Can Drug Safety be Predicted and Animal Experiments Reduced by Using Isolated Mitochondrial Fractions?

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Summary — Mitochondrial toxicity has resulted in the withdrawal of several drugs from the market. One particular example is nefazodone, an anti-depressant withdrawn in the USA due to hepatoxicity caused by drug-induced mitochondrial dysfunction. Drug development and safety testing can involve the use of large numbers of laboratory animals, which, without a decisive pre-screening for mitochondrial toxicity, are often unable to pre-empt higher mortality rates in some patient groups. The use of isolated mitochondria as a screening tool for drug safety can decrease the number of laboratory animals used in pre-clinical studies, thus improving animal welfare and healthcare outcomes and costs. Novel techniques involving high-throughput methods can be used to investigate whether a molecule is a mitochondrial toxicant. Moreover, these screens are mechanistically-based, since the effects of the drug on oxidative phosphorylation, calcium homeostasis and mitochondrial genetics can be assessed. This review is intended to demonstrate that isolated mitochondrial fractions are suitable for predicting drug and general chemical safety in toxicological screenings, thus contributing to the refinement and reduction of animal use in laboratory research.

Key words: *animal models, biosensor, mitochondria, toxicity.*

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Mitochondria and Toxicological Research

The potential toxicity of a newly-synthesised chemical compound is primarily scrutinised by *in vivo* testing, namely, by determining the LD50 (lethal dose) value — the concentration of a chemical that causes death in 50% of the exposed animals — as a measure of acute lethality. A variation of the LD50 test is the ED50 (effective dose) value, which measures the amount of a chemical that will produce a deleterious effect other than death in 50% of the test animals. Such tests are accompanied by several whole-animal assays, that include screening for chronic and subchronic toxicity, to provide information on mechanisms of action and target organs, and carcinogenicity, reproductive and developmental toxicity, special hypersensitivity and toxicokinetic studies (1). All of these tests require a large number of animals, are time-consuming, are extremely expensive to perform and frequently impose suffering on the animals used. For this reason, the tests that rely on the use of animals have been increasingly criticised by animal welfare groups, and have been subjected to ethical rejection by public opinion.

The complete toxicological evaluation of even one single chemical is complicated, very expensive, involves the sacrifice of thousands of animals, and may take several years. A great deal of progress has been made over the last few years in the development of high-throughput and cost-effective *in vitro* tests to fulfill testing requirements, and also to reduce the number of animals used in toxicity testing, particularly in the area of acute toxicity. *In vitro* testing includes the use of living systems, such as bacteria, frog embryos, fertilised chicken eggs, and cultured animal or human cells and tissues. These tests can contribute to a reduction in the number of animals that are subjected to testing, but the replacement of animal tests is somewhat limited by the relative simplicity of such methods, since, in general, the *in vivo* effects in humans or test species are due to complex physiological responses.

In vitro systems, despite their relative simplicity, are useful for predicting the cellular and molecular effects of a chemical. For this reason, toxicologists envisage the development of *in vitro* tests as a fruitful avenue for research, and as a possibility way of replacing many whole-animal methods in toxicity testing. In fact, *in vitro* tests can provide important preliminary screens for chemical toxicity, by identifying chemicals which have the lowest probability of toxicity, so that animals are exposed only to the least toxic chemicals.

During the last decade, many new drugs previously approved for the market by the regulatory agencies, have had to be withdrawn from the market, because of safety problems due to their toxic

impact on human health, and this has resulted in large financial losses (2). It has been shown that many of these drugs have deleterious effects on mitochondrial function (3–7). Furthermore, it has also been shown that mitochondria are one of the primary targets for toxic injury (8–12), which can also contribute to the induction of cell death. In this context, studies on mitochondria and their component proteins have revealed important toxicologically and pharmacologically relevant mechanisms for xenobiotics, providing a useful approach for the quantitative preliminary evaluation of a wide range of substances.

Mitochondrial Physiology within the Cell

Mitochondria are double-membraned cytoplasmic organelles, which synthesise most of the ATP used by eukaryotic cells. They possess a high degree of genetic and metabolic autonomy, although their function must always be seen as being integrated within the context of the cell as a whole. Mitochondria can vary in both shape and size, and can be spherical, elongated or even branched. The number of mitochondria can also vary, from a few discrete organelles per cell to a massive and dynamically fluctuating network, composed of an indefinable number of single, interconnected mitochondria in other cells. An outer mitochondrial membrane (OMM) encapsulates the organelle and is essentially permeable to ions and solutes up to 5kDa, which diffuse through transmembrane porin proteins. The inner mitochondrial membrane (IMM), by contrast, provides most of the mitochondrial barrier function, constituting an effective permeability barrier to cations, such as protons, potassium and calcium, as well as metabolites, which are transported through the membrane by specific proteins (8).

In addition to metabolite transporters, the IMM also contains the respiratory chain and the phosphorylation system complexes. The mitochondrial respiratory chain consists of diverse polypeptides and cofactors, including flavoproteins, iron–sulphur proteins, ubiquinone (a free lipid-soluble cofactor), cytochromes, and protein-bound copper. These components are arranged in three independent complexes (Complexes I, III, and IV; Figure 1). The respiratory chain catalyses the transfer of reducing-equivalent hydrogen atoms or electrons from respiratory substrates to molecular oxygen, a very active and harmful element, thus reducing the latter to water. The majority of respiratory substrates are oxidised via Complex I (NADH: ubiquinone reductase) that oxidises NADH produced by the tricarboxylic acid (TCA) cycle. The second entry point of the respiratory chain is Complex III (ubiquinol:cytochrome c reductase), which oxidises ubiquinol produced by succinate dehydrogenase, *s,n*-glycerophosphate, and the electron-transferring flavoprotein (ETF)-ubiquinone oxidoreductase, which transfers electrons from the flavin-linked β-oxidation of fatty acids. Reduced NADH transfers electrons to Complex I and then to ubiquinone, which shuttles electrons to Complex III. Cytochrome c, a relatively small water-soluble protein that rotates freely in the external face of the IMM, then shuttles electrons to Complex IV, the final electron acceptor. The end result is the reduction of molecular oxygen to water (13).

Complexes I, III, and IV are redox-driven proton pumps, in that they use the energy derived from oxidation–reduction reactions to translocate protons across the IMM, from the matrix into the intermembrane space. The proton pumping is coupled to the electron flow along the respiratory chain, so that there is no respiration without proton pumping, and *vice versa*. Succinate:ubiquinone reductase (Complex II) is not part of the respiratory chain, because its function is analogous to some other enzymatic activities in the IMM, which feed reducing equivalents into ubiquinone from various substrates, without being directly engaged in the conservation of energy (14).

The electrogenic pumping of protons by the three respiratory chain complexes establishes an electrochemical proton gradient ($\Delta\mu_H+$) across the IMM. The $\Delta\mu_H+$ consists of two components — an electrical membrane potential ($\Delta\Psi$) and a pH gradient provided by the transmembrane gradient of protons. Fully energised mitochondria from most mammalian tissues exhibit a proton-motive force (Δp) in the range of –180 to –200mV, with the ΔpH of 0.4–0.6 units (matrix pH of approximately 8; [15]).

As well as ATP production, mitochondria also participate in the initiation of apoptosis and necrotic cell death, and in maintaining calcium homeostasis (16). Excessive mitochondrial calcium loading and exposure to reactive oxygen (or nitrogen) species or high phosphate, may lead to a loss of $\Delta\Psi$, increase in IMM permeability, inhibition of ATP synthesis, and induction of mitochondrial damage, resulting in cell death. This is related to the opening of the mitochondrial permeability transition pore (MPTP; discussed in the following section).

Mitochondrial reactions are a major source of superoxide anion ($\cdot O_2^-$) and other reactive oxygen species (ROS) that may result from subsequent reactions of $\cdot O_2^-$ (17). During the transfer of electrons along the respiratory chain, single electrons occasionally leak out and react with molecular oxygen to form the superoxide anion. The latter is converted into hydrogen peroxide (H_2O_2) by the mitochondrial enzyme, superoxide dismutase (MnSOD). H_2O_2 is further metabolised by glu-

tathione peroxide (GPx) into H_2O. H_2O_2 may go on to generate the highly-reactive hydroxyl radical (·OH) in the presence of ferrous iron, via the Fenton reaction. $\cdot O_2^-$ can also react with nitric oxide to form the highly-reactive peroxynitrite anion ($ONOO^-$). Once mitochondrial enzymatic and non-enzymatic antioxidant systems are overwhelmed by ROS, oxidative damage and cell death can occur. Mitochondrial superoxide anion production can be significantly enhanced by functional impairment of the redox chain, especially of Complexes I and III (18).

Mitochondria contain their own DNA (mtDNA), which encodes thirteen mitochondrial peptides, all having the character of insoluble intrinsic membrane proteins. The mutation frequency of mtDNA is much higher than that of nuclear DNA, and these mutations can lead to mitochondrial diseases (19). Indeed, there are various disorders where mitochondrial alterations play an important role, including: Reye's syndrome, Wilson's disease, hereditary haemochromatosis, NASH (non-alcoholic steatohepatitis), Pearson's marrow pancreas syndrome, MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes), MERRF (myoclonic epilepsy associated with ragged-red fibres), LHON (Leber's hereditary optic neuropathy), and Kearns-Sayre syndrome (13).

There is growing evidence that xenobiotics with known organ toxicities are increasingly being found to directly inhibit and/or uncouple mitochondrial respiration (2, 4, 5, 17), to induce the opening of the mitochondrial permeability transition pore (MPTP; 20–22), inhibit mitochondrial membrane transporters, or increase the mitochondrial production of reactive oxygen (23) or nitrogen species (24). Molecules such as rotenone, antimycin, cyanide, oligomycin and mixothiazol, inhibit the respiratory chain, whilst others, such as amiodarone, perhexiline, flutamide and anthralin, also act as inhibitors, but do so by as-yet unresolved molecular mechanisms (25). There are several molecules which are considered to be typical Complex I inhibitors, such as papaverine, meperidine, cinnarizine, amyal, haloperidol, ketoconazole and its analogues, and other compounds used as therapeutic agents, including clofibric acid, bezafibrate, gemfibrozil and thiazolidiediones (25). Studies by Brunmair *et al.* (26) showed that the latter group of compounds inhibit Complex I, which results in metabolic consequences typical of their pharmacological activities (hypolipidaemic and hypoglycaemic effects). This also explains some of the toxic effects of the compounds, such as rhabdomyolysis and acute liver failure. Findings with various test compounds of clinical utility have yielded sufficient data to allow the prediction of different forms of clinical toxicity, based on the effects on tissue homeostasis of altering a precise aspect of mitochondrial physiology (26).

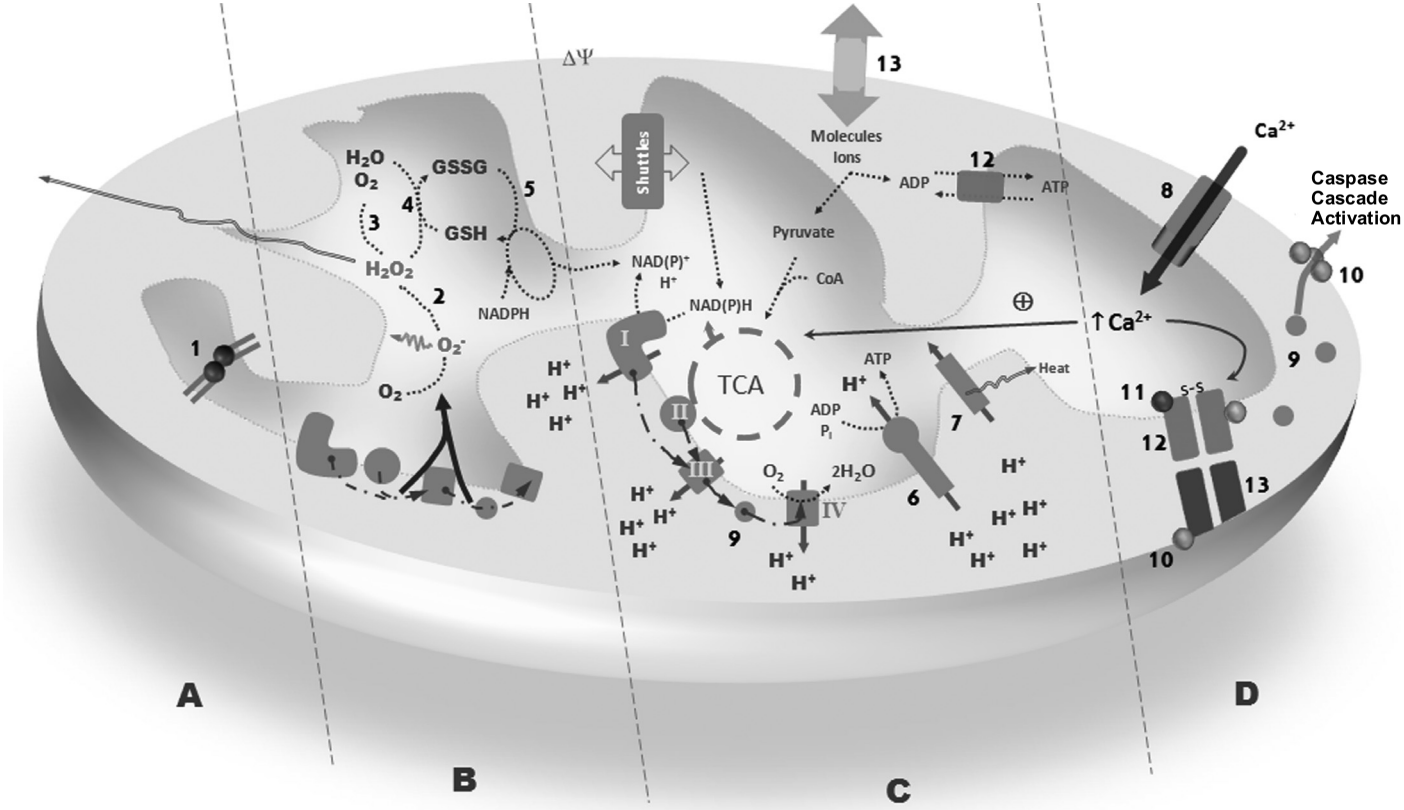
Mitochondrial Permeability Transition: The Toxicity Sensor in Cells?

When a stress stimulus reaches the death/survival threshold, a lethal outcome to the cell, with several changes in mitochondrial physiology and ultrastructure, can occur. Cell calcium overload, in addition to several stressors, including oxidants, can cause a sudden increase in the permeability of the IMM, a phenomenon called mitochondrial permeability transition (MPT) that is caused by the opening of the MPTPs. MPT was initially proposed by Haworth and Hunter in 1979, to be one result of the interaction between excessive calcium accumulation and the alteration of mitochondrial physiology (27). Up to that time, MPT was considered to be an *in vitro* artefact with little pathophysiological relevance. Today, however, it has received considerable attention as a potential mechanism for the induction of cell death, in, for example, neurodegeneration and cardiac disease (8, 13, 28).

MPT is normally associated with a deregulation of Ca^{2+} homeostasis. This phenomenon increases the IMM permeability to molecules of less than 1450Da (8, 22, 28, 29), and leads to a loss of ionic homeostasis, then to matrix swelling, OMM rupture and cell death. The MPTPs form at contact sites between the IMM and OMM (8, 22, 28). Based upon initial biochemical and pharmacological studies, it was proposed that the MPTPs contain a restricted set of proteins (Figure 1), including the unselective voltage-dependent anion channel (VDAC) in the OMM, the adenine nucleotide translocase (ANT) in the IMM, and cyclophilin D (CypD) in the matrix. Knockout studies, however, indicate that CypD is the only mandatory component of the MPTPs (8). Recently, a role for the phosphate transporter in the MPTP complex was proposed (29). The opening of MPTPs requires matrix Ca^{2+} and P_i, and is regulated by the H^+ electrochemical gradient, in the sense that depolarisation favours MPTP opening, while extra-mitochondrial acidification inhibits the phenomenon (30). Oxidative stress is also a known inducer of MPT, decreasing the calcium threshold for MPTP opening (13). Irrespective of the initiating mechanism, MPT-induced mitochondrial swelling causes matrix expansion and unfolding of the cristae, and when matrix expansion exceeds the viscoelastic resistance of the OMM, the latter will burst, and the release of pro-apoptotic intermembrane space proteins, most notably cytochrome c, SMAC/Diablo, and endonuclease-G (endoG), will occur. Apoptosis or necrosis may follow, depending on the local ATP levels (29).

Despite the fact that MPT has been implicated in the pathophysiology of several diseases, the phenomenon has been also correlated with cell damage caused by many toxicants, including: amphipathic anions (such as arachidonic acid),

Figure 1: Mitochondrial physiology within cells



*Mitochondria are dynamic organelles which can undergo fission and fusion, remodelling of the cristae, and alteration of the mitochondrial network (Panel A). These events are regulated by specific proteins, e.g. mitofusin (***1***), and several xenobiotics can interfere with their normal physiology. Although mitochondria are effective 'machines' in the conversion of O_2 to water, 4% of the O_2 used in respiration is converted to superoxide anion by electrons from the mitochondrial respiratory chain (MRC; lower Panel B). The superoxide radical can damage lipid membranes and proteins, or can be dismutated by the superoxide dismutase enzyme (SOD;* **2***) to hydrogen peroxide, which is capable of diffusing to remote areas of the cytoplasm. As a detoxification mechanism, mitochondria convert H_2O_2 to water by means of catalase (***3***) or glutathione peroxidase (***4***). The latter enzyme oxidises glutathione (GSH), which is recycled by glutathione reductase (***5***) through oxidation of NADPH. Panel C shows oxidative phosphorylation. Pyruvate is the bridge between glycolysis and the tricarboxylic acid (TCA) cycle, which produce substrates for the MRC. The electron flux from Complex I to Complex IV creates a proton-motive force which can be used to phosphorylate ADP by the ATP synthase (***6***), or can be dissipated by the uncoupling proteins (UCPs;* **7***). Mitochondria can also participate in calcium homeostasis (Panel D). After uptake by the mitochondrial calcium uniporter (***8***), matrix calcium accumulation can have two distinct effects: at lower physiological levels, calcium can stimulate dehydrogenases in the TCA cycle, while, at higher levels, it can be harmful and induce the opening of the mitochondrial permeability transition pore (MPTP). The consequence of this last event will be outer membrane rupture and consequent release of cytochrome c (***9***) into the cytoplasm and cell death. However, cytochrome c can also be released without the loss of membrane integrity, through the formation of Bax oligomers (***10***) in the outer membrane.* **11** *= cyclophilin D;* **12** *= adenine nucleotide translocase (ANT);* **13** *= voltage-dependent anion channel (VDAC).*

GD3-ganglioside, caffeic acid phenyl ester, arsenite, arsenic trioxide, pro-oxidants (like *tert*-butylhydroperoxide, diamide, phenylarsine oxide), several environmental pollutants (such as heavy metals, e.g. lead, mercury and cadmium), as well as acrolein (an air pollutant from cigarette smoke and other forms of pollution), and dichlorodiphenyltrichloroethane (DDT; 31, 32). MPT appears to occur in intact cells exposed to toxicants such as *tert*-butylhydroperoxide (33), honokiol (34), or malonate and gentamicin (31). In fact, many such compounds act by directly modulating the activity of different MPTP components, or by increasing mitochondrial oxidative stress, among other factors.

It is thus logical to use the susceptibility to calcium-induced MPT as a marker for compound toxicity in a given target organ, by using isolated mitochondrial fractions. The extent of MPT can easily be assessed by using methods such as determination of the alterations in light scattering by mitochondrial suspensions. This method is based on the observation that, when high-amplitude MPT occurs, the mitochondria swell considerably. This enlargement in size is accompanied by a decrease in opacity, resulting in a decrease in light scattering. Another common technique for measuring MPT induction is the direct measurement of calcium retention capacity, by using mitochondrial fluorescent calcium dyes (such as Calcium-Green 5N). The MPT can be studied indirectly by other methods, including measurements of mitochondrial $\Delta\Psi$ and oxygen consumption (8). One critical point is that all endpoints of MPT should be inhibited by cyclosporin A, which is the "gold standard" MPT inhibitor in isolated mitochondrial (13, 22, 28, 29).

As stated, if MPT occurs extensively in a cell, ATP will be depleted and the cell will die by necrosis. All the experimental evidence points to the fact that MPT is more than an *in vitro* artefact, and can indeed be used to predict the intrinsic toxicities of many different molecules.

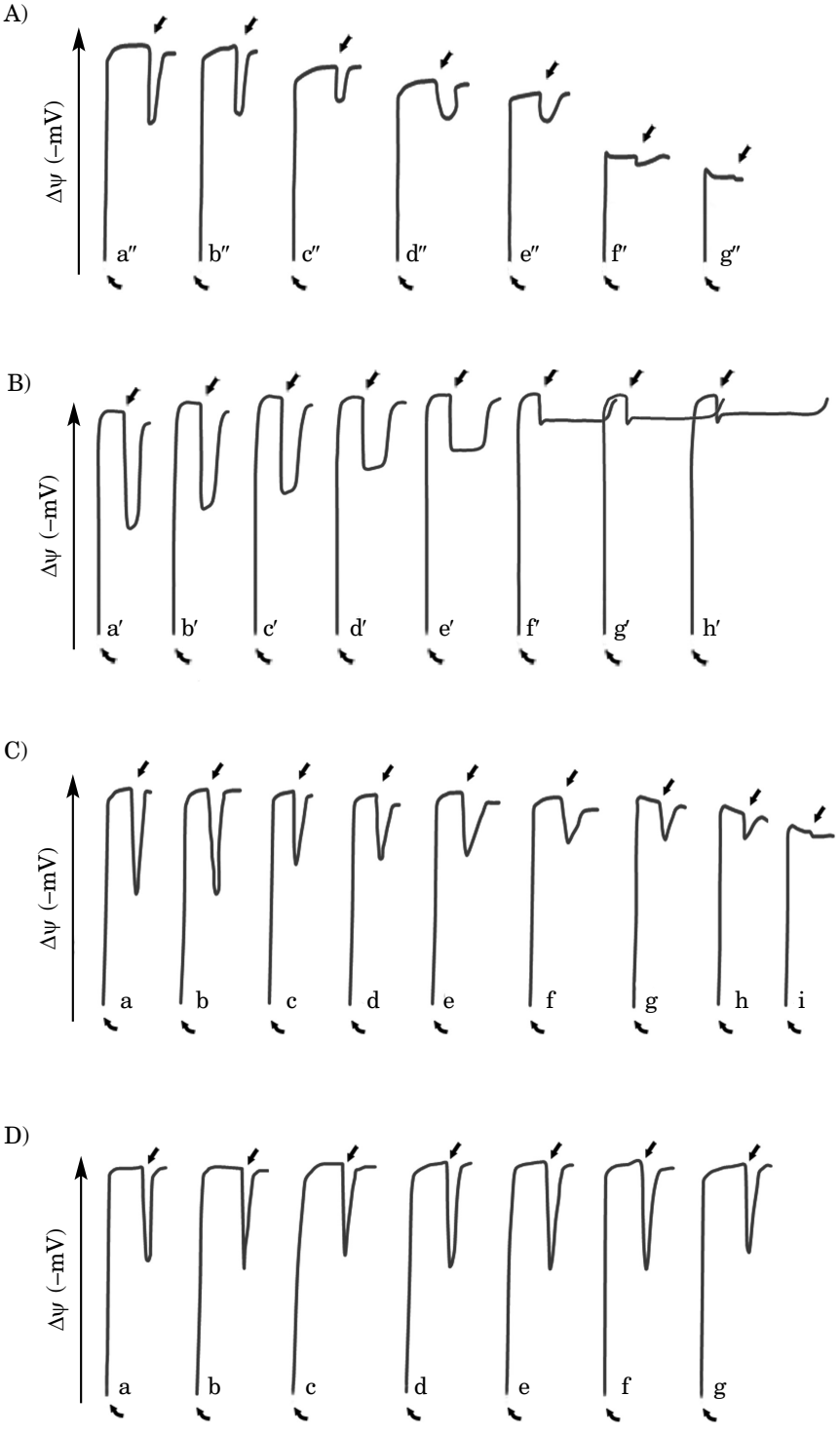
The Use of Mitochondrial Transmembrane Electric Potential ($\Delta\Psi$) as an Effective Biosensor in Toxicological Research

In our laboratory, isolated mitochondria from different sources are used to evaluate the toxicity of xenobiotics. We routinely monitor the fluctuations in mitochondrial transmembrane $\Delta\Psi$ which are associated with the energisation of mitochondria (e.g. by NADH-linked substrates and succinate) and the phosphorylative cycle, as indicators of mitochondrial toxicity. We employ a tetraphenylphosphonium ion (TPP^+)-selective electrode to estimate $\Delta\Psi$, according to previously-established methods (32, 35).

Isolated mitochondria, in the absence of toxicants, immediately develop a normal transmembrane $\Delta\Psi$ upon the addition of respiratory substrates (glutamate/malate or succinate) of between 210 and 220mV (negative inside), typical of State 2. Upon the addition of a known amount of ADP, which induces transition to State 3, the $\Delta\Psi$ immediately falls by about 30mV, since ATP synthase uses the $\Delta\Psi$ to phosphorylate the added ADP. After the phosphorylation cycle, the $\Delta\Psi$ returns nearly to its initial value (i.e. to State 4). In an assay in current use, we are testing the effects of increasing concentrations of the compound under study on the normal $\Delta\Psi$ profile. Increasing concentrations of endosulphan induced a consistent decrease in maximal substrate energisation, as compared to the control, which suggests that the mechanisms of $\Delta\Psi$ generation are affected (Figure 2, Panel A). Additionally, several changes are evident in the phosphorylation cycle profile. By contrast, tributyltin (TBT) profoundly affects the depolarisation induced by ADP, without affecting the maximal substrate energisation (Figure 2, Panel B). In the particular case of TBT, there is even a slight increase in $\Delta\Psi$ after substrate addition. Thus, selecting the most appropriate endpoint for this mitochondrial assay depends on: a) the capacity of mitochondria to develop $\Delta\Psi$ after substrate addition (a NADH-linked substrate or succinate) — for example, in the case of endosulphan; and b) the depolarisation induced by ADP — for example, in the case of TBT. Specific endpoints are then chosen, according to the different actions of the test compounds on oxidative phosphorylation. This mitochondrial assay can reveal the underlying mechanisms of xenobiotic toxicity. For instance, if a xenobiotic promotes a decrease in the maximal $\Delta\Psi$ developed, it might act by affecting the impermeability of the IMM to protons (e.g. uncouplers, inducers of the MPTP, or detergent-like test compounds), or act by inhibiting the respiratory chain (e.g. rotenone, antimycin, or compounds with cyanide-like effects). On the other hand, chemicals that only affect the depolarisation induced by ADP, act by inhibiting the phosphorylative system (ATP synthase, adenine nucleotide translocase or phosphate transporter).

This mitochondrial assay has also proven to be a convenient screen for fresh water or wastewater pollutants. In particular, it has shown some interesting advantages in comparison to other tests, such as high sensitivity to effluents, the ability to elucidate the mode of action of an effluent in the mitochondrial metabolism, and good reproducibility (36). In specific situations, the assay can be used to establish effluent toxicity management strategies. Figure 2 shows the application of the test in the toxicity evaluation of samples of local raw wastewater (influent; Panel C) and the treated wastewater (effluent; Panel D). Raw wastewater

Figure 2: The distinct effects of xenobiotics on typical tetraphenylphosphonium ion (TPP^+)-selective electrode measurements of mitochondrial membrane potential



Tributyltin (TBT) causes profound effects on the depolarisation induced by ADP (Panel B), unlike endosulphan (Panel A), whose effect is mostly apparent in the decrease in maximal substrate energisation ($\Delta\Psi$, negative inside). The curved arrows show the point of succinate addition and the straight arrows show the point of ADP addition.

Raw wastewater (influent; Panel C) decreases mitochondrial ADP depolarisation in a dose-dependent fashion that is abolished after water treatment (effluent; Panel D), confirming the efficacy of the water treatment in the clearance of the toxic components. The curved arrows show the point of succinate addition and the straight arrows show the point of ADP addition.

Figure 2: continued

E)

Key	Raw treated wastewater conc. (% v/v)	ADP depolarisation (mV)	Key	Endosulphan conc. (nmol/mg protein)	$\Delta\psi_{max}$ (–mV)
a	0.00	37	a″	0.0	211
b	1.15	36	b″	10.0	211
c	1.96	25	c″	14.0	205
d	2.72	23	d″	19.6	197
e	3.77	17	e″	27.4	186
f	5.20	13	f″	38.4	161
g	7.13	8	g″	53.8	145
h	9.60	5			
i	11.60	3			

Key	TBT conc. (nmol/mg protein)	ADP depolarisation (mV)
a′	0.0	65
b′	0.2	39
c′	0.4	26
d′	0.5	20
e′	0.6	15
f′	0.8	12
g′	1.0	12
h′	1.2	0

Panel E shows the concentrations of the xenobiotics used in the experiments referred to above (i.e. a–i, a´–h´, and a´´–g´´), and also the absolute values for the affected parameters. The half maximal effective concentration (EC50) values (expressed as the mean, n = 4, with the range in brackets) are: 24.66 (20.36–29.38) nmol/mg protein for endosulphan; 0.28 (0.17–0.42) nmol/mg protein for TBT; and 3.18 (2.48–3.97) %v/v for raw wastewater, where % v/v = volume of wastewater/volume of ultrapure water × 100. The figure is adapted from Silva et al. (37), with permission.

induces mitochondrial ADP depolarisation, without affecting the $\Delta\Psi$ generated by the respiratory substrate, succinate. After the treatment of the wastewater, it was demonstrated that the toxic compounds were removed, because the negative effects on mitochondrial bioenergetics were no longer apparent (37).

Owing to the central role of mitochondria in energy metabolism and their natural propensity to be affected by toxicants owing to their membrane structure, measuring mitochondrial activities can be a suitable toxicity biosensor. Furthermore, mitochondrial enzymes have been conserved by the evolutionary process, so a compound that is toxic in a mitochondrial assay is likely to be similarly toxic to all eukaryotes, including higher animals and humans. Thus, *in vitro* $\Delta\Psi$ assays can be envisaged as a valuable tool for environmental monitoring and preliminary screening of the acute toxicity of a variety of xenobiotics, including industrial chemicals and pharmaceutical products. However, complementary pharmacokinetic studies are required to gauge toxicity, since these *in vitro* studies cannot account for the uptake and excretion processes which occur *in vivo*.

Other mitochondrial toxicity tests include measurement of the enzymatic activity of submitochondrial particles (SMPs). These are isolated vesicles of the IMM, which are functionally equivalent to whole mitochondria (38). Both the $\Delta\Psi$ and the SMP assays are rapid, reliable and sensitive *in vitro* tests for the economical preliminary screening of chemical toxicity, and are appropriate alternatives to animal testing. One major limitation of this kind of assays is the relatively low-throughput of the methods themselves, which limits the number of compounds that can be tested per day, although one needs to take into account their advantages against *in vivo* studies (i.e. a lower number of animals used per compound tested).

Can We Speed Up the Screening Processes?

As illustrated in Figure 3, drug development and safety testing can involve the use of a large number of laboratory animals, which, without a decisive pre-screening for mitochondrial toxicity, often give data that translate poorly to the protection of human health and safety.

Faster novel techniques involving high-throughput methods are preferred for investigating whether a molecule is a mitochondrial toxicant. Moreover, such screens are mechanistically-based and allow effects on oxidative phosphorylation, calcium homeostasis and mitochondrial genetics to be assessed (13). As shown in Figure 3, such assays are sensitive and allow several types of data to be gathered. High-throughput methods, designed to evaluate the mitochondrial toxicities of test compounds, would also provide the necessary data for the construction of databases to contain information for determining whether mechanistically distinct types of mitochondrial toxicity can be attributed to common structural motifs. Theoretical methods based on data from compounds with established mitochondrial toxicity (namely, mitochondrial quantitative structure– activity relationships [mitoQSARs]), could permit the design of novel compounds with reduced risk to mitochondria, improving the success rates for new drug entities. Another possibility for such data is to assist with rational drug development, since structural motifs that are indicated in specific forms of mitochondrial toxicity can be avoided, masked or otherwise modified within drug candidates, whilst maintaining the desired pharmacological action.

As indicated, several high- throughput screening methods are used by the pharmaceutical industry to investigate thousands of new molecules for potential lead compounds. The new methods are essentially similar to methods that have been in use for decades in basic research laboratories to investigate mitochondrial toxicity, although the difference is in the potential number of compounds which can be analysed per day. As an example, one "gold standard" to evaluate mitochondrial function is the measurement of oxygen consumption. The normal protocol in a research laboratory involves testing several concentrations of a test compound from the same preparation, and repeating the experiment with three or more independent preparations. However, the conventional polarographic method used to measure mitochondrial oxygen consumption does not easily lend itself to high-throughput sample automation. In order to test the maximum range of compounds possible, the development of novel techniques is recommended. The measurement of mitochondrial oxygen consumption by using an optimised protocol that employs phosphorescent oxygen-sensitive probes and is suited to multi-well plate readout, is an important advance, as it permits the simultaneous determination of all the samples in a 96-well plate (39). Other techniques can also be used in a multi-well platform, including the assessment of mitochondrial swelling, or calcium-induced mitochondrial depolarisation and release.

Concluding Remarks

The opportunity to undertake specific dose–response studies on mitochondria, represents a step toward the minimisation of animal use in drug discovery and development, and thus should be suggested as a first step into the generation of safe pharmaceuticals. In a proposed flow-chart for assessing compound toxicity, the *in vitro* study of mitochondrial function would be the first step (8) after the selection of the group of compounds to be tested. It is possible to alter the type of studies to be performed, according to what it is known from previous data on compounds of the same family (e.g. whether a certain family of compounds is known to inhibit mitochondrial DNA transcription). The data obtained from the initial mitochondrial studies, besides eliminating compounds with higher toxicity, would also permit the prediction of idiosyncratic reactions of compounds with low mitochondrial toxicity in patients with diagnosed mitochondrial diseases, or in individuals that are known to be exposed to stress conditions that affect mitochondrial function in different tissues (e.g. tobacco or drug consumption, exposure to environmental toxins, ageing, or a history of endurance training).

Addressing the question posed by our title, it can be concluded that it is possible to use isolated mitochondrial fractions as a toxicological sensor to predict drug safety, and thereby to reduce and refine animal use in drug development and testing.

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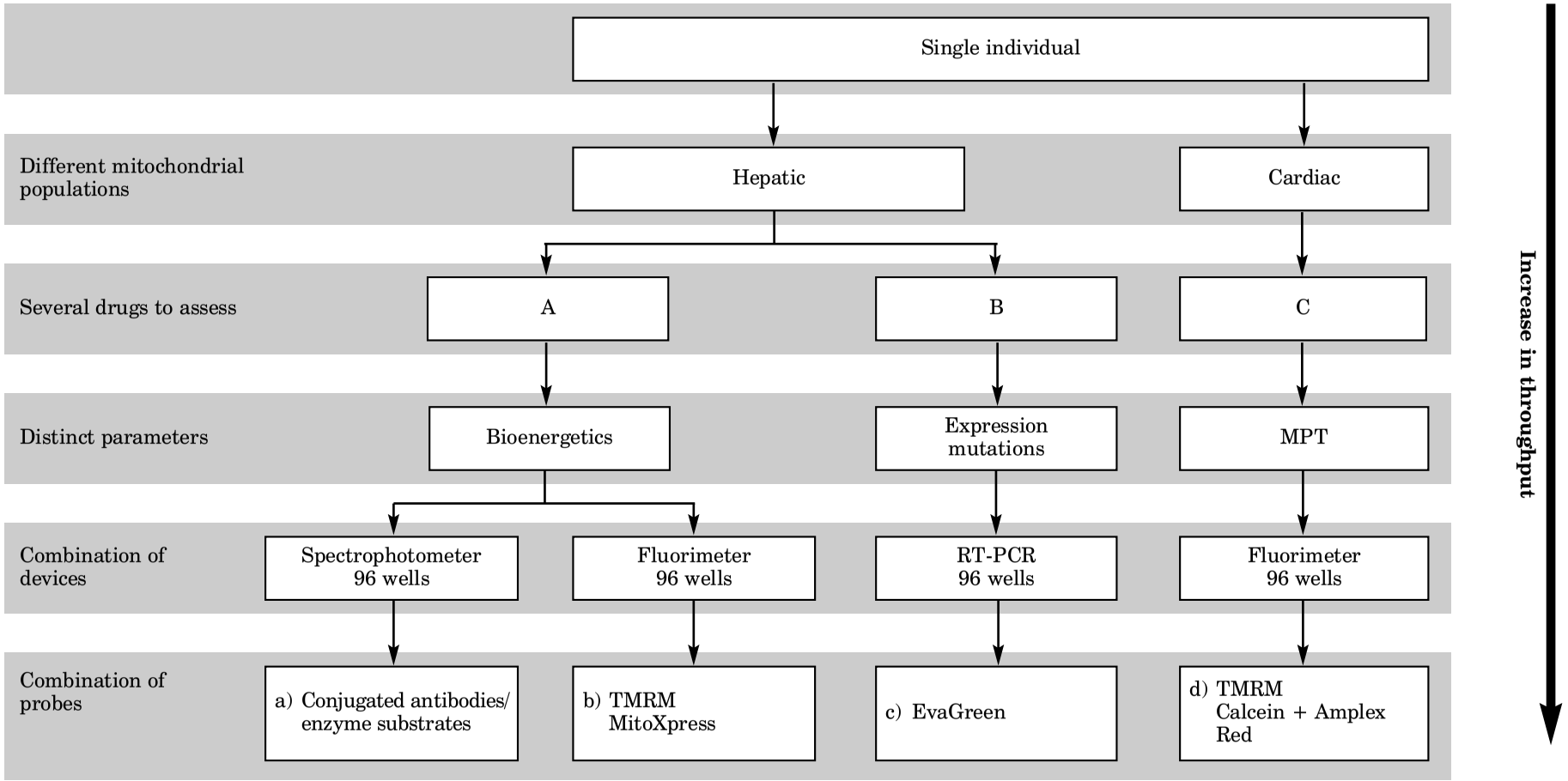
Figure 3: A typical example of how high-throughput techniques can be used to screen several drugs in different mitochondrial populations



Protein quantification and activity can be measured in multi-well plate assay format, by using antibodies that target mitochondrial respiratory chain complexes (a), taking advantage of the kinetics of degradation/production of certain mitochondrial metabolites. Alternatively, by using a combination of specific probes, such as tetramethylrhodamine methyl ester (TMRM) and MitoXpress, mitochondrial transmembrane electric potential and oxygen consumption, respectively, can be measured (b). By using RT-PCR to take advantage of the melting curve of the EvaGreen probe bound to DNA (c), mRNA levels, DNA copy number and DNA mutations of either the nuclear or mitochondrial genome can be evaluated. If mitochondrial dysfunction associated with mitochondrial permeability transition (MPT) is evaluated, different classical assays can be combined into a single well (d), as there is the potential to fluorimetrically scan at multiple wavelengths simultaneously. The number of compounds analysed at any one time can be increased by the utilisation of robotic handling. The hypothetical example in the figure shows that for several target organs, different parameters can be measured, according to the toxicity predicted in each case.

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