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BERBERINE MODULATION ON DOXORUBICIN-INDUCED CELL DEATH AND AUTOPHAGY

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Bioquímica, realizada sob a orientação científica do Doutor Paulo Oliveira (Centro de Neurociências e Biologia Celular, Universidade de Coimbra) e do Professor Doutor António Moreno (Universidade de Coimbra).

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I declare that this is a true copy of my dissertation, including any final revisions, as approved by my dissertation committee, and that this dissertation has not been submitted for a higher degree to any University or Institution.

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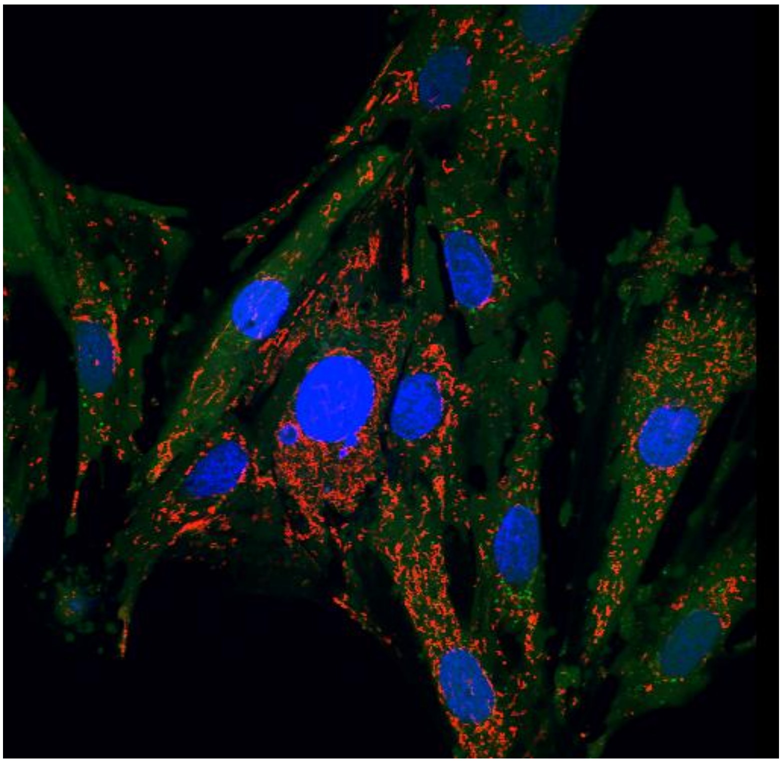

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[1] H9c2 cells treated with Berberine for 72 hours, co-treated with Doxorubicin in the last 24 hours.Images shows TMRM mitochondrial fluorescence, co-labeled with Hoechst 33342 (blue, nuclei) and calcein (green, viable cells). Image credit: Dr. Paulo Oliveira

“I’m not sure what I’ll do, but—well, I want to go places and see people. I want my mind to grow.”

The Ice Palace (1920), F. Scott Fitzgerald

ABSTRACT

Doxorubicin (DOX) is a widely prescribed and effective anti-cancer agent. The clinical use of DOX has been associated mainly with a cumulative and dose-specific cardiotoxicity that involves the development of congestive heart failure. DOX-induced delayed cardiomyopathy may occur or be aggravated through the decrease of cardiac progenitor cells pool in juvenile patients. In fact, has been reported that, DOX progressively decreases the pool of cardiac progenitor cells which combined with oxidative damage can lead to a progressive decrease in cardiomyocyte number. Oxidative stress is in fact a major event responsible for many alterations observed in normal cardiac cell function during DOX toxicity. Increased ROS generation induces alterations in ion homeostasis, alterations in iron metabolism, disruption of calcium homeostasis and mitochondrial dysfunction. DOX-induced free radical formation leads to pathological alterations in lipids, proteins, nucleic acids and biomolecules which can result in the mobilization of apoptotic machinery and consequent caspase activation. Autophagy may act as a compensatory mechanism that can control cell damage and restore energy homeostasis during DOX cardiac toxicity. If autophagy becomes uncontrolled this might lead cardiomyocyte to apoptosis, resulting in increased DOX toxicity. Because of the importance of DOX on anti-cancer therapy, new compounds that can inhibit the toxicity are needed. Berberine (BER) is a natural compound used in traditional chinese medicine that besides it cardioprotective properties, can modulate DOX-induced cardiotoxicity.

The objective of this work is to understand if BER can modulate cell death and autophagy in H9c2 cells treated with DOX. To understand the effect of BER on DOX-induced cell death and autophagy, H9c2 cells were pre-treated with BER 48 hours before DOX administration (24 hours). Cell viability, caspases and cathepsins activity, mitochondria-lysosome co-localization and autophagy markers were measured in this work.

Our results showed that BER inhibit DOX-induced caspases 9 and 3- like activation. In addition, BER appeared to inhibit autophagy in cells treated with

DOX. Moreover, BER was unable to prevent DOX-induced mitochondrial alterations. Nevertheless, mitochondrial biogenesis markers were upregulated by BER in the presence of DOX.

Although several mitochondrial alterations were not prevented by BER, apoptosis and autophagy were decreased by that alkaloid. Although more studies are needed, this combination could translate in future therapeutic strategies to allow the use of higher cumulative doses in DOX- cancer chemotherapy, proven that BER does not impair DOX anti-cancer efficacy.

Keywords: Berberine, Doxorubicin, Cardiotoxicity, Apoptosis, Autophagy

RESUMO [2]

A Doxorubicina (DOX) é um fármaco eficaz contra lesões neoplásticas globalmente prescrito. O uso prolongado da DOX tem sido associado a cardiotoxicidade que é dependente da dose utilizada e da sua acumulação no coração. A acumulação tecidual da DOX tem sido relacionada com o desenvolvimento de falha cardíaca congénita. A Cardiomiopatia induzida pela DOX que anos depois do último tratamento poderá ocorrer ou ser agravada devido à diminuição da quantidade de células progenitoras cardíacas em pacientes de idade pediátrica. Sabe-se que a DOX leva ao decréscimo progressivo da quantidade de células cardíacas progenitores presentes no coração o que combinado com o dano oxidativo pode levar à diminuição da quantidade de cardiomiócitos. O stresse oxidativo é o maior evento responsável pelas alterações na função normal em células cardíacas saudáveis quando exposta à toxicidade da DOX. O aumento da produção de espécies reactivas de oxigénio leva a alterações no metabolismo do ferro, alterações na homeostase iónica e à disfunção mitocondrial. A formação de radicais livres e o aumento do stresse oxidativo leva a sérias alterações nos lípidos, nos ácidos nucleicos e em biomoléculas importantes que resultam em acumulação de danos celulares e à mobilização de maquinaria apoptótica que culmina na activação de caspases. A autofagia pode actuar então como um mecanismo compensatório de controlo de danos celulares e de recuperação da homeostase energética durante a cardiotoxicidade induzida pela DOX. No entanto, se o fluxo autofágico se torna descontrolado pode levar ao desencadeamento de outros processos de morte celular, podendo agravar o desenvolvimento de cardiomiopatias. Tornou-se então importante a procura de novos compostos que inibissem a toxicidade provocada pela DOX, devido à sua importância clinica no tratamento contra o cancro. A Berberina, composto natural muito utilizado na medicina tradicional chinesa, apresenta propriedades cardioprotectoras que parecem modular a cardiotoxicidade provocada pela DOX.

O objectivo deste trabalho foi perceber em que se a BER consegue modular a morte celular e a autofagia em cardiomioblastos H9c2 tratados com DOX. Para perceber o seu efeito, as células H9c2 foram pré-tratadas com BER 48 horas

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[2] Por decisão da autora, não foi aplicado nesta dissertação o Acordo Ortográfico da Língua Portuguesa de 2011

antes da administração com DOX (24 horas). Foi avaliado o efeito da BER na viabilidade celular; na actividade de caspases e catepsinas; na co-localização entre mitocôndrias e lisossomas; e em marcadores de autofagia.

Os nossos resultados mostraram que a BER conseguia inibir a activação das caspases 3 e 9 provocada pelo tratamento com DOX em células H9c2. A BER também pareceu capaz de inibir a autofagia em células tratadas com DOX. A BER, no entanto, não pareceu induzir capaz de prevenir as alterações mitocondriais provocadas pela DOX. Apesar disso, os marcadores de biogénese mitocondrial estavam aumentados em células tratadas com BER e DOX.

Em suma, a BER não preveniu as alterações mitocondriais, apesar de ter prevenido a apoptose e autofagia. É interessante continuar a avaliar este efeito da BER e não é de descartar a hipótese da combinação de BER e DOX poder ter novas aplicações terapêuticas, permitindo o uso da DOX em doses cumulativas mais elevadas no tratamento contra o cancro, desde que não afecte a eficácia anti-neoplásica da DOX.

Palavras- chave: Berberina, Doxorubicina, Cardiotoxicidade, Apoptose, Autofagia

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ABBREVIATIONS

ADP	Adenosine Diphosphate
AIF	Apoptosis Inducing Factor
AMPK	AMP-activated Protein Kinase
ANT	Adenine Nucleotide Translocator
ATP	Adenosine Triphosphate
Bcl-2	B-cell Lymphoma 2
Bcl-xl	B-cell Lymphoma-extra Large
BER	Berberine
CHF	Congestive Heart Failure
CPC	Cardiac Progenitor Cells
CyP-D	Cyclophilin D
DNA	Deoxyribonucleic Acid
DOX	Doxorubicin
DOX$^{\bullet}$	Doxorubicin Semi-quinome Radical
DSB	DNA Double Stranded Breaks
DZX	Dexrazoxane
EPI	Epirubicin
ETC	Electron Transport Chain
FDA	Food and Drug Administration
HSP27	Heat shock Protein (molecular weight: 27 kDa)
hsc70	Heat Shock Chaperone (molecular weight: 70 kDa)
IMM	Inner Mitochondrial Membrane
LC3	Microtubule-associated Protein 1A/1B-light Chain 3
MAPK	Mitogen-activated Protein Kinase
MPT	Mitochondrial Permeability Transition
mtDNA	Mitochondrial DNA
mTOR	Mammalian Target of Rapamycin
mTORC1	Mammalian Target of Rapamycin Complex 1
NAD(P)H	Nicotinamide Adenine Nucleotide (Phosphate), reduced form

NADPH	Nicotinamide Adenine Nucleotide Phosphate, reduced form
NF-kβ	Nuclear Factor Kappa-light-chain-enhancer of Activated B Cells
OMM	Outer Mitochondrial Membrane
PERK	PKR-like ER kinase
PI3K	Phosphatidylinositol 3-Kinase
PINK1	Phosphatase and Tensin homolog–induced Putative Kinase 1
pNA	p-nitroanilide
PVDF	Polyvinylidene Difluoride
ROS	Reactive Oxygen Species
SDS- PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SIRT1	Sirtuin 1
SIRT3	Sirtuin 3
SOD	Superoxide Dismutase
Tfam	Mitochondrial Transcription Factor A
TOM20	Translocase of Outer Membrane (molecular weight: 20 kDa)
ULK	Atg1/unc-51-like Kinase
VDAC	Voltage-dependent Anion Channel
VPS	Vacuolar Protein Sorting
WHO	World Health Organization
$\Delta\Psi_m$	Mitochondrial Transmembrane Electric Potential

Part I

General Introduction

Chapter 1
Doxorubicin cardiotoxicity

Cancer is a leading cause of deaths worldwide with 7.6 million deaths in 2008 only. According to World Health Organization (WHO), the number of deaths are projected to continue rising with an expected account of 13.1 million deaths in 2030 (Cancer Research UK 2011, Cancer Research UK 2012, Cancer Research UK 2013). Anthracyclines are the most effective and widely prescribed cancer agents approved by the Food and Drug Administration (FDA) (Hrdina, Gersl et al. 2000, Wallace 2003, Carvalho, Santos et al. 2009).

Doxorubicin (Figure 1), (7S, 9S)-7-[(2R, 4S, 5S, 6S)-4-amino-5-hydroxy-6-methyloxan-2-yl]oxy-6, 9, 11-trihydroxy-9-(2-hydroxy-acetyl)-4-methoxy-8, 10-dihydro-7H-tetracene-5, 12-dione, is an antibiotic that belongs to anthracycline family. This compound was isolated in the late 1960s from a variant culture of *Streptomyces peucetius* var. *caesius* by aerobic fermentation after a mutagenic treatment (Arcamone, Cassinelli et al. 1969, Arcamone, Franceschi et al. 1969). DOX is characterized by a wide range of activity, being effective against several types of tumors (Hrdina, Gersl et al. 2000, Carvalho, Santos et al. 2009).

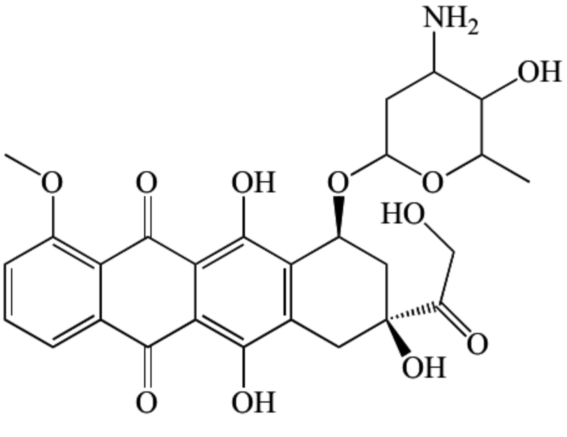

Figure 1: Chemical structure of DOX. DOX is an anthracycline constituted by a tetracyclic aglycone and the aminosugar daunosamine.

Structurally, DOX is a glycoside composed by a tetracyclic aglycone that possesses a substituted anthraquinone and the aminosugar daunosamine

(Arcamone, Franceschi et al. 1969). Similarly to many other anticancer agents, DOX specific chemical structure is responsible for the antineoplastic efficiency but also for its toxicity, which will be discussed further in this chapter (Carvalho, Santos et al. 2009, Pereira, Silva et al. 2011). In the past years, researchers have attempted to prevent toxicity which the development of second generation anthracyclines or the combined treatment with different agents including antioxidants have been the most studied (Minotti, Menna et al. 2004, Oliveira, Bjork et al. 2004, Berthiaume, Oliveira et al. 2005, Chua, Liu et al. 2006, Zhang, Feng et al. 2011, Chen, Hu et al. 2013).

1.1. Doxorubicin as a therapeutic agent

Since DOX discovery in 1960’s, the clinical use of this drug was extended to different types of cancers such as acute leukaemias and lymphomas, sarcomas, malignant neoplasms of bladder, breast, lung, ovary, stomach and thyroid being considered one of the most successful anticancer drugs available (Bonadonna, Monfardini et al. 1969, Carvalho, Santos et al. 2009).

Doxorubicin chemotherapy is generally administrated intravenously to patients at doses between of 15 and 90 mg/m^2, reaching initial DOX plasma concentration of approximately 1µM, until a maximum cumulative dose of 500-550 mg/m^2 (Gewirtz 1999). DOX binds to plasma proteins being widely distributed through tissues (Danesi, Fogli et al. 2002). Nevertheless, DOX clearance is slow taking almost 7 days to being totally secreted from the organism after treatment (Danesi, Fogli et al. 2002).

Despite of DOX chemotherapeutical potential, this drug causes toxicity in several tissues. Patients undergoing DOX treatments may present side effects such as alopecia, mucositis, leukopenia, fever, nausea, phlesitis and the most hazardous of all, cardiovascular complications (Bonadonna, Monfardini et al. 1969, Hrdina, Gersl et al. 2000). Although most clinical side effects supra-cited are clinically manageable, there is not a clinical available solution to prevent cardiovascular complications derived from DOX selective cardiotoxicity. Therefore, DOX usage as an antineoplastic agent has been limited to lower cumulative doses which affect its antineoplastic efficiency.

1.1.1 Mechanisms for antineoplastic activity

Doxorubicin enters tumor cells through either simple diffusion or carrier-mediated diffusion (Skovsgaard and Nissen 1982). There are two main mechanisms that may be involved in the antineoplastic activity of DOX: 1) production of reactive oxygen species (ROS) stimulated by DOX or its metabolites and 2) the inhibition of DNA synthesis by intercalation of the drug in the DNA or the inhibition of topoisomerase II activity (Tewey, Rowe et al. 1984, Gewirtz 1999, Kiyomiya, Matsuo et al. 2001).

Oxidative damage may in fact be an important mechanism that causes cellular damage and further death in tumor cells. DOX has the ability to augment the flow of electrons from NAD(P)H to O_2, generating a semiquinone radical. This process, that will be further discussed, is supported by a range of cellular oxidoreductases, including mitochondrial NADH dehydrogenase, NADPH cytochrome P450 reductase and xanthine oxidase. The semiquinone free radical then generates superoxide anion ($O_2^{\bullet-}$), which can be further dismuted to hydrogen peroxide (H_2O_2) (Bachur, Gordon et al. 1978, Doroshow 1983, Peters, Gordon et al. 1986, Yee and Pritsos 1997, Gewirtz 1999, Danesi, Fogli et al. 2002). DOX semiquinone can also become oxidized resulting in the formation of 7-deoxyaglycone that can intercalate into biologic membranes due to its increased lipid solubility, generating more ROS in the process (Minotti, Menna et al. 2004). The semiquinone free radical may have enough stability to enter the nucleus and bind DNA with high affinity and either react with or generate $O_2^{\bullet-}$ and H_2O_2, inducing extensive damage to DNA (Bachur, Gordon et al. 1978, Bachur, Gee et al. 1982).

Importantly, DOX can interfere with DNA-related functions such as DNA replication and RNA synthesis in cancer cells. Due to DOX capacity to bind tightly to DNA, Topoisomerase II binding activity may be blocked (Tewey, Rowe et al. 1984). On the other hand, DOX also inhibits the catalytic activity of Topoisomerase II by affecting the cleavage-binding reaction due so stabilization of the cleavage complex Topoisomerase II-DNA. This results in double-stranded breaks

(DSB) that may lead to cell death in case unrepaired damage (Liu, Rowe et al. 1983, Tewey, Rowe et al. 1984, Bodley, Liu et al. 1989, Duran, Lau et al. 1996, Swift, Rephaeli et al. 2006). Likewise, DOX is able to penetrate in the mitochondrial matrix and interact directly with mtDNA (Lebrecht, Setzer et al. 2003). Mitochondrial DNA-anthracycline intercalation induces nucleoid aggregation, inhibition of replication, cell cycle arrest and depletion which can also contribute to the antitumor action of this drug (Ashley and Poulton 2009). Moreover, DOX increased ROS generation induce cell damage which can result in cell death. All in all, DOX anti-proliferative mechanisms may result from both cell cycle arrest and cell death.

1.2 Doxorubicin-mediated Cardiotoxicity

The clinical use of DOX has been associated with the development of toxicity in healthy tissues being cardiotoxicity is the most known side effect.

Doxorubicin-induced cardiotoxicity can exert acute and chronic cardiovascular complications which can occur in different phases of the treatment and in some cases even several years after. Acute DOX cardiotoxicity generally occurs during or after a few days following the treatment and affects 11% of the patients. DOX acute effects includes symptoms as arrhythmias, hypotension and irregular electrocardiogram changes that are usually reversible and clinically manageable (Lefrak, Pitha et al. 1973, Hrdina, Gersl et al. 2000, Takemura and Fujiwara 2007). Chronic DOX-induced cardiac toxicity is dose-related and the probability to develop cardiac complications increase with the total dose administrated. Patients that receive total cumulative doses higher than 550 mg/m^2 may trigger the onset of congestive heart failure (CHF) and chronic cardiomyopathies (Lefrak, Pitha et al. 1973, Steinherz, Steinherz et al. 1991). DOX-induced cardiomyopathy and CHF incidence occurs in more than 4% of patients who received a total cumulative dose of 500- 550 mg/m^2 and increase for about 36% for doses higher than 600 mg/m^2 (Lefrak, Pitha et al. 1973, Takemura and Fujiwara 2007). Chronic cardiomyopathy, that can be developed within a month or past several years after the treatment (Takemura and Fujiwara 2007). Chronic cardiomyopathy and CHF include symptoms such as cardiac enlargement and arrhythmia which can lead to death in 50% of the patients to whom higher cumulative doses were administrated (Lefrak, Pitha et al. 1973, Steinherz, Steinherz et al. 1991, Takemura and Fujiwara 2007).

Nevertheless, DOX cardiotoxicity appears to be more related to the peak plasma concentration whereas antineoplastic efficiency which is higher with the increased drug exposure. (Senkus and Jassem 2011) (Gharib and Burnett 2002)

1.2.1 Why is the heart more affected?

There are several reasons that explain why DOX-induced toxicity affects mainly the heart. On one hand, the heart tissue has a large density of mitochondria due to his high energy demand, with DOX and its metabolites accumulate mostly in nuclei and in mitochondria (van Asperen, van Tellingen et al. 1999, Anderson and Arriaga 2004, Tokarska-Schlattner, Zaugg et al. 2005). DOX binds with high affinity to the inner mitochondrial membrane (IMM) phospholipid cardiolipin in a 2:1 molar ratio. The complex DOX-cardiolipin alters the membrane organization and results in the inhibition of complexes I -III and IV (Goormaghtigh, Huart et al. 1986, Nicolay and de Kruijff 1987, Goormaghtigh, Huart et al. 1990). Also, the proposed existence of a cardio-selective exogenous NADH dehydrogenase independent of Complex I may mediate direct reduction of DOX in the heart (Nohl 1987). Although, its existence is very polemic (Fraisse, Rey et al. 1993). Another possible explanation regards the low levels of antioxidants such as glutathione peroxidase in the heart, when compared with other tissues. Li and colleagues demonstrated that DOX induces downregulation of the copper-zinc SOD while other antioxidant enzymes such as glutathione peroxidase, catalase and manganese SOD were not affected. Therefore, the comparatively low antioxidant content may limit the protection against oxidative damage induced by DOX in the heart (Odom, Hatwig et al. 1992, Li, Danelisen et al. 2002). On the other hand, cardiomyocytes are post-mitotic cells which make cardiac recovery after DOX-induced damage very difficult. DOX not only affects cardiac post-mitotic cells but also cardiac progenitor cells (CPC) inhibiting their cell cycle which combined with oxidative damage leads to apoptosis (De Angelis, Piegari et al. 2010, Huang, Zhang et al. 2010). Ultimately, the decrease in the pool of CPCs may aggravate DOX-induced cardiomyopathy (De Angelis, Piegari et al. 2010).

Cardiotoxicity appears to result from different mechanisms from the ones responsible for DOX antineoplastic activity. Increased generation of ROS has been considered a major cause that leads to several apparently distinct events such as cellular loss, alterations in ion homeostasis, alterations in iron metabolism, disruption of calcium homeostasis and mitochondrial dysfunction (Jeyaseelan, Poizat et al. 1997, Jung and Reszka 2001, Berthiaume and Wallace 2007). The increase in ROS production results from the activation of DOX by reductive enzymes, especially mitochondrial NADH dehydrogenase, resulting, as described, in a DOX semi-quinone radical ($DOX^{\bullet}$) through one electron transfer. $DOX^{\bullet}$ then reduces O_2 regenerating the oxidized state and yielding $O_2^{\bullet -}$ (Davies, Doroshow et al. 1983). This reaction establishes a redox cycle that significantly increases the amount of $O_2^{\bullet -}$ generated (Bachur, Gordon et al. 1978, Berthiaume and Wallace 2007). The superoxide anion can still be converted by SOD in H_2O_2 and the hydroxyl radical ($^{-\bullet}OH$) may be yielded from iron-catalysed Haber-Weiss reaction (Davies, Doroshow et al. 1983). Doxorubicin also binds directly to iron (Xu, Persson et al. 2005), with the DOX-iron (III) complex being reduced to DOX-iron (II) complex by agents such as NADPH cytochrome P450 reductase, glutathione, and cysteine. In these reactions, the anthracycline quinone moiety is converted to a semiquinone radical coupled to the formation of $O_2^{\bullet -}$ (Xu, Persson et al. 2005). Therefore, DOX-induced free radical formation leads to pathological alterations in several cellular constituents such as lipids, proteins, nucleic acids and biomolecules, which ultimately may result in cell death (Doroshow 1983, Xu, Persson et al. 2005, Berthiaume and Wallace 2007).

1.2.2 Doxorubicin- induced Cardiotoxicity: the role of mitochondria

Mitochondrial oxidative damage and calcium overload are events that have been described to occur after DOX chronic treatment (Zhou, Heller et al. 2001, Zhou, Starkov et al. 2001) (Solem, Henry et al. 1994). Alterations in calcium homeostasis can result from the inhibition of important ionic pumps in the sarcoplasmic reticulum, mitochondria and sarcolemma such as the Na^+/Ca^{2+} exchanger and the Na^+/K^+ ATPase by DOX metabolite, doxorubicinol (Olson, Mushlin et al. 1988, Olson, Gambliel et al. 2005, Pereira and Oliveira 2008). This type of alterations causes calcium dysregulation, with mitochondria accumulating excessive calcium in the matrix. DOX and their metabolites also decrease the capacity of mitochondria to retain and accumulate calcium causing a secondary increase in ROS production (Pereira and Oliveira 2008). In addition, DOX induces stimulation of mitochondrial calcium cycling that cause energy depletion in the cell. This energy consuming calcium cycling constitutes a futile cycle of mitochondrial calcium release and re-uptake that can result in calcium-dependent depolarization of the mitochondrial membrane (Solem and Wallace 1993, Solem, Henry et al. 1994, Zhou, Heller et al. 2001). Failure of mitochondrial function increases bioenergetic stress because of the depression in ATP production (Zhou, Heller et al. 2001). Moreover, the enhancement of calcium release results into an alkalinization that is a fundamental requirement for mitochondrial permeability transition (MPT) induction (Solem, Henry et al. 1994).

The MPT is characterized by a progressive permeabilization of the IMM to protons, ions and small proteins through non-specific pores, the MPT pore. It was recently proposed that the MPT pore is formed by dimmers of the ATP synthase. Alavian and colleagues suggested that ATP synthase sub-unit c comprises the MPT pore exposed during ATP synthase uncoupling, being and regulating cell death (Alavian, Beutner et al. 2014). DOX-induced oxidative stress, already discussed above, may induce alterations in the redox state of specific proteins and therefore increase the sensibility to MPT pore induction. Increased oxidation of adenine nucleotide translocator (ANT) thiol groups may contribute to increased calcium-induced MPT pore opening and inhibition of state 3 of respiration

(Oliveira, Santos et al. 2006). There are evidences that ANT can also be inactivated due to peroxidative damage (Pereira and Oliveira 2008). Furthermore, DOX-induced decrease in the pool of functional ANT can contribute to decreased mitochondrial respiration (Oliveira and Wallace 2006). On the other hand, cardiolipin interacts tightly with the ANT in a stoichiometry of three cardiolipin molecules per protein monomer which stabilizes the dimeric structure. This kind of interaction can control the conformational changes and may have a role in regulating ADP/ATP exchange. DOX-induced free radical formation leads to damage in lipids, namely through peroxidation. Cardiolipin peroxidation provokes ANT conformational destabilization, leading also to MPT pore induction (Paradies, Petrosillo et al. 2009). In addition, cardiolipin molecules are an abundant IMM phospholipid that interacts with several proteins including electron transport chain (ETC) proteins. The established interactions are required for the optimal activity of Complex I, Complex III, Complex IV, ATP synthase as well as the formation and stabilization of supercomplexes (Paradies, Petrosillo et al. 2009). Thus, it is not surprising that mitochondrial dysfunction induced by lipid peroxidation may also result from the destabilization of ETC complexes (Pereira and Oliveira 2008, Paradies, Petrosillo et al. 2009).

Cardiac cells that are exposed to DOX chronic treatment are more susceptible to agents that cause an increase in cytosolic calcium concentration, including caffeine, which increase the probability of induction of MPT pore and leads to mitochondrial depolarization, energy depletion and cell death (Berthiaume and Wallace 2007).

1.3 H9c2 cells as a model to study Doxorubicin- induced Cardiotoxicity

The H9c2 cell line, developed by Kimes and Brandt, is a myoblastic heart-derived cell line and is derived from the original clonal embryonic cell line of the BD1X rat heart (Kimes and Brandt 1976). Morphologically, H9c2 cells present a spindle-to-stellate shaped form and might be mono- or multi-nucleated being similar to embryonic cardiomyocytes (Hescheler, Meyer et al. 1991, Sardao, Oliveira et al. 2009).

The H9c2 cell line has been a recurrent model not only to study DOX toxicity but also several compounds in a cardiac tissue surrogate. Several authors have been describing damage in H9c2 induced by DOX including DNA damage (L'Ecuyer, Sanjeev et al. 2006), mitochondrial dysfunction (Green and Leeuwenburgh 2002, Sardao, Oliveira et al. 2009) and cell death (Sardao, Oliveira et al. 2009, Chen, Wu et al. 2011). Moreover, this cell line has also been object of study of others compounds that might have some protective effect over DOX-induced toxicity such as flavonoids (Kaiserova, Simunek et al. 2007, Chen, Hu et al. 2013), carvedilol (Spallarossa, Garibaldi et al. 2004, Sgobbo, Pacelli et al. 2007) and pifithrin-α (Chua, Liu et al. 2006). Nonetheless H9c2 cell line is phenotypically more homogeneous than primary cultures and the whole tissue, DOX treatments leads to H9c2 hypertrophy being consistent to what were observed in *in vivo* experiments with rat adult cardiomyocytes during the development of the DOX-induced cardiomyopathy (Kimes and Brandt 1976, Lushnikova, Klinnikova et al. 2004, Merten, Jiang et al. 2006).

Due to the studies performed in the past few years on DOX-induced damage to H9c2 cells, this cell line is considered a good cellular model to perform studies related to DOX-induced cardiotoxicity.

Chapter 2
Doxorubicin-induced Cell Death and Autophagy

Mitochondrial dysfunction, alterations in calcium homeostasis, decrease of oxidative phosphorylation capacity and increase of MPT pore opening susceptibility are all events related with DOX-induced cardiotoxicity. These alterations to the normal cells function usual leads them to apoptotic cell death. However cardioprotective mechanisms can be activated allowing the elimination of damaged cellular components and the maintenance of energy homeostasis. The balance between cell death and protective autophagy may contribute to stop cardiomyopathy progression.

2.1 Doxorubicin-induced cell death

2.1.1 Apoptosis

Apoptosis is a “cell-suicide” mechanism in which the cell activates several signalling mechanisms in response to accumulated damage and culminates in cell death.

Apoptosis or programed cell death is characterized by the rounding-up of the cell with volume reduction, chromatin condensation, nuclear fragmentation and plasma membrane blebbing followed by phagocytation (Elmore 2007, Taylor, Cullen et al. 2008, Kroemer, Galluzzi et al. 2009). These morphological alterations are mainly due to the activation of a group of cysteinic proteases, caspases (Hengartner 2000, Kroemer, Galluzzi et al. 2009). There are over a dozen capases identified. The most relevant for apoptosis program are divided into two different cathegories: initiator caspases (caspases 8, 9, 10) and executioner caspases (caspases 3,6,7) (Elmore 2007, Taylor, Cullen et al. 2008). Caspases can be involved in two different apoptosis pathways, namely intrinsic and extrinsic. The intrinsic pathway is directly activated by intracelular damage, especially in mitochondria. Intracelular damage leads to events such as $\Delta\Psi_m$ loss which influence MPT pore opening, leading to the release of cytochrome c to cytosol, among others pro-apoptotic proteins. These events lead to the activation of caspase-dependent mitochondrial pathway in which cytochrome c binds to Apaf-1 and pro-caspase 9 which forms the apoptosome. Caspase 9 then activates caspase 3 that propagates the apoptotic signalling (Hengartner 2000, Elmore 2007, Taylor, Cullen et al. 2008).

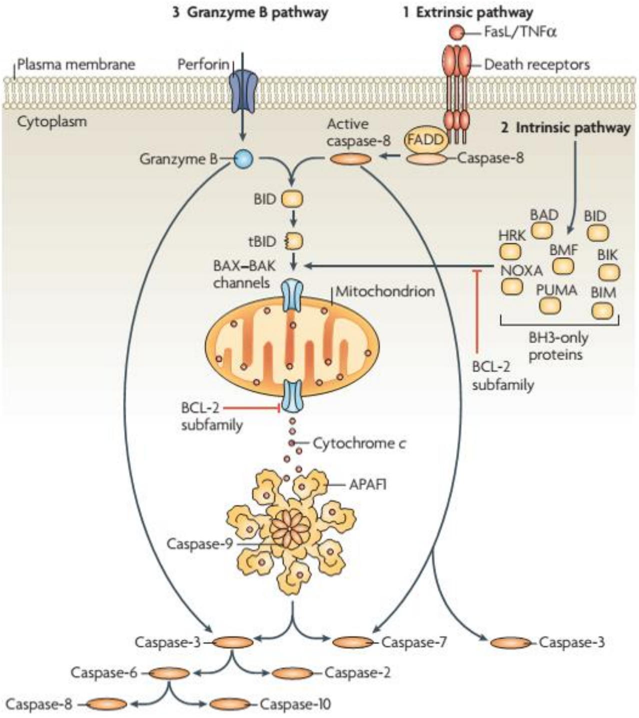
3 Granzyme B pathway
1 Extrinsic pathway
FasL/TNFα
Plasma membrane
Perforin
Death receptors
Cytoplasm
Active caspase-8
FADD
2 Intrinsic pathway
Granzyme B
Caspase-8
BID
tBID
BAX–BAK channels
Mitochondrion
BAD
BID
HRK
BMF
BIK
NOXA
PUMA
BIM
BH3-only proteins
BCL-2 subfamily
BCL-2 subfamily
Cytochrome c
APAF1
Caspase-9
Caspase-3
Caspase-7
Caspase-3
Caspase-6
Caspase-2
Caspase-8
Caspase-10

Figure 2: **Apoptotic cell death pathways.** The figure represents the extrinsic pathway which is initiated through the activation of death-receptors and leads to caspase 8 activation. The mechanisms that leads to the induction of intrinsic pathway and consequent caspase 3 activation is also represented. (Taylor, Cullen et al. 2008), used with permission (see annex).

The extrinsic pathway involves the activation of death receptor by death ligands, inducing the activation of death pathways. This type of mechanisms leads to caspase 8 activation that triggers apoptotic signalling (Hengartner 2000, Elmore 2007, Taylor, Cullen et al. 2008).

Moreover, mitochondria have the ability to modulate apoptosis. The shift of pro-apoptotic/ anti-apoptotic proteins towards anti-apoptotic proteins leads to apoptosis inhibition. Another protein that can interfere with apoptosis is p53. P53 is known as the genome guardian, since it regulates the activation of DNA-repair damage mechanisms. When damage is present in a great extent, p53 can directly activate apoptosis, inducing the transcription of pro-apoptotic genes (Fridman and Lowe 2003, Elmore 2007).

2.1.1.1 Doxorubicin-induced cell death

As discussed in section 1.2.2, DOX-induced extensive damage leads to $\Delta\Psi_m$ loss, which induces or is a consequence from MPT pore opening. These are key-events for the initiation of caspase-dependent mitochondrial apoptosis pathways. DOX treatments activate cardiomyocyte apoptosis (Arola, Saraste et al. 2000). DOX-induced apoptosis involves cytochrome c release with following apoptosome formation (Konorev, Vanamala et al. 2008). More data corroborates the activation of intrinsic apoptosis pathway by massive activation of caspase 9 and 3 (Konorev, Vanamala et al. 2008, Sardao, Oliveira et al. 2009, Sardao, Oliveira et al. 2009). Moreover, DOX-induced DNA damage leads to p53 activation and consequent cell death (L'Ecuyer, Sanjeev et al. 2006).

2.2 Autophagy

Autophagy is an evolutionary conserved catabolic process of cellular recycling in which cytosolic components such as proteins, lipids, sugars and even organelles are degraded through cellular self-digestion by lysosomal acidic hydrolases (Chen and Debnath 2010, Tanida 2011, Dutta, Calvani et al. 2012). There are three types of autophagy depending on the mechanisms that deliver the cellular components to lysosomes: macroautophagy, microautophagy, and chaperone-mediated autophagy (Dutta, Calvani et al. 2012). Macroautophagy, simply named autophagy, is the most well described type of autophagy in mammalians and involves the degradation of long-lived proteins and whole cellular organelles through their sequestration in the autophagosome that latter fuses with lysosomes (Kroemer, Marino et al. 2010, Dutta, Calvani et al. 2012). Micro-autophagy involves the direct sequestration of cytosolic components through invaginations of the lysosomal membrane and may serve mostly for the turnover of certain proteins. The last type of autophagy, chaperone-mediated autophagy, is a process that specifically degrades soluble cytosolic proteins (Dutta, Calvani et al. 2012). Chaperone-mediated autophagy differs from the other types of autophagy due to the specific mechanism of cargo selection which requires the targeting of the components by a cytosolic chaperone hsc70 to lysosomal degradation through interaction with a conserved aminoacid sequence (Arias and Cuervo 2011).

The autophagic process initiates when the cell receives a stimulus and a specific membrane-enclosed structure called phagophore is formed. The phagophore formation requires the assembly of a complex that is constituted by Beclin-1, VPS34, VPS15 and Ambra1 (Kroemer, Marino et al. 2010, Kubli and Gustafsson 2012). Then, the membrane system expands and this process is mediated by two ubiquitin-like conjugation systems: microtubule-associated LC3-I and 12-ATG5 which promote the assembly between ATG16L complex and the conjugation of LC3-I with phosphatidylethanolamine that origins LC3-II. The autophagosome is formed by a double-membrane structure resulting from the expansion of the phagophore. In the end, the autophagosome fuses with a lysosome resulting in the degradation of the cargo (Kubli and Gustafsson 2012).

There are several molecular mechanisms that regulate the autophagic process. Autophagy inducers modulate the interaction of mTOR complex 1 (mTORC1) with the ULK1/2 complex (Kroemer, Marino et al. 2010, Tanida 2011). In normal conditions, mTORC1 possesses kinase activity and interacts with a complex that contains ULK1/2. When the cell receives an autophagic stimuli, mTORC1 is inhibited and its dissociation from ULK1/2 complex occurs. Then, the inhibition of mTORC1 leads to ULK1/2 activation through (de)phosphorylation processes (Kroemer, Marino et al. 2010). Thus, mTORC1 inhibition results in autophagy induction and mTORC1 activation blocks the autophagic process (Kroemer, Marino et al. 2010, Tanida 2011). The ULK1/2 complex can also regulate the activity of Beclin 1/class III phosphatidylinositol 3-kinase (PI3K) complex through phosphorylation of Ambra1, a protein constituent of Beclin 1 complex, regulating the initial stages of autophagosome formation (Kroemer, Marino et al.

2010) (Kim, Rodriguez-Enriquez et al. 2007). Furthermore, autophagy can be induced or inhibited due to the interaction of Beclin 1 with other proteins. Anti-apoptotic family members such as Bcl-2 and Bcl-X_L can downregulate autophagy and the disruption of autophagy inhibition may involve several steps with pro-apopoptic proteins participating (Kim, Rodriguez-Enriquez et al. 2007, Gottlieb and Carreira 2010, Kroemer, Marino et al. 2010). Another important regulator of autophagy is AMPK. During a starvation stimulus, AMPK exerts a negative regulatory effect on mTOR and therefore induces autophagy (Gottlieb and Carreira 2010).

Several intracellular and extracellular stimuli can induce autophagic mechanisms including nutrient and energy stress, endoplasmatic reticulum stress, hypoxia, redox stress and mitochondrial damage. The stimulation of autophagy allows cells to eliminate components that can result in harm or damage to the cell (Kroemer, Marino et al. 2010, Tanida 2011).

2.2.1 Stress stimuli and induction of mitophagy

Mitochondria are a critical organelle from a metabolic point of view. Not only mitochondria perform essential cell functions such as energy production through oxidative phosphorylation, but are also involved in calcium homeostasis and ROS production. As discussed earlier, excessive ROS production can lead to oxidative stress and therefore to damage in several cellular constituents (Gottlieb and Carreira 2010, Scherz-Shouval and Elazar 2011). Nevertheless, physiological ROS production are usually involved in the regulation of multiple signalling pathways controlling intracellular ROS homeostasis. Physiologically, ROS act as sensors that activate redox mechanism to regulate antioxidants and other signalling pathways (Gius, Botero et al. 1999, D'Autreaux and Toledano 2007). One of the ROS-based mechanisms is the activation of nuclear transcription factors such as NF-kβ in response to cell oxidation (Gius, Botero et al. 1999).

Global or localized oxidative stress is a stimulus that promotes mitophagy through different mechanisms. ROS, more specifically H_2O_2, have the capacity to activate several proteins such as PERK that can stimulate other molecules. Ultimately, this cascade leads to an inhibition of mTOR (Kroemer, Marino et al. 2010).

Moreover, the cellular response to increased ROS may involve the activation of MAPKs which can also activate autophagy. DNA damage can also stimulate the expression of pro-autophagic p53-induced target genes (Kroemer, Marino et al. 2010, Scherz-Shouval and Elazar 2011).

Mitochondrial damage can lead to the opening of MPT pore, as described above, causing a secondary release of ROS and calcium which can activate caspases and consequently cell death. Thus, cells may primarily remove damaged mitochondria through a specific form of autophagy termed mitophagy (Gottlieb and Carreira 2010, Kroemer, Marino et al. 2010). Mitophagy is a highly selective process that has the capacity to induce the elimination of dysfunctional or unnecessary mitochondria (Dutta, Calvani et al. 2012). The loss of $\Delta\Psi_m$ is one of the events that can trigger mitophagy. Autophagic recognition for the removal of depolarized mitochondria involves a refined voltage sensor that includes PINK1. After mitochondrial depolarization, PINK1 accumulates in the mitochondrial surface and is involved in the recruitment of Parkin, a cytosolic E3-ubiquitin ligase that is selectively recruited to dysfunctional mitochondria and assists in their removal by mitophagy. Parkin has the capacity to ubiquitinate OMM proteins, including the VDAC and may recruit autophagy adaptor proteins such as p62 or NIX and BNIP3, targeting mitochondria for autophagy (Gottlieb and Carreira 2010, Kroemer, Marino et al. 2010, Dutta, Calvani et al. 2012).

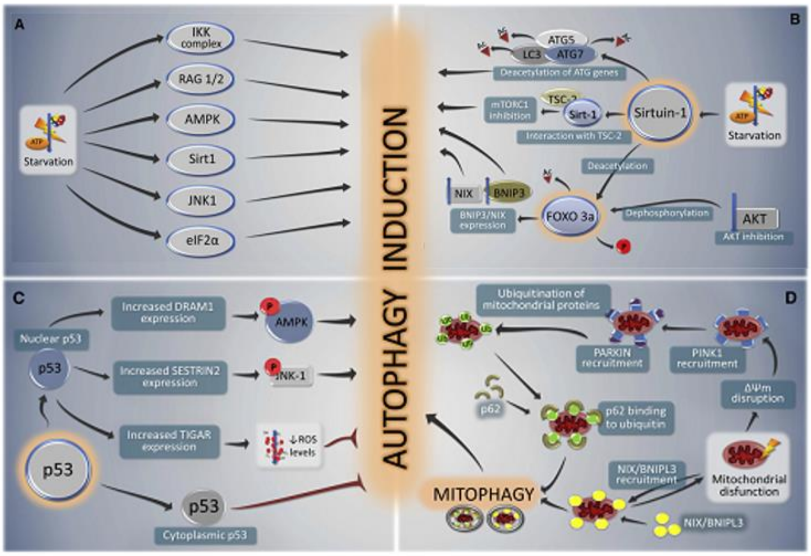
A
Starvation
ATP
IKK complex
RAG 1/2
AMPK
Sirt1
JNK1
eIF2α
AUTOPHAGY INDUCTION
B
ATG5
LC3
ATG7
Ac
Deacetylation of ATG genes
TSC-2
mTORC1 inhibition
Sirt-1
Sirtuin-1
Starvation
Interaction with TSC-2
Deacetylation
NIX
BNIP3
BNIP3/NIX expression
FOXO 3a
Dephosphorylation
AKT
AKT inhibition
P
C
Increased DRAM1 expression
AMPK
Nuclear p53
p53
Increased SESTRIN2 expression
JNK-1
Increased TIGAR expression
↓ROS levels
p53
p53
Cytoplasmic p53
D
Ubiquitination of mitochondrial proteins
PARKIN recruitment
PINK1 recruitment
ΔΨm disruption
p62
p62 binding to ubiquitin
NIX/BNIPL3 recruitment
Mitochondrial disfunction
MITOPHAGY
NIX/BNIPL3

Figure 3: Overview of selected stress pathways that induce autophagy. Pro-autophagic pathways are represented in panel A and B, perturbation of p53 system is represented in panel C and mitophagic pathways are represented in panel D. From(Kroemer, Marino et al. 2010) used with permission (see annex).

Mitochondria energy depletion may trigger starvation-induced autophagy. One of the most well studied mechanisms is the regulation of the starvation-induced autophagy by mTOR, AMPK and sirtuins. The induction of autophagy by starvation requires sirtuin-1 (SIRT1) protein. (Kroemer, Marino et al. 2010) This sirtuin can deacetylate several molecules inducing the activation autophagic related proteins and is regulated by AMPK which can regulate SIRT1 activity (Kroemer, Marino et al. 2010). Nonetheless, Gomes and *et al.* reported that SIRT1 is also involved in mechanisms of mitochondrial biogenesis leading to an improvement in mitochondrial function on skeletal muscle cells (Gomes, Duarte et al. 2012).

Autophagy appears to be a survival pathway, getting rid of organelles or structures that, because of damage, are no longer useful to the cell. Therefore, autophagy may serve as a cardioprotective response. Nevertheless, if this type of cardioprotective response is upregulated in an uncontrolled manner it may contribute to disease progression (Scherz-Shouval and Elazar 2011, Dutta, Calvani et al. 2012).

2.2.2 Doxorubicin-induced autophagy and cardiomyopathy

Doxorubicin-induced cardiotoxicity is characterized by several events such as redox stress, energy depletion and mitochondrial damage that may induce autophagy. Autophagy plays an important role in cardiomyocyte death, contributing to DOX-induced heart failure (Lu, Wu et al. 2009). Cardiac cell death resulting from autophagy results from autophagic degeneration, which involves mitochondrial damage concomitant with beclin-1 upregulation (Lu, Wu et al. 2009). Generally, autophagy in the myocardium occurs as a protective adaptation to cumulative cell damage (Goswami and Das 2006, Zhang, Shi et al. 2009, Sishi, Loos et al. 2013). Autophagy behaves as a self-renewable mechanism of cellular components which allows for the maintenance of energy homeostasis (Dutta, Calvani et al. 2012). In several pathologies including cardiomyopathy, autophagy is also substantially enhanced to eliminate protein aggregates and damaged organelles acting as a pro-survival mechanism (Zhang, Shi et al. 2009). Kawaguchi *et al.* suggested that DOX may impair autophagosome formation affecting the autophagic process (Kawaguchi, Takemura et al. 2012).

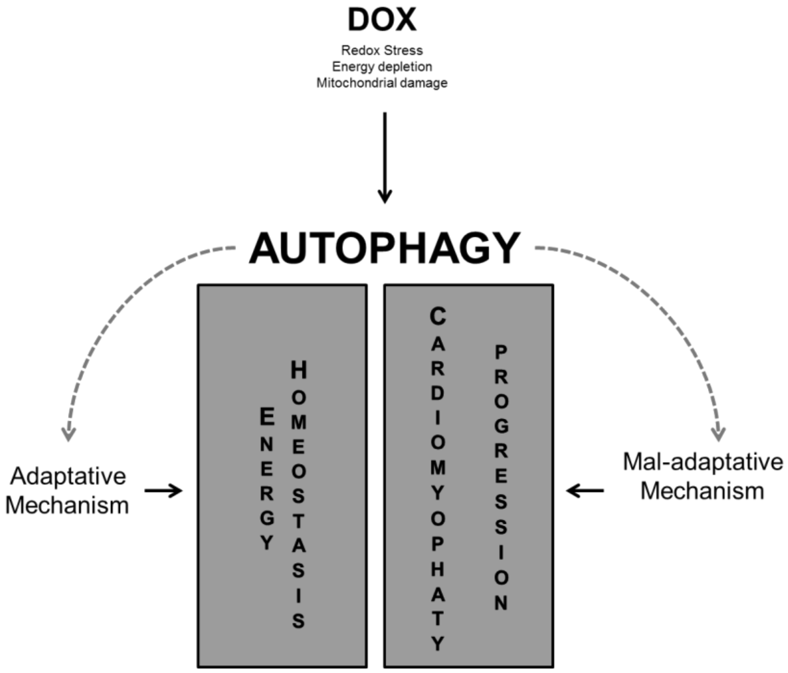
DOX
Redox Stress
Energy depletion
Mitochondrial damage
AUTOPHAGY
ENERGY HOMEOSTASIS
CARDIOMYOPHATY PROGRESSION
Adaptative Mechanism
Mal-adaptative Mechanism

Figure 4: **Doxorubicin-induced autophagy in the myocardium may act as a "double-edge sword" in a dose-dependent manner.** Autophagy in the myocardium occurs, generally, as a pro-survival mechanism that allows cells to restore energy homeostasis. Nonetheless, autophagy enhancement leads cardiomyocytes to death.

However, if autophagy becomes excessively active, cells may be committed to death (Dimitrakis, Romay-Ogando et al. 2012). Excessive autophagy induced by severe stimuli can also damage cytosol and organelles such as mitochondria and endoplasmatic reticulum. Then, lysosomal enzymes such as cathepsins or other cell death-inducing factors are released leading to apoptotic and necrotic cell death (Zhang, Shi et al. 2009). Progressive cell loss and impaired intracellular regeneration which leads to inefficient cardiomyocytes aggravates the heart condition (Semenov, Lushnikova et al. 2001). The data available suggest that DOX, in a dose dependent-manner, can result in induction (for low concentrations) or inhibition (for high concentrations) of autophagy, which may lead the cell from a protective to a deleterious phenotype (Zhang, Shi et al. 2009).

Chapter 3
Berberine: targeting Doxorubicin-induced cardiotoxicity

Cardiotoxicity is the most hazardous side effect of DOX antineoplastic treatments. Until today, chronic DOX-induced cardiac toxicity that lead to chronic cardiomyopathy and CHF does not have a suitable therapeutic approach to prevent the onset of these events. In the past few years, researchers have made efforts to find new ways to prevent or counteract DOX-induced cardiotoxicity by decreasing the cumulative dose administrated or by reducing drug plasma peak concentration. These new therapeutic approaches include combined treatments with antioxidants and second generation anthracyclines.

3.1 Targeting Doxorubicin-induced cardiotoxicity

3.1.1 New formulations

One possible solution to minimize DOX toxicity is by developing second generation anthracyclines. Epirubicin (EPI) is the most popular anthracycline derivatives among the ones developed. EPI is a semisynthetic DOX derivative that results from a conformational alteration of OH group from daunosamine (Danesi, Fogli et al. 2002, Minotti, Menna et al. 2004). Although this alteration has little effect on the mechanism of action, it introduces important alterations in pharmacokinetics as higher total body clearance and shorter terminal half-life (Danesi, Fogli et al. 2002, Minotti, Menna et al. 2004). These differences settle a new threshold to higher cumulative dose administrated to patients (900 mg/m^2) but do not eliminate the risk of development of cardiovascular complications (Ryberg, Nielsen et al. 1998).

3.1.1. Combined treatments

Oxidative stress is the major cause of DOX-induced cardiotoxicity. Controlling cellular damage induced by increased oxidative stress has been the objective of numerous investigations aimed at preventing or reducing the cardiotoxicity associated DOX treatments. The combination of DOX with other compounds as adjuvant has been extensively studied. One of them is dexrazoxane (DZX), the only FDA approved DOX- combined treatment. DZX has the ability to chelate free iron and displaces iron from DOX-iron complexes preventing iron catalysed ROS production (Swain, Whaley et al. 1997, Minotti, Menna et al. 2004, Carvalho, Santos et al. 2009, Pereira, Silva et al. 2011). Carvedilol, a β-blocker used in CHF treatments, also prevent cardiomyocyte apoptosis and has the ability to inhibit ROS formation and prevent endogenous antioxidant depletion (Oliveira, Bjork et al. 2004, Spallarossa, Garibaldi et al. 2004, Sgobbo, Pacelli et al. 2007).

Other approaches used pre-treatments with antioxidants to protect cardiac cells from DOX-induced damage. Akhlaghi and collegues showed that prior exposure of H9c2 cells to some flavonoids can act as a protection against oxidative stress and cell death (Akhlaghi and Bandy 2012). Pre-treatment of mice with berberine (BER), a phytoalkaloid, suggested that this drug might also protect against DOX-induced cardiotoxicity (Zhao, Zhang et al. 2011). Thus, preconditioning cells with a prior expose to brief oxidative stress allows them to regulate cell death pathways and endogenous antioxidant defences pathways, possibly acting as a pre-conditioning stimulus (Han, Wang et al. 2001, Akhlaghi and Bandy 2012).

3.2 Berberine: in the prevention of Doxorubicin-induced cardiotoxicity

Berberine (Figure 5), 5,6- dihydro-9,10-dimethoxy-benzo[g]- 1,3-benzodioxolo [5,6-α] quinolizinium, is an alkaloid isolated from plants of Berberidaceae family that has been extensively used in tradicional chinese medicine (Lau, Yao et al. 2001, Tillhon, Guaman Ortiz et al. 2012).

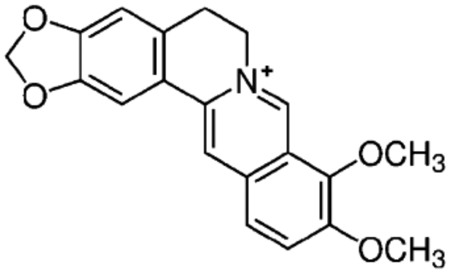

Figure 5: Chemical structure of BER. BER is quaternary ammonium salt from the protoberberine group of isoquinoline alkaloids.

Among the many pharmacological actions that have been attributed to BER antitumoral/antiproliferative (Letasiova, Jantova et al. 2005, Serafim, Oliveira et al. 2008, Tong, Zhang et al. 2012), antioxidant (Shirwaikar, Shirwaikar et al. 2006) and antiarrythmic (Hong, Hui et al. 2002) are the most important. These pharmacological properties potential led to the proposed use of BER as a cardiovascular agent and an antineoplastic agent.

The effect of BER in multiple cancer cell lines have been studied. BER inhibits cell growth and induces cell death processes in several cell lines including: lung cancer (Tong, Zhang et al. 2012, Fu, Chen et al. 2013), melanoma (Letasiova, Jantova et al. 2005, Pereira, Branco et al. 2007, Serafim, Oliveira et al. 2008, Mittal, Tabasum et al. 2014), cervix carcinoma (Tong, Zhang et al. 2012) and liver cancer (Wang, Feng et al. 2010, Hou, Tang et al. 2011, Tong, Zhang et al. 2012).

BER also demonstrated ability in reduce ventricular afterload and augment myocardial contractibility (Marin-Neto, Maciel et al. 1988, Lau, Yao et al. 2001). These properties claim BER as a cardioprotective agent that can offer a pharmacological advantage in the treatment of arrhythmias and heart failure. Facing this, one can ask whether BER can offer protection against DOX-induced toxicity and if such treatment can be useful in clinical practice. In fact, Zhang and colleagues suggested that BER offered protection against DOX-induced cardiotoxicity (Zhao, Zhang et al. 2011). Later Lv *et al*. showed that BER can impair DOX-induced toxicity by inhibiting apoptosis in cardiomyocyte primary cultures through modulation of the Bcl-2 expression, p53 phosphorylation and AMPKα inhibition (Lv, Yu et al. 2012). Still, no work so far focused on the regulation of mitophagy and autophagy by DOX has been done.

Chapter 4
Aims and Hypothesis of this dissertation

As discussed earlier, DOX is one of the most effective anti-cancer agents. Although showing large chemotherapeutic potential, DOX induces extensive damage in the heart, resulting in the development of cardiomyopathies. Increased oxidative stress is the responsible for DOX-induced heart damage. The accumulation of damage leads cells to activate repair mechanisms, such as autophagy, whether cell death is triggered if damage is extensive.

Autophagy behaves in a dual manner. On one hand, autophagy acts as a compensatory mechanism promoting energy homeostasis in cardiac cells, but if it becomes uncontrolled it can lead to cell death and impaired intracellular regeneration, leading to tissue damage. BER was already shown to have cardiovascular positive effects in patients with CHF. In order to counteract DOX-induced cardiotoxicity, BER may offer a potential protection through the regulation of autophagy.

The main objective of this project was to investigate if BER modulates DOX-induced cell death and autophagy. The effect of BER on DOX-induced cell death and autophagy in H9c2 cells was studied through:

1. The evaluation of the cellular density and cell viability in different conditions by the sulforhodamine B assay;
2. The evaluation of caspase-like activity by colorimetric assay;
3. The semi-quantification of key-proteins of certain events such as mitochondrial biogenesis and autophagy induction by western blotting;
4. The evaluation of autophagy and the relationship with cell death by lysosomal cathepsins B/D like-activity;
5. The measurement of simultaneous co-localization of mitochondrial and lysosomal markers by confocal microscopy.

The hypothesis of this dissertation is that BER can prevent DOX-induced toxicity through multiple mechanisms including activation of SIRT1 which may lead to mitochondrial biogenesis and expression of antioxidant defences, as well as by promoting protective autophagy.

Thus, this project might be relevant to consider BER as a compound able to counteract DOX-induced cardiotoxicity, which would then result in the use of this anti-cancer agent in higher cumulative dosages, that would be more effective as anti-cancer agents.

Part II

Experimental Results

Chapter 5
Materials and Methods

5.1 Reagents

All reagents were used at the highest grade of purity available. Aqueous solutions were prepared in ultra-pure water (MilliQ system, Millipore, Darmstadt, Germany). Non aqueous solutions were prepared in ethanol or DMSO. Doxorubicin hydrochloride, Berberine chloride, Sulforhodamine B, Z- Arg- Arg-N-methyl-coumarin and Hemoglobin were purchased from Sigma- Aldrich, St. Louis, MO, USA. Caspases substrates Ac-Asp-Glu-Val-Asp-pNA and Ac-Leu-Glu-His-Asp-pNA were purchased from Calbiochem, Darmstadt, Germany. The fluorescent substrate ECF™ were purchased from Healthcare Life Sciences, Buckinghamshire, UK. TMRM, Lysotracker Green DND26 and Hoescht were purchased from Invitrogen, Eugene, OR, USA.

5.2 Methods

The evaluation of the effect of BER on DOX-induced cell death and autophagy was performed *in vitro* in H9c2 cells. In order to evaluate this effect several assays were performed.

5.2.1 Cell culture

The H9c2 cell line, developed by Kimes and Brandt, is a sub-clone of the original clonal cell line derived from embryonic BD1X rat heart.(Kimes and Brandt 1976) H9c2 cells were purchased from America Tissue Type Collection (ATTC) and cultured in high glucose DMEM supplemented with 10% (vol/ vol) FBS, 1% (vol/ vol) penicilin/streptomycin, 1.8g/L sodium bicarbonate and 0,11 g/L sodium pyruvate at 37°C in a humidified atmosphere of 5% CO_2. Cells were fed at every 3 days and were subcultured once they reached 70-80% confluence. Then cells were harvested with trypsin/EDTA and seeded at a concentration of 35,000 cells/ mL. After 24 hours of cell attachment, H9c2 cells were incubated with BER for 72 hours and an equal volume of DMSO as a control. In the last 24 hours, cells were incubated with two different concentrations of DOX alone in presence of BER. Doxorubicin hydrochloride (D1515, Sigma- Aldrich, St. Louis, MO, USA) was prepared in an aqueous solution. Berberine chloride (B3251, Sigma- Aldrich, St. Louis, MO, USA) used for cell treatments was prepared in DMSO and total drug volume in cell media was always less than 0.1%. The timeline followed in all experiments is schematically represented in Figure 6.

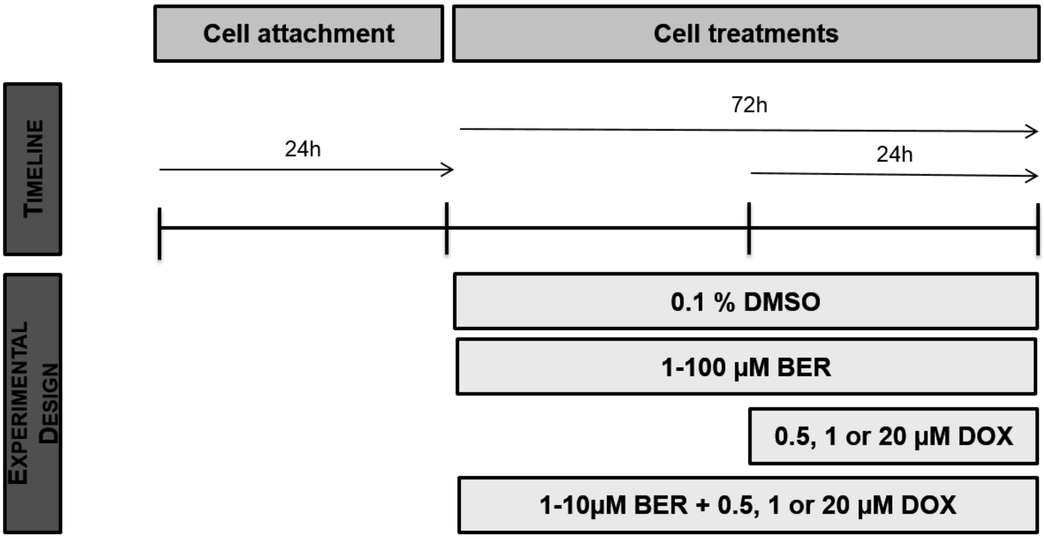

Figure 6: **Timeline of the experimental design for BER/ DOX treatments.** H9c2 cells were seeded and divided into different experimental of groups depending on the treatment that were applied: DMSO 72 hours, BER 72 hours, DOX in the last 24 hours of the 72 hours treatment, and BER and DOX in the last 24 hours of the 72 hours treatment. The effect of BER were measured in terms of the cell viability by SRB colorimetric assay, caspases activity by colorimetric assay, cathepsins activity by fluorometric and spectrophotometric detection, mitochondria-lysosome co-localization by confocal microscopy and mitochondrial and autophagy markers content by Western blotting.

5.2.2 Sulforhodamine B colorimetric assay

The evaluation of the cytotoxicity of BER and joint BER/ DOX treatments on H9c2 cell was performed using the Sulforhodamine B (SRB) colorimetric assay adapted from Vichai and Kirtikara (Vichai and Kirtikara 2006).

The SRB colorimetric assay was developed by Skehan *et al*. and allows an *in vitro* cytotoxicity screening (Skehan, Storeng et al. 1990). This method relies on the ability of SRB molecule to bind to protein components present in previously fixed cells. Structurally, SRB is an aminoxhantene dye with two sulfonic groups that allows the binding to basic amino acids in mild-acidic conditions and the dissociation under basic conditions. The amount of dye extracted from stained cells is directly proportional to the cell mass (Vichai and Kirtikara 2006).

To evaluate of the cytotoxicity of BER and BER/ DOX combination, H9c2 cells were cultured as previously described and seeded at a 35,000 cells/ mL concentration in a total volume of 500 µL in 48 multi-wells plates. Once cells were adherent to the plate, they were drugged for 72 hours with BER (1, 5, 10, 20, 50 or 100 µM) or vehicle in the last 24 hours with 0.5, 1 or 20 µM DOX. The culture medium was removed, the cells were washed with PBS and 200 µL of methanol/1% (vol/ vol) acetic acid was added in order to fix the cells to the bottom surface of the well. The cells were kept during 24 hours at -20ºC. Then, methanol/ 1% (vol/ vol) acetic acid solution was discarded and 200 µL of 0.05% (wt/ vol) of SRB in 1% (vol/ vol) of acetic acid was added during 1 hour at 37ºC. Stained-cells were quickly rinsed four times with 1% (vol/ vol) acetic acid to remove the excess of dye and were left to dry. One mL of 10 mM Tris pH 10.5 was added to solubilize the bound dye and the plate was maintained on a shaker for 10 minutes. To measure the optic density of he obtained solution, 100 µL of the solution was transferred to a 96 multi-well plate and the absorbance was read at 540 nm in a VICTOR X3 (Perkin Elmer, Inc.) microplate reader.

5.2.3 Caspase 3 and 9 like-activity assay

The evaluation of the apoptotic signalling pathway activation by BER/ DOX treatments in H9c2 cells was performed by the measurement of caspase 3 and caspase 9.

Caspase 3 and 9-like activities were measured through the spectrophotometric detection of the cromophore p-nitroanilide (pNA) after the cleavage of the substrate Ac-Asp-Glu-Val-Asp-pNA (Cat. #235400, Calbiochem, Darmstadt, Germany) and Ac-Leu-Glu-His-Asp-pNA (Cat. #218805, Calbiochem, Darmstadt, Germany), respectively.

H9c2 cells were cultured as previously described and seeded at a 35000 cells/ mL concentration in a total volume of 10mL at 100mm diameter dishes. After 24 hours of cell attachment, H9c2 cells were drugged for 72 hours with 1 µM or 10 µM of BER and with 0.5, 1 or 20 µM DOX in the last 24 hours. The culture medium was removed, the cells were washed with PBS and 200 µL of Lysis Buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% (wt/vol) CHAPS, 0.1 mM EDTA, 10% (vol/vol) glycerol and 10 mM DTT) was added at each dish. The dishes were scraped and total cell extracts were collected. Then, cell suspensions were frozen/ thawed 5 times and ruptured 30 strokes with a 25G needle. All samples were then centrifuged at 16 100 x g and the pellet were discarded.

The protein content was quantified by the Bradford method (described in 3.2.4 section), using BSA as standart. Caspase 3 and caspase 9 like- activities were measure in aliquots of cell extracts containing 25µg and 50µg respectively and incubated in Reaction Buffer containing 50mM HEPES pH 7.4, 100mM NaCl, 0.1% (wt/vol) CHAPS, 0.1mM EDTA, 10% (vol/vol) glycerol, 10mM DTT and 100µM of caspases 3 and 9 substrate (Ac-Asp-Glu-Val-Asp-pNA and Ac-Leu-Glu-His-Asp-pNA respectively) for 2 hours at 37ºC. Caspase-like activities were determined by the detection of pNA at 405 nm in VICTOR X3 (Perkin Elmer, Inc.) reader. The method was calibrated with known concentrations of pNA.

5.2.4 Western Blotting

The semi-quantification of key-proteins of mitochondrial biogenesis and autophagy pathways in BER/ DOX treatments in H9c2 cell were performed by Western Blotting.

To evaluate of the cytotoxicity of BER and BER/ DOX combination, H9c2 cells were cultured as previously described and seeded at a 35,000 cells/ mL concentration in a total volume of 28 mL at 150 mm diameter dishes. Once cells were adherent to the plate, they were drugged with 10µM BER for 72 hours and in the last 24 hours with 1 or 20 µM DOX. The culture media was collected and 5 mL of extraction buffer (PBS supplemented with 0.1 g/ L of EDTA) was added to each dish. Cells were then scraped and the cell suspension was collected and added to the correspondent cultured media.

Table I: Primary and Secondary antibodies used. Antibodies brand code, primary and secondary dilution are listed below.

Antibody	Brand Code	Primary dilution	Secondary dilution
LC-3 I/II	MBL PD014	1:1,000	Anti-rabbit 1:2,500
OXPHOS	Mito profile MS604	1:1,000	Anti-mouse 1:2,500
p53	Cell signalling 2524	1:1,000	Anti-mouse 1:2,500
p62	MBL PM045	1:1,000	Anti-rabbit 1:2,500
Sirt1	Abcam Ab110304	1:1,000	Anti-rabbit 1:2,500
Sirt3	Cell Signalling 5490	1:1,000	Anti-rabbit 1:2,500
Tfam	Santa Cruz sc23588	1:500	Anti-Goat 1:2,500
TOM 20	Santa Cruz Sc11415	1:1,000	Anti-rabbit 1:2,500

a) Primary antibodies were prepared in 2% (wt/vol) milk, except for LC3 I/II and Tfam that were prepared in 2% (wt/vol) BSA

b) Primary antibodies were recycled and used in a new membrane incubation for 5 times maximum.

c) Secondary antibodies were prepared in TBS-T. Tfam was prepared in 2% (wt/vol) milk and incubated at 4ºC for 3 hour.

The suspensions were centrifuged at 340 x g for 4 minutes, the pellet was collected and washed with PBS and centrifuged again at 340 x g for 4 minutes. Pellets were ressuspended in 100 µL of Cell Lysis Buffer (1x) (Cell Signalling Cat. #9803) supplemented with 0.01 mM PMSF. Samples were sonicated with short pulses and quantified by the Bradford method, using BSA as standard. Samples were stored at -20ºC for a short period of time until their use.

5.2.4.1 Protein quantification by the Bradford assay

The Bradford assay was adapted from the procedure developed in 1976 by Bradford and was used to quantify the total amount of protein present in the samples (Bradford 1976). The Bradford method relies on the binding of Coomassie Brilliant Blue G-250 dye to protein residues (Bradford 1976, Kruger 2009, Goldring 2012). Coomassie Brilliant Blue G-250 anionic form strongly binds to basic amino acids residues producing a blue colour with a maximum absorbance at 595 nm (Bradford 1976, Goldring 2012).

The reaction was initiated by adding 1 mL of Bradford reagent (0.1% (wt/ vol) Comassie Brilliant Blue G-250, 8.5% (vol/vol) of phosphoric acid, 5% (vol/vol) of 95% ethanol) to 3 µL of sample, previously diluted in 997 µL of H_2O. After 5 minutes incubation at room temperature the absorbance of the samples was read in a VICTOR X3 (Perkin Elmer, Inc.) multi-plate reach at 595nm. The standard curve was obtained from a BSA solution within the range 1.25 to 20 µg.mL^{-1}.

5.2.4.2 One- dimensional Sodium Dodecyl Sulphate- Polyacrylamide Gel Electrophoresis and Immunoblotting

One- dimensional Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS- PAGE) was used to separate the proteins from cell extracts according to their molecular size through a discontinuous polyacrylamide gel under denaturing conditions (Gallagher 2012). After sample protein separation by SDS-PAGE, they were transferred electroforetically to highly hydrophobic polyvinylidene difluoride (PVDF), membranes allowing the identification of specific antigens that can be recognized by polyclonal or monoclonal antibodies (Gallagher, Winston et al. 2008).

Samples were prepared in Laemelli Buffer (Biorad, Hercules, CA, USA) supplemented with 5% (vol/vol) of β-Mercaptoethanol, in a proportion of 1:1. Samples were denatured for 5 min at 95ºC and 30µg of protein were loaded into 8%, 10% or 16% poliacrilamide gels. SDS-PAGE was performed at room temperature in Mini Protean Cell system (Biorad, Hercules, CA, USA) at 150V for 45 minutes. After SDS-PAGE, proteins were transferred to PVDF membranes at 4ºC in Mini Protean system (Biorad, Hercules, CA, USA) at 100V for 1 hour and 30 minutes. The transfer of proteins to the membrane was monitored by Ponceau S (P3504, Sigma- Aldrich, St. Louis, MO, USA) staining. Membranes were blocked overnight at 4ºC with 5% (wt/vol) milk and incubated overnight at 4ºC with the primary antibody (Table I). Secondary incubation was performed at room temperature for 1 hour. Between incubations with primary and secondary antibodies and before immunodetection, membranes were washed 3 times with TBS-T for 5 minutes each.

5.2.4.3 Immunodetection

The detection of a certain protein is performed by exposing the membranes to a chemifluorescent substrate that will be cleaved by alkaline phosphatase coupled to the secondary antibody.(Gallagher, Winston et al. 2008)

Band detection was performed by incubating membranes with ECF™ (Healthcare Life Sciences, Buckinghamshire, UK), a chemifluorescent substrate, for 5 minutes maximum. Chemifluorescent reactive bands were visualized in Biosepctrum 500 imaging system (UVP, Upland, CA, USA). Data were recorded and band density quantification was performed in Image J (version 1.48, National Institute of Health, USA). Protein content of the desired proteins were expressed by the ratio between band and Ponceau S density and were represented in % of untreated H9c2 cells (control). Ponceau S normalization of gel loading control is considered a better strategy than the use of housekeeping proteins.

5.2.5 Cathepsin B and D- like activity assay

The evaluation of the autophagic fluxes and lysosomes viability were performed by the measurement of cathepsin B and D-like activity. Cathepsin B and D like-activity was measured through fluorimetric and spectrophotometric detection, respectively, adapted from Vega-Naredo and Coto-Montes (Vega-Naredo and Coto-Montes 2009).

H9c2 cells were cultured as previously described and seeded at a 35,000 cells/mL concentration in a total volume of 10 mL at 100mm diameter dishes. After 24 hours of cell attachment, H9c2 cells were incubated for 72 hours with 10 µM of BER and with 1 or 20 µM DOX in the last 24 hours. The culture media was collected and 3 mL of extraction buffer (PBS supplemented with 0.1g/L of EDTA) were added to each dish.

Cells were then scraped and the cell suspension was collected and added to the correspondent culture media. The suspensions were centrifuged at 340 x g for 4 minutes, the pellet was collected and washed with PBS and centrifuged again at 340 x g for 4 minutes. Pellets were ressuspended in 200 µL of Lysis Buffer (50 mM HEPES pH 7.4, 100 mM NaCl, 0.1% (wt/vol) CHAPS, 0.1 mM EDTA and 10 mM DTT). The cell suspensions were kept at -80ºC until used. The protein content was quantified by the Bradford method, using BSA as standard.

For Cathepsin B like-activity determination, aliquots of 50 µL of each sample were incubated with 40 µM of Z- Arg- Arg-N-methyl-coumarin (C5429, Sigma- Aldrich, St. Louis, MO, USA) in Incubation Buffer (100 mM Sodium Acetate pH 5.5, 1 mM EDTA, 5 mM DTT and 0.1% (vol/vol) Brij-35) at 37ºC for 20 minutes. After the incubation, 150 µL of Stopping Buffer (33mM Sodium Acetate pH 4.3, 33mM Sodium Chloroacetate) were used to stop the enzymatic reaction. Cathepsin B like- activity were determined by the detection of the N-methyl-coumarin (A9891, Sigma- Aldrich, St. Louis, MO, USA) fluorometrically at 360 nm excitation and 460nm emission in VICTOR X3 (Perkin Elmer, Inc.) reader. The method was calibrated with known concentrations of N-methyl-coumarin.

For Cathepsin D like-activity determination, aliquots of 75 µL of each sample were incubated with 125 µL of 3% (wt/vol) Hemoglobin (H2625, Sigma- Aldrich, St. Louis, MO, USA) in 200 mM Acetic acid at 37ºC for 30 minutes. After the incubation, 125 µL of 15% (vol/vol) TCA were added to the samples and they were kept at 4ºC for 30 minutes. Samples were then centrifuged at 13400 x g for 5 minutes. Cathepsin D like- activity was determined by the measurement optic density of 200 µL supernatant at 280 nm in a Cytation 3 (BioTek Instruments, Inc.) multi-plate reader.

5.2.6 Evaluation of mitochondrial and lysosomal markers localization

To evaluate the co-localization between lysosomes and mitochondria during BER+DOX treatments, H9c2 cells were cultured as previously described and seeded at a 15,000 cells/ mL concentration in a total volume of 2 mL at 6 multi-wells dishes. Once cells were adherent to the plate, they were incubated with 10 µM BER for 72 hours and with 1 or 20 µM DOX in the last 24 hours. The culture medium was removed and cells were incubated at 37ºC, at dark in a humidified atmosphere of 5% CO_2 for 30 minutes with 100 nM of TMRM (Invitrogen, Eugene, OR, USA), 75 nM of Lysotracker Green DND26 (Invitrogen, Eugene, OR, USA) and 1 µg/mL of Hoescht 33342 (Invitrogen, Eugene, OR, USA) prepared in DMEM medium. Images were obtained in Nikon C-1 laser scanning confocal microscope.

5.2.7 Statistical analysis

The results are presented as mean ± SEM of the described number of independent preparations. Statistical analyses were performed in Graph Pad Prism (version 6.01 for Windows, GraphPad Software, La Jolla California USA) using Kolmogorov-Smirnov test to assess normal distribution and One Way of Variance (ANOVA) followed by the Dunett or Bonferroni post-test for multiple comparisons. Statistical analysis was performed in comparison with the control (untreated cells) or between groups, as described in figure legends. Differences that presented p value inferior to 0.05 were considered statistically significant.

Chapter 6
Results

In order to evaluate BER modulation in DOX-induced cell death and autophagy, H9c2 cells were treated with BER and with a low (0.5 or 1µM) or with a high-DOX concentration (20µM DOX). The different concentrations of DOX allows the study of BER effects on clinically relevant concentrations (0.5 and 1µM) and in a supra-physiological dosage (20µM). It was previously described that highest DOX concentrations induce the appearance of cytosolic vacuoles possibly related with the blockage of autophagic processes (Sardao, Oliveira et al. 2009), allowing the investigation of certain mechanisms that could not be present at low concentrations.

6.1 Berberine inhibited caspase 3 and 9-like activity induced by DOX on H9c2 cells.

To evaluate if BER altered H9c2 cell proliferation, H9c2 cells were treated with several concentrations of BER (1, 5, 10, 20, 50 or 100 µM) for 72hours (Figure 7). Generally, we observed that BER induced a decrease in H9c2 cell mass. For lower BER concentrations (1 µM) cell mass decrease was minor compared with untreated H9c2 cells. For BER concentrations higher than 5 µM the decrease of cell viability were more pronounced and statistically relevant. For 5 µM and 10 µM BER, alterations in cell mass were not significantly different between each other (92% ± 2.8% and 88% ± 2.6% relatively to control) whereas for the highest BER concentration (100 µM) cell mass was decreased by half.

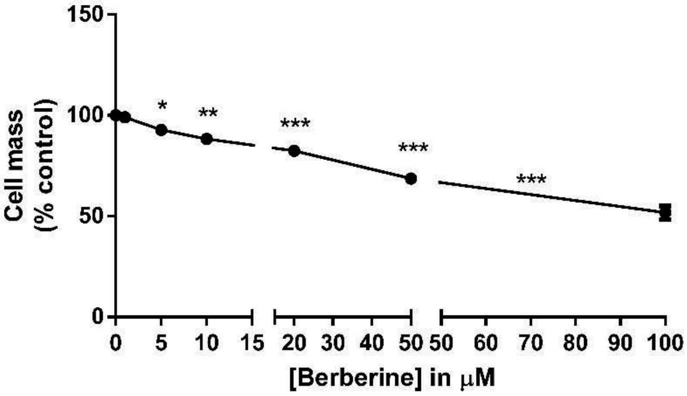

Figure 7: **Effect of Berberine in H9c2 cells.** H9c2 cells were incubated with BER (1, 5, 10, 20, 50 and 100µM) for 72 hours. BER cytotoxicity was measured by SRB colorimetric assay, described at section 3.2.2. The control (100%) was the sample which not receive BER treatment. Data were represented in mean ± SEM of 5 independent experiments. Most errors bars are hidden by graph plots. Comparisons were performed using One way ANOVA, followed by Dunett post-test (* - p< 0.05 vs. control; ** - p< 0.001; *** - p< 0.0001 vs. control).

In order to understand which BER concentration would be more suitable to be administered with DOX, determination of cell mass by the SRB assay and caspase 3 and 9 like-activity assay were performed.

First, H9c2 cells were treated for 72 hours with two different BER concentrations and in the last 24 hours with 0.5, 1 or 20 µM DOX. BER concentrations tested were the ones that did not decrease H9c2 cell viability: 1µM and 10µM.

The effect of combined BER/DOX treatments in H9c2 cells is represented in Figure 8. We observed that 0.5, 1 or 20 µM DOX induces decrease in H9c2 cell viability as previously described (Vichai and Kirtikara 2006). H9c2 cells that were treated with 1µM BER before 0.5, 1 or 20µM DOX administration did not show a significant protection when compared with the correspondent DOX treatment alone. Nevertheless, cells treated with 10µM BER and 1 or 20µM DOX presented a higher amount of cell mass, when compared with cells treated with 1 or 20 µM DOX alone. The same did not occur in cells treated with 10µM BER and 0.5 µM DOX, in which no protective effect was observed.

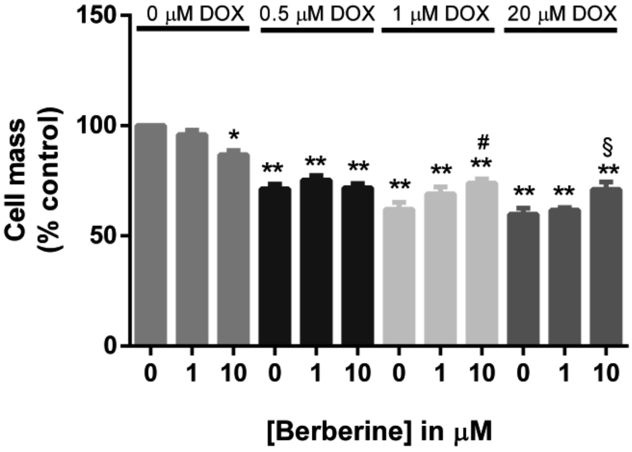

Figure 8: Effect of Berberine/Doxorubicin combined treatments in H9c2 cells. H9c2 cells were incubated for 72 hours with 1 µM or 10 µM BER for 72 hours and with 0.5,1 or 20 µM DOX in the last 24 hours. The evaluation of BER/ DOX cytotoxicity were measured by SRB colorimetric assay, described at section 3.2.2.. The control (100%) was the sample that did not receive BER and DOX treatments. Data were represented in mean ± SEM of 5 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (* - p< 0.01 vs. control; ** - p< 0.0001; # - p< 0.05 vs. 1µM DOX; § - p< 0.05 vs. 20µM DOX).

As previously described, activation of caspase 3 and 9 are involved in DOX-induced cell death in H9c2 cardiomyoblasts (Sardao, Oliveira et al. 2009, Sardao, Oliveira et al. 2009). To evaluate if BER decreases caspase 3 and 9 activation, we measured caspase 3 and 9 like-activity by using a colorimetric method, as described in section 3.2.3. H9c2 cells were treated for 72 hours with 1 or 10µM BER and with 0.5, 1 or 20µM of DOX in the last 24 hours. The effect of BER in caspase 3 and 9- like activity in H9c2 cells is represented in Figure 9.

We observed that, BER treatment *per se* did not significantly increased caspase 3 and 9 activity. As described in literature, all DOX treatments (0.5, 1 and 20µM DOX) showed increased both caspase 3 and 9-like activities (Sardao, Oliveira et al. 2009, Sardao, Oliveira et al. 2009). H9c2 cells treated with 1µM BER and DOX presented a decrease in caspase 3 and 9 activity, although that decrease was more evident in cells treated with 10 µM BER where a decrease in caspase 3-like activity after 0.5, 1 or 20µM DOX treatments was observed.

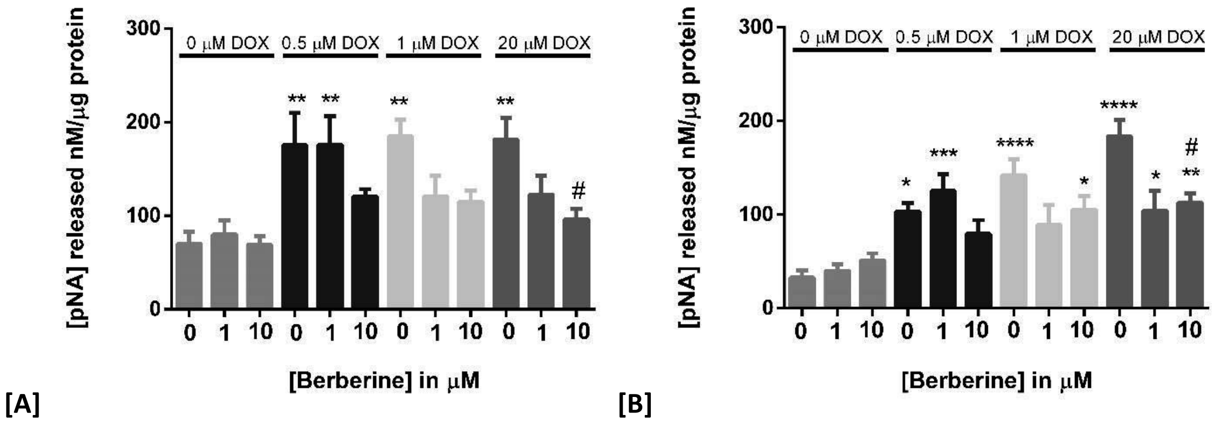

Figure 9: Effect of Berberine/Doxorubicin combined treatments on caspase 3 (Panel A) and 9 (Panel B) activation in H9c2 cells. H9c2 cells were incubated for 72 hours with 1 µM or 10 µM BER for 72 hours and with 0.5, 1 or 20µM DOX in the last 24 hours. The measurement of caspase 3 and 9 like- activity were performed by a colorimetric assay, described at section 3.2.3. Data were represented in mean ± SEM of 6 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (* - $p < 0.05$ vs. control; ** - $p < 0.01$ vs. control; *** - $p < 0.001$ vs. control; **** - $p < 0.0001$ vs. control; # - $p < 0.05$ vs. 20µM DOX.)

Although the decrease in caspase 3-like activity in cells treated with 10µM BER and 0.5 or 1µM DOX were not significant when compared with 0.5 and 1µM DOX, the increase of caspase 3-like activity when compared with untreated cells was not significant either. Samples that received treatments with 10 µM BER and 20 µM DOX had an evident and statistically significant decrease of caspase 3-like activity when compared to the respective DOX control. Curiously, cells treated with 1 µM BER and 0.5 µM DOX had an increase in caspase 3- like activity. The same pattern occur also in caspase 9-like activity. We observed that in general BER/DOX combined treatments decreased caspase- like activity when compared with the respective DOX treatment alone. Those results suggested that 10 µM is the best concentration tested to evaluate BER effects on DOX- induced cell death and autophagy.

Another protein that has been described to play an important role in apoptosis control is the transcription factor p53, which potentiates the transcription of pro-apoptotic genes that lead to an increase in the ratio of pro- to anti-apoptotic Bcl-2 proteins. The downstream effect is caspases activation can lead to apoptosis (Fridman and Lowe 2003, L'Ecuyer, Sanjeev et al. 2006). In order to assess if

BER can modify p53 content in DOX-treated H9c2 cells, p53 content was measured by Western Blotting. BER ability to modify p53 content in BER treated cells is presented in Figure 10.

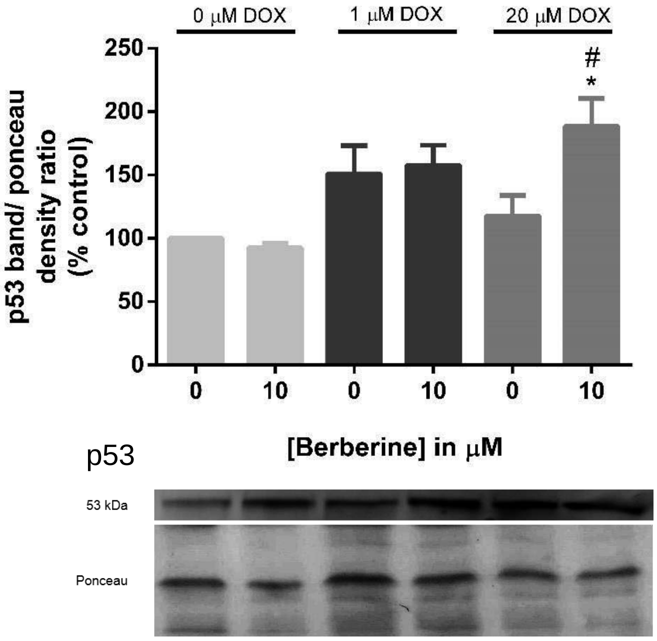

Figure 10: Effect of BER on p53 content in DOX-treated in H9c2 cells. H9c2 cells were incubated for 72 hours with 10µM BER and with 1 or 20µM DOX in the last 24 hours. p53 content was evaluated by Western blotting (section 3.2.4). Data were represented in mean ± SEM of 5 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (* - $p < 0.05$ vs. control; # - $p < 0.05$ vs. 20µM DOX).

As previously described (L'Ecuyer, Sanjeev et al. 2006, Sardao, Oliveira et al. 2009), DOX treatment increased p53 content in H9c2 cells, although because of sample variability those differences were not statistically significant. We observed that BER had no effect in p53 amount in H9c2 cells when compared with untreated cells and cells treated with 1 µM DOX. Contrarily to our expectations, BER increased p53 content in 20 µM DOX- treated cells, despite caspase 3 and 9 inhibition.

6.2 Berberine blocked autophagy on DOX-treated H9c2 cells

DOX-induced cell death results from apoptosis activation and autophagy impairment, playing the last one an important role in the development of heart conditions (Lu, Wu et al. 2009). In order to understand if BER has the capacity to modulate autophagy caused by DOX, we measured the content of LC3 I/II, p62 and, as well as cathepsin B and D like activity.

Figure 11 represent the effect of BER in LC3 I/II conversion on DOX-treated H9c2 cells.

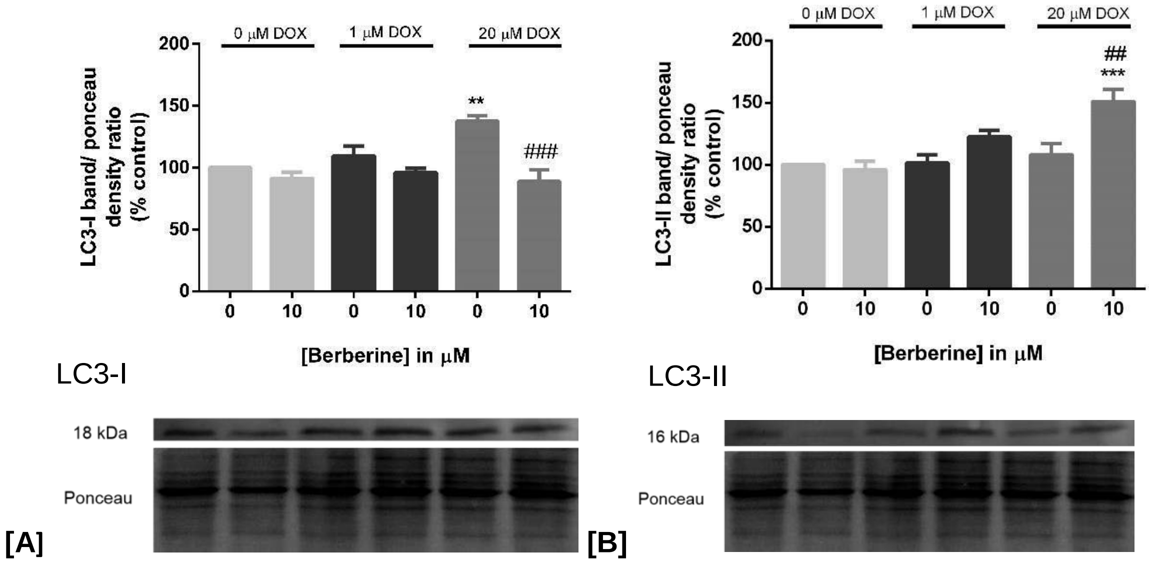

Figure 11: Effect of BER on LC3-I (panel A) conversion to LC3-II (panel B) in DOX-treated H9c2 cells. H9c2 cells were incubated for 72 hours with 10µM BER and in the last 24 hours with 1 or 20µM DOX. LC3-I/II content was evaluated by Western blotting (section 3.2.4). Data were represented in mean±SEM of 5 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (** - p< 0.01 vs. control; ****- p< 0.001 vs. control; ## - p< 0.01 vs. 20µM DOX; ### - p< 0.001 vs. 20µM DOX).

This results suggests that the presence of BER did not induce any changes in LC3-I -LC3-II conversion when compared with untreated cells. The presence of BER pre-treatment in H9c2 cells incubated with 1 µM DOX appeared to induce an increase (21.9% ± 6.2%) in LC3-II formation. However that result was not statistically significant. We also observed that the same BER pre-treatment induced a relevant conversion of LC3-I into LC3-II when both are compared with untreated cells and cells treated with 20 µM DOX. LC3-II amount was not altered in DOX-treated cells, but LC3-I was upregulated in the same group of cells.

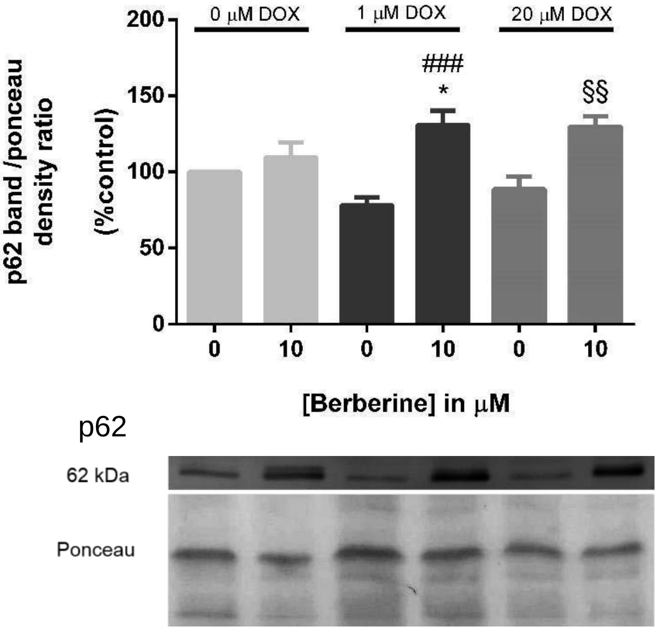

Figure 12: Effect of BER on p62 content in DOX-treated in H9c2 cells. H9c2 cells were incubated for 72 hours with 10 µM BER hours and in the last 24 hours with 1 or 20 µM DOX. P6 content was evaluated by Western blotting (section 3.2.4). Data were represented in mean±SEM of 5 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (* - p< 0.05 vs. control; ### - p< 0.001 vs. 1µM DOX; §§ - p<0.01 *vs* 20µM DOX.

The effect of BER in p62 content on DOX-treated H9c2 cells is represented in figure 12. We observed that p62 was down-regulated in both DOX-treated cells. Although results were not statistically significant, the decrease in p62 content was 20.1% ± 5.4% for cells treated with 1 µM DOX and 12.5% ± 6.6% in 20 µM DOX. BER slightly increased p62 content when compared with untreated H9c2 cells. In similar way, BER also induced p62 accumulation in DOX-treated cells. p62 cellular amount in increased 29.8% ± 7.8% relatively to untreated cells and 62.4% ± 8.9% when compared with cells treated with 1µM DOX. The increased p62 content in BER/ 20µM DOX combined treatments was also statistically significant when compared with the respective DOX concentration alone.

The capacity of BER to induce Cathepsin B or D activation on DOX-treated H9c2 cells is shown in Figure 13. We observed that BER appeared to have no effect in Cathepsin B and D activation on H9c2 cells. Despite BER or 1 µM DOX, alone or in combination, had no effect on cathepsin D activity, cathepsin B increased when 1 µM DOX was used in H9c2 cells (figure 11B). Interestingly, the ratio between cathepsin D /cathepsin B increased when 10 µM BER was incubated with 20 µM DOX (figure 13C).

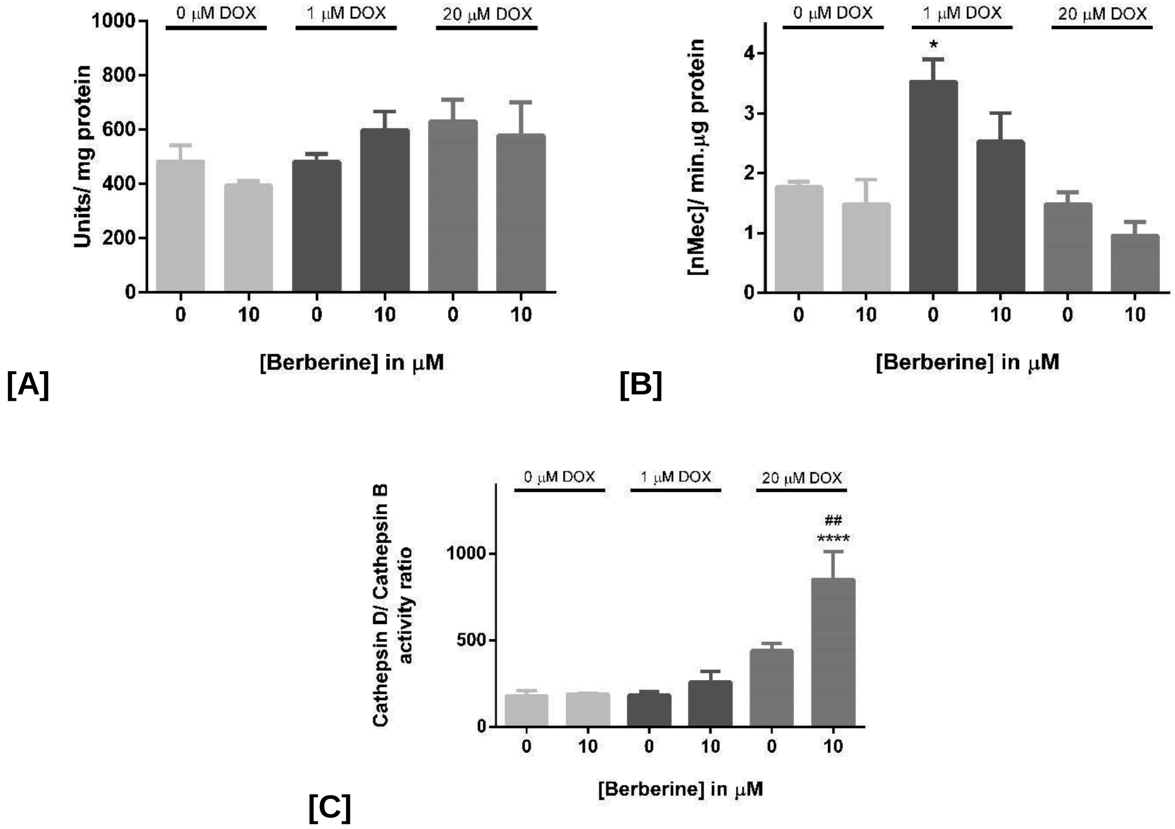

Figure 13: Effect of BER on Cathepsin D (panel A) and B (panel B) activation in DOX-treated H9c2 cells. The ratio between Cathepsin D and Cathepsin B is represented in panel C. The ratio between cathepsin D and cathepsin B is represented on panel C. H9c2 cells were incubated for 72 hours with 10µM BER and with 1 or 20µM DOX in the last 24 hours. The measurement of cathepsin B like- activity was measured fluorometrically while cathepsin D activity was measured spectometrically as described at section 3.2.5. Data were represented in mean±SEM of 5 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (* - p< 0.05 vs. control; **** - p< 0.0001 vs. control; ## - p< 0.01 *vs*. 20µM DOX).

6.3 Apparent modulation of DOX-induced mitophagy by BER

Mitophagy is a highly selective autophagic process that has the capacity to eliminate damaged and dysfunctional mitochondria. The loss of $\Delta\Psi_m$ is one of the events that can trigger mitophagy (Dutta, Calvani et al. 2012).

To evaluate if BER modulates mitophagy, we observed mitochondria and lysosome co-localization with Lysotracker Green and TMRM (Figure 14). We observed that untreated H9c2 cells presented normal cell morphology with a well-defined and filamentous mitochondrial network, as well as some lysosomal presence. In untreated control cells, little or no co-localization between the two organelles was found. BER did not appear to induce alterations in cell morphology. BER-treated H9c2 cells maintained the well-defined mitochondrial network and lysosome-mitochondria co-localization was minimal. DOX treatments induced changes in H9c2 cell morphology. Cells that were treated with low-DOX concentration manifested a mitochondrial network more fragmented and a significant increase of lysosomes. The lysosomal accumulation co-localized with labelled mitochondria possibly leading to their degradation through a mitophagic process (yellow bodies). High-DOX treated H9c2 cells presented a more fragmented mitochondrial network and an extensive accumulation of acidic vesicles. Despite that fact, we observed that lysosome- mitochondria co-localization was present, but in a much less extent that in low-DOX concentration. BER/DOX treatments appeared to exert different effects in low and high DOX concentrations. We observed that BER pre-treatment before the administration of 1 µM DOX led to a decrease on lysosomal-mitochondria co-localization. The opposite scenario happened in 20 µM DOX-treated cells. BER/DOX treatments with high- DOX concentration appeared to increase the co-localization between mitochondria and acid vesicles.

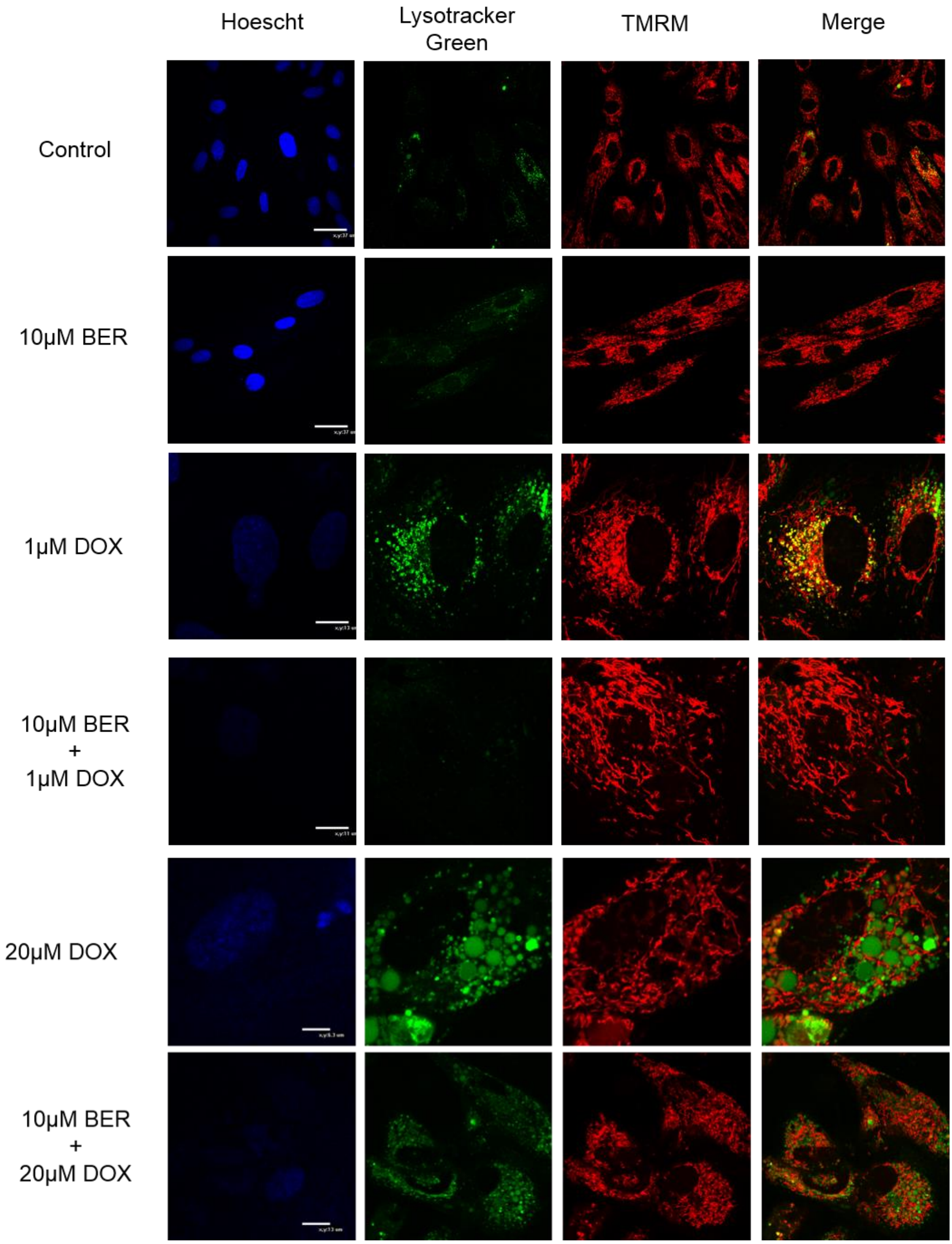
Hoescht
Lysotracker Green
TMRM
Merge
Control
10μM BER
1μM DOX
10μM BER + 1μM DOX
20μM DOX
10μM BER + 20μM DOX

Figure 14: Lysosomes and mitochondria co-localization in BER/DOX combined treatments in H9c2 cells. H9c2 cells were incubated for 72 hours with 10μM BER and with 1 and 20 μM DOX in the last 24 hours. Organelles staining were performed has described in section 3.2.6. Images represented have different magnification to allow a better identification of BER effects.

6.4 Berberine induced SIRT1 and SIRT3 upregulation on DOX-treated H9c2 cells.

Sirtuins are described to regulate mitochondrial metabolism, mitochondrial biogenesis, autophagy and cell death (Hariharan, Maejima et al. 2010, Nogueiras, Habegger et al. 2012, Pereira, Lebiedzinska et al. 2012). To see if BER can alter SIRT1 and SIRT3 cellular content, western blotting was performed. The effect of BER in SIRT1 and SIRT3 on DOX-treated cells is represented in Figure 15 and 16, respectively.

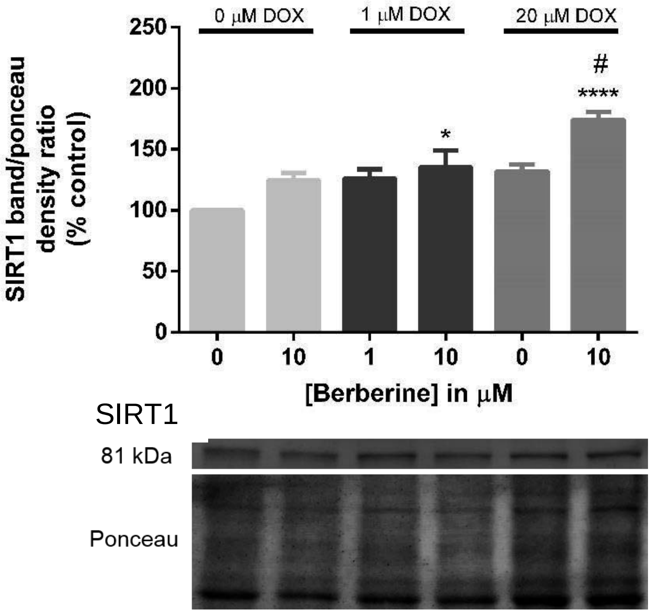

Figure 15: Effect of BER on SIRT1 content in DOX-treated H9c2 cells. H9c2 cells were incubated for 72 hours with 10µM BER and with 1 or 20µM DOX in the last 24 hours. SIRT1 content was evaluated by Western blotting (section 3.2.4.). Data were represented in mean ± SEM of 4 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (* - $p< 0.05$ vs. control; **** - $p< 0.0001$ vs. control; # - $p< 0.05$ vs. 20µM DOX).

We observed that DOX treatment increased SIRT1 amount in H9c2 cells, although the results were not statistically significant. Moreover, BER appeared to show capacity to increase SIRT1 content in H9c2 cells treated with 1 or 20 µM DOX. Despite that fact, SIRT1 increase in BER/ DOX combined treatments was only significant with the highest DOX concentration when compared with the respective DOX treatment alone.

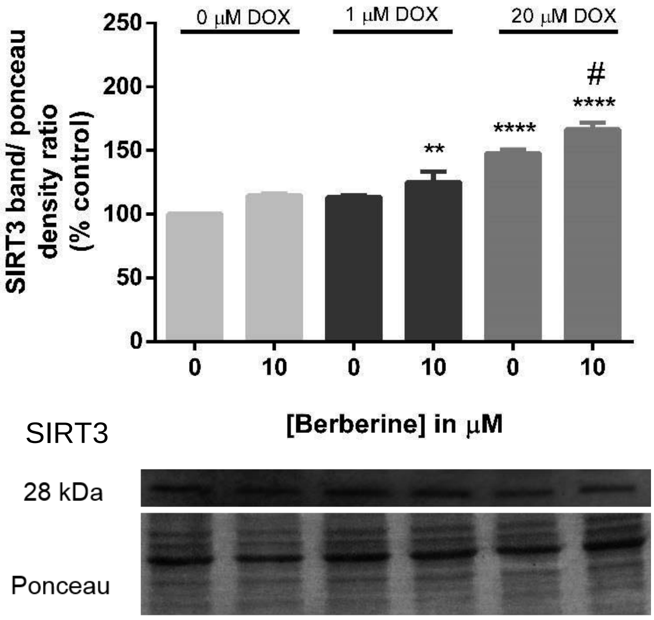

Figure 16: Effect of BER on SIRT3 content in DOX-treated H9c2 cells. H9c2 cells were incubated for 72 hours with 10µM BER and with 1 and 20 µM DOX in the last 24 hours. SIRT3 content was evaluated by Western blotting (section 3.2.4.). Data were represented in mean ± SEM of 4 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (** - p< 0.01 vs. control; **** - p< 0.0001 vs. control; # - p< 0.05 vs. 20µM DOX).

We observed that 20 µM induced an increase in SIRT3 content. In addition, the combination of BER (10 µM) with 20 µM DOX resulted in increased SIRT3 content when compared with DOX alone. Ten µM BER combined with 1 µM also resulted in increased SIRT3 content.

6.5 Berberine did not protect against DOX-induced mitochondrial alterations on H9c2 cells.

Increased oxidative stress is one of the mechanisms responsible for DOX-induced damage. DOX capacity to inhibit complex I, III and IV and the accumulation of damage is several biomolecules leads to activation of mechanisms that results in mitochondrial dysfunction (Goormaghtigh, Huart et al. 1986, Nicolay and de Kruijff 1987). In order to investigate mitochondrial oxidative phosphorylation components, we measured the content of Mitochondrial Complex III and ATP-synthase α by Western Blotting. The protein content is represented in Figure 17.

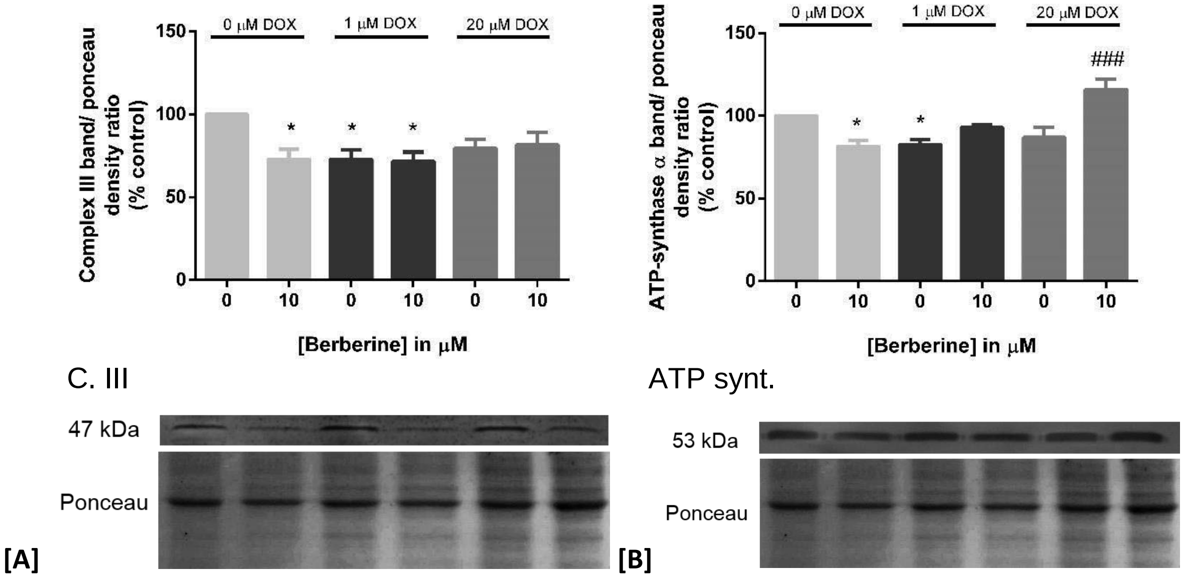

Figure 17: Effect of BER on Mitochondrial Complex III (Panel A) and ATP-synthase α (Panel B) in DOX-treated H9c2 cells. H9c2 cells were incubated for 72 hours with 10 μM BER or with 1 and 20 μM DOX in the last 24 hours. Complex III and ATP-synthase α content were evaluated by Western blotting (section 3.2.4.). Data were represented in mean ± SEM of 5 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (* - $p< 0.05$ vs. control; ## - $p< 0.01$ vs. 20μM DOX).

DOX, namely the lowest concentration, induced Complex III and ATP synthase α subunit downregulation. We observed that both complex III and ATP-synthase α cellular amount were decreased on BER treatments alone (10 μM). Moreover, BER did not rescue the decrease in Complex III and ATP- synthase subunits induced by DOX. An exception was the ATP-synthase α upregulation on BER/DOX treatment with 20μM DOX when compared with 20μM DOX control.

6.6 Berberine increases mitochondrial biogenesis markers on DOX-treated H9c2 cells.

SIRT1 activation by BER is described to be involved in mitochondrial biogenesis mechanisms in skeletal muscle cells resulting in an improvement of mitochondrial function (Gomes, Duarte et al. 2012). To verify if mitochondrial biogenesis mechanisms are active in BER/ DOX combined treatments, we measured

TOM20 and Tfam protein content in H9c2 cells. Tfam is a protein which regulates the replication of mtDNA and is involved in biogenesis (Ventura-Clapier, Garnier et al. 2008, Scarpulla, Vega et al. 2012). The effect of BER in mitochondrial mass was measured indirectly by TOM20 content in H9c2 cells and is represented in Figure 18.

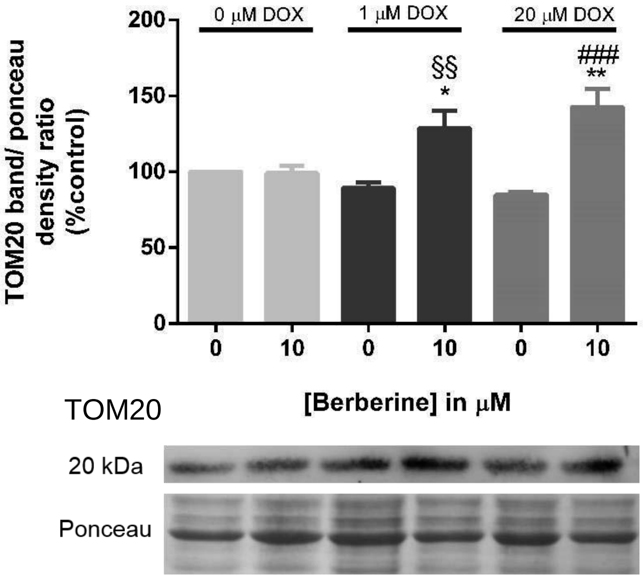

Figure 18: Effect of BER on TOM20 in DOX-treated H9c2 cells. H9c2 cells were incubated for 72 hours with 10 µM BER and with 1 or 20 µM DOX in the last 24 hours. TOM20 content was evaluated by Western blotting (section 3.2.4.). Data were represented in mean ± SEM of 4 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (** - $p< 0.01$ vs. control; **** - $p< 0.0001$ vs. control; # - $p< 0.05$ vs. 20µM DOX).

We observed that DOX induced a minor non- significant decrease in TOM20 content. BER pre-treatment by itself did not alter TOM20 content. However, BER induce an increase in TOM20 content in DOX-treated cells, when compared with the respective DOX control.

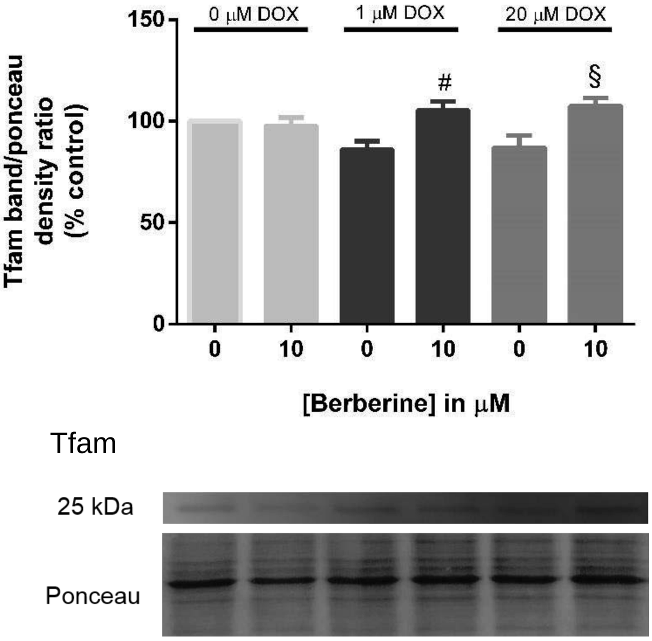

Figure 19: Effect of BER on Tfam in DOX-treated H9c2 cells. H9c2 cells were incubated for 72 hours with 10μM BER and with 1 or 20 μM DOX in the last 24 hours. Tfam content was evaluated by Western blotting (section 3.2.4.). Data were represented in mean ± SEM of 4 independent experiments. Comparisons were performed using One way ANOVA, followed by Bonferroni post-test (#- $p < 0.05$ vs. 1μM; § - $p < 0.05$ vs. 20μM DOX).

The effect of BER on Tfam in DOX-treated H9c2 cells is represented in Figure 19. BER modulation of Tfam transcription factor followed the same pattern of TOM20. We observed that Tfam content suffered a minor, although non- significant decrease on DOX-treated H9c2 cells. In another way, BER administration *per se* did not change Tfam content but when H9c2 cells were pre-treated with BER before DOX administration Tfam content increased significantly when compared with the respectively DOX control.

Chapter 7

Discussion

Berberine is a natural compound that presents cardioprotective and anti-tumoral properties that afforded protection against DOX-induced cardiotoxicity (Marin-Neto, Maciel et al. 1988, Lau, Yao et al. 2001, Zhao, Zhang et al. 2011). It was demonstrated that BER protected against DOX-induced cell death and mitochondrial dysfunction through modulation of the Bcl-2 expression, p53 phosphorylation and AMPKα inhibition (Lv, Yu et al. 2012). Our hypothesis was that BER provides protection against DOX-induced cardiotoxicity in H9c2 cells though SIRT1 activation and oxidative cell pre-conditioning. We observed that BER protected H9c2 cells against DOX-induced caspase activation, although the protection against the loss of cell mass was less evident. This decrease in caspase 9 and 3- like activity is in concordance with previously described (Lv, Yu et al. 2012). Caspase- dependent mitochondrial apoptosis pathway requires the formation of apoptosome and further caspase 9 and 3 activation, which initiates irreversibly apoptosis (Elmore 2007, Taylor, Cullen et al. 2008). BER capacity to modulate this pathway by inhibiting caspase 9 and 3 may play a role in H9c2 cells protection. BER may manifest capacity to inhibit the formation of apoptosome and therefore control caspase-dependent mitochondrial apoptosis. However, BER did not induce a decrease in p53 content. p53 is involved in many cellular regulation processes, and regulates apoptosis and autophagy (Fridman and Lowe 2003, Kroemer, Marino et al. 2010). p53 capacity to directly influence the transcription of pro-apoptotic genes leads to a control of apoptosis (Fridman and Lowe 2003). The results suggest that BER induces p53 upregulation in high-DOX treatments. p53 may participate in different mechanisms that might translate in different cell fates (Fridman and Lowe 2003, Brooks and Gu 2010). One of them is that p53 may be involved in caspase-independent cell apoptosis by activating downstream effectors of apoptosis that can modulate $\Delta\Psi_m$ inducing the release of the apoptosis-inducing factor (AIF) that causes chromatin condensation (Youn, Kim et al.

2005). p53 activation is due to pos-translational modifications, such as phosphorylation and acetylation, which influences its transactivation capacity (Brooks and Gu 2010). In addition, p53 might have a role in mtDNA repair after DOX-induced DNA damage in order to maintain normal mitochondria function (Nithipongvanitch, Ittarat et al. 2007). p53, in a similar mechanism of nuclear DNA repair, might accumulate in mitochondria to rescue mtDNA from damage induce by ROS (Nithipongvanitch, Ittarat et al. 2007). However, if the damage is irreversible p53 can induce pro-apoptotic gene transcription and induce apoptosis.(Nithipongvanitch, Ittarat et al. 2007, Brooks and Gu 2010) Cell senescence and cell cycle arrest may also be regulated by p53 (Brooks and Gu 2010). Activation of HSP27 can modulate p53 transactivation and control p53-dependent cell death in DOX-treated H9c2 cells (Venkatakrishnan, Dunsmore et al. 2008). HSP27 can bind to p53, leading to p21 upregulation and the decrease of pro-apoptotic/ anti-apoptotic ratio leading to cell cycle arrest. Cell cycle in G_2/M phase allows DNA repair and the promotion of cell survival (Venkatakrishnan, Dunsmore et al. 2008). p53 regulates autophagy in a sub-cellular dependent manner. Different treatments that induces different cellular responses will possibly have different p53 locations. p53 localization can also act as an inducer or an inhibitor of autophagy (Kroemer, Marino et al. 2010, Marino, Niso-Santano et al. 2014). On one hand, cytosolic p53 pool can repress autophagy but on the other hand p53 phosphorylation and its nuclear translocation decreases cytosolic p53 facilitating autophagy induction (Kroemer, Galluzzi et al. 2009, Marino, Niso-Santano et al. 2014). BER combined with low-DOX treatments may lead to autophagy inhibition, possibly resulting from p53 accumulation in the cytoplasm whereas in high DOX treatments it might be localized in the nucleus. Nuclear p53 allows the transcription of autophagy related genes that includes AMPK, mTORC, autophagy pathway and lysosomal proteins (Marino, Niso-Santano et al. 2014). To further understand, the role of p53 in the regulation of cell death and autophagy by BER more experiments needs to be done.

Autophagy plays a role in the development of DOX-induced cardiomyopathy (Lu, Wu et al. 2009, Zhu, Soonpaa et al. 2009). DOX-induced autophagy is a double-edged sword. DOX induces autophagy in low concentrations and inhibits autophagy in higher concentrations (Zhang, Shi et al. 2009). Autophagy can act as a cardioprotective mechanism allowing the elimination of damaged cellular components and the maintenance of energy homeostasis (Cecconi and Levine 2008, Dutta, Calvani et al. 2012) However, if autophagy becomes excessively active or uncontrolled, cell death follows resulting in the progression of cardiomyopathy (Dutta, Calvani et al. 2012). Autophagy starts with the engulfment of the cargo targeted for degradation by the phagophore. The assembly of several proteins and the conversion of LC3-I to LC3-II formes the autophagosome (Kroemer, Marino et al. 2010). The autophagic flux goes on and the fusion of the lysosome with the autophasosome results in cargo degradation by lysosomal enzymes such as Cathepsins. (Gottlieb and Carreira 2010, Tanida 2011) Our results showed autophagossome and p62 accumulation in BER- treated cells for low-DOX treatments. Low p62 turnover and autophagosome accumulation leads to autophagy impairment where an inability to degrade the cargo exists (Rusten and Stenmark 2010). This inability is much possible to arise from the blockage of the fusion between the autophagosome and the lysosome. Lysosomal viability can be indirectly measured by cathepsin activity data and it is related to high cathepsin B and low cathepsin D activity (Vega-Naredo, Caballero et al. 2009). We observed a cathepsin B inhibition with BER administration in 1 µM DOX treatment without a significative increase of cathepsin D that might result in lysosomal integrity preservation. High-DOX concentrations also showed p62 and autophagosome accumulation. In addition, the huge accumulation of acidic vesicles that we observed by confocal microscopy imaging may be due to an activation of a protective autophagy mechanism in order to eliminate protein damage that accumulates which cannot be predicted with the data available (Zhou, Tan et al. 2013). In some way, the accumulation of cellular damage was so extensive that mechanisms may be activated to eliminate all the damaged structures. Nevertheless, the capacity to eliminate that cellular damage may be inferior to the rate of its targeting explaining the apparent lysosome and autophagosome accumulation. On another hand, cathepsin B inhibition led to a massive increase of cathepsin D/ cathepsin B ratio. This might indicate that BER modulate lysosomal cathepsin B in order to have the capacity to degrade the cargo targeted for elimination despite the possible inability of autophagosome/ lysosome fusion. Moreover, lysosomal integrity may possibly be decreased due to high cathepsin D activity that may damage the lysosomal membrane. The loss of lysosomal integrity might results in the leakage of lysosomal cathepsins to the cytosol leading to lysosomal-related cell death (Aits and Jaattela 2013). Lysosomal- related cell death may be caspase-independent and can possibly justify the modest protection afforded by BER (Aits and Jaattela 2013).

Although the autophagy may be blocked, mitophagy can be playing a role in elimination of damage mitochondria.(Kubli and Gustafsson 2012) Confocal microsocope imaging results showed some co-localization between mitochondria and lysosomes in DOX-treated cells, indicating that BER may apparently blocked mitophagy in low-DOX treatments. In high-DOX treatments, we did not see mitochondria-lysosome co-localization and BER pre-treatment did not apparently induce a significative increase in the elimination of damage mitochondria. However, this data must be interpreted carefully. It is known that Lysotracker Green only stains acid vesicules and TMRM only accumulates on polarized mitochondria. Mitophagy is normally triggered by loss of $\Delta\Psi_m$ and after cargo degradation lysosome vesicles loose acidic pH. Then our data may show initial mitophagy steps only. More experiments need to be done in order to fully understand which role BER plays in elimination of DOX-induced damaged mitochondria.

Doxorubicin-induced damage results in mitochondrial dysfunction at an early state (Green and Leeuwenburgh 2002). Moreover, DOX has the capacity to inhibit Complex I, III and IV leading to unpaired mitochondrial respiration (Goormaghtigh, Huart et al. 1986, Nicolay and de Kruijff 1987). Our results suggests that BER did not rescue the content of Complex III and ATP- synthase in H9c2 cells. However, BER administration on high-DOX treatments led to upregulation of ATP-synthase when compared with only high-DOX treatment *per se*. The mitochondrial complexes content in cells appears to be unaltered with BER pre-incubation, suggesting that BER may not rescue DOX-treated H9c2 cells from mitochondrial alterations at that level. However, again more experiments need to be done to confirm this result. Removal of dysfunctional mitochondria and the biogenesis of new organelles may lead to a renewable state of energy homeostasis. Mitochondrial biogenesis is characterized by the growth of new mitochondria or the dividing of the pre-existing ones leading to variations on mitochondrial size, number and mass (Ventura-Clapier, Garnier et al. 2008, Scarpulla, Vega et al. 2012). Our results showed an increase in mitochondrial TOM20 which suggests that increased mitochondrial mass may occur with joint DOX/ BER treatments. The increase in mitochondrial mass by BER-treatment is possibly related with the biogenesis of new functional mitochondria. Still, it has to be also considered that the increase of mitochondrial mass might be influenced by the accumulation of the damaged ones that cannot be eliminated by mitophagy. BER modulation of SIRT1 has the ability to activate mechanisms of mitochondrial biogenesis on skeletal muscle cells (Gomes, Duarte et al. 2012). BER pre-treatment in H9c2 cells resulted in SIRT1 and Tfam upregulation in both DOX-treatments. SIRT1 might activate mitochondrial biogenesis in BER treatments in H9c2 cells and possibly lead to the downstream activation of PGC-1 and consequently Tfam. Tfam activation induces the transcription and the replication of mtDNA which translates in more functional mitochondria (Ventura-Clapier, Garnier et al. 2008, Scarpulla, Vega et al. 2012). PGC-1 has another downstream effects despite mitochondrial biogenesis. SIRT3 can be regulated by PGC-1 activation and it may lead to upregulation of ROS-detoxifying (Kong, Wang et al. 2010). It is possible that BER can upregulate SIRT3 expression in both DOX treatments. This might represent the induction of another ROS protective machinery to protect H9c2 cells against DOX-induced oxidative damage.

Chapter 8

Conclusion

In this work, we evaluated the effect of BER in DOX-induced cell death and autophagy. BER pre-conditioning was able to confer protection against DOX-induced cell death cells by inhibiting caspase-dependent mitochondrial apoptosis pathway. Moreover, BER might being regulating DOX-induced cell death by alternatives death pathways that were not considered until now such as lysosomal-induced death. Although autophagy might represent itself as a protective mechanism, its uncontrolled upregulation might led to a mal-adaptive mechanism and the induction of cell death. BER were able to block autophagy in DOX-treated cells. Nevertheless more data is necessary to evaluate the autophagic flux in high-DOX treatments and understand if it is a protective or a deleterious mechanism for the cell. BER appeared to induce mitochondrial biogenesis and increased SIRT1 content, although it is still early to identify the relationship between both. The activation of SIRT1 might led to downstream events such as PGC-1 activation that is involved in mitochondrial biogenesis and SIRT3 activation. More experiments need to be performed to understand the modulation of BER in some mechanisms underlying DOX-induced cell death and autophagy and whereas the effects on the H9c2 cell line also occur in vivo.

In conclusion, BER appears to have a protective effect on DOX-induced cardiotoxicity, controlling mechanisms of cellular death and autophagy. Although this topic needs further investigation, co-administration of BER and DOX might be a promising approach to counteract DOX-induced cardiotoxicty.

Chapter 9

Future Directions

To understand completely how BER can modulate cell death processes and autophagy, more work has to be done to clarify this mechanism. On one hand, we observed that BER appeared to inhibit caspases-dependent cell death. Moreover, BER blocked autophagy for both low and high-DOX concentration treatments. It has been known that p53 has either the ability to induce apoptosis, autophagy or cell arrest depending of it cellular localization and it activation status. To understand which effect p53 upregulation induce in BER/DOX treatments it could be interesting to analyse p53 localization, it degree of acetylation, as well as p21 content.

We also observed in this work that BER capacity to modulate autophagy might be dependent of DOX concentration. Although BER appears to block autophagy and mitophagy in 1µM DOX-treated cells, its effect on 20µM DOX-treated cells appeared to slightly increase mitophagy. To complement the results obtained, the quantification of $\Delta\Psi_m$ will be a result that could corroborate confocal microscopy images. In addition, measurement of carbonilated protein content could help to understand if the autophagy that occurs in BER/DOX treatments with 20µM DOX is a protective or a defective mechanism.

BER did not appear to reverse DOX-induced mitochondrial alterations. However, the decrease in ETC protein content might not translate in activity loss. To verify if there are a direct correlation between protein content loss and loss of activity the measurement of mitochondrial complexes activity or global respiration might be essential to fully understand that results.

We observed that BER appears to induce mitochondrial biogenesis pathways. To confirm the results that we already discussed it could be important to quantify mtDNA copy number in BER/DOX treatments in H9c2 cells, as a direct measure of mitochondrial biogenesis. Moreover, the analysis of PGC-1α content in BER/DOX treatments will also provide a fully clarification of this mechanism.

Finally, it would be interesting to understand if the activation of all BER induced cell mechanisms may result from AMPK activation. To evaluate this AMPK/AMPK phosphorylated content ratio might be a good indicator.

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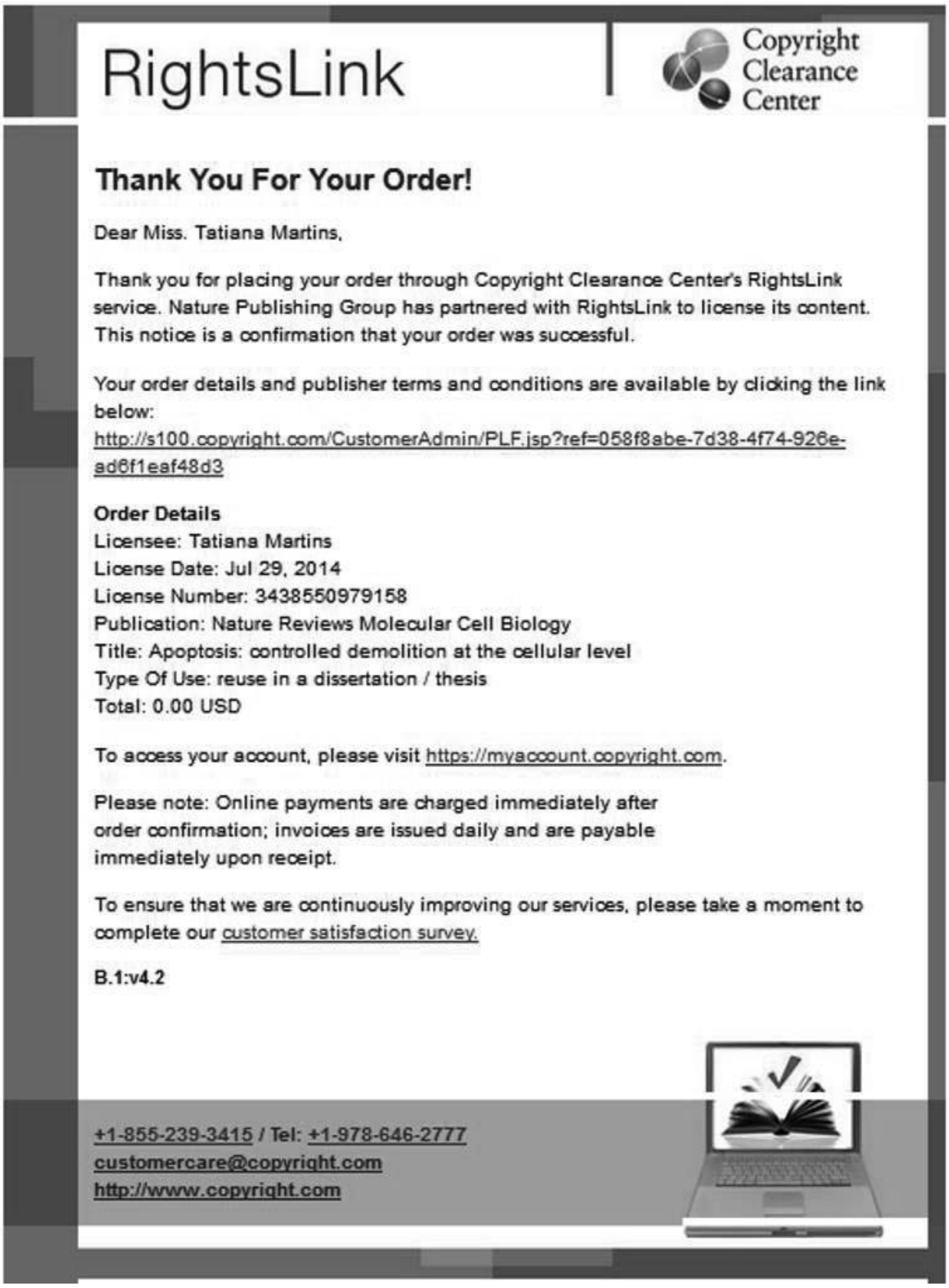

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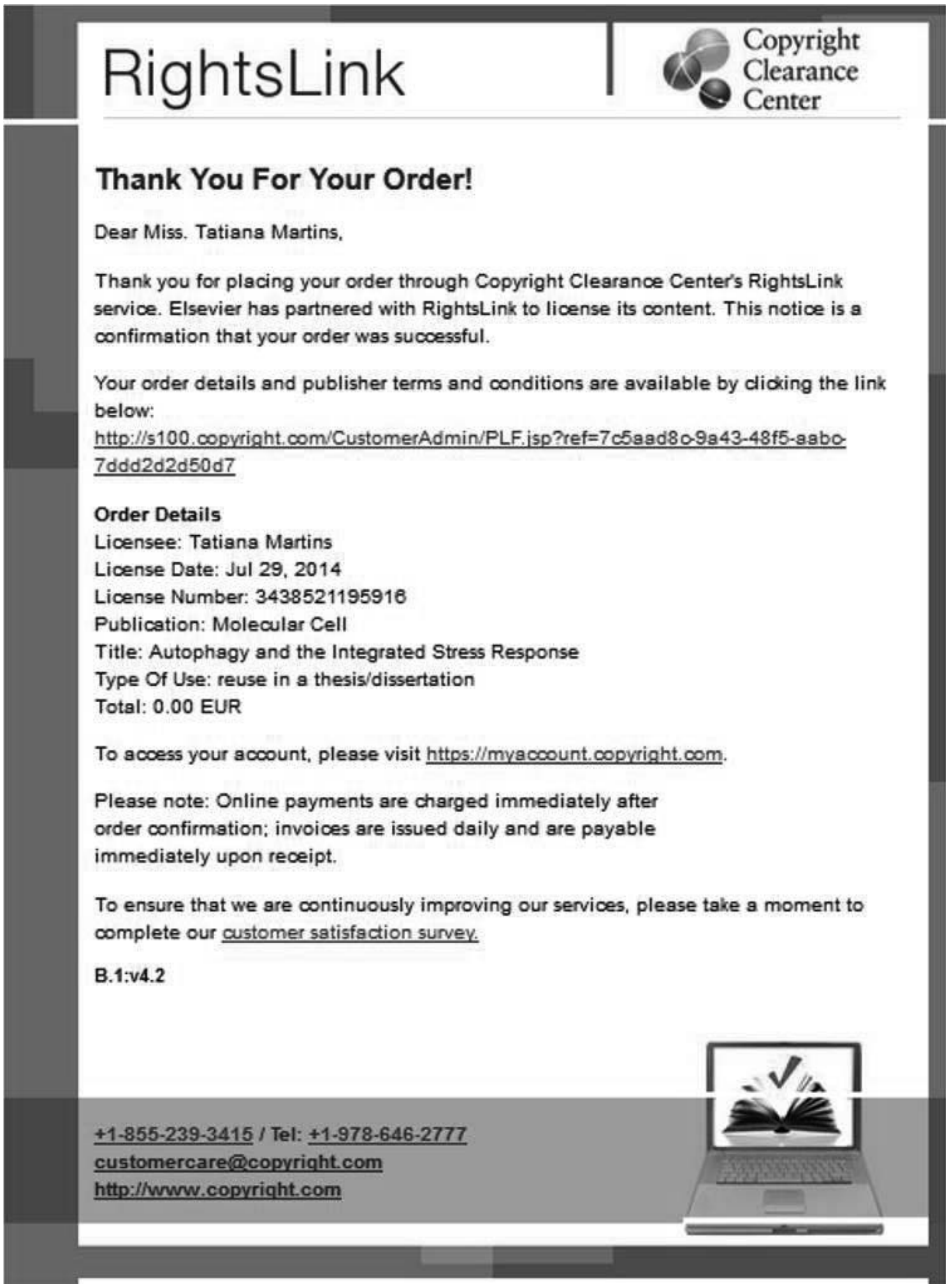

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