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The significance of mitochondrial toxicity testing in drug development

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Mitochondrial dysfunction is increasingly implicated in the etiology of drug-induced toxicities. Members of diverse drug classes undermine mitochondrial function, and among the most potent are drugs that have been withdrawn from the market, or have received Black Box warnings from the FDA. To avoid mitochondrial liabilities, routine screens need to be positioned within the drug-development process. Assays for mitochondrial function, cell models that better report mitochondrial impairment, and new animal models that more faithfully reflect clinical manifestations of mitochondrial dysfunction are discussed in the context of how such data can reduce late stage attrition of drug candidates and can yield safer drugs in the future.

Attrition of drugs causes humanitarian and financial losses

Of the new drugs approved by the US Food and Drug Administration (FDA) between 1994 and 2006, 38 were later withdrawn from the market because of safety concerns, the majority being hepatotoxic and cardiotoxic (Figure 1) [1]. Although this represents only 2–3% of the total, such failures to predict adverse drug reactions result in unacceptable human suffering, erode trust in the regulatory process and pharmaceutical industry, and impose immense financial losses.

A decade ago, 40% of drug-development programs were abandoned pre-clinically because of poor pharmacokinetics (PK). Much effort has improved understanding of PK so that today the number one reason for pre-clinical attrition of nascent drugs is organ toxicity [1]. Similarly, pre-clinical drug failure due to lack of efficacy has also drastically declined because of improved *in vitro* and animal models. However, this has not been the case for drug safety where outdated tools and discordance between animal models and the clinic highlight the weaknesses of our predictive capabilities [2,3].

How is it that toxicity often remains undetected until large numbers of patients have been exposed? First, toxicity is often missed precisely because it is such a rare occurrence, and, as such, might not be revealed by even a large Phase III trial; if a particular drug yields an adverse event in 0.1% of patients, more than 10 000 patients would have to be exposed before the probability of the event occurring becomes realistic. Secondly, the nascent drug would have been discontinued had the animal models revealed its toxicity before the clinic [4]. As such, toxicity is unanticipated and can remain undetected until severe or frequent enough to rise above the noise.

The notion that mitochondrial impairment could be an inadvertent 'off-target' effect of drug exposure that contributes to the etiology of various organ toxicities has only recently become more widely acknowledged. In fact, ample evidence indicates that mitochondrial dysfunction played a role in the toxicity that forced the withdrawal of troglitazone, cerivastatin, and tolcapone, though the latter is again on the market with severe restrictions (Figure 1) [5–15]. This review covers possible clinical signs of mitochondrial toxicity, improvements in pre-clinical assays that have helped illuminate possible mechanisms, and how such assays fit within the drug-development process better to predict toxicity, correspondingly reducing late stage drug attrition and improving drug safety.

Function of normal mitochondria

The dominant function of mitochondria is the production of >90% of the cell's energy in form of adenosine triphosphate (ATP). This double-membraned organelle contains its own DNA

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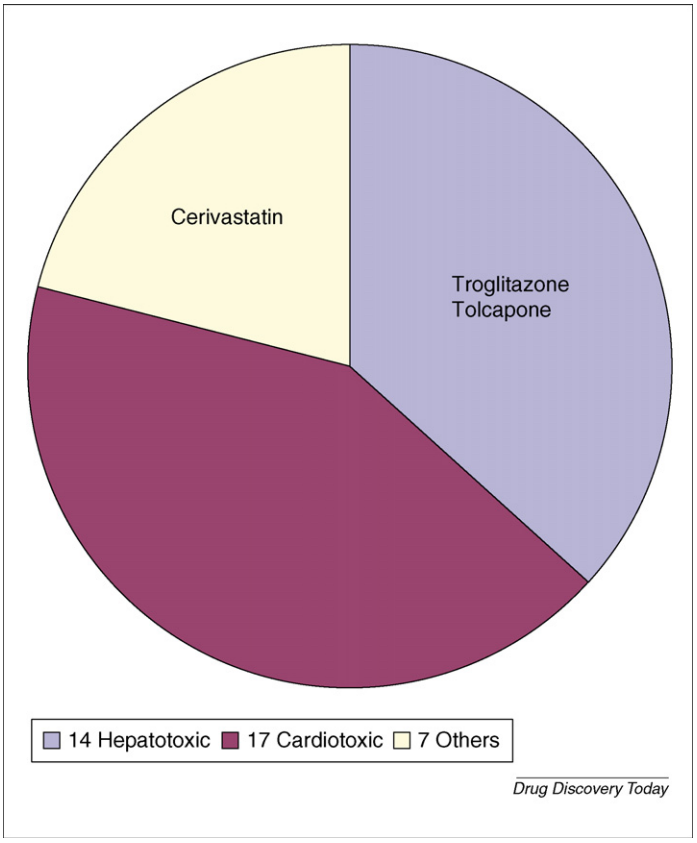

FIGURE 1

Of the 38 drugs withdrawn from the market between 1994 and 2006, the majority was toxic to liver or heart. To date, mitochondrial impairment has been implicated in the observed toxicities by cerivastatin, troglitazone, and tolcapone.

(mitochondrial, or mtDNA) with a genetic code that differs from that in nuclear DNA. Mitochondria also contain the requisite mechanisms to replicate and express their genome. Mitochondria replicate independently of cell division with a typical half-life of five days to several weeks depending on the tissue. Although mitochondria are classically depicted as individual organelles of various shapes, including spherical, rod-like, or kidney bean, *in vivo* mitochondria often merge to form a reticulated network. Contrary to popular belief, mitochondrial phenotype varies widely in different tissues, though the basic coupling of ETC (electron transport chain) with phosphorylation of ADP to form ATP (oxidative phosphorylation, or OXPHOS) is highly conserved [16].

The substrates used to generate ATP include breakdown products of glucose and fatty acid metabolism that will ultimately be oxidized to water by the electron transport system (ETS). The outer mitochondrial membrane is freely permeable to a wide variety of substrates and metabolites, whereas the inner membrane, where ETS is located, is highly impermeable, necessitating specific protein carriers to translocate molecules. For example, long-chain fatty acids are imported by binding to coenzyme A and carnitine. In the matrix, pyruvate and fatty acids are converted to acetyl-CoA, which enters the Krebs cycle. Electrons are sequentially removed in this cycle, and these reducing equivalents cascade down the electron transport system consisting of four redox active respiratory complexes. The energy released during this process pumps protons across the inner membrane into the intramembrane compartment resulting in an inside-out negative membrane potential ($\Delta\Psi$) that is used to phosphorylate ADP to ATP [16]. Many lipophilic cationic drugs can accumulate in the matrix in response to $\Delta\Psi$ greatly amplifying their local concentration.

Other functions of mitochondria are related to the cell type in which they are found. Mitochondria are involved in hemoglobin synthesis, production of estrogen, and testosterone and are required for cholesterol metabolism. Mitochondrial function is physiologically regulated by complex interactions, reflecting bioenergetics within the cell as well as external influences (Figure 2). For example, mitochondrial biomass increases in response to exercise, various uncoupling proteins responsive to adenylate state are implicated in the dissipation of the transmembrane potential as heat, and nitric oxide acts an endogenous regulator of OXPHOS [17–19]. Conversely, mitochondrial dysfunction is induced by physiologically relevant factors, including excessive Ca^{2+} loading and exposure to reactive nitrogen-centered and oxygen-centered free radicals (reactive oxygen species, ROS). For example, when exposed to elevated Ca^{2+}, mitochondria can undergo an irreversible collapse of membrane potential that releases various apoptotic initiators. Such 'permeability transition' (PT) is attributed to formation of a 'pore' made up of several proteins, including adenine nucleotide translocator (ANT), among others. PT is inhibited by cyclosporine A and several inhibitors of ANT. It should be noted, in this context, that rapid loss of $\Delta\Psi$ need not always be due to PT; any loss of the impermeability of the inner membrane to protons will dissipate $\Delta\Psi$ and result in diminished ATP production (Figure 2) [20,21].

Drug-induced mitochondrial toxicity

Mitochondrial replication requires the capacity to replicate and express mtDNA, and mitochondrial function depends predominantly on the impermeability of the inner membrane and the catalytic integrity of the respiratory complexes. It was not until the launch of NRTIs (nucleotide reverse transcriptase inhibitors) that mitochondrial toxicity was widely accepted as causing organ toxicities. Many NRTI's inhibit the polymerase that replicates mtDNA, thereby preventing mitochondrial replication. This gradually reduces mitochondrial function in various tissues, resulting in muscle and liver toxicity, as well as lipodystrophy and lipoatrophy [22–24]. Other drugs, such as certain aminoglycoside antibiotics, induce long-term mitochondrial dysfunction by impairing protein synthesis in the organelle, resulting in ototoxicity and nephrotoxicity [25].

In addition to these longer term effects, other drugs, such as some fibrates and thiazolidinediones, also acutely undermine mitochondrial function by directly inhibiting ETC [26–28], or by uncoupling electron transport from ATP synthesis, as shown by NSAIDs [29,30]. In both cases, $\Delta\Psi$ is dissipated, and ATP production is diminished, or abolished, depending on severity. Other drugs, such as acetaminophen, doxorubicin, and ethanol impose oxidative stress via redox cycling or via glutathione depletion, cyp2E1-derived ROS generation, or reactive metabolite formation [31–33]. Such free radicals can directly inactivate several of the electron transport complexes, but more circuitous routes to mitochondrial failure are also important. For example, acetaminophen-induced hepatotoxicity is forestalled *in vivo* by leflunomide, implicating c-jun NH_2-terminal protein kinase (JNK) activation and subsequent Bcl-2 and Bcl-xL inactivation in the

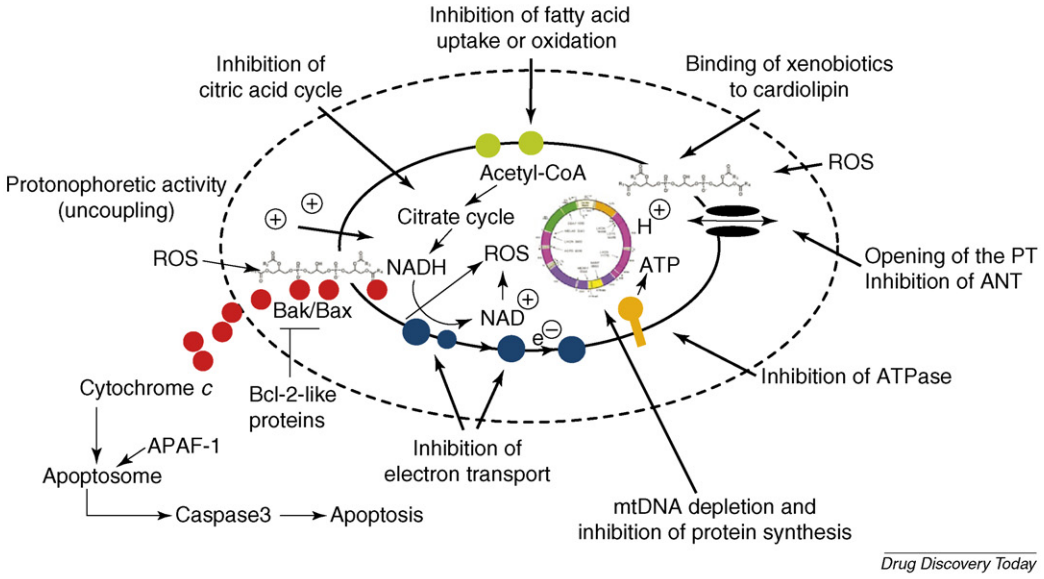

FIGURE 2

Mitochondrial function can be inhibited in many ways in addition to inhibition of electron transport and uncoupling of it from the membrane potential. For example, impairment of exchange of requisite substrates via membrane transporters, inhibition of metabolic pathways that fuel respiration, and direct effects of drugs on cardiolipin can acutely undermine mitochondrial function. Such acute effects frequently accelerate autoxidation of electron transport components that yields oxygen-centered and nitrogen-centered free radicals. By contrast, drugs that impair DNA replication or protein synthesis will diminish mitochondrial and hence bioenergetic capacity over a longer time period. In addition, many drugs can precipitate irreversible mitochondrial collapse via formation of the permeability transition pore leading to release of pro-apoptotic factors such as cytochrome *c*. Drugs that alter the normal equilibrium between pro-apoptotic and anti-apoptotic proteins, such as Bak/Bax and Bcl-2, among many others, can also induce mitochondrial failure.

mitochondrial failure [34]. Some thiazolidinediones and statins induce mitochondrial permeability transition leading to irreversible collapse of the transmembrane potential and release of pro-apoptotic factors [5–10], and such impairment can be prevented by Bcl-xL overexpression [11]. In addition, inhibition of metabolic pathways, such as fatty acid p-oxidation and Kreb's cycle, that fuel OXPHS will impair ATP production [35,36], as will inhibition of any of the many mitochondrial membrane transporters that exchange metabolites across the impermeable inner membrane [37].

Clinical presentation of drug-induced mitochondrial toxicity

Mitochondrial impairment typically affects the most aerobically poised tissues, such as kidney and heart, or tissues exposed to higher concentrations of the drug, such as the liver, because of hepatoportal absorption of oral drugs and robust capacity for bioactivation. Similarly, bio-accumulation of statins by fast twitch skeletal muscle cells puts this fiber type at particular risk of mitochondrially induced rhabdomyolysis [38].

As mitochondrial function declines, cells respond to the loss of ATP production capacity by accelerating glycolysis, resulting in increasing production of lactate. As a result, serum lactic acidosis is a hallmark of mitochondrial impairment. Clinical signs of lactic acidosis include initial gastrointestinal symptoms such as nausea, vomiting, and abdominal pain, and often malaise is also present. Lactic acidosis can be presaged by dyspnea or tachypnea, and laboratory findings show increases in anion gap, lactic acid levels, and changes in lactate/pyruvate.

Insights into the connections between lactic acidosis and mitochondrial impairment metabolic dysregulation were accelerated by studies of long-term effects of antiviral therapies that interfere with mitochondrial replication, most of which now carry Black Box warnings (see below) [39]. In a continuum, adverse responses to other drugs, such as cerivastatin, forced examination of toxicity and/or were withdrawn from the market when sporadic but severe organ toxicity to heart, liver, or muscle became apparent. In many cases, mitochondrial failure underlies this toxicity, or contributes to it. Similarly, sporadic, but relatively benign, adverse events have fostered expanded evaluation of mitochondrial impairment by members of drug classes containing drugs that have been withdrawn or received a Black Box warning. Thus, the clinical signs of drug-induced mitochondrial impairment range from obvious and severe to more subtle reflections of modest loss of mitochondrial capacity such as exercise intolerance, malaise, and mild lactic acidosis.

In this light, increased serum liver enzymes, lactic acidosis, and sporadic liver failure are readily attributable to the threshold nature of progressive mitochondrial failure. Cells can tolerate diminished mitochondrial membrane potential, as long as a minimal capacity is maintained. As loss of mitochondrial capacity approaches this threshold, the cell is increasingly unable to respond to stressors, that is, physiological scope is progressively lost, but the cell remains viable. However, once the threshold of insufficient mitochondrial capacity is exceeded, the cell precipitously fails, succumbing to apoptosis or necrosis, depending on the rate of decline. The notion that such catastrophic failure was merely the end stage of a gradual progression was obscured by the

efforts to elucidate why one patient was afflicted, while others were clinically acceptable. Such a model also explains the lack of correlation between toxicity and drug dose. Clearly, previous organ history and genetics are also prime determinants of the sporadic and/or idiosyncratic appearance of frank toxicity, but these are merely contributors that conspire to reflect the underlying loss of mitochondrial capacity. Indeed, polymorphisms in manganese superoxide dismutase (Mn-SOD), the mitochondrial form of the enzyme, are more susceptible to drug-induced liver injury [40]. The genetic component in establishing such a threshold is not restricted to nuclear genes, and mitochondrial genotype can also alter susceptibility. For example, individuals with an A1555G mutation in mtDNA are unusually susceptible to aminoglycoside-induced ototoxicity [25], and lactic acidosis has been reported in patients carrying mtDNA polymorphisms in the16SrRNAgene [41].

Pre-clinical detection of drug-induced mitochondrial toxicity

Appreciation of the importance of drug-induced mitochondrial toxicity has recently accelerated, fostered by the development of techniques conducive to drug-development efforts. For example, the vast majority of immortalized cell lines used to evaluate drug toxicity pre-clinically are derived from cancers and are typically grown under culture conditions of supraphysiological glucose concentrations [42,43]. However, despite the presence of metabolically competent mitochondria, cells grown under these conditions derive ATP almost exclusively via glycolysis, not via oxidative phosphorylation. Oxygen consumption in these cells is characteristically low and rates of media acidification, a direct reflection of excessive glycolytic flux yielding lactate, correspondingly elevated. As such, these anaerobically poised cells are resistant to xenobiotics that impair mitochondrial function and grow quite well in the presence of rotenone, antimycin, oligomycin, and many other mitochondrial toxicants [42,43].

To generate cell models that are capable of detecting mitochondrial impairment, Marroquin *et al*. replaced glucose in the growth media with galactose. The latter requires an investment of two ATPs in order to enter glycolysis. Since complete glycolysis to pyruvate (or lactate) yields two ATPs, the net yield with galactose is zero. In these circumstances, the cells must use OXPHOS in order to obtain ATP. Cells grown in galactose become susceptible to mitochondrial toxicants, and correspondingly reveal a wide variety of drugs that impair and/or uncouple OXPHOS [43].

Mitochondrial status has classically been monitored via oxygen consumption using low-throughput polarographic electrodes. More recently, a higher throughput method has been developed that uses a phosphorescent probe in 96-well format to report mitochondrial respiration [44,45]. Although suitable for intact cells, this technology has primarily been used with isolated mitochondria. An assay using isolated organelles potentially overpredicts drug effects because compounds have unrestricted access and not subject to metabolism (either activation or degradation). Nevertheless, data from this assay indicate that many drugs with various adverse events are acute mitochondrial toxicants, and potency tracks well with severity and frequency of such events.

Several other techniques have been used for drug discovery campaigns that could easily be adapted for mitochondrial toxicity screening. Fluorescent dyes that report $\Delta\Psi$ are readily adapted to 96 (or higher) well formats, either singly or in fluorescence resonance energy transfer formats [47–49], though these assays cannot distinguish whether such a loss is due to inhibition, uncoupling, or mitochondrial permeability transition (MPT). Indeed, MPT is induced by many anticancer drugs and is probably not only a key event in the therapeutic effect (killing cells) but also a key contributor to concomitant toxicities, such as described for tamoxifen [50]. Similarly, some thiazolidinediones have been reported to induce the MPT, which at the least contributes to the observed hepatotoxicity [8]. Assays have also been developed to detect MPT specifically [51,52].

Electron transport components can be directly inhibited by xenobiotics, as is the case for several fibrates and biguanides [27,44,53]. High-throughput measurements of respiratory complex activities are now possible using immunocapture technology, facilitating detection of such direct drug effects [28,46]. In addition, inhibitors of protein synthesis, as well as mtDNA replication (antibiotics and NRTIs) have been investigated using radiolabeled deoxynucleotide triphosphates (dNTPs) [54], and [^{35}S]-methionine incorporation has been used to test for impairment of protein synthesis [55]. With only minor modifications, all such assays could be used to assess drug-induced mitochondrial dysfunction in cells or with isolated organelles.

Mitochondrial impairment induced by many drugs has also been detected in isolated organ models. For example, anterograde perfusion of rat liver with clofibrate decreases oxygen consumption by 40% within 15 min and yields selective killing of aerobically poised periportal cells [56]. This selective toxicity is exacerbated by hyperoxia and is also apparent in isolated cylinders of periportal and pericentral tissue, eliminating selective drug delivery as an explanation [56]. The cardiotoxicity of adriamycin is readily apparent in isolated perfused rat hearts [57], where its autofluorescence readily localizes it to both cardiomyocyte nuclei and mitochondria [58]. Isolated perfused heart has also been used in SAR studies of amiodarone and its analogues [59].

It is axiomatic in drug development that human data trump animal models, which in turn trump organ models, which trump cell models, which in turn trump organelle models. However, the pre-clinical animal models failed to reveal idiosyncratic adverse events and frequently did not report mitochondrial impairment. Thus, there is a disconnect between the cell and organelle studies showing mitochondrial failure and the clinical findings of frank organ toxicity; tissue injury via drug-induced mitochondrial failure is a well-justified inference, but it remains just that unless animal models that are predictive of the clinical outcome can be developed.

To that end, several transgenic animal models have been generated with a phenotype that includes mitochondrial impairment, such as mice with deficiencies in uncoupling protein, mitochondrial transcription factor A (*tfam*), glutathione peroxidase-1, γ-ghitamyl transpeptidase, and adenine nucleotide translocase (ANT) [60–69]. Some of these models have been used to explore drug toxicity, but not with the goal of detecting drug-induced mitochondrial toxicity. A promising model is a heterozygous knockout mouse where expression of the mitochondrial isoform of the antioxidant Mn-SOD is halved. The hepatotoxicity that forced the market withdrawal of troglitazone is not detected in

wild-type mice but is readily apparent in these $SOD2^{+/-}$ animals [5]. Similarly, nimesulide, an NSAID that potently uncouples respiration *in vitro* [70], also elicits liver mitochondrial damage, loss of ATP, and ensuing apoptosis significantly more in the knockout animals compared with wild-type rodents [71].

Mitochondrial testing in drug development

In order to increase drug safety and reduce attrition rates, pharmaceutical companies have started to explore and adopt newer *in vivo*, *in vitro*, and *in silico* tools, as well as new technologies such as the 'omics' platforms [72]. In addition, many limited duration teams and consortia have been formed to tackle specific issues such as hepatotoxicity, carcinogenicity, and vasculitis [http://www.fda.gov/bbs/topics/news/2006/NEW01337.html, http://rarediseasesnetwork.epi.usf.edu].

In large pharmaceutical companies, drug safety is traditionally considered fairly late in the drug discovery process. However, it is increasingly acknowledged that safety testing should begin during lead selection, when there is a wider variety of compounds to support structure–activity relationship (SAR) approaches. Postponing safety evaluations until after a lead molecule has been nominated – on the basis of potency on target and *in vivo* efficacy – is the primary cause of late stage attrition. For example, an ideal program would include a Tier I *in vitro* high-throughput screen (HTS) for mitochondrial dysfunction, and when needed, Tier II mechanistic studies, to inform lead selection.

To underscore the utility of mitochondrial screening, of the 38 drugs withdrawn from the market because of safety reasons, at least three, troglitazone, cerivastatin, and tolcapone, have *in vitro* or *in vivo* mitochondrial liabilities that caused or exacerbated the observed organ toxicities. For example, troglitazone dissipates $\Delta\Psi$, induces mitochondrial swelling, and irreversible mitochondrial permeability transition in mouse liver mitochondria [6–8,44]. As previously discussed, troglitazone causes hepatic necrosis in $MnSOD^{+/-}$ mice, a model with silent mitochondrial abnormalities [5]. Similarly, cerivastatin also collapses $\Delta\Psi$ and represses glutamate-driven respiration in isolated rat skeletal muscle mitochondria [9] and causes ultrastructural changes in muscle [73]. Tolcapone has been shown to be a strong uncoupler *in vitro* [12] leading to reduced ATP production and increased heat production [13]. Tolcapone was also shown to decrease mitochondrial membrane potential *in vitro* and *in vivo* where it induced hepatic necrosis, increased respiration, diminished ATP/ADP ratios in liver tissue, and elevated temperature [14,15].

More frequently than market withdrawal, adverse events elicit a Black Box Warning from the FDA, for inclusion in the patient

BOX 1

Drugs with FDA Black Box Warnings for hepatotoxicity and cardiovascular toxicity. Those with documented mitochondrial liabilities are highlighted in red. All major indications are listed and these include ~80% of the drugs in this group. The remaining are single representatives of biologics, hormones, immuno-suppressants, anabolic steroids, anticoagulants, and steroids

Black Box Warning for Hepatotoxicity		Black Box Warning for Cardiovascular Toxicity	
Antivirals[39,74-76]	**Antibiotics[79-81]**	**Anthracyclines [83-85]**	**Anti-Cancer 90-91]**
Abacavir	Isoniazid	Daunorubicin	Arsenic Trioxide
Didanosine	Ketoconazole (oral)	Doxorubicin	Cetuximab
Emtricitabine	Streptozocin	Epirubicin	Denileukin diftitox
Entecavir	Trovafloxacin	Idarubicin	Mitoxantrone
Emtricitabine			Tamoxifen
Lamivudine	**CNS [28,82]**	**NSAIDs[86-89]**	
Nevirapine	Dantrolene	Celecoxib	**Beta-Blocker [92]**
Telbivudine	Divalproex Sodium	Diclofenac	Atenolol
Tenofovir	Felbamate	Diflunisal	
Tipranavir	Naltrexone	Etodolac	**Antiarhythmic [93]**
Stavudine	Nefazodone	Fenoprofen	Amiodarone (oral)
Zalcitabine	Valproic Acid/	Ibuprofen	Disopyramide
Zidovudine		Indomethacin	Dofetilide
	Hypertension	Ketoprofen	Ibutilide
Anti-Cancer [43,44,77,78]	Bosentan	Mefenamic acid	
Flutamide		Meloxicam	**CNS [94]**
Dacarbazine		Naproxen	Amphetamines
Gemtuzumab		Nabumetone	Atomoxetin
Methotrexate		Oxaprozin	Droperidol
Pentostatin		Piroxicam	Methamphetamine
Tamoxifen		Salsalate	Pergolide
		Sulindac	
		Thioridazine	**Anaesthetic [95]**
		Tolmetin	Bupivacaine
			Diabetes [8,27,44]
			Pioglitazone
			Rosiglitazone

insert. Although often triggered by organ toxicity to liver, heart, kidney, and CNS, other mechanisms such as drug–drug interactions will also elicit such warnings. A retrospective analysis of such Black Box Warnings indicates that some 385 have been issued up to 2006 [http://www.formularyproductions.com/master/showpage.php? dir=blackbox&whichpage=9], and those with warnings primarily for hepatotoxicity or cardiac toxicity are listed in Box 1.

As is evident from Box 1, organ toxicity crosses drug classes but can also be a consistent caution for a whole class. For example, antivirals, many of which impede mitochondrial replication, are all included in the Black Box Warning. Hepatotoxicity is a common finding for NRTIs, anticancer drugs, antibiotics, drugs used in various CNS applications, as well as biologics, immuno-supressants, and steroids. Cardiac toxicity is associated with anthracyclins, NSAIDs, anticancer drugs, antiarrhythmic, and CNS-related drugs. Such divisions are not absolute, and many drugs with a primary organ toxicity for which the warning was issued also impose adverse events in other tissues. For example, the NSAIDS and thiazolidinediones have a Black Box Warning for cardiovascular events, but they also show hepatotoxicity [46,96].

Importantly, mitochondrial toxicity has been described for many members of these classes (highlighted in red), but note that not all members have equal mitochondrial effects. For example, much evidence indicates that the cardiac toxicity caused by doxorubicin results from oxidative stress that undermines mitochondrial function [83–85]. By contrast, daunorubicin, epirubicin, and idarubicin show less direct mitochondrial toxicity and are correspondingly less cardiotoxic in the clinic [85]. The same is true for the NSAIDs, some of which are potent uncouplers, while others have no reported mitochondrial liabilities [86–89]. Various combinations of *in vitro* screens for ROS production, cell death (including long term to detect agents that impair mitochondrial replication), and mitochondrial respiration reveal such liabilities.

To date, assessment of drug-induced mitochondrial toxicity has been retrospective, examining drugs with organ toxicity and known adverse effects for potential mitochondrial liabilities. But such examinations are needed to establish limits of predictability, such as determining how severe a mitochondrial impair-

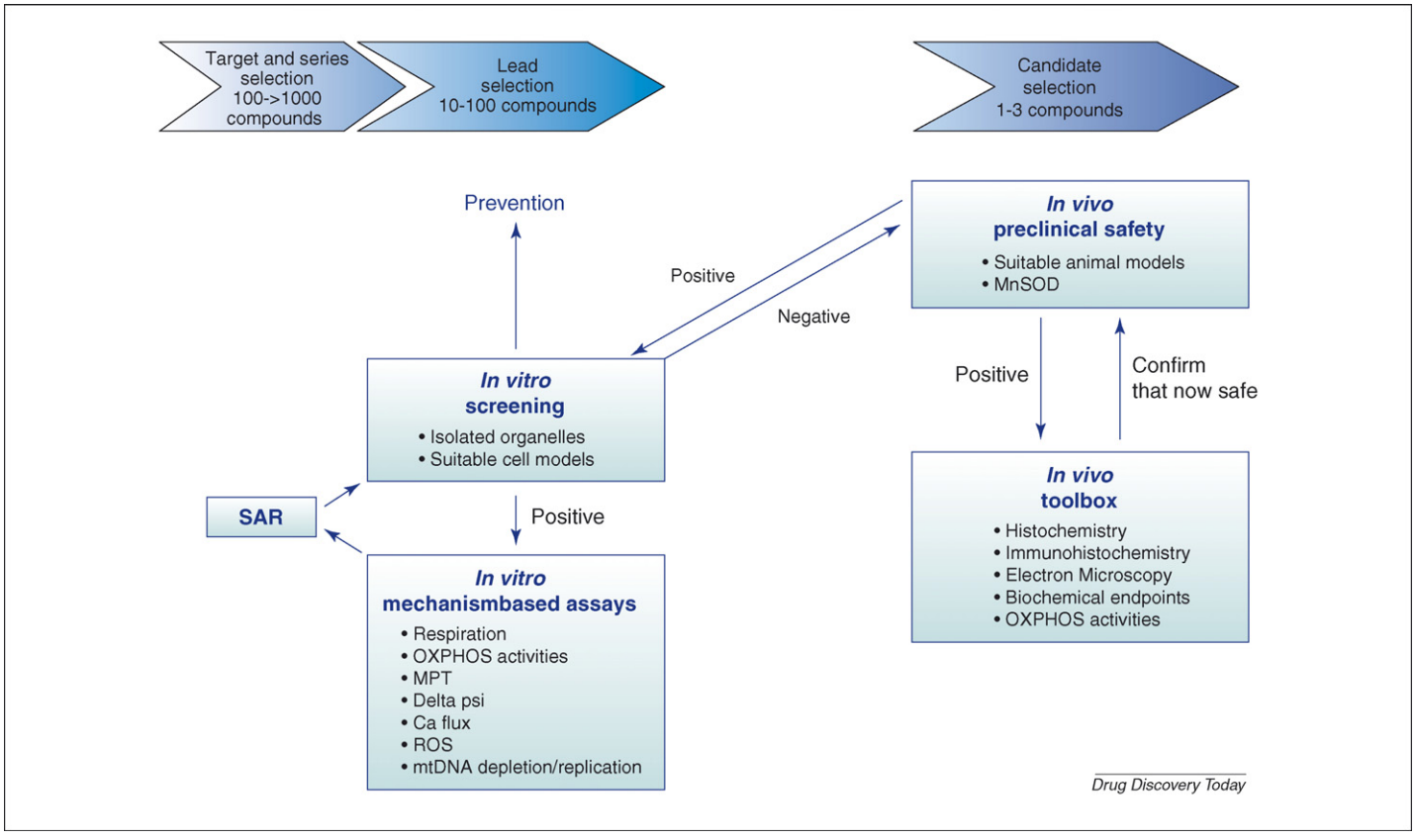

FIGURE 3

Model of mitochondrial toxicity screening. Early identification of mitochondrial liabilities during compound series selection and lead selection using *in vitro* screens allows for structure–activity relationship (SAR) studies to circumvent it. Cell-based screening assays are available for initial identification of general cytotoxicity. If a mitochondrial etiology is suspected, assessment of isolated mitochondrial function can also be performed in screening mode. If positive, additional *in vitro* mechanistic studies can examine effects on OXPHOS respiration, permeability transition, reactive oxygen and nitrogen centered (ROS), membrane potential ($\Delta\Psi$m), and mitochondrial DNA (mtDNA) status, among others. Such data enable structure–activity relationship (SAR) studies necessary to circumvent mitochondrial toxicity. Mitochondrial assessment should be completed before a compound moves into further development and is elevated to drug candidate level but should certainly also be triggered if a compound has a negative response in an animal model. Classical assays such as electron microscopy and enzyme-linked assays of the individual respiratory complexes are available for assessing mitochondrial status in tissues. In addition, newer histochemical and immunohistochemical techniques are being developed assays that will help illuminate mechanism of mitochondrial impairment. Fostered by the organelle and cell studies, animal models are also being developed that better reveal mitochondrial toxicity, and hence better predict clinical outcome. One such model is a manganese superoxide dismutase (MnSOD) knockdown mouse that more sensitively detects drug-induced mitochondrial toxicity [71].

ment has to be before it yields frank toxicity *in vivo*. Projections based on *in vitro* data also need to consider safety margins at the anticipated Cmax, which is usually not defined at the earlier stages of drug development. Of course, such predictions can be thwarted by bio-accumulation of drug. For example, mitochondrial toxicity leading to rhabdomyolysis is well documented for statins, but *in vitro* mitochondrial effects are not detectable until doses of approximately 2000 times Cmax [9]. However, transporters on the surface of fast twitch muscle fibers concentrate these drugs sufficiently to yield mitochondrial failure [38]. The complexity of mitochondrial biology with respect to differences in tissue, heteroplasmy, aging, and accumulation of mutations also confounds predictions, but eventually the assays and models will be refined enough to provide robust *in vivo* projections.

Nevertheless, identifying mitochondrial liabilities early in the development process increases the likelihood that they can be avoided (Figure 3). Depending on the targeted disease, severe *in vitro* mitochondrial impairment might be sufficient to abandon a nascent drug, whereas mild impairment may be acceptable. This is an iterative process, and as drug parameters become better defined during development, the accuracy of *in vivo* predictions improves. If a mitochondrial liability is known for a compound, use of animal models more sensitive to mitochondrial impairment, with additional attention to likely sites or signs of organ toxicity, and/or use of specialized staining techniques during pathology examination, will improve clinical predictions.

Once in the clinic, drugs with mitochondrial liabilities require increased vigilance. Although lactic acidosis (or abnormal lactate-to-pyruvate ratio) can be considered a hallmark of mitochondrial insufficiency, it is typically not included in basic clinical chemistry panels. The care provider has to order the test separately, but without knowing that many drugs undermine mitochondrial function, lactate assessment would not be routinely justified. Moreover, although venous lactate assays can readily detect advanced mitochondrial impairment, they may not be sensitive enough to reveal the gradual erosion of mitochondrial capacity associated with long-term drug exposures.

The diagnostic procedure for assessing mitochondrial impairment, either congenital or iatrogenic, is biopsy and histopathology. Although this been the 'gold standard' for diagnosis of mitochondrial diseases, it has not been used in the drug safety arena. Several non-invasive or minimally invasive technologies to monitor mitochondrial status are being developed to circumvent repeated biopsies, and these will help illuminate xenobiotic effects in larger populations. For example, metabolic intermediates containing non-radioactive ^{13}C atoms can be given orally and rate of $^{13}CO_2$ exhalation monitored using isotope ratio mass spectrometry. Several groups have shown that [^{13}C]-methionine, an essential amino acid, serves as a reporter of hepatic mitochondrial dysfunction induced by alcohol and by antiviral therapy in HIV patients [97,98]. Moreover, repression of methionine decarboxylation detected with this stable isotope technique is highly significantly correlated with mtDNA content of peripheral blood leukocytes. Different metabolic pathways can be probed by placing the ^{13}C in different positions, or on a methyl group. Thus far, this technology has been demonstrated to report loss of mitochondrial capacity induced by antiviral therapies. It remains to be seen whether this will also hold for direct impairment of mitochondrial function by other xenobiotics, particularly those where hepatic injury is a not the primary toxicity, such as the statins where skeletal muscle is predominantly at risk. Another technique being used to monitor mitochondrial effects of the antivirals is by monitoring leukocyte mtDNA and respiratory complex activity from a peripheral blood sample that provides sufficient resolution to presage NRTI-induced lactic acidosis [99]. As above, this technique could also have utility for monitoring mitochondrial status in response to antibiotics, and potentially other drugs.

As a result of such efforts, an expanding series of pre-clinical and clinical assays and models now more faithfully reflect mitochondrial involvement in tissue toxicity, and is correspondingly increasing the concordance between organelle, cell, and animal data models with the clinical outcome. Although still in progress, the retrospective analysis of correlations between adverse drug events and mitochondrial toxicity corroborates the notion that such toxicity is important to etiology, and hence worthy of avoidance. Conversely, such an analysis will help define the range of mitochondrial impairment that can be tolerated clinically. It is becoming apparent that many beneficial drugs have mitochondrial liabilities, but if the latter is modest it may not be clinically significant in terms of the risk-to-benefit ratio. Moreover, assays using isolated mitochondria that lack cytochrome P-450 enzymes and other detoxifying capacities will probably 'over report' drug effects. As such, depending on potency, finding a drug-induced mitochondrial liability is cause for increased safety vigilance, not necessarily cause for ceasing development. It also bears reiteration that screening at standard concentrations (often a multiple of anticipated maximum serum concentration) may or may not reflect physiological reality. Some drugs, particularly cations, can accumulate in mitochondrial as a function of the membrane potential, while others bio-accumulate in specific subpopulations of cells of tissues because of surface transporters [100].

It is apparent that organelle and cell models that more faithfully report mitochondrial liabilities will serve the earlier stages of drug-development programs by directing away from molecules and molecular motifs that undermine mitochondrial function. Animal models that more faithfully predict mitochondrial failure *in vivo* will reduce unanticipated safety issues arising from such impairment in the clinic. Non-invasive or minimally invasive clinical assessments of mitochondrial function will provide additional safety for those drugs with known mitochondrial toxicity. When considered as a whole, a suite of assays capable of detecting impairment of mitochondrial function and/or replication will lower attrition rates in late-stage pre-clinical programs, and yield safer drugs in the future.

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