Drug-induced Cardiac Mitochondrial Toxicity and Protection: From Doxorubicin to Carvedilol

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Abstract: Mitochondria have long been involved in several cellular processes beyond its role in energy production. The importance of this organelle for cardiac tissue homeostasis has been greatly investigated and its impairment can lead to cell death and consequent organ failure. Several compounds have been described in the literature as having direct effects on cardiac mitochondria which can provide a mechanistic explanation for their toxicological or pharmacological effects. The present review describes one classic example of drug-induced cardiac mitochondrial toxicity and another case of drug-induced mitochondrial protection. For the former, we present the case of doxorubicin, an anticancer agent whose treatment is associated with a cumulative and dose-dependent cardiomyopathy with a mitochondrial etiology. Following this, we present the case of carvedilol, a β-blocker with intrinsic antioxidant activity, which has been described to protect cardiac mitochondria from oxidative injury. The final part of the review integrates information from the previous chapters, demonstrating how carvedilol can contribute to reduce doxorubicin toxicity on cardiac mitochondria. The two referred examples result in important take-home messages: a) drug-induced cardiac mitochondrial dysfunction is an important contributor for drug-associated organ failure, b) protection of mitochondrial function is involved in the beneficial impact of some clinically-used drugs and c) a more accurate prediction of toxic vs. beneficial effects should be an important component of drug development by the pharmaceutical industry.

Keywords: Cardioprotection, carvedilol, doxorubicin, heart, mitochondria, toxicity.

1. MITOCHONDRIA AS A DRUG TARGET: PROTECTION VS. TOXICITY

Mitochondria are usually considered the power plants of the cells, supplying most of the energy needed. Isolated mitochondrial fractions from different tissues, more frequently from liver, proved to be good in vitro systems to predict drug toxicity before more complex and expensive in vivo experiments [1]. Isolated mitochondrial fractions were used to obtain toxicological parameters such as IC₅₀ in order to establish a ranking of compound toxicity, however, one obvious disadvantage is the lack of complexity of the biological system. Nevertheless, isolated mitochondrial fractions can be used as a good indicator to predict drug safety, being in fact in use by several major pharmaceutical companies [2, 3].

But why is mitochondrial toxicity screening that important? For example, after extensive research mitochondria began to be linked with several pathologies such as Huntington’s and Parkinson’s disease [4], diabetes [5] and several cardiovascular disorders, including atherosclerosis, cardiac failure and ischemic heart disease [6] which demonstrates that mitochondrial function is critical for tissue homeostasis. Organs such as cardiac and skeletal muscles and the brain are metabolically more active, implying a higher dependence of mitochondrial-produced energy [7], with a consequent increase in tissue damage resulting from drug-induced mitochondrial failure.

At a first glance, mitochondria may appear an organelle mostly dedicated to the production of cell energy. In fact, not only mitochondria constitute a dynamic filamentous network that can undergo remodeling by fusion and fission (as opposed to the classic “peanut” representation in the text books), but also mitochondria are much more than simple batteries. Mitochondria play an important role in the regulation of cell survival, proliferation [8] and death [9], calcium signaling [10], as well as redox homeostasis [11].

This initial section will briefly describe the physiology and function of mitochondria in the context of cardiomyocyte homeostasis, serving as framework to justify the need to preserve cardiac mitochondrial performance during drug-induced toxicity. Excellent reviews are available focusing in detail alterations of cardiac mitochondrial energetic during stress [12, 13].

1.1. Mitochondrial Energy Production by Oxidative Phosphorylation

Mitochondria is one of the endomembrane systems found in cells, being constituted by two lipid bilayer membranes: an outer membrane containing cholesterol and highly permeable to ions due to the presence of non-specific high conducting channels and an inner membrane, rich in cardiolipin and mostly impermeable, which allows for selective ion and metabolite transport as well as to formation of ion gradients. Two other spaces are generally considered in mitochondria, the intermembrane space and the matrix, the gel-like organelle interior moiety [14, 15].

The degree of internal mitochondrial structure varies from tissue to tissue, depending on their metabolic states [16]. Even within the same tissue, mitochondrial heterogeneity can occur. One example occurs in the cardiomyocyte, where two distinct mitochondrial populations exist, one close to the T-tubules and sarcoplasmic reticulum and another one closely associated with contractile filaments. In adult cardiomyocytes, both populations are largely associated with cytoskeleton and, therefore, the dynamic properties of the organelle are limited [17]. However, the highly energy demanding character of the heart requires a bioenergetic state of the highest level which can only be achieved if refined quality control is verified. In this case, damaged or non-functional mitochondria are removed by autophagy and new mitochondria are formed by biogenesis. Both situations depend on fission/fusion events but the spatial constrains and crystal-like pattern of those mitochondria, which are found packed between the myofibrils, has generated discussion about the possibility of such events to occur [16, 17]. Supporting the notion of mitochondrial fusion and fission in the myocardium is the fact that cardiomyocytes have all the necessary machinery for the two events and, in certain pathological situations very small
mitochondria (e.g., dilated cardiomyopathy, cardiac rhabdomyomais and ventricular-associated congenital heart diseases) or abnormally large and defective mitochondria (e.g., senescent/aged cardiomyocytes) can be found [16, 17]. Studies not only in cardiomyocytes but also in neonatal cardiomyocytes have demonstrated a high degree of motility and morphological changes [17, 18]. However, although this type of cell lines shares some characteristics of differentiated cardiac cells, they lack the well organized and dense packing that is typical of adult cardiomyocytes. Likewise, studies where mRNA or protein levels are quantified in whole heart content should be interpreted cautiously since the organ consists in several types of cells besides cardiomyocytes [16]. The ultimate answer would be live imaging of adult cardiomyocytes but this carries some technical difficulties: myocytes are difficult to transfect and the spatial resolution of confocal microscopy may not be optimal [16, 17]. Nevertheless, an interesting work compared the dynamics of mitochondria between cell lines and adult cardiomyocytes showing, that under normal conditions, although fusion and fission do not occur, mitochondrial movements were observed which were attributed to transitions between the orthodox and condensed form and not to the formation of a continuous network [18].

Depending on the tissue, several energy substrates are primarily used to produce adenosine triphosphate (ATP). In muscle tissue, preferential substrates are fatty acids which are metabolized by β-oxidation in mitochondria and peroxisomes [19]. Fatty acid oxidation generates substrates for the mitochondrial electron transport chain (ETC). The transference of electrons among several multi-subunit proteins (Complexes I-IV) is driven by the decrease in the redox potential, until reaching oxygen as the final acceptor. Electron transfer is coupled to proton ejection by specific sites of the ETC, generating a proton gradient along the two sides of the inner mitochondrial membrane. The proton gradient, or proton motive force, is comprised by two main components: an electric factor, which is the major component in the energy transduction system, and a pH-dependent element. The proton gradient allows the phosphorylation of adenosine triphosphate (ADP) by the enzyme ATP synthase; ATP is then translocated back to the cytosol which is the major component in the energy transduction system, and a pH-dependent element. The proton gradient allows the phosphorylation of adenosine triphosphate (ADP) by the enzyme ATP synthase; ATP is then translocated back to the cytosol via the inner mitochondrial membrane adenine nucleotide translocator (ANT) and the outer membrane voltage-dependent anion channel (VDAC) [11]. The overall process of ATP generation due to substrate oxidation coupled to ADP phosphorylation is called oxidative phosphorylation (OXPHOS) [20].

Since mitochondria are responsible for the production of about 90% of the energy used by the cardiomyocyte [7], impairment of mitochondrial function will likely affect normal organ homeostasis. Therefore, it is not surprising that cardiac mitochondria, and specifically OXPHOS, are a preferential target for several clinically used drugs and other xenobiotics including local anesthetics [21, 22], anti-cancer agents [23, 24] and non-reverse transcriptase inhibitors [25], among others [26].

1.2. Calcium Homeostasis and the Mitochondrial Permeability Transition

Mitochondrial calcium concentration is one of the key factors regulating mitochondrial function and ATP synthesis [27]. Calcium enters mitochondria through a specific non-ATP dependent unipporter and exits by exchanging either with Na⁺ through the Na⁺/Ca²⁺ carrier (e.g. heart) or with the Ca²⁺/H⁺ antiporter (e.g. liver) to create a continuous cycle of calcium across the inner membrane [11]. Matrix calcium accumulation is electrophoretically driven by the proton gradient generated by the respiratory chain [28].

In excitable cells such as cardiomyocytes, the close proximity between the sarcoplasmic reticulum and the mitochondria allows the latter to act as a cytoplasmic calcium buffer when the ion is released from intracellular stores during contraction, being able to spatially and temporally modulate calcium signaling [29]. Because such events are intrinsically regulated, disturbances on calcium homeostasis can lead to organ failure, as for example during cardiac ischemia and reperfusion [27].

During stressful events, including those resulting from drug-induced toxicity, excessive mitochondrial calcium accumulation can result in the induction of the mitochondrial permeability transition (MPT). This phenomenon is characterized by a sudden increase in the permeability of the inner mitochondrial membrane. It is accepted that the MPT originates due to the opening of non-selective pores in the inner membrane which allows the free passage of any molecule smaller than 1.5 kDa [30]. When the permeability barrier is disrupted, low molecular weight solutes move freely through the membrane while larger proteins do not, resulting in a colloidal osmotic pressure that leads to mitochondrial swelling [30]. If the MPT pores remain open indefinitely, as usually occurs during pathological events, mitochondrial structural alterations will cause the expansion of the inner membrane and consequent mechanical disruption of the outer membrane, which has a smaller area. Other consequences for the mitochondrion during the MPT, include mitochondrial membrane depolarization and OXPHOS uncoupling, inhibition of respiration due to loss of co-factors and/or cytochrome c and increase in oxidative stress. Also, mitochondrial membrane depolarization leads to reversal of the ATP synthase activity, which starts hydrolyzing ATP instead of synthesizing it, in an attempt to re-establish the lost transmembrane potential [30].

As described above, mitochondrial dysfunction induced by MPT is a consequence of mitochondrial calcium overload [28], particularly when accompanied by oxidative stress [31], mitochondrial depolarization, elevated phosphate concentrations and adenosine nucleotide depletion [30]. In opposition, inhibition of the MPT pore can be achieved with cyclosporin A (CsA), Mg²⁺, ADP and antioxidants [32, 33]. A number of compounds and deleterious conditions increase the sensitivity to cardiac MPT pore opening in vitro as well as in vivo and that peculiarity is often used to explain their deleterious effects in the organ. Examples include the anti-cancer doxorubicin (DOX, [34-36] and below) and ischemia and reperfusion [37, 38]. Several strategies to prevent such actions are usually applied in the form of antioxidants, calcium chelators or specific inhibitors of the MPT pore which sometimes yield positive results [27].

1.3. Mitochondrial Oxidative Stress and Role in Cell Death

Mitochondrial respiration is generally identified as the prime source of reactive oxygen species (ROS) [39]. ROS generation by Complex I and III [31] is increased under pathological conditions such as ischemia and reperfusion [39]. Increased mitochondrial oxidative stress results in mitochondrial proteins damage and lipid peroxidation [31] which consequently promotes loss of mitochondrial functional integrity [39]. Another consequence of increased mitochondrial oxidative stress is damage of the genetic apparatus of the organelle. In fact, accumulation of oxidized mtDNA has been reported in several human pathologies and lead ultimately to a decrease in mtDNA copy number and progressive loss of proteins involved in energy production [40]. During normal mitochondrial activity, about 1% of oxygen is converted in superoxide radical anion (O₂⁻*) [41] which is further converted into hydrogen peroxide (H₂O₂) [42], a relatively inert molecule but which is able to cross membranes and react with free metal ions originating a more dangerous ROS, the hydroxyl radical (HO*) [43]. Cardiac mitochondria are equipped with a wide range of enzymatic defenses, including manganese-superoxide dismutase (Mn-SOD), glutathione reductase, glutathione peroxidase and peroxiredoxin, among others, which are modulated to answer to alterations in the redox balance [44]. A chronic increase of mitochondrial free radical production in the heart, which is not counteracted by either enzymatic or non-enzymatic defenses, can have further consequences besides oxidative damage to mitochondrial proteins, lipids and DNA. Myocardial
remodeling can also constitute a maladaptive consequence of increased ROS generation through activation of specific proteins including metalloproteinases [44, 45].

If mitochondrial ROS generation is too extensive and overload the antioxidant network, one consequence would be the removal of damaged mitochondria by autophagy [46], or a more drastic solution, the triggering of cell death [42]. If the imbalance in oxidative stress is present concomitantly with alterations in cytosolic and mitochondrial calcium, the MPT may occur, with the rupture of the outer membrane leading to the release of several apoptotic initiators usually enclosed in the inter-membrane space [2], e.g., cytochrome c, the apoptosis-inducing factor (AIF) and the second mitochondria-derived activator of caspase/direct inhibitor of apoptosis (IAP)-binding protein with low isoelectric point (SMAC/DIABLO) protein. Alternatively to the MPT pore pathway, pro-apoptotic factor release from mitochondria can also occur through outer membrane permeabilization by channels formed by specific pro-apoptotic proteins including the Bcl-2-associated protein (BAX), Bcl-2 homologous antagonist killer (BAK) and the truncated form of BH3 interacting domain death agonist (BID) [47]. After its release, cytochrome c interacts with several proteins in the cytosol, forming the apoptosome complex and consequently activating the caspases cascade [33], which leads to cleavage of a number of targets and apoptosis. Several other excellent reviews are available concerning the role of mitochondria in cell death in different tissues [9, 48, 49].

Various links between mitochondrial oxidative stress and protein-mediated pro-apoptotic permeabilization of the mitochondrial outer membrane have already been described [50, 51]. Furthermore, oxidative stress can lead to the oxidation of cardiolipin residues, which decreases the affinity of this phospholipid for cytochrome c, contributing for its release to the cytosol after outer mitochondrial permeabilization due to a pro-apoptotic stimuli [52, 53].

As consequence of the post-mitotic nature of cardiomyocytes, loss of these cells by apoptosis may have deleterious effects in the organ. For example, it has been described that apoptosis or necrosis can follow ischemia and reperfusion damage in the heart, which can seriously limit the recovery of the myocardium [54]. Apoptosis in the heart has been detected in different conditions including diabetes [55] and heart failure [56].

The above sections describe how the disruption of cardiac mitochondrial balance can result in a grave danger to the cell, ultimately ending in organ failure. The next two chapters will provide one clear example of drug-induced mitochondrial toxicity (DOX) and drug-induced mitochondrial protection (carvedilol). Ultimately, we will focus on the work available of how one of the compounds can prevent the damage caused by the other. Further reviews on drug-induced cardiac mitochondrial dysfunction can be found in the literature [57].

2. DRUG-INDUCED CARDIAC MITOCHONDRIAL TOXICITY: THE CASE FOR DOXORUBICIN

DOX (Fig. 1), [(7S, 9S)-7-[(2R, 4S, 5S, 6S)-4-amino-5-hydroxy-6-methylloxan-2-yl]oxo-6, 9, 11-trihydroxy-9-(2-hydroxyacetyl)-4-methoxy-8, 10-dihydro-7H-tetracene-5, 12-dione], was discovered in the late 1960s by the pharmaceutical company “Farmitalia Research Laboratories” and initially named Adriamycin, due to the site of discovery (the Adriatic Sea). DOX is an antibiotic belonging to the anthracycline family and was isolated from a variant culture of the Streptomyces peucetius (Streptomyces peucetius caesius) by aerobic fermentation after mutagenic treatment [58, 59]. The new molecule was chemically characterized and found to possess an anthraquinone moiety connected to a glycoside group [59, 60]. The specific chemical structure is responsible not only for the antineoplastic activity but also for its toxicity, and will be discussed further on in this review.

Initial studies have shown that DOX is able to stop the progression of tumors in rats [61] and its use in clinical trials achieved great success [62, 63]. However, it was later described that patients under DOX treatment developed signs of cardiac toxicity and cardiomyopathy [63, 64], hence a cardio-selective toxicity was soon considered one effect of DOX-chemotherapy. Nevertheless, the clinical use of DOX continued and still nowadays is probably one of the most potent anticancer agents in the clinical practice [65].

2.1. Clinical Use and Side Effects

DOX is commonly used to treat several types of human and non-human tumors, from leukemia and lymphomas to soft-tissue sarcomas and solid tumors, including: breast carcinoma, osteosarcoma, Kaposi’s sarcoma, Hodgkin’s and non-Hodgkin’s lymphomas [62, 63]. Patients subjected to DOX-chemotherapy usually exhibit symptoms such as nausea, vomiting, alopecia, myelosuppression, stomatitis and gastrointestinal disturbances [66, 67], which are all clinically manageable side effects. Nevertheless, cardiotoxicity is a completely different story, being probably the most hazardous side-effect associated with DOX treatment, sometimes reaching 50% mortality for the highest cumulative dosages. Acute toxicity accounts for 11% of all cases [68] with symptoms such as myopericarditis, sinus tachycardia, reversible arrhythmics, prolonged QT interval and flattening of the T wave [68], occurring during or right after the last administered dose, and disappearing right after discontinuation of the treatment. Alternatively, chronic DOX-induced cardiotoxicity can be observed within one month or even years later after the treatment ceases [64]. Although the incidence of this type of delayed toxicity is much lower (around 1.7%) it is rather dose-dependent, i.e., the probability of developing cardiomyopathy increases with the total cumulative dose [69]. Total dosages between 500-550 mg/m² have a 5% probability of inducing cardiac heart failure [69] and, therefore, this threshold was established as the maximum dosage of DOX tolerable in chemotherapy. Naturally, the downside of such approach is a decrease in the performance of the drug as an anti-cancer agent. In addition to the cumulative dose, other risk factors can lower the toxicity threshold, including co-treatment with other drugs, mediastinal radiation therapy, age, gender, as well as a previous history of cardiovascular problems, hypertension and liver disease [66, 68]. Still, not all patients show signs of cardiomyopathy even when high doses of DOX are applied suggesting intrinsic differences between subjects, at the genetic background. The idea of genetic polymorphisms in DOX-induced toxicity has been previously suggested [70] with other clinical situations using this type of approach to deliver a more selective therapeutically dosage to patients [71]. Although so far there are no experiments performed regarding polymorphism associated with DOX toxicity, isolated experiments with knock-out or genetically engineered animals give new understandings about the topic of polymorphism or SNPs and its relationship with drug-induced toxicity. In a relatively recent report [72], authors reviewed these aspects and pointed out genes related to drug efflux transporters, antioxidant and detoxification enzymes as well as some enzymes capable of reducing DOX as possible targets for this type of analysis. Nevertheless, the authors mention that further work is needed to better address this important question.

2.2. Monitoring DOX-induced Cardiotoxicity

Presently, there is no effective approach that fully prevents or even treats DOX-associated toxicity and, therefore, the best strategy is the early detection of cardiomyopathy end-points. However, the idea may not be so straightforward because clinical manifestation of DOX-induced toxicity share symptoms with other cardio and non-cardiovascular illnesses and the current methods used to follow heart function lacks sensitivity or specificity [66]. Currently, from all the techniques available [66, 70] only two of them constitute the
best methodology to follow-up the progression of cardiomyopathy during and/or after DOX treatment: serial echocardiography and endomyocardial biopsy. Although the latter is the most sensitive and specific method it is also the more invasive and less available for the clinician. Echocardiography has its advantages over other methods because it is widely available, noninvasive and highly reliable being the study of ejection fraction to detect signs of early cardiomyopathy one of the main advantages of the technique [66, 68], the sensitivity of which can be increased when associated with some particular protocols (e.g., exercise) or Doppler imaging [73, 74]. Measurement of cardiac enzymes or humoral factors, such as troponin I and T and B-type natriuretic peptide, can be an additional strategy to detect early signs of DOX-induced cardiotoxicity, however these markers represent general cardiac tissue damage and are not specific of DOX-induced damage [75]. Nevertheless, the combined use of specific cardiac damage markers together with imaging techniques can increase the sensitivity of the detection and demonstrate that marker levels are directly proportional to cardiomyopathy score.

Unfortunately, there is no effective treatment available for patients with established cardiomyopathy even though the mortality rate is incredibly high once the prognosis is revealed [69]. The reason for that situation is probably related to the fact that standard therapy for cardiac heart failure fails to relieve the symptoms of DOX-treated patients [70, 76]. The only solution is cardiac transplantation but besides its radical character, it has also specific disadvantages in addition to the drawbacks of general cardiac transplantation [76]. First, the immunosuppressive condition after transplantation may increase the risk of cancer recurrence. Second, patients qualified as able to receive a new heart must provide evidences that are cancer-free for at least a period of 5 years. However, because the incidence of cancer-related death is so high in patients receiving DOX therapy, the majority of them die before the time frame is complete. Still, the use of ventricular-assist devices may be a temporary alternative for this group of patients until the requirements for cardiac transplantation are met [76].

### 2.3. Mechanisms for DOX Antineoplastic Activity

Despite the existing controversy, it is being more widely accepted that DOX-induced cardiotoxicity is completely independent from its anticancer activity. The idea gets further ground if one agrees that cardiomyocytes, as terminal differentiated cells, should not be as sensitive to the primary antineoplastic activity, which is related with cell cycle blockage. Nevertheless, the inhibition of transcription of certain proteins can contribute to the cardiomyopathy, although a causal relationship between both still needs to be addressed.

The planar structure of DOX anthraquinone moiety allows the intercalation of the drug into the DNA double strand, which will not only unwind nucleic acids, as it will also cause a stereochemical disorder and consequent block transcription and replication processes [77]. Meanwhile, topoisomerase II is also inhibited and downstream effects are triggered due to the formation of DNA double-strand breaks. All these effects result in cell cycle arrest that culminates in the activation of the apoptosis machinery leading to the death of cancer cells and tumor growth arrest. Obviously, one could not ignore the hypothesis that DNA interference may also occur in cardiomyocytes. However, other DNA intercalating compounds such as cisplatin or actinomycin D do not cause cardiotoxicity [78] and therefore the mechanism described is not likely responsible for degeneration of cardiac function.

### 2.4. Mitochondria and DOX-induced Cardiotoxicity

The cardiac tissue has a high energy demand and consequently any interference in the energy production machinery will clearly affect the whole physiology and heart contraction. Cardiomyocytes...
depend approximately 90% on ATP production by the mitochondrial OXPHOS, thus mitochondria appear as one attractive target for DOX toxicity. In fact, it is widely recognized that DOX-induced cardiotoxicity has a strong mitochondrial component [79, 80]. Mitochondria from DOX-treated animals show traces of the drug [81], which could be due to its affinity for particular biomolecules within the organelle. As with nuclear DNA, DOX also forms adducts with mitochondrial DNA (mtDNA) [82], while complexes between DOX and cardiolipin, one of the most abundant phospholipid in the inner mitochondrial membrane, have also been described [83]. Once accumulated in the mitochondrion, DOX can initiate its direct and deleterious effects which include: a) stimulation of ROS production, b) induction of MTP and c) inhibition of OXPHOS.

As pointed out earlier, the chemical structure of DOX, namely the anthraquinone group, also accounts for the toxic response of the drug. The quinone at the central ring (Fig. 1) is prone to reduction with a redox potential of approximately ~320 mV, similar to reduced nicotinamide adenine dinucleotide (NADH) [84]. Therefore, DOX can divert electrons from Complex I as well as from several other cellular dehydrogenases, namely, NADH transdehydrogenase, xanthine oxidase and cytochrome P450, being converted into a semiquinone radical along the process (Fig. 2) [84]. Afterwards, in the presence of molecular oxygen, DOX univalent reduced form, can donate its unpaired electron resulting in the formation of O₂⁺, regenerating the parent drug in the process [85]. The fact that in vitro studies with Complex I-specific inhibitor rotenone exacerbated ROS formation suggests that the site of DOX activation in the enzyme is upstream to the binding site of the inhibitor [86]. Since, theoretically, no net consumption of DOX occurs, the redox-cycle can take place endlessly.

In addition to the possible action of iron as a catalyst for production of HO² through Fenton reaction with DOX semiquinone participating by recycling ferric anion, the metal ion may also establish complexes with DOX (Fig. 2), stimulating the production of H₂O₂ [82, 87]. However, significant levels of free iron are unlikely to be observed in cells during physiological conditions [82, 88] and therefore, it is possible that DOX may induce first a dysregulation of iron homeostasis. Aconitase is known to be very sensitive to variations in oxidative stress and it is possible that when damaged, iron from aconitase iron-sulfur clusters can be displaced thus increasing free iron in mitochondria. The most important aspect is the fact that this enzyme has a double role, since in its iron-free form, it can act as an iron-regulating protein, facilitating iron uptake over iron sequestration hence increasing the pool of free iron available to react with DOX [82, 87].

In the context of reactive radical species, nitric oxide (NO) plays a particular important role in the cardiovascular system, being responsible for the increase in contraction, myocardial relaxation and diastolic function, Frank-Starling response, heart rate, β-adrenergic response, myocardial energetics and substrate metabolism [89, 90]. For this basal action to occur, cardiomyocytes and endothelial cells constitutively express two different isoforms, neuronal NO synthase (nNOS) and endothelial NO synthase (eNOS). Still, a third isoform with higher output, the inducible NO synthase (iNOS), whose abundance increases upon several stimuli, was proposed to be linked to different cardiac pathologies [91]. During DOX administration, transcription of eNOS in endothelial cells and iNOS in cardiomyocytes was shown to be increased, resulting in unbalanced amounts of NO and consequent deleterious effects [68, 90]. Moreover, DOX can be directly metabolized by all NO isoforms similarly to the mentioned reductases, uncoupling NO production and increasing O₂⁺ [68, 90]. In fact, the turnover of DOX cycle for iNOS is ~10 fold higher than for nNOS and eNOS, which alone have a Kₘ about ~100 fold lower than NADH-dehydrogenase [68, 90]. During the deregulated production of NO and O₂⁺ both molecules can react decreasing their cellular amount. Although this may seem like a protective mechanism, in fact it leads to the production of peroxynitrite (ONOO⁻), a much more reactive molecule when compared to NO which is relatively inert [90]. The importance of NO in the cardiac toxicity induced by DOX was confirmed when in vivo studies performed by inhibiting iNOS, or by using ONOO⁻ scavengers, delayed or prevented DOX-induced cardiomyopathy [90, 92]. Nevertheless, some contradictory information is reported in the literature regarding the impact of NO production inhibition during DOX treatment [89, 93]. One view suggested that the protection afforded by NO was mediated by O²⁻ since both the radical species can react together, which synergizes with superoxide dismutase (SOD) in the removal of O₂⁺ [89]. In fact, overexpression of Mn-SOD in NO null mice prevented the deleterious effects of DOX [89]. A second study demonstrated that iNOS overexpression or delivered as a bolus increased the toxicity of DOX on human breast cancer cells in culture but not on H9c2 cardiomyoblasts, suggesting that NO can act to prevent DOX toxicity in cardiac cells but can act to improve the clinical efficacy of DOX as an anti-tumor agent in cancer cells [93].

DOX-increased oxidative stress mediated by oxygen and nitrogen species results in damage to several biomolecules, including DNA, lipids and proteins. Biomolecule oxidation can directly affect mitochondrial function, or alternatively lead to secondary responses. For instance, oxidation of mtDNA can result in a defective respiratory chain, which will not only be unable to respond to high energy demands but will also increase electron leak to molecular oxygen, increasing ROS production [94]. ROS can directly inhibit the respiratory complexes of OXPHOS, e.g., cytochrome c oxidase (COX) [95], but alternatively the oxidation of certain residues or functional groups can induce conformational changes inhibiting protein function. One particular example is thiol groups, which are very vulnerable to oxidation. Several mitochondrial proteins show oxidation of this group after DOX treatment which forms disulfide bonds upon oxidation [94, 96]. One of such proteins is the ANT, in which the authors showed that oxidative damage lowers the formation of dimers which are crucial for the formation and opening of MPT pores [97]. In fact, mitochondria treated in vitro with DOX show increased susceptibility to the MPT [35, 36] when treated with inhibitors and modulators of the MPT pore, e.g., ATP, dithiothreitol (DTT), butylated hydroxytoluene (BHT) and CsA, this phenomenon was prevented. The same authors showed that secondary metabolites, such as DOX aglycones, are even more powerful in the induction of the MPT [36]. The in vitro MPT-inducing effects may actually be due to increased oxidative stress, oxidation of pyridine nucleotides, depletion of high energy adenine nucleotides and decrease in membrane potential, while increased sensitivity to MPT observed in cardiac mitochondria from DOX-treated animals [34] is probably due to the synergistic action of such effects with calcium homeostasis dysregulation. In fact, DOX has been described to interfere with the flux of calcium from the sarcoplasmic reticulum by increasing the opening state probability of calcium release channels [98]. Additionally, DOX also interferes with the Na⁺/Ca²⁺ and Na⁺/K⁺ pumps which can contribute for enhanced cytoplasmic calcium accumulation and, therefore contributing to augment mitochondrial calcium accumulation [98]. Similarly to in vitro experiments, cardiac mitochondria from DOX-treated animals have a lower capacity to accumulate and retain calcium [34, 79, 96, 99-101]. The effect is more pronounced if the cumulative dose of DOX is increased and persists for long periods after the end of the treatment [34]. Therefore, the loss of mitochondrial calcium loading capacity can be classified as a sensitive marker for DOX-induced cardiotoxicity [102]. The co-administration of CsA in vivo was capable to prevent DOX-induced cardiomyopathy demonstrating the importance of calcium homeostasis and induction of MPT in the toxicity mechanism of the drug. Nonetheless, caution should be taken when inferring the results, since FK-506 also inhibited DOX cardiotoxicity and this compound is not a MPT inhibitor, being in fact an immunosuppressant similarly as CsA [101]. An improved knowledge on this issue could be accomplished if specific
MPT inhibitors were used instead [103, 104]. Another intriguing aspect is the ex vivo use of CsA on cardiac mitochondria from DOX-treated animals resulted in restoration of respiration to control values, which suggests that the MPT pore or cyclophilin D, the target of CsA in mitochondria, are involved in DOX-induced inhibition of mitochondrial respiration [96]. The authors suggest that binding of CsA to cyclophilin D may cause a disassembling of pre-formed pores allowing crucial components such as the ANT to become available again to participate in OXPHOS. Although there is currently some controversy about the structural characteristic of the MPT pore [105], it is likely that the ANT may have at least a regulatory effect. Agreeing with the possible role of the ANT on DOX-induced toxicity, a decrease in functional ANT was detected after DOX treatment, suggesting a genetic or biochemical interference of DOX with this protein which results in a reduction of bioenergetic capacity and decrease mitochondrial calcium loading capacity [100]. Nevertheless, an evident direct interaction between drug-enzyme is still to be described in the literature for almost all OXPHOS enzymes, as pointed before [83], and the described inhibitory effects are due to increased oxidative stress or to the displacement of lipid-enzyme environment, as is in the case of DOX-induced inhibition of COX [83].

A decrease on state 3 respiration also seems to be another mitochondrial marker of DOX-induced cardiomyopathy, as it was al-

Fig. (2). Different mechanisms proposed for DOX activation and stimulation of ROS production. DOX can suffer univalent reduction by several cellular NAD(P)H-dependent flavoproteins originating a semiquinone free radical intermediate. Under aerobic conditions (A), molecular oxygen is not limiting and DOX semiquinone undergoes a futile cycle generating ROS in the process. However, under anaerobic conditions (B), the semiquinone undergoes aglycosilation and further reduction giving rise to the deoxy-aglycone of DOX which intercalates into DNA. Alternatively, DOX can form complexes with cellular free-iron (C and D). DOX-Fe³⁺ complexes can be enzymatically reduced by different flavoproteins or by low-molecular-weight compounds such as glutathione (C). In the absence of reducing agents, the ferric ion component of the drug-metal complex can be reduced at the expense of intramolecular oxidation of DOX (D). In the presence of oxygen, oxidation of DOX can continue until the fully oxidized end product of DOX (9-COOH-DOX) is reached, releasing ROS in the process. Both in A and C, futile cycles will lead to pyrimidine nucleotides and antioxidant defenses depletion. The reactions indicated as a can also be catalyzed by H₂O₂ with concomitant production of HO⁺. Adapted from [87, 199]. Partial molecular structure of DOX is presented in some schemes for ease of comprehension. Abbreviations: SOD, superoxide dismutase; FP, flavoprotein; FPH₂, reduced flavoprotein; GSH, reduced glutathione; GSSG, oxidized glutathione.
ready been described in several models of chronic exposure to the drug. The same effect was observed \textit{in vitro} together with a slight stimulation of state 4 [106], usually attributed to the diversion of electrons from the respiratory chain to DOX during its redox cycle with concomitant increase of oxidation. However, is the \textit{ex vivo} effect of DTT on mitochondrial respiration from DOX-treated rats that allows to draw major conclusions since it reverses respiratory state 3 inhibition, supporting the idea that DOX oxidation of thiol groups causes several of the mitochondrial alterations observed [96]. Nevertheless, as has been already pointed out [79], one must have some moderation in mind when analysis and conclusions are inferred from two distinct models of toxicity. \textit{In vitro} effects are mainly due to the direct interaction of the drug with mitochondria and its metabolites and may result from direct production of ROS by drug redox cycling. The changes in bioenergetics observed after the \textit{in vivo} treatment may reflect alterations that are persistent, even after the drug is washed out from the system (memory effect). This may also result, among other possibilities, from genetic alterations.

Exposure to DOX has been described to inhibit several OXPHOS enzymes, including NADH dehydrogenase. Rieske iron-sulfur protein, succinate dehydrogenase, COX, creatine kinase (CK), carnitine palmitoyl transferase, fatty acid β-oxidation-related enzymes as well as the translocation of phosphate and pyruvate to the mitochondrial matrix ([79] and references therein). A proteomic profile of sub-chronic anthracycline-induced cardiotoxicity was recently performed [107]. In order to perform the experiments, authors used the well established rabbit model [108] and chose the parent compound of DOX, daunorubicin, to induce cardiac toxicity. Although interpretations should be taken cautiously, since the degree of toxicity between the two compounds is slightly different [109], we can infer that the most drastic changes found in the study can be shared between both drugs. The authors showed that most of the observed alterations were in proteins related with the cytoskeleton (OxPHOS) and may result from direct production of ROS which are being covered in this review and which are considered typical markers of DOX-toxicity. One limitation of the work is related with the end-stage of the experiment and, therefore, one cannot discern cause from consequence. Nevertheless, daunorubicin caused a tremendous specific increase of desmin in cardiomyocytes which was suggested to act as rescue event for the loss of myofibril stability and for reposition of energy production and consumption sites since proteins related to energy production and channeling (see below) were decreased. Interestingly, desmin abundance was directly proportional to the extension of DOX-induced cardiac dysfunction and increased levels of this protein were detected even in animals without congestive heart failure and thus might be used as another early marker of DOX-toxicity progression. An additional aspect is the dichotomy between cytoplasmic and mitochondrial abundance of SOD, which is increased in the former and decreased in the latter, suggesting the possible compartmentalization of oxidative stress, as suggested by the authors. Supporting this study, another previous work investigated the variation of transcripts in the heart over time after a single treatment with DOX [110].

Contrarily to the increased expression and activity of COX observed in some works, the expression of at least some of its subunits as well as its activity appear diminished in other reports [95], which makes this respiratory complex another possible target during DOX toxicity. Interestingly, an analysis of different metabolic pathways (including OXPHOS) through the use of microwaves and quantitative PCR showed that despite the variety of altered enzymes, the most affected biochemical networks are fatty acids β-oxidation and glycolysis [111]. This result suggests alterations on substrate utilization, an event that is usually associated with myocardial remodeling and the development of cardiac pathologies. In fact, decrease in cardiac fatty acid utilization was already reported in DOX-treated animals, occurring before any functional change [112]. More recently, 13C-isotopomer analysis of isolated perfused hearts from DOX-treated rats showed a metabolic shift from fatty acid oxidation towards glycolysis [113]. In this particular study, the use of the cardioprotector dextrazoxane proved to be capable of reversing the observed shift, shedding some light once again in the role of the imbalance of oxidative stress. Nevertheless, it remains to be elucidated if it is the impairment of the mitochondrial function that results in the metabolic remodeling. If indeed is this the case, the inability of the cardiac tissue to sustain energy production by glycolysis may be due to the fact that DOX treatment results in alterations of glucose intracellular transport and initial metabolism [114]. Following proteomic studies, treatment with anthracyclines induces a decrease in the fatty acid binding protein responsible for the import of such molecule fuels into cardiomycocytes [107]. Such decrease, in addition to the increased abundance of the rate limiting enzyme pyruvate dehydrogenase [107] might explain the metabolic shift observed during DOX-treatment. In addition, the same study attempts to link heart failure to metabolic remodeling since it was also found that anthracyclines decrease the triosephosphate isomerase enzyme important in the glucose metabolism and which was previous found to be sensitive to oxidative stress and associated with cardiomycocytes and mitochondrial degeneration [107].

Another problematic issue identified in cardiomycocytes after exposure to DOX is the interference with the channeling of ATP and phosphocreatine (PCr) from mitochondria to the cytosol, i.e., the energy shuttle between production and consumption sites. The easily diffusible creatine (Cr) and its phosphorylated form PCr act like global or local buffers of ATP/ADP ratios, and are extremely important in heart physiology [115]. The term “channeling” is generally used when the CK is associated with ATP-consuming or -consuming transporters, pumps or enzymes. In the heart, different CK isoenzymes can be found free in the cytosol or bound to sarcoplasmic or mitochondrial membranes. The mitochondrial isosform (mtCK) has high affinity for cardiolipin forming complexes with it and releasing a string of mitochondrial components allowing not only the regeneration of intramitochondrial ADP but also the generation of the diffusible energy molecule PCr from ATP and Cr [115]. As it was mentioned before, DOX competes for cardiolipin binding sites and therefore, mtCK is a very likely target. In fact, \textit{in vitro} assays showed that the drug was not only able to displace the complexes between the enzyme and phospholipids, but it was also capable to cause a direct oxidation of mtCK, namely in its cysteine residues [114]. \textit{In vivo}, DOX also decreases the activity of mtCK and induces a shift of the expression of the isoenzymes towards an embryonic profile, an event that was already observed in several other cardiac pathologies [116]. All these effects are likely to be responsible for the decrease in the cellular PCr pool as assessed by nuclear magnetic resonance spectroscopy [117].

All adverse effects here stated, can drive the cardiomycocyte to undergo signaling resulting into cell death; being post-mitotic cells, cardiomycocytes cannot be regenerated leading to cardiac failure. There are several apoptotic markers present in individuals after DOX treatment which are covered elsewhere [118]. Several studies indicate that the transcription factor p53 is also involved in DOX cardiotoxicity. Targeted disruption of p53, either in knockout mice [119] or by using pifithrin-α, a specific p53 inhibitor on cardiomyoblasts in culture [120, 121] prevents DOX cardiotoxicity. Mitochondrial dysfunction has been attributed to induction of BAX expression and mitochondrial translocation following initial nuclear DOX accumulation and DNA damage in cardiomycoblasts [121, 122]. Furthermore, DOX induces mitochondrial p53 translocation [121] but it is however unclear whether this translocation is involved in the apoptotic process or rather is part of a defense mechanism [123]. DOX-induced cell death also results in depletion of cardiac stem cell pool which is critical for cardioprotection and repair [124].

Lastly, the importance of autophagy in mitochondrial quality control in the heart should also been taken into account due to the
intensive work accomplished in this area in the last decades [46, 125-131]. Autophagy is the process by which the cell seeks to recycle damaged macromolecules or non-functional organelles and therefore promote cell survival. This is particularly important in the cardiac tissue due to its high energy and terminal differentiated state. Nevertheless, autophagy is a two-edged sword because when disproportionate, it can lead to cell death [125], being in fact augmented under certain pathological conditions; however, the precise event responsible for that dichotomy remains to be elucidated. Autophagy might be triggered by starvation, i.e. energy depletion, ROS, accumulation of misfolded/oxidized proteins, mitochondrial depolarization and MPT induction [127-130], all of which are present after DOX treatment. Therefore, it is reasonable to consider that the treatment would activate the autophagic pathway in a protective manner to remove damaged mitochondria but interestingly the few works performed so far show the opposite [132, 133]. Although at this time is difficult to discriminate if autophagy is directly activated by DOX or it is induced as a consequence of mitochondrial dysfunction it arises as another putative pathway for cardiomyocyte death, (in)dependent of apoptosis or necrosis. Kobayashi and co-workers stressed out the role of the heart-specific transcription factor GATA4 in the progression of DOX-toxicity and found that its abundance is inversely proportional to the autophagic flux, which is modulated by the expression of Bcl-2 related genes [132]. However, further investigations should be carried out to prove that the direct or indirect inhibition of the autophagic pathway is actually protective during DOX treatment.

2.5. DOX Toxicity in Other Organs – Why is the Heart More Affected?

Despite all the knowledge acquired over the years, one important doubt remains about DOX toxicity - why is the heart the most affected organ? Obviously, several authors have tried to explain the observed organ selectivity, however, most ideas still remain as simple hypothesis with little practical support. Different drug accumulation in tissues was previously proposed [134]. Apparently, during its systemic circulation, DOX accumulates slowly in the tissues being the liver the organ showing the highest accumulation, perhaps because biliary secretion constitutes the major route of DOX elimination [135]. However, after several DOX administrations, its levels in the cardiac tissue can increase up to 6-fold without changes in the rate of accumulation in other tissues [134]. It could be a specific cellular accumulation or differences in drug efflux as it was pointed earlier in this section. Another possible explanation is that the cardiac tissue apparently shows decreased levels of antioxidant defenses when compared with other organs, e.g. the liver [136]. This would make the heart less capable of dealing with the increased oxidative stress induced by DOX. One author also suggested the existence of a cardio-selective external NADH dehydrogenase, alternative to OXPHOS Complex I and similar to those found in plants [86]. The presence of this enzyme in the outer leaflet of the inner mitochondrial membrane would exacerbate the DOX redox cycle. Also, the fact that myocardial tissue possesses a higher mitochondrial content [137] due to higher dependence on mitochondrial ATP could also contribute to enhanced DOX activation. Finally, one could point to the fact that different tissues possess different energy demands and therefore different dependence on mitochondrial bioenergetics. In addition, some tissues can present higher reserves of certain important proteins for mitochondrial function, being consequently less sensitive to damage or decline in expression of those same proteins or respiratory complex [138]. This idea is usually presented to explain the severity of certain mitochondrial hereditary disorders in specific tissues but it might also be applied to DOX toxicity.

Despite the fact that the heart is particularly affected by DOX, other organs do present signs of DOX toxicity as well. As has previously reviewed [67], DOX also affects the liver, kidney and brain although it must be stressed that DOX itself cannot cross the blood-brain barrier. Therefore, DOX-induced effects in the brain are probably indirect and mediated through cytokines, mainly tumor necrosis factor-alpha (TNF-α). The role of the cytokine on brain injury gains further ground when treatment is accompanied by an anti-TNF-α, decreasing the high levels of the circulating cytokine usually observed in patients and preventing brain damage [67]. In the brain, DOX indirectly induces an increase in oxidative stress seen as increased levels of malondialdehyde, thiobarbituric acid reactive substances (TBARS) and protein carbonyl groups [67]. Once more, the mechanism underlying DOX-induced toxicity appears to be related to the stimulation of ROS production. Mitochondria fractions isolated from this tissue also present increased sensitivity to the MPT, a phenomenon clearly modulated by the redox balance of the organelle, supporting the previous idea [139].

During DOX treatment, liver tissue accumulates high amounts of DOX and its metabolites, since it is one of the principal organs responsible for detoxification in the organism. The liver is also responsible for drug clearance since bile is the major route for drug elimination. Certainly is not a surprise that about 40% of patients present signs of liver failure after treatment [67]. Besides inducing changes in the expression of antioxidant enzymes and decreases the energy charge of hepatocytes, DOX also increases the basal production of ROS since one can find several other NADPH-oxidoreductases in the tissue [67]. As it was observed in the heart, mtDNA from hepatic mitochondria is oxidized after DOX treatment although in a reduced manner when compared to cardiac mitochondria [140]. Other effects on mitochondrial function include a slight decrease in the respiratory control ratio (RCR) and decrease calcium loading capacity which contributes to the observed mitochondrial vacuolization and swelling pattern in histology [67, 141].

Recently, our lab has found that sub-chronic treatment with DOX does not induce alterations in the levels of apoptotic and mitochondrial membrane permeabilization proteins in the lung but exhibit markers of lipid peroxidation, however if it is a direct effect of the drug or rather an indirect effect as observed in the brain tissue remains to be elucidated [142]. Nephrotoxicity sometimes accompanies the treatment and its cause is probably related to apoptosis of glomerular podocytes [67]. If the extent of injury is mild to high, the kidneys filtration process is likely to be affected and proteinuria can be observed. One of the mechanisms proposed for renal toxicity is the formation of iron-DOX complexes, i.e., increased ROS production which will cause mtDNA damage and the production of a defective respiratory chain [67].

2.6. Prevention of DOX-Induced Cardiotoxicity

As the cure for DOX-induced cardiac heart failure is not presently available, prevention remains the best therapeutic. The idea is not only to counteract DOX-induced toxicity but to increase the therapeutic efficacy of the drug either by allowing the use of higher dosage (>500 mg/m²) or to decrease the toxic response without interfering with the antineoplastic activity. However, the benefits from all preventive approaches currently available are limited. Nevertheless, a preventive approach can be achieved either by pharmacological or non- pharmaco- logically means. One obvious approach is achieved by limiting the cumulative dose of DOX to less than 450 mg/m² or by decreasing the drug peak plasma results in a reduction of the toxicity. A precise schedule for drug administration and/or choosing a continuous bolus infusion over a single infusion has proven to attain less damage to the heart and therefore decrease the probability of heart failure since both strategies can decrease drug concentration in the plasma [82, 98].

Alternatively, new anthracyclines analogs that have the same or better therapeutically effect but lack the toxic activity has been proposed. The rationale behind this idea goes back to late 1960s, since DOX is derived from its parent compound, daunorubicin, by the substitution of a hydrogen atom by a hydroxyl group, increasing the therapeutic efficacy (Fig. 1) [59]. Due to the tetracyclic ring and
amino-sugar moiety, several modifications can be made. However, from the almost 2000 analogues investigated in the last 4 decades, only a few have reached clinical approval [82, 98].

Use of liposome-encapsulated or tumor-targeted delivery of DOX is probably one of the most investigated strategies in the past decade [143]. The fact that vasculature around tumors is more permeable, i.e., higher fenestration, allows for higher accumulation of liposomes at the tumor site, therefore decreasing systemic drug concentration and improving the targeting of the treatment. Liposomal formulations of DOX have proven improved efficacy over free DOX and are currently used in the clinic [70, 82, 98].

If no plausible replacement is obtained for DOX, one sounding strategy is to spare the non-target organ by co-administering DOX with compounds capable of prevent side effects, e.g. by preventing DOX-induced cardiomyopathy. Along the years, strategies used included antioxidants, iron chelators, lipid-lowering drugs and β-blockers. Several antioxidants either naturally occurring or synthetic have been co-administered with DOX [144, 145]. Whereas some in vitro studies demonstrated positive effects caused by direct antioxidant activity or indirectly, by stimulating the production of natural antioxidant defenses, in vivo results were disappointing [146]. The lack of positive outcome might be due to the poor bioavailability of the protective compounds after oral administration or to a limited availability in the critical sites where DOX exerts its toxic effects, one example being mitochondria. Related to this latter point, one solution, suggested previously [98], would be the specific mitochondrial targeting of such antioxidants. Currently, two similar approaches are proposed to achieve such objective: linking antioxidants to the lipophilic cationic ion tetraphenilphosphonium, e.g., coenzyme Q₁₀ forming the molecule MitoQ [147] or plastoquinone in the case of Skulachev ions [148]. Due to the positively charge nature of these compounds and higher negative-inside membrane potential of mitochondria, the compounds can accumulate up to 200-fold in the matrix. Besides, the natural character of such molecules enables the recycling of the molecule by OXPHOS after scavenging the radical species and, theoretically, the process can continue indefinitely. Regarding DOX-induced oxidative stress, MitoQ was recently reported to confer protection against DOX toxicity [95]. This cationic molecule was capable to prevent alterations on the echocardiographic profile and the extension of fibrosis in cardiac tissue. Moreover, MitoQ restored the expression of COX subunits and therefore the activity of the enzyme [95], although no further end-points of mitochondrial dysfunction were investigated.

Dexrazoxane is currently the best therapeutic approach of choice, reaching significant efficacy in counteracting DOX-associated toxicity [82]. In fact, dexrazoxane is the only approved drug used in co-administration with DOX with the ultimate aim of preventing DOX-induced cardiac damage. The mechanism of action involves its ability to chelate free iron and to displace iron from DOX-iron complexes. Once accumulated into cardiomyocytes, dexrazoxane is hydrolyzed to form an open-ring which has strong chelating characteristics. However, some caution should be taken when using this compound since it can also deplete iron from intracellular stores and interfere with iron transport [149]. Moreover, it possesses myelosuppressive action which can aggravate the same suppressive action of DOX [150].

Another approach would be to decrease the rate of cardiac contraction. The rationale behind this strategy is that by decreasing the energy demand of a myocardium with impaired mitochondrial capacity, one can maintain heart function with a limited mitochondrial supply. β-blockers are commonly used to decrease hypertension and can be used to control cardiomyopathies although some caution should be taken when interpreting results or long treatment periods [151]. Carvedilol is a β-adrenergic receptor antagonist, which has demonstrated efficacy in preventing DOX-induced cardiac toxicity [102]. It has been described that the protective effects of carvedilol against DOX-induced cardiotoxicity go beyond hemodynamic maintenance and directly involve maintenance of mitochondrial function, as we will see briefly.

Finally, reports exist that exercise can prevent DOX-induced cardiac damage [152-154] and specifically protect cardiac mitochondria [99, 155] from the deleterious effects of that anti-neoplastic agent. This subject is explored elsewhere [156].

3. DRUG-INDUCED MITOCHONDRIAL PROTECTION - CARVEDILOL

Carvedilol (Fig. 1), 1-[9H-carbazol-4-yloxy]-3-[2-(2-methoxyphenoxy)ethylamino]prop-2-01 is a non-selective β-blocker with both β₁- and β₂- and α₁-adrenoceptor blocking properties [157] which was initially used in hypertension patients [158]. In addition to vasodilator effects [159] which are probably due to its α-blockade activity, carvedilol also presents antioxidant properties, including the ability to inhibit lipid peroxidation in myocardial cells and the preservation of antioxidant systems [160]. Carvedilol is currently used as an anti-hypertensive drug [157] to manage several cardiac pathologies, e.g. congestive heart failure or angina pectoris [161]. More recently, it has been shown in models of myocardial ischemia-reperfusion that carvedilol can reduce infarct size and the number of apoptotic myocytes in the ischemic area [160] demonstrating the cardioprotective action of the compound in myocardial infarction.

Cardiac dysfunction is often correlated with changes in mitochondrial bioenergetics [162] and since mitochondria has a critical role in myocyte biology several studies about the interaction carvedilol-mitochondria were already performed [39] in order to explain why carvedilol presented a higher protective impact when compared with other similar compounds. Similar β-blockers have the ability to decrease cardiac rhythm or to decrease systemic blood pressure contributing to improvements in after load and offering cardiac protection by the Frank-Starling law theory, nevertheless carvedilol always presented higher protective impact [102, 163, 164]. One rationale to explain the discrepancy lies in the fact that carvedilol is a very lipophilic compound with a high partition coefficient and therefore, can easily cross cell membranes reaching much higher concentration in cytoplasm and even in mitochondria, exerting intracellular responses unrelated with its hemodynamic effects [164].

3.1. Antioxidant Protection of Cardiac Mitochondrial Function by Carvedilol

In several in vitro systems, carvedilol was demonstrated to have intrinsic antioxidant activity. Several mechanisms have been proposed for carvedilol antioxidant activity. Yue et al. [164] demonstrated that carvedilol was able to inhibit iron-mediated lipid peroxidation and α-tocopherol depletion for concentrations much lower than other β-adrenergic antagonists counterparts. The authors proposed a direct free radical scavenging mechanism that was mainly resident in the carbazole moiety and potentiated by the introduction of hydroxyl groups in specific positions of the molecule [164]. This was later confirmed by the same authors by providing evidence that SB 211475 (4-[2-hydroxy-3-[2-(2-methoxyphenoxy)ethylamino]propoxy]-9H-carbazol-3-01), one of carvedilol hydroxylated derivatives, was more effective than carvedilol against iron and hydroxyl-mediated lipid peroxidation, macrophage-induced oxidation of low density lipoproteins and xanthine oxidase/xanthine-related damage to endothelial cells [165]. Aruoma provided specific evidence that carvedilol react rapidly with peroxyl radicals, which provides support for its anti-lipid peroxidation effects [166]. In opposition, it was also proposed that carvedilol antioxidant effect on lipid peroxidation is not due to direct scavenging of free radicals but instead to a chelating effect on Fe³⁺, a species that is a catalyst in the process [167]. An apparent support for both theories was later published [168] and in fact, based on the entire set of data collected on carvedilol antioxidant activity, this seems to be a more reason-
able explanation for the superior antioxidant properties of this β-adrenergic antagonist. The clinical relevance of carvedilol antioxidant effects was confirmed by demonstrating that carvedilol was able to decrease elevated oxidative stress in the failing human myocardium [169]. With this as background, antioxidant protection of mitochondrial function during deleterious events was investigated [170].

As described in the previous section, Nohl et al. suggested the existence of an organo-specific NADH dehydrogenase in heart mitochondria [171]. The oxidation of external NADH promoted by this very controversial and hypothetical [172] NADH dehydrogenase located in the external side of the inner membrane of mitochondria is linked to a high respiratory rate even though no ADP phosphorylation occurs [173]. The enzyme has also been associated with pathological increased ROS production [173]. Oliveira et al. demonstrated that carvedilol inhibits the respiratory activity attributed to the proposed exogenous NADH dehydrogenase in rat heart mitochondria, diminishing its activity and therefore contributing to protect cardiac cells from oxidative damage [173]. Also, protection against cardiac mitochondrial oxidative damage caused by O$_2^*$ production through the xanthine oxidase/hypoxanthine system was achieved by carvedilol [174]. Carvedilol improved mitochondrial respiratory activity in state 3 and was able to prevent the loss of mitochondrial respiratory coupling [174].

As with several other compounds, carvedilol is metabolized after entering the systemic circulation. Between all its metabolites, BM-910228 (Fig. 1) [1,3-dihydroxy-3-(4-oxo-3-(2-methoxyphenoxy-ethyl)amino-propanol-(2)], is one of the most important and results from the addition of a hydroxyl group at the position 3 of the carbazole group [175]. BM-910228 showed a better antioxidant activity, when compared with carvedilol since it is effective at concentrations 30 times lower than the parent compound [175]. In a work performed with hepatic mitochondrial fractions, both compounds inhibited lipid peroxidation and prevented the collapse of transmembranar electric potential [175]. Moreover, no toxic effect was observed on mitochondrial bioenergetics for all the concentrations effective as antioxidants [175]. One can infer therefore that some of carvedilol metabolites, namely BM-910228 further contribute to the antioxidant and therapeutic activity of the drug. Also, the antioxidant and scavenger properties of carvedilol are primarily attributed to the carbazole moiety [164] and as previously mentioned hydroxylation at certain positions of the phenyl ring increases its antioxidant activity.

The antioxidant properties of carvedilol suggest that its hydroxylated metabolites may be responsible for the cardioprotective action in vivo. After administration, carvedilol is distributed to the tissues and the concentration on plasma is low, but yet effective [176]. Keeping this in mind, it is reasonable to think that the amount of drug available is capable of protecting mitochondrial membranes from oxidative injuries, including lipid peroxidation and to prevent deleterious effects. Abreu et al. supported this idea by providing data where it was shown that the toxicity of carvedilol was verified for concentrations above 40 μM [175]. However, such concentrations are not reached in vivo after an oral dose of 50mg since the absolute bioavailability only reaches 24% [176].

### 3.2. Carvedilol Effects on the Respiratory Chain – protonophore vs. Complex I Inhibitor

Several studies investigated the mechanism by which carvedilol causes a mild decrease in mitochondrial transmembrane potential on isolated heart mitochondria [177]. Results suggest that carvedilol depresses mitochondrial membrane potential through a weak protonophoretic mechanism, as demonstrated by the induction of a pH-dependent increased proton permeability without corresponding increasing potassium permeability across the mitochondrial membrane [177]. Accordingly, carvedilol increases state 4 respiration in succinate-energized cardiac, which is a hallmark shared by classical protonophores. However, Monteiro et al. did not obtain such effect when cardiac mitochondria were isolated from hearts perfused with carvedilol and submitted to ischemia/reperfusion [178]. Nevertheless, under these experimental conditions, carvedilol promoted mitochondrial protection against ischemia and reperfusion damage.

Since mitochondrial membrane potential is critical for mitochondrial basic functions, such as being the driving force for the overall ionic fluxes and ATP synthesis, one should ask whether a mild mitochondrial depolarization is beneficial or hazardous. For example, a mild decrease in mitochondrial membrane potential can prevent calcium overload during pathological events, thus reducing the damaging effects of that cation in mitochondria. Also, it has been described that a small decrease in the membrane potential promotes a reduction of ROS produced by the mitochondrial respiratory chain [179], which can result in further antioxidant activity by carvedilol.

In an attempt to analyze the interaction of carvedilol with the components of the OXPHOS system, Cocco et al. performed specific assays in mitochondria isolated from several rat organs [39]. The results obtained showed that carvedilol inhibited respiration supported by NAD-dependent substrates through decreasing the activity of respiratory chain Complex I, inducing toxic effects on mitochondrial function for high concentrations but without apparent tissue selectivity. In an apparent paradox, besides its intrinsic antioxidant activity, carvedilol appears to induce the production of ROS as a consequence of Complex I inhibition [39]. Similar effects were observed in another in vitro model, the cardiomyoblast cell line H9c2, where a consistent decline of the activity of mitochondrial Complex I was observed concomitantly with an increase in mitochondrial H$_2$O$_2$ production and total glutathione and protein thiol content [157]. Nonetheless, the use of an oxidant insult such as H$_2$O$_2$ in cells pre-treated with carvedilol did not result in further cell damage; in fact, carvedilol prevented oxidative damage, suggesting that despite its effects on Complex I, carvedilol can provide some preconditioning mechanism that allows cells to be more resistant to oxidative damage [157].

#### 3.3. Inhibitory Effect of Carvedilol on the Cardiac Mitochondrial Permeability Transition

Carvedilol and BM-910228 were also tested for their in vitro protective effects on heart MPT, due to the role of this phenomenon on cardiac damage [27]. Several experimental protocols were used to induce the MPT in order to investigate the protective effect of carvedilol and its metabolite. Isolated rat heart mitochondria [180] pre-incubated with carvedilol had shown inhibition of mitochondrial thiol groups oxidation induced by calcium/phosphate [180], the golden-standard for MPT induction. It was described that carvedilol had a dual action on mitochondrial calcium accumulation: a negligible effect when calcium concentrations were below the threshold for pore opening and a protective effect when calcium concentration reaches the pore activation threshold level. In this way, carvedilol is proposed to be an inhibitor of the high-conductance state of the MPT pore, acting on mitochondrial swelling and calcium efflux without preventing mitochondrial membrane depolarization or swelling in an ionic media [180]. Later [181, 182] it was described that carvedilol was effective only when MPT was triggered by a primary oxidative process which leads to protein thiol-groups oxidation and consequently induce MPT [181, 182]. To better understand how carvedilol manages to prevent MPT induction, it is important to consider that the drug does not prevent pore opening when the event relies on structural changes of some pore components. In contrast, when primary oxidation of mitochondrial thiol groups occurs, carvedilol is able to exert is protective role, as demonstrated when incubating mitochondria with the oxidative pair calcium/phosphate as opposed to results achieved with carboxyatractysoside, a specific ANT inhibitor and MPT inducer [183]. This idea reinforces once more that the enhanced pro-
tective action of carvedilol is due to its antioxidant activity. Other studies confirm the same effects in events of cardiac stress, such as during cardiac ischemia/reperfusion, where heart mitochondria suffer from increased oxidative injury and accumulation of calcium and phosphate [182]. Moreover, the inhibitory effect of carvedilol decreases MPT-related cytochrome c release, which is relevant in the reduction of apoptosis in myocardium [177, 184]. One particular work demonstrated that carvedilol ameliorates the overload of mitochondrial and cytosolic calcium, an effect that is completely dependent on the antioxidant properties of the compound rather than its adrenergic blocker activity since other β-blockers lacking antioxidant activity, such as metoprolol, had no protective effect [184].

Interestingly, carvedilol, but not its metabolite, prevented membrane depolarization, osmotic swelling and release of matrix calcium when the MPT was induced by chenodeoxycholic acid (CDCA), which strengthens the role of carvedilol in the protection of mitochondrial function [185]. Nevertheless, inhibition of CDCA-induced MPT may not be due to its antioxidant activity, as the hydroxylated metabolite does not offer such protection. Moreover, in this study, one cannot ignore the possible interaction between the bile acid and the drug or even the competition for potential binding sites and the ability of the carvedilol to insert into the membrane causing both protein-protein and lipid-protein disturbance which could ultimately interfere with the kinetic of the MPT pore, as was suggested by the authors [185].

3.4. Non-mitochondrial Targets for Carvedilol in the Heart

So far, several evidences that carvedilol also targets other structures outside mitochondria have been reported. For example, carvedilol and its metabolite attenuated H$_2$O$_2$-mediated decrease in sarcoplasmic reticulum Ca$^{2+}$-ATPase isoform 2 (SERCA2) mRNA and protein levels in rat primary cardiomyocytes. Furthermore, carvedilol significantly enhanced SERCA2 gene transcription, suggesting that this compound specifically restores SERCA2 gene transcription being therefore suggested as a subjacent mechanism of the beneficial effects of carvedilol on cardiac function [186].

Yue et al. explored the cardioprotective effects of carvedilol in the ischemic myocardium and to understand its mechanism of action using rabbits subjected to coronary artery occlusion followed by reperfusion [187]. When carvedilol was administered before reperfusion, a decrease of over 77% in the number of apoptotic myocytes in the ischemic area was observed. Ischemic cardiomyocytes from rabbit hearts revealed an upregulation of Fas protein which carvedilol was able to reduce. The ischemia/reperfusion process induces fast activation of stress-activated protein kinase (SAPK) in the ischemic area and carvedilol was also capable to inhibit the activation of this protein by approximately 50%. In this case, the protective action of carvedilol against ischemia/reperfusion injury is thought to be due to the downregulation of the SAPK signaling pathway, through the inhibition of Fas receptor expression, and by β-adrenergic blockade instead of its antioxidant activity [187].

4. FIGHTING DRUG-INDUCED MITOCHONDRIAL TOXICITY BY USING DRUG-INDUCED MITOCHONDRIAL PROTECTION: CARVEDILOL VS. DOXORUBICIN

The main goal of introducing a cardioprotective substance concomitantly during DOX-chemotherapeutic program is to prevent the deleterious effects of the anti-cancer drug (Fig. 3), especially at the mitochondrial level, with few or no toxic side effects. Carvedilol, with its intrinsic antioxidant activity in addition to its β-blocker properties, was a logical choice in an attempt to prevent DOX cardiac and mitochondrial damage by multiple mechanisms.

An early study by Fazio et al. [188] was the first to demonstrate that carvedilol was able to induce complete recovery in a patient undergoing DOX treatment and used to show signs of congestive heart failure. Following this work, an animal model of DOX-induced cardiac toxicity showed a remarkable protective effect of carvedilol, attributed to its antioxidant and lipid-lowering properties [189]. Nevertheless, it remained to be known whether the protective effect of carvedilol on DOX-induced cardiac toxicity originated from the prevention of cardiac mitochondrial degeneration.

One work by Santos et al. demonstrated that carvedilol protects against cardiac and hepatic mitochondrial bioenergetic dysfunction associated with a sub-chronic DOX toxicity [190]. Co-administration of carvedilol decreased the extent of cellular vacuolization in cardiac myocytes and prevented the inhibitory effect of DOX on mitochondrial respiration in both heart and liver. DOX decreased mitochondrial calcium loading capacity in ~50% and only 10% in liver. Interestingly, co-treatment with carvedilol protected cardiac mitochondria from DOX-induced decrease in that parameter but did not protect liver mitochondria [190].

One logical concern would be that carvedilol, besides preventing DOX mitochondrialopathy, may also decrease the anti-neoplastic activity of DOX. Nevertheless, it was observed that carvedilol reduces P-glycoprotein activity increasing therefore the sensitivity to DOX cytotoxicity in cell cultures of two human breast cell sub-lines [191].

In cultured cardiomyoblast H9c2 cells, carvedilol inhibited free radical production, apoptotic pathways and the hallmarks associated with those events induced by DOX and consequently increased cell viability [163]. However, that effect was specific to carvedilol since atenolol, another β-blocker, did not prevent DOX-induced increase in the BAX:Bcl-2 ratio. Confirming, once more that the cardioprotective effect of carvedilol is a consequence of its antioxidant activity and not to its β-blocking function. The possible toxic effect of carvedilol per se was not confirmed since carvedilol did not increase cell apoptosis.

Further in vivo studies with DOX-treated rats have confirmed that carvedilol is able to prevent cardiac mitochondrialopathy through an antioxidant mechanism [192]. Rats submitted to long-term treatment with DOX present several mitochondrial alterations, including decreased calcium loading capacity [102]. Although both compounds were able to preserve ATP levels in the tissue, only carvedilol was capable to preserve mitochondrial function by preventing the loss of mitochondrial calcium loading capacity and by inhibiting the appearance of protein carbonyl groups. The protective effect of carvedilol was not shared by atenolol, once more demonstrating the importance of carvedilol antioxidant activity [102]. Another common consequence of DOX-induced increase in oxidative stress is lipid peroxidation and once more, carvedilol was able to avoid such harmful occurrence in DOX-co-treated animals as assessed by the levels of TBARS [189].

Soon, clinical studies in humans have started in an attempt to confirm improvement of cardiac function in DOX-treated patients. Kalay et al. [193] performed the first clinical trial with 50 patients undergoing anthracyclines therapy, with half of them receiving carvedilol at the same time. The carvedilol group showed preserved left ventricular diastolic and systolic function, showing cardiac function enhancement. However, further clinical trials need to be performed with larger study groups.

One should apply the acquired knowledge regarding mitochondrial studies to design superior approaches using carvedilol or similar molecules to protect cardiac function without compromising the antitumor activity of DOX.

CONCLUDING REMARKS

The cardiac muscle is highly dependent on ATP production from mitochondria, which also makes this tissue highly susceptible to mitochondrial failure caused by different pathologies or drugs [194-197].
The present review supplies two classical cases of drug-induced cardiac mitochondrial dysfunction and protection, not only demonstrating that organ degeneration can occur due to drug-induced mitochondrial toxicity, but also that pharmacological strategies can guide mitochondria from different tissues is enormous [1, 57, 198] and so, the development of mitochondrial quantitative structure-activity relationship (mito-QSAR) libraries might constitute a useful tool to specifically address which chemical moieties, or structural combinations result in deleterious effects on different mitochondrial targets and also how different selective mitochondrial antioxidants can be designed to accumulate in the sites expected to be disturbed by drug actions. Novel pharmaceutical design, coupled to testing of mitochondrial drug safety in early drug development [2], will allow for the development of a new mitochondrially-safe generation of cardiovascular pharmaceuticals.

**ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ADP</td>
<td>Adenine nucleotide diphosphate</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis-inducing factor</td>
</tr>
<tr>
<td>ANT</td>
<td>Adenine nucleotide translocator</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenine nucleotide triphosphate</td>
</tr>
<tr>
<td>BAX</td>
<td>Bcl-2 associated protein</td>
</tr>
<tr>
<td>BAK</td>
<td>Bcl-2 homologous antagonist killer</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylatedhydroxytoluene</td>
</tr>
<tr>
<td>CDCA</td>
<td>Chenodeoxycholic acid</td>
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<td>CK</td>
<td>Creatine kinase</td>
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</table>
ACKNOWLEDGMENTS

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Doxorubicin, Carvedilol and Cardiac Mitochondria


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Doxorubicin, Carvedilol and Cardiac Mitochondria


Review

Regulation and protection of mitochondrial physiology by sirtuins

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Abstract

The link between sirtuin activity and mitochondrial biology has recently emerged as an important field. This conserved family of NAD⁺-dependent deacetylase proteins has been described to be particularly involved in metabolism and longevity. Recent studies on protein acetylation have uncovered a high number of acetylated mitochondrial proteins indicating that acetylation/deacetylation processes may be important not only for the regulation of mitochondrial homeostasis but also for metabolic dysfunction in the context of various diseases such as metabolic syndrome/diabetes and cancer. The functional involvement of sirtuins as sensors of the redox/nutritional state of mitochondria and their role in mitochondrial protection against stress are hereby described, suggesting that pharmacological manipulation of sirtuins is a viable strategy against several pathologies.

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Abbreviations: Acetyl-CoA, acetyl-coenzyme A; AceCS2, acetyl-coenzyme A synthethase 2; ACC, Acetyl-CoA carboxylase; ADP, adenosine diphosphate; AIF, apoptosis-inducing factor; AMPK, 5′ adenosine monophosphate-activated protein kinase; ANT, adenine nucleotide translocator; ATP, adenosine triphosphate; BER, base excision repair; CPS1, Carbamoyl phosphate synthase; CR, calorie restriction; CREB, cAMP response element-binding; CypD, cyclophilin D; DNA-PK, DNA-dependent protein kinase; DSB, double-strand break; ERK, Estrogen-related receptor alpha; FAO, fatty acid oxidation; FOXO, Forkhead box O; GDH, glutamate dehydrogenase; H4, histone 4; HDL, high-density lipoprotein; HFD, high fat diet; HIF1α, Hypoxia-inducible factor 1, alpha subunit; HMGS2, 3-hydroxy-3-methylglutaryl CoA synthase 2 Km, Michaelis-Menten constant; LCAD, long chain acyl coenzyme A dehydrogenase; MFMs, mouse embryonic fibroblasts; MPP, mitochondrial processing peptidase; mPTP, mitochondrial permeability transition pore; MitoSOD, manganese superoxide dismutase; MEFs, mouse embryonic fibroblasts; NDUFA9, NADH dehydrogenase [ubiquinone]-1 alpha subcomplex subunit; OSCC, oral squamous cell carcinoma; OTC, ornithine transcarbamoylase; PPARγ, peroxisome proliferator-activated receptor; PARP-1, Poly [ADP-ribose] polymerase 1; PGC-1α, peroxisome proliferator-activated receptor gamma coactivator 1-alpha; Pol1, polimerase1; ROS, reactive oxygen species; SIRT, sirtuin; Sir2, Silent Information Regulator Two (Sir2) protein; SOD2, superoxide dismutase 2; TNF, Tumor necrosis factor; UCP2, uncoupling protein 2.

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1. Sirtuins: function and families

1.1. Introduction

The involvement of mitochondria in multiple fundamental cellular processes places these organelles under the highlight of many researchers. In fact, mitochondria are now considered one major target for many therapeutic approaches. Oxidative stress has been a particular fulcrum of interest since mitochondrial oxidative damage has been recognized as being involved in many diseases and in the aging process itself. Also, disrupted mitochondrial metabolism may be one of the critical elements leading to cancer, diabetes, age-associated and neurodegenerative disorders (Campisi and Yaswen, 2009; Harman, 1956; Sultana and Butterfield, 2010; Weber and Reichert, 2010; Zhu and Chu, 2010). In this fascinating area of mitochondrial disease and protection, sirtuins are being specially focused, since these proteins are able to regulate stress responses and cell survival (Canto and Auwerx, 2009; Gan and Mucke, 2008). One of the most intensively investigated compound, resveratrol, is a known sirtuin 1 (SIRT1) activator, shown to have a positive effect on mitochondrial metabolism and to delay the aging process (Alcain and Villalba, 2009; Finkel et al., 2009). The present review comprises our recent knowledge in the field of sirtuins with a special focus on the most recent and breakthrough studies related with the protection and regulation of mitochondrial physiology by SIRT3, SIRT4 and SIRT5. Several other reviews on the subject are available, including the very interesting work by Huang et al.(2010). The present review expands the previous works by incorporating novel and exciting results, which allows the reader to get a full picture of how sirtuins in general, and mitochondrial sirtuins in particular, contribute to cellular and mitochondrial protection.

1.2. Classification of sirtuins

Sirtuins, or Silent information regulator proteins (Sir), and their homologs are present in a very wide range of organisms, from bacteria to humans, forming a conserved family of proteins (Denu, 2005). Up to date, seven sirtuin homologues have been identified in mammalian cells. These have different intracellular localization as well as various roles in cell physiology (Blander and Guarente, 2004; Tanny et al., 1999). Based on the phylogenetic analysis of the core domain, mammalian sirtuins have been classified into four classes together with other Sir2-related proteins widely distributed in eukaryotes and prokaryotes (Frye, 2000; Smith et al., 2000). Mammalian SIRT1 (62.0 kDa), SIRT2 (41.5 kDa) and SIRT3 (43.6 kDa) are considered Class I sirtuins, while SIRT4 (35.2 kDa) is a Class II sirtuin. Class III SIRT5 (33.9 kDa) and Class IV SIRT6 (39.1 kDa) and SIRT7 (44.8 kDa) are other examples. All the described sirtuins contain a conserved 275 amino acid catalytic core domain together with N-terminal and/or C-terminal domains. Additionally, a novel class ("U") has been created to include sirtuins with unique features, such as gram-positive bacteria andTermoga maritima sirtuins. An appropriate Class affiliation of the individual Sir2-related proteins has been described in the comprehensive review article by Michan and Sinclair(2007). A list of the different sirtuins, plus other details on their physiology/activity, can be seen in Table 1.

1.3. Sirtuin enzymatic activity

All sirtuins, with only one exception (SIRT4), catalyze protein deacetylation in which the lysine acetyl group is transferred from the target protein to the ADP-ribose component of NAD⁺, which leads to their full dependence on NAD⁺ availability and indicates that sirtuins can be sensors of the cellular redox state. As a consequence, sirtuin activity leads to the generation of deacetylated proteins, 2′-O-acetyl-ADP ribose and nicotinamide (Sauve, 2010). Moreover, SIRT6 additionally demonstrates ADP-ribosyl transferase activity while SIRT4 (as mentioned earlier) demonstrates ADP-ribose transferase activity only. As described above, sirtuin activity is regulated by NAD⁺ availability. On the other hand, nicotinamide noncompetitively inhibits sirtuins suggesting that the deacetylation reaction product can also act as an endogenous regulator of sirtuin activity. Interestingly, isonicotinamide, which binds to the nicotinamide pocket of yeast sirtuin (Sir2), only inhibits the base exchange, with the deacetylation activity remaining unaffected or even being increased. This fact indicates that chemical compounds such as isonicotinamide can act as potent sirtuin activators in mammalian cells. Additionally, it has been demonstrated that activity of yeast sirtuin Hst2 (a Class I member) can be regulated by homo-oligomerization of the enzyme. Another well known and currently intensively studied sirtuin activator is resveratrol, a polyphenol present in different sources, including for example grapes skin and red wine (Lagouge et al., 2006). It has been demonstrated that polyphenols and especially resveratrol, reduce Km values for sirtuin substrates, resulting in different biological effects as increased resistance to apoptosis and to stress stimuli. Apart from nicotinamide, an inhibitory effect on sirtuins has also been described for several other compounds. The most well-known are sirtinol, splitomicin (specific for yeast sirtuins Hst1 and Sir2) and dehydrospilomicin (specific mostly to sirtuin Hst1). Human sirtuins are not inhibited significantly by splitomicin as well as by dehydrospilomicin. More about the mechanism of protein deacetylation carried out by sirtuins as well as detail on their inhibitors and activators can be found in the review of Sauve et al. (Sauve et al., 2006).

1.4. Sirtuin protein targets

Potential sirtuins substrates are various acetylated proteins involved in cell metabolism, apoptosis and regulation of gene transcription. Sirtuins can thus determine the ability of cells to adapt to different conditions (Finkel et al., 2009; Haigis and Guarente, 2006; Vaquer, 2009). Published data suggest a significant role of deacetylation in metabolic responses to fasting or caloric restriction as well as in the response to different stress stimuli, including for example oxidative stress (Choudhary et al., 2009; Schwer et al., 2009). Among the several proteins which are deacetylated by sirtuins, histones and transcription factors such as p53 (Li et al., 2010), FOXO (Brunet et al., 2004), peroxisome proliferator activated receptor γ (PPARγ) (Picard et al., 2004), nuclear factor-κB (NF-κB) (Kawahara et al., 2009) and PGC-1α (Sugden et al., 2010) can be found. Sirtuins are also able to deacetylate α-tubulin (Tang and Chua, 2008) and acetyl-CoA synthetase (Hallows et al., 2006). Another important protein for metabolism, glutamate dehydrogenase (GDH) is a well-known example of ADP-riboseylation substrates for SIRT4 (Haigis et al., 2006). The variety of sirtuin substrates, either being crucial enzymes or gene regulatory elements, indicates the possibility that multiple sirtuins may modulate cell physiology and metabolism through interacting with distinct targets regulated under a wide range of physiological conditions.

1.5. Tissue specificity and intracellular localization of mammalian sirtuins

Molecular analysis revealed that SIRT1, 2, 3, 5 and 6 are ubiquitously expressed in different tissues and organs. The highest amount of SIRT6 was detected in muscles, brain and heart, while SIRT4 is mostly present in muscle, kidney, testis and liver (Michishita et al., 2005). By its turn, SIRT7 has been found in brain, kidney, liver, lung and adipose tissue (Michishita et al., 2005). Further detailed studies determined subcellular localization of several sirtuins. Among seven mammalian sirtuins, SIRT1 and SIRT2 show both cytoplasmic and nuclear localization, while SIRT6 and SIRT7 are only located in the nucleus and nucleoli, respectively (Michishita et al., 2005). SIRT3, SIRT4 and SIRT5 are mitochondrial proteins, although SIRT3...
<table>
<thead>
<tr>
<th>Class</th>
<th>HDACIII</th>
<th>Sirtuin</th>
<th>Localization</th>
<th>Substrates</th>
<th>Catalytic activity</th>
<th>Function</th>
<th>Modulators</th>
<th>Knockout outcome</th>
<th>References</th>
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<tr>
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<td></td>
<td></td>
<td></td>
<td>Activators</td>
<td>Inhibitors</td>
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</tr>
<tr>
<td>Class I</td>
<td>SIRT1</td>
<td>Cytoplasm, nucleus</td>
<td>PG C1α, eNOS, FOXO, p53, MyoD, NF-κB, histone H3 and H4</td>
<td>NAD⁺-dependent protein deacetylation</td>
<td>Cell survival, insulin signaling, inflammation, metabolism regulation, oxidative stress response, lifespan regulation</td>
<td>Resveratrol, AROS, HIC1, splitomicins, quercetin, dihydropyridine</td>
<td>DBC1, NAD⁺, iso-nicotinamide, Salermide</td>
<td>High pre-natal mortality, metabolic abnormalities, heart and bone defects, shortened lifespan</td>
<td>(Alcain and Villalba, 2009; Aquilano et al., 2010; Bordone et al., 2006, 2007; Borra et al., 2005; Brunet et al., 2004; Canto et al., 2010; Feige et al., 2008)</td>
</tr>
<tr>
<td></td>
<td>SIRT2</td>
<td>Cytoplasm, nucleus</td>
<td>α-tubulin, histone H4</td>
<td>NAD⁺-dependent protein deacetylation</td>
<td>Cell cycle regulation, nervous system development</td>
<td>Dihydropyridine</td>
<td>Sirtinol, NAD⁺, iso-nicotinamide, Salermide</td>
<td>Aberrances during mitosis, defective development</td>
<td>(Garske et al., 2007; Harting and Knoll, 2010; Hiratsuka et al., 2003; Nahhas et al., 2007; Southwood et al., 2007; Wang et al., 2007)</td>
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<tr>
<td></td>
<td>SIRT3</td>
<td>Mitochondria, nucleus</td>
<td>AceCS2, IDH2, ShdhA</td>
<td>NAD⁺-dependent protein deacetylation</td>
<td>Regulation of mitochondrial energetic metabolism</td>
<td>Dihydropyridine</td>
<td>NAD⁺, iso-nicotinamide, dihydrocoumarin</td>
<td>Affected mitochondrial metabolism, respiratory chain complex I inhibition</td>
<td>(Ahuja et al., 2008; Ahhazzazi et al., 2011; Allison and Milner, 2007; Cimen et al., 2010; Someya et al., 2010)</td>
</tr>
<tr>
<td>Class II</td>
<td>SIRT4</td>
<td>Mitochondria</td>
<td>GDH</td>
<td>Mono-ADP-ribosyl transferase</td>
<td>Regulation of mitochondrial energetic metabolism, insulin secretion</td>
<td>NAD⁺, iso-nicotinamide</td>
<td>Increased GDH activity</td>
<td>(Ahuja et al., 2007; Argmann and Auer, 2006; Haigis et al., 2006)</td>
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<tr>
<td>Class III</td>
<td>SIRT5</td>
<td>Mitochondria</td>
<td>Histone H4, CP51, cyt c</td>
<td>NAD⁺-dependent protein deacetylation</td>
<td>Urea cycle regulation, apoptosis</td>
<td>NAD⁺, iso-nicotinamide</td>
<td>Altered mitochondrial metabolism</td>
<td>(Nakagawa et al., 2009; Nakagawa and Guarente, 2009)</td>
<td></td>
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<tr>
<td>Class IV</td>
<td>SIRT6</td>
<td>Nucleus</td>
<td>Histone H3K9</td>
<td>NAD⁺-dependent protein deacetylation, mono-ADP-ribosyl transferase</td>
<td>Genome stability, DNA repair, nutrient-dependent metabolism regulation</td>
<td>NAD⁺, iso-nicotinamide</td>
<td>Chromosomal aberrations, tumorigenesis, premature senescence, decreased rate of mitochondrial respiration</td>
<td>(Kawahara et al., 2009; Mahlknecht et al., 2006b; McCord et al., 2009; Michishita et al., 2008; Mostoslavsky et al., 2006)</td>
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<tr>
<td></td>
<td>SIRT7</td>
<td>Nucleus</td>
<td>RNA pol I, p53</td>
<td>NAD⁺-dependent protein deacetylation</td>
<td>Regulation of rRNA transcription, cell cycle regulation</td>
<td>NAD⁺, iso-nicotinamide</td>
<td>Proliferation arrest, increased rate of apoptosis, heart abnormalities, fibrosis, shortened lifespan</td>
<td>(Ford et al., 2006; Michishita et al., 2005; Vakhrusheva et al., 2008; Voelter-Mahlknecht et al., 2006)</td>
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</table>

Abbreviations: mt, mitochondrial; n, nuclear; HDACIII, histone deacetylases class III; GDH, glutamate dehydrogenase; PG C1α, Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; e-NOS, epithelial nitric oxide synthase; FOXO, forkhead transcription factors; NF-κB, nuclear factor κB; Myo D, Myogenic differentiation 1 factor; CPS1, carbamoyl synthetase 1; cytc, cytochrome c; IDH2, isocitrate dehydrogenase; ShdhA, succinate dehydrogenase flavoprotein; AceCS2, acetylcoenzyme synthetase 2. For more details and references, please refer to the text.
can also be found in the nucleus. (Schwer and Verdin, 2008; Shoba et al., 2009).

1.6. Cytosolic and nuclear sirtuins

SIRT1, the closest ortholog of yeast Sir2, is the most well studied mammalian sirtuin, harboring both cytosolic and nuclear localization (although absent from nucleoli). SIRT1 has multiple functions and its role was confirmed in the regulation of energy metabolism (Yu and Auwerx, 2010), embryonic development (Mayanil et al., 2006; Saunders et al., 2010), myocyte differentiation (Fulco et al., 2003), cell survival and apoptosis (Guo et al., 2010; Yi and Luo, 2010) as well as in the apoptosis of gene transcription by histone deacetylation (Zhang and Kraus, 2010). SIRT1 is also known to mediate the effects of caloric restriction (Gillum et al., in press) and to play an important role in response to different stress stimuli (Leiser and Kaerberlein, 2010). Additionally, SIRT1 may have an impact on the aging rate (Donmez and Guarente, 2010). The nuclear fraction of SIRT1 is also localized to promyelocytic leukemia protein (PML) bodies, where it interacts with the tumor suppressor p53 which carries several acetylation sites. Deacetylation of lysine residues in the p53 protein processed by SIRT1 decreases its transactivation activity, which in turn can suppress apoptosis initiated by DNA damage or oxidative stress (Kume et al., 2006; Sedding, 2008; Yi and Luo, 2010). The effect of SIRT1 on cell survival can be also mediated by FOXO transcription factors that play an important role in cell adaptation to stress conditions including Foxo1, Foxo3a and Foxo4. Under oxidative stress, deacetylation of Foxo3a induces its translocation from cytosol to the nucleus where it forms a complex with SIRT1, regulating the expression of some antioxidant enzymes, such as mitochondrial superoxide dismutase (SOD2) and catalase. At the same time, FOXO3a increases the expression of cell-cycle checkpoint and DNA repair genes, arresting the cell cycle (Brunet et al., 2004; Jacobs et al., 2008; Motta et al., 2004; Wang et al., 2007). FOXO1-mediated inhibition of apoptosis occurs via FOXO4 activity regulation. In transformed epithelial cells, deacetylated FOXO4 suppresses two proapoptotic caspases, caspase-3 and caspase-7, leading to an arrest of apoptosis (Ford et al., 2005; Frescas et al., 2005). In turn, deacetylation of FOXO1 results in the expression of gluconeogenesis genes under control of FOXO1 (Frescas et al., 2005).

Other SIRT1 targets such as PPARγ and PGC-1α are important elements controlling energetic balance, especially fatty acid and glucose metabolism (Michan and Sinclair, 2007; Sugden et al., 2010). SIRT1 content and activity is increased under starvation and caloric restriction (CR) (Chen et al., 2008; Nisoli et al., 2005). Deacetylation of PGC-1α by SIRT1 also stimulates mitochondrial biogenesis. Experiments by using transgenic animals showed that not all effects of CR are mediated by SIRT1 but it is noteworthy that both SIRT1 overexpression and caloric restriction are manifested by lower body weight, reduced cholesterol, glucose and insulin serum level and also by improved physical performance (Chen et al., 2005; Nisoli et al., 2005). Lower fat accumulation in SIRT1-overexpressing animals is associated with PPARγ transcriptional regulation which results in the inhibition of adipogenesis (Picard et al., 2004). In the liver, SIRT1 regulates glycolysis, gluconeogenesis and fatty acid oxidation through PGC-1α deacetylation (Rodgers and Puigserver, 2007). Lipid metabolism (HDIL biogenesis) in the liver is also stimulated by SIRT1-dependent Liver’s X receptor deacetylation (Li et al., 2007). Deacetylation of PGC-1α by SIRT1 also stimulates mitochondrial biogenesis and induces oxidative phosphorylation (Lagouge et al., 2006). Similarly, activation of SIRT1 by resveratrol is connected to enhanced mitochondrial biogenesis and to more efficient metabolism in the skeletal muscle (Aquilano et al., 2010; Chabi et al., 2009). SIRT1 is also found to be involved in insulin secretion in pancreatic β-cells (Chen et al., 2010; Liang et al., 2009). The most convincing proof of concept comes from experiments on SIRT1-overexpressing mice which present more efficient energy metabolism of pancreatic β-cells than wild-type individuals (Bordone et al., 2006). This is caused by the repression of uncoupling protein 2 (UCP2) gene expression by SIRT1, resulting in decreased content of mitochondrial UCP2 in pancreatic β-cells, higher mitochondrial coupling and higher rate of ATP production, which loops to enhanced insulin secretion by pancreatic β-cells (Bordone et al., 2006). SIRT1 knockout animals showed a lower level of ATP in pancreatic β-cells as a result of increased UCP2 level and they exhibited a decrease insulin secretion in the response to glucose. As indicated above, the feedback between mitochondrial ATP and insulin secretion is enhanced in SIRT1-overexpressing mice (Bordone et al., 2007; Chen et al., 2005).

SIRT2 is a mainly cytosolic protein, although a small amount was also detected in the nucleus, where it carries out histone deacetylation. In vitro studies demonstrated that SIRT2, similarly to SIRT1, preferentially deacetylates histone H4 (Vaqueiro et al., 2007). In the cytosol, SIRT2 was found to be associated with microtubules, where it deacetylates α-tubulin (Harting and Knoll, 2010). Based on the observations that SIRT2 increases during the mitotic phase and co-localizes with chromatin during transition from G0 to M phase, it is thought that this sirtuin can be involved in the regulation of the cell cycle (Dryden et al., 2003). More detailed studies on sirtuins showed that SIRT2 plays an important role in glial cells development, microtubule dynamics in oligodendrocytes and maintenance of axonal integrity (Michan and Sinclair, 2007; Tang and Chua, 2008), which makes this isoform an attractive therapeutic target in neurodegenerative diseases (Wu et al., 2010). SIRT2 was also found to be involved in Parkinson’s and Alzheimer’s disease and also in a Huntington’s disease model induced in Drosophila (Pallos et al., 2008). Additionally, the development and progression of many brain tumors are connected with a disturbance in SIRT2 deacetylase activity (Hirotsuka et al., 2003). Thus, many reports describe attempts to modulate SIRT2 activity with the objective of treating several malignancies (Harting and Knoll, 2010).

Less published data exists for SIRT6 and SIRT7. Nonetheless, it has been demonstrated that SIRT6 is similar to SIRT1 regarding the maintenance of genome integrity, since deletion of this protein leads to different chromosomal aberrations, impairs cells proliferation and increases the rate of DNA damage accumulation (Michishita et al., 2008). There is growing evidence indicating that SIRT6 is involved in base excision repair (BER) of single-stranded DNA breaks. Moreover, SIRT6 is directly involved in DNA repair by forming a macromolecular complex with the DNA double-strand break (DSB) repair factor, DNA-PK (DNA-dependent protein kinase) promoting DSB repair. Cells lacking SIRT6 are more sensitive to DNA damaging agents (McCord et al., 2009). Moreover, SIRT6 can be considered also as a regulator of metabolism under variable nutrient availability (Kanfi et al., 2008). Although SIRT6 has weak deacetylase activity, it can deacetylate histone H3K9, which makes SIRT6 involved in the stability of telomeric chromatin structure (Michishita et al., 2008). SIRT6 depletion is associated with premature senescence due to abnormal telomere structure. It was found that interaction of WRN, a factor mutated in human premature aging syndrome ( Werner syndrome), with histone H3K9 requires proper SIRT6 activity (Michishita et al., 2008). By deacetylating histone H3K9, SIRT6 may additionally regulate expression of glycolytic genes, especially because it co-represses transcription of Hif1α, which is crucial for the response to nutrient deprivation. In SIRT6 knockout cells, increased glucose uptake and higher glycolysis rate is followed by a decrease in mitochondrial respiration. All these metabolic changes are associated with a higher Hif1α activity. The regulatory role seems to be important in several pathologic states associated with diabetes and obesity (Zhong et al., 2010). Moreover, SIRT6 has been associated with the production of pro-inflammatory cytokines such as TNF-α by immune cells (Van Gool et al., 2009). Recently, it was described that SIRT6 plays a role in the control of somatic growth and in the prevention of obesity.
by modulating neural chromatin structure and gene activity (Schwer et al., 2010).

SIRT7 was found to regulate transcription of ribosomal genes in the nucleus by interacting and regulating RNA polymerase (Pol1) activity which means that this sirtuin can have an important impact on multiple cellular processes (Ford et al., 2006). In fact, it has been shown that transcription of rRNA directly depends on SIRT7 content (Ford et al., 2006; Mostoslavsky et al., 2006), with the highest SIRT7 level found in blood and CD33+ myeloid bone marrow precursor cells. On the other hand, lower SIRT7 levels were observed in ovaries and mammalian skeletal muscle (Voelter-Mahlknecht et al., 2006). In case of SIRT7 ablation, cells do not proliferate and enter the apoptotic pathway (Ford et al., 2006), as opposed to an increased SIRT7 level, which has been associated with carcinogenesis. In fact, SIRT7 overexpression was found to occur in breast cancer cells (Ashraf et al., 2006). Therefore, the role of SIRT7 in the regulation of cell cycle is important for cell adaptation processes under stress conditions. Interestingly, SIRT7 knockout animals suffer from heart hypertrophy, fibrosis and inflammatory cardiomyopathy and shortened lifespan (Vakhrusheva et al., 2008).

1.7. Mitochondrial sirtuins: characterization and activity

1.7.1. Localization and processing

Among the seven sirtuins already described, three (SIRT3, SIRT4 and SIRT5) are present inside mitochondria, regulating several metabolic pathways through protein deacetylation (Fig. 1). It has been shown that about 1/5 of mitochondrial proteins undergo acetylation, being one of the highest pools in the cell. Proteomic studies of mice liver mitochondria revealed almost 300 lysine residues that can be acetylated in 133 proteins involved in different metabolic pathways, including Krebs and urea cycle, fatty acid beta-oxidation and oxidative phosphorylation (Kim et al., 2006). Lysine acetylation profile of mitochondrial proteins demonstrates some similarities with prokaryotic organisms, which appears to support the symbiotic origin of mitochondria hypothesis (Huang et al., 2010). All three mitochondrial sirtuins are located in the mitochondrial matrix (Huang et al., 2010); however, SIRT5 was also found in the mitochondrial intermembrane space (Nakamura et al., 2008; Schlicker et al., 2008). Interestingly, SIRT5 overexpression does not increase the mitochondrial intermembrane pool of this protein but results instead into a preferential accumulation in the mitochondrial matrix (Nakagawa et al., 2009). There are some controversial data showing that in COS7 cells co-expressing SIRT3 and SIRT5, the 44 kDa isoform of SIRT3 can be found in the nucleus, although its function still waits to be elucidated (Nakamura et al., 2008; Scher et al., 2007). It was speculated that under normal conditions, both a mitochondrial and nuclear localization of SIRT3 is possible, although under stressful conditions, most of the nuclear pool moves to mitochondria (Scher et al., 2007). Mitochondrial sirtuins, especially SIRT3 and SIRT4, are responsible for sensing NAD+/NADH balance, which by being disrupted by several factors may affect cellular redox homeostasis (Yang et al., 2007; Yu and Auwerx, 2009).

All three mitochondrial sirtuins are encoded by nuclear DNA and contain a mitochondrial targeting sequence located on their

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SIRT4 is a 35 kDa protein with an ADP-ribosyltransferase activity present in mitochondrial matrix. SIRT4 transfers ADP-ribose from NAD+ to target proteins. In vitro studies showed no detectable deacetylase activity of SIRT4 but it cannot be excluded that a very specific substrate would have to be present in order to observe some relevant activity (Huang et al., 2010). Similarly to SIRT3, SIRT4 is also processed in the mitochondrial matrix where a 28 amino acid sequence is cleaved when pro-SIRT4 reaches its destination (Schwer et al., 2002).

The 33.0 kDa SIRT5 is present in the mitochondrial matrix, but when overexpressed, it can also be found in the mitochondrial intermembrane space. SIRT5 N-terminal 36 amino acids sequence is immediately cleaved after import into the mitochondrial matrix (Mahlknecht et al., 2006a; Michishita et al., 2005).

1.7.2. Regulation of mitochondrial metabolism by sirtuins

As described above, reversible protein acetylation allows cells to adjust to a changing environment. A key question is why and where do mitochondrial proteins become acetylated? This question has not been answered in detail yet but it has been confirmed that protein acetylation does occur in mitochondria (Guan and Xiong, 2011) and, in fact, it is also suggested that the acetylation mechanism in mitochondrial proteins is different from cytosolic and nuclear proteases (Kim et al., 2006). Studies in knockout SIRT4 and SIRT5 mice showed that there was no change in the acetylation level of mitochondrial proteins, but studies on SIRT3 showed a significant increase in protein acetylation (Huang et al., 2010; Lombard et al., 2007). This finding suggests that SIRT3 seems to play a central role in protein deacetylation in mitochondria. It is known that sirtuins are directly or indirectly involved in many different metabolic pathways including lipid metabolism (Lombard et al., 2010), calorie restriction conditions/insulin uptake (Ahuja et al., 2007; Guarente, 2008; Qiu et al., 2010b), urea cycle (Nakagawa et al., 2009; Nakagawa and Guarente, 2009), glycolysis, gluconeogenesis and Krebs cycle (Huang et al., 2010; Verdin et al., 2010).

Nowadays, major threats to human health are cancer, neurodegenerative diseases, immune dysfunction and, certainly, the metabolic syndrome (Guarente, 2006). In the US, around 32% of adults and 17% of children and adolescents are obese (Elliott and Jirousek, 2008). The human body is not adapted to process the excess of calories. During calorie restriction, animals up-regulate fatty-acid oxidation and switch fuel usage from glucose to fatty-acids. At a cellular level, increased mitochondrial biogenesis in calorie-restricted tissues suggests a tissue-specific increase in metabolic rate of the affected animals (Qiu et al., 2010b). Calorie-restricted animals have low levels of blood insulin in response to limited food intake. Low insulin levels suppress gluconeogenesis and maintain blood glucose levels. As described above, SIRT1 suppresses UCP2 in pancreatic b-cells, which allows for a more efficient ATP production (Bordone et al., 2006). Nevertheless, the full spectrum of calorie restriction response seems to be mitochondrial activation which allows a metabolic adaptation to chronic energy deficit demand (Qiu et al., 2010b). Here is where sirtuins have now the spotlight. Fig. 1 shows a summary of the interplay between mitochondrial and in-house sirtuins.

1.7.2.1. SIRT3 and regulation of mitochondrial metabolism. SIRT3 is a unique mitochondrial sirtuin once it is the only one that was related with extended lifespan in humans (Rose et al., 2003). SIRT3 may be critical in sensing NAD+ levels in the mitochondria since increased NAD+ would trigger a regulatory pathway that would activate SIRT3 leading to the deacetylation of specific targets. It has been demonstrated that mice deficient in SIRT3 present hyper protein acetylation (Lombard et al., 2007), including of the metabolic enzyme glutamate dehydrogenase (GDH) suggesting that this sirtuin may have an important impact in metabolic control (Schlicker et al., 2008). Lombard et al. concluded that SIRT3 deficient mice are metabolically unremarkable under both fed and fasted conditions, including normal thermogenesis (Lombard et al., 2007). Another study demonstrated that SIRT3 was able to deacetylate and activate the mitochondrial enzyme acetyl-CoA synthase 2 (Hallows et al., 2006), an enzyme that catalyzes the formation of Acetyl-CoA from acetate (Hallows et al., 2006; North and Sinclair, 2007). Under ketogenic conditions, such as during calorie restriction period, the liver releases a large amount of acetoacetate to the blood. The heart and the muscles express acetyl-CoA synthase 2 and use acetate in an efficient way as an energy source. Deacetylation of acetyl-CoA synthetase switches on its activity so it is relevant that SIRT3 is indeed activated during CR (Hirshey et al., 2010). A study by Ahn et al. (2008) stressed the importance of NAD+-dependent deacetylation in the regulation of energy homeostasis and also provided evidence for SIRT3-dependent regulation of global mitochondrial function. The authors of this paper showed that SIRT3 is an important regulator of basal ATP levels and observed that SIRT3 can physically interact with at least one of the subunits of complex I, the 39-KDa protein NDUF9, although in a reversible manner (Ahn et al., 2008). Other studies demonstrated that mitochondria from SIRT3−/− animals display a selective inhibition of complex I activity and altered basal ATP content (Ahn et al., 2008). Another target of SIRT3 is also the enzyme isocitrate dehydrogenase 2 (Schlicker et al., 2008), which promotes regeneration of antioxidants and catalyzes a key regulation point of the citric acid cycle. Schlicker C et al. reported that the N- and C-terminal regions of SIRT3 regulate its activity against glutamate dehydrogenase and a peptide substrate, indicating roles of these regions in substrate recognition and sirtuin regulation (Schlicker et al., 2008). Cimen et al. identified that the succinate dehydrogenase flavoprotein (ShdhA) subunit is also another SIRT3 target in the mitochondrial respiratory chain (Cimen et al., 2010). It was demonstrated that ShdhA is highly acetylated in SIRT3 knockout mice and also that the activation of complex II was dependent on SIRT3 both in wild-type mice and in cells over-expressing SIRT3. The regulation of complex II by reversible acetylation is actually an important point of control since it is a crossroad between oxidative phosphorylation and the Krebs cycle, acting on the regulation of metabolism in mammalian mitochondria.

The skeletal muscle is a metabolically active organ and crucial for insulin-mediated disposal and lipid catabolism. Palacios et al. demonstrated that exercise signals can regulate SIRT3 in skeletal muscle, with a dynamic response of that sirtuin to coordinate...
mediated by deacetylation of two critical lysine residues in SOD2, the protective effects of CR against oxidative stress and damage are essential player in enhancing the mitochondrial glutathione antioxidant (Someya et al., 2010). Under caloric restriction conditions, al. relates age-related hearing loss and calorie restriction with this 2010). Although it is not new that under certain conditions SIRT3 dysfunction and cardiac hypertrophy during aging (Hafner et al., 2010). In an elegant study, the authors reported that SIRT3 can regulate the mPTP through deacetylation with cyclophilin D also inducing the detachment of cyclosporin A, a CypD inhibitor. Cardiac myocytes from SIRT3 mice lacking SIRT3 presented hallmarks of fatty-acid oxidation disorders during fasting, including reduced ATP levels and intolerance to cold exposure (Hirsche et al., 2010). This study identified acetylation as a novel regulatory mechanism for mitochondrial fatty acid oxidation and demonstrates that SIRT3 modulates mitochondrial intermediary metabolism and fatty-acid use during fasting. Interestingly, it was also reported that SIRT3 deacetylates cyclophilin D (Shulga et al., 2010), diminishing its activity and inducing its dissociation from the adenine nucleotide translocator (ANT). In addition, SIRT3-induced interaction with cyclophilin D also induces the detachment of hexokinase II from mitochondria. These findings might be important for the role of SIRT3 in the metabolism of cancer cells and their susceptibility to toxicity by foreign agents. Recently, Hafner et al. showed that SIRT3 can regulate the mPTP through the deacetylation of CypD at lysine 166, suppressing age-related cardiac hypertrophy (Hafner et al., 2010). In an elegant study, the authors reported that SIRT3 deacetylates the regulatory component of the mPTP, cyclophilin D (CypD), at lysine 166, which is adjacent to the binding site of cyclosporin A, a CypD inhibitor. Cardiac myocytes from SIRT3 knockout mice showed increased mitochondrial swelling due to the mPTP opening and accelerated signs of cardiac aging including cardiac hypertrophy and fibrosis at 13 months of age (Hafner et al., 2010). All together, the data shows that SIRT3 controls the mPTP and that a loss of SIRT3 promotes mitochondrial alterations resulting in enhanced ROS production and cell dysfunction. The results are also a clear evidence that SIRT3 activity is necessary to prevent mitochondrial dysfunction and cardiac hypertrophy during aging (Hafner et al., 2010). Although it is not new that under certain conditions SIRT3 mediates the reduction of oxidative damage, a study from Someya et al. relates age-related hearing loss and calorie restriction with this topic (Someya et al., 2010). Under caloric restriction conditions, a reduction of oxidative damage in multiple tissues and a decrease of age-related hearing loss in WT mice were observed, as opposed to mice lacking SIRT3. The authors of this study identified SIRT3 as an essential player in enhancing the mitochondrial glutathione antioxidant defense system during caloric restriction and concluded that mitochondrial adaptations and aging retardation in mammals may be dependent on SIRT3 (Someya et al., 2010). Another paper showed that the protective effects of CR against oxidative stress and damage are diminished in mice lacking SIRT3, with the effects of this protein being mediated by deacetylation of two critical lysine residues in SOD2, enhancing its antioxidant activity (Qiu et al., 2010a).

Several other SIRT3 targets involved in metabolism have been recently described. Shimazu et al. identified 3-hydroxy-3-methylglutaryl CoA synthase 2 (HMGCS2) as another SIRT3 deacetylated protein (Shimazu et al., 2010). Mice lacking SIRT3 present a decrease in β-hydroxybutyrate levels during fasting, which can demonstrate that SIRT3 regulates ketone body production during fasting (Shimazu et al., 2010). Also, Hallows et al. identified the urea cycle enzyme ornithine transcarbamoylase (OTC) and other enzymes involved in β-oxidation as likely SIRT3 targets. Fasted mice lacking SIRT3 revealed alterations in β-oxidation and in the urea cycle, demonstrating a direct role of SIRT3 in the regulation of the two important metabolic pathways during CR (Hallows et al., 2011). The results also suggested that under low energy input conditions, SIRT3 modulates mitochondria by promoting amino acid catabolism and β-oxidation (Hallows et al., 2011). Recently, it has been shown that livers from mice maintained on a high fat diet (HFD) exhibited reduced SIRT3 activity, a 3-fold decrease in hepatic NAD⁺ levels, and increased mitochondrial protein oxidation. In contrast, neither SIRT1 nor histone acetyltransferase activities were altered, suggesting SIRT3 as a crucial factor contributing to the observed phenotype (Kendrick et al., 2011). In SIRT3 mice lacking SIRT3, HFD further increased the acetylation status of liver proteins and reduced the activity of respiratory complexes III and IV. This study identified acetylation patterns in liver proteins from HFD-fed mice and the results suggest that SIRT3 is an integral regulator of mitochondrial function, with its depletion resulting in hyperacetylation of critical mitochondrial proteins that protect against liver lipotoxicity under conditions of nutrient excess (Kendrick et al., 2011).

1.7.2.2. SIRT4 and regulation of mitochondrial metabolism. The mitochondrial isoform of SIRT4 does not show detectable deacetylase activity but possesses NAD-dependent mono-ADP-ribosyltransferase activity (Ahuja et al., 2007; Yamamoto et al., 2007). It is known that glutamate dehydrogenase (GDH) is a substrate of SIRT4 (Hagis et al., 2006). By ADP-ribosylation GDH, SIRT4 suppresses its activity and prevents the usage of amino acids as an energy source. During calorie restriction, although NAD⁺ levels are increased in mitochondria, SIRT4 expression is decreased. The decrease in SIRT4 activity results in the activation of GDH and it induces the usage of glutamate and glutamine in order to generate ATP (Hagis et al., 2006). This mechanism is important in pancreatic β-cells of calorie-restricted mice. Due to low blood glucose levels, glucose-stimulated insulin secretion is suppressed. However, the activation of GDH allows amino-acid stimulated insulin secretion and thus maintain basal levels of insulin (Argmann and Auwerx, 2006). GDH can also be deacetylated by SIRT3, although the functional link between acetylation and ADP-ribosylation of this important enzyme is not yet well understood. Also, a new role for mitochondrial SIRT4 in the regulation of insulin secretion was identified, with SIRT4 considered as a protein that negatively regulates insulin secretion (Ahuja et al., 2007). Mass spectrometry analysis of proteins that co-immunoprecipitate with SIRT4 identified insulin-degrading enzyme and the adenine nucleotide translocator isoforms ANT2 and ANT3 (Ahuja et al., 2007). It was demonstrated that depletion of SIRT4 from insulin-producing INS-1E cells results in increased insulin secretion in response to glucose (Ahuja et al., 2007). Nasrin et al. investigated whether the depletion of SIRT4 would enhance liver and muscle metabolism (Nasrin et al., 2010). An increase in gene expression of mitochondrial and fatty acid metabolism enzymes was found in hepatocytes SIRT4 KO cells (Nasrin et al., 2010). Interestingly, it was also found out that SIRT1 mRNA levels and protein expression were increased after SIRT4 knockout (Nasrin et al., 2010). Interestingly, the loss of activity of SIRT4 in primary hepatocytes and in whole liver led to an increased expression of SIRT3. Fatty acid oxidation (FAO) was increased in SIRT4 KO primary hepatocytes and this effect was dependent on SIRT1 (Nasrin et al., 2010). In SIRT4 KO primary myotubes, increased fatty acid oxidation, cellular respiration and pAMPK levels were detected. The findings demonstrate that SIRT4 inhibition increases fat oxidative
capacity in liver and mitochondrial function in skeletal muscle. Taken together, it seems like SIRT4 is a negative regulator of oxidative metabolism, in contrast with the functions of SIRT1 and SIRT3, which enhance the oxidative capacity of tissues (Nasrin et al., 2010).

1.7.2.3. SIRT5 and mitochondrial metabolism. As compared with other sirtuins, there is a lot still to be discovered about SIRT5. For example, it is known that SIRT5 deacetylizes and activates carbomoyl phosphatase synthetase 1 (CPS1), an enzyme that catalyzes the first step of the urea cycle (Gertz and Steegborn, 2010; Michishita et al., 2005; Nakagawa et al., 2009). The expression levels of SIRT5 are unaltered in the urea cycle (Gertz and Steegborn, 2010; Michishita et al., 2005; Nakagawa et al., 2009). This increase in the activity of SIRT5 might regulate the urea cycle during caloric restriction conditions by deacetylation and activation of CPS1. Schilicker et al. reported that SIRT5 did not deacetylate any of the mitochondrial matrix proteins tested (Schlicker et al., 2008). The surprising result was that SIRT5 can deacetylate cytochrome c (Schlicker et al., 2008). By using a mitochondrial import assay, the authors determined that SIRT5 can be translocated not only to the mitochondrial intermembrane space but also to the matrix, indicating that localization might contribute to SIRT5 regulation and substrate selection (Schlicker et al., 2008). In vivo, SIRT5 appears to co-localize with cytochrome c in the intermembrane space. The reversible acetylation of cytochrome c could either affect its function in respiration or in apoptosis, or both. In cerebellar granule neurons overexpressing SIRT5, a pro-apoptotic function for this sirtuin was pointed out (Gertz and Steegborn, 2010). Several other target proteins for SIRT5 are possible due to the intermembrane space localization of the sirtuin. One of them is the apoptosis inducing factor (AIF), which was already reported to be acetylated depending on the feeding status (Gertz and Steegborn, 2010). Further and exciting studies are thus needed to understand the physiological role of SIRT5 as a mitochondrial inter-membrane space deacetylase.

1.7.3. Protection of mitochondrial function by sirtuins

The role of mitochondria in cell physiology and survival, as well as in drug-induced toxicity is well established (Pereira et al., 2009a,b; Sardao et al., 2008). Besides control of mitochondrial metabolism, sirtuins are also involved in the lines of defense of that organelle. In fact, there is actually evidence that at least SIRT3 can protect mitochondria from exogenous and endogenous stresses (Kong et al., 2010; Pillai et al., 2010; Scher et al., 2007; Sundaresan et al., 2008). Mitochondria are an important site of reactive oxygen species (ROS) production in a cell, which is why a tight ROS production by that organelle must be exerted by different mechanisms in order to prevent structural damage and accelerated aging (Guarente, 2008). The role of mitochondrial sirtuins in the protection against mitochondrial damage is now beginning to be clarified. One starting point was the finding that NAD⁺ levels dictate cell survival (Yang et al., 2007). It is well known that one of the major causes for cell death due to genotoxic stress is the hyperactivation of PARP-1 that depletes nuclear and cytosolic NAD⁺ causing the translocation of the AIF from the mitochondrial membrane to the nucleus. Yang et al. identified Nampt as a stress- and nutrient-responsive gene that increases mitochondrial NAD⁺ levels (Yang et al., 2007). It has been demonstrated that increased mitochondrial NAD⁺ levels improve cell survival during genotoxic stress and that this protection is dependent on SIRT3 and SIRT4, which means that Nampt-mediated cell protection requires mitochondrial sirtuins (Yang et al., 2007). Nampt increased SIRT3 activity since Nampt overexpression decreased the acetylation level of Accs2. Allison et al. exploited apoptosis through Bcl-2/p53 regulation and verified a SIRT3 involvement in this process (Allison and Milner, 2007). Bcl-2 and SIRT3 were silenced separately and in combination in human epithelial cancer and non-cancer cells. It was demonstrated that SIRT3 is required for apoptosis under basal conditions, by selective silencing of Bcl-2 in HCT116 human epithelial cancer cells. SIRT3 is dispensable for stress-induced apoptosis in HCT116 human epithelial cancer cells but it is an essential pro-apoptotic mediator for both Bcl-2/p53-regulated apoptosis. Interestingly, SIRT3 functions in JNK2-regulated apoptosis but it is dispensable for both SIRT-1 regulated and stress-induced apoptosis. It is then concluded that SIRT3 is a pro-apoptotic protein that participates in distinct basal apoptotic pathways (Allison and Milner, 2007). Nevertheless, this paper does not really point to a direct link between SIRT3 and protection against apoptosis under stress conditions in contrast to many other papers. For example, it has been shown that SIRT3 protects the mouse heart by blocking the cardiac hypertrophic response (Sundaresan et al., 2009). In cardiomyocytes, SIRT3 prevented cardiac hypertrophy activation by activating the fork head box 03a-dependent (Foxo3a-dependent), anti-oxidant superoxide dismutase and catalase genes and decreasing cellular levels of ROS (Sundaresan et al., 2009). The results demonstrate that SIRT3 is an endogenous negative regulator of cardiac hypertrophy by the suppression of ROS levels. Another study showed that SIRT3 levels are increased under stress not only in mitochondria but also in the nuclei of cardiomyocytes (Sundaresan et al., 2008). This particular paper identified Ku70 as a new SIRT3 target, since SIRT3 physically binds to Ku70 and deacetylates the latter, promoting its interaction with Bax. Under stress, SIRT3 overexpression protects cardiomyocytes by partially preventing the translocation of Bax to mitochondria. This study points an essential role of SIRT3 in the survival of cardiomyocytes under stress conditions. Recently, SIRT3 was pointed out as a tumor-suppressor mitochondrial-localized protein (Kim et al., 2010). In this study, it has been shown that the expression of a single oncogene (Myc or Ras) in SIRT3 knockout MEFs results in in vitro transformation and altered intracellular metabolism. In addition, SIRT3 knockout mice developed ER/PR-positive mammary tumors (Kim et al., 2010). In this work, it is also shown that several human cancer tissues exhibit reduced SIRT3 levels, thus leading the authors to propose SIRT3 as a mitochondrial fidelity protein. In this regard, loss of SIRT3 results in a decrease in the antioxidant defenses, resulting in an increase of ROS production. It is also speculated that a pro-oxidant environment may be permissive for in vivo carcinogenesis. It is known that PCG-1α is an important inducer of detoxifying enzymes, even though the molecular mechanism is not clearly understood. Kong et al. showed that PCG-1α activated the mouse SIRT3 promoter mediated by an estrogen-related receptor binding element (ERRE) (Kong et al., 2010). The knockdown of ERRα reduced the induction of SIRT3 by PCG-1α in C2C12 myotubes. In the same work, it was found out that SIRT3 was crucial for PCG-1α-dependent induction of ROS-detoxifying enzymes and other components of the mitochondrial respiratory chain, such as glutathione peroxidase-1, superoxide dismutase-2, ATP synthase 5c and cytochrome c (Kong et al., 2010). Both overexpression of SIRT3 and PCG-1α decreased basal ROS levels in myotubes. SIRT3 stimulated mitochondrial biogenesis and might provide a novel target for treating ROS-related diseases. Another interesting study focused on SIRT3 capacity of reducing lipid accumulation via AMPK activation in human hepatic cells (Shi et al., 2010). The knockdown of SIRT3 downregulated the phosphorylation of AMPK and acetyl coenzyme A carboxylase (ACC), promoting increased lipid accumulation. It was concluded that the capacity of SIRT3 to activate AMPK is dependent on its deacetylase activity (Shi et al., 2010). A very interesting paper correlates p53, SIRT3 and protection in vitro fertilized mouse against oxidative stress (Kawamura et al., 2010). During pre-implantation development, mitochondrial dysfunction or increased levels of oxidative stress adversely affect the developmental outcome. In this study, it was shown that SIRT3 inactivation increases mitochondrial ROS production leading to p53 up-regulation and alterations in downstream gene expression. The findings indicate that SIRT3 might play a protective role in pre-implantation of embryos under stress conditions during
in vitro fertilization and culture, pointing out a new and interesting future clinical application related with manipulation of SIRT3 and post-implantation success.

Recently, another study involved SIRT3 in oral cancer (Alhazzazi et al., 2011). The authors demonstrated for the first time that SIRT3 is overexpressed in oral squamous cell carcinoma (OSCC) in vitro and in vivo, when compared with other sirtuins. Down-regulation of SIRT3 inhibited cell growth, increased cell sensitivity to chemotherapy and reduced tumor burden in vivo, demonstrating that SIRT3 can be a novel potential therapeutic target for oral cancer (Alhazzazi et al., 2011). Another paper showed that SIRT3-mediated deacetylation of evolutionary conserved lysine 122 in MnSOD results into increased activity in response to stress. SIRT3-knockout results in increased mitochondrial superoxide, formation of a tumor-permissive environment and finally, enhanced mammary carcinogenesis (Tao et al., 2010).

To the best of our knowledge, there is not much information about SIRT4 or even SIRT5 but it seems very likely that both sirtuins can also be important in mitochondrial protection against genotoxic stress suggesting that they can contribute to apoptosis in tumor-suppressive or stress resistant conditions (Verdin et al., 2010). Interestingly, SIRT4 is apparently involved in mitochondrial oxidative metabolism. SIRT4 KO primary hepatocytes and myotubes were used in order to study fatty acid oxidation and oxygen consumption under these conditions (Nasrin et al., 2010). As described above, the findings from this study suggest that SIRT4 is a negative regulator of oxidative metabolism, in contrast with SIRT3 and SIRT1, which means that a functional interplay between different sirtuins must exist in order to coordinate the flow of energy during a given metabolic state. It seems that SIRT4 inhibition may improve hepatic insulin sensitivity via increased fat oxidative capacity and hence may be beneficial for the treatment of type 2 diabetes.

Despite the limited knowledge on in vivo substrates for SIRT5, the contribution of this sirtuin in disease has been suggested. Repetitive elements in its gene structure suggested possible genomic instabilities and malignant diseases (Gertz and Steegborn, 2010). Furthermore, one particular study indicates that SIRT5 is decreased after alcohol exposure in rats, which increased hepatic mRNA expression of FoxO1 and p53 (Lieber et al., 2008). Thus, alcohol consumption compromises nuclear mitochondrial interactions by post-translational modifications which contribute to alteration of mitochondrial biogenesis through the newly discovered decrease in SIRT5. It seems that SIRT5 contributes to liver damage induced through chronic exposure to alcohol but would also be interesting to understand the role of this sirtuin under other stress conditions and understand its role in mitochondrial protection against various stressors. 1.8. Future perspectives: are sirtuins good therapeutic targets?

It is plausible that drugs aimed at modulating sirtuins activity and/or expression may have important consequences in cellular responses to stress and life span. It is extremely important to fully identify and characterize targets of these exciting class of proteins and understand how the manipulation of their enzymatic activity or protein expression can be involved in the protection, not only of mitochondria but also of the entire cell. It seems that sirtuins are strategically positioned along the different organs and cellular compartments and that they may play distinct roles (Schwer and Strubel, 2008). Furthermore, it would be very interesting to understand how sirtuins communicate within each other and explore their communication network code that should in theory, contribute to protect the cell. What do these proteins have in common? How do they interact with each other? Is the stoichiometry of the different sirtuins in mitochondria constant? How does it change there and in other cellular locations upon different stimuli? Many of these questions remain unanswered. Mitochondrial sirtuins might contribute to the decrease of “unhealthy” mitochondria and some of them (e.g. SIRT3) might even be protective against drug-induced toxicity (Scher et al., 2007; Sundaresh et al., 2008, 2009) which is why it is fundamental to understand the mechanism behind sirtuin-mediated mitochondrial protection and how targeting sirtuins can be a valid future therapeutic approach for several different diseases. Although many recent reviews have provided new directions in this field, there is still much to be elucidated. There is still a gap of information regarding SIRT4 and SIRT5 functions that warrants further studies in order to understand the roles of these important players in the regulation and protection of mitochondrial function. This review provided an overall view of sirtuin function with a special relevance on the most recent findings on the function and relevance of mitochondrial sirtuins. Furthermore, the new and exciting data opens a completely new road for novel pharmaceutical applications of inhibitors and inducers of this class of proteins.

Acknowledgments

Work in the authors' laboratory is funded by the Portuguese Foundation for Science and Technology (FCT) (research grant PTDC/SUA-TOX/110952/2009 to Paulo Oliveira). Claudia Pereira is the recipient of a Ph.D. fellowship from the FCT (SFRH/BD/48029/2008). The work was also partially supported by the Polish Ministry of Science and Higher Education under grant NN407 075 137 for Magda Lebiedzidzki and Mariusz R. Wieckowski.

References


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Drug-induced cardiac mitochondrial dysfunction can progressively result into organ degeneration. Classical examples of drug-induced cardiac mitochondrionopathy include nucleoside reverse transcriptase inhibitors, local anesthetics and anthracyclines. Doxorubicin (adriamycin, DOX) is a clear study case as a well known pharmaceutical can lead to progressive degeneration of cardiac mitochondrial function. DOX is a potent anthracycline antineoplastic agent, whose clinical use is limited by a dose-dependent and cumulative cardiotoxicity, with a clear mitochondrial component. In this particular case, protection of cardiac mitochondrial function during DOX treatment appears to be critical for preventing the maintenance of the myocyte bioenergetics.

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FEATURED SPEAKERS

Thomas Force, M.D.
Professor of Medicine and Clinical Director of the Center for Translational Medicine, Thomas Jefferson University

Stephen Furlong, Ph.D.

Paul B. Watkins, M.D.
Director, Hamner-UNC Institute for Drug Safety Sciences, Verne S. Caviness Distinguished Professor of Medicine, University of North Carolina at Chapel Hill

SHORT COURSES
Monday, June 6
- Use of Stem Cells for Safety Screening
- Advanced Topics in Drug Metabolism
- Translating Safety Biomarkers from the Lab to the Clinic
- Addressing Safety Concerns for Biological Drugs

Wednesday, June 8
- Mechanistic Insights into Hepatotoxicity

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WORLD PHARMA CONGRESS CONFERENCE-AT-A-GLANCE

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WPC Short Courses*

MONDAY, JUNE 6 (9 AM - 12 PM)

ANIMAL MODELS OF PAIN: PROGRESS AND CHALLENGES
Due to frustration with translational progress, animal models of pain are currently being reconsidered. This course will cover:
• Implementation of classical models of acute, tonic and chronic pain
• Limitations of these classical models
• Refinement of classical models via a consideration of modulatory factors (sex, genetics, testing environment, social modulation)
• Development of new animal models (e.g., operant methods, spontaneous behaviors)
Course Instructor:
Jeffrey S. Mogil, Ph.D., E.P. Taylor Professor of Pain Studies, McGill University

USE OF STEM CELLS FOR SAFETY SCREENING
The course provides new insights into the use of embryonic and pluripotent stem cells for drug safety testing, especially cardiac safety.
• Differentiation of human stem cells into cardiac myocytes
• Comparison of electrophysiology and pharmacology
• Overcoming technical challenges related to working with stem cells
• Methodologies to maintain and use stem cells for predictive safety testing
Course Instructor:
Craig T. January, M.D., Ph.D., Professor, Medicine and Physiology, Division of Cardiovascular Medicine, University of Wisconsin, Madison

ADVANCED TOPICS IN DRUG METABOLISM
The purpose of this course is to cover advanced topics related to drug metabolism with a focus on newer developments in the field.
• In vitro tools to study drug metabolism
• New biotransformation pathways including some that lead to reactive metabolites
• Evidence linking reactive metabolites and idiosyncratic drug toxicity
• In silico tools to predict metabolism
Course Instructor:
John C. Erve, Ph.D., Investigator III, Analytical Sciences, Novartis Institutes for Biomedical Research

MONDAY, JUNE 6 (2 PM - 5 PM)

TRANSLATING SAFETY BIOMARKERS FROM THE LAB TO THE CLINIC
The course offers a unique and practical perspective for successfully translating the pre-clinical work done for testing and validating safety biomarkers to the clinic.
• Design and implementation of studies to identify new biomarkers
• Designing clinical studies to test and validate biomarkers
• Clinical methodologies for cost-effective and reliable decision-making
• Bridging the gap between pre-clinical and clinical findings
• Practical considerations when using biomarkers in the clinic
• Points to consider for a successful transfer from the lab to the clinic
Course Instructors:
William B. Mattes, Ph.D., DABT, Independent Consultant, PharmPoint Consulting

ADDRESSING SAFETY CONCERNS FOR BIOLOGICAL DRUGS
The course offers guidance from experts in the field on what is being used and looked at for early safety assessments for biological molecules, and how these early predictions are then being applied for clinical testing.
• Overview of challenges pertaining to the safety of biologics
• Tools, markers and assays for early safety predictions
• Assessing immunogenicity and off-target effects
• Regulatory guidelines and their interpretations
• Criteria for determining what needs to be tested and when
Course Instructors:
Lisa M. Plitnick, Ph.D., Senior Investigator, Safety Assessment, Merck & Co., Inc.
Noël Dybdal, Ph.D., D.V.M., Associate Director, Principal Scientist, Safety Assessment, Genentech, Inc.
Vivek Kadambi, Ph.D., Senior Director, Drug Safety Evaluation, Millennium, The Takeda Oncology Company

PRACTICAL APPLICATION OF PLATE-BASED LABEL-FREE BIOSENSORS: GETTING THE BASICS & DETAILS YOU NEED FOR DECISION MAKING
This course will provide the attendee with all the necessary information to select the most appropriate label free method for their work. A wide variety of technologies are available, each with specific advantages depending on the scope of individual projects. This course will walk the attendee through evaluating instrumentation, comparing and selecting methodologies, as well as learning the basics of each technology. All current label free methods will be presented.
• Basic understanding of current technologies
• Evaluating instruments
• Latest applications in biochemical measurements of proteins and small molecules
• Latest applications in cell based assays from target validation through inter/intra cell signaling and cardiac toxicity
• Optimization of your label-free assays
Course Instructor:
Lance Laing, Ph.D., Executive Director, Business Development, ACEA Biosciences

WEDNESDAY, JUNE 8 (6 PM - 9 PM)

MOLECULAR IMAGING IN DRUG DISCOVERY AND DEVELOPMENT: BACK TO BASICS.
This course will provide knowledge needed to choose the appropriate imaging modality for a pre-clinical study and the basic requirements for generation of imaging agents for optical, MR, and nuclear imaging. It will consist of two parts:
• Strengths and limitations of imaging modalities
• Imaging agent design and synthesis
Course Instructors:
Thomas Krucker, Ph.D., Head, Molecular Imaging, Global Imaging Group, Novartis Institutes for Biomedical Research, Inc.
Hisataka Kobayashi, M.D., Ph.D., Chief Scientist, Molecular Imaging Program, NC/NIH
Vania Kenanova, Ph.D., Head, Pre-Clinical PET/SPECT/CT Imaging Laboratory, Novartis Institutes for Biomedical Research, Inc.

MECHANISTIC INSIGHTS INTO HEPATOTOXICITY
The course is designed for both pre-clinical and clinical scientists looking to better understand the mechanisms underlying drug-induced liver injury or DILI, to help in the development of early predictive technologies for hepatotoxicity including mechanism-based assays. It provides an overview of cellular pathways involved in:
• Mitochondrial dysfunction and oxidative stress
• Inflammation
• Excessive generation of reactive metabolites
• Inhibition of bile salt efflux protein and involvement of hepatic transporters in drug-induced hepatotoxicity
Course Instructors:
Dylan P. Hartley, Ph.D., Senior Scientist, Investigative Toxicology, Genentech, Inc.
José E. Manautou, Ph.D., Associate Professor of Toxicology, Department of Pharmaceutical Sciences, University of Connecticut
Robert A. Roth, Ph.D., DABT, Professor, Pharmacology and Toxicology, Director, Graduate Program in Environmental and Integrative Toxicological Sciences, Michigan State University
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

*Separate Registration Required
TUESDAY, JUNE 7

7:45 am Registration and Morning Coffee

EARLY IN VITRO MODELS AND MARKERS FOR CARDIAC SAFETY PREDICTIONS

8:45 Chairperson’s Opening Remarks
Peter Hoffmann, M.D., Ph.D., Executive Director, Pre-Clinical Safety, Novartis Institutes for BioMedical Research

8:55 Expanding in vitro Biochemical and Cellular Models for Earlier Drug Safety Assessment
Mary Ellen Cvic, Ph.D., Principal Scientist, Lead Evaluation, Molecular Sciences and Candidate Optimization, Bristol-Myers Squibb Co.
We have developed a high-throughput in vitro assay panel including target classes such as GPCRs, kinases, transporters, ion channels, and nuclear hormone receptors to determine which critical targets can be used to best flag potential cardiac liability issues before compounds advance to in vivo or late-stage drug safety evaluation. We have investigated which in vitro assays offer more sensitive and physiologically-relevant read-outs for assessing compound safety profiles and have identified current gaps in harnessing state-of-art technology platforms. We can now address how to establish a comprehensive pharmacologic liability tool kit for structure liability relationship studies and how to build connectivity between cause and effect.

9:25 Stem Cell Cardiomyocyte Screening
Craig T. January, M.D., Ph.D., Professor, Medicine and Physiology, Division of Cardiovascular Medicine, University of Wisconsin, Madison

9:55 Networking Coffee Break

10:25 Development of Translatable Biomarkers for Cardiovascular Safety
Jennifer Colangelo, Ph.D., Associate Director, Drug Safety R&D, Pfizer Global Research and Development
Safety biomarkers are an integral part of the decision-making process for drug development at all stages, aiding in compound selection for early pre-clinical studies and ensuring patient safety in clinical trials. Mass spectrometry is one of the critical tools for safety biomarker discovery, development and deployment, providing assays that are often easily translatable between species. This presentation will provide examples of mass spectrometry applications to the development of biomarkers for drug-induced cardiovascular toxicity, including MS-based metabolomics for biomarker discovery and stable label approaches for the quantitation of novel proteins.

10:55 Pre-Clinical Strategies for De-Risking the Potential of Cardiovascular Toxicity
Peter Hoffmann, M.D., Ph.D., Executive Director, Pre-Clinical Safety and Co-Chair, Translational Cardiovascular Advisory Team, Novartis Institutes for BioMedical Research
The introduction of in vitro and in vivo cardiovascular safety tests as suggested by guidelines S7A and S7B was successful in preventing acute and catastrophic effects in Phase 1 studies, primarily in healthy male volunteers. On the contrary, recent experiences show that the manifestation of human cardiovascular adverse effects during late stage clinical development or post-marketing is poorly predicted. The presentation summarizes current status and emerging trends of preclinical strategies for de-risking the potential of cardiovascular toxicity in the target population, e.g., diabetic patients.

11:25 Biologics and Cardiac Toxicity Risk: Relating Toxicity to Mechanism of Action
Noël Dybdal, Ph.D., D.V.M., D.A.C.V.P, Associate Director, Principal Scientist, Safety Assessment, Genentech, Inc.
High molecular weight biological therapeutics in general and monoclonal antibodies specifically are highly targeted in their activity and risk of off-target toxicity is low. Adverse cardiovascular effects associated with these drugs to-date result from on-target pharmacology and relate to their mechanism of action. Species specificity of biologics presents challenges that limit the extent to which cardiotoxicity risk can be assessed preclinically. However, novel approaches including in vitro strategies are increasingly offering better opportunities for focused safety assessments. This presentation will include case studies as examples of preclinical cardiac toxicity assessments for biologics of various types.

11:55 Luncheon Presentation
Speaker to be Announced

12:25 pm Luncheon Presentation II
(Sponsorship Opportunity Available)

CHALLENGES CORRELATING IN VITRO AND IN VIVO DATA

1:30 Chairperson’s Remarks
Vivek Kadambi, Ph.D., Senior Director, Drug Safety Evaluation, Millennium, The Takeda Oncology Company

1:35 Evolving Trends in Pre-Clinical Cardiovascular Safety: Gazing into the Crystal Ball
Gary Gintant, Ph.D., Senior Group Leader, Integrative Pharmacology, Global Pharmaceutical Research & Development, Abbott Laboratories
Increasing emphasis on the safety of novel drug candidates demands more efficient discovery and development efforts with a balanced focus on risk/benefit considerations. This goal will be realized only with a) early “frontloading” of appropriate assays to derisk compounds early in discovery, b) an understanding of the strengths and limitations of present (and evolving) assays, and c) an appreciation of present and emerging cardiac safety issues within the context of drug efficacy in development. This presentation will provide some instructive preclinical examples of where we have succeeded present-day gaps in our understanding, and future challenges for pre-clinical cardiac safety.

2:05 Does it Help or Hurt to Know Cardiovascular Biology?
Douglas B. Sawyer, M.D., Ph.D., Lisa M. Jacobson Professor of Medicine and Chief, Cardiovascular Division, Vanderbilt University Medical Center
It is important to search for potential risks of drugs in subjects possessing the clinical substrates for which they are indicated. Heart failure, ventricular hypertrophy, and diabetes are present in many persons manifesting drug toxicity, therefore it appears reasonable to search for potential toxicity in animals afflicted with them. Furthermore it is essential to interrogate all physiological parameters that if affected by a drug may translate to morbidity and/or mortality. In models of rabbits and dogs possessing hypertrophy and heart failure, sensitivity to detect drug toxicity is improved dramatically over studies conducted in normals, and specificity does not appear to be reduced significantly.

2:35 You Don’t Give Drugs to Normal People, so Why Search for Toxicity in Normal Animals?
Robert Hamlin, Ph.D., Professor, Veterinary Biosciences, Ohio State University

3:05 Sponsored Presentations (Opportunities Available)

3:35 Grand Opening Refreshment Break in the Exhibit Hall
Despite intense interest in strategies to predict which tyrosine kinase inhibitor (TKI) cancer therapeutics may be associated with cardiotoxicity, current approaches are inadequate. Herein I will explore a variety of pre-clinical models, starting with “best guess” approaches (virtual cardiotoxicity) based on what we do, and do not know about the role of protein kinases in maintaining homeostasis in the heart. I will also discuss various pre-clinical models, highlighting pros and cons of each. Finally, I will focus on the zebrafish as a pre-clinical model, and present some recent data suggesting it may be a viable tool for prediction and for defining mechanisms of cardiotoxicity.

5:05 PANEL DISCUSSION: Minimizing the Disconnect Between the In Vitro and In Vivo Worlds
Moderator: Vivek Kadambi, Ph.D., Senior Director, Drug Safety Evaluation, Millennium, The Takeda Oncology Company

5:35 - 6:30 Happy Hour in the Exhibit Hall

WEDNESDAY, JUNE 8

7:30 am Continental Breakfast Breakout Discussions

7:30: Chairperson’s Remarks
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

8:30 Breakout Discussion Topics:
• Trends in Safety Screening for Small Molecule Drugs Versus Biologics
• New Approaches and Insights for Early Pre-Clinical Safety Testing

8:40 INTRODUCTION TO MITOCHONDRIAL INVOLVEMENT IN CARDIAC, RENAL AND LIVER TOXICITY

0:40: Chairperson’s Remarks
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

8:40 INTRODUCTION TO MITOCHONDRIAL INVOLVEMENT IN CARDIAC, RENAL AND LIVER TOXICITY

(Joint session for Cardiotoxicity and Nephrotoxicity tracks)

9:00 Breakout Discussion Topics:
• Trends in Safety Screening for Small Molecule Drugs Versus Biologics
• New Approaches and Insights for Early Pre-Clinical Safety Testing

9:10 TALES OF BROKEN MITOCHONDRIA: DRUG-INDUCED CARDIAC MITOCHONDRIONOPATHY
Paulo Oliveira, Ph.D., Group Leader, Mitochondrial Toxicology and Disease, Center for Neuroscience and Cell Biology, University of Coimbra, Portugal and Visiting Research Associate, University of Minnesota Medical School

Drug-induced cardiac mitochondrial dysfunction can progressively result in organ degeneration. Classical examples of drug-induced cardiac mitochondrialopathy include nucleoside reverse transcriptase inhibitors, local anesthetics and anthracyclines. Doxorubicin (adriamycin, DOX) is a clear case study of a well known pharmaceutical that can lead to progressive degeneration of cardiac mitochondrial function. DOX is a potent anthracycline anti-neoplastic agent, whose clinical use is limited by a dose-dependent and cumulative cardiotoxicity, with a clear mitochondrial component. In this case, protection of cardiac mitochondrial function during DOX treatment appears to be critical for preventing the maintenance of the myocyte bioenergetics.

9:40 SPONSORED PRESENTATIONS (Opportunities Available)

10:10 NETWORKING COFFEE BREAK IN THE EXHIBIT HALL

10:50 MITOCHONDRIAL HOMEOSTASIS IN ACUTE KIDNEY INJURY
Rick Schnellmann, Ph.D., Professor, Chair, Pharmaceutical and Biomedical Sciences, South Carolina College of Pharmacy

Mitochondrial damage is a major contributor to the initiation of tubular cell injury and the progression of acute kidney injury (AKI) produced by drugs, toxicants, and ischemia. To understand the role of mitochondria in organ damage and repair, we think that mitochondria need to be examined holistically by measuring mitochondrial homeostasis. This includes changes in mitochondrial loss, fission/fusion, mitophagy, and biogenesis over time. Using this approach, temporal differences in mitochondrial loss, dynamics and biogenesis were observed with mitochondrial loss occurring early and changes in mitochondrial fission/fusion and biogenesis occurring later after AKI.

11:20 MECHANISTIC INSIGHTS INTO MITOCHONDRIAL-BASED ORGAN TOXICITY
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

Previous speakers have elucidated in detail on the contribution of mitochondrial impairment to different organ toxicities. It is apparent that in order to avoid late stage attrition due to mitochondrial toxicity, early predictive screens need to be deployed early in the drug discovery process. Here I will show organelle and cell-based HTS applicable screens to detect such liabilities. I will describe the screens using examples from different drug classes such as antidiabetics/antilipidemics, antivirals, antibiotics, and NSAIDs.

11:50 PANEL DISCUSSION: STRATEGIES FOR ASSESSING MITOCHONDRIAL INVOLVEMENT IN DRUG-INDUCED ORGAN TOXICITIES
Moderator: Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

12:20 PM END OF CONFERENCE

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1:55 Chairperson’s Opening Remarks
Arie Regev, M.D., Hepatology Consultant and Chair, Liver and GI Safety Committee, Global Patient Safety, Eli Lilly and Company

2:00 How Good are Currently Available Biomarkers for Idiosyncratic DILI, and Which New Biomarkers Should We Look for?
Arie Regev, M.D., Chair, Liver and GI Safety Advisory Committee, Global Patient Safety, Eli Lilly and Company; Adjunct Associate Professor of Medicine, Indiana University School of Medicine

Efforts to develop new biomarkers for IDILI have been largely unsuccessful due to limitations such as, incomplete understanding of the mechanisms, lack of effective in-vitro models, absence of universally accepted animal models, and often, lacking scientific exchange between clinicians and basic science researchers. One important prerequisite for the facilitation of new IDILI biomarkers is enhanced understanding on the types of questions that can and should be addressed. This presentation will outline pertinent clinical issues that could theoretically be addressed by specific diagnostic or predictive biomarkers.

2:30 FEATURED PRESENTATION: Genetic Basis of Susceptibility to DILI
Paul B. Watkins, M.D., Director, Hamner-UNC Institute for Drug Safety Sciences, Verne S. Caviness Distinguished Professor of Medicine, University of North Carolina at Chapel Hill

Over the last several years, some pharmaceutical companies have successfully used genome wide association (GWA) to find genetic susceptibility loci for hepatotoxicity observed in clinical trials. In one case, this approach has lead to a proposal to reintroduce with routine genetic testing a drug that has been withdrawn from worldwide markets due to DILI. Genotyping data from the largest DILI genebanks (the International Severe Adverse Events Consortium and the U.S. based Drug Induced Liver Injury Network) have been recently pooled. GWA analysis of this dataset, which reflects DILI due to over 200 different drugs, is providing fresh insight into mechanisms underlying DILI and should inform the hunt for epigenetic and environmental factors underlying DILI.

3:00 Sponsored Presentations (Opportunities Available)

3:30 Networking Refreshment Break in the Exhibit Hall

4:30 Pediatric Drug Induced Liver Injury- Children Are Not Just Small Adults
William Salminen, Ph.D., DABT, Director, Center for Hepatotoxicity, U.S. FDA, National Center for Toxicological Research
Children are not simply small adults and it follows that children may exhibit differential sensitivity to drug-induced adverse events. This also applies to drug-induced liver injury (DILI). As an embryo develops, leading to the birth of a child, and eventually maturation into an adult, the human body goes through many different development phases. Various factors involved with the developmental phases may make the developing human more or less susceptible to DILI when compared to adults. This presentation will review the major developmental phases of the maturing liver with an emphasis on phases that may pose unique sensitivities to DILI.

5:00 PANEL DISCUSSION: Early Prediction of Idiosyncratic DILI: Recent Progress and Interesting Trends
Moderator: Arie Regev, M.D., Hepatology Consultant and Chair, Liver and GI Safety Committee, Global Patient Safety, Eli Lilly and Company

5:30 End of Day

THURSDAY, JUNE 9

7:20 am Continental Breakfast Breakout Discussions
Breakout Discussion Topics:
- Utilization of Appropriate Models and Markers for Predicting Liver Injury
- Promising in vitro Tools for Early Pre-Clinical Testing
- Challenges with Predicting and Monitoring Liver Injury in the Clinic

EFFECTIVE USE OF MODELS AND BIOMARKERS FOR PREDICTING LIVER INJURY

8:20 Chairperson’s Remarks
Robert A. Roth, Ph.D., DABT, Professor, Pharmacology and Toxicology, Director, Graduate Program in Environmental and Integrative Toxicological Sciences, Michigan State University

8:30 DILI-sim: An in silico Approach to Understanding and Predicting Drug-Induced Liver Injury
Brett A. Howell, Ph.D., Research Scientist, The Hamner-University of North Carolina Institute for Drug Safety Sciences

Drug-induced liver injury (DILI) is the adverse drug event that most frequently leads to termination of clinical development programs and regulatory actions on drugs. A predictive model has been developed based on physiological processes involved in DILI. The model initially focuses on acetaminophen and includes multiple scales, spanning from the organ/tissue level to the molecular and cellular levels. The model accurately reproduced acetaminophen pharmacokinetic and other measures for rats, mice, and humans. The use of N-acetyl-cysteine (NAC) as a treatment for acetaminophen overdose was analyzed to predict optimal use. Finally, the model successfully predicted the species differences in hepatotoxicity of methapyrilene using in vitro to in vivo extrapolation.

9:00 Virtual Liver: Integrating in vitro and in vivo Data to Predict Chemical-induced Toxicity
Imran Shah, Ph.D., Head, Computational Systems Biology, Natl .Ctr. for Computational Toxicology, U.S. Environmental Protection Agency

It is difficult to assess the health impact of long-term exposure to low levels of contaminants from animal studies. Current methods for testing the toxicity of a single chemical can cost millions of dollars, take up to two years and sacrifice thousands of animals. In vitro models offer a more efficient and humane alternative, however, translating chemical-induced molecular changes in cell cultures to clinical outcomes remains an open problem. This talk will explain how the EPA Virtual Liver aims to bridge in vitro data from the EPA ToxCast Project to in vivo effects through a novel cellular systems model of hepatic tissues. This abstract does not necessarily reflect US EPA policy.

9:30 Inflammatory Stress Responses and Animal Models for Idiosyncratic DILI
Robert A. Roth, Ph.D., DABT, Professor, Pharmacology and Toxicology, Director, Graduate Program in Environmental and Integrative Toxicological Sciences, Michigan State University

A modest inflammatory stress in animal models can render the liver sensitive to injury from numerous drugs that cause idiosyncratic toxicity in humans. This enhanced sensitivity is associated with expression of proinflammatory cytokines, other mediators of inflammation and with enhanced sensitivity of hepatocytes to their damaging influence. In particular, prolonged generation of tumor necrosis factor-alpha (TNF) appears to be important for the development of injury. Understanding mechanisms of drug-inflammation...
interactions could lead to models to predict preclinically the potential of some drug candidates to cause idiosyncratic liver injury in humans.

10:00 Networking Coffee Break in the Exhibit Hall

MECHANISMS UNDERLYING HEPATOTOXICITY

10:45 Prediction of Immune-Mediated Drug-Induced Liver Injury in Pre-Clinical Drug Development
Tsunoshi Yokoi, Ph.D., Professor, Drug Metabolism and Toxicology, Kanazawa University

Drug-induced liver injury (DILI) is a major problem in drug development and clinical drug therapy. The pathogenesis of DILI usually involves the participation of the parent drug or metabolites that either directly affect the cell biochemistry or elicit an immune response. However, in most cases the mechanisms are still unknown, thus it is difficult to predict and prevent these reactions. Recently, we demonstrated that halothane- and alpha-naphthylisothiocyanate (ANIT)-induced liver injury is mediated by interleukin-17 in mice, and carbamazepine-induced liver injury also mediated by IL-17. Dicloxacillin-, methimazole-, and flutamide-induced liver injury is mediated by IL-4. Continued advances in our understanding of immune-mediated DILI will lead to earlier prediction of hepatotoxic potential of drug under development.

11:15 Hepatoprotective Effect of Peroxisome Proliferators is Associated with Induction of Vanin-1 Gene Expression
José E. Manautou, Ph.D., Associate Professor of Toxicology, Pharmaceutical Sciences, University of Connecticut

Clofibrate (CBF) is a peroxisome proliferator, hypolipidemic drug that affords protection against acetaminophen (APAP) hepatotoxicity. The mechanism of this protection is still unknown but thought to be dependent on peroxisome proliferator-activated receptor alpha (PPARα) function. A gene array analysis revealed that the expression of Vanin 1 (Vnn1) is greatly increased in animals exhibiting CFB-mediated resistance to APAP toxicity. In this presentation in vivo and in vitro approaches to examine the role of Vnn1 in APAP hepatotoxicity and hepatoprotection by CFB, and for the development of new therapeutics approaches to minimize acute liver failure produced by APAP overdose will be discussed.

11:45 Luncheon Presentations (Sponsorship Opportunities Available) or Lunch on Your Own

EARLY PRE-CLINICAL PREDICTIONS OF LIVER INJURY

(Joint session for Hepatotoxicity and ADME/DMPK tracks)

1:15 pm Chairperson’s Remarks
Eric Blomme, D.V.M., Ph.D., D.A.C.V.P, Sr Project Leader, Abbott Labs

1:25 Current Toolbox for the Prediction of Hepatotoxicity
Eric Blomme, D.V.M., Ph.D., D.A.C.V.P, Senior Project Leader, Abbott Laboratories

Hepatotoxicity represents an important cause of failure in drug discovery and development, and improved approaches to predict and characterize this toxicity could significantly impact pharmaceutical R&D. This presentation will provide an overview of the current status of several technologies used to improve hepatotoxicity prediction during drug discovery. Examples will be used to illustrate the strengths, limitations and optimal application of these technologies during lead optimization and candidate selection.

1:55 In vitro Strategies: High Content Mechanistic Screening, Mitochondrial Toxicity, and Transporter Assessment
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

In recent years we have developed several assays that can be deployed early in the drug discovery process. These include 1. An oxygen consumption assay for mitochondrial toxicity, 2. Several assays for ERs stress and 3. A High Content Mechanistic Screening approach for multiple assay endpoints in the presence and absence of cytokine addition. Here, I will describe these screens using examples of hepatotoxic drugs. I will discuss advantages and limitations of each in vitro screen with respect to predicting hepatotoxicity.

2:25 Ice Cream Refreshment Break in the Exhibit Hall

3:05 Approaching Hepatotoxicity in Drug Discovery as a Lead Optimization Problem
Dylan P. Hartley, Ph.D., Senior Scientist, Investigative Toxicology, Genentech, Inc.

Given the demands on drug discovery teams to produce molecules devoid of hepatotoxicity, toxicologists are now integral members of these teams with new responsibilities geared toward lead optimization. Toxicologists are now expected to assess risk for hepatotoxicity in a compound in predictive manner, or ascribe a mechanism to early hepatotoxicity findings, link the findings to an offending moiety within the structure, and define the chemical lead optimization path. Structure-based strategies to predict and/or attenuate hepatotoxicity will be presented with respect to various hepatotoxic signals.

3:35 The Utility of Emerging Biomarkers of Liver Injury in Pre-Clinical and Clinical Drug Development
Shell Schomaker, Principal Scientist, Drug Safety R&D, Pfizer, Inc.

While alanine aminotransferase (ALT) activity remains the gold standard biomarker of liver injury, the correlation between increased ALT levels and morphological liver findings is rather imperfect. These discrepancies could be due to adaptive responses, altered liver membrane permeability, extrahepatic injury, or treatment-related effects on ALT enzyme activity. This presentation will focus on an evaluation of malate dehydrogenase, purine nucleoside phosphorylase and glutamate dehydrogenase for the detection of liver injury when ALT activity is limited and will demonstrate the utility of this alternative biomarker approach for improving confidence.

4:05 From Mild Pre-Clinical Transaminase Elevations to Idiosyncratic Liver Injury in One Easy Lesson
Paul Vancutsem, D.V.M., Ph.D., Director, Pre-Clinical Safety; Senior Member, Novartis Internal Liver Experts Team, Novartis Pharmaceuticals

Even if we do not fully understand idiosyncratic liver injury (IDILI), it appears to necessitate events involving an often subtle local liver insult, an immune system imbalance and a specific genetic make-up (HLA alleles). Integration of these traditionally unconnected areas of toxicology will lead to an improved prediction of IDILI. This presentation proposes a template for integration using a pragmatic approach.

4:35 End of Conference

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TUESDAY, JUNE 7

7:45 am Registration and Morning Coffee

UNDERSTANDING MECHANISMS OF NEPHROTOXICITY

8:45 Chairperson’s Opening Remarks

8:55 Cisplatin Nephrotoxicity and Renal Protective Strategies
Zheng Dong, Ph.D., Professor, Department of Cellular Biology and Anatomy, Medical College of Georgia
Cisplatin, a widely used chemotherapy drug, has major side effects in normal tissues, notably nephrotoxicity in kidneys. Research during last few years has delineated several signaling pathways leading to renal tubular damage and kidney injury in cisplatin nephrotoxicity, including a rapid DNA damage response. Importantly, cisplatin may activate different signaling pathways in normal tissues and tumors. Targeting these pathways may uncover clinically effective strategies for kidney protection without diminishing the chemotherapy efficacy of cisplatin in tumors.

9:25 Primary Cell Cultures from Human and Rat Proximal Tubule as Models to Study Mechanisms of Acute Kidney Injury
Lawrence H. Lash, Ph.D., Professor, Associate Chair, Pharmacology, School of Medicine, Wayne State University
Our laboratory has developed several in vitro models from rat and human kidney, using both non-specific toxic chemicals and specific nephrotoxicants, including halogenated solvents, analgesics, and antibiotics. One focus has been the metabolism and toxicity of the halogenated solvent trichloroethylene (TCA), which is a significant environmental contaminant that has the kidney as one target organ. Molecular approaches and proteomics have been integrated into our in vitro toxicology models to explore the role of specific proteins and examine the influence of disease processes, such as diabetic nephropathy and reduced nephron mass, on renal toxicological responses.

9:55 Networking Coffee Break

MONITORING AND ASSESSING KIDNEY INJURY

10:25 Current Use of Renal Biomarkers in Early Drug Development
Diann Weddle, Ph.D., D.V.M., Senior Pathologist, Pre-Clinical Safety, Abbott Laboratories
An overview of renal toxicology will be presented including a discussion of use and limitations of current biomarkers. Key concepts will be further emphasized through case examples. Within the last year, the Predictive Safety Testing Consortium’s Nephrotoxicity Working Group submitted a qualification package for multiple renal biomarkers to the FDA and EMEA and received clearance for limited use in nonclinical and clinical drug development. Conclusions and recommendations from the submission will be summarized.

10:55 Pre-Clinical Biomarkers of Nephrotoxicity: Applications in Drug Discovery and Development
Eric Bloomme, D.V.M., Ph.D., D.A.C.V.P., Senior Project Leader, Abbott Laboratories
This presentation will build upon the preceding presentation discussing the current use of renal biomarkers in early drug development. Specifically, several case examples will be used to illustrate how biomarkers of renal toxicity can be applied in drug discovery and development to increase probability of success and make better decisions on compounds at earlier stages. Data on performance characteristics for several of these biomarkers in rats will be presented.

11:25 Integrative Assessment of Drug-Induced Kidney Function Changes and Acute Injury Using an Automated Blood Sampling and Telemetry (ABST) System
Yafei Chen, M.D., M.S., Scientist Safety Pharmacology, Global Safety Assessment, AstraZeneca Pharmaceuticals
~20% of hospital admissions are caused by nephrotoxic drugs due to acute kidney injury. Current preclinical methods are insufficient to detect and predict drug-induced changes in kidney function and/or kidney injury (DIKI). We will describe an integrated pharma-cology platform representing a convergence of automated blood sampling with telemetry (ABST) for simultaneous assessment of cardiovascular, renal hemodynamic and excretory functions, and nephron site-specific DIKI biomarkers in surgically prepared conscious rats. This integrated preclinical approach provides multiple translational markers for risk management in early clinical development.

11:55 Luncheon Presentations (Sponsorship Opportunities Available) or Lunch on Your Own

KIDNEY MARKERS: FROM BENCH TO BEDSIDE

1:30 pm Chairperson’s Remarks
W. Brian Reeves, M.D., F.A.C.P., Chief, Division of Nephrology, Professor and Vice Chair, Medicine, Penn State College of Medicine

1:35 FEATURED PRESENTATION: Establishing the Context for Introducing New Safety Biomarkers into Clinical Trials
There has been rapid progress in developing new safety biomarkers for monitoring organ toxicity. Some of these new markers have already been qualified for use in supporting pre-clinical studies. Efforts to qualify these new safety biomarkers for use in human clinical trials are also underway. This presentation will explore recent experiences with organ-specific markers and strategies and challenges for progressing these markers for support of clinical trials.

2:05 Clinical Evaluation and Qualification of Kidney Safety Biomarkers: A Collaboration between Two Consortia
Maria Vassileva, Ph.D., Scientific Program Manager, The Biomarkers Consortium, Foundation for the NIH
The Biomarkers Consortium (BC), a public private partnership managed by the Foundation for the NIH, is preparing to launch an important project expected to generate the data needed to advance the regulatory acceptance of new biomarkers appropriate for monitoring kidney safety in the clinic, and reaching alignment on how these biomarkers could improve clinical diagnoses of drug-induced acute kidney injury during drug development and patient therapy with aminoglycosides in patients with cystic fibrosis and cisplatin in patients with head and neck cancer. This project is a collaboration between the BC and the Predictive Safety Testing Consortium of the Critical Path Institute.

2:35 Urinary Cytokines as Biomarkers of Nephrotoxicity
W. Brian Reeves, M.D., F.A.C.P., Chief, Division of Nephrology, Professor and Vice Chair, Medicine, Penn State College of Medicine
Inflammation is a critical component of drug-induced acute kidney injury. In response to injury, renal epithelial cells elaborate a variety of chemokines and cytokines which alter epithelial cell function and also lead to the recruitment and/or activation of inflammatory cells in the kidney. Levels of cytokines increase in the urine early after kidney injury and may...
hepatotoxicity. Emerging animal models will be discussed with an emphasis on transporters that facilitate bio-accumulate of drugs into the mitochondria. Compound chemistry, but also of genetic diversity in plasma membrane drug-induced organ toxicity. These off-target effects are a function of mitochondrial effects that contribute to the etiology of idiosyncratic bioenergetic capacity of the cell. Many drugs have direct and deleterious membrane potential it generates from phosphorylation, reduce the poised cells, plus the majority of potentially injurious, free radicals. Mitochondria typically produce more than 90% of the ATP in aerobically filtered FITC dextran. In less than one hour accurate GFRs are obtained by using a non-filterable large red fluorescent dextran and a small kidney freely filterable FITC dextran. In less than one hour accurate GFRs are obtained in many conditions including acute kidney injury (AKI). We believe this has clinically important diagnostic and therapeutic advantages in AKI and chronic kidney disease.

5:05 PANEL DISCUSSION: Renal Injury Markers and How Effectively Can They Be Used?
Moderator: W. Brian Reeves, M.D., F.A.C.P., Chief, Nephrology; Professor and Vice Chair, Medicine, Penn State College of Medicine

5:35 - 6:30 Happy Hour in the Exhibit Hall

WEDNESDAY, JUNE 8

7:30 am Continental Breakfast Breakout Discussions
Breakout Discussion Topics:
- Biomarkers for Organ Toxicity and Their Effective Use in Pre-Clinical and Clinical Development
- Monitoring Mitochondria and Their Impact on Organ Toxicities

MITOCHONDRIAL INVOLVEMENT IN CARDIAC, RENAL AND LIVER TOXICITY
(Joint session for Cardiotoxicity and Nephrotoxicity tracks)

8:30 am Chairperson’s Remarks
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

8:40 Introduction to Mitochondrial Function and Drug-Induced Dysfunction
James Dykens, Ph.D., CEO, EyeCyte Therapeutics
Mitochondria typically produce more than 90% of the ATP in aerobically poised cells, plus the majority of potentially injurious, free radicals. Inhibition of the electron transfer system, or the uncoupling of the membrane potential it generates from phosphorylation, reduce the bioenergetic capacity of the cell. Many drugs have direct and deleterious mitochondrial effects that contribute to the etiology of idiosyncratic drug-induced organ toxicity. These off-target effects are a function of compound chemistry, but also of genetic diversity in plasma membrane transporters that facilitate bio-accumulate of drugs into the mitochondria. Emerging animal models will be discussed with an emphasis on hepatotoxicity.

9:00 Tales of Broken Mitochondria: Drug-Induced Cardiac Mitochondrionopathy
Paulo Oliveira, Ph.D., Group Leader, Mitochondrial Toxicology and Disease, Center for Neuroscience and Cell Biology, University of Coimbra, Portugal and Visiting Research Associate, University of Minnesota Medical School
Drug-induced cardiac mitochondrial dysfunction can progressively result in organ degeneration. Classical examples of drug-induced cardiac mitochondrialopathy include nucleoside reverse transcriptase inhibitors, local anesthetics and anthracyclines. Doxorubicin (adriamycin, DOX) is a clear case study of a well known pharmaceutical that can lead to progressive degeneration of cardiac mitochondrial function. DOX is a potent anthracycline anti-neoplastic agent, whose clinical use is limited by a dose-dependent and cumulative cardiotoxicity, with a clear mitochondrial component. In this case, protection of cardiac mitochondrial function during DOX treatment appears to be critical for preventing the maintenance of the myocyte bioenergetics.

9:40 Sponsored Presentations (Opportunities Available)

10:00 Networking Coffee Break in the Exhibit Hall

10:50 Mitochondrial Homeostasis in Acute Kidney Injury
Rick Schnellmann, Ph.D., Professor, Chair, Pharmaceutical and Biomedical Sciences, South Carolina College of Pharmacy
Mitochondrial damage is a major contributor to the initiation of tubular cell injury and the progression of acute kidney injury (AKI) produced by drugs, toxicants, and ischemia. To understand the role of mitochondria in organ damage and repair, we think that mitochondria need to be examined holistically by measuring mitochondrial homeostasis. This includes changes in mitochondrial loss, fission/fusion, mitophagy, and biogenesis over time. Using this approach, temporal differences in mitochondrial loss, dynamics and biogenesis were observed with mitochondrial loss occurring early and changes in mitochondrial fission/fusion and biogenesis occurring later after AKI.

11:20 Mechanistic Insights into Mitochondrial-Based Organ Toxicity
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D
Previous speakers have elucidated in detail on the contribution of mitochondrial impairment to different organ toxicities. It is apparent that in order to avoid late stage attrition due to mitochondrial toxicity, early predictive screens need to be deployed early in the drug discovery process. Here I will show organelle and cell-based HTS applicable screens to detect such liabilities. I will describe the screens using examples from different drug classes such as anti-diabetics/antilipidemics, antivirals, antibiotics, and NSAIDs.

11:50 PANEL: Strategies for Assessing Mitochondrial Involvement in Drug-Induced Organ Toxicities
Moderator: Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

12:20 pm End of Conference
comparing DDI assay design in the discovery and development, features that influence experimental design for clinical attrition rates. This presentation will focus on identifying the key interactions (DDI) is critical for designing safer therapeutics and reducing risk of transporter-mediated clinical effects has the potential to significantly reduce the attrition rates and help progress the right compounds through development. The talk will highlight current industry strategies for transporter tools for assessing the impact of these transporter strategies will also be discussed.

Examples will be presented in which in vitro-in vivo correlations and differences in species sensitivity can be better understood by considering target organ exposures. Systematic analysis of tissue drug levels for a group of structurally diverse small molecules and consideration of their pharmacokinetic parameters indicates that tissue distribution can be roughly predicted from the volume of distribution (Vss) and the clearance (CLp) of a particular molecule. These findings suggest that consideration of these pharmacokinetic parameters could help provide relevant and translatable information.

Pharmaceuticals

Over the past decade, or so, we have seen the implementation of early screening for ADME and PK become a standard approach in the drug discovery armamentarium. In addition, there is growing interest in the earlier application of predictive PK and PK/PD modeling. More recently, we have seen and increasing focus on improving the screening approaches for toxicity. This presentation will discuss our strategy for the early application of ADME/PK and toxicology screening and the application of this data to the rapid transition from Discovery into Development.

Strategic role of ADME, PK and Toxicology

Over the past several years our laboratory has focused on establishing a series of in vitro assays that provide rapid and efficient screening for genotoxicity. However, the tests often show limited specificity. Positive results in these assays to predict genotoxic liabilities have high throughput and require very small amounts of compound. However, they are limited by lower predictivity for the outcome of regulatory OECD guideline assays (including limitations), with emphasis in practical applications in pharmaceutical drug development. Screening strategies for genotoxicity lead optimization and their rationale will be discussed, as well as investigational approaches around positive findings.

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Prediction, Pfizer Global R&D
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety
Mitochondrial Toxicity, and Transporter Assessment

1:55
Optimization and candidate selection.
This presentation will provide an overview of the current status of the "safety" of various chemicals proposed for registration.

Eric Blomme, D.V.M., Ph.D., D.A.C.V.P., Sr Project Leader, Abbott Labs

1:25
Current Toolbox for the Prediction of Hepatotoxicity
Eric Blomme, D.V.M., Ph.D., D.A.C.V.P., Sr Project Leader, Abbott Labs
This presentation will provide an overview of the current status of several technologies used to improve hepatotoxicity prediction during drug discovery. Examples will be used to illustrate the strengths, limitations and optional application of these technologies during lead optimization and candidate selection.

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In vitro Strategies: High Content Mechanistic Screening, Mitochondrial Toxicity, and Transporter Assessment
Yvonne Will, Ph.D., Associate Research Fellow, Compound Safety Prediction, Pfizer Global R&D

2:25 Ice Cream Refreshment Break in the Exhibit Hall
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Dylan P. Hartley, Ph.D., Senior Scientist, Investigative Toxicology, Genentech, Inc.

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3:35 The Utility of Emerging Biomarkers of Liver Injury in Pre-Clinical and Clinical Drug Development
Shelli Schomaker, Principal Scientist, Drug Safety R&D, Pfizer, Inc.

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4:05 From Mild Pre-Clinical Transaminase Elevations to Idiosyncratic Liver Injury in One Easy Lesson
Paul Vancutsem, D.V.M., Ph.D., Director, Pre-Clinical Safety and Senior Member of Novartis Internal Liver Experts Team, Novartis Pharmaceuticals
Even if we do not fully understand idiosyncratic liver injury (IDILI), it appears to necessitate events involving an often subtle local liver insult, an immune system imbalance and a specific genetic make-up (HLA alleles). Integration of these traditionally unconnected areas of toxicology will lead to an improved prediction of IDILI. This presentation proposes a template for integration using a pragmatic approach.

4:35 End of Conference
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NUTRITIONAL MODULATION OF SIRTUIN EXPRESSION IS MEDIATED BY REDOX CHANGES

GABINII J 1, MACHADO NG 2, INGLES M 1, LÓPEZ - GRUESO R 1, BONET - COSTA V 1, ABDELAZIZ KM 1, EL ALAMI M 1, EDO R 1, DROMANT M 1, OLASO G 1, BORGAS C 1, OLIVEIRA PJ 2 and VIÑA J 1.

1 Department of Physiology, Faculty of Medicine, University of Valencia, Valencia, Spain.
2 Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal

Sirtuins are NAD+-dependent deacetylases that modulate metabolism, the rate of ageing and other critical physiological parameters. The role of the NAD+/NADH redox ratio in the regulation of sirtuins has been proposed. To test this idea, researchers have measured NAD+ and NADH in several cellular models. However, in a pioneer study Krebs and co-workers showed that direct measurement of tissue content of NAD+ and NADH do not supply the required information: they fail to differentiate between free and bound nucleotides.

We have used cell culture and exercised vertebrates (mice) models to study the regulation of sirtuins by redox modulation.

We have found that buffering NAD+/NADH pair with known concentrations of lactate and pyruvate regulate sirtuin expression in 3T3 fibroblasts cell culture. Ethanol, which affects NADH/NAD+ ratio, also up-regulates sirtuin expression in this model. Exercise causes a significant increase in lactate-pyruvate ratio and thus changes in NAD+/NADH.

The importance of this study lies in the fact that sirtuin expression does not depend on total free [NAD+] or [NADH] but rather on the ratio of these co-enzymes in each tissue. This ratio can be modulated by several physiological manipulations such as exercise or moderate ethanol intake. We have observed that these metabolic changes may play a role on the life span of these models through regulation of sirtuin system. The present work advances our current knowledge of how oxidation state of a certain tissue affects the aging process.

This work has been supported by BFU2007-65803/BFI and ISCIII2006-RED13-027 from the Spanish “Red Temática de investigación cooperativa en envejecimiento y fragilidad” (RETICER RD06/003-027) to J.V., by grants GV/2007/263 to C.B and GVPRE/2008/138 and Catholic University 2008-011-002 to J. G. and also by grants SFRH/BDE/56178/2009 to NGM and PTDC/SAS-TOX/110952/2009 to PJO from the Foundation for Science and Technology, Portugal.