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REVIEW ARTICLE

Pharmacological strategies to counteract doxorubicin-induced cardiotoxicity: the role of mitochondria

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ABSTRACT

Since the discovery of doxorubicin (DOX) as a potential anticancer agent, different types of human cancers have been successfully treated. However, the use of the drug leads to an irreversible and dose-dependent cumulative cardiotoxicity thus restraining the treatment efficacy. Previous studies pointed out mitochondrial dysfunction as the main mechanism for drug cardiotoxicity, although the story still remains incomplete. Several approaches have been proposed in order to prevent DOX selective cardiotoxicity, some of which specifically target mitochondria. The present review performs a brief overview of the mitochondrial targets of DOX toxicity and the pharmacological strategies designed to decrease it, with a special focus on agents that act on mitochondria. This review is thus divided in to three main sections: a) the effects of DOX on mitochondrial physiology and bioenergetics as well as the consequences at the myocyte level; b) non-pharmacological strategies already demonstrated to counteract DOX-induced cardiotoxicity and, c) damage-preventing approaches involving mitochondria and their mechanism. Our objective was to review the state-of-art regarding the mechanisms of DOX-induced cardiotoxicity, especially focusing on mitochondria, and to describe strategies aimed at preventing such toxicity. The present review can help on the development of new strategies to counteract DOX toxicity focusing on the mitochondria as the target.

Key words

Doxorubicin; Heart; Mechanism of action; Mitochondria; Prevention; Toxic potential

RÉSUMÉ

La découverte de la doxorubicine (DOX) comme agent anticancéreux potentiel a permis de traiter avec succès différents types de cancers humains. L'utilisation répétée de cette médication cause cependant une cardiotoxicité dose-dépendante et irréversible qui limite son usage thérapeutique. Des études précédentes ont identifié une dysfonction mitochondriale comme mécanisme principal de cette cardiotoxicité, mais ce n'est qu'une partie de l'histoire. Différentes approches ont été proposées pour prévenir cette toxicité sélective, certaines visant spécifiquement les mitochondries. Cet article effectue une brève revue des cibles mitochondriales de toxicité de la DOX et des stratégies pharmacologiques conçues pour la réduire, avec un accent sur les molécules agissant sur les mitochondries. Cette revue est divisée en trois sections: a) les effets de la DOX sur la physiologie et la bioénergétique mitochondriale, et leurs conséquences au niveau des myocytes; b) les stratégies non-pharmacologiques contrecarrant la cardiotoxicité induite, et c) les approches préventives impliquant les mitochondries et leur mécanisme. L'objectif était la revue du "state-of-art" concernant les mécanismes de la cardiotoxicité induite par la DOX avec un accent sur les mitochondries et la description des stratégies visant à prévenir cette toxicité. Cette revue devrait aider à développer de nouvelles stratégies pour contrecarrer cette toxicité en mettant l'accent sur les mitochondries.

Mots clés

Doxorubicine; Cœur; Mécanisme d'action; Mitochondries; Prévention; Potentiel toxique

RESUMEN

Desde el descubrimiento de la doxorubicina (DOX) como un posible compuesto anticancerígeno, diferentes tipos de cáncer han sido tratados exitosamente. Sin embargo, el uso de este fármaco puede conducir a una cardiotoxicidad irreversible, dosis-dependiente y acumulativa, limitando su eficacia terapéutica. Estudios anteriores propusieron a la disfunción mitocondrial como el principal mecanismo de cardiotoxicidad; sin embargo, aún son necesarios más estudios. Se han propuesto varias alternativas para prevenir la cardiotoxicidad selectiva de DOX, algunas dirigidas específicamente a la mitocondria. Este artículo revisa en forma breve la toxicidad mitocondrial inducida por DOX y propone estrategias farmacológicas orientadas a disminuirla, en especial con agentes que actúan a nivel mitocondrial. Esta revisión se divide en tres secciones principales: a) los efectos de DOX en la fisiología mitocondrial y su bioenergética así como las consecuencias a nivel del miocito; b) la descripción de estrategias no farmacológicas para contrarrestar la cardiotoxicidad de la DOX, y c) acciones preventivas del daño que involucran a la mitocondria en su mecanismo de acción. Nuestro objetivo fue revisar el conocimiento actual de los mecanismos de cardiotoxicidad inducida por DOX y su prevención, enfocados en forma particular en la mitocondria. Esta revisión puede ayudar en el desarrollo de nuevas estrategias para contrarrestar la toxicidad mitocondrial inducida por DOX.

Palabras clave

Doxoribicina; Corazón; Mecanismo de acción; Mitocondria; Prevención; Toxicidad potencial

INTRODUCTION

Doxorubicin (DOX, Adriamycin) (Figure 1) is an anthracycline antibiotic discovered and isolated from *Streptomyces peucetius* in the early 1960's [1]. Presently, it is one of the most important antineoplastic agents used against a broad spectrum of cancer types, such as lymphomas, leukemias and solid tumors. The anticancer activity of DOX is explained by its ability to bind and/or intercalate the DNA double strand causing a stereochemical disorder and thus inhibiting the synthesis of DNA, RNA, and proteins, eventually leading to cell death [2].

Despite the potent antineoplastic activity of DOX, its clinical use is restrained by an irreversible and dose-dependent cumulative cardiotoxicity. Acute effects may arise immediately after treatment and are clinically characterized by arrhythmias, hypotension, pericarditis, diastolic functional impairment (first) and systolic function alterations (later) [3].

The reported effects are transient and disappear as soon as the patient discontinues the treatment. However, chronic effects, which are characterized by progressive left ventricular dysfunction, lead to irreversible heart failure and are therefore more serious and difficult to manage [3]. Morphological alterations usually include myofibrillar loss, vacuolization of cytoplasm, dilatation of sarcoplasmic reticulum and swollen mitochondria [4] [5].

DOX-cardiotoxicity markers generally include an increase of cardiac-specific and non-specific protein levels in serum, such as lactate dehydrogenase (LDH), cardiac troponin I (cTnI), creatine phosphokinase (CPK), glutamate oxaloacetate transaminase (GOT) and glutamyltransferase (GTP) [6]. The correlation between DOX cumulative doses and the risk of heart failure is well known. It has become widely accepted that cumulative doses up to 550 mg m^{-2} exponentially increase the probability of DOX-induced cardiomiopathy [7]. Dosages above this level are avoided in order to prevent DOX-induced damage. Besides the cumulative effect of DOX, another important issue is the peak plasma concentration of drug delivered to the heart during treatment. It was hypothesized that DOX toxicity is primarily related to the peak dose concentration while the antitumor efficacy is more related to total drug exposure [4].

Although several intracellular targets of DOX have been already indentified, the complete mechanism of DOX-induced cardiotoxicity remains unclear. Over the years, different hypotheses were proposed for such enhanced cardiotoxicity. Mitochondria have received particular attention in this regard due to their role producing energy in heart tissue by synthesizing ATP through oxidative phosphorylation; by producing reactive oxygen species (ROS), which can act as second messengers, in some cases, or be deleterious if in excess [8] [9]; and, by regulating cytosolic calcium [8]. DOX has been reported to inhibit some of the respiratory complexes, to decrease the ATP content in cardiomyocytes, to stimulate ROS production, and to decrease mitochondrial calcium-loading capacity through induction of the mitochondrial permeability transition (MPT) [3] [4] [10] [11] [12]. Together with morphological alterations, the data suggests mitochondrial dysfunction as one of the main causes for DOX-induced cardiomiopathy.

Due to the limitation imposed by the DOX-cumulative effect, different approaches appeared over the years as an attempt to prevent or to counteract DOX-induced cardiotoxicity. These approaches rely on the different hypotheses explaining DOX toxicity and, in fact, some were shown to be capable of decreasing the damage induced by DOX. The different data obtained also suggests that the mechanism behind DOX cardiotoxicity is most likely a multifactorial event rather than one isolated effect.

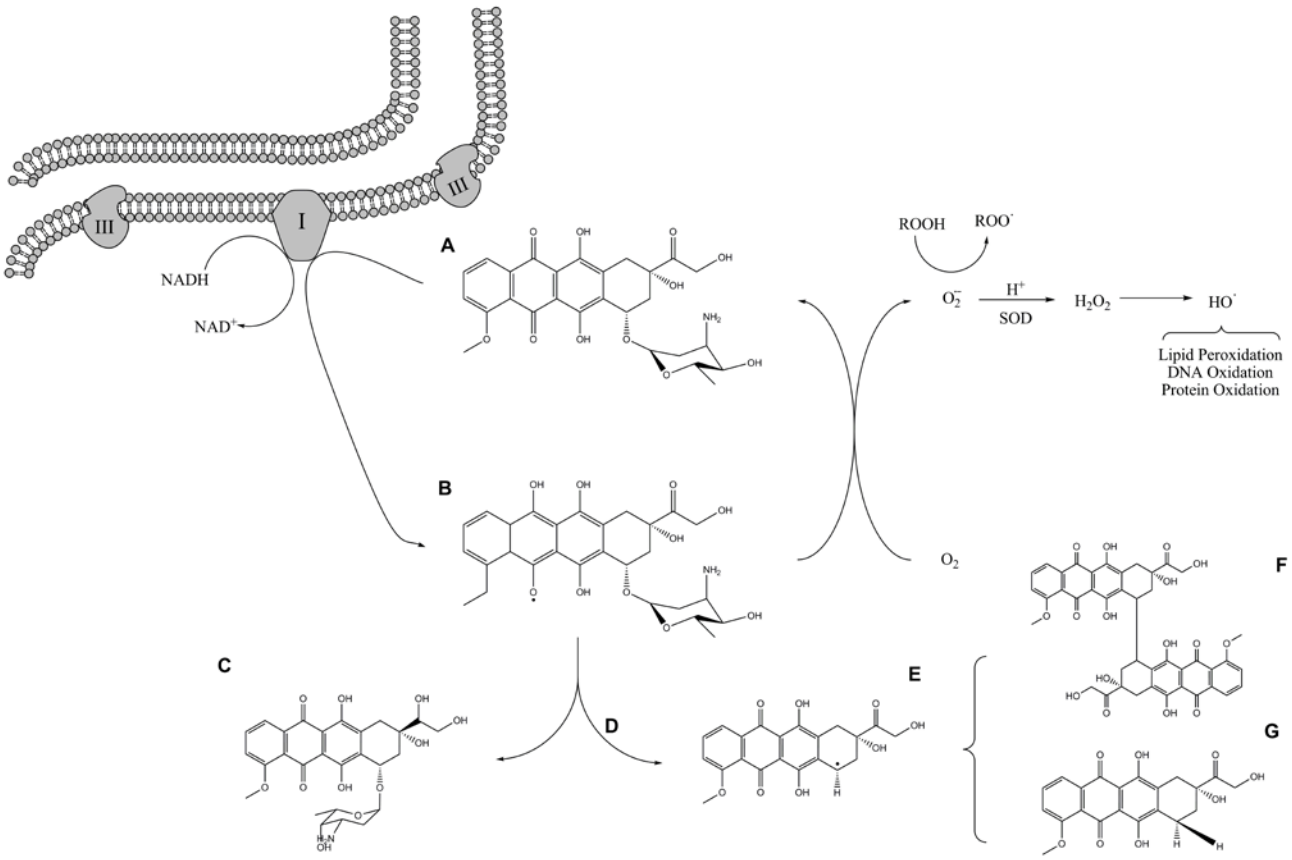

Figure 1 **Bioactivation of DOX by mitochondrial complex I. DOX (A) can be activated/reduced by several oxyreductase enzymes, here represented by the mitochondrial respiratory chain NADH dehydrogenase. NADH acts as an electron donor for the reduction of DOX producing a semiquinone radical (B) which can therefore be re-oxidized in the presence of molecular oxygen, consequently regenerating DOX. This process is known as DOX redox cycle and increases the production of superoxide anion which can directly damage lipids and proteins or can also be converted to other ROS. Alternatively, in the absence of molecular oxygen, DOX semiquinone can be simply converted to secondary metabolite doxorubicinol (C) or can undergo deglycosylation (D) forming a C7 radical (DNA alkylation) (E) which can form dimers (F) or be converted to a 7-deoxyaglycone (G) (DNA intercalation).**

It should be stressed, however, that strategies should minimize the cardiotoxicity imparted by DOX while preserving the antineoplastic efficacy of the drug. As mitochondria are a particular target in DOX toxicity, new approaches should be focused on this particular cellular structure in order to circumvent DOX cardiotoxicity.

CELLULAR EFFECTS OF DOX-INDUCED TOXICITY – THE ROLE OF MITOCHONDRIA

Cardiomyocytes are terminally differentiated muscle cells with little or no capacity to proliferate. As the endpoint of DOX toxicity involves cardiomyocyte death, repetitive and cumulative induction of cell death may lead to irreversible cardiotoxicity and consequently heart failure. Over the years, different hypotheses have been proposed in order to explain DOX-induced cardiotoxicity.

DOX-induced myocyte cell loss through induction of apoptosis includes up-regulation of proapoptotic proteins (Bax, caspases and cytochrome c) [13] [14] [15] with or without down-regulation of antiapoptotic proteins (Bcl2, Akt) [13] [15], making mitochondrial-mediated apoptosis an attractive mechanism.

However, it is not clear if apoptosis is a cause or a consequence of drug effects. Since DOX binds to DNA, it should be expected that this particular effect

would be related to the cardiotoxicity induced by the drug. However, cardiomyocytes are terminally differentiated cells with low replication/division activity. Thus, DOX-induced impairment of DNA replication is unlikely to trigger cardiomyopathy, although inhibition of DNA transcription can also contribute to cardiac toxicity. In fact, other anticancer agents that kill cancer cells through binding/intercalation in DNA, such as cisplatin and actinomycin D, do not cause cardiotoxicity.

Another interesting fact is that p53, a DNA damage-activated protein, is active in cardiomyocytes after treatment with DOX [16]. p53 activation may reflect a link between the redox cycle/activation of DOX and the increase in oxidative stress with the transcriptional activation of Bax by p53. Alternatively, DOX-stimulated oxidative stress may also reflect the direct opening of the MPT pore, cytochrome c release, and consequent apoptosis triggering.

Furthermore, it has been demonstrated that the gene profile of cardiomyocytes can be altered by DOX treatment [17] [18]. Along with other cellular proteins, the levels of some mitochondrial enzymes are decreased in cardiomyocytes exposed to DOX which can partly explain the altered mitochondrial bioenergetics observed *in vivo* [17] [18] [19] [20] [21]. In fact, transcripts and/or protein levels of the adenine nucleotide translocator and Rieske iron-sulfur protein are decreased after DOX treatment [17] [22]. The results appear to indicate that although DOX-induced cancer elimination and cardiomyopathy involve distinct mechanisms, direct DOX effects on nuclear DNA, which has been proposed by some authors as responsible for early events in cardiomyocyte death, is likely not to be the major cause of toxicity [16].

The reason for the increased susceptibility of the cardiac muscle for DOX toxicity is still under debate. Although a full comparative study between different human organs is still to be performed, some authors have proposed that DOX-induced oxidative stress may preferentially affect the heart due to the lower antioxidant capacity of the cardiac muscle [23]. Another, very controversial, point of view is the possibility of the existence of an organoselective mitochondrial NADH dehydrogenase, located in the outer leaflet of the inner mitochondrial membrane which would promote DOX-induced oxidative stress [24]. However, no further investigations have been performed exploring this possibility.

Although the mechanism for such enhanced toxicity in the cardiac muscle remains unclear, DOX affinity for some macromolecules can explain the effects on different organelles. As already referred, DOX has high affinity to DNA and therefore the nuclei are a particular target. DOX has also a high affinity for cardiolipin (CL) [25] [26], a membrane phospholipid predominately present in mitochondrial membranes.

The inner mitochondrial membrane is by far the richest cellular structure containing CL and this phospholipid acts as a co-factor for several enzymes [27]. CL-protein complexes may be crucial in several mitochondrial processes including oxidative phosphorylation, since cytochrome c oxidase (COX) requires the presence of CL [27] [28]. Interestingly, DOX is capable of inhibiting COX [29], most likely due to drug-lipid complexes rather than direct drug-protein interactions [25]. DOX-interaction with mitochondrial membranes, including lipid peroxidation, due to increases in oxidative stress are also mechanisms in the toxicity induced by DOX.

More than 90% of the ATP utilized by cardiomyocytes is produced by mitochondrial respiration and therefore any alteration on mitochondrial function will have a strong impact in the energy of cardiomyocytes subsequently inducing cardiotoxicity. DOX has been reported to interfere with mitochondrial respiration decreasing both intracellular pools of ATP and phosphocreatine (PCr) [3].

The inhibitory effect on mitochondrial respiration is translated into a decrease in State 3 and sporadic stimulation of State 4 respiration [19] [30]. Surprisingly, the decrease in State 3 respiration was reversed by addition of dithiothreitol (DTT) suggesting that DOX induces specific alterations in protein thiol groups [20]. Other effects observed on isolated mitochondrial fractions include a decrease in the respiratory control ratio and ADP/O [31]. The increase in State 4 respiration is thought to be due to proton leak from the inner membrane as a consequence of drug-lipid interaction [25] [30]. Respiratory complexes are plausible targets in DOX toxicity action, and inactivation of several mitochondrial enzymes was already reported. DOX sensitive sites were mainly located in complex I, III and IV [25] [30] [32] [33]. NADH-dehydrogenase and COX activity were particularly impaired after DOX treatment [31]. Although DOX inhibits ATP synthase activity, the crucial targets of DOX seem to be peripheral proteolipid complexes involved in the channeling of ATP and PCr from mitochondria to the cytosol (ANT, VDAC and MtCK) [3] [31].

The large variability in the percentage of inhibition in the activity of isolated respiratory complexes and/or

intact mitochondria respiration, reported in different *in vivo* studies, can be explained by different DOX whole body distribution, binding specificity, and metabolism. Alternatively, the differences may indicate that mitochondrial oxygen consumption is not a sensitive method to evaluate mitochondrial dysfunction, as has already been pointed out [12]. It is proposed instead that altering mitochondrial calcium-loading capacity is a more sensitive and quantifiable indicator of mitochondrial dysfunction upon DOX treatment [10] [12] [19] [20] [34]. DOX interferes with the sarcoplasmic reticulum, increasing the open state probability of calcium release channels interfering with intracellular calcium homeostasis [35].

The increase in cytoplasmatic calcium concentration can be potentiated by inhibition of Na^+/Ca^{2+} and Na^+/K^+ pumps [36] [37]. As calcium is the second most common messenger in cells, DOX-induced increases in cytoplasmatic calcium concentrations can trigger numerous cell responses affecting normal cell behavior. Cytosolic calcium overload is more serious when calcium increases in microdomains between mitochondria and the reticulum. Upon calcium dysregulation, mitochondria accumulate an excess of calcium in the matrix. In fact, several *in vitro* and *in vivo* studies show that DOX, or even its metabolites, decrease the capacity of mitochondria to accumulate and retain calcium [10] [19] [38].

Calcium over-accumulation on mitochondria has been reported to increase the production of reactive oxygen species (ROS) [39] and can therefore be a link between oxidative stress and alterations in calcium homeostasis. Mitochondrial calcium dysregulation became an important component of DOX toxicity; in fact, ruthenium red, an inhibitor of mitochondrial calcium uptake, not only decreased the production of ROS but also increased cell viability [40]. Although the relationship between calcium release from the sarcoplasmic reticulum and mitochondrial dysfunction has not yet been fully explored, different studies have proposed that ROS production and cytosolic calcium dysregulation are reciprocally regulated in cardiomyocytes exposed to DOX.

The fact that altered mitochondrial calcium regulation is completely blocked by cyclosporin A (CsA), a specific inhibitor of the MPT pore [41], implicates induction of MPT by DOX as another potential cause for DOX-induced cardiotoxicity [20] [38]. The MPT pore can be defined as a voltage-dependent high-conductance channel in the inner mitochondrial membrane. The pores are suggested to be between 2-3 nm in diameter and to exclude molecules with molecular weights higher than 145 kDa [42].

Although the identities of the individual components of the MPT pore remain controversial, it is agreed that the adenine nucleotide translocator (ANT), voltage-dependent anion channel (VDAC), and cyclophilin-D are elements of the pore complex or at least are involved in its formation or regulation. Matrix alkalinization, oxidative stress, and calcium overload in the matrix are known to increase the susceptibility of mitochondria to MPT pore opening [43] [44]. In the majority of *in vitro* studies, pore opening is accompanied by mitochondrial depolarization, respiratory inhibition or stimulation, matrix swelling, pyridine nucleotides depletion and release of intermembrane proteins, including cytochrome c. In fact, all these effects were already reported in heart mitochondria exposed *in vitro* or *in vivo* to DOX [10] [12] [19] [20].

In vitro studies show that induction of MPT is increased when DOX metabolites, such as aglycones and secondary alcohols, are used instead of the parent compound [38]. DOX metabolites appear to be more deleterious to mitochondria than DOX itself. MPT pore induction by DOX is thought to be due to increasing production of ROS leading to the oxidation of specific critical sulfhydryl groups [10] [31]. The model agrees with the ability of DOX to generate free radicals through a redox cycle mechanism [11] [45].

Besides slower respiration, mitochondria from DOX-treated rats exhibit an enhanced sensitivity to MPT and therefore the calcium loading capacity is decreased [19] [30] [46] [47]. Curiously, this aspect of calcium dysregulation may still persist after the end of the treatment [48]. It was demonstrated that addition of CsA to a suspension of isolated cardiac mitochondrial fractions from DOX-treated rats was able to reverse the DOX-induced decrease in calcium loading capacity [20]; CsA was also able to revert the inhibition of respiration induced by DOX. Both observations indicate that the decrease in mitochondrial calcium-loading capacity is due to an increase in sensitivity to the MPT and that the inhibition of oxidative phosphorylation by DOX is a secondary effect of MPT induction. It was proposed that probable binding of CsA to cyclophilin-D might cause a disassembly of pre-formed MPT pores allowing ANT, which is crucial to oxidative phosphorylation, to be functional again. Alternatively, it was also demonstrated that the ANT is depressed both in content and function in DOX-treated rats, which can contribute to decreased respiration and increased MPT occurrence [17].

DOX-induced oxidative stress appears to be the main factor responsible for drug cardiotoxicity. DOX can

stimulate the production of ROS by two distinct mechanisms, one involving enzymatic reactions and the other through an iron-dependent mechanism [4] [7]. In the first one, the DOX quinone moiety undergoes a univalent reduction to yield a semiquinone free radical.

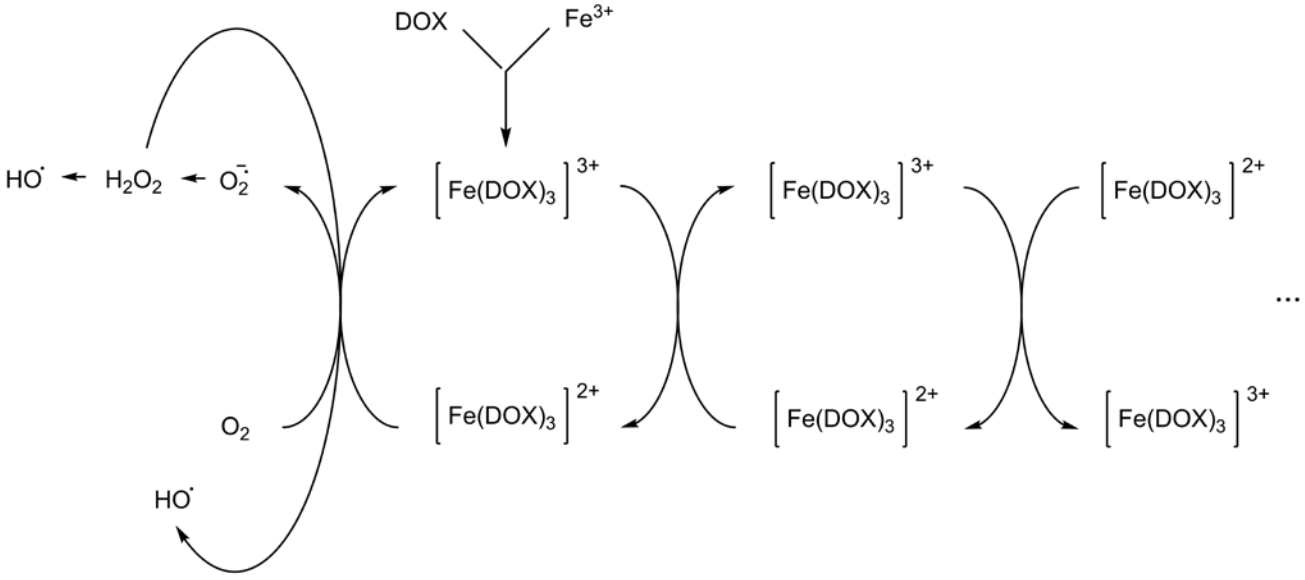

Figure 2 Doxorubicin-stimulated ROS production through free-iron coordination. DOX can complex with free iron in a 1:3 ratio which can then be reduced to a Fe^{2+}-complex by the cellular reducing system, or alternatively by another Fe^{2+}-complex, allowing the cycle to continue indefinitely, which can cause the formation of ROS and cell oxidative stress.

The reduction process can be performed by several oxyreductases; the mitochondrial NADH dehydrogenase (also known as Complex I) is one important example [11]. After DOX activation, the semiquinone free radical donates its unpaired electron to molecular oxygen (O_2), which is converted into a free radical – superoxide anion ($O_2^{\cdot-}$) – and regenerates the parent quinone in the process (Figure 1). The DOX redox-cycle is probably the principal source of mitochondrial ROS production.

DOX also chelates free iron to form a stable complex with a Fe^{3+} to DOX ratio of 1:3 (Figure 2). The complex can then be reduced to a Fe^{2+}-DOX complex by the cellular reducing system or even by another Fe^{2+}-DOX complex. The new Fe^{2+}-DOX complex can univalently reduce O_2, increasing $O_2^{\cdot-}$ levels and regenerating Fe^{3+}-DOX complexes in the process. Alternatively, Fe^{2+}-DOX complexes can convert hydrogen peroxide (H_2O_2), formed by dismutation of the $O_2^{\cdot-}$ produced by previous Fe^{3+}-DOX complexes, resulting in a far more deleterious free radical – the highly reactive hydroxyl radical (HO·). In both mechanisms, the increase in ROS can cause lipid peroxidation and DNA or protein oxidation observed on cardiomyocytes exposed to DOX [25].

STRATEGIES TO COUNTERACT DOXORUBICIN-INDUCED CARDIOTOXICITY

Strategies to prevent DOX toxicity are necessary so higher doses and, therefore, enhanced therapeutic efficacy can be achieved. Different strategies should be capable of minimizing DOX-induced toxicity and should not interfere with antineoplastic activity.

Non-pharmacological strategies, including decreasing the peak drug plasma concentration or decreasing the amount of drug targeted to the heart, can prevent DOX-induced cardiomyopathy. A careful choice on administration schedule showed that weekly administrations of DOX decrease the incidence of heart failure compared to the standard schedule in which DOX is administered every three weeks [4]. Another attempt to decrease DOX cardiotoxicity was by administering DOX by continuous infusion over a 48 h period rather than the standard 15 min bolus infusion [4]. This approach was capable of reducing the drug peak plasma and also the damage in cardiac tissue as accessed by biopsy [49]. However, this procedure was later discarded when it was found that continuous infusion might also reduce the antitumor efficacy probably due to a decrease in the initial concentration at tumor level [50].

Protection against DOX-induced heart damage might also involve the development of new strategies to achieve tumor-targeted delivery of DOX and thereby reduce systemic and cardiac toxicity. The microvasculature in tumors is typically discontinuous and it contains pores big enough for liposomes to move in from the blood to the space surrounding tumor cells. The use of liposomal formulations has already been proven to be capable of enhancing the treatment efficacy with improved pharmacokinetic indexes and lower toxic profile [6] [7] .

The improvement can be explained by several factors including prolonged half-life, prevention of metabolic conversion of DOX to inactive forms or even to aglycones and secondary alcohol metabolites, preferential distribution and prolonged release of the drug within the tumor mass and, finally, limited accumulation in healthy tissues [4]. Currently, two different preparations of DOX-liposomal formulations with superior action and fewer side effects when compared with free DOX have been assessed in experimental models and clinical trials. The first one is a polyethyleneglycol-coated (pegylated) liposomal DOX that has more favorable pharmacokinetics than free DOX and is also capable of overcoming the blood-brain barrier [4] [7]. The other preparation involves uncoated citrate-containing liposomal DOX formulation, where citrate is included to increase DOX encapsulation. The results showed less pronounced pharmacokinetics indexes than pegylated liposomal DOX [51].

The search for novel DOX structure analogues that may offer lower toxicity but have the same clinical efficacy is very active. It was initially observed that different anthracyclines had similar antitumor efficacy and only differed in a few functional groups. The knowledge that the amino-sugar moiety plays a crucial role in DNA intercalation process has enabled the development of new analogues [4] [6]. As a result of the search for 'better anthracyclines', nearly 200 analogues have emerged. This fact should not be surprising, if we consider the number of possible chemical modifications that can be made on the tetracyclic ring or the amino-sugar moiety. However, only a few of those analogues have reached the clinical approval. Epirubicin has been one of the most extensively investigated DOX analogues and is now widely used as an alternative to DOX [4] [7]. Epirubicin shows the same spectrum of antitumoral efficacy although with different pharmacokinetics and metabolism. The toxicity effects are the same in quality but quantitatively less when compared to DOX, which enabled an increase in cumulative doses to almost double of those of DOX (900 to 1000 mg m^{-2}).

In the previous section it was mentioned that apoptosis is one possible endpoint for DOX toxicity. Apoptosis can be initiated by extrinsic or intrinsic signals [43]. However, in both pathways, cytochrome c released from mitochondria seems to be a crucial step. For that reason, mitochondria have a central role in the cell death process. The release of cytochrome c from the mitochondrial intermembrane space allows this protein to interact with monomeric APAF-1 leading to its oligomerization and recruitment of caspase-9 to form the apoptosome [52]. The activated caspase-9 cleaves and activates executioner caspases (caspase-3 and -7). If this event occurs without any restraint the cell will inevitably be conducted to death [52] [53]. Usually, cytochrome c is involved in mitochondrial oxidative phosphorylation and is located primarily in intercristae membrane space, as well as in the mitochondrial intermembrane space [54]. Mitochondrial outer membrane permeabilization (MOMP) is necessary so that the release of this protein to the cytosol can occur [55]. There are two possible mechanisms for this event. The first involves the permeabilization of the inner membrane due to the formation of MPT pore, leading to mitochondrial swelling and outer membrane rupture [44]. The second event involves the oligomerization of Bax within the outer mitochondrial membrane, forming small channels which allow proteins, such as cytochrome c, to access the cytosol [56]. In this case, respiration, as well as ATP production, is not primarily affected because the inner membrane is still intact.

For all the reasons described so far, strategies in which the objective is to maintain normal mitochondrial homeostasis are plausible routes to counteract or at least diminish the extent of apoptosis in cardiomyocytes exposed to DOX. Nevertheless, there are quite few strategies that focus on apoptosis machinery itself. *In vitro* studies show that DOX-induced apoptosis of cardiac cells is prevented by several compounds such as pifithrin-alpha, a p53 inhibitor; survival factors signaled by phosphoinositide kinase; IGF-1; L-carnitine or metallothioneins [4] [55] [57] [58] [59]. Antioxidant compounds also show capacity to decrease apoptosis. This effect may be due to a direct effect on oxidative stress rather than, contrarily to pifithrin-alpha, a direct effect on the apoptosis machinery. Directly blocking DOX-induced cardiac apoptosis with antioxidants may be a double-edged sword in clinical practice since DOX-induced apoptosis of cancer cells would likely be inhibited as well. However, different approaches aimed at preventing oxidative stress, cytosolic calcium dysregulation and DNA damage, which can all trigger cell death, demonstrate the capacity to increase cardiomyocyte viability after DOX treatment [12] [19] [60]. In fact, a

promising approach would consist in the direct and selective inhibition of apoptosis in the heart of DOX treated patients. Other approaches differ from what has been described so far because they attempt to circumvent DOX-induced cardiotoxicity with a special focus on mitochondrial-mediated events. Based on the data described before, prevention of DOX toxicity can also be achieved by the use of iron chelators. Many agents able to remove iron from a complex with DOX have been investigated as possible cardioprotectors. The most effective in clinical trials is dexrazoxane (DEX) which belongs to the bisdioxopiperazine family. The molecule is an analogue of ethylene diamine tetraacetic acid (EDTA) and was initially designed as an antitumor agent [7]. After diffusing to cardiomyocytes, DEX is hydrolyzed to its open-ring form which can strongly chelate iron, thereby displacing Fe^{3+} from its complexes with DOX. Interestingly, the one-ring open intermediate demonstrates the same capacity to remove Fe^{3+} from DOX complexes as DEX [4] [7]. However, DEX can also chelate Fe^{3+} from transferrin, with ferritin depleting iron from storage and transport proteins [61]. Furthermore, DEX is also myelosuppressive and can increase the myelosuppressive effects of DOX [62] [63]. Thus, despite the cardioprotective capacity of DEX, its clinical use is not free of risks and other iron-chelating agents should be developed and tested.

Another approach in the prevention of DOX-increased oxidative stress would be increasing the activity of the cell antioxidant system. The elimination of hydrogen peroxide is one critical step in the detoxification process because it can lead to formation of the hydroxyl radical, a highly reactive molecule. Hydrogen peroxide can be inactivated by two enzymes, catalase, which converts it into water and oxygen, or by glutathione peroxidase, which reduces hydrogen peroxide to water producing oxidized glutathione in the process. As previously described, antioxidant enzymes levels in the heart are relatively low comparative to liver or kidney, which can in part explain the enhanced susceptibility of the heart to DOX toxicity. One possible approach is to increase expression of specific antioxidant enzymes. In fact, transgenic mice overexpressing catalase or glutathione peroxidase, or even superoxide dismutase (SOD), were demonstrated to decrease cardiac damage after DOX treatment [64]. Overexpression of the radical scavenger metallothionein in the hearts of transgenic mice also prevented DOX-induced cardiotoxicity [64] [65], supporting the concept that detoxification by overexpressing antioxidant proteins might be a plausible strategy to circumvent DOX toxicity. However, this expression may not always be restricted to the heart, thus making it necessary to first design a specific cardiac overexpression system. Alternatively, the use of antioxidant agents administered either in the diet or together with DOX may enhance protection of the heart. These compounds can be precursors of antioxidant proteins due to their high thiol content.

One important molecule responsible for the removal of hydrogen peroxide is glutathione. Cellular levels of this protein can be increased by thiol-containing agents such as N-acetylcysteine (NAC), a molecule with high bioavailability which, upon hydrolysis, releases cysteine, a precursor of glutathione biosynthesis [66] [67]. In the same way, cystamine or its reduced form cysteamine, as well as metallothionein, are able to increase cellular glutathione content [64] [68]. The use of these compounds both *in vitro* and *in vivo* has decreased the toxicity of DOX [64]. Unfortunately, the use of NAC had no success in clinical trials even when high doses were administered to patients [69]. The use of antioxidant nutrients have been shown to prevent some damage on cardiac tissue caused by DOX treatment [70]. Vitamins (E, C and A), coenzyme Q, and flavonoids/polyphenols normally found in the diet increased the cellular contents of glutathione and SOD and also decreased malonyldialdehyde and lipid peroxidation demonstrating the capacity to prevent DOX toxicity. In the case of vitamin E (α-tocopherol), this antioxidant agent decreased oxidative stress induced by DOX without preventing the mitochondrial dysfunction associated with the treatment [19].

In one particular work, a diet enriched in vitamin E did not afford protection against DOX-induced State 3 and State 4 inhibitions and decreased calcium loading capacity. However, protein carbonyl content, a marker of oxidized proteins, was decreased in the DOX-treated group fed with an α-tocopherol enriched diet. As reported in the previous case, protection against DOX with antioxidants is not always achieved perhaps due to difference between doses, pre-treatment times, and the type and schedule of DOX-treatment. Also, the ineffective delivery of antioxidants to critical subcellular compartments, such as mitochondrial membranes, might explain the unsatisfactory results observed. Based on this, antioxidants alone might not be a robust strategy to prevent DOX toxicity but when administered together with other agents might improve the treatment efficacy. To overcome the problem of target-specific subcellular compartments, new strategies involving the use of compounds of interest attached to specific molecules (which can be recognized by themselves or even accumulated within those structures) should be developed allowing for specific accumula-

tion of bio-active molecules. In fact, a similar strategy has already been explored in the prevention of lipid peroxidation taking the advantage of using triphenylphosphonium (TPP) cation linked to α-tocopherol (MitoE) or ubiquinone (MitoQ) [71]. TPP is a positively charged lipophilic cation with high oral bioavailability which can easily pass through phospholipid bilayers without a specific uptake mechanism, and accumulate in the cell, driven by the plasma membrane potential (Figure 3, Panel B). Further substantial accumulation within mitochondria occurs, driven by the negative-inside mitochondrial transmembrane potential. The approach allows the accumulation of high concentrations of antioxidants within mitochondria thus decreasing oxidative damage (Figure 3, Panel C). Furthermore, the antioxidant can be recycled back to its active form through the respiratory chain [71].

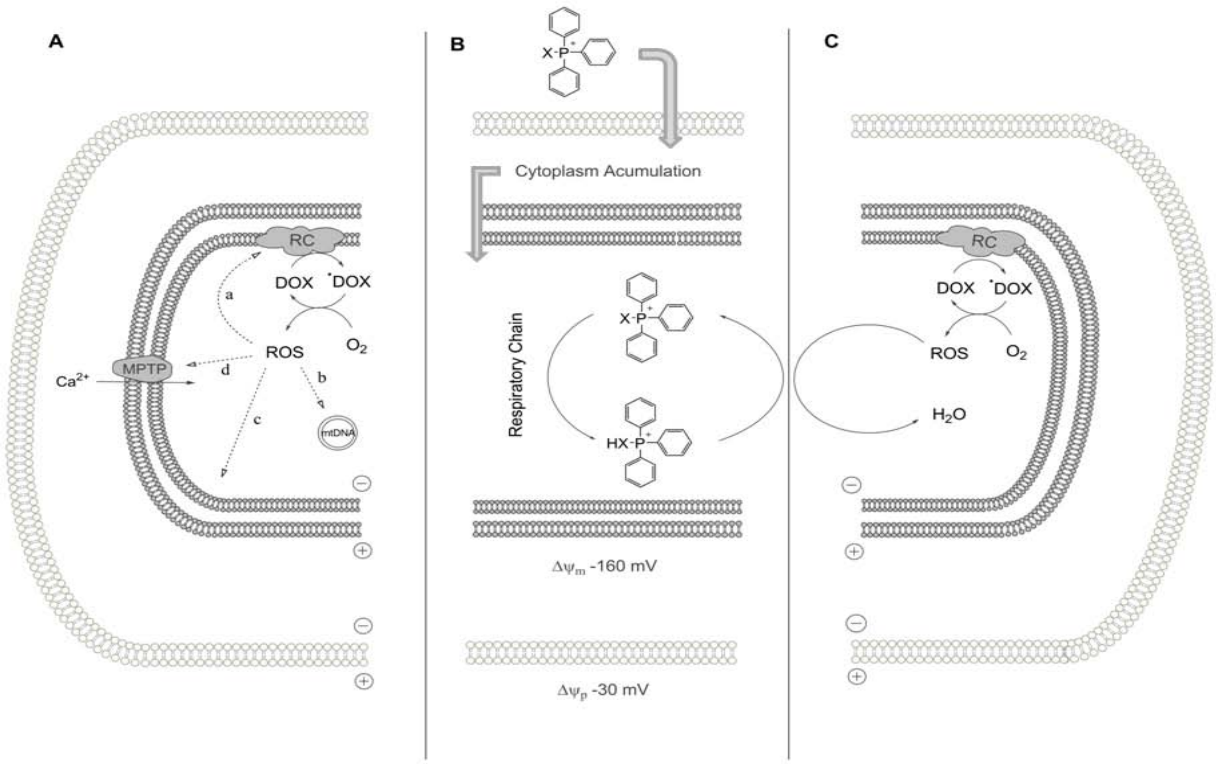

Figure 3 Doxorubicin toxicity and a possible strategy to counteract it. For the sake of simplicity, a single cell, surrounded by a light colored bilayer, contains only one mitochondrial body, surrounded by a dark lipid bilayer. A) Summary of DOX targets and effects. DOX redox cycling at Complex I results in the generation of oxidative stress, which contributes to the oxidation and decline in function of critical mitochondrial proteins (a), DNA (b), membrane lipids (c) and to the formation and opening of the MPT pore (d). B) Cellular accumulation of a bioactive molecule (X) linked to TPP which is a lipophilic cation and can, therefore, accumulate 5-fold in the cytoplasm due membrane potential. Moreover, the high negative mitochondrial membrane potential allows for a 200-fold accumulation inside the organelle. If the linked molecule behaves as an antioxidant, it is possible to have it regenerated by the mitochondrial respiratory chain. Adapted from Murphy et al. [71]. C) Hypothetical strategy to overcome DOX-increased ROS production. If a construct TPP-antioxidant is actively accumulated in mitochondria, it would be possible in theory to counteract DOX-induced oxidative stress, by scavenging free radicals resulting from DOX redox cycle.
Abbreviations: RC: respiratory chain; MPTP: MPT pore.

Several lipophilic cations, such as rhodamine, JC-1 and MitoTracker compounds, share the membrane permeabilization and mitochondrial accumulation capacity of TPP. However, TPP-modified cations are actually the most synthesized and evaluated, probably because TPP is a well known molecule in terms of bioavailability and toxicology and also because the interactions with mitochondria are well understood. Theoretically, any molecule can be linked to TPP moiety to satisfy different targeting strategies. Several antioxidants targeted to mitochondria have been developed, including MitoSOD ($O_2^{\cdot-}$), MitoPeroxidase (H_2O_2), MitoTEMPOL ($^{\cdot}OH$), MitoE2 and MitoQ10 (lipid peroxidation) [71].

Besides their beneficial effects, the toxicity of these compounds should be evaluated as higher concentrations of mitochondrial-targeted compounds can disrupt membrane integrity or inhibit respiration and ATP synthesis [71]. It is clear that the compound of interest should display protective effects at low concentrations in order to prevent cell damaging effects.

A particular compound that can be co-administered with DOX is carvedilol [Kredex, Coreg, (1-[carbazolyl-(4)-oxy]-3-[(2-methoxyphenoxyethyl)amino] -propanol-(2)]. Carvedilol is a β-adrenergic receptor antagonist which blocks β_1, β_2 and α_1 receptors and consequently slows the heart rhythm without increasing blood pressure [72]. The drug is used to treat cases of hypertension and has also demonstrated to be helpful in the management of cardiomyopathies. Carvedilol is primarily used to prevent heart failure, one endpoint of DOX toxicity [72]. In fact, DOX-treated patients showed significant improvement of left ventricular dysfunction after carvedilol treatment [73]. However, not all generic β-blockers are equally helpful against DOX toxicity. In fact, the β-blockers propanolol and atenolol are not as efficient as carvedilol in circumventing DOX-toxicity [12]. Although both compounds are equally effective in reducing blood pressure, they were not capable of preventing mitochondrial dysfunction [12]. Different reports suggest that the greater cardioprotection achieved by carvedilol may be due to its potent antioxidant activity which is unshared by other β-blockers [72]. The antioxidant mechanism of carvedilol was attributed to free radical scavenging through its carbazole moiety [74]. In addition it was also demonstrated that carvedilol can sequester ferric ions, thereby preventing ferric ion-induced oxidation [75].

As mentioned above, an exogenous NADH-dehydrogenase was proposed as the main site of DOX activation in the heart [24]. Interestingly, carvedilol has been shown to inhibit the so-called external NADH-dehydrogenase [76], with the pure β-blocker propanolol showing reduced effects [77]. Although some discussion may arise on the particular site of DOX-activation at complex I level, the existence of a specific inhibitor of DOX activation site can be of great value in the prevention of DOX side effects on the heart. At the cellular level, carvedilol has shown the capacity to prevent most of the toxic effects of DOX in a sub-chronic model of mitochondrionopathy. Carvedilol decreases the extent of cellular vacuolization, and prevents the inhibition of cardiac mitochondrial respiration, the inhibition of complex I of heart mitochondria, and the decrease in calcium loading capacity, all associated with DOX treatment [30]. In other models of pro-oxidant-induced mitochondrial damage, carvedilol was able to inhibit the MPT and reduce cytochrome c release due to inhibition of the oxidation of mitochondrial thiol groups [34]. However, carvedilol was only capable of inhibiting the high-conductance form of the MPT; a subform of the MPT is still active after carvedilol action [34] [78].

The fact that carvedilol was only effective against the MPT induced by oxidative stress [78] demonstrates not only that the antioxidant properties of carvedilol are crucial for its cardioprotective effect but also that this action might be due, in part, to a selective inhibition of a deleterious high-conductance state of the MPT pore [79]. In a sub-chronic model of DOX toxicity (2 mg Kg^{-1} per week to a total of 14 mg Kg^{-1}), carvedilol prevented oxidative damage to mitochondrial complexes and increased mitochondrial calcium loading capacity in DOX-treated animals [30]. Carvedilol possesses a protonophore activity, which is translated into a decrease of mitochondrial transmembrane potential, not shared by propanolol [80]. The protonophoretic mechanism of carvedilol was independent of potassium fluxes across mitochondrial membranes [77]. Raman and theoretical *ab-initio* calculations indicated that the amino side chain group was responsible for protonation/deprotonation cycles [81] [82]. Carvedilol protonophoretic activity might be of extreme importance since a 'mild uncoupling' can induce a reduction on ROS production [83] or prevent mitochondrial calcium overload. Nonetheless, it has been described that carvedilol has no effect on total calcium accumulation by mitochondria but may induce a decrease of ROS produced by mitochondrial respiratory chain [72]. As mentioned elsewhere [84], carvedilol increases the cytotoxicity of DOX as a consequence of inhibition of multidrug resistance protein (MDR). Consequently, the antineoplastic activity of DOX on tumor cells is not impaired, indicating that carvedilol antioxidant and β-receptor antagonism activity could be a very useful tool to counteract DOX-induced cardiotoxicity.

Another important end-point of DOX-induced mitochondrionopathy is calcium homeostasis dysregulation, particularly the stimulation of MPT. Therefore, one plausible approach is the use of a specific inhibitor of the pore – cyclosporin A (CsA) – in a similar fashion to *in vitro* experiments. CsA inhibits the MPT pore through the binding of cyclophilin (CyP) D, a matrix peptidyl-prolyl cis-trans isomerase [42]; the role of CyP-D on the MPT pore physiology is thought to be as a regulator rather than structural. Taken together, CyP-D normally binds to the adenine nucleotide translocator regulating the opening of the

pore which explains why CsA inhibits the MPT. *In vivo* experiments showed that CsA is capable of preventing DOX-induced toxicity end-points, including increased serum LDH and transaminases and decreased calcium loading capacity [60]; tracolimus (FK506) prevented DOX cardiotoxicity as well. FK506 and CsA are both immunosuppressants, although the former does not bind CyP-D, thus not affecting MPT opening. The immunosuppressive effects are caused by the Ca^{2+}-calmodulin-dependent inhibition of calcineurin through the complex between CsA or FK506 and CyP-A [42]. Although direct effects on calcineurin activity are not usually involved on MPT inhibition, calcineurin may affect mitochondrial functions through dephosphorylation of BAD releasing pro-apoptotic factors [42].

Taken together, the immunosuppressive effects of the drugs could prevent cardiomyocyte death through interaction with CyP-A, without explaining the prevention by FK506 of DOX-induced loss calcium loading capacity. More research is necessary to understand the effects of FK506 in the prevention of DOX-induced calcium homeostasis dysregulation, as well as to understand the systemic effects due to the use of immunosuppressants as cardio-protection therapy. It would also be of interest to develop and to evaluate the protective effects of CsA derivatives, without immunosuppressive activity but with the capacity to bind to CyP-D, against DOX-induced cardiotoxicity.

Although some caution should be taken, ischemic events and DOX-induced cardiomyopathy are fairly similar. For example, in both processes increased ROS production and calcium accumulation are observed, resulting (in some cases) in the stimulation of MPT. Previous studies have suggested that mitochondrial ATP-sensitive K+ channels (mitoKATP) play a role in the mechanism of ischemic preconditioning mediated cardioprotection [85]. This mechanism is probably due to mild uncoupling and increased matrix volume after mitoKATP opening which leads to a decrease in ROS production and ATP hydrolysis. A decrease in transmembrane membrane potential, which decreases matrix calcium accumulation, also contributes to energy conservation and prevention of the MPT. Based on this data, it is acceptable to think that mitoKATP opening may attenuate DOX-induced toxicity and, in fact, this approach was already explored showing promising preliminary results [86] [87].

However, most mitoKATP agonists and antagonists also present other effects on mitochondrial and cellular function, even for micromolar concentrations [85]. One of the strategies used combined sildenafil and a selective inhibitor of cGMP specific phosphodiesterase-5, which protected against oxidative stress injury through opening mitoKATP in a chronic model of DOX cardiotoxicity [86]. In this model, sildenafil was capable of attenuating cardiomyocyte apoptosis, of maintaining mitochondrial membrane potential, and preventing left ventricular dysfunction and ST prolongation. All protective effects were abolished in the presence of mitoKATP inhibitors. As also referred by the authors, sildenafil has proved to be a safe drug for different pathologies, including erectile dysfunction and pulmonary hypotension, which makes it acceptable to counteract DOX-induced cardiotoxicity. Although its effects on DOX neoplastic activity were not evaluated, sildenafil would allow the use of increased doses of DOX improving its treatment efficacy.

CONCLUDING REMARKS

Doxorubicin is a powerful antineoplastic agent and therefore it is prescribed for several human and animal cancer treatments. However, together with its high potential efficacy the treatment is accompanied by a selective and cumulative cardiotoxicity which, ultimately, may escalate into heart failure. Since its discovery, efforts to counteract DOX-induced cardiotoxicity have been made. However, none of the preventative measures are fully efficient, possibly because the full extent of DOX toxicity mechanism still remains to be discovered. One particular target of DOX is the mitochondrial compartment. DOX is activated in mitochondria by undergoing a redox-cycle in mitochondrial Complex I, which results into ROS production. Although increased ROS production upon DOX treatment is thought to be one of the major events responsible for DOX toxicity, calcium homeostasis dysregulation has also received particular attention.

Since the mitochondrial function is extremely important for the cardiac tissue due to a high energy demand, preventing mitochondrial dysfunction would have exceptional importance to prevent toxicity. The most important strategies which have mitochondria as their main target involve antioxidants or inhibitors of the MPT pore. However, few of those have shown high efficacy within *in vivo* models, probably because mitochondria targeting was not effective. It is therefore necessary to develop strategies involving molecules such as MitoQ or MitoE which have lipophilic cations attached to molecules of interest and are selectively accumulated within mitochondria.

CONCLUSION

Mitochondrial-targeted strategies have shown capacity to prevent DOX-induced cardiotoxicity and might become the most efficient means to decrease DOX cardiotoxicity. The discovery of every mechanism of DOX toxicity and the development of new strategies to counteract them should coincide.

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CONFLICT OF INTERESTS/DISCLAIMERS

PJ Oliveira is member of the Editorial Board of the journal. There are no other conflicts of interest to declare.

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