Investigating Drug-induced Mitochondrial Toxicity: A Biosensor to Increase Drug Safety?

Cláudia V. Pereira, Ana C. Moreira, Susana P. Pereira, Nuno G. Machado, Filipa S. Carvalho, Vilma A. Sardão and Paulo J. Oliveira[*]

Center for Neurosciences and Cell Biology, Department of Zoology, University of Coimbra, Portugal

Abstract: Mitochondria are recognized as the producers of the majority of energy cells need for their normal activity. After the initial comprehension of how mitochondrial oxidative phosphorylation produces energy, mitochondrial research was not a priority for most cell biologists until novel mitochondrial functions were identified. In fact, it is now known that mitochondria are not only involved in cell calcium homeostasis, intermediate metabolism and free radical generation but are also a crucial crossroad for several cell death pathways. The notion that several clinically used drugs and other xenobiotics induce organ degeneration through damaging mitochondrial bioenergetics led to the use of the organelle as an effective and reliable bio-sensor to predict drug safety. Classic methods used to test the toxicity of a wide range of compounds on isolated mitochondrial fractions were later replaced by novel high-throughput methods to investigate the safety of a very large number of new molecules. Without surprise, the assessment of "mitochondrial safety" for new discovered molecules is of clear interest for pharmaceutical companies which can now select compounds lacking mitochondrial toxicity to undergo further trials, thus avoiding the possibility of later human toxicity due to mitochondrial liabilities.

Keywords: Mitochondria, drug safety, toxicity, high-throughput methods, biosensor.

INTRODUCTION

1. Mitochondria: More than Just Cell Furnaces

In the majority of human tissues, mitochondria play different and crucial roles. It has been estimated that almost 90% of oxygen consumption by mammals occurs in mitochondria with the ultimate objective of synthesizing ATP [1].

After determining that mitochondria produce energy by chemiosmosis [2], the organelle was mostly considered as the cell furnace, whose operation involved membrane charge separation. In the 80's and 90's, mitochondria again took the spotlight with the breakthrough discovery that the organelle not only provided cell energy but was also involved in calcium homeostasis [3], in intermediate metabolism, in the generation of reactive oxygen species (ROS) and most importantly, in the progression or initiation of cell death [4-6]. The central role mitochondria play in cell life and death and its involvement in a wide range of diseases including cancer [7], diabetes [8] or cardiovascular [8] and neurodegenerative diseases [8], increased the scientific interest in the regulation of mitochondrial bioenergetics in cells.

Advances in analytical methods aimed at investigating compounds that damage mitochondrial function have been a major thrust for the design and development of drugs that would specifically target mitochondria for therapeutic purposes. In fact, drug safety is now one of the most important issues for most if not all pharmaceutical companies. Improving the pharmacological effect of a drug in the organism and diminishing the toxicological effects is a major concern in

drug development. Since mitochondrial dysfunction is increasingly implicated in drug-induced toxicity, mitochondrial models are now used to reduce late stage attrition of drug candidates and to allow the design of safer drugs [9].

The interest on mitochondrial toxicology and pharmacology is not unexpected as the importance and interest on the regulation of mitochondrial physiology and dysfunction has increased in the last years. Searching PUBMED database (http://www.ncbi.nlm.nih.gov/sites/entrez/) using the keyword "mitochondria" resulted in 125,733 published papers from 1950 until July 2008. A further increase in the number of publications per year is coincident with the discovery of the relationship between apoptosis and mitochondria (Fig. **1**). The increasing trend still goes on, fuelled by exciting and novel discoveries in mitochondrial physiology.

The present review demonstrates the importance of mitochondria on organ viability and exemplifies several cases of drug-induced mitochondrial toxicity. An overview on the use of mitochondria as a preliminary bio-sensor for the improvement of drug safety in many different target organs is also provided, together with a description of traditional and novel methods available to investigate drug-induced alterations of mitochondrial function.

1.1. Mitochondrial Energy Production

The discovery of mitochondria occurred in the late 19th century, the organelle being described as a collection of cytosolic free-floating individual vesicles forming threads inside cells. The early descriptions were confirmed by electron microscopy observations of tissue sections, which revealed the existence of a mitochondrial network [10] also denominated "mitochondrial reticulum" [11, 12].

Mitochondrial energy production is achieved by electron transfer in the respiratory chain by using a process called
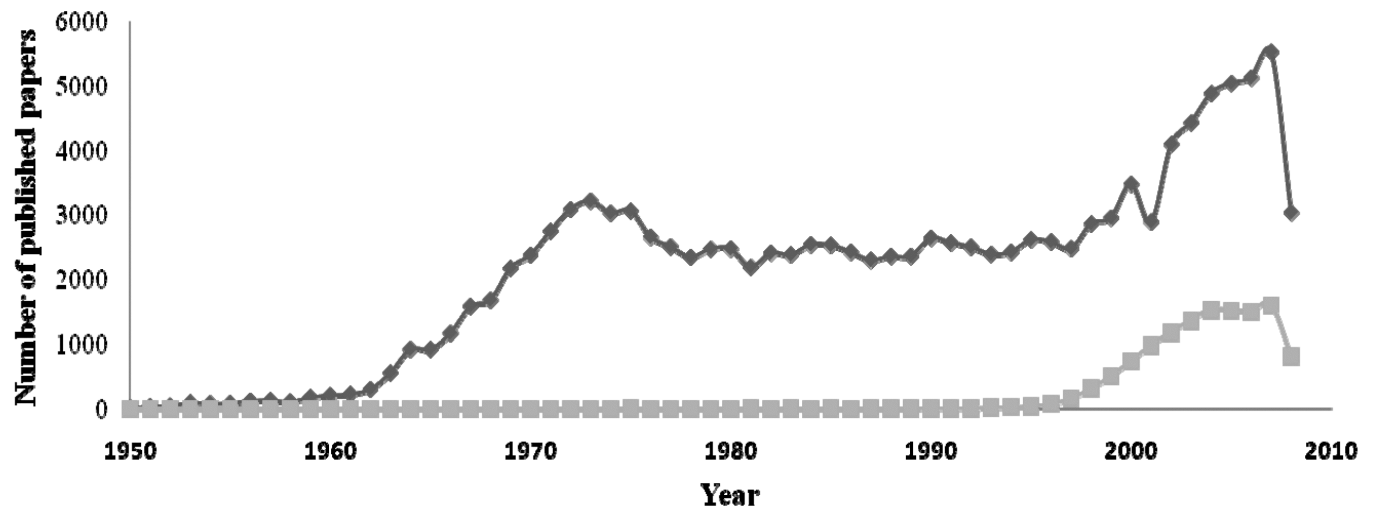
Fig. (1). The increasing number of published papers in *PUBMED* (http://www.ncbi.nlm.nih.gov/sites/entrez/) archive between 1950 and July 2008 The trend shows an increased interest on mitochondrial, especially when apoptosis was related with the organelle. The dark grey line above represents the number of publications found after searching using the keyword "mitochondria". The light grey line below represents the number of papers found with the keywords "mitochondria and apoptosis". Notice the increase in published papers when mitochondria become associated with the apoptotic process.

oxidative phosphorylation (OXPHOS, Fig. **2A**) [2, 13]. Mitochondria are also the arena for other metabolic pathways, the most well known being fatty acid β-oxidation and the Krebs cycle (Fig. **2B**). Under anaerobic conditions, pyruvate produced during glycolysis can undergo alcoholic or lactate fermentation, while under aerobiose, pyruvate is converted in the mitochondrial matrix by pyruvate dehydrogenase into acetyl coenzyme A (acetyl-CoA) [14]. In the mitochondrial matrix, the Krebs cycle occurs, which consists in a series of chemical reactions involving enzymes with the final objective of oxidizing acetyl CoA, producing molecules capable of supplying electrons to the MRC or replenishing intermediates for other pathways. The final result of the Krebs cycle is the formation of two molecules of CO_2, three molecules of NADH, and one molecule of GTP and acetyl CoA (Fig. **2B**). Additionally, succinate formed in the cycle can be oxidized by complex II of the MRC. An alternative way of obtaining energy through oxidative phosphorylation is by using lipids stored under the form of fatty acids in triglycerides. Fatty acids are degraded by a catabolic process known as β-oxidation in the mitochondrial matrix. The process consists in the subtraction of several units of acetyl-CoA by oxidation; the triglycerides are hydrolyzed by lipases resulting in glycerol and fatty acids. Glycerol can follow the glycolytic pathway while fatty acids suffer β-oxidation. Inside the mitochondrial matrix, chemical reactions such as dehydration, hydration and oxidation occur, converting fatty acids in several products used in the mitochondrial metabolism including succinyl-CoA which enters the Krebs cycle. The reduced equivalents obtained from the different pathways are then used by the mitochondrial respiratory chain to generate ATP by OXPHOS. Electrons from different origins are transferred between different proteins which form the electron transport chain (ETC), finally being delivered to molecular oxygen, which acts as the final acceptor. The ETC is composed of several proteins located in the inner mitochondrial membrane and in structures called cristae [15]. According to the chemiosmotic teory, the proton gradient that is

formed between the matrix and the intermembrane space has two components, one being the transmembrane electric potential ($\Delta\Psi$) and the other one being the pH gradient (ΔpH). Mitochondria are unique cellular organelles which can build up a transmembrane electric potential of up to -180 mV [2, 5] (Fig. **2**). Mitochondrial energy production can alternate between two steady-states. Respiration is slower during the so-called state 4 respiration as no ATP production occurs, leading to the maintenance of a high $\Delta\Psi$ value. By its turn, state 3 respiration is faster as ATP is being generated by the ATP synthase, with concomitant use of the $\Delta\Psi$. In particular occasions, when the inner membrane becomes permeable or when a protonophore is used, the $\Delta\Psi$ can be completely dissipated. Respiration is then increased and ATP production decreases due to the formation of a futile proton cycle that causes mitochondria to produce heat, instead of ATP. The inhibition of the ETC also generally decreases $\Delta\Psi$ with a simultaneous decrease in respiration. Under physiological conditions, it is considered that mitochondrial ATP production occurs in an intermediate state between state 3 and state 4. Synthesized ATP is then exported by the adenine nucleotide translocator (ANT) to the cytosol in exchange for ADP.

1.2. Calcium Homeostasis and the Mitochondrial Permeability Transition Pore

Changes in cytosolic Ca^{2+} concentration provide signals to control important events such as muscle contraction, neurotransmitter release, alterations in gene transcription and even cell death, among other phenomenon [16-19]. In the last years, a wide range of evidence demonstrated a crucial role for mitochondria in shaping cellular calcium signaling, which converted mitochondria into an organelle of interest when studying temporal and spatial regulation of calcium spikes in cells. The capacity of isolated mitochondria to accumulate calcium in the matrix depending on the proton gradient was first described in 1962 [20]. According to the Nernst equation, calcium should be accumulated inside the mitochondrial matrix with a 1 million fold concentration
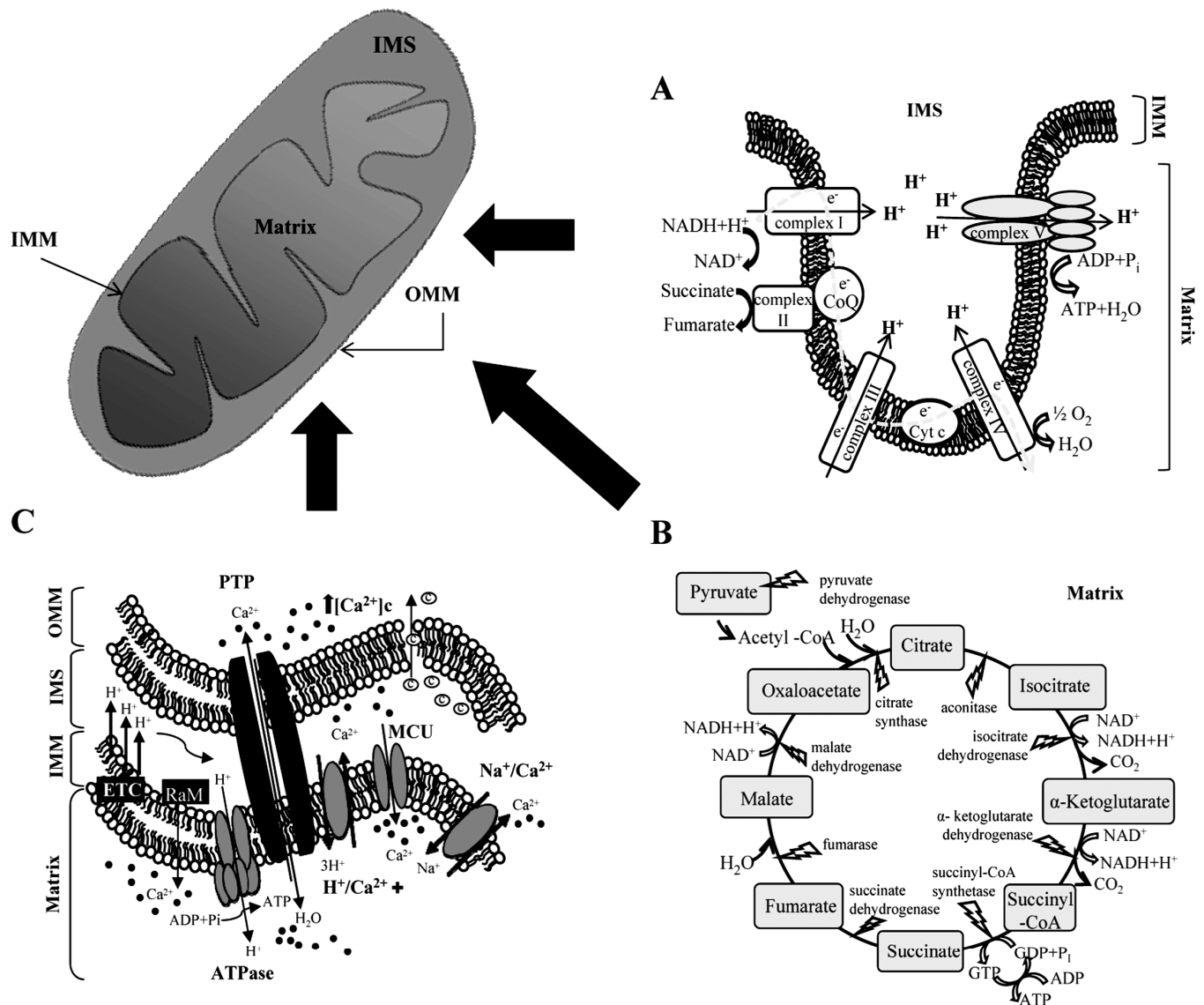
Fig. (2). Mitochondria: the center of bioenergetics, calcium homeostasis and cell death. The figure represents different functions of mito-chondria: (**A**) Oxidative phosphorylation (OXPHOS). Substrates from Krebs cycle are oxidized by the mitochondrial respiratory chain in order to establish an electrochemical gradient of protons that is used by the ATP synthase to produce ATP. The chain of reactions involves five enzymatic complexes, the NADH-ubiquinone oxireductase (complex I); the succinate-ubiquinone oxireductase (complex II); the ubiqui-nol-ferrocytochrome c oxireductase or cytochrome c reductase (complex III), cytochrome c oxidase (complex IV) and finally, complex V or F_1Fo ATP synthase. Other crucial components of the electron transfer reactions are two electron carriers, the lipid soluble coenzyme Q (CoQ) and the soluble protein cytochrome c (cyt c.). The ETC complexes are composed of several subunits encoded by the mitochondrial or nuclear DNA, with the exception of complex II, which is encoded by the nuclear DNA only. In the presence of substrates (NADH or succi-nate), the transfer of electrons from complex I and/or complex II to complex IV occurs simultaneously with the pumping of protons from the matrix to the intermembrane space, generating an proton electrochemical gradient ($\Delta\mu H^+$) which is ultimately used by complex V to phos-phorylate adenosine diphosphate (ADP) to ATP, the energetic fuel of the cells. (**B**) Krebs cycle, which occur inside the mitochondrial matrix and where the complete oxidation of pyruvate leads to the production to NADH and succinate under aerobic conditions. (**C**) Calcium homeo-stasis and the permeability transition pore: calcium concentration inside the mitochondrial matrix depends not only of an electrogenic mito-chondrial calcium uniporter (MCU) but also on antiporters (Na^+/Ca^{2+} and H^+/Ca^{2+}). Inside mitochondria, calcium modulates the activity of several important enzymes, some of which part of the Krebs cycle, and also stimulates ATP synthase. An excess of calcium accumulation in the matrix leads to the formation of the mitochondrial permeability transition pore, which spans the inner and outer mitochondrial membrane and whose opening leads to the collapse of the transmembrane electric potential, ultimately leading to mitochondrial and cellular dysfunc-tion. Legend for figure: IMM – inner mitochondrial membrane, OMM – outer mitochondrial membrane, IMS – intermembrane space, PTP – permeability transition pore, MCU – mitochondrial calcium uniporter.

than that in the cytosol [21]. However, this was against all the experimental measurements of intramitochondrial cal-cium concentrations. The paradox was eventually solved

when it was demonstrated that calcium accumulation inside the mitochondrial matrix depends not only of an electrogenic uniporter or mitochondrial calcium uniporter (MCU) but also

on antiporters (Na^+/Ca^{2+} or H^+/Ca^{2+}) which export Ca^{2+} from mitochondria (Fig. **2C**). Upon accumulation, mitochondria slowly release calcium back to the cytosol *via* the antiporters [22, 23]. Mitochondria can thus act as localized cytosolic calcium buffering organelles, modulating several events of Ca^{2+} feedback inhibition or activation [24, 25]. Within mitochondria, calcium increases the activity of several enzymes that have a crucial role on the Krebs cycle and ATP production, such as pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and NAD^+-isocitrate dehydrogenase [26] as well as the ATP synthase [27]. The increase of mitochondrial calcium ($[Ca^{2+}]_m$) activates mitochondrial metabolism, increasing the supply of ATP under aerobic conditions for a number of energy-consuming processes.

The mitochondrial efflux mechanisms assisted by the matrix Ca^{2+} buffering activity, maintain a stable $[Ca^{2+}]_m$, allowing mitochondria to accumulate 700-1000 nmol Ca^{2+}/mg of protein before bioenergetic breakdown occurs. The mitochondrial buffering capacity make these organelles in efficient controllers of the spatial and temporal shape of global cellular Ca^{2+} signals, cooperating with the endoplasmic reticulum in calcium-signaling processes [28]. It has been reported that mitochondrial calcium accumulation also occurs through a "rapid uptake mode" (RaM) mechanism which acts on a millisecond timescale, as well as through a mitochondrial isoform of the ryanodine receptor which has been described in some excitable cells [29] (Fig. **2C**).

Moderate mitochondrial Ca^{2+} uptake activates metabolism while excessive Ca^{2+} accumulation can induce radically different effects. Calcium may synergize apoptotic mediators and induce a large-scale mitochondrial morphological alteration, which can cause cell death [30].

The deregulation of mitochondrial calcium homeostasis is now recognized to play a crucial role in several pathologies. In addition to the above-described mitochondrial Ca^{2+} transport mechanisms, it has been shown that some alternative forms of the mitochondrial permeability transition (MPT, see below) may also participate in mitochondrial Ca^{2+} homeostasis. A low conductance form of the calcium-induced MPT leads to calcium extrusion from the mitochondrial matrix, which in particular systems may serve as an amplification loop for Ca^{2+}-induced Ca^{2+} release from the ER/SR calcium stores [31]. In general terms, the MPT is a sudden non-selective increase in the permeability of the inner mitochondrial membrane to solutes of molecular mass less than ≈1500 KDa (Fig. **2C**). The MPT occurs when mitochondria are treated *in vitro* with an excess of calcium or several reagents that increase oxidative stress [32]. The MPT results in a loss of mitochondrial membrane potential and in a decrease of ATP production. It is now known that the MPT is not a consequence of nonspecific mitochondrial membrane damage, but instead the result of the opening of membrane channels, the so called permeability transition pores (PTP) [33, 34], which are inhibited by the immunosuppressant cyclosporin A (CsA) [35].

The nature of the structural components of the PTP has been a topic of debate. Three main components have been proposed to constitute the structure of the PTP: the adenine nucleotide translocator (ANT), the voltage-dependent anion channel (VDAC), the most abundant protein in the outer mitochondrial membrane, and also the matrix chaperone cyclophilin D (CyP-D). The PTP complex is proposed to be located at contact sites between the inner and outer mitochondrial membrane. The reconstitution of PTP-like activity in planar bilayers and proteoliposomes from preparations containing complexes with the VDAC, the ANT, the cytosolic hexokinase, and the mitochondrial creatine kinase [36], suggests that kinases are also involved in MPT regulation.

The role of the ANT in the MPT is supported by the inhibition or activation of the MPT by bongkrekic acid and atractyloside, respectively, which are specific ligands of the ANT [37]. Cyclophilin D shows peptidyl prolyl-cis, trans-isomerase (PPIase) activity and has a very important role in protein folding [38]. The presumed role of Cyp-D as a regulatory component of the MPT is based on the observation that CsA, a specific inhibitor of the cyclophilin family, blocks the MPT [39].. It is suggested that Cyp-D associates with the inner mitochondrial membrane during the MPT to induce a conformational change of a critical pore component, most likely the ANT, leading to an increase of inner membrane permeability. Experimental evidence for a direct role of VDAC in the MPT has been provided by studies using anti-VDAC antibodies that inhibit VDAC activity [40] and also the calcium-induced MPT [41].

Some forms of apoptosis (see below) are inhibited by CsA, suggesting a role of the MPT in cell death [42]. The observation is also supported by the fact that apoptosis can in some cases be inhibited by bongkrekic acid [37, 43]. Interestingly, anti-apoptotic members of the Bcl-2 family (Bcl-2 and Bcl-x_L) can inhibit the Bax/Bak-dependent increase of mitochondrial membrane permeability by direct interaction with some of the PTP components, including the VDAC (see Fig. **2D**) [44].

Understanding the molecular structure of the PTP can provide new understanding of the mechanisms that are critical for cell life and death and also present an important target for a novel class of drugs. Interestingly, an increasing new volume of experimental data antagonizes the classical model view of the PTP. Genetic knockout experiments have contradicted the long-established "dogma" concerning the main identity of the PTP. Some experimental evidence now exists against the role of the VDAC [33, 45-47] and the ANT [33, 48] as critical pore components. Also, the role of hexokinase II as a PTP regulator is not yet fully understood [33, 36, 49, 50]. Only the role of cyclophilin D as an important PTP regulator was confirmed and clarified [33, 51-53]. Nevertheless, none of the published data is completely clear in excluding pore components.

The traditional ideas about the role of Ca^{2+} on MPT induction also require particular attention. Although it is accepted that the MPT in intact cells is triggered by an increase in mitochondrial calcium concentration [21, 34, 54], some studies suggest that calcium might not be the key element to MPT induction in cardiac myocytes and neurons [33]. Emerging evidence suggests that after an injury-producing stress, it is the generation of reactive oxygen species and not calcium the responsible for pore induction [33]. An interesting study [55] on the role of ANT as a mitochondrial calcium sensor may help explaining some contradictory results. The results indicate that overexpression of different ANT isoforms induces different alterations in calcium homeostasis [55].

1.3. Mitochondrial Oxidative Stress

Reactive oxygen species (ROS) generation by mitochondria is a continuous and physiologic process that occurs in aerobic conditions [56] and which increases with ageing [57]. A small but constant leak of electrons from the mitochondrial respiratory chain induces a monoelectronic reduction of molecular oxygen, forming superoxide anion ($O_2^{\bullet-}$). Nearly 2-4% of the total oxygen consumed by mitochondria is not totally reduced to water and results in the formation of ROS. Although superoxide anion has moderated chemical reactivity, it can be converted in more reactive species [58]. Superoxide anions generated on mitochondria are rapidly converted by the intramitochondrial superoxide dismutase ($_{mit}SOD$) to hydrogen peroxide (H_2O_2), also classified as ROS due to its oxidative power [58]. Deregulation of cellular calcium can also increase the production of superoxide anion by the MRC. The binding of calcium to the phospholipid cardiolipin induces alterations in mitochondrial membranes which compromise the conformation and consequently the functionality of membrane enzymes, facilitating electron leak from the respiratory chain [59]. The production of superoxide anion occurs primarily in the NADH dehydrogenase (complex I) and in the CoQ cycle in complex III [60]. The formation of superoxide anion in the iron-sulfur centers in complex I is increased if several NADH-linked substrates such as malate, glutamate and pyruvate, are used [61]. Rotenone, an inhibitor of the electron transfer from complex I to CoQ stimulates the production of superoxide anion [62]. The CoQ cycle is also prone to electron leakage and consequent ROS production. The formation of superoxide anion probably occurs due to electron donation from semiquinone anions ($UQ^{\bullet}H$) to molecular oxygen. The electron leak at this level may be stimulated by succinate (complex II substrate), antimycin A (complex III inhibitor) and cyanide (complex IV inhibitor). Antimycin A blocks the formation of semiquinone anions in the matricial face of the inner mitochondrial membrane, promoting the accumulation of semiquinonic anions previously formed in the cytosolic face, facilitating electron leak and ROS production [63].

Although ROS production by mitochondria is a continuous process in physiological conditions, this organelle has an efficient antioxidant defense network [64]. Mitochondrial superoxide dismutase, glutathione peroxidase, glutathione redutase, α-tocopherol and cytochrome c are some examples of mitochondrial antioxidant defenses [64]. The methionine residues of mitochondrial proteins are also an effective antioxidant protection to prevent the oxidation of protein cysteine residues. The reduction of methionine residues induces the formation of methionine sulphoxide, which does not compromise the protein functionality [58].

As described above, the majority of cellular ROS production occurs in the MRC, which explains why lipids and proteins from the mitochondrial inner membrane are more susceptible to be oxidized. However, mitochondrial DNA can also be a preferential target for ROS since it is located in close proximity with the inner membrane [65, 66]. The oxidation of mitochondrial lipids, protein and DNA are responsible for ROS-induced mitochondrial dysfunction. Oxidative modifications of mitochondrial electron transport chain proteins compromise normal activity, resulting into a further increase in ROS production and oxidative damage, contributing for further mitochondrial dysfunction [57]. Nonfunctional mitochondria may be eliminated by autophagy, a cellular process involving the degradation of non-functional cellular components. Several studies have proposed that ROS may be also involved in the induction of cellular autophagy, eliminating non-functional and oxidative modified structures, including organelles [67, 68].

In situations of mild oxidative stress, mitochondrial ROS production may also be beneficial. For example, during a brief episode of cardiac ischemia/reperfusion, ROS can contribute for pre-conditioning of the myocardium, making the heart more resistant to longer periods of ischemia and reperfusion [69]. Furthermore, when at low levels, mitochondria ROS may also act as a signaling molecules in various intracellular processes [70], activating and improving cellular antioxidant defenses.

1.4. Mitochondria and Cell Death

Until recent years, scientists believed that cell death was a passive and a degenerative process. Cells would only die when injured by external factors, such as physical agents (mechanical action, temperature, radiation or magnetic effects), chemicals (alcohol, drugs or detergents) or biological agents (viral, bacterial and parasitic infections). It is now known that cell death can occur under different processes, the most characterized of which being apoptosis, or programmed cell death, and necrosis.

During necrosis cells suffer from damage that results in increased volume, aggregation of chromatin, disorganization of the cytoplasm, loss of plasma membrane integrity and overall cell disruption. During necrosis, most of the cell contents are released, causing damage to neighboring cells, local inflammatory reactions and also irreversible alterations in the affected tissue and/or organ [71]. Although necrosis is considered a response against passive cellular injury, recent studies suggest that the phenomenon can also be regulated. In this process of cell death, several studies have shown several alterations in mitochondrial function. Cell death by necrosis can occur due to activation of the MPT that compromises ATP production, since the mitochondrial inner membrane becomes freely permeable to protons leading to the uncoupling of oxidative phosphorylation (OXPHOS). The F_1Fo-ATP synthase can reverse its activity and hydrolyze ATP in order to maintain the mitochondrial membrane potential ($\Delta\Psi m$), resulting into a further decline of intracellular ATP concentrations [34].

Unlike cell retraction observed in apoptosis, cell swelling occurs during necrosis due to alterations in the cytoskeleton and inhibition of the Na^+/K^+ pump, causing the loss of the selective permeability of the membrane [72]. It has been described that the loss of homeostasis during necrosis involves the cell respiratory system (mitochondria), the digestive enzymatic system (lysosomes) and the cell membranes, which seem to have a crucial role for the establishment of irreversible lesions in the cell [72].

Apoptosis (Fig. **3**) requires the interaction of many factors and involves the activation of a group of cysteine proteases called "caspases" (cysteine-dependent aspartate-specific proteases), forming a complex cascade of events that connect the initiating stimuli to the final termination of the cell. Caspases are categorized into initiators (caspase-2,-8,-9,-10),
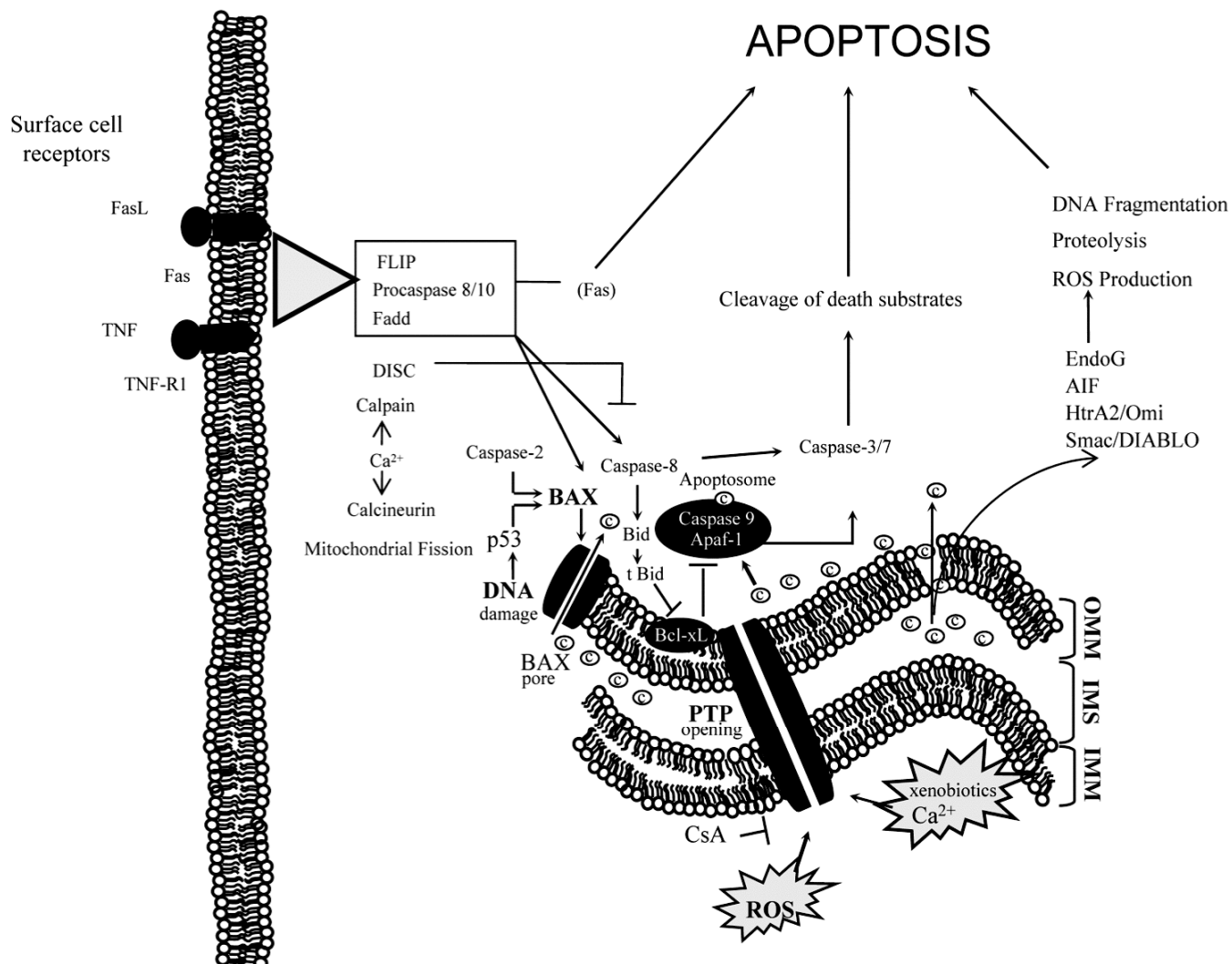
Fig. (3). Simplified scheme of the intrinsic and extrinsic pathways for apoptosis. Programmed cell death in response to cytotoxic stress or extracellular stimulus can occur *via* extrinsic (receptor-mediated) and intrinsic (mitochondria-dependent) pathways. These two routes can interact with each other at the mitochondrial level, which leads to signal intensification. Apoptosis is dependent on ATP levels and is characterized by several morphological cell alterations, such as cell shrinkage and nuclear DNA fragmentation. In the intrinsic pathway, several "death signals" (ROS, DNA damage, excessive mitochondrial calcium influx, etc) promote the release of cytochrome c and other pro-apoptotic factors, including the apoptosis factor (AIF) from the intermembrane space, by two basic mechanisms: one involves opening of the permeability transition pore (PTP), leading to mitochondrial swelling and burst of the outer membrane. The second mechanism involves the formation of pores in the outer membrane by pro-apoptotic proteins, including Bax. Several clinically drugs activate cell death pathways, some of which involve a direct effect on mitochondria. Legend for figure: IMM – inner mitochondrial membrane, OMM – outer mitochondrial membrane, IMS – intermembrane space, PTP – permeability transition pore, CsA – cyclosporin A.

effectors or executioners (caspase-3,-6,-7) and inflammatory (caspase-1,-4,-5) [73, 74]. In general, two partly interdependent routes may lead to apoptosis, the extrinsic and intrinsic pathways [75, 76]. In the extrinsic pathway, also known as "death receptor pathway", apoptosis is triggered by ligand-induced activation of death receptors at the cell surface (such as the tumor necrosis factor receptor (TNF), CD95/Fas, and TRAIL death receptors). The intrinsic pathway is generally characterized by intracellular apoptotic stimuli, with mitochondria having a leading role in the process, either by acting as initiators or propagators of the signal [77, 78]. One of the early events leading to apoptosis is the formation of the cytosolic apoptosome composed of Apaf-1, procaspase 9 and mitochondrial cytochrome c [77, 79].

The important role of mitochondria in the control of cell death is now widely accepted. Upon different apoptotic stimuli, mitochondria release different proteins such as cytochrome c, Smac/DIABLO and the AIF (apoptotic-inducing factor), which contribute to the apoptotic phenotype [80]. Protein release from mitochondria is complex and includes the oligomerization on the outer mitochondrial membrane of the pro-apoptotic proteins Bax and Bak, which form a channel permeable to cytochrome c. Nevertheless, despite the initial assumption that the MPT is only involved in necrosis, it appears that PTP opening is a strong candidate to mediate Ca^{2+}-dependent induction of apoptosis [78, 81]. *In vitro* studies performed by using HeLa cells [82] showed that apoptotic stimuli can induce the release of Ca^{2+} from the ER and the uptake by mitochondria. Eventually, mitochondria
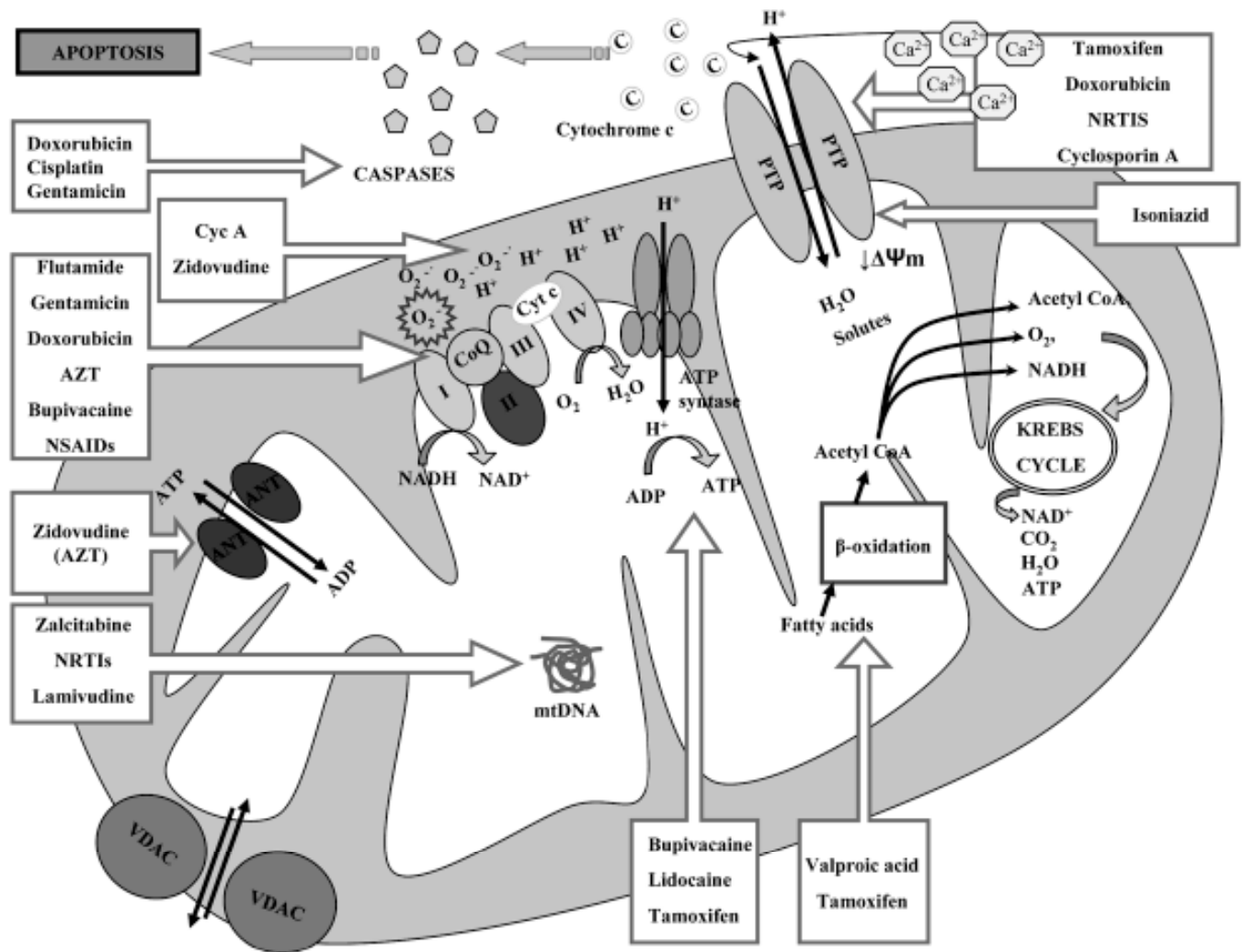
Fig. (4). Mitochondria as a mediator of drug-induced toxicity. A) OXPHOS systems, the mitochondrial permeability transition (MPT) and mtDNA are the major targets of drug-induced mitochondrial dysfunction. The induction of apoptosis by anti-cancer drugs such as tamoxifen, doxorubicin or flutamide has been observed in humans and laboratory animals. The mechanism appears to include PTP induction and oxidative stress. Other class of drugs such as the local anesthetics bupivacaine and lidocaine, as well as NSAIDs also interfere with proteins involved in OXPHOS, inducing uncoupling of respiration in some cases. Antiretroviral therapeutic using zalcitabine and lamivudine, among others, interfere with mitochondrial DNA polymerase gamma, which is responsible for mtDNA replication. Legend for figure: DOX – doxorubicin, VDAC – voltage-dependent anion channel, NRTIS - nucleoside reverse transcriptase inhibitors, ANT – adenine nucleotide translocator, mtDNA – mitochondrial DNA, PTP – permeability transition pore.

swell and fragment, releasing cytochrome c in the process. These changes were prevented by Bcl-2 expression as well as by experimental conditions that prevented the rise in cytosolic Ca^{2+} [83]. The alteration of the Ca^{2+} signal reaching mitochondria and/or the combined action of apoptotic agents or pathophysiological conditions (i.e. oxidative stress) can induce a profound alteration of the organelle structure and function [82, 84]. Zamzami and collaborators found that mitochondrial fragmentation during apoptosis was closely related with the collapse of the mitochondrial membrane potential ($\Delta\Psi m$). The collapse of $\Delta\Psi m$ was considered a point of no return in the death cascade [85], with the integrity and function of outer mitochondrial membrane regulated by proteins of the Bcl-2 family [86, 87].

Bcl-2 family members regulate mitochondrial outer membrane permeabilization resulting in the release of cyto-chrome c, Smac/DIABLO and Omi/HtrA2 and subsequent caspase activation. The Bcl-2 family includes pro- and anti-apoptotic proteins [88] with anti-apoptotic proteins, including Bcl-2 or Bcl-XL, inhibiting the function of pro-apoptotic proteins, such as Bax or Bak. An important subgroup of pro-apoptotic Bcl-2 members is the 'BH3-only' proteins (Bik, Bid, Bim, Bad, Puma) which have a pro-apoptotic role by either activating pro-apoptotic proteins (Bax and Bak) or inhibiting anti-apoptotic members (Bcl-2, Bcl-XL). The mechanisms by which the pro-apoptotic Bcl-2 family members regulate the permeabilization of the outer mitochondrial membrane remains controversial [89].

2. Drug-Induced Mitochondrial Toxicology

The first part of the present review addressed basic aspects of mitochondrial physiology which can be altered by different cellular conditions or by drug treatments. Identifi-

Table 1. Examples of Drugs with Black Box Warnings for Mitochondrial Toxicity

Cardiovascular Toxicity	Hepatic Toxicity	Renal Toxicity
- Nucleoside reverse transcriptase inhibitors (NRTIs)	- Isoniazid	- Doxorubicin (DOX)
- Zidovudine (AZT)	- Valproic acid	- Cysplatin
- Bupivacaine	- Tamoxifen	- Gentamicin
- Lidocaine	- Flutamide	- Cyclosporin A
- Thiazolidinediones (TZD)	- Lamivudine	- Ifosfamide
- Doxorubicin (DOX)	- Zidovudine (AZT)	- Statins
- Sorafenib	- Zalcitabine	- Tenofovir
- Daunorubicin	- Phenoformin	
- Epirubicin	- Metformin	
- Idarubicin	- Nefazodone	
- Celecoxib	- Abacavir	
- Diclofenac	- Didanosine	
- Ibuprofen	- Nevirapine	
- Indomethacin	- Tenofovir	
- Mefenamic acid	- Stavudine	
- Meloxicam	- Ketoconazole	
- Naproxen	- Divalproex Sodium	
- Piroxicam		
- Sulindac		
- Atenolol		
- Pioglitazone		
- Rosiglitazone		

The most frequent targets in drug-induced mitochondrial dysfunction are the heart, liver and kidneys, although other organs can also be affected. The present table describes several clinically used drugs whose mechanisms of toxicity are described to involve disturbance of mitochondrial physiology. Refer to text for further details on mechanisms of action.

cation of mitochondria as a primary or secondary target of drug-induced toxicity may help us to better understand the mechanism of action of a drug and open new perspectives for its application (Fig. **4**, Table **1**). The use of mitochondria for testing drug safety can also provide a mean of screening many prodrugs or chemical molecules which may be mitochondrial toxicants in many different types of tissues. Mitochondrial toxicity is most frequently evaluated in the cardiac, hepatic and renal tissue, although studies in the brain and testis are also commonly found. Molecular mechanisms of mitochondrial effects of some drugs, including a few harboring FDA (US Food and Drugs Administration) "Black Box" warnings for hepatotoxicity and cardiovascular toxicity will be described next.

2.1. Mitochondrial Cardiovascular Toxicity

In some cases, the clinical applications of a drug is compromised due to the cardiotoxicity associated with its administration. Mitochondrial dysfunction is occasionally associated with the observed cardiotoxicity. For example, combinations of nucleoside reverse transcriptase inhibitors (NRTIs), prescribed during Acquired Immune Deficiency Syndrome (AIDS) therapy, interfere with mtDNA replication [90, 91]. Due to the similarities with substrates for the mitochondrial enzyme DNA polymerase gamma, NRTIs also inhibit the enzyme [92], which may result into a decrease in mtDNA copy number. NRTIs were also demonstrated to directly interfere with mitochondrial bioenergetics, as inhibition of mitochondrial respiration, decreased membrane potential and decreased calcium accumulation were all observed in isolated cardiac mitochondria after treatment with

different NRTIs [91]. Zidovudine (AZT) is the most well known NRTI and it is characterized as a mitochondrial poison. Competitive inhibition of thymidine phosphorylation [93, 94], induction of superoxide anion formation [95, 96] and inhibition of the adenine nucleotide translocator [97] are some effects observed when isolated mitochondria are incubated with AZT and other NRTIs. This particular family of compounds is also able to inhibit the regulation of mitochondrial complex I by cyclic adenosine monophosphate (cAMP) which may explain disturbances in the $NADH/NAD^+$ ratio, generation of free radicals and increase in lactate observed in patients treated with this class of drugs [98].

Local anesthetics, which inhibit reversible sodium influx through voltage-gated sodium channels in neuronal cells [99], and which cause analgesia and paralysis after local application, can also induce cardiac toxicity if the exposure is systemic and in excessive quantities. Two examples of local anesthetics are bupivacaine and lidocaine. Bupivacaine is a local anesthetic used during small surgeries and administrated by epidural injection. However, when administrated in higher dosages, bupivacaine induces hypotension, bradycardia, arrhythmias and/or cardiac arrest [100]. Mitochondria also appear to be a target for the action of bupivacaine in the heart muscle [101]. Uncoupling of cardiac mitochondrial oxidative phosphorylation through a protonophore-like mechanism [101] was found to be dependent on the mitochondrial respiration state [102]. Bupivacaine acts as a protonophoretic uncoupler during state 4 respiration while inducing a change in the proton pump stoichiometry, or decoupling, during state 3 respiration [102]. Higher concentra-

tions of bupivacaine also inhibit the mitochondrial respiratory chain, with consequent decrease in mitochondrial ATP synthesis [103, 104]. Complex I appears to be the main target [103] for bupivacaine action. No stereospecific effects of bupivacaine enantiomers were observed in the inhibition of complex I activity or uncoupling of oxidative phosphorylation [105].

Lidocaine is normally used as a dental and topic local anesthetic. In certain circumstances, lidocaine may also be used as an antiarrhythmic compound during myocardial ischemia [106], although an excessive exposure to lidocaine results in hypotension, bradycardia and/or cardiac arrest [107] (Fig. **5B**). Lidocaine appears to interfere with cardiac mitochondrial function, inducing an increase in state 4 oxygen consumption [108], a non-competitive and partial inhibition of ATP synthase activity [109] and also an inhibition of the activity of the mitochondrial lactate dehydrogenase [110]. Lipophilicity and membrane stabilizing activity of lidocaine may justify the effects observed in ATP synthase and lactate dehydrogenase activity [110]. Furthermore, an impairment of mitochondrial oxidation mediated by mitochondrial K_{ATP} channels was also observed in ventricular myocytes after exposure to lidocaine [111].

Thiazolidinediones (TZD), also known as glitazones, are a class of oral antihyperglycemic compounds that increases insulin-stimulated glucose removal and which have been used as an adjunctive therapy for *diabetes mellitus* [112, 113]. TZDs lower glucose levels in models of insulin resistance without elevating pancreatic insulin production, which justifies its denomination as insulin sensitizers [114]. However, studies reveal that TZDs may also increase the risk of heart failure [115], which limit their clinical application. Once again, mitochondrial dysfunction appears to accompany TZD toxicity. Disruption of NADH oxidation at mitochondrial complex I is one consequence of TZD action, although the toxicity effect may also be the basis for the pharmacological benefits [116]. In a larger scale, the inhibition of complex I activity can lead to ATP depletion, oxidative stress and ultimately cell death [117].

The number of patients subjected to chemotherapy has been increasing nowadays, with the anthracycline doxorubicin (DOX) being one of the most potent antineoplastic agents. In fact, anthracyclines are among the most active and broad-spectrum antineoplastic agents used in the treatment of several cancers [118]. However, their use is associated with significant side effects, of which cardiotoxicity is the most important. Clinically, the cardiotoxicity is expressed as a dose-dependent and cumulative cardiomyopathy and ultimately in high mortality risk [119]. The risk of cardiotoxicity is higher in individuals with a previous history of cardiomyopathy, or mediastinal irradiation with previous heart disease [120]. Due to cardiac toxicity, the clinical application of DOX and similar compounds is limited by cumulative, dose-related, progressive myocardial damage that may lead to congestive heart failure [121, 122]. Thus, a balance between the beneficial (i.e., anticancer) and the risk of cardiac toxicity should be attained by the clinician. Besides DOX, daunorubicin (DNR) is another well known anthracycline. The only difference between DOX and DNR is that the side chain of DOX terminates with a primary alcohol, whereas that of

DNR terminates with a methyl group. The minor difference reflects on a different spectrum of activity of DOX and DNR against different types of cancer [122]. The ultrastructural features of anthracycline-induced cardiomyopathy, include the loss of myofibrils, dilation of the sarcoplasmic reticulum, microtubule damage, cytoplasmic vacuolization, mitochondrial swelling and increased number of lysosomes [123, 124]. Several mechanisms explain the cardiotoxicity induced by DOX and related molecules, but the exact mechanism and the metabolic consequences are still not clear. Some mitochondrial hypothesis to explain the cardiac toxicity of DOX have been proposed: oxidative stress associated with the DOX redox-cycling, mitochondrial damage and consequent deterioration of myocardial energy, deregulation of mitochondrial and cellular Ca^{2+} homeostasis and disruption of mitochondrial gene expression [125]. Biochemical and physiological data strongly suggests that DOX is primarily toxic to the heart muscle through increased formation of ROS [126], which oxidizes proteins [127], nucleic acids [128] and stimulates lipid peroxidation [129], thus altering the integrity of the cell membrane [126, 130-132]. Data from the literature indicates that cardiac mitochondria are especially damaged by DOX-induced oxidative stress [118, 133-135]; in fact, the cardiac tissue is particularly susceptible to ROS due to reduced levels of enzymatic antioxidants defenses when compared with other tissues [118].. DOX-induced oxidative stress can also be part of the mechanism of increased MPT which has been implicated in mitochondrial and cell dysfunction induced by DOX [136-141]. Biochemical and biophysical measurements in cardiac mitochondrial preparations showed that DOX quinone moiety was reduced by mitochondrial complex I originating superoxide anion by a redox-cycling mechanism [142, 143], which implicates cardiac mitochondria as the origin and the target of DOX-induced cardiotoxicity (Fig. **5A**). Published data also showed the association of a cardiac-selective exogenous NADH dehydrogenase with mitochondrial complex I, which would participate in the transfer of electrons to DOX, promoting the formation of ROS through DOX redox-cycle [144, 145]. Attempts to find new anthracyclines with lower toxicity than daunorubicin and doxorubicin resulted in the discovery of a new generation of anthracyclines: epirubicin and idarubicin.

The list of compounds currently used in clinical therapy that present cardiac toxicity as a side effect is vast. In this past section, only a few examples where evidence for the role of mitochondria as mediator of toxicity exists were presented. It is expected that tissues with higher energy demand, such as the cardiac muscle, will be first to suffer from chemical-induced mitochondrial dysfunction.

2.2. Mitochondrial Hepatic Toxicity

Hepatotoxicity is defined as liver injury caused by drugs or chemicals and it can be accompanied by clinical symptoms such as fever or jaundice [146]. However, such drugs only cause toxicity if administered at high doses or alternatively at low doses but in hepatic compromised patients. The present section will focus on some examples of hepatoxicants which are known to affect mitochondrial bioenergetics.

Isoniazid, also called isonicotinyl hydrazine, is the first anti-tuberculosis medication used in the treatment of
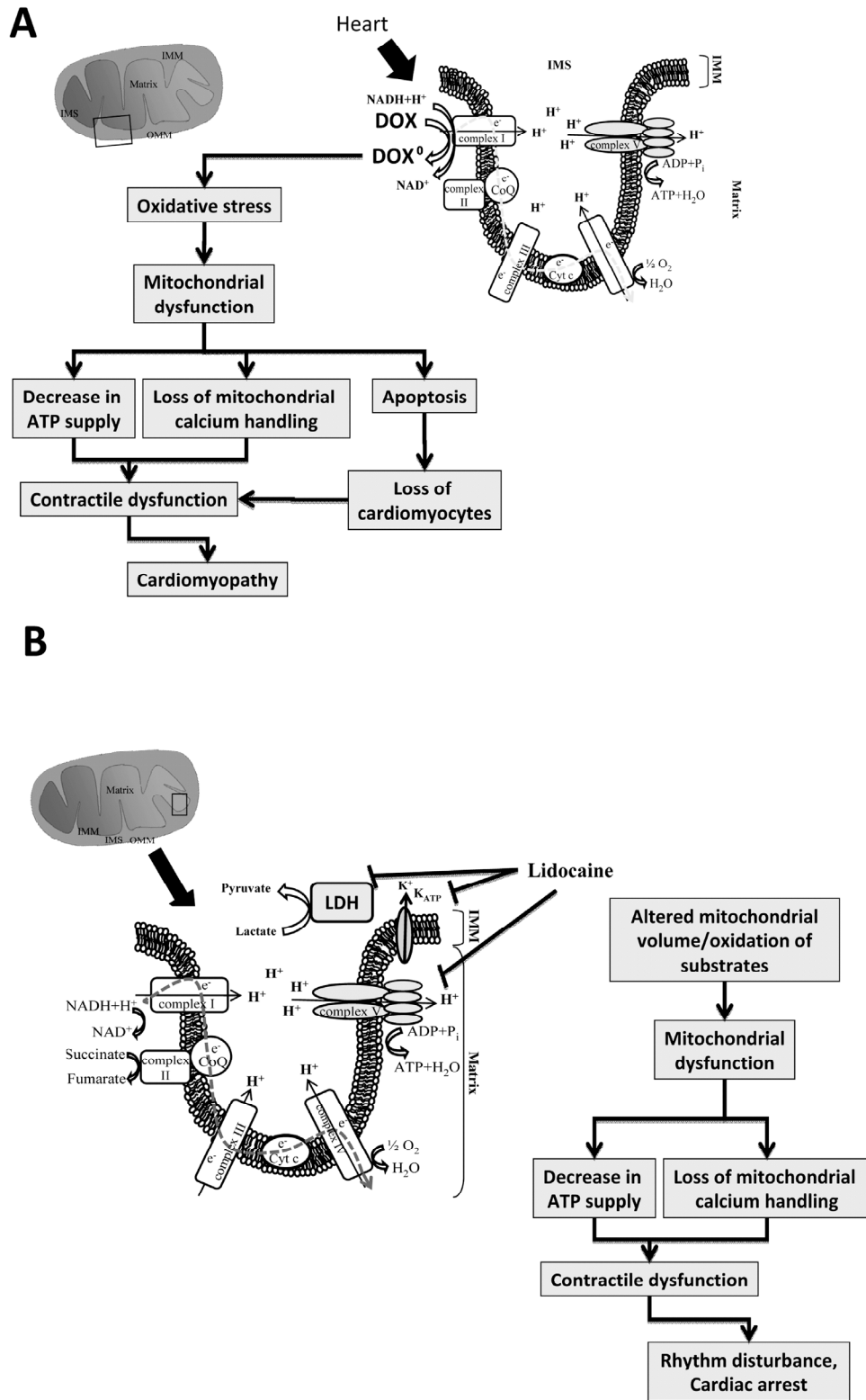
Fig. (5). Two selected drugs which present cardiac mitochondrial toxicity, the antineoplastic doxorubicin (DOX) and the anesthetic lidocaine. A) DOX causes a dose-dependent cardiomyopathy by a mechanism that appears to involve increased mitochondrial oxidative stress in the heart tissue, resulting in MPT induction and overall mitochondrial dysfunction, which can in some cases compromise cardiomyocyte survival. Inhibited mitochondrial respiration, increased MPT induction and decreased mitochondrial ATP synthesis after DOX treatment will disturb, among other cell processes, the maintenance of ionic homeostasis and heart contractility. B) Lidocaine-induced mitochondrial dysfunction. Lidocaine has been described to interfere with the function of several mitochondrial proteins including the ATP synthase, the mitochondrial lactate dehydrogenase (LDH) and mitochondrial K_{ATP} channels. Disturbance of mitochondrial oxidation of substrates and inhibition of the mitochondrial ATP synthase can result into decreased cardiac ATP concentration and consequent loss of ion homeostasis, which can explain cardiac rhythm disturbances observed in some patients.
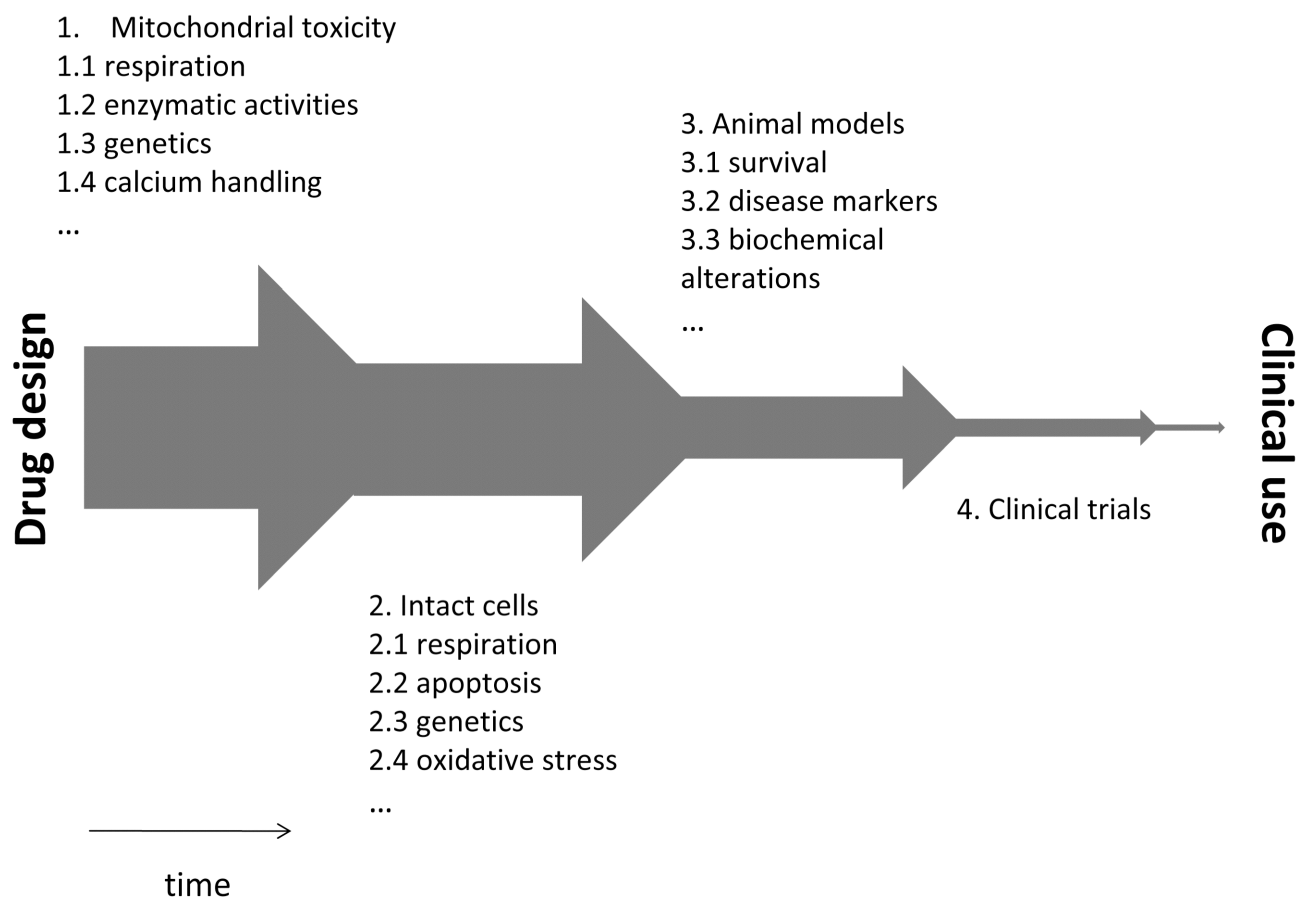
1. Mitochondrial toxicity
1.1 respiration
1.2 enzymatic activities
1.3 genetics
1.4 calcium handling
...

3. Animal models
3.1 survival
3.2 disease markers
3.3 biochemical alterations
...

Drug design

Clinical use

4. Clinical trials

2. Intact cells
2.1 respiration
2.2 apoptosis
2.3 genetics
2.4 oxidative stress
...

time

Fig. (6). Basic scheme of the drug development process involving mitochondria as an important marker for drug-induced toxicity. The thickness of each arrow exemplifies the number of different molecules in each evaluation stage. Drug toxicity on mitochondria is proposed as the bottleneck step in decision-making. When facing a compound that presents mitochondrial toxicity, the industry must face a difficult choice whether to drop the molecule altogether from further assays or in alternative use other *in vitro* and *in vivo* models.

mycobacterial infection. The molecule is metabolized in the liver *via* acetylation and dehydrazination [147], with the N-acetylhydrazine metabolite being responsible for the hepatotoxic effects by causing several symptoms including nausea, vomit, dark urine, fatigue, pain, melancholy and weakness. The interaction with cytochrome P450 system and increased oxidative stress have been associated with mitochondrial permeability alterations [133, 148].

Valproic acid is an antiepileptic drug that can provoke hepatotoxicity when used in inappropriate doses. The molecule is metabolized in the liver, *via* mitochondrial β-oxidation and gluocoronic acid conjugation, producing multiples metabolites. Valproic acid is activated and binds to reduced acetyl-CoA, which allows its translocation to the mitochondrial matrix, and the consequent inhibition of several mitochondrial enzymes and decreased fatty acid β-oxidation [149].

Tamoxifen is a non-steroidal anti-estrogen agent, which has been successfully used as a post-operative adjuvant therapy for breast cancer [150]. Tamoxifen is generally well tolerated with few side effects, especially when used at a typical dose of 10 mg twice daily [150]. Reports of tamoxifen-induced hepatotoxicity [150] may be explained by results obtained on isolated liver mitochondria. One particular study observed that tamoxifen and/ or estradiol lead(s) to mitochondrial failure by acting on the flavin mononucleotide

(FMN) site of mitochondrial complex I [151]. The data provided mechanistic bases to understand the multiple cytotoxic effects of tamoxifen and also why tamoxifen-resistant breast cancer can revert to tamoxifen-sensitive with the use of estradiol at the apropriate time. Tamoxifen (25 μM) alone induced a significant increase in hydrogen peroxide production and state 4 respiration. In addition, a significant decrease in the respiratory control ratio and in the transmembrane electric potential were also observed. All of the deleterious effects induced by tamoxifen were highly exacerbated in the presence of estradiol [151]. Tamoxifen-induced inhibition of topoisomerases, mitochondrial DNA depletion and also the triggering of steatosis in mouse liver was also previously demonstrated [152]. The study demonstrated that tamoxifen is electrophoretically accumulated inside hepatic mitochondria, where it acutely impairs β-oxidation and respiration. It has also been demonstrated that tamoxifen can decrease fat removal from the liver and steatosis, despite a secondary down-regulation of hepatic fatty acid synthase expression [152]. Another report confirmated that tamoxifen may cause toxicity through a mitochondrial mechanism since it demonstrated tamoxifen-induced oxidative stress and mitochondrial apoptosis *via* the stimulation of mitochondrial nitric oxide synthase [153]. Interestingly, the report showed that tamoxifen increased intramitochondrial Ca^{2+} and stimulated NO synthase activity in mitochondria from rat liver and human breast cancer MCF-7 cells. In the same study, ta-

moxifen was confirmed to inhibit mitochondrial respiration, induce cytochrome c release and increase mitochondrial lipid peroxidation and tyrosine nitration of mitochondrial proteins. The enzymatic activity of succinyl-CoA:3-oxoacid CoA-transferase was decreased and mitochondrial aggregation was augmented [153].

Flutamide (2-methyl-N-[4-nitoro-3(trifluoromethyl) phenyl]propamide) is a non-steroidal antiandrogen which behaves as a competitive agonist of the androgen receptor, being considered a good treatment for prostate cancer when used in combination with luteinizing hormone-releasing hormone agonists or in orchiectomy [154]. Flutamide-induced hepatic dysfunction is considered to be a consequence of its biotransformation by cytochrome P450. The resulting electrophilic metabolites bind to microsomal proteins leading to hepatitis. A higher incidence of hepatotoxicity was found in Japanese patients, the difference being attributable to ethnic differences in the frequency of genetic polymorphism in drug metabolizing enzymes [154]. The complete mechanism of flutamide-induced hepatotoxicity has not yet been precisely elucidated. Studies with rat liver mitochondria incubated with 50 μM flutamide indicated that this drug inhibited respiration, mainly by decreasing complex I activity [155]. Higher concentrations of flutamide (1 mM) decreased ATP levels in isolated male rat hepatocytes [155]. The authors concluded that flutamide is toxic to rat hepatocytes as a result of cytochrome P450-mediated formation of electrophilic metabolites, which also contributed to the inhibitory effect of flutamide on mitochondrial respiration and ATP formation [155].

Besides cardiotoxicity (see above), hepatotoxicity is also one of the most serious complications of highly active antiretroviral therapy (HAART). A wide range of nucleoside analogs used in Human Immunodeficiency Virus (HIV) treatment exhibits a delayed clinical toxicity limiting their usage. As for the heart, the toxicity of nucleoside analogs may be related to mitochondrial toxicity, mediated by the DNA mitochondrial polymerase gamma. Among the anti-HIV drugs in clinical use, two are modified cytosine analogs, such as Zalcitabine (ddC) and Lamivudine ((-)3TC) [156]. Feng *et al.* (2001) performed structure/function relationships of mitochondrial DNA polymerase inhibitors and demonstrated that the D-isomer of Lamivudine (+3TC) is a more potent inhibitor of human mitochondrial DNA Polγ than the (-) isomer, the difference being related with tighter binding and faster incorporation rate into the DNA double helix, which leads to greater toxicity [156]. AZT (3'-Azido-3'-deoxythymidine), a thymidine analogue, inhibits the viral reverse transcriptase, which blocks the life-cycle of HIV and slows the progression of the disease [93]. When given for long time periods and at high doses, AZT is known to cause toxicity in many tissues, including the liver. The results showed that in isolated rat liver mitochondria, the phosphorylation of thymidine to TMP exhibit higher V_{max} and k_m than in heart mitochondria. AZT is phosphorylated to AZTMP, but no further derivatives were detected. Another study [157] demonstrated that AZT treatment resulted in metabolic disruption (increased lactate and superoxide production) and increased liver hepatoma HepG2 mortality with decreased proliferation, while mtDNA remained unchanged or even increased. Zalcitabine (ddC) caused pronounced mtDNA depletion in HepG2 cells but not in myoblast H9c2

cells and increased cell mortality, but no metabolic disruption, in either cell type [157].

Of relevance for hepatic mitochondrial toxicity is the existence of a cytochrome P450 system (CYP) in mitochondrial membranes, which can be regulated by phosphorylation [158]. Mitochondrial CYP systems have been overlooked when considering the mechanisms of toxicity of several xenobiotics, because their real function is still a subject of debate [159]. Nevertheless, some studies indicate that it is the activity of the mitochondrial CYP isoforms that increase the toxicity of some xenobiotics, including dioxins [160]. If demonstrated that mitochondrial CYP actively participate in the liver and in other tissues in drug conversion processes, the use of inhibitors is a two-edged sword, since the degree of mitochondrial toxicity of the parent compound *vs* metabolites should be first evaluated.

2.3. Renal Mitochondrial Toxicity

Apoptosis has an important role not only in the physiological processes of kidney growth and remodeling but also in various renal diseases and drug-induced nephrotoxicity. Apoptosis in the kidney is a double-edged sword because not only it leads to tissue loss and dysfunction but also contributes to eliminate intoxicated cells and to control proliferative responses [161].

Regarding anti-neoplastic renal toxicity, two compounds are considered classic examples: doxorubicin (DOX, see above) and cisplatin. The toxicity of DOX on mitochondria was shown to involve release of cytochrome c and activation of caspase-9 in rat proximal tubular cells [162]. Nephrotoxicity is also a severe consequence of aggressive therapy with cisplatin [163]. This chemotherapeutic agent accumulates in cells in all nephron segments but it is especially found in S1 and S3 segments of the proximal and distal tubules [164]. Cisplatin toxicity appears to involve inhibition of protein synthesis, mitochondrial injury and DNA damage causing blockage of DNA replication and gene transcription due to the formation of single and double strand DNA breaks. Cisplatin treatment results in renal mitochondrial translocation of the pro-apoptotic molecule Bax [165] and activation of the initiator caspases-8, -9, -2, and executioner caspase-3 in cultured tubular cells and in *in vivo* models [166, 167]. It was demonstrated that cisplatin-induced renal apoptosis is mediated by p53 and caspase 3-activation independently of caspase 8 and 9 [168]. Hydroxyl radicals were found responsible for cisplatin-induced apoptosis as hydroxyl radical scavengers inhibited cytochrome c and caspase activation [169]. When renal collecting duct-derived cells were incubated with inhibitors of the mitochondrial respiratory chain or ATP-synthase, cisplatin-induced apoptosis was strongly enhanced showing that intact mitochondria are essential to prevent cisplatin-induced apoptosis [170]. Interestingly, it was shown that cisplatin-induced renal cell death is mediated by the mitochondrial protein Omi/HtrA2 [171].

Aminoglycoside antibiotics including gentamicin are widely used in the treatment of gram-negative infections. Gentamicin is able to alter mitochondrial respiration, causing state 4 stimulation and state 3 inhibition [172]. It is known that gentamicin leads to the formation of hydrogen peroxide by renal cortical mitochondria; also, radical scavengers and iron chelators provided functional and histological protection

against renal failure in gentamicin treated rats [173]. Gentamicin has been shown to release iron from renal cortical mitochondria. This was supported by *in vitro* studies in which iron chelators protected against gentamicin acute renal failure [174]. The results strongly support the importance of hydroxyl radicals or a similar oxidant in gentamicin-induced renal failure. Gentamicin-induced mitochondrial dysfunction involves the release of cytochrome c and the activation of caspase-3 [175], which can be prevented by overexpression of Bcl-2. Cytosolic gentamicin can act on mitochondria by a direct effect or indirectly by inhibiting Bax proteosomal degradation. The later was demonstrated by an increase of ubiquitinated Bax, which was correlated with increase bonding of gentamicin to the ß type 9 subunit of the proteosome [161].

Cyclosporin A has been used as the major advance in transplantation, enhancing graft and patient survival; its use, however, is restrained by nephrotoxicity. Chronic cyclosporin A nephrotoxicity, characterized by tubular atrophy and interstitial fibrosis with progressive renal impairment, contributes to chronic kidney problems [161]. Apoptosis induced by cyclosporin A on tubular epithelial cells is related to the translocation of Bax to mitochondria as Bax antisense oligodeoxynucleotides prevented cyclosporin A-induced apoptosis [176]. It was also previously documented that cyclosporin A-induced nephrotoxicity is related to an alteration of Ca^{2+} intracellular homeostasis [177], including increased Ca^{2+} accumulation into the matrix and delay of efflux [178], as well as increased ROS production [179].

3. Mitochondria as a Traditional Bio-Sensor for Toxicological Studies

The present section is aimed at the description of isolated mitochondrial fractions as an *in vitro* model for studying xenobiotic toxicity. Most studies were performed in research laboratories by using mitochondrial fractions from different sources, especially the liver, and were performed in a low throughput manner, with only a few compounds tested per day. Nevertheless, there was already a general understanding that mitochondria could be a reliable, inexpensive bio-sensor to gather preliminary data on drug-induced toxicity.

3.1. Traditional Methodology

Various analytical techniques can be employed to investigate mitochondrial function *in vitro*, including the use of absorbance spectroscopy to assess the redox state of mitochondrial cytochromes, fluorescent calcium dyes (such as Calcium-Green 5N), measurement of ATP levels, assessment of reactive oxygen species (ROS) production, and measurements of mitochondrial membrane potential ($\Delta\Psi$) and oxygen consumption. One of the most informative techniques for assessing mitochondrial function is the screening of mitochondrial oxygen consumption [180-184]. Detected changes in mitochondrial function after the addition of a test chemical could be correlated to its toxic effects. Polarography using the Clark-type oxygen electrode [185] has been the main technique for measuring pO_2 and hence establish oxygen consumption, and is still widely used in the laboratory [186-188]. Although experimental approach has proven very useful, the methodology is associated with a number of inherent limitations. These include the invasive nature of measurement, intrinsic oxygen consumption by the elec-

trode, sensitivity to mass exchange (stirring requirements), sterility and reuse issues, electrode poisoning and signal drift [120].

The measurement of mitochondrial protonmotive force (Δp) is another important end-point for mitochondrial function. All quantitative methods to measure Δp and its components rely directly or indirectly on the measurement of the equilibrium distribution of positively charged probes across the mitochondrial inner membrane. It is important to maintain a low probe concentration, otherwise its movement across the membrane will have a significant effect on the gradient that is being measured [185]. The accumulation of the probe molecules is monitored by following some appropriate change in absorbance or fluorescence, by the use of radiolabels, or by measurement of the external concentration with ion-selective electrodes. Distribution of tetraphenylphosphonium cations (TPP^+) can also be monitored with electrodes that report the extramitochondrial concentration. From the change in internal concentration and the volume of the mitochondrial matrix it is possible to calculate the intramitochondrial concentration, and hence the $\Delta\Psi$ value. But to obtain Δp, both $\Delta\Psi$ and ΔpH should be measured. It is sometimes convenient to avoid the need to measure ΔpH by clamping it to zero, so that Δp is equivalent to $\Delta\Psi$. Some of the inherent limitations are quite the same as those of the Clark type oxygen-electrode [185]. However, the primary limitations in the context of toxicity assessment within drug discovery are the low sample throughput and the lack of flexibility and automation associated with electrode-based systems [189].

Mitochondrial ROS production can be assessed by loading mitochondria with dyes, which, by reacting with radical species, can produce fluorescent derivatives (e.g. reduced xanthene dyes such as dichlorohydrofluorescein) [190]. This approach facilitates analysis of ROS providing an insight into the activity of the ETC, but can be limited due to the nonspecific nature of product formation. In addition, it has been noticed that fluorescent potential-sensitive probes themselves may interfere with mitochondrial function [93, 190, 191].

Another type of mitochondrial function end-point studied by traditional methodology is mitochondrial calcium retention capacity. Calcium Green-5N is a fluorescent dye that is used to assess Ca^{2+} retention capacity of isolated mitochondria [192]. Other mitochondrial calcium probes exist and can be used on a spectrophotometer or fluorimeter [193, 194]. The use of probes to measure ion movement is always troubled because of the possibility of artifacts arising from incomplete organelle loading, toxicity of the probe itself or even damage from the excitation light. One way of avoiding some of those problems is by using calcium-selective electrodes [195], although problems inherent to the use of electrodes still exist (see above).

The ability of mitochondria to swell under a wide variety of conditions, including PTP induction, is a property which has been the subject of intensive investigation for more than a decade [196, 197]. Light scattering of mitochondrial suspensions has become the technique of choice to detect mitochondrial size change [198]. This technique, however, has certain limitations. First, this technique cannot provide information on the situation *in situ*. Second, robust isolation of

mitochondria in potassium-free sucrose medium probably damages mitochondrial membranes, disrupting contact sites and leading to dramatic matrix contraction [199]. Changes in mitochondrial light scattering may not always reflect changes in matrix volume. A recent study demonstrated that light scattering can give positive results when no changes are seen by isotope techniques, and suggested that light scattering might be sensitive to conformational changes of the mitochondrial adenine nucleotide carrier [200]. Moreover, the results of several fluorescent microscopy studies *in situ* do not fully support results obtained earlier by light scattering [201-206]. On the other hand, the microscopy techniques have their disadvantages. Limited spatial resolution of actual fluorescence microscopy precludes 3D imaging of submicron scale cellular compartments. Electron microscopy provides the necessary resolution, but does not enable the study of functioning mitochondria; besides, the fixation procedure itself affects mitochondrial volume.

There are other methods to measure mitochondrial toxicity that have been used in early screening of compounds, including measurement of mitochondrial dehydrogenase activities using Alamar Blue, determination of ATP production using the luciferin-luciferase luminescent assay [207] or the assessment of mitochondrial membrane potential using flow cytometry [208]. The problem inherent with most of the methods above is that the amount of test compounds to study each day is limited, which increases the span of time required for correctly assessing the safety of a family of compounds.

3.2. Validity of the Use of Mitochondria as an In vitro Bio-sensor

The widespread use of mitochondria as a toxicological test was initiated at the end of the 80's, when phosphorylating submitochondrial particles were used as *in vitro* monitors of water quality [182], although many molecules had been already tested on isolated mitochondrial fractions. Among the different techniques used to investigate mitochondrial toxicity the TPP^+-electrode was one of the most used even in alternative systems as synaptosomes [209] or yeast mitochondria [210]. Another experimental protocol used in environmental toxicology was the use of reverse electron transfer which allowed for a sensitive quantification of acute toxicity by measuring the rate of NAD^+ reduction [182]. The correlation between drug concentrations that altered enzyme function or membrane stability in electron transport submitochondrial particles and plasma concentrations associated with whole organism toxicity suggested that such mitochondrial tests could be helpful to use in the human or environmental risk assessment. Tests with submitochondrial particles were easier, faster and cheaper than whole animal test and culture analysis. By comparing mitochondrial data with serum drug concentrations, it was possible to partially validate the use of mitochondria as a bio-sensor to assess the human health risks related to the exposure to a chemical agent [211]. In addition, alterations of mitochondrial respiration could be another marker for mitochondrial toxicity. Mitochondria are highly sensitive to ions such as calcium and iron. False toxicity assessments would result if these ions are present with the test compound. Because of this, another mitochondrial system was developed. Yeast, which has higher life-time than mitochondria, can be easily har-

vested without damage to the mitochondrial respiratory chain and are not affected by the presence of calcium and iron ions. A dissolved oxygen electrode was used to assess toxicity by monitoring alterations in the respiratory rate of yeast cells. Despite its capacity to screen contaminants in the environment due to its quickness and short price, it is less sensitive than isolated mitochondria to indicate a toxic response [212].

The use of mitochondrial electron transport enzymes for preliminary drug safety tests permitted the prediction of chemical effects for a large variety of species because of the high conservation of these proteins during evolution. The analytical protocols are easily performed and only require a spectrophotometer, pH meter and a household refrigerator [211]. Isolated mitochondria and further preparation of submitochondrial particles was also fairly easy, furthermore it was possible to freeze mitochondria or submitochondrial particles at different temperatures for a future use. Although several function are lost during freezing (no generation of membrane potential is a serious drawback), toxicity of a certain drug on the activity of a complex of the MRC would still be possible to analyze. The method could be also used to assess the percentage of inhibition of the respiratory chain by a certain toxic substance found in water [213].

From its use as a toxicological bio-sensor to a promising large-scale drug safety *in vitro* model, only a short jump was necessary, but that same jump meant the appearing of new fast track, high-throughput methods to evaluate thousands of compounds in a very short time.

4. Mitochondria as a Drug Safety Bio-sensor: The New Experimental Strategies

Since 2006, the FDA approved the fewest number of drugs in the past 24 years, with only 19 new drugs being accepted. Above 75 new and revised "black box" warnings appeared since 2004, according to the report of The Wall Street Journal (published online July 2nd, 2008).

Apparently, drug safety is more and more an issue of concern for many pharmaceutical companies. The toxicological effects of several drugs are only detected at a large phase III trial, the clinical phase of drug safety assessment and development. This is obviously a serious problem not only for patient safety but also for pharmaceutical companies which see more frequent FDA Black Box warnings in their labels, not mentioning that some may see their pharmaceuticals withdrawn from the market.

For many pharmaceutical companies, drug safety assessments are still made fairly late in the drug discovery process. However, it is clear that safety testing should be one of the first steps in the selection of lead compounds, especially when a great variety of compounds exist to support the creation of structure-activity relationships (SAR). The adoption of newer *in vivo*, *in vitro* and *in silico* tools and the use of new and promising technologies must be the priority for pharmaceutical companies in order to increase drug safety [9].

Despite the extensive use of isolated mitochondrial fractions by several research groups as a model for the toxicological action of several molecules, the industry kept its low interest in the use of such *in vitro* model to predict drug

toxicity. The change in opinion came when it was demonstrated that several pharmaceuticals had to be withdrawn from the market due to intrinsic mitochondrial toxicity, thus causing an enormous economical burden in the companies and decreasing the people trust in how pharmaceutics safety is assessed [9]. Furthermore, it soon became clear that mitochondrial effects were part of the pharmacological mechanism of action of several pharmaceuticals, one example of which being glitazones, which act to decrease blood glucose levels [214]. A growing body of evidence suggests that inhibition of mitochondrial complex I can be part of their mechanism of action [214]. On the other hand, the capacity of some natural compounds (such as berberine) to induce the permeability transition pore and apoptosis can enable its usage as potential anti-cancer drugs [125, 215, 216].

In order to prevent such dramatic and negative effects of drugs on mitochondria, a wide range of cell lines are used to evaluate pre-clinical drug toxicity. However, despite of the presence of metabolic active mitochondria, cells are grown under enriched glucose culture media and produce ATP mainly through glycolysis, and not through OXPHOS. Oxygen consumption in these cells is low and cells are resistant to xenobiotics that impair mitochondrial function. By replacing media glucose with galactose, Marroquin *et al.* created an experimental model capable of detecting mitochondrial injury [189]. After the substrate alteration, cells must use oxidative phosphorylation in order to obtain ATP, becoming more susceptible to toxicants that inhibit or uncouple mitochondria [189].

High-throughput methods designed to evaluate mitochondrial toxicity of a certain molecule would allow the construction of a database, where a certain mitochondrial toxic effect could be attributed to common structural motives. Theoretical methods (viz. quantitative structure-activity relationships, QSARs) based on data from compounds with established mitochondrial toxicity could permit the design of novel compounds with decreased mitochondrial toxicity. Once the precise mechanism of drug mitochondrial toxicity is known, structural modifications in the molecule can be performed in order to alter its physical-chemistry properties, although still maintaining the desired pharmacological action. Several high-throughput screening methods are now available to industry R&D to investigate the toxicity of the thousand of new molecules that appear every year. One particular example is the small-scale immunopurification of cytochrome c oxidase which can be used to investigate both direct effects of molecules on the activity of the isolated enzyme and to evaluate long-term effects on cytochrome c oxidase expression [217], which is particularly useful because many toxic molecules act by hindering mitochondria genetics. Immunocapture of mitochondrial Complexes I-V can also allow the precise identification of the site of action of a determined compound, as opposed to an "average" effect on the entire respiratory chain [218]. The feasibility of the present method was confirmed by testing molecules with well known sites of action, including classical inhibitors of the respiratory chain [218].

Fluorescent dyes that report $\Delta\Psi$ can also be adapted to 96 (or higher) well formats [219], although none of the assays is conclusive whether a compound is causing inhibition, uncoupling or induction of the MPT. Also, as described elsewhere, one golden standard to evaluate mitochondrial function is the measurement of oxygen consumption. The normal protocol in a research laboratory is to test several concentrations of a test compound in the same preparation and repeat the experiment in 3 or more independent preparations. However, the conventional polarographic method to measuring mitochondrial oxygen consumption is not satisfactory to high sample automation. In order to test a full range of compounds, the development of novel techniques was almost mandatory. Mitochondrial oxygen consumption optimized for multiwell plate reader detection using phosphorescent oxygen-sensitive probes is an important advance as it allows to simultaneously measure all the samples in a 96-well plate [190, 220]. In fact, it has been described that the method allows for the measurement of 200 compounds/day using one concentration or alternatively generate IC50 values for approximately 25 compounds. Such technique has been successful in the assessment of mitochondrial toxicity of fibrates, statins and of the hepatotoxicants nefazodone, trazodone, and buspirone and has been described equally effective if used in cells, isolated mitochondria, enzymes, tissues and organisms [190, 220]. High-throughput measurements of respiratory complex activities and mitochondrial mtDNA replication and protein synthesis are also now possible using immunocapture technology, radiolabeled deoxynucleotide triphosphates (dNTPs) and S-methionine incorporation [221, 222].

Also, as described above, opening of the calcium-induced PTP can be a sensitive and early marker of mitochondrial dysfunction [139]. Although not widely used to measure the toxicity of novel compounds, high throughput methods using a multi-well plate are available and can be used when required to investigate if a certain molecule triggers mitochondrial dysfunction through decreasing mitochondrial calcium tolerance [223].

Besides organelle and cell studies, animal models that better reveal mitochondrial toxicity are also being developed. One such model is the manganese superoxide dismutase (MnSOD) knockdown mouse that is more sensible in detecting drug-induced mitochondrial impairment. Several other transgenic animal models have been generated with a phenotype that includes mitochondrial damage, such as mice with deficiencies in uncoupling proteins, mitochondrial transcription factor A (tfam), glutathione peroxidase-1, γ-ghitamyl transpeptidase or in the ANT [224].

The available diagnostic procedures for assessing mitochondrial damages are biopsy and histopathology which are invasive medical procedures. Non-invasive or minimally invasive technologies are being developed such as metabolic intermediates containing non-radioactive ^{13}C atoms that can be orally administrated with the rate of $^{13}CO_2$ exhalation monitored using isotope ratio mass spectrometry.

5. Can We Use Mitochondrial Research to Increase Drug Safety?

The logical answer to the question is "Yes, we can and we should". From the previous sections, it is perfectly clear that mitochondria are critical components of cell physiology and that several clinically used drugs harbor toxic effects on mitochondria in different target and non-target organs. It is also clear that the pharmaceutical industry now has the tools

to investigate in a more throughput manner the direct interactions between different molecules and mitochondrial models. Identifying mitochondrial toxicity as soon as possible in the drug development process increases the likelihood that toxicity during clinical practice will be avoided. Depending on the targeted disease, severe *in vitro* mitochondrial impairment may be enough to abandon a promising or nascent drug. Pharmaceutical companies have also now a new dilemma, which is to know how much of the supposedly mitochondrial toxicity is a component of the pharmacological effect. Just to cite one example, it has been demonstrated that metformin, an anti-diabetic agent, inhibit mitochondrial complex I, which appears to be part of its pharmacological mechanism [214].

On the other hand, it may be a difficult choice to discard compounds showing a certain degree of mitochondrial toxicity in *in vitro* tests but with a very significant pharmacological effect. Pharmaceutical companies may choose to push the compound forward for further *in vivo* assays in order to also identify ways of decreasing undesirable mitochondrial toxicity. Different pharmacotherapeutic strategies can be used to decrease mitochondrial toxicity. The cardiac toxicity of doxorubicin (DOX) is a classical example. One possibility to decrease drug-induced mitochondrial toxicity would be by improving drug targeting, reducing the amount of drug that reach non-target organs. One particular example of this strategy is the use of pegylated liposomal DOX, which has a distinct pharmacokinetic profile characterized by an extended circulation time and a reduced volume of distribution [225]. The alternative formulation was also, in fact, found to decrease the incidence of cardiac toxicity [225], although studies are still lacking in order to understand if cardiac mitochondrial toxicity is also minimized. Optimizing the dosage regimen is also attractive, as lower, although still pharmacologically effective dosages may decrease mitochondrial accumulation of lipophilic drugs in some tissues. Another possibility is the co-administration of mitochondrial protective agents, one example of which being the preventive role of the beta-adrenergic antagonist carvedilol on DOX-induced cardiac mitochondrial dysfunction [226]. Once in the clinic, drugs with mitochondrial liabilities require increased vigilance, especially if several pharmaceuticals are used at the same time [9].

The refinement of different methodologies results into higher success rates in the isolation of functional mitochondrial fractions from different organs, which can be used to assess mechanisms of tissue-specific drug-induced mitochondrial toxicity. Nevertheless, studies with isolated mitochondria lack the complexity associated with experiments in intact cells, in whole organs or even in *in vivo* studies. Nevertheless, it is also true that the use of isolated mitochondrial fractions allows pinpointing precise sites of action of the molecule on mitochondria. In a perfect world, the perfect drug safety assessment would correlate data in isolated mitochondria with data gathered in intact cells and in *in vivo* (Fig. **4**).

One important point in this entire discussion regards what can be gained by using mitochondria as a bio-sensor to investigate drug safety. One particular example: nefazodone, an anti-depressant. This drug was withdrawn from the market in 2004, after several reports of serious hepatotoxicity

[227, 228]. Would the use of mitochondria as predictors of drug safety helped in avoiding the entry of the drug in the market? The answer is, maybe so. Dykens *et al.* demonstrated that nefazodone is highly toxic to isolated liver mitochondria, human hepatocytes and HepG2 cells, showing a clear mitochondrial toxicity even when cell lines were used [229]. If an initial assessment of mitochondrial toxicity had been done, it is very unlikely that nefazodone would have entered further clinical trials.

Industry-sponsored studies investigating drug-induced mitochondrial toxicity are know becoming more numerous. Examples include anti-depressants [229], anti-hyperglycemic agents [230], tyrosine kinase inhibitors [231] or thiazolidinediones, fibrates and statins [232], among others (reviewed in [9]). More industry R&D will for sure follow the same example in order to identify, as early as possible, mitochondrial toxicity of new agents under development.

To further complicate things, idiosyncratic drug toxicity is also a problem during drug development. Host-dependent drug-toxicity is not easily predictable during *in vitro* assays or during most clinical trials. Industry is now also focusing on a possible mitochondrial role on the mechanism behind idiosyncratic drug toxicity. One example is trovafloxacin, whose idiosyncratic toxicity was proposed to involve mitochondrial damage [233]. In an industry-sponsored study, unique gene changes induced by trovafloxacin and involved in mitochondrial damage were detected [233].

It is now apparent that mitochondrial toxicology has become an area of interest to the industry, since a primary assessment of mitochondrial toxicity of a range of compounds can be performed in a fast and relatively inexpensive way, avoiding some later human toxicity problems that may arise during subsequent testing stages or even during clinical use. We should expect to see in the future an increasing number of pharmaceutical companies establishing protocols to assess mitochondrial toxicity of novel molecules under study in a fast and inexpensive way. Some companies will focus more on investigating direct drug-induced mitochondrial dysfunction, others will rather measure drug-induced alterations in mitochondrial-relevant genes. Whatever the chosen strategy is, the final outcome is the prediction of drug safety based on a mitochondrial end-point.

ACKNOWLEDGEMENTS

REFERENCES

[1]   Benard G, Rossignol R. Ultrastructure of the mitochondrion and its bearing on function and bioenergetics. Antioxid Redox Signal 2008; 10(8): 1313-42.
[2]   Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature 1961; 191: 144-8.
[3]   Yi M, Weaver D, Hajnoczky G. Control of mitochondrial motility and distribution by the calcium signal: a homeostatic circuit. J Cell Biol 2004; 167(4): 661-72.
[4]   Liu X, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. Cell 1996; 86(1): 147-57.

[5]   Mitchell P, Moyle J. Respiration-driven proton translocation in rat liver mitochondria. Biochem J 1967; 105(3): 1147-62.

[6]   Kroemer G. Mitochondrial control of apoptosis: an overview. Biochem Soc Symp 1999; 66: 1-15.

[7]   Costantini P, Jacotot E, Decaudin D, Kroemer G. Mitochondrion as a novel target of anticancer chemotherapy. J Natl Cancer Inst 2000; 92(13): 1042-53.

[8]   Armstrong JS. Mitochondrial medicine: pharmacological targeting of mitochondria in disease. Br J Pharmacol 2007; 151(8): 1154-65.

[9]   Dykens JA, Will Y. The significance of mitochondrial toxicity testing in drug development. Drug Discov Today 2007; 12(17-18): 777-85.

[10]   Lewis MR, Lewis WH. Mitochondria in tissue culture. Science 1914; 39(1000): 330-3.

[11]   Bakeeva LE, Chentsov Yu S, Skulachev VP. Mitochondrial framework (reticulum mitochondriale) in rat diaphragm muscle. Biochim Biophys Acta 1978; 501(3): 349-69.

[12]   Poliakova IA, Zorov DB, Leikina MI. [Polarographic study of cell respiration in a tissue culture]. Tsitologiia 1983; 25(2): 162-7.

[13]   Chance B, Williams GR. The respiratory chain and oxidative phosphorylation. Adv Enzymol Relat Subj Biochem 1956; 17: 65-134.

[14]   Rich PR. The molecular machinery of Keilin's respiratory chain. Biochem Soc Trans 2003; 31(Pt 6): 1095-105.

[15]   Gilkerson RW, Selker JM, Capaldi RA. The cristal membrane of mitochondria is the principal site of oxidative phosphorylation. FEBS Lett 2003; 546(2-3): 355-8.

[16]   Bootman MD, Collins TJ, Peppiatt CM, et al. Calcium signalling--an overview. Semin Cell Dev Biol 2001; 12(1): 3-10.

[17]   Berridge MJ, Lipp P, Bootman MD. The versatility and universality of calcium signalling. Nat Rev Mol Cell Biol 2000; 1(1): 11-21.

[18]   Berridge MJ, Bootman MD, Lipp P. Calcium--a life and death signal. Nature 1998; 395(6703): 645-8.

[19]   Orrenius S, Zhivotovsky B, Nicotera P. Regulation of cell death: the calcium-apoptosis link. Nat Rev Mol Cell Biol 2003; 4(7): 552-65.

[20]   Vasington FD, Murphy JV. Ca ion uptake by rat kidney mitochondria and its dependence on respiration and phosphorylation. J Biol Chem 1962; 237: 2670-7.

[21]   Giacomello M, Drago I, Pizzo P, Pozzan T. Mitochondrial Ca2+ as a key regulator of cell life and death. Cell Death Differ 2007; 14(7): 1267-74.

[22]   Crompton M, Heid I. The cycling of calcium, sodium, and protons across the inner membrane of cardiac mitochondria. Eur J Biochem 1978; 91(2): 599-608.

[23]   Rottenberg H, Marbach M. The Na(+)-independent Ca2+ efflux system in mitochondria is a Ca2+/2H+ exchange system. FEBS Lett 1990; 274(1-2): 65-8.

[24]   Hajnoczky G, Hager R, Thomas AP. Mitochondria suppress local feedback activation of inositol 1,4, 5-trisphosphate receptors by Ca2+. J Biol Chem 1999; 274(20): 14157-62.

[25]   Mackenzie L, Roderick HL, Berridge MJ, Conway SJ, Bootman MD. The spatial pattern of atrial cardiomyocyte calcium signalling modulates contraction. J Cell Sci 2004; 117(Pt 26): 6327-37.

[26]   McCormack JG, Denton RM. The activation of isocitrate dehydrogenase (NAD+) by Ca2+ within intact uncoupled rat brown adipose tissue mitochondria incubated in the presence and absence of albumin [proceedings]. Biochem Soc Trans 1980; 8(3): 339.

[27]   Territo PR, Mootha VK, French SA, Balaban RS. Ca(2+) activation of heart mitochondrial oxidative phosphorylation: role of the F(0)/F(1)-ATPase. Am J Physiol Cell Physiol 2000; 278(2): C423-35.

[28]   Rizzuto R, Pinton P, Carrington W, et al. Close contacts with the endoplasmic reticulum as determinants of mitochondrial Ca2+ responses. Science 1998; 280(5370): 1763-6.

[29]   Altschafl BA, Beutner G, Sharma VK, Sheu SS, Valdivia HH. The mitochondrial ryanodine receptor in rat heart: a pharmaco-kinetic profile. Biochim Biophys Acta 2007; 1768(7): 1784-95.

[30]   Pinton P, Rizzuto R. Bcl-2 and Ca2+ homeostasis in the endoplasmic reticulum. Cell Death Differ 2006; 13(8): 1409-18.

[31]   Ichas F, Mazat JP. From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. Biochim Biophys Acta 1998; 1366(1-2): 33-50.

[32]   Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Rev 2007; 87(1): 99-163.

[33]   Juhaszova M, Wang S, Zorov DB, Nuss HB, Gleichmann M, Mattson MP, et al. The identity and regulation of the mitochondrial permeability transition pore: where the known meets the unknown. Ann N Y Acad Sci 2008; 1123: 197-212.

[34]   Armstrong JS. The role of the mitochondrial permeability transition in cell death. Mitochondrion 2006; 6(5): 225-34.

[35]   Scarpa M, Colombo A, Sorgato P, De Renzi E. The incidence of aphasia and global aphasia in left brain-damaged patients. Cortex 1987; 23(2): 331-6.

[36]   Beutner G, Ruck A, Riede B, Brdiczka D. Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. Biochim Biophys Acta 1998; 1368(1): 7-18.

[37]   Zamzami N, Maisse C, Metivier D, Kroemer G. Measurement of membrane permeability and permeability transition of mitochondria. Methods Cell Biol 2001; 65: 147-58.

[38]   Galat A, Metcalfe SM. Peptidylproline cis/trans isomerases. Prog Biophys Mol Biol 1995; 63(1): 67-118.

[39]   Broekemeier KM, Dempsey ME, Pfeiffer DR. Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. J Biol Chem 1989; 264(14): 7826-30.

[40]   Shimizu S, Matsuoka Y, Shinohara Y, Yoneda Y, Tsujimoto Y. Essential role of voltage-dependent anion channel in various forms of apoptosis in mammalian cells. J Cell Biol 2001; 152(2): 237-50.

[41]   Crompton M, Virji S, Ward JM. Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. Eur J Biochem 1998; 258(2): 729-35.

[42]   Crompton M. On the involvement of mitochondrial intermembrane junctional complexes in apoptosis. Curr Med Chem 2003; 10(16): 1473-84.

[43]   Zamzami N, Marchetti P, Castedo M, et al. Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. FEBS Lett 1996; 384(1): 53-7.

[44]   Reed JC, Zha H, Aime-Sempe C, Takayama S, Wang HG. Structure-function analysis of Bcl-2 family proteins. Regulators of programmed cell death. Adv Exp Med Biol 1996; 406: 99-112.

[45]   Yagoda N, von Rechenberg M, Zaganjor E, et al. RAS-RAF-MEK-dependent oxidative cell death involving voltage-dependent anion channels. Nature 2007; 447(7146): 864-8.

[46]   Krauskopf A, Eriksson O, Craigen WJ, Forte MA, Bernardi P. Properties of the permeability transition in VDAC1(-/-) mitochondria. Biochim Biophys Acta 2006; 1757(5-6): 590-5.

[47]   Baines CP, Kaiser RA, Sheiko T, Craigen WJ, Molkentin JD. Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. Nat Cell Biol 2007; 9(5): 550-5.

[48]   Kokoszka JE, Waymire KG, Levy SE, et al. The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. Nature 2004; 427(6973): 461-5.

[49]   Pastorino JG, Shulga N, Hoek JB. Mitochondrial binding of hexokinase II inhibits Bax-induced cytochrome c release and apoptosis. J Biol Chem 2002; 277(9): 7610-8.

[50]   Pastorino JG, Hoek JB, Shulga N. Activation of glycogen synthase kinase 3beta disrupts the binding of hexokinase II to mitochondria by phosphorylating voltage-dependent anion channel and potentiates chemotherapy-induced cytotoxicity. Cancer Res 2005; 65(22): 10545-54.

[51]   Baines CP, Kaiser RA, Purcell NH, et al. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. Nature 2005; 434(7033): 658-62.

[52]   Schinzel AC, Takeuchi O, Huang Z, et al. Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. Proc Natl Acad Sci U S A 2005; 102(34): 12005-10.

[53]   Basso E, Fante L, Fowlkes J, Petronilli V, Forte MA, Bernardi P. Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. J Biol Chem 2005; 280(19): 18558-61.

[54]   Tsujimoto Y, Nakagawa T, Shimizu S. Mitochondrial membrane permeability transition and cell death. Biochim Biophys Acta 2006; 1757(9-10): 1297-300.

[55]   Wieckowski MR, Szabadkai G, Wasilewski M, Pinton P, Duszynski J, Rizzuto R. Overexpression of adenine nucleotide translocase reduces Ca2+ signal transmission between the ER and mitochondria. Biochim Biophys Res Commun 2006; 348(2): 393-9.

[56]   Miwa S, Brand MD. Mitochondrial matrix reactive oxygen species production is very sensitive to mild uncoupling. Biochem Soc Trans 2003; 31(Pt 6): 1300-1.

[57]   Choksi KB, Papaconstantinou J. Age-related alterations in oxidatively damaged proteins of mouse heart mitochondrial electron transport chain complexes. Free Radic Biol Med 2008; 44(10): 1795-805.

[58]   Kowaltowski AJ, Vercesi AE. Mitochondrial damage induced by conditions of oxidative stress. Free Radic Biol Med 1999; 26(3-4): 463-71.

[59]   Grijalba MT, Vercesi AE, Schreier S. Ca2+-induced increased lipid packing and domain formation in submitochondrial particles. A possible early step in the mechanism of Ca2+-stimulated generation of reactive oxygen species by the respiratory chain. Biochemistry 1999; 38(40): 13279-87.

[60]   Turrens JF, Boveris A. Generation of superoxide anion by the NADH dehydrogenase of bovine heart mitochondria. Biochem J 1980; 191(2): 421-7.

[61]   Muller FL, Liu Y, Abdul-Ghani MA, *et al*. High rates of superoxide production in skeletal-muscle mitochondria respiring on both complex I- and complex II-linked substrates. Biochem J 2008; 409(2): 491-9.

[62]   Grivennikova VG, Vinogradov AD. Generation of superoxide by the mitochondrial Complex I. Biochim Biophys Acta 2006; 1757(5-6): 553-61.

[63]   Han D, Williams E, Cadenas E. Mitochondrial respiratory chain-dependent generation of superoxide anion and its release into the intermembrane space. Biochem J 2001; 353(Pt 2): 411-6.

[64]   Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. Mitochondria, oxidative stress and cell death. Apoptosis 2007; 12(5): 913-22.

[65]   Meissner C. Mutations of mitochondrial DNA - cause or consequence of the ageing process? Z Gerontol Geriatr 2007; 40(5): 325-33.

[66]   Pang CY, Ma YS, Wei YU. MtDNA mutations, functional decline and turnover of mitochondria in aging. Front Biosci 2008; 13: 3661-75.

[67]   Chen Y, Gibson SB. Is mitochondrial generation of reactive oxygen species a trigger for autophagy? Autophagy 2008; 4(2): 246-8.

[68]   Chen Y, McMillan-Ward E, Kong J, Israels SJ, Gibson SB. Oxidative stress induces autophagic cell death independent of apoptosis in transformed and cancer cells. Cell Death Differ 2008; 15(1): 171-82.

[69]   Halestrap AP, Clarke SJ, Khaliulin I. The role of mitochondria in protection of the heart by preconditioning. Biochim Biophys Acta 2007; 1767(8): 1007-31.

[70]   Scherz-Shouval R, Elazar Z. ROS, mitochondria and the regulation of autophagy. Trends Cell Biol 2007; 17(9): 422-7.

[71]   Curtin JF, Donovan M, Cotter TG. Regulation and measurement of oxidative stress in apoptosis. J Immunol Methods 2002; 265(1-2): 49-72.

[72]   McConkey DJ. Biochemical determinants of apoptosis and necrosis. Toxicol Lett 1998; 99(3): 157-68.

[73]   Cohen JJ. Programmed cell death in the immune system. Adv Immunol 1991; 50: 55-85.

[74]   Rai NK, Tripathi K, Sharma D, Shukla VK. Apoptosis: a basic physiologic process in wound healing. Int J Low Extrem Wounds 2005; 4(3): 138-44.

[75]   Hengartner MO. The biochemistry of apoptosis. Nature 2000; 407(6805): 770-6.

[76]   Kaufmann SH, Earnshaw WC. Induction of apoptosis by cancer chemotherapy. Exp Cell Res 2000; 256(1): 42-9.

[77]   Martinou JC, Desagher S, Antonsson B. Cytochrome c release from mitochondria: all or nothing. Nat Cell Biol 2000; 2(3): E41-3.

[78]   Halestrap AP, Doran E, Gillespie JP, O'Toole A. Mitochondria and cell death. Biochem Soc Trans 2000; 28(2): 170-7.

[79]   Yang J, Liu X, Bhalla K, *et al*. Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. Science 1997; 275(5303): 1129-32.

[80]   Kroemer G, Reed JC. Mitochondrial control of cell death. Nat Med 2000; 6(5): 513-9.

[81]   Petronilli V, Penzo D, Scorrano L, Bernardi P, Di Lisa F. The mitochondrial permeability transition, release of cytochrome c and cell death. Correlation with the duration of pore openings in situ. J Biol Chem 2001; 276(15): 12030-4.

[82]   Pinton P, Ferrari D, Rapizzi E, Di Virgilio F, Pozzan T, Rizzuto R. The Ca2+ concentration of the endoplasmic reticulum is a key determinant of ceramide-induced apoptosis: significance for the molecular mechanism of Bcl-2 action. Embo J 2001; 20(11): 2690-701.

[83]   Pinton P, Ferrari D, Rapizzi E, Di Virgilio F, Pozzan T, Rizzuto R. A role for calcium in Bcl-2 action? Biochimie 2002; 84(2-3): 195-201.

[84]   Biagioli M, Pifferi S, Ragghianti M, Bucci S, Rizzuto R, Pinton P. Endoplasmic reticulum stress and alteration in calcium homeostasis are involved in cadmium-induced apoptosis. Cell Calcium 2008; 43(2): 184-95.

[85]   Zamzami N, Marchetti P, Castedo M, *et al*. Reduction in mitochondrial potential constitutes an early irreversible step of programmed lymphocyte death *in vivo*. J Exp Med 1995; 181(5): 1661-72.

[86]   Kelekar A, Thompson CB. Bcl-2-family proteins: the role of the BH3 domain in apoptosis. Trends Cell Biol 1998; 8(8): 324-30.

[87]   Harris MH, Thompson CB. The role of the Bcl-2 family in the regulation of outer mitochondrial membrane permeability. Cell Death Differ 2000; 7(12): 1182-91.

[88]   Gross A, McDonnell JM, Korsmeyer SJ. BCL-2 family members and the mitochondria in apoptosis. Genes Dev 1999; 13(15): 1899-911.

[89]   Green DR, Kroemer G. The pathophysiology of mitochondrial cell death. Science 2004; 305(5684): 626-9.

[90]   Lewis W, Kohler JJ, Hosseini SH, *et al*. Antiretroviral nucleosides, deoxynucleotide carrier and mitochondrial DNA: evidence supporting the DNA pol gamma hypothesis. Aids 2006; 20(5): 675-84.

[91]   Lund KC, Wallace KB. Direct, DNA pol-gamma-independent effects of nucleoside reverse transcriptase inhibitors on mitochondrial bioenergetics. Cardiovasc Toxicol 2004; 4(3): 217-28.

[92]   Lewis W, Simpson JF, Meyer RR. Cardiac mitochondrial DNA polymerase-gamma is inhibited competitively and noncompetitively by phosphorylated zidovudine. Circ Res 1994; 74(2): 344-8.

[93]   Lynx MD, Bentley AT, McKee EE. 3'-Azido-3'-deoxythymidine (AZT) inhibits thymidine phosphorylation in isolated rat liver mitochondria: a possible mechanism of AZT hepatotoxicity. Biochem Pharmacol 2006; 71(9): 1342-8.

[94]   Lynx MD, McKee EE. 3'-Azido-3'-deoxythymidine (AZT) is a competitive inhibitor of thymidine phosphorylation in isolated rat heart and liver mitochondria. Biochem Pharmacol 2006; 72(2): 239-43.

[95]   de la Asuncion JG, Del Olmo ML, Gomez-Cambronero LG, Sastre J, Pallardo FV, Vina J. AZT induces oxidative damage to cardiac mitochondria: protective effect of vitamins C and E. Life Sci 2004; 76(1): 47-56.

[96]   Szabados E, Fischer GM, Toth K, *et al*. Role of reactive oxygen species and poly-ADP-ribose polymerase in the development of AZT-induced cardiomyopathy in rat. Free Radic Biol Med 1999; 26(3-4): 309-17.

[97]   Valenti D, Barile M, Passarella S. AZT inhibition of the ADP/ATP antiport in isolated rat heart mitochondria. Int J Mol Med 2000; 6(1): 93-6.

[98]   Lund KC, Wallace KB. Adenosine 3',5'-cyclic monophosphate (cAMP)-dependent phosphoregulation of mitochondrial complex I is inhibited by nucleoside reverse transcriptase inhibitors. Toxicol Appl Pharmacol 2008; 226(1): 94-106.

[99]   Amir R, Argoff CE, Bennett GJ, *et al*. The role of sodium channels in chronic inflammatory and neuropathic pain. J Pain 2006; 7(5 Suppl 3): S1-29.

[100]   Heavner JE. Cardiac toxicity of local anesthetics in the intact isolated heart model: a review. Reg Anesth Pain Med 2002; 27(6): 545-55.

[101]   Schonfeld P, Sztark F, Slimani M, Dabadie P, Mazat JP. Is bupivacaine a decoupler, a protonophore or a proton-leak-inducer? FEBS Lett 1992; 304(2-3): 273-6.

[102]   Sztark F, Ouhabi R, Dabadie P, Mazat JP. Effects of the local anesthetic bupivacaine on mitochondrial energy metabolism: change from uncoupling to decoupling depending on the respiration state. Biochem Mol Biol Int 1997; 43(5): 997-1003.

[103]   Sztark F, Malgat M, Dabadie P, Mazat JP. Comparison of the effects of bupivacaine and ropivacaine on heart cell mitochondrial bioenergetics. Anesthesiology 1998; 88(5): 1340-9.

[104]   Zhang S, Yao S, Li Q. Effects of ropivacaine and bupivacaine on rabbit myocardial energetic metabolism and mitochondria oxidation. J Huazhong Univ Sci Technolog Med Sci 2003; 23(2): 178-9, 83.

[105] Sztark F, Nouette-Gaulain K, Malgat M, Dabadie P, Mazat JP. Absence of stereospecific effects of bupivacaine isomers on heart mitochondrial bioenergetics. Anesthesiology 2000; 93(2): 456-62.

[106] Jaffe AS. The use of antiarrhythmics in advanced cardiac life support. Ann Emerg Med 1993; 22(2 Pt 2): 307-16.

[107] Centini F, Fiore C, Riezzo I, Rossi G, Fineschi V. Suicide due to oral ingestion of lidocaine: a case report and review of the literature. Forensic Sci Int 2007; 171(1): 57-62.

[108] Alto LE, Dhalla NS. Effects of some antiarrhythmic agents on dog heart mitochondrial oxidative phosphorylation. Eur J Pharmacol 1979; 59(3-4): 311-4.

[109] Chazotte B, Vanderkooi G, Chignell D. Further studies on F1-ATPase inhibition by local anesthetics. Biochim Biophys Acta 1982; 680(3): 310-6.

[110] Dzimiri N, Almotrefi AA. Investigation of class I anti-arrhythmic drug actions on guinea-pig cardiac mitochondrial lactate dehydrogenase activity. Clin Exp Pharmacol Physiol 1993; 20(4): 201-6.

[111] Tsutsumi Y, Oshita S, Kawano T, *et al*. Lidocaine and mexiletine inhibit mitochondrial oxidation in rat ventricular myocytes. Anesthesiology 2001; 95(3): 766-70.

[112] Mudaliar S, Henry RR. New oral therapies for type 2 diabetes mellitus: The glitazones or insulin sensitizers. Annu Rev Med 2001; 52: 239-57.

[113] Petersen KF, Krssak M, Inzucchi S, Cline GW, Dufour S, Shulman GI. Mechanism of troglitazone action in type 2 diabetes. Diabetes 2000; 49(5): 827-31.

[114] Kliewer SA, Xu HE, Lambert MH, Willson TM. Peroxisome proliferator-activated receptors: from genes to physiology. Recent Prog Horm Res 2001; 56: 239-63.

[115] Delea TE, Edelsberg JS, Hagiwara M, Oster G, Phillips LS. Use of thiazolidinediones and risk of heart failure in people with type 2 diabetes: a retrospective cohort study. Diabetes Care 2003; 26(11): 2983-9.

[116] Scatena R, Martorana GE, Bottoni P, Giardina B. Mitochondrial dysfunction by synthetic ligands of peroxisome proliferator activated receptors (PPARs). IUBMB Life 2004; 56(8): 477-82.

[117] Li N, Ragheb K, Lawler G, *et al*. Mitochondrial complex I inhibitor rotenone induces apoptosis through enhancing mitochondrial reactive oxygen species production. J Biol Chem 2003; 278(10): 8516-25.

[118] Hrdina R, Gersl V, Klimtova I, Simunek T, Machackova J, Adamcova M. Anthracycline-induced cardiotoxicity. Acta Medica (Hradec Kralove) 2000; 43(3): 75-82.

[119] Allen A. The cardiotoxicity of chemotherapeutic drugs. Semin Oncol 1992; 19(5): 529-42.

[120] Papkovsky DB. Methods in optical oxygen sensing: protocols and critical analyses. Methods Enzymol 2004; 381: 715-35.

[121] Lefrak EA, Pitha J, Rosenheim S, Gottlieb JA. A clinicopathologic analysis of adriamycin cardiotoxicity. Cancer 1973; 32(2): 302-14.

[122] Minotti G, Menna P, Salvatorelli E, Cairo G, Gianni L. Anthracyclines: molecular advances and pharmacologic developments in antitumor activity and cardiotoxicity. Pharmacol Rev 2004; 56(2): 185-229.

[123] Saeki K, Obi I, Ogiku N, Shigekawa M, Imagawa T, Matsumoto T. Doxorubicin directly binds to the cardiac-type ryanodine receptor. Life Sci 2002; 70(20): 2377-89.

[124] Cassidy SC, Chan DP, Rowland DG, Allen HD. Effects of doxorubicin on diastolic function, contractile reserve, and ventricular-vascular coupling in piglets. Pediatr Cardiol 1998; 19(6): 450-7.

[125] Serafim TL, Oliveira PJ, Sardao VA, Perkins E, Parke D, Holy J. Different concentrations of berberine result in distinct cellular localization patterns and cell cycle effects in a melanoma cell line. Cancer Chemother Pharmacol 2008; 61(6): 1007-18.

[126] Myers CE, McGuire WP, Liss RH, Ifrim I, Grotzinger K, Young RC. Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. Science 1977; 197(4299): 165-7.

[127] Demant EJ. Inactivation of cytochrome c oxidase activity in mitochondrial membranes during redox cycling of doxorubicin. Biochem Pharmacol 1991; 41(4): 543-52.

[128] Palmeira CM, Serrano J, Kuehl DW, Wallace KB. Preferential oxidation of cardiac mitochondrial DNA following acute intoxication with doxorubicin. Biochim Biophys Acta 1997; 1321(2): 101-6.

[129] Mimnaugh EG, Trush MA, Bhatnagar M, Gram TE. Enhancement of reactive oxygen-dependent mitochondrial membrane lipid per-

[130] Singal PK, Deally CM, Weinberg LE. Subcellular effects of adriamycin in the heart: a concise review. J Mol Cell Cardiol 1987; 19(8): 817-28.

[131] Doroshow JH. Effect of anthracycline antibiotics on oxygen radical formation in rat heart. Cancer Res 1983; 43(2): 460-72.

[132] Kang YJ, Chen Y, Yu A, Voss-McCowan M, Epstein PN. Overexpression of metallothionein in the heart of transgenic mice suppresses doxorubicin cardiotoxicity. J Clin Invest 1997; 100(6): 1501-6.

[133] Sardao VA, Oliveira PJ, Holy J, Oliveira CR, Wallace KB. Morphological alterations induced by doxorubicin on H9c2 myoblasts: nuclear, mitochondrial, and cytoskeletal targets. Cell Biol Toxicol 2008.

[134] Singal PK, Iliskovic N, Li T, Kumar D. Adriamycin cardiomyopathy: pathophysiology and prevention. Faseb J 1997; 11(12): 931-6.

[135] Childs AC, Phaneuf SL, Dirks AJ, Phillips T, Leeuwenburgh C. Doxorubicin treatment *in vivo* causes cytochrome C release and cardiomyocyte apoptosis, as well as increased mitochondrial efficiency, superoxide dismutase activity, and Bcl-2:Bax ratio. Cancer Res 2002; 62(16): 4592-8.

[136] Oliveira PJ, Wallace KB. Depletion of adenine nucleotide translocator protein in heart mitochondria from doxorubicin-treated rats--relevance for mitochondrial dysfunction. Toxicology 2006; 220(2-3): 160-8.

[137] Solem LE, Heller LJ, Wallace KB. Dose-dependent increase in sensitivity to calcium-induced mitochondrial dysfunction and cardiomyocyte cell injury by doxorubicin. J Mol Cell Cardiol 1996; 28(5): 1023-32.

[138] Zhou S, Starkov A, Froberg MK, Leino RL, Wallace KB. Cumulative and irreversible cardiac mitochondrial dysfunction induced by doxorubicin. Cancer Res 2001; 61(2): 771-7.

[139] Zhou S, Heller LJ, Wallace KB. Interference with calcium-dependent mitochondrial bioenergetics in cardiac myocytes isolated from doxorubicin-treated rats. Toxicol Appl Pharmacol 2001; 175(1): 60-7.

[140] Sokolove PM, Shinaberry RG. Na+-independent release of Ca2+ from rat heart mitochondria. Induction by adriamycin aglycone. Biochem Pharmacol 1988; 37(5): 803-12.

[141] Solem LE, Henry TR, Wallace KB. Disruption of mitochondrial calcium homeostasis following chronic doxorubicin administration. Toxicol Appl Pharmacol 1994; 129(2): 214-22.

[142] Davies KJ, Doroshow JH. Redox cycling of anthracyclines by cardiac mitochondria. I. Anthracycline radical formation by NADH dehydrogenase. J Biol Chem 1986; 261(7): 3060-7.

[143] Kalyanaraman B, Perez-Reyes E, Mason RP. Spin-trapping and direct electron spin resonance investigations of the redox metabolism of quinone anticancer drugs. Biochim Biophys Acta 1980; 630(1): 119-30.

[144] Nohl H. A novel superoxide radical generator in heart mitochondria. FEBS Lett 1987; 214(2): 269-73.

[145] Nohl H, Gille L, Staniek K. The exogenous NADH dehydrogenase of heart mitochondria is the key enzyme responsible for selective cardiotoxicity of anthracyclines. Z Naturforsch [C] 1998; 53(3-4): 279-85.

[146] Pineiro-Carrero VM, Pineiro EO. Liver. Pediatrics 2004; 113(4 Suppl): 1097-106.

[147] Timmins GS, Deretic V. Mechanisms of action of isoniazid. Mol Microbiol 2006; 62(5): 1220-7.

[148] Chowdhury A, Santra A, Bhattacharjee K, Ghatak S, Saha DR, Dhali GK. Mitochondrial oxidative stress and permeability transition in isoniazid and rifampicin induced liver injury in mice. J Hepatol 2006; 45(1): 117-26.

[149] Lheureux PE, Penaloza A, Zahir S, Gris M. Science review: carnitine in the treatment of valproic acid-induced toxicity - what is the evidence? Crit Care 2005; 9(5): 431-40.

[150] Floren LC, Hebert MF, Venook AP, Jordan VC, Cisneros A, Somberg KA. Tamoxifen in liver disease: potential exacerbation of hepatic dysfunction. Ann Oncol 1998; 9(10): 1123-6.

[151] Moreira PI, Custodio J, Moreno A, Oliveira CR, Santos MS. Tamoxifen and estradiol interact with the flavin mononucleotide site of complex I leading to mitochondrial failure. J Biol Chem 2006; 281(15): 10143-52.

[152] Larosche I, Letteron P, Fromenty B, *et al*. Tamoxifen inhibits topoisomerases, depletes mitochondrial DNA, and triggers steatosis in mouse liver. J Pharmacol Exp Ther 2007; 321(2): 526-35.

[153] Nazarewicz RR, Zenebe WJ, Parihar A, *et al*. Tamoxifen induces oxidative stress and mitochondrial apoptosis *via* stimulating mitochondrial nitric oxide synthase. Cancer Res 2007; 67(3): 1282-90.

[154] Takashima E, Iguchi K, Usui S, Yamamoto H, Hirano K. Metabolite profiles of flutamide in serum from patients with flutamide-induced hepatic dysfunction. Biol Pharm Bull 2003; 26(10): 1455-60.

[155] Fau D, Eugene D, Berson A, *et al*. Toxicity of the antiandrogen flutamide in isolated rat hepatocytes. J Pharmacol Exp Ther 1994; 269(3): 954-62.

[156] Feng JY, Johnson AA, Johnson KA, Anderson KS. Insights into the molecular mechanism of mitochondrial toxicity by AIDS drugs. J Biol Chem 2001; 276(26): 23832-7.

[157] Lund KC, Peterson LL, Wallace KB. Absence of a universal mechanism of mitochondrial toxicity by nucleoside analogs. Antimicrob Agents Chemother 2007; 51(7): 2531-9.

[158] Redlich G, Zanger UM, Riedmaier S, *et al*. Distinction between Human Cytochrome P450 (CYP) Isoforms and Identification of New Phosphorylation Sites by Mass Spectrometry. J Proteome Res 2008. (in press)

[159] Omura T. Mitochondrial P450s. Chem Biol Interact 2006; 163(1-2): 86-93.

[160] Genter MB, Clay CD, Dalton TP, Dong H, Nebert DW, Shertzer HG. Comparison of mouse hepatic mitochondrial versus microsomal cytochromes P450 following TCDD treatment. Biochem Biophys Res Commun 2006; 342(4): 1375-81.

[161] Servais H, Ortiz A, Devuyst O, Denamur S, Tulkens PM, Mingeot-Leclercq MP. Renal cell apoptosis induced by nephrotoxic drugs: cellular and molecular mechanisms and potential approaches to modulation. Apoptosis 2008; 13(1): 11-32.

[162] Chen CH, Lin H, Hsu YH, *et al*. The protective effect of prostacyclin on adriamycin-induced apoptosis in rat renal tubular cells. Eur J Pharmacol 2006; 529(1-3): 8-15.

[163] Ciarimboli G, Ludwig T, Lang D, *et al*. Cisplatin nephrotoxicity is critically mediated *via* the human organic cation transporter 2. Am J Pathol 2005; 167(6): 1477-84.

[164] Kroning R, Katz D, Lichtenstein AK, Nagami GT. Differential effects of cisplatin in proximal and distal renal tubule epithelial cell lines. Br J Cancer 1999; 79(2): 293-9.

[165] Park MS, De Leon M, Devarajan P. Cisplatin induces apoptosis in LLC-PK1 cells *via* activation of mitochondrial pathways. J Am Soc Nephrol 2002; 13(4): 858-65.

[166] Wei Q, Dong G, Franklin J, Dong Z. The pathological role of Bax in cisplatin nephrotoxicity. Kidney Int 2007; 72(1): 53-62.

[167] Kaushal GP, Kaushal V, Hong X, Shah SV. Role and regulation of activation of caspases in cisplatin-induced injury to renal tubular epithelial cells. Kidney Int 2001; 60(5): 1726-36.

[168] Cummings BS, Schnellmann RG. Cisplatin-induced renal cell apoptosis: caspase 3-dependent and -independent pathways. J Pharmacol Exp Ther 2002; 302(1): 8-17.

[169] Baek SM, Kwon CH, Kim JH, Woo JS, Jung JS, Kim YK. Differential roles of hydrogen peroxide and hydroxyl radical in cisplatin-induced cell death in renal proximal tubular epithelial cells. J Lab Clin Med 2003; 142(3): 178-86.

[170] Schwerdt G, Freudinger R, Schuster C, Weber F, Thews O, Gekle M. Cisplatin-induced apoptosis is enhanced by hypoxia and by inhibition of mitochondria in renal collecting duct cells. Toxicol Sci 2005; 85(1): 735-42.

[171] Cilenti L, Kyriazis GA, Soundarapandian MM, *et al*. Omi/HtrA2 protease mediates cisplatin-induced cell death in renal cells. Am J Physiol Renal Physiol 2005; 288(2): F371-9.

[172] Walker PD, Shah SV. Gentamicin enhanced production of hydrogen peroxide by renal cortical mitochondria. Am J Physiol 1987; 253(4 Pt 1): C495-9.

[173] Walker PD, Shah SV. Evidence suggesting a role for hydroxyl radical in gentamicin-induced acute renal failure in rats. J Clin Invest 1988; 81(2): 334-41.

[174] Walker PD, Barri Y, Shah SV. Oxidant mechanisms in gentamicin nephrotoxicity. Ren Fail 1999; 21(3-4): 433-42.

[175] Servais H, Van Der Smissen P, Thirion G, *et al*. Gentamicin-induced apoptosis in LLC-PK1 cells: involvement of lysosomes and mitochondria. Toxicol Appl Pharmacol 2005; 206(3): 321-33.

[176] Justo P, Lorz C, Sanz A, Egido J, Ortiz A. Intracellular mechanisms of cyclosporin A-induced tubular cell apoptosis. J Am Soc Nephrol 2003; 14(12): 3072-80.

[177] Salducci MD, Chauvet-Monges AM, Tillement JP, *et al*. Trimetazidine reverses calcium accumulation and impairment of phosphorylation induced by cyclosporine A in isolated rat liver mitochondria. J Pharmacol Exp Ther 1996; 277(1): 417-22.

[178] Simon N, Morin C, Bruguerolle B, Tillement JP. [Effects of trimetazidine on altered functions of rat kidney induced by cyclosporine]. Therapie 2001; 56(5): 583-7.

[179] Jeon SH, Piao YJ, Choi KJ, *et al*. Prednisolone suppresses cyclosporin A-induced apoptosis but not cell cycle arrest in MDCK cells. Arch Biochem Biophys 2005; 435(2): 382-92.

[180] Khan HM, Cutkomp LK. Effects of DDT, DDE, and PCBs on mitochondrial respiration. Bull Environ Contam Toxicol 1982; 29(5): 577-85.

[181] Masini A, Ceccarelli-Stanzani D, Muscatello U. The effect of oligomycin on rat liver mitochondria respiring in state 4. FEBS Lett 1983; 160(1-2): 137-40.

[182] Blondin GA, Knobeloch LM, Read HW, Harkin JM. Mammalian mitochondria as *in vitro* monitors of water quality. Bull Environ Contam Toxicol 1987; 38(3): 467-74.

[183] Ogata M, Mori T, Izushi F, Etoh K, Sakai R, Meguro T, *et al*. Classification of potentially toxic chemicals based on their effects on mitochondrial respiration. Physiol Chem Phys Med NMR 1983; 15(3): 229-32.

[184] Pisani DE, Elliott AJ, Hinman DR, Aaronson LM, Pardini RS. Relationship between inhibition of mitochondrial respiration by naphthoquinones, their antitumor activity, and their redox potential. Biochem Pharmacol 1986; 35(21): 3791-8.

[185] Kamo N, Muratsugu M, Hongoh R, Kobatake Y. Membrane potential of mitochondria measured with an electrode sensitive to tetraphenyl phosphonium and relationship between proton electrochemical potential and phosphorylation potential in steady state. J Membr Biol 1979; 49(2): 105-21.

[186] Gnaiger E, Lassnig B, Kuznetsov AV, Margreiter R. Mitochondrial respiration in the low oxygen environment of the cell. Effect of ADP on oxygen kinetics. Biochim Biophys Acta 1998; 1365(1-2): 249-54.

[187] Gnaiger E. Oxygen conformance of cellular respiration. A perspective of mitochondrial physiology. Adv Exp Med Biol 2003; 543: 39-55.

[188] Lemasters JJ. The ATP-to-oxygen stoichiometries of oxidative phosphorylation by rat liver mitochondria. An analysis of ADP-induced oxygen jumps by linear nonequilibrium thermodynamics. J Biol Chem 1984; 259(21): 13123-30.

[189] Marroquin LD, Hynes J, Dykens JA, Jamieson JD, Will Y. Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. Toxicol Sci 2007; 97(2): 539-47.

[190] Hynes J, Marroquin LD, Ogurtsov VI, *et al*. Investigation of drug-induced mitochondrial toxicity using fluorescence-based oxygen-sensitive probes. Toxicol Sci 2006; 92(1): 186-200.

[191] Scaduto RC, Jr., Grotyohann LW. Measurement of mitochondrial membrane potential using fluorescent rhodamine derivatives. Biophys J 1999; 76(1 Pt 1): 469-77.

[192] Etter EF, Kuhn MA, Fay FS. Detection of changes in near-membrane Ca2+ concentration using a novel membrane-associated Ca2+ indicator. J Biol Chem 1994; 269(13): 10141-9.

[193] Gerasimenko O, Tepikin A. How to measure Ca2+ in cellular organelles? Cell Calcium 2005; 38(3-4): 201-11.

[194] Vanden Berghe P. Fluorescent molecules as tools to study Ca2+ signaling, mitochondrial dynamics and synaptic function in enteric neurons. Verh K Acad Geneeskd Belg 2004; 66(5-6): 407-25.

[195] Madeira VM. A rapid and ultrasensitive method to measure Ca++ movements across biological membranes. Biochem Biophys Res Commun 1975; 64(3): 870-6.

[196] Lehninger AL. Water uptake and extrusion by mitochondria in relation to oxidative phosphorylation. Physiol Rev 1962; 42: 467-517.

[197] Neubert D, Foster GV, Lehninger AL. Effect of temperature on uptake and extrusion of water by isolated rat-liver mitochondria. Biochim Biophys Acta 1962; 60: 492-8.

[198] Tedeschi H, Harris DL. Some observations on the photometric estimation of mitochondrial volume. Biochim Biophys Acta 1958; 28(2): 392-402.

[199]   Garlid KD, Paucek P. Mitochondrial potassium transport: the K(+) cycle. Biochim Biophys Acta 2003; 1606(1-3): 23-41.

[200]   Das M, Parker JE, Halestrap AP. Matrix volume measurements challenge the existence of diazoxide/glibencamide-sensitive KATP channels in rat mitochondria. J Physiol 2003; 547(Pt 3): 893-902.

[201]   Lyamzaev KG, Izyumov DS, Avetisyan AV, Yang F, Pletjushkina OY, Chernyak BV. Inhibition of mitochondrial bioenergetics: the effects on structure of mitochondria in the cell and on apoptosis. Acta Biochim Pol 2004; 51(2): 553-62.

[202]   Okamoto K, Shaw JM. Mitochondrial morphology and dynamics in yeast and multicellular eukaryotes. Annu Rev Genet 2005; 39: 503-36.

[203]   Poppe M, Reimertz C, Dussmann H, *et al*. Dissipation of potassium and proton gradients inhibits mitochondrial hyperpolarization and cytochrome c release during neural apoptosis. J Neurosci 2001; 21(13): 4551-63.

[204]   Rintoul GL, Filiano AJ, Brocard JB, Kress GJ, Reynolds IJ. Glutamate decreases mitochondrial size and movement in primary forebrain neurons. J Neurosci 2003; 23(21): 7881-8.

[205]   Gao W, Pu Y, Luo KQ, Chang DC. Temporal relationship between cytochrome c release and mitochondrial swelling during UV-induced apoptosis in living HeLa cells. J Cell Sci 2001; 114(Pt 15): 2855-62.

[206]   Kahlert S, Reiser G. Swelling of mitochondria in cultured rat hippocampal astrocytes is induced by high cytosolic Ca(2+) load, but not by mitochondrial depolarization. FEBS Lett 2002; 529(2-3): 351-5.

[207]   Schoonen WG, de Roos JA, Westerink WM, Debiton E. Cytotoxic effects of 110 reference compounds on HepG2 cells and for 60 compounds on HeLa, ECC-1 and CHO cells. II mechanistic assays on NAD(P)H, ATP and DNA contents. Toxicol *In Vitro* 2005; 19(4): 491-503.

[208]   Ludovico P, Sansonetty F, Corte-Real M. Assessment of mitochondrial membrane potential in yeast cell populations by flow cytometry. Microbiology 2001; 147(Pt 12): 3335-43.

[209]   Aiuchi T, Matsunaga M, Nakaya K, Nakamura Y. Calculation of membrane potential in synaptosomes with use of a lipophilic cation (tetraphenylphosphonium). Chem Pharm Bull (Tokyo) 1989; 37(12): 3333-7.

[210]   Pereira-da-Silva L, Sherman M, Lundin M, Baltscheffsky H. Inorganic pyrophosphate gives a membrane potential in yeast mitochondria, as measured with the permeant cation tetraphenylphosphonium. Arch Biochem Biophys 1993; 304(2): 310-3.

[211]   Knobeloch LM, Blondin GA, Read HW, Harkin JM. Assessment of chemical toxicity using mammalian mitochondrial electron transport particles. Arch Environ Contam Toxicol 1990; 19(6): 828-35.

[212]   Haubenstricker ME, Holodnick SE, Mancy KH, Brabec MJ. Rapid toxicity testing based on mitochondrial respiratory activity. Bull Environ Contam Toxicol 1990; 44(5): 675-80.

[213]   Bragadin M, Dell'Antone P. A new *in vitro* toxicity test based on the response to toxic substances in solutions of mitochondria from beef heart. Arch Environ Contam Toxicol 1994; 27(3): 410-4.

[214]   Brunmair B, Staniek K, Gras F, *et al*. Thiazolidinediones, like metformin, inhibit respiratory complex I: a common mechanism contributing to their antidiabetic actions? Diabetes 2004; 53(4): 1052-9.

[215]   Pereira CV, Machado NG, Oliveira PJ. Mechanisms of Berberine [Natural Yellow 18, 5,6-dihydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo (5,6-a) quinolizinium]-induced Mitochondrial Dysfunction: Interaction with the Adenine Nucleotide Translocator. Toxicol Sci 2008.

[216]   Pereira GC, Branco AF, Matos JA, *et al*. Mitochondrially targeted effects of berberine [Natural Yellow 18, 5,6-dihydro-9,10-dimethoxybenzo(g)-1,3-benzodioxolo(5,6-a) quinolizinium] on K1735-M2 mouse melanoma cells: comparison with direct effects on isolated mitochondrial fractions. J Pharmacol Exp Ther 2007; 323(2): 636-49.

[217]   Murray J, Schilling B, Row RH, *et al*. Small-scale immunopurification of cytochrome c oxidase for a high-throughput multiplexing analysis of enzyme activity and amount. Biotechnol Appl Biochem 2007; 48(Pt 4): 167-78.

[218]   Nadanaciva S, Bernal A, Aggeler R, Capaldi R, Will Y. Target identification of drug induced mitochondrial toxicity using immunocapture based OXPHOS activity assays. Toxicol *In Vitro* 2007; 21(5): 902-11.

[219]   Dykens JA, Stout AK. Assessment of mitochondrial membrane potential in situ using single potentiometric dyes and a novel fluorescence resonance energy transfer technique. Methods Cell Biol 2001; 65: 285-309.

[220]   Papkovsky DB, Hynes J, Will Y. Respirometric Screening Technology for ADME-Tox studies. Expert Opin Drug Metab Toxicol 2006; 2(2): 313-23.

[221]   Ashley N, Adams S, Slama A, *et al*. Defects in maintenance of mitochondrial DNA are associated with intramitochondrial nucleotide imbalances. Hum Mol Genet 2007; 16(12): 1400-11.

[222]   Nagiec EE, Wu L, Swaney SM, *et al*. Oxazolidinones inhibit cellular proliferation *via* inhibition of mitochondrial protein synthesis. Antimicrob Agents Chemother 2005; 49(9): 3896-902.

[223]   Blattner JR, He L, Lemasters JJ. Screening assays for the mitochondrial permeability transition using a fluorescence multiwell plate reader. Anal Biochem 2001; 295(2): 220-6.

[224]   Habib GM, Shi ZZ, Ou CN, Kala G, Kala SV, Lieberman MW. Altered gene expression in the liver of gamma-glutamyl transpeptidase-deficient mice. Hepatology 2000; 32(3): 556-62.

[225]   Soloman R, Gabizon AA. Clinical pharmacology of liposomal anthracyclines: focus on pegylated liposomal Doxorubicin. Clin Lymphoma Myeloma 2008; 8(1): 21-32.

[226]   Oliveira PJ, Bjork JA, Santos MS, *et al*. Carvedilol-mediated antioxidant protection against doxorubicin-induced cardiac mitochondrial toxicity. Toxicol Appl Pharmacol 2004; 200(2): 159-68.

[227]   Aranda-Michel J, Koehler A, Bejarano PA, *et al*. Nefazodone-induced liver failure: report of three cases. Ann Intern Med 1999; 130(4 Pt 1): 285-8.

[228]   Lucena MI, Carvajal A, Andrade RJ, Velasco A. Antidepressant-induced hepatotoxicity. Expert Opin Drug Saf 2003; 2(3): 249-62.

[229]   Dykens JA, Jamieson JD, Marroquin LD, *et al*. *In vitro* assessment of mitochondrial dysfunction and cytotoxicity of nefazodone, trazodone, and buspirone. Toxicol Sci 2008; 103(2): 335-45.

[230]   Dykens JA, Jamieson J, Marroquin L, Nadanaciva S, Billis PA, Will Y. Biguanide-induced mitochondrial dysfunction yields increased lactate production and cytotoxicity of aerobically-poised HepG2 cells and human hepatocytes *in vitro*. Toxicol Appl Pharmacol 2008.

[231]   Will Y, Dykens JA, Nadanaciva S, *et al*. Effect of the multitargeted tyrosine kinase inhibitors imatinib, dasatinib, sunitinib, and sorafenib on mitochondrial function in isolated rat heart mitochondria and H9c2 cells. Toxicol Sci 2008; 106(1): 153-61.

[232]   Nadanaciva S, Dykens JA, Bernal A, Capaldi RA, Will Y. Mitochondrial impairment by PPAR agonists and statins identified *via* immunocaptured OXPHOS complex activities and respiration. Toxicol Appl Pharmacol 2007; 223(3): 277-87.

[233]   Liguori MJ, Anderson MG, Bukofzer S, *et al*. Microarray analysis in human hepatocytes suggests a mechanism for hepatotoxicity induced by trovafloxacin. Hepatology 2005; 41(1): 177-86.