally incorporated in drug development pipelines at present, and where they are used, the approach taken can vary considerably. One of the more commonly applied early screening methods is the glucose-galactose assay, which is based on the observation that cells obtain less ATP from glycolysis under galactose conditions (Will and Dykens, 2014). Accordingly, cells rely much more on mitochondrial metabolism, which may render them more susceptible to mitochondrial toxicants. Nonetheless, only 2% to 5% of all mitochondrial toxicants are detected by this assay, underscoring its limited predictive value (Hynes et al., 2013) as a stand-alone approach. To improve the predictive value, additional assays for mitochondrial activity are required (Wagner et al., 2008; Wills et al., 2015). To conclude, a combination of low-cost assays with medium- to high-throughput capacity can be seen as a first tier of our strategy to demonstrate whether mitochondrial function is affected.

B. Tier 2: Key Metabolic Profiling During Lead Development

Upon suspicion of a mitochondrial disease, based on clinical signs and symptoms and clinical chemistry findings, a more detailed biochemical diagnosis is requested. Here, a combination of conventional and complimentary techniques is used to assess several biochemical features associated with mitochondrial diseases. Such histopathological or biochemical analyses are often performed in muscle biopsies in specialized laboratories. Histopathological alterations include morphologic structural changes and altered enzyme-based stainings (e.g., cytochrome C oxidase, NADH reductase, succinic dehydrogenase). Biochemical measures most often include determination of ATP production and substrate oxidation rates, as well as analysis of the individual activities of the OXPHOS complexes (Rodenburg, 2011). Additionally, oxygen consumption and OXPHOS complex assembly can be determined as a follow-up strategy. These contribute to a robust insight into whether mitochondrial function is truly impaired. The aims are very similar to those of mitochondrial assessment during lead development (i.e., to confirm activity and help to identify the most potent compounds) (Hughes et al., 2011) by generating concentration-response curves and subsequent IC50s (inhibitory concentration 50%) or minimal effect concentration. Such a rigorous assessment of mitochondrial function would be relevant for those compounds that have demonstrated a mitochondrial activity flag in Tier 1 but are still interesting drug candidates for further development by virtue of a favorable profile (e.g., high pharmacological potency for the primary target, efficacy in human-derived disease model assays, or good projected pharmacokinetic properties).

Several methods to detect mitochondrial activity described earlier (e.g., mitochondrial membrane potential, ROS, oxygen consumption measurements using the Seahorse platform) could also be used to provide an initial understanding of the underlying mechanisms. Subsequently, more comprehensive techniques can be applied to further define mechanisms, including systems pharmacology approaches. Hereinto, we propose to follow the classic systems biology cycle (Fig. 3), starting with data collection. Which type of data to collect depends on the chosen systems pharmacology approach: network-based or structure-based. Data of metabolic networks can consist of transcriptomic, proteomic, or metabolomic data of cells exposed to a concentration of the candidate compound, which resulted in mitochondrial dysfunction in Tier 1 and 2 assays. Structure-based data includes X-ray protein structures, homology models derived from similar structures, or ligand-based pharmacophores. Subsequently, the various systems pharmacology approaches described previously can be applied to explore mitochondrial drug off-targets.

C. Tier 3: Mechanistic Studies During Lead Optimization

Upon biochemical diagnosis of a mitochondrial disorder, further insight into the disease etiology is provided by next-generation sequencing. The introduction of genetic screens, including whole exome sequencing, resulted in the association of more than 1,500 nuclear genes with mitochondrial diseases (Wallace et al., 1988; Goto et al., 1990; Lodi et al., 2000; Van Goethem et al., 2001; Winterthun et al., 2005; McCormick et al., 2013; Wortmann et al., 2015; Theunissen et al., 2018; Panneman et al., 2020). Various gene panels, based on suspected strength of a mitochondrial disease in Tier 1, are used (Wortmann et al., 2015). Panels cover variants known to directly disturb activity of the electron transport chain (van den Heuvel et al., 1998; Koopman et al., 2012; Nouws et al., 2012; Jonckheere et al., 2013; Hallmann et al., 2016), which is most evidently linked to mitochondrial dysfunction (DiMauro et al., 1999; Dimauro, et al., 2004). Moreover, these panels include many other genes associated with mitochondrial diseases (Wortmann et al., 2015) encoding for mitochondrial carriers (e.g., SLC25A3, MPC1), proteins involved in mtDNA maintenance (e.g., POLG), mitochondrial fission and fusion (e.g., OPA1, MFN2), and mitochondrial phospholipid metabolism (e.g., SERAC1). In analogy to these steps in the diagnosis of mitochondrial disease, more detailed insights into causal molecular mechanisms underlying drug-induced mitochondrial dysfunction are required next. Applying a systems pharmacology approach at the end of Tier 2 would be a valuable starting point. To validate such etiologic relevance of an off-target for the observed...
Fig. 5. A tiered approach for the implementation of systems pharmacology to detect and ameliorate mitochondrial activity during drug development. We propose a step-by-step strategy to incorporate systems pharmacology in the drug development pipeline, based on a similar approach used to diagnose mitochondrial diseases. The first tier in this diagnostic workflow consists of phenotypic observations, which are comparable with phenotypic screening methods to detect mitochondrial dysfunction in early drug development stages like hit identification. The second tier in patients consists of clinical chemistry and can in drug development be compared with the lead development phase. This tier could consist of more in-depth phenotypic characterization of the previously observed mitochondrial liability. In this tier we suggest incorporating systems pharmacology to aid identification of the mechanisms underlying drug-induced mitochondrial dysfunction. These approaches can consist of network-based or structure-based modeling to identify off-target mechanisms. Subsequent computational techniques of systems biology and corresponding in vitro evaluation (e.g., biochemical and cellular assays) would result in an optimized lead or clinical candidate. If mitochondrial activity is though observed, the systems biology cycle can be reinitiated, with slight chemical adjustments to the potential lead. The third tier of the diagnosis of a mitochondrial disease is genetic screening. In drug development this phase would compare with the lead optimization phase, in which only a small number of compounds is considered as well. Here, more mechanistic insights into the underlying off-target mechanism could be obtained using more advanced techniques including medium- and high-throughput and microscopic imaging and the used of knockout strategies like CRISPR-Cas9. The fourth and last tier compares to functional in situ studies as performed in clinical diagnosis of mitochondrial diseases, which can in drug development be used to assess a drug's effect on mitochondrial function in vivo and most likely be used as an in vivo validation of the previously applied systems pharmacology approaches to attenuate mitochondrial dysfunction.
mitochondrial activity, various cell biologic methods could be applied, including the use of a knockout or overexpression model of common mitochondrial off-targets generated preemptively using techniques such as CRISPR-Cas9 or using RNAi-mediated knockdown (i.e., siRNA, shRNA) and selected off-the-shelf as required. Ideally, such approaches are combined with high-content microscopic imaging to simultaneously investigate various mitochondrial and cellular parameters. To integrate these parameters, this can be combined with machine learning techniques, as described before, along with specific bespoke mechanism of action investigations driven by hypotheses derived from the machine learning output. Implications for mitochondrial function can be further validated in vivo in Tier 4, as described next.

**D. Tier 4: Functional In Situ Studies Versus In Vivo Efficacy and Safety Studies**

Once the genetic cause of a mitochondrial disorder is identified, deeper understanding of molecular and cellular pathogenesis can be obtained through mechanistic studies in patient-derived fibroblasts or even induced pluripotent stem cells. Moreover, these studies are essential to validate the etiologic role of the identified genetic polymorphisms or mutations. Besides the notion that these cells can be obtained less invasively via skin biopsy and have a proliferative capacity, they carry all patient-specific mutations. Although biologic properties at the cellular level are therefore preserved (Saada, 2011; Hu et al., 2019), potential tissue-specific differences in mtDNA heteroplasmy levels should be considered. A relevant addition is complementation studies, in which wild-type DNA of the suspected disease-causing mutated gene is introduced into these patient-derived cells by viral transduction or transient transfection (Kirby et al., 2004; Hoefs et al., 2008; Jonckheere et al., 2011; Koopman et al., 2016), followed by functional confirmation of pathogenicity using protein expression levels or enzymatic activity (Hoefs et al., 2008; Jonckheere et al., 2013; Koopman et al., 2016). A known pathogenesis will help in the identification and development of potential therapeutic targets. Although complementation studies are comparable to the cellular validation steps performed as part of the systems pharmacology design, clearly for compound development an in vivo validation is required. A validation step using a relevant animal model could also closely resemble the use of patient-derived cells as a model for the patient’s cellular response. To investigate whether the proposed approach to lead optimization indeed helped reduce the intrinsic mitochondrial toxicity hazard of the lead and that the mechanistic insights gained in vitro in Tier 4 have allowed the risk posed by any residual mitochondrial activity to be correctly assessed, one needs robust methodologies to measure mitochondrial function in vivo. In this respect some recently developed methods significantly enhance the capabilities to monitor in vivo mitochondrial function at the molecular level. Development of the MITO-Tag Mice, for example, enabled the rapid determination of mitochondrial metabolites in various tissues (Bayraktar et al., 2019). High-resolution Fourier-transform mass spectrometry on isolated mitochondria provides an alternative approach (Go et al., 2014). Application of the same hemagglutinin-tag–based rapid mitochondrial isolation technique has also been previously applied in animal and plant cells (Chen et al., 2016; Kuhnert et al., 2020), which also demonstrated its ability for enzymatic evaluation of OXPHOS complex activity. Even without the isolation of organelles, functional analysis of mitochondrial complexes in permeabilized muscle fibers, tissues, and cells has been demonstrated previously (Kuznetsov et al., 2008). In addition, injection of an exogenous probe, followed by its evaluation ex vivo, enabled the in vivo determination of superoxide, hydrogen sulfide, hydrogen peroxide, and

<table>
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<th>Evaluation strategy</th>
<th>Assay examples</th>
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<td><strong>Tier 1</strong> Phenotypic observations</td>
<td>Glucose-galactose assay, cellular viability (fluorescence live/dead stain), respirometry assessment (e.g., OCR using Seahorse XF Bioscience), ATP levels (e.g., bioluminescent ATP or CellTiter-Glo), lactic acid (colorimetric assays).</td>
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<tr>
<td><strong>Tier 2</strong> Clinical chemistry</td>
<td>Drug concentration-response curves (IC₅₀/MEC), enzymatic activity (e.g., cytochrome C oxidase, NADH reductase, or succinic dehydrogenase), ATP production rates (using Seahorse XF), OXPHOS complex activity or assembly (e.g., BN-PAGE), MMP (fluorescence, e.g., TMRM or JC-1 or flow cytometry, e.g., MitoTracker Green FM), ROS production (fluorescence, e.g., H₂DCFDA and MitoSOX), network or structure-based model construction (e.g., “omics” or X-ray protein structures/pharmacophore prediction using ProBiS/KRIPO).</td>
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<tr>
<td><strong>Tier 3</strong> Cellular studies</td>
<td>Protein overexpression or knockout (CRISPR/Cas9, RNAi, e.g., shRNA or siRNA) combined with cellular/metabolic parameters (e.g., cellular viability, OXPHOS activity, and ATP production rates).</td>
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<tr>
<td><strong>Tier 4</strong> In vivo studies</td>
<td>In vitro genetic complementation using patient-derived fibroblasts: protein expression (e.g., Western blot), metabolic parameters (e.g., OXPHOS complex activity, MMP) and in vivo evaluation of mitochondrial function (e.g., MITO-Tag mice, MS, ROS, and ATP levels).</td>
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BN-PAGE, blue native polyacrylamide gel electrophoresis; H₂DCFDA, 2',7'-dichlorodihydrofluorescein diacetate; KRIPO, key representations of interaction in pockets; MEC, minimal effect concentration; MMP, mitochondrial membrane potential; MS, mass spectrometry; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, small interfering RNA; TMRM, tetramethyl rhodamine methyl ester.
the mitochondrial membrane potential (Cochemé et al., 2011; Logan et al., 2016; Arndt et al., 2017; Shcheципova et al., 2017). Other assays use genetically encoded fluorescent markers and two-photon imaging to measure in vivo ROS and ATP production (van Hameren et al., 2019). The latter could provide real-time imaging of these levels, and if costs are justified by results, the dedicated equipment required may not limit its use in drug development.

V. Concluding Remarks and Future Perspectives

Consciousness of potential mitochondrial off-target effects is key during design and development of new drugs. Implementation of systems pharmacology in the drug developmental process is expected to significantly enhance the detection and prevention of drug-induced mitochondrial dysfunction. The proposed tiered strategy aims to reduce drug-induced mitochondrial dysfunction before entering clinical drug mitochondrial stages (see also Fig. 5) and follows a workflow similar to that applied in the clinical setting to detect inherited mitochondrial disorders.

The ultimate aim is to deploy systems pharmacology approaches early enough in compound development such that the chemistry of the lead molecules can be adjusted (e.g., compound structure) to remove or substantially diminish the intrinsic mitochondrial activity hazard, thereby negating or reducing the risk of later mitochondrial toxicity. An example of such an adaptive systems pharmacology method has been demonstrated by our group for the potential antibiotic drug ibipinabant (Schirris et al., 2015b). Using a structure-based approach, we demonstrated inhibition of mitochondrial ADP/ATP exchange as off-target mechanism explaining the observed muscle toxicity, which could be reversed upon minor chemical modification of ibipinabant. As the capabilities in each tier mature, supported by systems pharmacology, applied methods could be moved progressively earlier. Then in silico strategies like molecular docking and pharmacophore modeling could offer an appropriate starting point in drug design, with subsequent testing in enzymatic or cellular assays to evaluate the potential off-target effects of early compound leads as part of an iterative chemistry development effort.

We propose the implementation of systems pharmacology in early stages of drug development (e.g., lead development) to reduce drug-related adverse effects and to enable the early detection of molecules with mitochondrial liabilities, thereby minimizing the number of drug attritions in later development phases and improving patient safety.

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Authorship Contributions

Participated in research design: Hoogstraten, Schirris. Performed data analysis: Hoogstraten, Schirris. Wrote or contributed to the writing of the manuscript: Hoogstraten, Lyon, Smeitink, Russell, Schirris.

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