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In vivo assessment of mitochondrial toxicity

Bradley F. Hamilton, Alan H. Stokes, Jon Lyon and Rick R. Adler

GlaxoSmithKline, 5 Moore Drive, PO Box 13398, Research Triangle Park, NC 27709-3398, USA

Drug effects on mitochondrial function are frequently characterized *in vitro*. Whether these findings are relevant pharmacologically or toxicologically is generally unclear. Methods for *in vivo* assessment of mitochondrial function would help establish biological significance, but none is widely accepted or readily available. Ideally, these methods would be sensitive and specific, noninvasive, predictive of efficacy or toxicity, translatable from preclinical to clinical studies and localizable to the target organ. Although not fully developed or validated, several approaches to *in vivo* mitochondrial assessment show promise. Collaboration between scientists from academia, the pharmaceutical industry, and regulatory agencies will be required to develop and apply these methods.

Mitochondria play a complex role in health and disease. The nexus of metabolic pathways involving these organelles has grown more complicated as our understanding of mitochondrial biology evolves. In addition to their central role in energy metabolism, mitochondria are integrally involved in embryonic development, cell signaling activities, cell-cycle control, and cell death [1,2].

Although some drugs interact unintentionally with mitochondrial targets and have the potential to cause adverse effects, many medicines have side effects associated with mitochondrial perturbation that are manageable in relation to the therapeutic benefit the drug provides [3,4]. There is also growing interest in intentionally altering mitochondrial function with new drugs for the treatment of obesity, diabetes, neurodegenerative disease, and cancer [5]. Given the multiple metabolic pathways potentially affected by a change in mitochondrial function, however, development of drugs with mitochondrial effects requires judicious safety assessment and risk management [6].

Reliance on traditional drug development methods may be insufficient. Standard endpoints used for safety testing in preclinical and clinical development, such as clinical signs, histopathology, hematology, and serum chemistry, are very important filters for detecting compounds with unintended effects, but they are relatively imprecise and insensitive for detecting alterations in mitochondrial function. For drugs that perturb mitochondrial function, development studies may need to be augmented with more specialized or targeted evaluations. Specific biomarkers would be helpful but have not been developed, at least not for ready application *in vivo*. As one of many challenges facing drug development companies, FDA's Critical Path Opportunities Report emphasizes the need for new and better safety biomarkers that are predictive and translatable. Ideally, new biomarkers will assure safety by improving the selection of initial human doses and enabling focused monitoring for early toxicity (see: http://www.fda.gov/oc/initiatives/criticalpath/reports/opp_report.pdf).

The risks associated with compounds that alter mitochondrial function are significant. Dintrophenol (DNP), a potent mitochondrial uncoupler, is a classic example. Sold in the 1930s as a weight-loss drug, numerous DNP associated side effects were eventually recognized, and it was removed from the market, but not before fatalities had occurred [7]. DNP would never make it out of the preclinical laboratory today, but its potent effects underscore the potential risks encountered with drugs causing mitochondrial toxicity. In a contemporary example, the antiviral drug fialuridine, a nucleoside analog, caused delayed, fatal, multi-systemic toxicity in a Phase II clinical trial. The toxicity was ultimately linked to deleterious effects on mitochondrial function [8]. Mitochondrial effects associated with other marketed nucleoside analogs, such as hepatotoxicity and lactic acidosis putatively associated with mtDNA depletion, were not novel at the time, but preclinical studies with fialuridine did not adequately predict the risk at hand.

Corresponding author: Hamilton, B.F. (brad.f.hamilton@gsk.com)

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Drug-induced mitochondrial dysfunction can also cause more subtle effects, develop slowly over a period of months and may be confounded by multiple complicating interactions involving the underlying disease state (such as HIV infection and diabetes), genetically determined predispositions, tissue-dependent exposure, tissue-specific mitochondrial heterogeneity, and functional reserve and interactions with other co-administered drugs. The anti-neoplastic drug, doxorubicin, can cause a cardiomyopathy pursuant to mitochondrial dysfunction that develops over a period of weeks and months [9]. HIV-infected patients given nucleoside reverse transcriptase inhibitors (NRTIs) have many potentially significant side effects attributed to drug-induced mitochondrial toxicity, but NRTI-specific effects are complicated by the well-recognized direct mitochondrial effects of the HIV virus itself, as well as interactions with other drugs used in highly active antiretroviral therapy (HAART) [10]. Some cases of idiosyncratic hepatotoxicity may be caused by drug-induced mitochondrial dysfunction when combined with various other susceptibility factors [11].

In vitro techniques using cell culture or isolated mitochondria are commonly used to evaluate the effects of xenobiotics on mitochondria. They are crucial for identifying targets of drug toxicity such as distinguishing effects on the electron transport chain (oxidation) from effects on phosphorylation efficiency (uncoupling) [3]. Although identifying mechanisms of toxicity using *in vitro* screens can undoubtedly inform drug development in early discovery when such issues can possibly be resolved by altering synthetic chemistry, they are of uncertain relevance *in vivo* [12,13]. Indeed, it seems unlikely that *in vitro* assessments can adequately define the relevance of mitochondrial dysfunction *in vivo* with the confidence necessary for robust decision-making regarding dose selection, safety monitoring, and risk.

The inability to account for *in vivo* variables (like distribution, metabolism, length of exposure, target organ specificity, hormonal, and other systemic interactions) remains an obstacle. Even in traditional preclinical toxicology studies with laboratory animals, commonly employed endpoints of mitochondrial health, such as ultrastructural morphology and blood lactate, are very limited in their ability to inform subsequent clinical development. Obtaining tissue for ultrastructural examination is invasive and not suited to repeated sampling for clinical safety monitoring. Blood lactate varies considerably in its sensitivity and specificity, and by itself, would not adequately ensure safety [8,14,15]. Robust and reliable methods for *in vivo* assessment of mitochondrial function are needed.

The objective of this article is to review promising methods for *in vivo* assessment of mitochondrial function. Although mitochondrial biology is far more complex than just production of high-energy phosphates for cellular energy needs [1,2], the methods discussed here measure endpoints related specifically to energy metabolism. This includes magnetic resonance spectroscopy (MRS), positron emission tomography (PET), breath testing, and evaluation of blood, urine, or surrogate cell populations such as peripheral blood mononuclear cells (PBMCs). These techniques share characteristics that make them attractive for use in drug development: noninvasive, target organ specific, predictive, and translatable.

Magnetic resonance spectroscopy

Magnetic resonance spectroscopy (MRS), based on the principle of nuclear magnetic resonance, measures the magnetic properties of elemental nuclei with an odd number of neutrons or protons inside a strong magnetic field. The resulting spectra are analyzed to determine the concentration of metabolites containing the relevant nuclear species. In evaluating mitochondrial function, detection of ^{13}C- and ^{31}P-containing molecules permit direct evaluation of energy metabolite concentrations and flux in tissues such as skeletal muscle, heart, and brain [16–19]. ^{1}H-MRS can be used for assessment of tissue lipids (both intracellular and extracellular), which could be of interest in evaluating the extent of mitochondrial dysfunction in a tissue [20].

^{13}C-MRS measures carbon flux through energy metabolic pathways after administration of an appropriate substrate [17]. For example, after infusion of glucose, 3-hydroxybutyrate, or acetate labeled with ^{13}C, the rate of flow (flux) of labeled substrate through glycolysis, ketone body metabolism or the TCA cycle can be followed, respectively [19]. Measurement of the carbon flux of these metabolic intermediates can be localized to individual tissues to give an organ-specific estimation of substrate utilization. Furthermore, oxygen consumption can be estimated because of the stoichiometric relationship between acetate consumption by the TCA cycle and oxidative phosphorylation [21].

^{31}P-MRS measures concentrations of inorganic phosphate (Pi), adenosine triphosphate (ATP) and phosphocreatine (PCr), and related metabolite concentrations, such as ADP, can be calculated. ^{31}P is endogenous, so manufacture of labeled markers is not required. By using ^{31}P-MRS and ^{13}C-MRS simultaneously, TCA flux (acetate oxidation) as an index of oxygen consumption can be related to ATP synthesis to calculate mitochondrial coupling efficiency [21]. Alternatively, a recent technical advance using optical spectroscopy to measure oxygen saturation dynamically may be simpler and more quantitative [22]. Again, mitochondrial coupling efficiency can be measured by concurrently measuring ATP synthesis assessed with ^{31}P-MRS [22–24].

In vivo MRS, to evaluate mitochondrial function, has been investigated in skeletal muscle in both laboratory animals and humans. Skeletal muscle in rats given triiodo-L-thyronine (T_3) to induce expression of mitochondrial uncoupling protein, or a subtoxic dose of the uncoupler DNP, was analyzed with combined ^{13}C-MRS and ^{31}P-MRS [21]. Under basal or 'resting state' conditions in awake rats, T_3 and DNP treatment resulted in a 1.5× and 4× increase in TCA cycle flux (acetate oxidation), respectively, but no significant difference in ATP synthesis. This resulted in a decrease in the ATP synthesis/TCA cycle flux ratio by 60% and 80% with T3 and DNP, respectively. These results, indicating a requirement for increased substrate oxidation to maintain a given rate of ATP synthesis, confirmed that treatment resulted in a significant decrease in mitochondrial coupling efficiency. Using ^{31}P-MRS combined with optical spectroscopy in anesthetized mice, similar results were obtained in skeletal muscle after treatment with DNP [23].

Using a standardized exercise protocol in NRTI-treated patients, changes in PCr depletion and recovery measured with ^{31}P-MRS in skeletal muscle were considered indicative of altered mitochondrial function. In HIV-infected patients given zidovudine for an average of 33 months who developed clinical myopathic

symptoms, PCr depletion, and postexercise PCr recovery times were increased compared with HIV-infected patients not exposed to zidovudine [25]. In a similar study, patients given zidovudine for 7–20 months, with no evidence of clinical myopathy, also had increased postexercise PCr recovery times compared with untreated, HIV-negative volunteers [26]. In an investigation with stavudine, short-term treatment of healthy volunteers resulted in a decreased postexercise PCr recovery rate compared with untreated controls [27]. Although these findings indicate ^{31}P-MRS can detect NRTI-related effects on energy metabolism in skeletal muscle, it remains unproven whether it can also predict development of mitochondrial dysfunction sufficient to cause clinical myopathy.

In a Phase I clinical trial with a candidate anti-cancer drug (MKT077), ^{31}P-MRS was used to assess mitochondrial function in skeletal muscle to monitor subclinical toxicity [28]. MKT077 was shown to decrease mitochondrial oxygen consumption significantly in preclinical studies [29]. The clinical trial was curtailed after one to four treatment cycles because of renal toxicity, so the MRS evaluation was limited. No MRS changes were found in patients receiving one or two treatment cycles. One patient received 11 treatment cycles before consent was withdrawn because of clinical myalgia. In this patient, there was increased postexercise depletion of PCr (measured after the 11th treatment) that was considered indicative of mitochondrial dysfunction associated with MKT077. Although these findings suggest MRS could be useful in monitoring altered mitochondrial function, predictiveness remains unproven. Additionally, in this particular clinical trial, renal toxicity was the limiting factor, so the ability to monitor mitochondrial function in kidney would have been of greater benefit.

In HIV-infected adults with varying clinical and antiretroviral treatment histories, liver fat content was determined using ^{1}H-MRS to look for associations with insulin resistance, body fat distribution, and other factors [30]. Hepatic steatosis defined as $\geq$5% liver fat content was present in 42% of subjects. A significant correlation was observed between liver fat content, insulin resistance, and increased visceral adiposity. There was no correlation with duration of HIV infection or with antiretroviral exposure. No specific mitochondrial endpoints were measured in this study, but use of ^{1}H-MRS to evaluate tissue lipid content could potentially be valuable as a measure of mitochondrial toxicity in cases where there is a proven mechanistic link between fat accumulation in a tissue and mitochondrial dysfuncton.

Positron emission tomography

Positron emission tomography (PET) measures the uptake and washout kinetics of tracer molecules in target tissues [31]. The tracer molecules, either metabolic substrates or xenobiotics, are labeled with short-lived radioactive isotopes such as ^{18}F, ^{11}C, ^{13}N, or ^{15}O. As with MRS, metabolic substrates such as glucose, acetate, and lactate are potentially useful for evaluating changes in energy metabolism. The tissue washout kinetics of ^{11}C-acetate measured with PET are used to assess the rate of TCA cycle substrate oxidation and oxidative phosphorylation. Cardiac PET studies have been used to evaluate the relationship between the rate of substrate oxidation and the heart rate pressure product (RPP), a measure of cardiac work. Also, lipophilic cations have recently been used to assess mitochondrial membrane potential (MMP) with PET. It does not appear that PET has been evaluated specifically for detection of mitochondrial toxicity.

In normal human subjects, the ratio of the acetate clearance rate constant to RPP (K_{mono}/RPP) at rest and after dobutamine infusion are similar [32]. Substrate oxidation (acetate washout) and cardiac work (RPP) increased with dobutamine infusion and the ratio of the two remained unchanged as expected with normal mitochondrial coupling efficiency. In hyperthyroid patients given dobutamine, K_{mono}/RPP is increased [33]. There is increased substrate oxidation compared with cardiac work, which the authors suggest indicates 'myocardial oxygen wastage' due to direct effects of thyroid hormones on the myocardium [33]. This result is similar to that obtained with MRS in rats given T_3 [21]. Evaluation of acetate clearance and RPP in patients with type 2 diabetes mellitus also suggested increased oxidative metabolism relative to cardiac work [34].

Although originally designed for evaluation of myocardial perfusion, ^{18}F-fluorobenzyl triphenylphosphonium cation (^{18}F-FBnTP), and technetium-99m sestambibi (MIBI) may be useful as indicators of mitochondrial function in the heart. ^{18}F-FBnTP and MIBI are lipophilic cations that accumulate in cells in proportion to the MMP [35,36]. The uptake and washout kinetics, therefore, would be expected to reflect the integrity of mitochondrial function. In chick myocardial cells, MIBI uptake and retention *in vitro* was predictably altered by treatment with various mitochondrial toxins that either depolarize or hyperpolarize the MMP [37]. Biodistribution studies with ^{18}F-FBnTP in CD1 mice and in dogs indicate accumulation in kidney, heart, and liver [36,38]. In the heart, there is rapid accumulation in the myocardium with a plateau in a few minutes and then prolonged retention. In one clinical study with congestive heart failure patients over a period up to 19 months, deterioration in functional cardiac endpoints, increased natriuretic peptide, and increased cardiac events correlated with an increased rate of MIBI washout [39].

Breath tests

Methionine and ketoisocaproic acid (KICA) breath tests have been used to evaluate hepatic mitochondrial function. Exhaled $^{13}CO_2$ is measured by mass spectroscopy in serial samples after administration of ^{13}C-labeled methionine or KICA. Because methionine and KICA metabolism is largely restricted to hepatic mitochondria, the kinetics of labeled CO_2 elimination is believed to be a marker of mitochondrial function in hepatocytes. This does not appear to have been verified directly by correlation with standard mitochondrial energy endpoints; however, experimental results suggest breath tests may be a valid way to assess hepatic mitochondria.

The link between hepatic mitochondrial energy metabolism and KICA or methionine metabolism is related to the NAD+/NADH redox state [40,41]. Increased NAD+ availability (oxidative stress) results in increased labeled CO_2 exhalation, and decreased NAD+ availability (reductive stress) decreases labeled CO_2 exhalation. Ethanol and acetylsalicylic acid have been used to investigate methionine and KICA breath tests. In rats and humans, ethanol decreased and acetylsalicylic acid increased the rate and cumulative elimination of labeled CO_2 [40–42]. Although not concurrently evaluated in these studies, ethanol oxidation in the liver results in increased NADH availability (decreased NAD+) in both

the cytosol and mitochondria [43]. By contrast, acetylsalicylic acid, a purported mitochondrial uncoupler, causes decreased NADH availability (increased NAD+) [44].

In HIV-infected patients, decreased labeled CO_2 excretion with the methionine breath test was attributed to NRTI-related mitochondrial toxicity. Excretion was decreased more in those with hyperlactatemia than those with normal lactate [45]. In patients with normal lactate, excretion was decreased in patients with lipoatrophy compared with healthy controls and asymptomatic patients [46]. Although these studies suggest there is potential for breath testing to aid evaluation of metabolic toxicity associated with NRTI treatment, the ability of breath testing to improve clinical safety remains unproven.

Metabolic profiling of body fluids

Metabolic profiling of blood and urine using magnetic resonance spectroscopy or mass spectroscopy (MS) is increasingly used for analysis of disease mechanisms and to search for potential fluid biomarkers [47]. Metabolites of interest in blood and/or urine as indicators of mitochondrial dysfunction include glucose, lactate, pyruvate, ketone bodies (3-hydroxybutyrate, acetoacetate), TCA cycle intermediates, and dicarboxylic acids, such as suberic acid and adipic acid.

Metabolic profiling has been used in a limited manner and with limited success for diagnosis of inherited mitochondrial disorders [48,49]. For detection of mitochondrial effects due to xenobiotics, various studies report alterations in biofluid metabolites that were attributed to altered mitochondrial function. For example, studies in rats given allyl formate [50], acetominophen [51], and a synthetic antiangiogenic compound [52] caused fluctuations in TCA cycle intermediates (acetate, citrate, succinate, alpha-ketoglutarate, 2-oxoglutarate), lactate, pyruvate, glucose, 3-hydroxybutyrate, and various lipidic metabolites. Although mitochondrial dysfunction is implicated in the toxicity of each compound [52–54], concurrent assessment of mitochondrial endpoints in target tissues to confirm the relevance of the measured biofluid responses was not conducted. This is important because physiological alterations associated with diet, gender, reproductive status, circadian rhythm, and nonspecific stress-related responses can significantly alter many of these metabolites as well [48,55].

Surrogate cell populations

Mitochondrial endpoints in peripheral blood mononuclear lymphocytes (PBMCs) have been investigated as surrogate marker of toxicity associated with NRTIs with conflicting results [56-58]. Some of these differences may be due to the complexity of mitochondrial effects in HIV-infected patients: the direct effect of the HIV virus on mitochondria, drug variations, and multi-drug interactions typical of highly active antiretroviral therapy (HAART) and the relative balance of these various factors [59,60]. Apparently little or no research has been done with PBMCs in animals treated with NRTIs or with other mitochondrial toxicants.

Although not strictly a functional endpoint, mtDNA content in PBMCs from HIV-infected patients have been measured to look for correlations with NRTI treatment and with clinical events attributed to mitochondrial toxicity. In a recent study, PBMC mtDNA measured during the first 12 months of treatment with different NRTI combinations found an association between mtDNA depletion at 12 months and the subsequent development of lipodystrophy at 30 months [61]. In another study, NRTI treatment was the major determinant of mtDNA depletion in PBMCs and subcutaneous fat in patients on various HAART regimens although the association was tissue and drug specific [62].

Mitochondrial membrane potential (MMP) and oxygen consumption measured in PBMCs may be a reasonable surrogate for evaluating systemic mitochondrial effects in some cases. There was decreased oxygen consumption (measured polarographically) in PBMCs from HIV-infected patients given HAART comprising drugs with a high mitochondrial toxicity profile compared with measurements in the same patients after switching to HAART with a low toxicity profile [63]. In another study, MMP in peripheral blood lymphocytes was lower in stavudine-treated patients than those with no treatment [64]. Consistent with direct effects of HIV on mitochondria, MMP was significantly reduced in untreated HIV-infected patients compared with uninfected patients, and it increased significantly after starting therapy [65] similar to the effects found with mtDNA [59,60].

Capabilities and limitations

When mitochondrial toxicity is a limiting factor in the development of a candidate drug, the methods discussed here may be useful in defining a safe path forward in the clinic. At present, however, none of these methods are commonly used, and all need further development and validation to understand their capabilities and limitations and to gain clinical and regulatory acceptance.

An overarching disparity between capabilities and needs in developing drugs with mitochondrial effects concerns the ability to make distinctions between nonadverse drug effects that are physiologic, pharmacologic, or adaptive, and 'pre-adverse' effects that predict liability for unacceptable dysfunction and toxicity. The sensitivity and specificity of individual endpoints must be characterized. The studies presented in this review were not generally designed to investigate these issues or to address efficacy or safety concerns in general. Focused and comprehensive investigation is required if these technologies are to be applied in development of drugs with mitochondrial effects whether these effects are intended or undesired.

Preclinical studies are important in filling the gap since it is crucially important to connect sequential *in vivo* assessments of mitochondrial endpoints with the development of adverse target organ toxicity. This is necessary to confirm the relevance of mitochondrial dysfunction to toxicity and to assess the predictive ability of the individual endpoints. Methods that are adaptable for use in the clinic are required since it typically does little good to characterize toxicity in animal studies without the ability to monitor for effects in the clinic before expression of the toxicity. In both preclinical and clinical studies, correlation with *in vitro* studies would help characterize mechanism of action and improve the utility of *in vitro* data in future.

Despite these limitations and challenges, the methods outlined in this review have features that suggest utility in assessment of mitochondrial function, dysfunction, and toxicity *in vivo*. An important feature of MRS and PET is the capability for target organ localization. These methods also give a relatively direct assessment of mitochondrial function by specifically probing pertinent metabolic endpoints similar to those usually examined *in vitro*: TCA

cycle flux; oxygen consumption; ATP synthesis; recovery of high-energy phosphate compounds after stress and measurement of MMP. Although examples are limited, a variety of pharmacologic and toxicologic effects on mitochondrial function have been successfully measured with MRS and PET. These methods are, however, perhaps the most technically demanding in terms of equipment and expertise [17].

The methionine and KICA breath tests may be relatively sensitive methods for the detection of altered mitochondrial function, since pharmacologic doses of ethanol and acetylsalicylic acid are sufficient to detect a change [41,42]. Testing with other mitochondrial toxins has not been reported. The testing procedures are relatively simple, with minimal imposition on the test subject. Although the metabolic link between methionine or KICA metabolism and hepatic mitochondrial function is logical, this has not been directly validated by concurrent measurements *in vivo*. At present, these tests are limited to assessment of mitochondrial function in the liver.

Metabolic profilings of blood or urine to examine the systemic effects of xenobiotics are fairly numerous, although the capability to detect and distinguish mitochondrial toxicity is largely unproven. The findings in some studies have been attributed to mitochondrial derangement, but there has been no validation by concurrent analysis of mitochondrial endpoints in target tissue. Whether or not mitochondrial dysfunction in a tissue will be sensitively and specifically reflected in blood or urine will probably vary, depending on the target tissue and the specific drug in each case. One advantage of technologies like MRS and MS is that these are 'open' systems that do not require prior selection of a limited panel of analytes. It seems unlikely that a few individual analytes will adequately signal mitochondrial dysfunction, but a complex response pattern determined by evaluating many analytes holds promise. The data sets acquired in metabolic profiling studies are large and require considerable data processing and analytical skills. Interpretive expertise is crucial to decipher the pathophysiologic implications of complex metabolite patterns.

Use of PBMCs as a surrogate cell population is limited to NRTI-induced mitochondrial effects in human patients, and the results are mixed. Some studies report positive correlations that will undoubtedly lead to further exploration in HIV-infected patients given HAART since detection and management of mitochondrial toxicity is an ongoing problem. Like metabolic profiling, the utility of PBMCs will need to be investigated and validated on a case-by-case basis with individual drug candidates.

Conclusion

In vivo assessment of mitochondrial function could facilitate the development of safe and effective medicines, but methods are currently undeveloped and unvalidated for practical application in preclinical and clinical studies. There has been no rigorous comprehensive effort to correlate *in vitro* and *in vivo* functional endpoints with conventional measures of efficacy and toxicity. Prospective planning and investment is required to establish a base of experience and knowledge and to begin to answer difficult questions such as: Which mitochondrial endpoints are most relevant to assess safety or efficacy? Are different mechanism-specific endpoints required to detect different varieties of drug-mitochondria interaction (for example, uncoupling *vs.* respiratory chain inhibition), or would a short list of endpoints that measure integrated function (like ATP production or oxygen consumption) be adequate? What changes are physiologic, adaptive and nonadverse? Which changes or what magnitude of change portends toxicity? And ultimately, can *in vivo* assessment of mitochondrial function permit study designs with more sensitive and specific measures of efficacy and safety than otherwise possible? There will be a need of concerted effort and collaboration across discovery, preclinical, and clinical phases of drug development to begin answering these questions.

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