REVIEW MANUSCRIPT

Time to change: a systems pharmacology approach to disentangle mechanisms of drug-induced mitochondrial toxicity

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Running Title:

Systems pharmacology in drug-induced mitochondrial toxicity

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List of non-standard abbreviations

AD	Alzheimer's disease
AMPK	AMP-activated protein kinase
AOP	adverse outcome pathway
CYP	cytochrome P450
ETC	electron transport chain
FA(O)	fatty acid (β-oxidation)
GEM	genome-scale metabolic model

IEM	inborn errors of metabolism
KEGG	Kyoto encyclopedia of genes and genomes
MFN	Mitofusin
MMP	mitochondrial membrane potential
mPTP	mitochondrial permeability transition pore
mtDNA	mitochondrial DNA
OPA	optic atrophy
OXPHOS	oxidative phosphorylation
ROS	reactive oxygen species
TCA	tricarboxylic acid

ABSTRACT

An increasing number of commonly prescribed drugs are known to interfere with mitochondrial function, which is associated with almost half of all FDA 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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I | MITOCHONDRIAL DYSFUNCTION AS A MAJOR DETERMINANT IN ADVERSE DRUG REACTIONS

Mitochondria are well-known for their classical role in cellular energy production, as they harbor many central metabolic pathways, including the tricarboxylic acid (TCA) cycle and the oxidative phosphorylation (OXPHOS, **Figure 1**). Consequently, they generate the majority of cellular adenosine triphosphate (ATP) (Galluzzi et al., 2012a; Galluzzi et al., 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 (CAC), driving β-oxidation and leading to the production of acetyl-CoA that fuels the TCA (**Figure 1**). Although fatty acid β-oxidation (FAO) 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-sulphur 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; Riley and Tait, 2020; Tiku et al., 2020; Weinberg et al., 2015). 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 (Alam and Rahman, 2014; Galluzzi et al., 2013; Murphy and Hartley, 2018; Rao et al., 2014; Sivitz and Yorek, 2010; Walters et al., 2012; Weinberg and Chandel, 2015). Hence, mitochondria have gained much interest as therapeutic targets (Lanzillotta et al., 2019; Patel et al., 2019; Roth et al., 2020; Seo et al., 2019).

In addition, an increasing number of commonly prescribed drugs are known to interfere with mitochondrial function (*e.g.,* cholesterol-lowering and anti-diabetic drugs, antibiotics,

chemotherapeutics and immunosuppressants). Accordingly, these drugs often affect tissues with a high energy demand, including central nervous system, skeletal muscle, heart, liver and kidneys (Amacher, 2005; Begriche et al., 2011; Montaigne et al., 2012; Pessayre et al., 2012; Rolo et al., 2004; Schirris et al., 2015a; Wallace et al., 2020). The relevance of mitochondrial toxicity as targets for adverse drug effect is exemplified by the observation that approximately 50 percent of all FDA black box warnings are associated 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 percent 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 ~1200 mitochondrial proteins (Calvo et al., 2016), off-target mechanisms are generally categorized as: (I) inhibition of multi-subunit OXPHOS complexes (Fosslien, 2001; Schirris et al., 2015a), (II) respiratory uncoupling (Madiraju et al., 2014), (III) permeability transition pore opening, (IV) suppression of fatty acid β-oxidation and carnitine shuttling pathways for several drugs, including diclofenac, ibuprofen and zidovudine (Console et al., 2020; EI-Gharbawy and Vockley, 2018; Massart et al., 2013), (V) mitochondrial transporter inhibition (Divakaruni et al., 2013; Dolce et al., 2001; Kalghatgi et al., 2013), and (VI) affected mitochondrial DNA replication, transcription or translation (Brinkman et al., 1998; Chan et al., 2005; Dykens, 2008; McGill et al., 2012; Payne et al., 2011). Functionally, these mechanisms are often associated with 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 its therapeutic efficacy (Lin et al., 2015). The anti-diabetic 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 (AMP) levels result in a decreased hepatic gluconeogenesis, via respective positive feedback signaling and AMP-activated protein kinase (AMPK) activation, explaining its use as first-line therapy in type II diabetes mellitus (Madiraju et al., 2014; Owen et al., 2000). The ability of metformin to inhibit mitochondrial function also led to the exploration of its potential use in several cancer types (Kheirandish et al., 2018; Thakur et al., 2018; Viollet et al., 2012). 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) (Babaei et al., 2020; Canta et al., 2015; Dykens, 2008; Jamil et al., 2015; Kwon et al., 2002; Lebrecht et al., 2010; Quintanilla et al., 2013; Swain et al., 2003; Yadav et al., 2015).

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 (Bova et al., 2005; Bridges et al., 2014; Dykens, 2008; Furberg and Pitt, 2001; Kaufmann et al., 2006; Masubuchi et al., 2006; Seachrist et al., 2005; Segawa et al., 2018; Tirmenstein et al., 2002; Totten et al., 2021; Westwood et al., 2005). 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 (Amoedo

et al., 2017; Bellance et al., 2020; Dykens, 2008; Meyer et al., 2018; Rana et al., 2019). A comprehensive overview of novel cases of mitochondrial toxicity, including enhanced insights into the underlying pathomechanisms, have been reviewed by others (Leuthner and Meyer, 2021; Will et al., 2019; Wu et al., 2020). To understand the physiological effects of a drug or compound on mitochondrial function and correlate this mechanistic, biological, 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 1400 small molecules and drugs and aims to integrate knowledge on mitochondria-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, 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 figure 2A (Lin et al., 2021). In this review, we will focus on specific mitochondrial characteristics that explain why mitochondria are particularly prone to adverse drug effects (**Figure 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 (MCU), 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 (Pathak and Trebak, 2018; Salimi et al., 2017). Fourth, the highly negative electrochemical membrane potential (~120-180 mV) (Griffiths, 2000) over the mitochondrial inner membrane facilitates a strong

accumulation (~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 to 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 to 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 non-coding sequences that are particularly involved in regulation of gene expression prevents this 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., 2018; Meyer et al., 2017; Ramachandran et al., 2018; Sharma et al., 2019; Zhou and Huang, 2018; Zhou et al., 2020). Seventh, mitochondria harbor several cytochrome P450 (CYP) 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 (Bereiter-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 (Ramachandran et al., 2021; Tilokani et al., 2018). 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 (Mishra and Chan, 2016; Mitra et al., 2009; 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; Sidarala et al., 2022; Silva Ramos et al., 2019). 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; Ramachandran et al., 2021; Tilokani et al., 2018).

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 (Abdelwahid, 2017; Mishra and Chan, 2016; Wai and Langer, 2016). Consequently, mitochondrial biogenesis, typically occurring in response to loss of functional mitochondria is fundamental to maintain 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 (DRP) 1, which is a fundamental component of mitochondrial fission, resulting in inhibition of fusion and promotion of fission (Li et al., 2014; Osataphan et al., 2020; Tang et al., 2017). 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; MMP, 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 (Kwok et al., 2022; Nemade et al., 2018; Nomura et al., 2017; 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 pathological irreversibly fragmented mitochondria (Hanawa et al., 2008; Hu et al., 2016; Kon et al., 2004; 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 rely on healthy animals which have a high metabolic reserve capacity and can easily adapt to moderate metabolic challenge 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; Fannin et al., 2010; Lee et al., 2013; Schirris et al., 2015b; Wagner et al., 2008). This review aims to provide an overview of these strategies. We propose implementation of a tiered systems pharmacology approach to aid 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, 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 (Beeson et al., 2010; Hynes et al., 2006; Hynes et al., 2009). The importance of measuring respiratory capacity of the mitochondrial energy generating system is based on the notion that virtually all mitochondrial bio-energetic pathways converge in the OXPHOS system. Moreover, OXPHOS complexes are often observed as important off-targets involved in adverse effects of drugs (Hargreaves et al., 2016; Nadanaciva et al., 2007) 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 is 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-H_2DCFDA, 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 (cyYFP), HyPer and reduction-oxidation sensitive green fluorescent protein (roGFP). It is important to note, that each of these probes can be used to get insight into the formation of ROS molecules, which are known to have different origins. For example, the primary mitochondrial ROS molecule $O_2^{\cdot -}$ 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 (Dussmann et al., 2017; Ferrick et al., 2008; Iannetti et al., 2016; Wagner et al., 2008; 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 (Megjhani et al., 2017; Valm et al., 2016). 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., 2019; Iannetti et al., 2016). 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 physiological system, as described in more detail in section 4, tier 4.

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 a 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 above, systems biology integrates data on multiple levels, including experimental (*e.g.,* mechanistic studies), omics and predictive bioinformatics datasets. 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 **Figure 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 (IEM) and mitochondrial disease (Bonte et al., 2019; Buzkova et al., 2018; Coene et al., 2018; Hoegen et al., 2021; Miller et al., 2015; Tebani et al., 2016a; Tebani et al., 2016b; 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), 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 biological systems. The three modeling approaches that are characteristically applied are discussed in detail in figure 4 and below. 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 above in section II. If model refinement is needed, the cycle is reinitiated. Systems biology typically employs top-down, bottom-up, or middle out models (**Figure 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 (Bludau and Aebersold, 2020; Rolland et al., 2014; Wan et al., 2015). The use of these 'omics' datasets enables the construction of biological 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.* (Maldonado et al., 2019; Suomalainen et al., 2011). 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, it is 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 (Cortassa et al., 2019; Klipp et al., 2009; Marin-Hernandez et al., 2020; Teusink et al., 2000) 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, 2020). The construction of genome-scale metabolic models (GEMs) using pre-existing databases combined with literature input are also powerful in modeling biological 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 (Brunk et al., 2018; Maldonado et al., 2019; O'Brien et al., 2015). As described above 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.,* GEM), 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 multi-omics approaches have already demonstrated to be a valuable tool in improving patient care (Maldonado et al., 2019). However, as the integration of large omics datasets can lead to modelling problems, methods such as similarity network fusion (SNF) have been developed to aggregate and analyze multiple complex omics datasets on a genomic scale (Wang et al., 2014). xMWAS is an approach that identifies associations and correlations between molecules based on multi-omics data and allows integration of more than two datasets (Hu et al., 2020; Uppal et al., 2018). Expansion of these knowledge bases, including xMWAS and MitoCarta, is an essential next step towards more efficient integration of multi-omics 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 biological 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 biological systems (Albert, 2007; Sun et al., 2018) (**Figure 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 (Kristensen et al., 2012; Ng et al., 2017; Soler-Lopez et al., 2011; Wang et al., 2018;

Zhang et al., 2014). 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 (Hartung et al., 2017; Kongsbak et al., 2014; Turner et al., 2015; Yahya et al., 2021). To evaluate the dynamic interaction between drugs and biological networks, physiochemical-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 predict 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 biological specimen goes beyond the scope of standard clinical laboratory techniques and allows precise analyses of hundreds to thousands of compounds. Application of techniques like liquid/gas-chromatography and mass-spectrometry (L/GC-MS) provide 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 benefited 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 (AAR) 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; van der Stel et al., 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, like the OXPHOS system, the TCA cycle, or metabolite transport, have been constructed (Bolaji O, 2018; Heiske et al., 2017; Wu et al., 2007). 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 (Huang et al., 2019; Kanehisa, 1997). Over the years, more human metabolic network models have become available, like Edinburgh Human Metabolic Network (EHMN; (Ma et al., 2007)), Human Metabolic Reaction (HMR; (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 steatogenic 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.,* poly-pharmacology). 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 (Ferreira et al., 2015; Xie et al., 2011). Although all examine binding pocket similarity, different methods are applied, like 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 anti-microbial 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, like 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, like 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 (SILAC), combined with affinity chromatography and mass spectrometry (MS) (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 biological 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 toxin-target associations described in scientific journals and electronic databases are included (Lin et al., 2021). It correlates chemical, biological 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 (PBPK) models, which demonstrated phenotype-specific cases of drug-induced metabolic perturbations (Cordes et al., 2018). Lastly, integration of data from experiments, modelling prediction, and exposure assessment in adverse outcome pathways (AOPs) has aided toxicological risk assessment, and demonstrated 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 chemicals and structurally related substances for potential mitochondrial respiratory chain-mediated neurotoxicity hazards was described by van der Stel *et al.* (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 pertubation of mitochondrial function (Watkins, 2020; Yang et al., 2015). 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 having a mitochondrial liability (Wills et al., 2015) and the serious consequences if this translates into mitochondrial toxicty (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 labour 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 pre-clinical 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 below, in **Figure 5** and **Table 2**. It is important to note that the proposal below 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 increases to the point where early chemistry decisions can be influenced to remove or significantly reduce the intrinsic hazard of mitochondrial activity.

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 are 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 percent of mitochondrial diseased children present with elevated venous lactate levels (Munnich 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 (Dykens 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 associate 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 percent 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 or not mitochondrial function is affected.

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 morphological structural changes and altered enzyme-based stainings (*e.g.,* cytochrome C oxidase (COX), 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 IC_{50}s (inhibitory concentration 50%) or MECs (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 above (*e.g.,* mitochondrial membrane potential, ROS, oxygen consumption measurements using the Seahorse platform) could also be used to provide 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 classical systems biology cycle (**Figure 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 above can be applied to explore mitochondrial drug off-targets.

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 (WES), resulted in the association of more than 1,500 nuclear genes with mitochondrial diseases (Goto et al., 1990; Lodi et al., 2000; McCormick et al., 2013; Panneman et al., 2020; Theunissen et al., 2018; Van Goethem et al., 2001; Wallace et al., 1988; Winterthun et al., 2005; Wortmann et al., 2015). 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 (Hallmann et al., 2016; Jonckheere et al., 2013; Koopman et al., 2012; Nouws et al., 2012; van den Heuvel et al., 1998), 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 mitochondrial activity, various cell biological methods could be applied, including the use of a knockout or overexpression model of common mitochondrial off-targets generated preemptively using techniques like 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 below.

Tier 4: Functional *in situ* studies *vs*. *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, biological properties at the cellular level are therefore preserved (Hu et al., 2019; Saada, 2011), potential tissue-specific differences in mtDNA heteroplasmy levels should be considered. A relevant addition are 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 (Hoefs et al., 2008; Jonckheere et al., 2011; Kirby et al., 2004; 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 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 3 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 enhanced 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 HA-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 sulphide, hydrogen peroxide and the mitochondrial membrane potential (Arndt et al., 2017; Cocheme et al., 2011; Logan et al., 2016; Shchepinova 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 development stages (see also **Figure 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 anti-obesity 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 modelling 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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AUTHOR CONTRIBUTIONS

Participated in research design: Hoogstraten, Schirris

Performed data analysis: Hoogstraten, Schirris

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FOOTNOTES

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FIGURE LEGENDS

Figure 1 | The mitochondrion and its main characteristics. Mitochondrial ATP production from glucose starts with the import of glycolytic pyruvate and conversion into acetyl-coenzyme A (acetyl-CoA) by the pyruvate dehydrogenase complex. Subsequently, acetyl-CoA enters the TCA cycle producing reduced nicotinamide adenine dinucleotide (NADH) and reduced flavin adenine dinucleotide ($FADH_2$), which function as substrates for the first and second multi-subunit enzyme complexes of the mitochondrial respiratory chain, respectively. This enables the transfer of protons from the mitochondrial matrix into the intermembrane space by the respiratory chain complexes I, III and IV, while oxygen is consumed at complex IV. The resulting electrochemical membrane potential is used by the F_1F_0-ATP synthase, also known as complex V, to generate the majority of cellular ATP from ADP. In addition, the matrix harbors various other metabolic pathways, placing mitochondria in the center of cellular catabolic and anabolic pathways. β-Oxidation accounts, for example, for the production of acetyl-CoA and NADH from fatty acids, imported through the carnitine transport system (Hoppel, 1982) and is used by the TCA cycle and OXPHOS system, respectively. Other metabolic pathways located in the mitochondrial matrix include heme biosynthesis, steroidogenesis (and the first steps of cholesterol synthesis), part of amino acid metabolism, iron-sulphur cluster assembly, part of gluconeogenesis, and calcium storage. Inside the mitochondrial matrix, mtDNA resides and may be subjected to damage from reactive oxygen species (ROS).

Figure 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 (~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, Tfam and mitochondrial single-strand DNA-binding protein (SSB). The non-coding displacement or D-Loop region acts as promotor 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 (CYP) enzymes that facilitate the conversion of xenobiotics into toxic and reactive metabolites, which could accumulate in the matrix, but also directly damage mitochondrial proteins, DNA, and lipids.

Figure 3 | Representation of the classical systems biology cycle. Systems biology strategies are typically initiated with biological data collection, which can originate from a variety of sources as indicated. Subsequently, collected data is integrated in computational models to simulate conditions of interest. Depending on available data and outcomes, different types of models can be applied, including top-down, bottom-up or middle-out modeling (also see Figure 4). Next, resulting predictions are experimentally verified *in vitro* and *in vivo*. After validation, new knowledge and insights originate and could also reinitiate the cycle to further adjust and refine the model. In this way, the predictive power of simulations can be improved.

Figure 4 | Overview of different types of systems biology approaches. Systems biology approaches can be categorized as either top-down, bottom-up or middle-out strategies. Top-down methods are initiated by the collection of big data sets often obtained using omics approaches (*e.g.,* proteomics, genomics, transcriptomics and metabolomics) to construct network models representing the interactions between genes, transcripts, proteins and metabolites in a biological system. In this modeling methodology, the nodes represent the molecular targets (*viz.* interacting molecules in a biological network). Interactions between nodes are represented by edges. Hubs are defined as nodes that pose interactions with other nodes. In contrast, bottom-up approaches use hypothesis-driven data and is applied to simulate a smaller set of interactions. For the generation of such dynamic models, biochemical data is preferentially used. This type of data is often modeled as ordinary and partial differential equations, which represent the dynamics of the molecular interactions involved. These models are based on experimental data, including Michaelis-Menten kinetics (as shown in the equation). 'v' describes the reaction rate, related to the substrate concentrations 's'. At saturating substrate concentration, V_{max} represents the maximum reaction rate, while K_m is the substrate concentration at which half V_{max} is reached. The middle-out strategy uses elements from both approaches in a dynamic network model that aims to implement data from different levels of complexity. This modeling strategy connects the network to the dynamic behavior of the system by describing how known interactions among defined elements determine the state of the elements, and how the whole system may change over time under different conditions (Albert, 2007).

Figure 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 to 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 to the lead optimization phase, in which also only a small number of compounds is considered. 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 drugs 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.

TABLES

Table 1. Overview of example drugs with FDA black box warnings for cardiovascular, renal and hepatic toxicity. Drugs for which the toxicity is related to mitochondrial liabilities are highlighted in grey. The reported toxic effects on mitochondrial function are indicated.

Drug class	Drug	Toxicity	Mitochondrial toxic effects	Reference
Alkylating agents	Cisplatin	Renal	Complex I and IV inhibition, declined MMP, low mtDNA, lower FAO, inhibition of protein synthesis	(Miller et al., 2010; Pereira et al., 2009; Santos et al., 2007; Zsengellér et al., 2012)
	Ifosfamide	Renal	Complex I inhibition	(Nissim et al., 2006)
Anesthetic	Bupivacaine	Cardiovascular	OCR reduction, mitochondrial swelling	(Hiller et al., 2013)
Antiarrhythmic	Amiodarone	Cardiovascular	Complex I inhibition, reduction in ATP, OXPHOS uncoupling, MMP dissipation	(Karkhanis et al., 2018)
	Disopyramide	Cardiovascular	-	
	Dofetilide	Cardiovascular	-	
	Ibutilide	Cardiovascular	-	
Anthracyclines	Daunorubicin	Cardiovascular	MMP dissipation, ROS elevation, lipid peroxidation, inhibition of topoisomerase II (mtDNA)	(Bloom et al., 2016; Luo et al., 2009; Wu et al., 2014)
	Doxorubicin	Cardiovascular/ renal	Loss of cytochrome C, downregulation TCA protein expression, lipid	(Benzer et al., 2018; Brandão et al., 2021;

			peroxidation, decreased mtDNA content, oxidative stress	Gnanapragasam et al., 2007; Lahoti et al., 2012; Lebrecht et al., 2010; Oz et al., 2006; Pereira et al., 2016)
	Epirubicin	Cardiovascular	Nitrosative stress	(Guven et al., 2007)
	Idarubicin	Cardiovascular	Mitochondrial swelling, inhibition of antioxidant enzymes, ROS elevation, lipid peroxidation, inhibition of topoisomerase II (mtDNA)	(Bloom et al., 2016; Kalender et al., 2002)
Antibiotics	Gentamicin	Renal	Decreased MMP, reduced mtDNA, ROS elevation, complex II inhibition	(Chen et al., 2017b; Gai et al., 2020)
	Isoniazid	Hepatic	ROS elevation through complex I-III inhibition, increased lipid peroxidation, dissipation MMP, mitochondrial swelling, cytochrome C release	(Ahadpour et al., 2016)
	Ketoconazole	Hepatic	Complex I-IV inhibition, ATP depletion, decreased mtDNA, decreased MMP, superoxide accumulation	(Haegler et al., 2017; Rodriguez and Acosta, 1996)
	Streptozocin	Hepatic	-	
	Trovafloxacin	Hepatic	-	

Anti-cancer	Arsenic trioxide	Cardiovascular	Structural mitochondrial damage, abnormal mPTP opening, ROS elevation, downregulation mitochondrial biogenesis	(Zhang et al., 2018a)
	Cetuximab	Cardiovascular	-	
	Dacarbazine	Hepatic	-	
	Denileukin diftitox	Cardiovascular	-	
	Flutamide	Hepatic	MMP dissipation, ATP depletion, complex I inhibition	(Ball et al., 2016; Coe et al., 2007; Fau et al., 1994; Zhang et al., 2018b)
	Gemtuzumab	Hepatic	-	
	Mitoxantrone	Cardiovascular	-	
	Methotrexate	Hepatic	-	
	Pentostatin	Hepatic	-	
	Tamoxifen	Cardiovascular/ hepatic	OXPHOS uncoupling, inhibition of complex III and IV, inhibition FAO, mtDNA depletion	(Gudbrandsen et al., 2006; Larosche et al., 2007; Lelliott et al., 2005; Satapathy et al., 2015; Tuquet et al., 2000)
Antivirals	Abacavir	Hepatic	Inhibition of mtDNA polymerase gamma	(Brinkman and Kakuda, 2000)
	Didanosine	Hepatic	mtDNA depletion and inhibition of mtDNA polymerase gamma	(Igoudjil et al., 2006; Mihajlovic and Vinken, 2022;

				Walker et al., 2004)
	Emtricitabine	Hepatic		
	Entecavir	Hepatic		
	Emtricitabine	Hepatic		
	Lamivudine	Hepatic	mtDNA depletion and inhibition of mtDNA polymerase gamma	(Igoudjil et al., 2006)
	Nevirapine	Hepatic	MMP dissipation,	(Paemanee et al., 2017)
	Telbivudine	Hepatic		
	Tipranavir	Hepatic		
	Stavudine	Hepatic	mtDNA depletion and inhibition mtDNA polymerase gamma	(Walker et al., 2004)
	Zalcitabine	Hepatic	Depletion of mtDNA	(Walker et al., 2004)
	Zidovudine	Hepatic	Mitochondrial swelling, inhibition complex II, MMP dissipation, loss of cytochrome C, ROS elevation, mtDNA depletion	(Elimadi et al., 1997; Igoudjil et al., 2006; Lewis et al., 1994; Mihajlovic and Vinken, 2022; Scruggs and Dirks Naylor, 2008)
Beta-blockers	Atenolol	Cardiovascular	Mitochondrial swelling, inhibition complex II, MMP dissipation, loss of cytochrome C, ROS elevation	(Seydi et al., 2020)

CNS agents	Amphetamines	Cardiovascular	Impaired OXPHOS, ROS elevation	(Chen et al., 2017a)
	Atomoxetin	Cardiovascular	-	
	Dantrolene	Hepatic	-	
	Droperidol	Cardiovascular	-	
	Felbamate	Hepatic	-	
	Methamphetamine	Cardiovascular	-	
	Naltrexone	Hepatic	-	
	Nefazodone	Hepatic	Inhibition of complex I and IV, collapse mitochondrial membrane potential, imposed oxidative stress	(Dykens et al., 2008)
	Pergolide	Cardiovascular	-	
	Valproic acid/ Divalproex sodium	Hepatic	mPTP opening, inhibition FAO enzymes and sequestration FAO cofactors	(Aires et al., 2010; Li et al., 2015; Silva et al., 2008)
Diabetes	Pioglitazone	Cardiovascular	Mitochondrial swelling, MMP dissipation, loss of cytochrome C, ROS elevation	(Seydi et al., 2020)
	Rosiglitazone	Cardiovascular	Inhibition of complex I and IV, uncoupling OXPHOS, increase mitochondrial oxidative stress, impairment mitochondrial bioenergetics	(He et al., 2014)
Hypertension	Bosentan	Hepatic	-	
Immunosuppressants	Cyclosporin A	Renal	Decreased MMP, ROS elevation, increased mitochondrial fission, liberation of cytochrome C	(de Arriba et al., 2013)

NtRTIs	Tenofovir	Hepatic/Renal	Inhibition mtDNA polymerase gamma	(Kohler et al., 2009)
NSAIDs	Celecoxib	Cardiovascular	Mitochondrial swelling, inhibition complex IV, reduction in ATP content, MMP dissipation, decreased antioxidant enzyme level, ROS elevation, lipid peroxidation	(Atashbar et al., 2022; Salimi et al., 2019)
	Diclofenac	Cardiovascular	Mitochondrial swelling, complex II and III inhibition, reduction in ATP content, OXPHOS uncoupling, MMP dissipation, decrease antioxidant enzyme level, ROS elevation, lipid peroxidation, inhibition of adenine nucleotide translocase (ANT)	(Brandolini et al., 2020; Ghosh et al., 2016a; Khezri et al., 2020; Moreno-Sánchez et al., 1999; Salimi et al., 2019; Thai et al., 2021)
	Diflunisal	Cardiovascular	-	
	Etodolac	Cardiovascular	-	
	Fenoprofen	Cardiovascular	-	
	Ibuprofen	Cardiovascular	OXPHOS uncoupling	(Satapathy et al., 2015)
	Indomethacin	Cardiovascular	Reduction in ATP content, OXHPOS uncoupling, MMP dissipation	(Moreno-Sánchez et al., 1999)
	Ketoprofen	Cardiovascular	-	
	Mefenamic acid	Cardiovascular	Induction of mPTP opening	(Olszewska and Szewczyk, 2013;

				Uyemura et al., 1997)
	Meloxicam	Cardiovascular	Reduction in ATP content, OXHPOS uncoupling, MMP dissipation	(Moreno-Sánchez et al., 1999)
	Naproxen	Cardiovascular	Mitochondrial swelling, complex I and II inhibition, reduction in ATP content, MMP dissipation, decrease antioxidant enzyme level, ROS elevation, lipid peroxidation	(Ghosh et al., 2016b; Salimi et al., 2019)
	Nabumetone	Cardiovascular	-	
	Oxaprozin	Cardiovascular	-	
	Piroxicam	Cardiovascular	Reduction in ATP content, OXHPOS uncoupling, MMP dissipation	(Moreno-Sánchez et al., 1999)
	Salsalate	Cardiovascular	-	
	Sulindac	Cardiovascular	Mitochondrial uncoupling, membrane dissipation, ATP depletion	(Leite et al., 2006)
	Tolmetin	Cardiovascular	-	

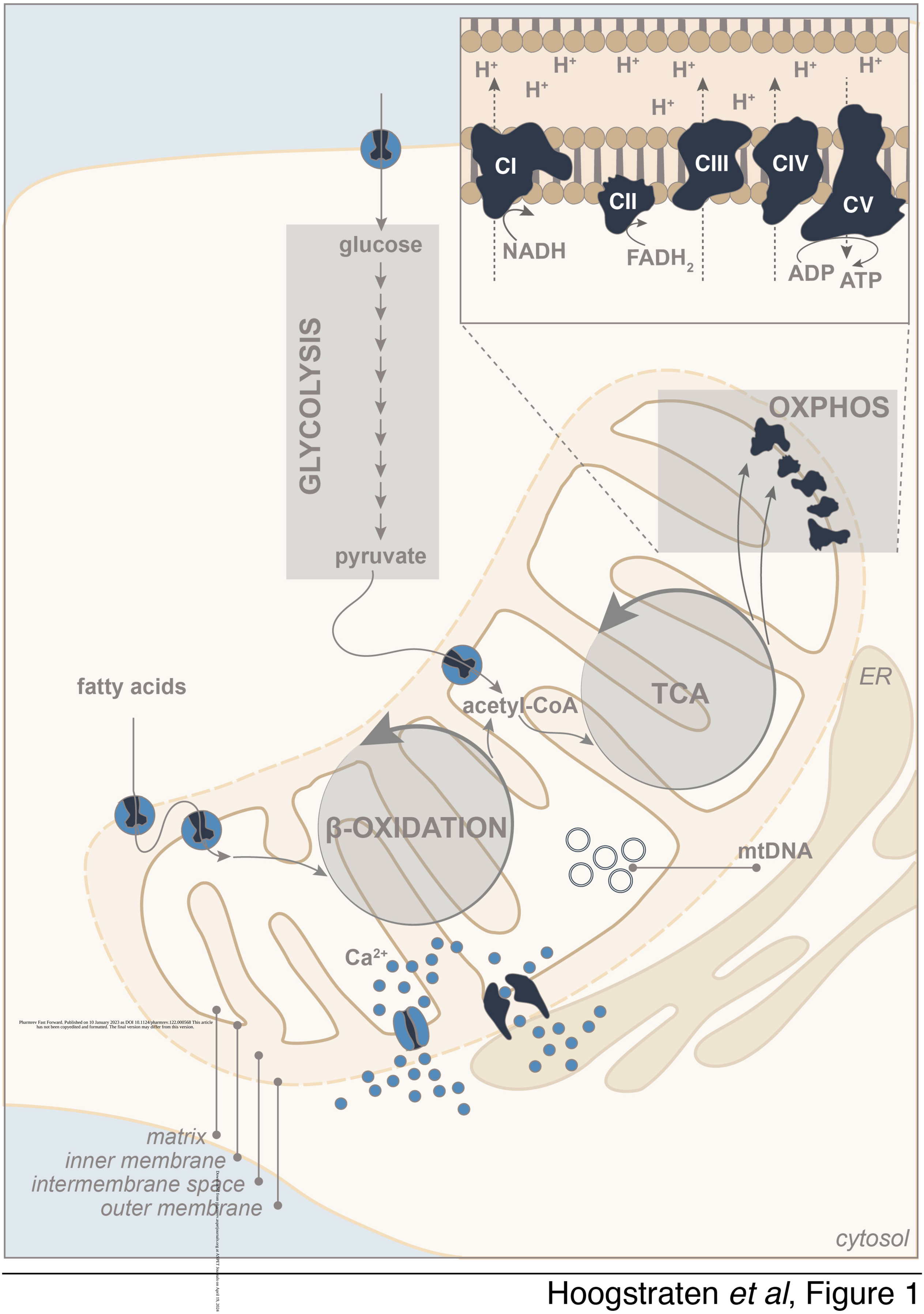
Abbreviations: ATP: adenosine 5'-triphosphate, CNS: central nervous system, FAO: fatty acid β-oxidation, MMP: mitochondrial membrane potential, mPTP: mitochondrial permeability transition pore, mtDNA: mitochondrial DNA, NSAID: non-steroidal anti-inflammatory drug, NtRTI: nucleotide reverse transcriptase inhibitor, OCR: oxygen consumption rate, OXPHOS: oxidative phosphorylation, ROS: reactive oxygen species, TCA: tricarboxylic acid.

Table 2. Overview of example assays that can be applied in our proposed tiered evaluation of drug-induced mitochondrial dysfunction, based on methodologies used in the clinical investigation of inherited mitochondrial disease.

	Evaluation strategy	**Assay examples**
Tier 1	Phenotypic observations	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).
Tier 2	Clinical chemistry	Drug concentration-response curves (IC_{50}/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 flowcytometry, *e.g.* MitoTracker Green FM), ROS production (fluorescence, *e.g.* H_2DCFDA and MitoSOX), network or structure-based model construction (*e.g.* 'omics' or X-ray protein structures/pharmacophore prediction using ProBiS/KRIPO).
Tier 3	Cellular studies	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).
Tier 4	*In vivo* studies	*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).

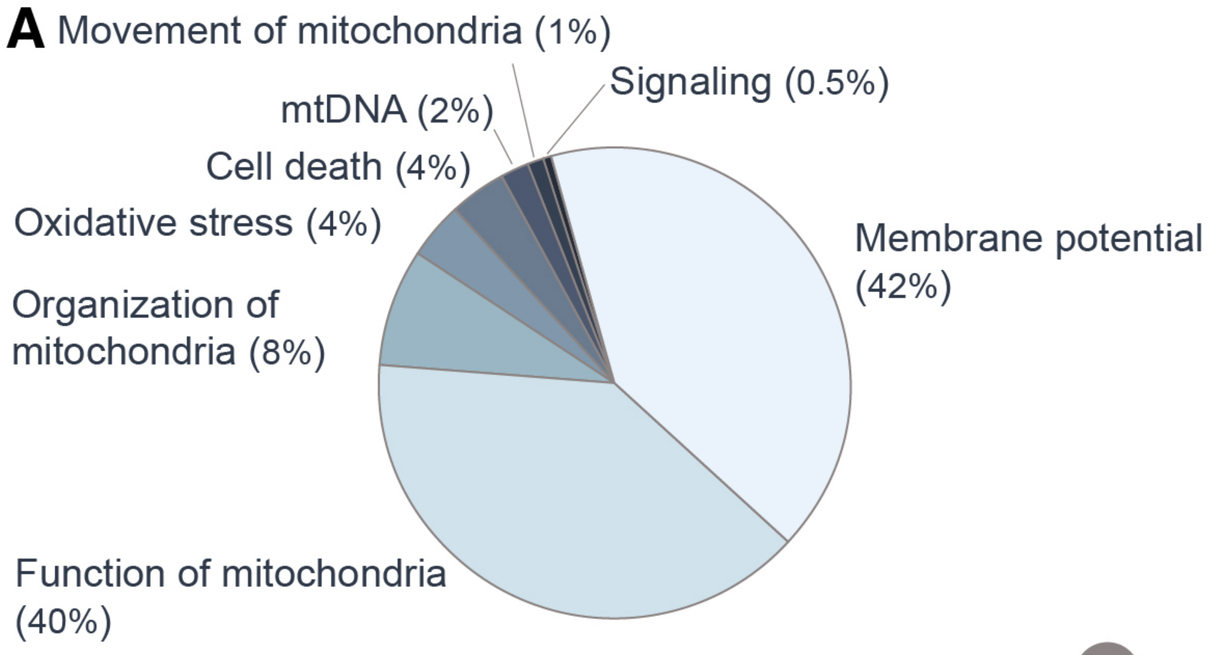
Abbreviations: ATP: adenosine 5'-triphosphate, BN-PAGE: blue native polyacrylamide gel electrophoresis, CRISPR: clustered regularly interspaced short palindromic repeats, H_2DCFDA: 2',7'-dichlorodihydrofluorescein diacetate, IC_{50}: inhibitory concentration 50%, KRIPO: key representations

of interaction in pockets, MEC: minimal effect concentration, MMP: mitochondrial membrane potential, MS: mass spectrometry, NADH: nicotinamide-adenine dinucleotide reduced form, OCR: oxygen consumption rate, OXPHOS: oxidative phosphorylation, RNAi: RNA interference, ROS: reactive oxygen species, shRNA: short hairpin RNA, siRNA: small interfering RNA, TCA: tricarboxylic acid, TMRM: tetramethyl rhodamine methyl ester.



Hoogstraten *et al*, Figure 1

A



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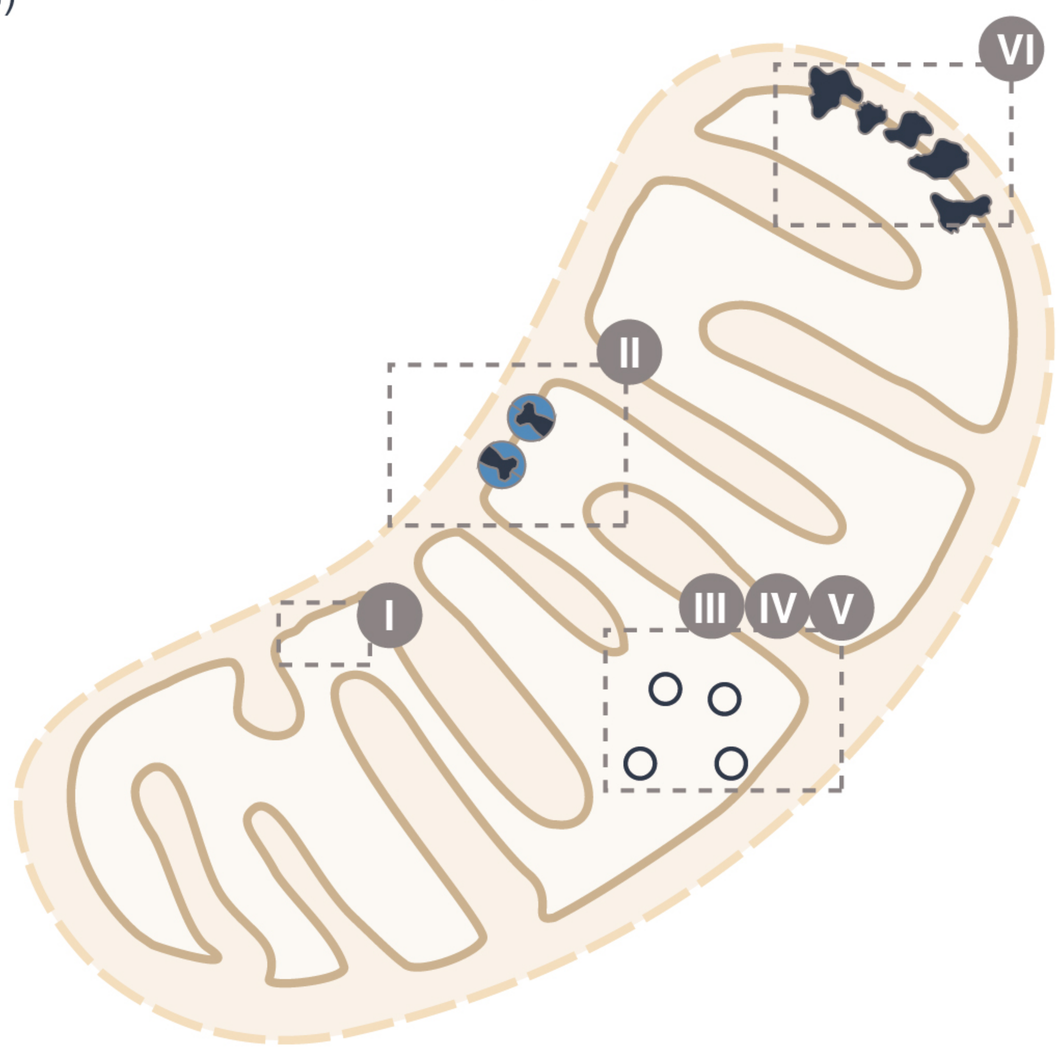
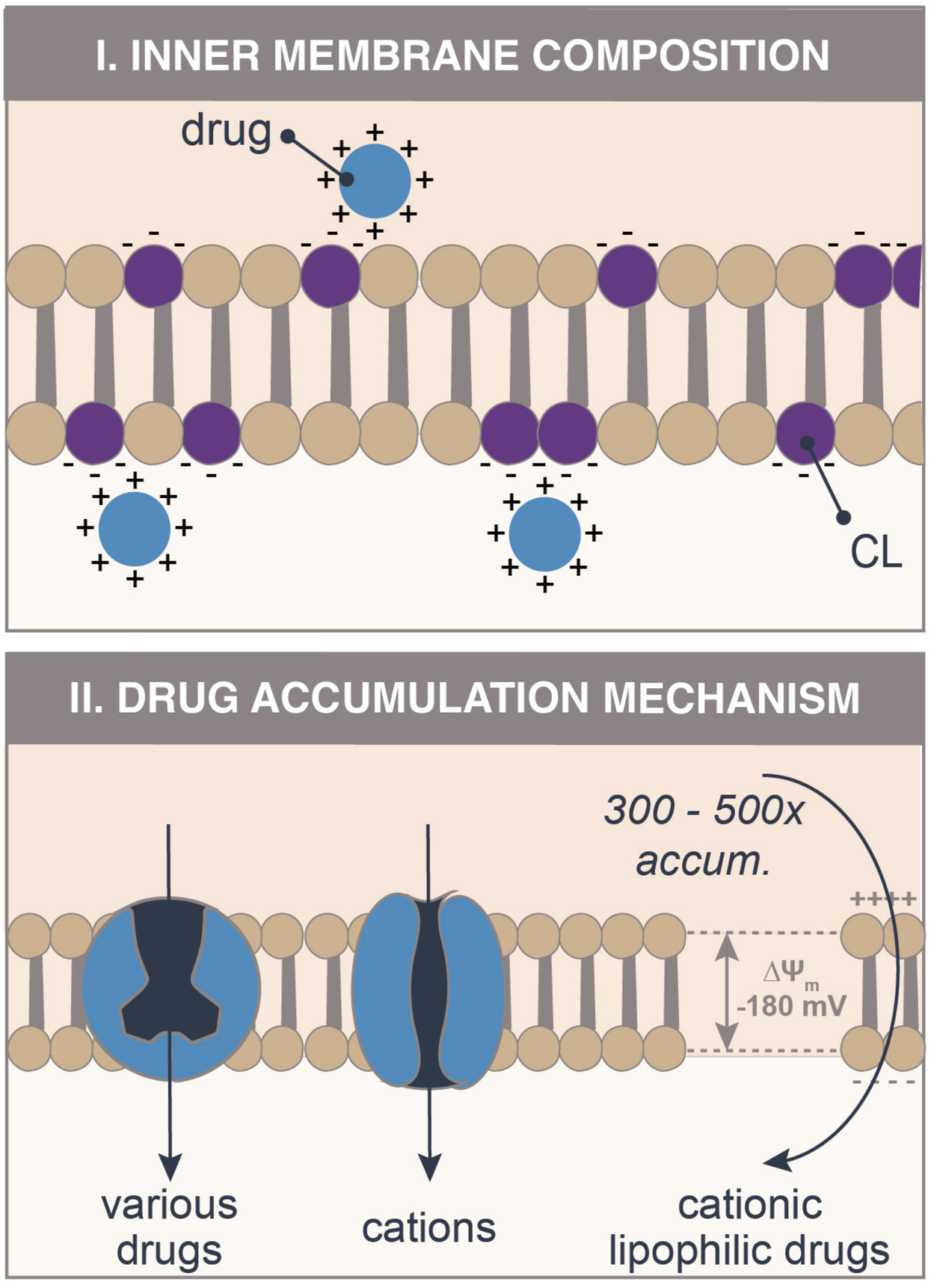
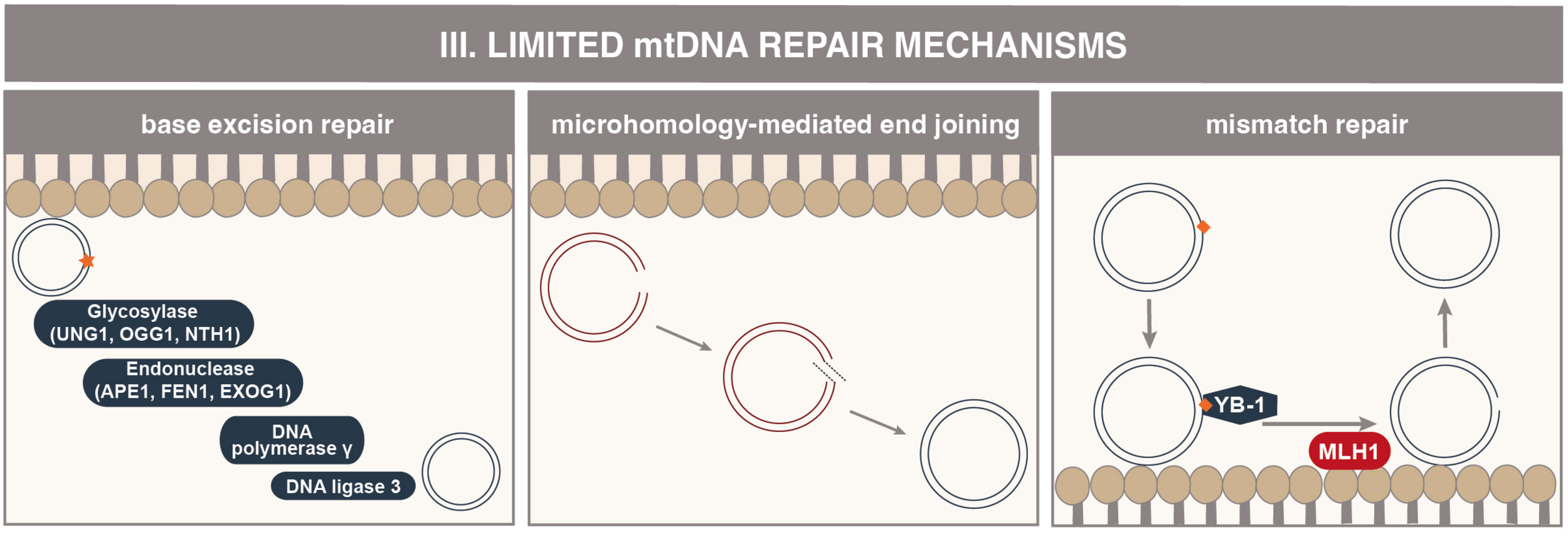
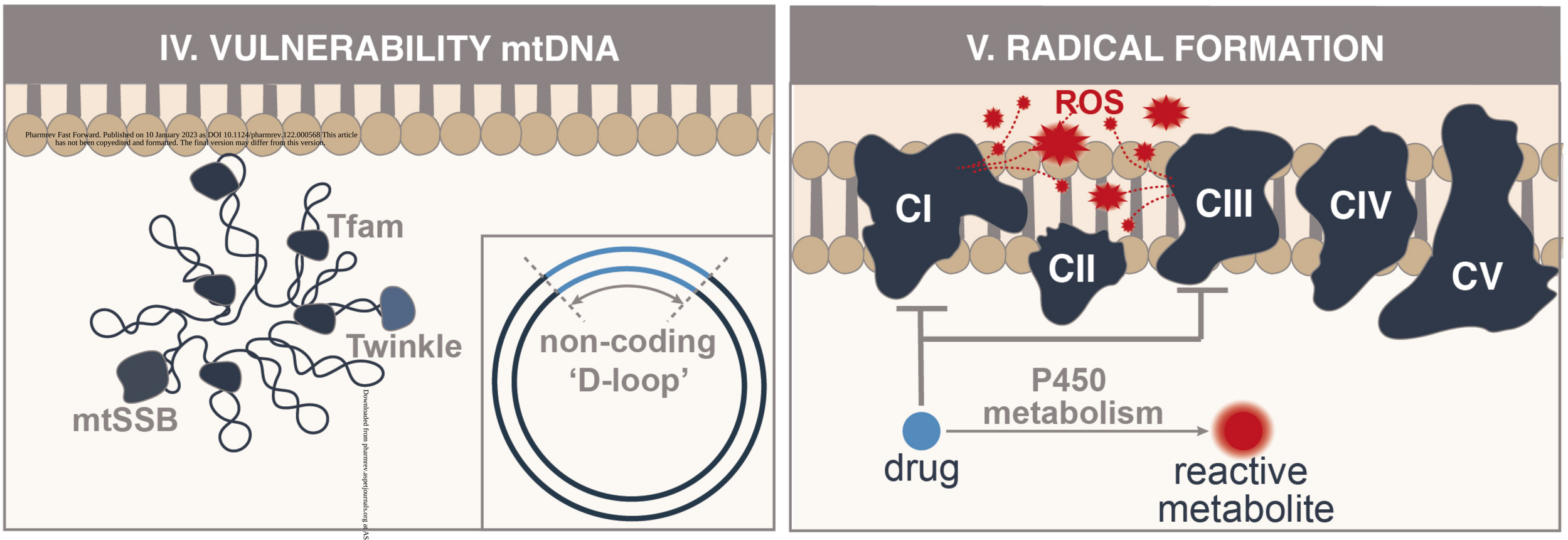
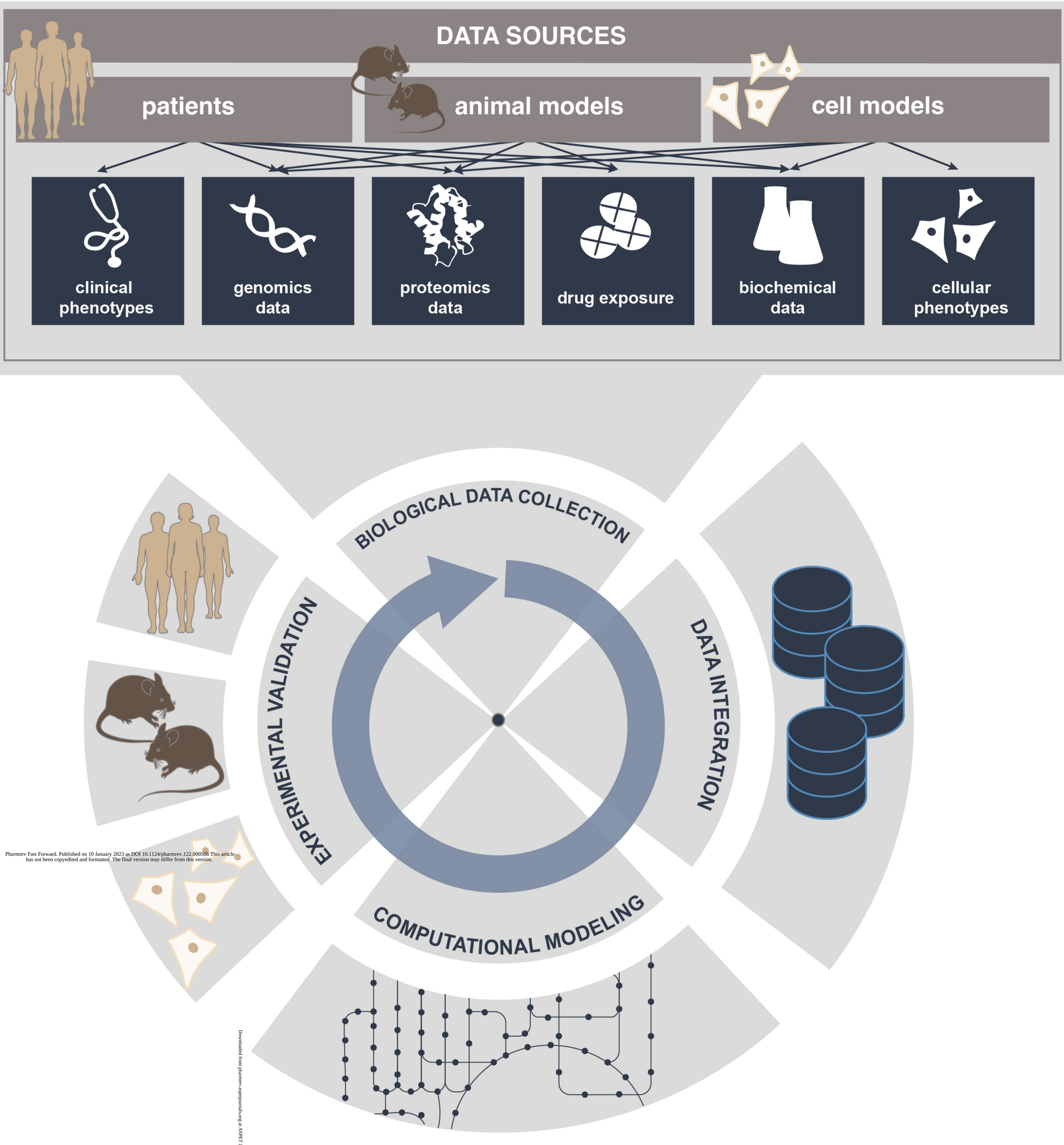








Hoogstraten *et al*, Figure 2



Hoogstraten *et al*, Figure 3

APPROACHES IN SYSTEMS BIOLOGY

	top-down strategy	middle-out strategy	bottom-up strategy
data acq.	**holistic** (*i.e.* **'omics')**	**holistic + hypothesis-driven**	**hypothesis-driven** (***i.e.*** **metabolic pathways)**
data type	proteomics, transciptomics, metabolomics, genomics	[metabolite], enzyme activities, drug-drug interactions, protein-protein interactions, 'omics', metabolite flux	metabolite flux, enzyme activities, [metabolite], drug-drug interactions, protein-protein interactions
	network model	dynamic network model	dynamic model
modeling app.	holistic	holistic	holistic
modeling methodology	hub (*e.g. gene, protein*); node (*e.g. gene, protein*); edge (*e.g. chemical transformation*)	V_1 … V_8	$S_0 \xrightarrow{V_1} S_1$; $S_0 \xrightarrow{V_2} S_2 \xrightarrow{V_3} S_3$; $S_3 \xrightarrow{V_4} S_4$; $S_3 \xrightarrow{V_5} S_5$; $V_n = \frac{V_{max}[S_{n-1}]}{K_m + [S_{n-1}]}$

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