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DEPARTAMENTO DE CIÊNCIAS DA VIDA

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Mitochondria-directed Antioxidants as Anticancer Agents

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 Jorge G. S. da Silva Oliveira (Centro de Neurociências e Biologia Celular) e com supervisão académica do Professor Doutor António Joaquim Matos Moreno (Departamento de Ciências da Vida, Universidade de Coimbra)

Katia Queirós Fernandes dos Santos

2015

Valeu a pena? Tudo vale a pena
Se a alma não é pequena
Fernando Pessoa, *Mensagem*

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Abstract

Cancer is one of the most widespread and fatal diseases of the past decades. Due to its multifactorial character, this disease has been extremely difficult to control. Current drugs used in chemotherapy are extremely toxic to healthy tissue since they have poor specific mechanisms of action, leading to unspecific action in healthy tissues. In addition, the lack of efficacy of these compounds may be due to acquired or intrinsic resistance. Therefore, clinical research has been increasing in order to develop a drug, which may be termed as the "magic bullet", more specific to a potential therapeutic target. The compounds used in this study were developed to be directed to the mitochondria, given the importance of this organelle for the normal functioning of eukaryotic cells. Mitochondria are important organelles for cell homeostasis as they control the production of ATP by oxidative phosphorylation. For this process, the escape of electrons through complexes I and III can lead to the formation of reactive oxygen species (ROS) such as superoxide anion. Generally speaking, cancer cells produce more ROS than healthy cells, which justifies considering mitochondria as potential targets for treating cancers. With this purpose in mind, two molecules derived from caffeic acid and vitamin K, MitoXT1 and MitoXT2 respectively, were developed at the Faculty of Science, University of Porto. These compounds have a tetraphenyl phosphonium group that increases the targeting of these compounds into mitochondria and demonstrated antioxidant activity in a series of preliminary experiments. The present work has two tandem objectives: a) investigate whether MitoXT1 or MitoXT2 increase the cytotoxicity of classic anti-cancer agents, doxorubicin (DOX), cisplatin (CIS) and etoposide, on human lung cancer A549 cells and b) investigate whether the same agents protect lung MRC-5 fibroblasts from the toxicity of the above mentioned anti-cancer agents. The study was performed with a pre-treatment with antioxidants for 24h followed by incubation for 24 or 48 h with corresponding anti-cancer agent. Cell viability, caspase activity, superoxide production and cell cycle were assessed in order to investigate the effect of these mitochondria-directed antioxidants in both cell lines studied in the presence and absence of the anti-cancer agents.

The results obtain suggest that the compound MitoXT2 protect MRC-5 cells from cell death resulting from DOX toxicity, while still causing cytotoxicity to A549 cells in conjugation with that snit-cancer agent. The conjugation of the same antioxidant with CIS also resulted in significant toxicity against A549 cells while showing little, if any toxicity against MRC-5 cells. Interestingly, MitoXT2 slightly, but significantly, decreased DOX toxicity on H9c2 cardiomyoblast. Regarding MitoXT1, the effects were somehow lower than MitoXT2 with

no increased cytotoxicity resulting from MitoXT1 in conjugation with etoposide and CIS. When measuring caspase-like activities (caspase 3 and 9) MitoXT2 increase caspase 3 activity when added before DOX to A549 cells but interestingly decrease that same activity in MRC-5 cells, again suggesting some protection to the non-tumour cell line. Surprisingly, the combination of MitoXT1 with CIS increased caspase 3 and 9-like activities in the tumour cell line, while decreasing those specific activities in the non-tumour cell line. Live/Dead assays by flow cytometry, although less robust in terms of differences, shows an apparent small increase in dead cells when the antioxidant were used in combination in tumour cells and the inverse in non-tumour-cells. Vital epifluorescence microscopy using nuclear and mitochondrial dyes appear to show that mitochondrial depolarization and chromatin condensation are associated to the cytotoxicity presented by the difference molecules, alone or in conjugation. Data was also obtained regarding cell cycle analysis which appear to be compound and cell type-dependent but the reduced number of experiments does not allow any conclusions. Finally, results obtained by using the mitochondrial superoxide anion specific dye MitoSOX suggest that the combination of agents may contribute to increase the production of the reactive oxygen species. In conclusion, although preliminary, the present work demonstrates that the novel mitochondria-directed molecule may be used to increase the cytotoxicity of anti-cancer agents as seen in a cell model for lung cancer. Interestingly, some of the data obtained suggested that the same approach resulted in a lower toxicity for non-tumour cells. Furthermore, the present work highlights a previously unknown feature of the two novel molecules: their ability to increase mitochondrial superoxide anion production, which can contribute to a hormesis-type effect. More work must be performed to distinguish the mechanisms involved and whether these are extended to others cancer cell lines.

Keywords: mitochondrial-directed antioxidants; cisplatin; doxorubicin; oxidative stress; mitochondria; lung cancer

Resumo

O cancro é uma das doenças mais disseminada e fatal das últimas décadas. Devido ao seu carácter multifatorial, esta doença tem sido extremamente difícil de controlar. Os fármacos existentes para o tratamento desta doença são extremamente tóxicos para o tecido saudável, uma vez que possuem mecanismos de ação muito pouco específicos, podendo interagir tanto com células tumorais como com células saudáveis. Para além disso, as células tumorais podem desenvolver resistência aos fármacos usados na quimioterapia. Por esse motivo, a investigação clínica tem vindo a aumentar no sentido de desenvolver um fármaco, algo que podemos denominar como a "bala mágica", que seria capaz de se ligar especificamente a um potencial alvo terapêutico. Os compostos usados neste trabalho foram desenvolvidos de modo a ter a mitocôndria como alvo, dada a importância desta para o funcionamento normal de células eucarióticas. A mitocôndria é um organelo importante para a homeostasia celular pois controla a produção de ATP através da fosforilação oxidativa. Relativamente a este a processo, a fuga de eletrões através dos complexos I e III pode levar à formação de espécies reativas de oxigénio (ERO), como é o caso do anião superóxido. Geralmente, as células tumorais produzem mais ERO do que as células saudáveis, o que justifica a consideração desta como um potencial alvo para o tratamento de cancros. Com esse propósito em mente, foram desenvolvidos na Faculdade de Ciências da Universidade do Porto, dois compostos derivados do ácido cafeico e da vitamina K, MitoXT1 e MitoXT2, respetivamente. Estes compostos possuem um grupo tetrafenifosfónio capaz de aumentar o redireccionamento destes compostos para o interior da mitocôndria, tendo-se demonstrado em ensaios preliminares que possuem atividade antioxidante. O presente trabalho tem dois objetivo principais: a) investigar se os compostos MitoXT1 ou MitoXT2 irão aumentar a citotoxicidade de anticancerígenos clássicos como doxorrubicina (DOX), cisplatina (CIS) e etoposídeo em células do cancro do pulmão A549 e b) investigar se os mesmos compostos irão proteger os fibroblastos do pulmão MRC-5 dos efeitos tóxicos dos efeitos tóxicos dos anticancerígenos mencionados acima. O estudo foi realizado com um pré-tratamento de 24h com os antioxidantes seguido de uma incubação de 24 ou 48h com as correspondentes drogas. A viabilidade celular, a atividade de caspases, a produção de superóxido e o ciclo celular foram avaliados de modo a tirar conclusões sobre o efeito destes antioxidantes dirigidos para a mitocôndria nas duas linhas celulares em estudo na presença ou ausência dos agentes anticancerígenos. Os resultados obtidos sugerem que o composto MitoXT2 protege as células MRC-5 da morte celular resultante de toxicidade de DOX enquanto, em células A549, causa citotoxicidade quando combinado com esse agente anticancerígeno. A

conjugação do mesmo antioxidante com CIS aumentou significativamente a toxicidade contra as células A549 enquanto em células MRC-5 mostra pouca ou nenhuma toxicidade. Curiosamente, MitoXT2 diminui pouco mas significativamente a toxicidade induzida por DOX em cardiomioblastos H9c2. Quanto aos efeitos de MitoXT1, são menores do que MitoXT2 com nenhum aumento na citotoxicidade resultando do tratamento conjunto de MitoXT1 com etoposídeo e CIS. Ao medir as atividades das caspases 3 e 9, verifica-se que MitoXT2 aumenta a atividade da caspase 3 quando adicionado antes de DOX mas, interessantemente, decresce a mesma atividade em células MRC-5, sugerindo novamente alguma proteção para a linha celular não tumoral. Surpreendentemente, a combinação de MitoXT1 com CIS aumentou a atividade das caspases 3 e 9, na linha celular tumoral, enquanto diminui essa atividade na linha celular não tumoral. O ensaio de *Live/Dead* realizado por citometria de fluxo, embora menos robusto em termos de diferenças, mostra um pequeno aumento aparente na quantidade de células mortas quando o antioxidante foi usado em combinação nas células tumorais, verificando-se o oposto nas células não tumorais. Utilizando sondas vitais nucleares e mitocondriais, verificou-se por microscopia de epifluorescência uma despolarização mitocondrial e condensação da cromatina, estando estes dois processos associados à citotoxicidade apresentado pelas moléculas de diferença, sozinhas ou em combinação. Em relação à análise dos dados obtidos para o ensaio do ciclo celular, estes parecem depender do tipo de célula e do tipo de composto mas o reduzido número de experiências não permitiu tirar nenhuma conclusão. Finalmente, os resultados obtidos usando a sonda MitoSOX específica para o anião superóxido mitocondrial sugerem que a combinação de agentes pode contribuir na produção de espécies reativas de oxigénio. Em conclusão, embora preliminar, o presente trabalho demonstra que novas moléculas dirigidas à mitocôndria podem ser usadas para aumentar a citotoxicidade de agentes anticancerígenos, tal como foi verificado no modelo de células do cancro de pulmão. Curiosamente, algumas das avaliações dos dados obtidos pela mesma abordagem resultaram numa menor toxicidade para as células não tumorais. Além disso, o presente trabalho demonstra a existência de uma característica até então desconhecida das duas novas moléculas; a sua capacidade para aumentar a sua produção de anião superóxido mitocondrial, o que pode contribuir para um efeito do tipo hormese. Mais trabalho deve ser realizado de modo a identificar os mecanismos envolvidos nestas e noutras linhas celulares tumorais.

Palavras-chave: antioxidantes dirigidos a mitocôndria; cis-platina; doxorrubicina; *stress* oxidativo; mitocôndria; cancro do pulmão

Abbreviations

Δψ	Mitochondrial transmembrane electric potential
ΔpH	pH component of proton motive force
ADP	Adenosine Diphosphate
AIF	Apoptosis Inducer Factor
ANT	Adenine Nucleotide Translocator
APAF-1	Apoptotic Protease Activating Factor-1
ATP	Adenosine Triphosphate
Bad	Bcl-2-associated Death Promoter
Bak	Bcl-2 Homologous Antagonist Killer
Bax	Bcl-2-associated X protein
Bcl-2	B-cell lymphoma 2
Bcl-XL	B-cell Lymphoma-extra-large
BSA	Bovine Serum Albumin
CIS	Cisplatin
COX	Cycloyigenase
Cyt c	Cytochrome C
DMEM	Dulbecco's Modified Eagle's Medium
DOX	Doxorubicin
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic Acid
EH-1	Ethidium Homodimer-1
EPO	Eosinophil Peroxidase
FAD	Flavin Adenine Dinucleotide
FBS	Foetal Bovine Serum
GPx	Glutathione Peroxidase
HIF	Hypoxia Inducible Factor
LOX	Lipoxygenase
MCU	Mitochondrial Calcium Uniporter
MIM	Mitochondrial Inner Membrane
MitoXT1	New molecule derived from caffeic acid
MitoXT2	New molecule derived from Vitamin K
MOM	Mitochondrial Outer Membrane

MAO	Monoamine Oxidase
MPO	Mieloperoxidase
MPT	Mitochondrial Permeability Transition
MRC	Mitochondrial Respiratory Chain
mtDNA	Mitochondrial Deoxyribonucleic Acid
NADH	Nicotinamide Adenine Nucleotide , Reduced Form
NADPH	Nicotinamide Adenine Nucleotide Phosphate, Reduced Form
NOS	Nitric oxide Synthase
NOX (1)	NADH Oxidase (1)
NSCLC	Non Small Cancer Lung Cell
$\bullet O_2^-$	Superoxide Anion
$\bullet ONOO^-$	Peroxynitrite
OXPHOS	Oxidative Phosphorylation
PBS	Phosphate Buffer Saline
pNA	p-Nitroanilide
PPP	Pentose Phosphate Pathway
PMF	Proton Motive Force
PI	Propidium Iodide
SOD	Superoxide Dismutase
SRB	Sulforhodamine B
TCA	Tricarboxilic Acid
TKT	Transketolase
TMRE	Tetramethylrhodamine, Ethyl Ester
ROS	Reactive Oxygen Species
VDAC	Voltage Dependente Anion Channels
XO	Xanthine Oxidase

Index

Acknowledgements ix
Abstract xi
Resumo xiii
Abbreviations xv
Index of Figures xxi
Chapter 1-Introduction 1
1.1General Consideration about Cancer 1
1.1.1 Properties of Lung Cancer 2
1.2 The Role of Mitochondria in Cancer 4
1.2.1Mitochondria Structure and Physiology 4
1.2.2 Contribution of Oxidative Stress for Disease 6
Reactive Species of Oxygen 6
Importance of the Right Equilibrium Between Oxidative Stress and Antioxidant Compounds in Cancer 7
1.2.3 Switch in Mitochondria Regulation in Cancer 10
1.2.4 Cell Death and Mitochondria 12
1.2.5 Mitochondria as a New Important Target for Therapy 14
1.3 Cancer Treatment 15
1.3.1 Anti-cancer Agents 16
1.3.1.1 Doxorubicin and its Chemotherapeutic Effects 16
DOX Anti-cancer Mechanisms of Action 17
Cardiac Toxicity 18
DOX-induced Lung Cancer Resistance 19
1.3.1.2 Cisplatin and Cancer Treatment 20
Involvement of Cisplatin in ROS Production 22
Antioxidant as a Protective CIS Induced-toxicity 22
1.2.1.3 Etoposide as a cancer treatment 24
1.3.2 Antioxidant compounds 25
1.3.2.1 Caffeic acid and derivatives 26
1.3.2.2 Vitamin K and Derivatives 27
Anticancer Properties of Vitamin K 28
Chapter 2- Main Goal 30
Chapter 3- Material and Methods 35
3.1 Common Reagents 37

3.2 Solution Preparation 37
3.2.1 Cell Culture Media 37
3.2.2 Phosphate Buffered Saline (PBS) 37
3.2.3 Phosphate Buffered Saline –Tween (PBS-T) 37
3.2.4 Vital Microscopy Media 38
3.2.5 Sulforhodamine B Solution 38
3.2.6 Lysis Buffer 38
3.2.7. Bradford Reagent (2X) 38
3.3 Cell Lines 38
3.3.1 A549 Lung Carcinoma Cell Line 38
3.3.2 MRC-5 Lung Fibroblast Cell Line 39
3.3.3 H9c2 Cardiomyoblast Cell Line 39
3.3.4 Initiating a New Cell Culture 39
3.3.5 Assessments of Cell Proliferation 40
3.3.6 Cell Counting 40
3.4 Assays Planning 41
3.5 Specific Methods 43
3.5.1 Resazurin Assay 43
3.5.2 Sulforhodamine B Assay 43
3.5.3 Protein Quantification 44
3.5.4 Determination of Caspase 3 and 9-Like Activities 44
3.5.5 Live/Dead Assays by Flow Cytometry 45
3.5.6 Cellular Cycle Measurements 46
3.5.7 Vital Epifluorescence Microscopy 46
3.5.8 MitoSOX Based Detection of Mitochondrial Superoxide Anion 47
3.5.9 Statistical Analysis 47
Chapter 4- Experimental Results 49
4.1 Effects of Tested Antioxidants and Anti-Cancer Agents On A549 and Mrc-5 Cell Mass and Metabolic Activity 51
4.2 Effects of Mitoxt2 Against Dox-induced Cytotoxicity on H9c2 Cardiomyoblasts 55
4.3 Effects of Mitoxt1 Against Dox-induced Cytotoxicity on H9c2 Cardiomyoblasts 57
4.4 Effects of MitoXT2 on Caspase 3 and 9-like Activities Induced by DOX on A549 and MRC-5 Cells 58
4.5 Effects of MitoXT1 on Caspase 3 and 9-like Activities Induced by CIS on A549 and MRC-5 Cells 59
4.6 Effect of Tested Antioxidant on Anti-cancer Agents-induced Toxicity on A549 and MRC-5 Cell 60

4.6.1 MitoXT1 Pre-treatment Combined with CIS Cytotoxicity on A549 and MRC-5 Cell Death 60
4.6.2 DOX and MitoXT2 Combined Effects on A549 and MRC-5 Cell Death 61
4.7 Mitochondrial and Nuclear Alterations Resulting from Anti-cancer Treatments 62
4.8 Cell Cycle Measurements on A549 and MRC-5 Cells Treated with DOX and/or MitoXT2 67
4.9 Cell Cycle Measurements on MRC-5 and A549 Cells Treated with MitoXT1 and/or CIS 68
4.10 Pre-treatment with Antioxidants and Drugs Increase Superoxide Production in Cancer Cells 70
Chapter 5- Discussion 73
Chapter 6- Conclusion and Future Directions 81
6.1 Conclusion 83
6.2 Future Directions 85
References 87
Annex 105
Copyright Permission for Figure 1 107
Copyright Permission for Figure 2 109

Index of figures

Figure 1: Mitochondrial structure and metabolism **6**

Figure 2: ROS metabolism throughout the cell **8**

Figure 3: Schematic illustration of the "Warburg effect" in cancer cells **12**

Figure 4: Morphology of A549 and MRC-5 ..... **40**

Figure 5: Time Line and experimental drug design for all the assays performed **43**

Figure 6 A: Molecular structure of calcein- AM **45**

Figure 6 B: Ethidium monodimer-1 molecular structure **45**

Figure 7: Effects of MitoXT2 pre-treatment followed by DOX treatment on cell mass and metabolic activity, on A549 and MRC-5 cell lines **52**

Figure 8: Effects of MitoXT1 pre-treatment followed by CIS treatment on cell mass and metabolic activity on A549 and MRC-5 cell lines. **52**

Figure 9: Effects of MitoXT2 pre-treatment followed by CIS treatment on cell mass and metabolic activity, on A549 and MRC-5 cell lines **53**

Figure 10: Effects of MitoXT1 pre-treatment followed etoposide treatment on cell mass and metabolic activity on A549 and MRC-5 cell lines **53**

Figure 11: Effect of different concentrations of mitochondria-directed antioxidants MitoXT1 and MitoXT2 in H9c2 cells.: **56**

Figure 12: Protection by MitoXT2 against DOX-induced toxicity on H9c2 cells. **56**

Figure 13: Protection by MitoXT1 against DOX-induced toxicity on H9c2 cells. **57**

Figure 14: Effects of MitoXT2 pre-treatment on DOX induced caspase 3 and 9-like activities, assay performed on both the cell lines A549 and MRC-5. On the top left and right panels: **58**

Figure 15: Effects of MitoXT1 pre-treatment on CIS induced caspase 3 and 9-like activities, assay performed on both the cell lines A549 and MRC-5 ..... **59**

Figure16: Live/Dead assay of A549 and MRC-5 cells in the presence of CIS and MitoXT1 ..... **60**

Figure 17: Live/Dead assay of A549 and MRC-5 cells in the presence of DOX and MitoXT2 ..... **61**

Figure 18: Nucleus and mitochondrial network morphology under MitoXT2 and DOX treatment on A549 cells **63**

Figure 19: Nucleus and mitochondrial network morphology under MitoXT2 and DOX treatment on MRC-5 cells **64**

Figure 20: Nucleus and mitochondrial network co-localization under MitoXT1 and CIS treatment on A549 cells : **65**

Figure 21: Nucleus and mitochondrial network co-localization under MitoXT1 and CIS treatment on MRC-5 cells **66**

Figure 22: Cell cycle effect of DOX combined with MitoXT2 treatment on MRC and A549 cells **67**

Figure 23: Cell cycle effect of CIS combined with MitoXT1 treatment on MRC and A549 cells.....**68**

Figure 24: Histograms of cell cycle distribution of A549 and MRC-5 cells after the combined MitoXT2 and DOX treatments **69**

Figure 25: Histograms of cell cycle distribution of A549 and MRC-5 cells after the combined MitoXT1 and CIS treatments **69**

Figure 26: Superoxide production through MitoXT2 24h pre-treatment followed by a 3h DOX incubation on A549 and MRC-5 cells : **71**

Figure 27: Superoxide production through MitoXT1 24h conditioning followed by a 3h CIS treatment on A549 and MRC-5 cells. **71**

Chapter 1
Introduction

Introduction

1.1 General Considerations about Cancer

Cancer is a potentially fatal disease which targets a large percentage of the human population all over the world, with a higher prevalence in developing countries. In 2002, over ten million new cases of cancer were diagnosed. In addition, around 24.6 million patients, which had been diagnosed within the previous 5 years, were living with cancer and, beyond that, an estimate of six to seven million of deaths were measured [1]. For the last few years, the number of new cancer cases remained constant. For example, in 2014, almost the same number of new cancer cases were estimated as in 2010, about 1,600,000 in the United States of America, together with almost 600,000 deaths [2, 3]. It is estimated that until 2020, the number of new reports of cases of cancer will increase to a total of 15 million and the number of deaths may reach 12 million [4].These facts reveal the importance of researching novel, more effective, anticancer drugs, which are may be key to reduce cancer mortality. The amount of research regarding this matter has been growing and numerous studies have been performed in order to test the efficiency and toxicity of new drugs, or adjuvants, in the treatment of cancer [4].

Cancer is a complex disease which is extremely hard to understand due to it having many possible causes, where environmental factors such as tobacco, chemical compounds, radiation or even infectious organisms are some of the most commons examples, or intrinsic causes which include genetic mutations and mutations that occur due to metabolism, hormone dysregulation and immune conditions [2]. In addition, several different mechanisms can alter the normal functioning of cancer cells, leading them to replicate uncontrollably, ignoring all growth control regulation mechanisms.

It has been reported that the types of cancer to which an individual may be more susceptible is dependent on their background, more specifically on where the patient was born and grew up. In developed countries, prostate and lung cancers are most widespread among men with breast, colon and rectum the most persistent types of cancer in women. On the contrary, in developing countries, the most common types of cancers in men are lung and stomach while in women, breast and uterine are the most prevalent types of cancer [5].

Hanahan and Weinberg proposed a total of eight hallmarks with the purpose of better understanding cancer. These are the persistence of proliferation, release of growth factors,

induction of metastases, inhibition of the cell death mechanisms, induction of angiogenesis, immortal replication, adaptation of the energetic metabolism and avoiding immune suppression. The last two points need more researches to be accept Likewise, these Hallmarks might be fundamental for extrapolating new forms of diagnosis, prognosis and treatment of cancer [6, 7].

1.1.1 Properties of Lung Cancer

Lung cancer is the most deadly form of cancer in both sexes, although it is not the most widespread. [5] In 2014, in the U.S, the estimated new cases of lung cancer are about 220,000 with 163,510 (small and non-small cell included) deaths[2]. In2008, in Europe, the third most frequent type of cancer was lung cancer with 391,000 cases and it was also the most lethal form of cancer, with 342,000 deaths [8]. This form of cancer is generally not detected at an early stage, this being the main reason for the five-year survival rate being under 15% at the time of diagnosis [9]. Despite all the advances in the diagnosis and treatment of cancer, the number of cases of lung cancer continues to increase. As a way to counter this tendency, most research has been focused on developing a target-specific agent and to identify new biomarkers [9]. Although the majority of cases that suffer from this disease are due to smoking, the rates of lung cancer in non-smoking patients is increasing [10]. Adding to this, even as rates of smoking are decreasing, the number of new cases of lung cancer is still increasing and it is estimated that, for the next 50 years, it may be considered to be one of the greatest health challenges [9]. For this reason, many studies have been performed to identify biochemical mechanisms responsible for lung cancer, allowing for the development of specific therapeutic drugs directed at these mechanisms [9]. However, it is important to note that changes in the genetic and molecular constitution of lung cancer cells, or in any other of the patient's cells might influence the biology of tumours [11]. Even so, intensive research in this area in the last decade led to a better understanding on to classify tumors [12], has improved the prognosis of patients [13], and provided specific treatments or even new targets for more specific therapies.

Lung cancer is divided into two groups, small and non-small lung cancer cells, in order to obtain a better clinical understanding of this condition [2]. Non-small cell lung cancer (NSCLC) is the most common subtype, representing about 85% of all lung cancer cases, while small cell lung cancer represents only about 15% of all lung cancer cases [8]. However, this classification of lung cancer is far too simple and does not represent the complexity and

peculiarity of this illness, since as it does not take into account the histological pattern of this type of cancer [14].

A549 cells are derived from NSCLC that mimics lung cancer cells, representing a good model to study the effect of anticancer drugs, such as doxorubicin, cisplatin (CIS) etoposide or the novel compounds MitoXT1 and MitoXT2. MRC-5 was used a healthy cell line in order to compare the results obtain in the cancer model.

1.2 The Role of Mitochondria in Cancer

Mitochondria Structure and Physiology

The mitochondrion is a small organelle present in eukaryotic cells which plays a very important role in the regulation of several intracellular pathways, such as the production of adenosine triphosphate (ATP), through oxidative phosphorylation (OXPHOS), calcium homeostasis, and regulation of oxidative status as well as induction of apoptosis. Lynn Margulies proposed in 1967 that mitochondria have their origins on proteobacterium through a mechanism of symbiosis which allowed this organelle to specify itself in the production of energy [15].

Mitochondria are constituted by two lipid bilayer membranes, the inner membrane (MIM), which has numerous invaginations to provide a higher contact surface, and the outer membrane (MOM), wherein lies the mitochondrial matrix in which the multiple chain reactions of oxidative phosphorylation occur [16]. Cholesterol is present in the MOM and this membrane is more permeable to molecules since as it contains voltage-dependent anion channels (VDAC), which are non-specific [17]. Regarding MIM, this membrane has a high content in cardiolipin that is very important to the protein activity of the supercomplexes which constitute the mitochondrial respiratory chain (MRC). Additionally, this membrane is very selective as it contains substrate specific shuttles [17, 18]. MIM is fundamental to mitochondrial activity as it provides a compartment isolated from the matrix and maintains the electrochemical proton gradient necessary for ATP production through the ATP synthase. This gradient, also called the proton motive force (PMF), formed through the proton pump from the mitochondrial matrix to the inner membrane space, is composed by two components, an electrical component $\Delta\psi$, coupled to a pH component ΔpH [19].

Another transporter important for maintaining calcium homeostasis in cells is the mitochondrial calcium uniporter (MCU).

Mitochondria are present in all nucleated cells and produces the energy currency in all aerobic in-vivo organisms, ATP. The hydrolysis of ATP (1) has a negative variation of Gibbs free energy (ΔG) that makes this reaction favourable and is able to provide sufficient energy to activate other endergonic reactions, making them favourable to life [20].

$$ATP \rightarrow ADP + Pi \quad (1)$$

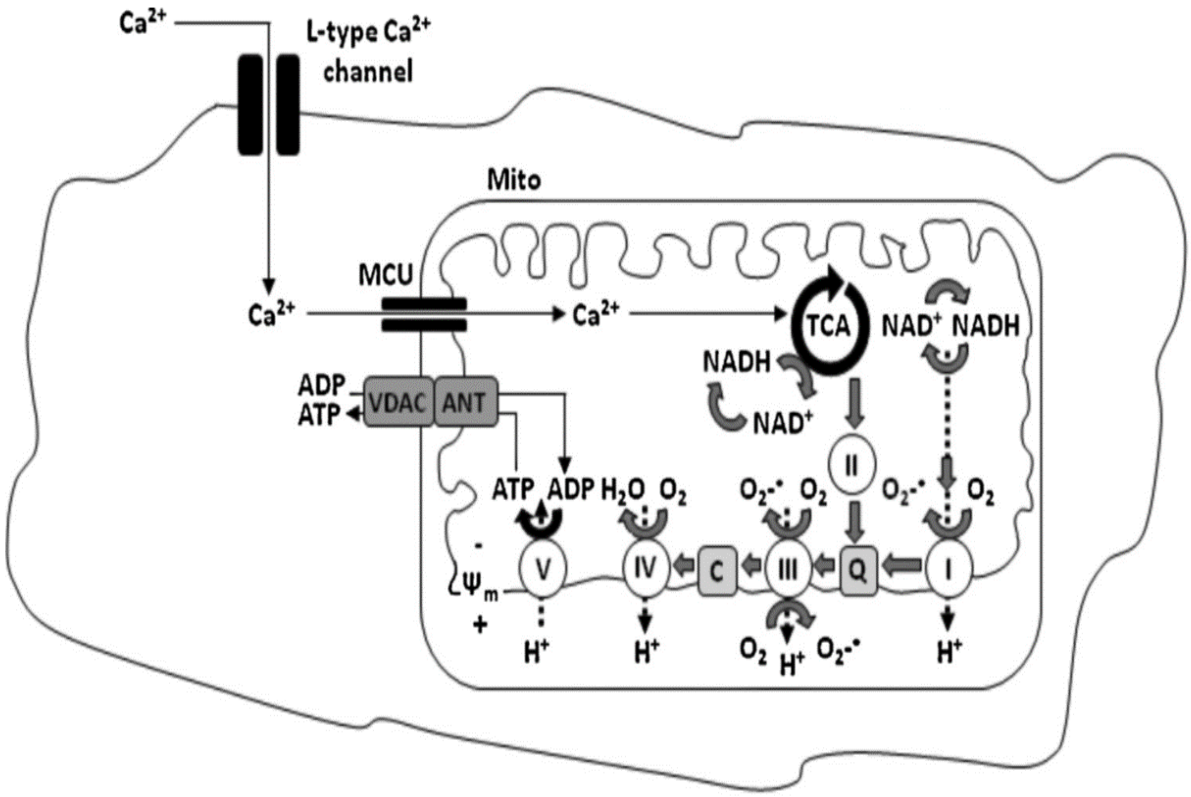

Figure 1: Mitochondria structure and metabolism. The production of superoxide anion radical occurs in the complexes I and III. The tricarboxylic acid cycle occurs in the mitochondrial matrix and provides the electron potential requirement for the activity of OXPHOS. Several tricarboxylic acid cycle proteins are dependent of calcium, including α-ketoglutarate dehydrogenese and pyruvate dehydrogenase. Calcium is an important signalling transducer and enters the cell by a specific L-type Ca^{2+} channel, with the mitochondrial calcium uniporter carrying this ion inside the mitochondrial matrix. Voltage dependent anion channels are responsible for the regulation of ATP and metabolite transport as well as calcium. Adenine nucleotide translocator regulates the homeostasis between ADP and ATP in the mitochondrial matrix. Figure taken from [224], with permission (see Annex I).

The main requirements for this process to occur are the abundance of glucose and the presence of oxygen. It is important to note that, while oxygen is important to OXPHOS due to its high reduction potential (ΔE^{o}), this also means that this molecule may oxidize almost everything in our cells, which may lead to oxidative injuries. This high tendency by oxygen to receive electrons and, adding the fact that oxygen is widely available to cells, leads this molecule to be the first choice as the final acceptor of electrons in OXPHOS, being reduced to water at the end of this chain reaction.

The catabolism of carbohydrates and lipids trough glycolysis and fatty acid β-oxidation performed in the cytosol provides an electron potential in the form of nicotinamide adenine dinucleotide (reduced form) NADH, that is transferred to ubiquinone through complex I [21]. The tricarboxylic acid cycle is also connected with OXPHOS by the intermediary flavin adenine dinucleotide, $FADH_2$, a component of the succinate dehydrogenase enzyme (complex II) that convert succinate to fumarate and transfers electrons to ubiquinone. Electrons of the reduced ubiquinone are then transferred to cytochrome c reductase or complex III and cytochrome c mediates the electron transfer to cytochrome c oxidase (complex IV), coupled to proton ejection in three sites of the MRC [21] (Figure 1). In the end, the high reduction potential accumulated through the re-flow of protons from the intermembrane space is used to phosphorylate ADP to ATP by the ATP synthase or complex V (Figure 1).

1.2.2 Contribution of Oxidative Stress to Disease

Reactive Oxygen Species

Oxygen has it was previously described is a widespread molecule with an imperative role in control of redox homeostasis [22, 23]. O_2, due to its two unpaired electrons can be a source of superoxide anion ($\bullet O_2^-$) through a semi-reduction [22, 24]. This molecule is highly reactive in the presence of other superoxide anion molecules through the activity of antioxidant enzyme superoxide dismutase (SOD). This reaction originates a more stable molecule, hydrogen peroxide, although it still is a potential oxidative species that can be controlled by catalase and glutathione peroxidase [25]. H_2O_2 can also undergo Fenton reaction in the presence of transition metals such as iron, to generating hydroxyl radical (•OH) [26, 27]. To regulate the antioxidant status, cells developed antioxidant defences such as enzymatic and non-enzymatic antioxidants. Regarding enzymatic antioxidants, glutathione peroxidase,

catalase and superoxide dismutase (SOD) can be included. There are two forms of glutathione peroxidase (GPX), in which one is dependent of selenium and is responsible for the elimination of peroxides substrates, required for the Fenton reaction, while the other is independent of selenium (glutathione-S-transferase) and is capable of reducing free hydrogen superoxide to water by the oxidation of glutathione (GSH) to glutathione disulphide. GPX are essential for eliminating hydrogen peroxide as catalase also reacts with H_2O_2 [28]. Catalase is highly effective against hydrogen peroxide since it can reduce over a million water molecules per minute [29]. SOD, as it was previously described manages the reaction of superoxide anion dismutation, leading to the formation of hydrogen peroxide and molecular oxygen. There are three primary forms of SOD, Cu/Zn SOD predominantly present in the cytosol, Mn-SOD found in the mitochondria and extra-cellular SOD. SOD is a radical scavenger and acts by a mechanism of redox cycling, causing a transition on the charge of metal ions present on the active sites of the enzyme [29].

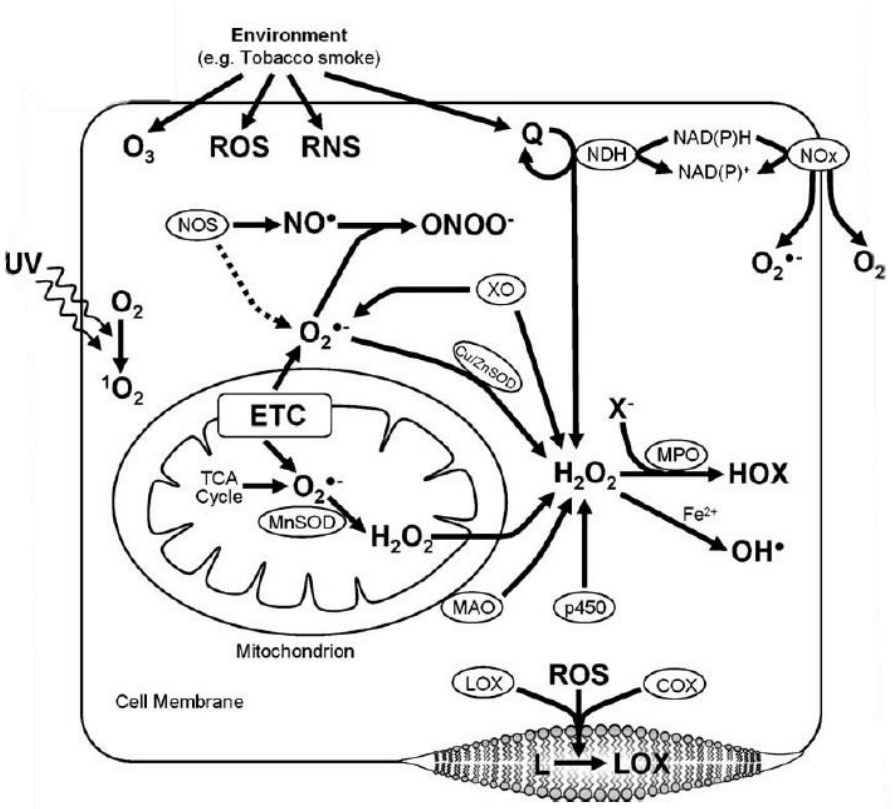

Figure 2: ROS metabolism throughout the cell. ROS can be produced in mitochondria by OXPHOS, tricarboxylic acid cycle and by monoamine oxidase (MAO) present in the MOM. In mitochondria MnSOD converts $\bullet O_2^-$ in the lipophilic H_2O_2, while in the cytosol Cu/Zn SOD is the enzyme involved in the process. In the cytosol, xanthine oxidase (XO), cytochrome p450 and NADPH oxidase (NOx) are the principal sources of ROS. Extrinsic factors may also produce ROS such as O_3, UV radiation, and other toxic chemicals. Peroxynitrite is obtained by the reaction of nitric oxide produced by nitric oxide synthase (NOS) with superoxide anion. NOS may also produce $\bullet O_2^-$. H_2O_2 may be involved in the production of •OH by the Fenton reaction in the presence of a metal transition ion. ROS lipoxygenase (LOX) and cyclooxygenase (COX) undergoes lipid peroxidation (LOX) in the membrane bilayer. Hypochlorus acids such as HOCl and HOBr are produced by myeloperoxidase (MPO) and eosinophil peroxidase (EPO). Imaged used under permission, view annex [33].

Importance of the Right Balance between Oxidants and Antioxidant Network in Cancer

Reactive oxygen species, ROS, are extremely reactive and can be responsible for severe molecular damage. Oxidative stress occurs when a dysregulation between ROS production and the antioxidant network occurs [30]. Interestingly, it appears that a mild increase in oxidative stress plays an important role in intracellular signalling regarding cellular senescence, apoptosis and the control of infection by bacteria through neutrophils and macrophages [31–33]. Indeed, ROS production mediates cellular senescence and apoptosis and acts as an anti-tumorigenic effects. A huge imbalance in oxidative stress originates an uncontrolled oxidation of lipids, proteins, carbohydrates, and DNA, which can lead to gene overexpression and cellular dysfunction [34] [35]. Thus, mitochondrial activity may also be affected by an overproduction of ROS. Moreover, it is well known that ROS are related with many pathologies, such as Parkinson's disease [36], Alzheimer's disease, diabetes and cancer [37, 38]

Reactive oxygen species can be naturally produced by several different mechanisms in cells and by other external factors which may also be associated with ROS production, such as UV radiation, ozone and other pollutants for the metabolism, redox cycling of quinones and nitroaromatic molecules. (Figure 2) It is believed that less than 1% of all oxygen consumed is converted to ROS, with mitochondria being a source of these species. ROS levels are widely controlled by the antioxidant barrier protection because of their double face. On one hand, ROS are essential for many signalling processes but on the other hand when present in excess, ROS can lead uncontrolled oxidation of biomolecules leading to oxidative injuries in the cell [39].

Oxidative phosphorylation is an important redox homeostatic mechanism in the cell, being recognized as an important generator of ROS. The first description of the production of ROS through the mitochondrial respiratory chain goes back to 1966 when Chance *et al.* [40] observed that isolated mitochondria are able to produce hydrogen peroxide. This was the beginning of a new insight regarding ROS production and its purpose in cell signalling. Even taking into account that mitochondria may not be the primary source of ROS, as explained by Brown and Borutaite [41], several reports show that mitochondria have a preponderant role in the ROS formation, as complexes I and III can leak electrons to molecular oxygen, thereby producing superoxide[42] [33]. Monamine oxidase (MAO) in mitochondria also plays an important role in H_2O_2 production, as this flavoenzyme uses oxygen to inactivate

neurotransmitters through oxidation of monamines [43]. ROS also have their origin on the tricarboxylic acid cycle (TCA), in which the enzymes α-ketoglutarate dehydrogenase and the pyruvate dehydrogenase complex might be responsible for producing H_2O_2 and $\bullet O_2^-$ [44]. Superoxide anion is also generated by NADPH oxidase (NOx), which is found in macrophages [45], and by xanthine dehydrogenase/oxidase (XO) responsible for the metabolism of pentoses [46]. Another important enzyme relevant for the protection against microorganism invasions is myeloperoxidase (MPO), which produces hypochlorous acid, also considered as potent oxidative species inside the phagosome [47]. Reactive nitrogen species are also highly reactive to the cells and can lead to cell injury and even cell death. The enzyme nitric oxidase synthase originates nitric oxide (NO) which in turn can react with $\bullet O_2^-$, leading to the formation of peroxynitrite ($\bullet ONOO^-$) [33]. Another source of oxidative stress is p66Sch protein, which acts through the inhibition of MnSOD [48]. p53 modulation might also lead to the increase of ROS in cells, as part of its pro-apoptotic action leading to cell death [48]. Moreover, ROS can be produced by other compartments in the cell, such as the endoplasmic reticulum, peroxisomes, cytosol and the plasma membrane [33]. In cancer cells, several pathways trigger the production of ROS, such as through the activation of oncogenes that lead to enzymatic alterations, mitochondrial dysfunction and metabolic switch, infiltration by macrophage and the increase in oxygen due to angiogenesis [49].

It is well known from previous studies that most, although not all cancer cells have a higher level of oxidative stress than normal cells [50, 51]. The antioxidant content in cancer cells has been debatable [39]. The primary difficulties for data analysis are due to intratumoral heterogeneity, pools of enzymes in different cell compartments, decrease in enzyme activity in cancer cells, lack of reference when comparing with healthy tissues, artificial conditions of cell culture *in vitro*, among other different factors. Nonetheless, in general, there is a consensus that there are some specific alterations in cell physiology in almost all cancers, which include the capacity to overcome apoptosis by inhibiting the pro-apoptotic pathway, managing its own growth, unbounded potential for replication, angiogenesis and the ability to induce metastasis [6]. This is in agreement with the fact that many steps in the cancer process are affected by regulatory factors, such as hypoxia and oxidative stress[39, 52]. During cancer, several mechanisms are activated and lead to a more oxidative status. In fact, the elevated ROS production can stabilize the hypoxia-inducible factor 1-α (HIF-1α), a subunit of the transcription factor HIF, signalling the reduction of oxygen throughout the cell and promoting the expression of glycolytic enzymes [53]. Moreover, ROS signalling is indispensable to trigger angiogenesis and, adding to this, evidence shows that the levels of

H_2O_2 and NOX1 (NADH oxidase 1) in human prostate cancer seems to increase when in comparison with healthy tissue [54]. ROS can also be triggers for carcinogenesis, causing permanent damage to DNA, mutations in transcription factors and by modulating some important enzymes, favouring the rise in cell proliferation and migration sustaining tumour progression. Although the elevated oxidative stress can be extremely prejudicial in healthy cells, cancer cells develop some strategies to be able to overcome this barrier and take advantage of this condition for proliferation [6]. Indeed, a moderate and sustained production of ROS within cancer cells can also activate secondary survival signalling pathways such as the phosphatidylinositol 3-kinases Akt (PI3K–Akt), ERK MAPK, and NF-κB pathways, which mediate and increase cell survival capabilities [55]. Thus, in a hypoxia and acidosis condition, ROS can also downregulate p53, leading to an increase in uncontrolled cell proliferation [56]. As it was already highlighted, a hypoxic environment leads to a rise in HIF, which increase glycolytic pathway activity instead of OXPHOS, leading to an alteration in mitochondrial biogenesis, diminishing mtDNA content, decreasing ATP synthesis, oxygen consumption and mitochondrial protein synthesis [57].

1.2.3 Switch in Mitochondrial Regulation in Cancer

Numerous studies showed how mitochondrial dynamics can influence the correct behaviour of the cell, since metabolic dysfunction in mitochondria can lead to various pathologies and conditions as, for example, neurodegenerative diseases, diabetes and aging, and even cancer.

Otto Warburg proposed in 1927 that cancer cells switch their metabolism to produce ATP from glucose by glycolysis, instead of using OXPHOS to produce energy [58–60]. Mammalian cells obtain their energy from two main metabolic processes depending on the available substrate. Under anaerobic stressful conditions, which occur in highly proliferative tissue, such as in cancer, cells prefer to use glucose as substrate up-regulated glycolysis to produce two molecules of ATP for each glucose molecule catalysed. Under aerobic conditions, cells oxidize glucose completely and all other substrates to CO_2 trough glycolysis, Krebs cycle and OXPHOS, seeing as this process is much more profitable to the cell when compared to glycolysis [20]. The Crabtree effect is described as the decrease of mitochondrial respiration caused by an increase of the glycolysis pathway, as this was reported not only in cancer cells but also in a fast growing non-tumour cells [61]. The changes in metabolism that occur cancers are, however, still inexplicable. Irreversible alterations of mitochondrial metabolism was pointed by Warburg to be the main cause of cancer. However, we know now that often this is not true and that cancer has several has several distinct hallmarks [7].

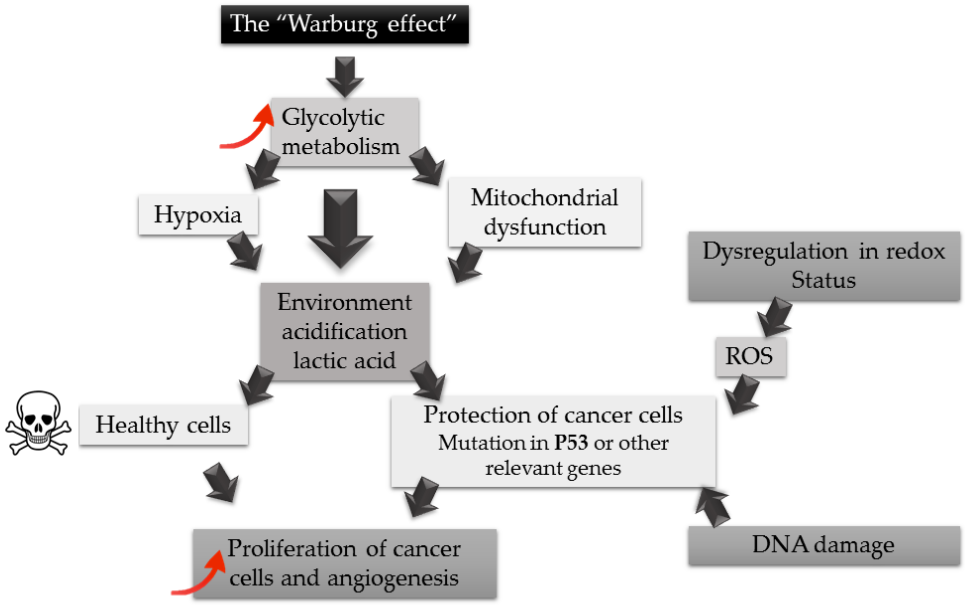

Figure 3: Schematic illustration of the "Warburg effect" in cancer cells. The switch of metabolism in cancer cells is mainly due to hypoxia and mitochondrial dysfunction. The increase in the glycolytic metabolism generates an increase in environment acidification through the production of lactic acid, which leads to the death of healthy cells. Cancer cells in turn are able to grow and proliferate due to the lack of antioxidant protection increasing the redox status and to DNA damage, leading to several mutation in apoptotic proteins such as p53.This increase the proliferation of cancer cells and the angiogenesis.

Cancer cell proliferation leads to a decrease in oxygen in the centre of tumours, which can be considered as a possible explanation for the glycolytic metabolism preference of these types of cells. On the hand, acidosis caused by cancer cells through the increase of lactic acid production leads to the death of proximal healthy cells around the tumour, which is an advantage for cancer cells as it can increase malignant proliferation [49]. As previously described, cancer cells develop mechanisms to manage this acidic environment and continue to proliferate [49]. However, studies have demonstrated a good correlation between mitochondrial dysfunction and the upregulation of glycolysis, which has also been observed in *in vivo* models. A study performed in 2014 demonstrated through proteomic techniques such as mass spectrometry and Western blotting that many enzymes belonging to glycolysis pathway are up-regulated in A549 lung adenocarcinoma, which is in agreement with previous studies [62]. An up-regulation in 6-phosphogluconate dehydrogenase (6PGD) and transketolase (TKT), two important enzymes in the pentose phosphate pathway (PPP) were also demonstrated [62]. Despite of this, when the glycolysis pathway was supressed in the two human lung cancer cell lines H460 and A549, the mitochondrial metabolism was not

increased which is an indication that these cells may have an intrinsic mitochondrial dysfunction [62]. Mitochondria have been extensively studied over the years in cancers and it has been established that targeting mitochondria is a new strategy to induce apoptotic mechanisms in cancer cells [61]. As such, targeting mitochondria through the use of antioxidants may prove to be one such strategy.

1.2.4 Cell Death and Mitochondria

To regulate the correct growth and to protect cells against external and internal stress, multiple forms of death can occur in the cell, such as apoptosis, necrosis and mitophagy [63]. Each of these mechanisms of cells death can occur, but the availability of ATP seems to be a limiting factor [64]. Usually, necrosis is associated with an increase in MPT pore opening, which in turns leads to OXPHOS uncoupling [64]. Necrosis causes inflammation and is normally associated with hypoxia. Apoptosis or cellular programed death is an important mechanism for regulation of tissue growth. However, although this process is present in a large variety of cells, it is not known if it is a common event in all cells. Series of specific events accompany apoptosis, such as the rounding up of cells and their decrease in volume, chromatin condensation and fragmentation of the nucleus, modification of the morphology of cytoplasmic organelles, blebbing of the plasma membrane without loss of integrity ,which occurs until the phagocytes eliminate the rest of the [65, 66]. This process may be triggered by two distinct pathways, the extrinsic apoptosis pathway and the intrinsic apoptosis pathway. Imbalances between levels of apoptosis induction and non-activation of programmed cell death can cause numerous diseases, such as neurodegenerative diseases, ischemic damage, autoimmune disorders and many types of cancer [67]. The intrinsic pathway, also called the mitochondrial pathway can be activated by a significant increase in ROS, viral infection, damage caused by several toxins or even radiation [66]. DNA damage may also activate this specific pathway. The intrinsic pathway, contrarily to the extrinsic one, involves intracellular signals that induce the mitochondria to initiate apoptosis and does not involve specific receptors. The mitochondrial outer membrane is very sensitive to variations in the oxidative status in the normal metabolism of cells. The protein family Bcl-2 regulates the MOM by triggering a loss of transmembrane potential, through the formation of specific pores, though where cytochrome c, apoptosis factor (AIF) and SMAC/DIABLO can be released, causing loss of mitochondrial functions essential for cell survival [68]. Bax (Bcl-2 associated X protein) and Bak (Bcl-2 homologous antagonist killer) pro-apoptotic proteins

are responsible for the release of the apoptotic factors, through the stabilization of VDAC and/or ANT pore increasing the membrane permeability. The tumour suppressor protein p53 is essential in the regulation of pro-apoptotic events and, when DNA suffers any type of damage, this transcription factor is overexpressed, inducing the regulation of NOXA and PUMA, Bcl-Xl and Bax [69]. Several pathways that regulate the activity of the p53 protein are also potential targets for anticancer therapies[70]. NOXA and PUMA are two proteins responsible for regulating cell death since they mediate the inhibition of anti-apoptotic protein [71]. Cytochrome c release leads to the association of pro-caspase 9 and APAF-1 (apoptotic protease activating factor 1), resulting in the formation of the apoptosome. This complex activates the caspase 9 initiator, which in turn activates effector caspase through the cleavage of caspase 3 [72]. This caspase subtype, together with caspase 6 and 7 are effectors and act in a myriad of targets such as, for example, nuclear PARP and lamins in the process of apoptosis [70]. Although the activation of caspases is necessary for apoptosis, this observation by itself is not sufficient for establishing a conclusion regarding their role in cellular death. In fact, caspase activity can also be associated with non-lethal biological processes [68] [73]. In healthy cells, the B-cell lymphoma 2 (Bcl-2) family is expressed and prevent the induction of pro-apoptotic factors, so the inhibition of these proteins may be a possible trigger for cancer treatment, causing a decrease in cell viability [68]. Regarding the extrinsic pathway, this also involves a caspase cascade but it is triggered by a complex cell membrane receptor, the death receptor, which activate initiator caspase 8 leading to apoptosis. The extrinsic pathway is also associated with the mitochondrial pathway through the activation of BID protein, leading to the release of mitochondrial apoptotic factors [70].

Cancer cells can easily overcome the apoptosis regulation because of several mutations in some of the most important proteins for the regulation of this process. For example p53, tumour suppressor gene is muted in almost half the cases of human cancers, leading to an alteration of the rheostat of the cell [74]. Moreover, deregulation in Bcl-2 protein family is also common in cancer. A possible way to reverse the intrinsic or acquired resistance that human cancer cells have against apoptosis and involves the activation of the pathways responsible for managing cell survival or the inhibition of the anti-apoptotic Bcl-2 family proteins [75].

1.2.5 Mitochondria as an Important Target for Therapy

As it is well known, ROS exert important signalling functions throughout the cell. However, an uncontrolled variation in redox status causes cell damage, leading to several diseases, as earlier described. Hence, it is relevant to better understand how antioxidant compounds can be a solution for radical scavenging, because if not correctly controlled, the excess of antioxidants may become harmful. Thus, a steady state of ROS is required for the homeostasis of cells, which may be a clue for explaining antioxidant therapy failures [70]. Mitochondria have an important homeostatic function in the regulation of energy production, Ca^{2+}regulation, cell signalling and in cell death [76]. These relevant functions of mitochondria made this organelle a potential target for drug delivery. Therefore, mitochondria-targeted antioxidants emerged as a new subject of discussion and some strategies have been developed for achieving this goal and discover an efficient antioxidant directed towards the mitochondria. One approach can be the use of specific agents that interact specifically with mitochondrial proteins. Untargeted mitochondrial drugs have also been a possibility in antioxidant delivering. However, until now the best way to target compounds to mitochondria was the use of lipophilic cations such as TPP^+ that are well known to cross mitochondria membrane trough the electrochemical gradient due to the negative potential of mitochondria [77, 78]. MitoQ is a synthetic antioxidant that targets mitochondria based on ubiquinone antioxidant properties, and it was demonstrated to have some beneficial properties at low concentrations [26]. In our work, we used synthetic molecules developed specially for accumulating in mitochondria, by binding them to TPP^+. These formulations increase by 5 to 10 times the accumulation of antioxidants in the cells and even more in mitochondria, reaching an accumulation of 500 times superior to the basal value [79, 80]. The molecules here tested were characterized as antioxidants due to their effects in cell-free systems and by inhibiting lipid peroxidation of mitochondrial membranes (preliminary data not shown). The compounds are being the subject of intellectual property, hence their structure is not shown.

1.3 Treatment/Therapies

Variation on cancer treatment has being studied for decades since it is a very complicated disease with many hallmarks. For this reason, the research of a specific target for cancer that could induce a toxic response only to malignant cells has an important role in anticancer drug discovery. This magic bullet should be specific, with a large action spectrum, well metabolized and with reduced toxicity for normal cells [81]. Therefore, development of novel selective drugs is an important and perplexing mission, and to reach this goal it is essential to understand all the biological differences between cancer and normal cells [51]. Nowadays, it seems reasonable to consider that the combination between novel clinical agents (such as antioxidants, for example) and the current chemotherapy may be a relevant outcome in the field of cancer treatment. Furthermore, this kind of combination between drug therapies and antioxidant adjuvants has shown an increase in the cancer cells susceptibility to drug treatment, and appears to increase the specificity for malignant cells [82]. This analysis was also performed in animals and several studies demonstrated a reduction in tumour size and/or an increase in lifetime when a combination of classic chemotherapy and antioxidants was used [83–87]. However, antioxidants may interfere with the oxidative mechanisms of alkylating agents, resulting in necrosis [88]. It is important to known whether chemotherapeutic agents induce programed cell death [66] and antioxidants might increase these mechanisms[89]. Livingston *et al.* highlighted six possible mechanisms of action for predicting interaction between chemotherapeutic agents and antioxidant compounds, relatively to the importance of ROS production in the effectiveness of the drug, the form and characteristics of ROS produced by the anti-cancer agent, dose of drug administration that can mediate an optimal concentration of ROS, features of the antioxidant (regarding pharmacokinetic), concentration of the antioxidant and, finally, the temporal interaction between the antioxidant and the chemotherapeutics agents [88]. Nevertheless, many doubts still persist on the effectiveness of antioxidant adjunctive in cancer therapies which demonstrated that this subject needs to be carefully considered [90].

In this section, a brief description of all the drugs used in this work and their primary means of action will be discussed, together with their respective toxicity. In addition, the antioxidants used in this study will also be briefly described, as well as recent studies involving these drugs will be highlighted.

1.3.1 Anti-cancer Agents

In the past decade, cancer patients have seen a large rise in their quality of life due to the numerous advances in cancer therapies, even when considering the use of antineoplasic agents, which are non-specific and have a large action spectra, increasing the risk of toxicity. Additionally, ADMET (Administration, distribution, metabolism, excretion of drug and toxicity) may have significant importance in drug effectiveness and all these parameters need to be thoroughly studied [81]. This kind of treatment can normally trigger different sites in cancer cells such as mitochondria, replication of DNA, protein synthesis and cellular division. Their means of action are varied, ranging from DNA intercalation, topoisomerase II inhibition, hormonal manipulation, and proteosome degradation, to an increase in ROS production. So as to maximize the effects of the drugs on cancer and reduce side effects, the administrated doses are strictly controlled to not undergo critical toxicity effects. Another reason for this level of control lies on the fact that cancer cells themselves can developed several mechanisms of resistance such as multidrug-resistance, which involves the expression of a P-glycoprotein that mediates an energy dependent drug extrusion [91]. It was shown that calcium homeostasis may have an important role in cytotoxic drug resistance, since when cells were treated with a specific calcium channel blocker, the cytotoxicity of drug increases, while Ca^{2+} intracellular concentration decreases[91, 92].

In this work, we used doxorubicin (DOX), cisplatin (CIS) and etoposide as these drugs are currently widely used against a large variety of cancers, in combination with two mitochondria-directed molecules with antioxidant properties synthetized in the laboratory of Dra. Fernanda Borges in the Faculty of Science at the University of Porto.

1.3.1.1 Doxorubicin (DOX) and its Chemotherapeutic Effects

DOX is an antitumoral drug of the large anthracycline family, which is used since the 1960's. This antibiotic has one of the widest action spectra and is therefore one of the most used anticancer drugs [93]. In particularly, DOX is used in many cases of cancer treatment such as leukemia, lymphoma, lung cancer and other solid tumours [94–97]. DOX was first isolated from *Streptococcus peucetius var caesius* by Arcamone in 1969, U.S patent 3,590,028 (1971) [98]. The general structure of this compound is very simple, wherein it has a chromophore group antraquinone linked to a daunosamine glycoside [98]. However, despite the high therapeutic potential of this drug, DOX is poorly specific to cancer cells and this leads to acute side

effects that can appear between 2-3 days after the treatment, such as alopecia, mucositis, mielossupression, fever, nausea, phlebitis and chronic cardiovascular complications [95]. There are other, dose-dependent, secondary effect that can appear later such as a cumulative and dose-dependent cardiotoxicity, as described in several reviews [93, 99–102]. Moreover, various mechanisms of spontaneous or acquired resistance have been described, and DOX can become ineffective in cancer treatment [103]. On account of this, lower cumulative doses of DOX have been used, interfering with the effectiveness of the drug against cancer. It is fundamental to investigate a new way to make DOX less toxic to normal tissue without losing its effectiveness against cancer cells in order to treat cancer more efficiently.

Anti-cancer Mechanisms of Action of Doxorubicin

Doxorubicin is able to cross tumours cell membranes through simple diffusion or by carrier mediated diffusion, as shown by Skovsgaard and Nissen [104]. More recently, it has been observed that DOX undergo slow flip-flop mechanisms across the lipid core of the membrane [105]. The mechanism by which DOX is able to induce cancer cell death is not yet established, since it involves diverse mechanisms of action and these can act together. Nevertheless, multiples studies have been published on this subject and nowadays it is known that DOX can act by itself, intercalating DNA, interfering with the correct DNA replication and protein synthesis, and even blocking topoisomerase 2. This way, DOX can induce oxidative stress through itself or through its metabolites, causing cell membrane and DNA damage [100, 106, 107].

Doxorubicin intercalates DNA *in vitro*, while also causing, interfering with normal DNA replication and RNA transcription [108]. The catalytic activity of topoisomerase II seems to be inhibited by DOX trough a specific stabilization of the cleavage complex topoisomerase II-DNA [109, 110]. On another hand, DOX also inhibits the catalytic activity of Topoisomerase II by affecting the cleavage-binding reaction due to stabilization of the cleavage complex Topoisomerase II-DNA. DOX also induces loss of DNA mismatch repair function, interfering with some important genes, which can result in an increase in cell resistance [68]. DNA damage caused by DOX interference with topoisomerase II is followed by cell [109, 111]. The oncogene p53 has been implicated in DOX-induced programed cell death, and some studies present an upregulation of this protein after DOX treatments [112]. p53 post-translational modifications have been associated with an increase in oxidative status. Recently, it was shown that p53 inhibition lead to a decrease in the rates of apoptosis, modulating the activity of Bax and MDM 2 (murine double minute 2) protein [113]. On the

other hand, ROS might also be the initiator for the DOX-induced apoptosis, even without the presence of p53 [114]. In the same manner as with the induction of apoptosis, antracyclines anti-proliferative effect may be triggered by cell cycle arrest [115]. DOX can also induce mitochondrial DNA (mtDNA) intercalation, stimulating nucleoid aggregation because of its capacity to penetrate the mitochondrial matrix [116, 117] Likewise, the fact that DOX is capable of establishing interstrand links when the DNA is unwinded or to interfere with the helicase activity, can cause DNA damage, such as fragmentation and single-strands breaks, and block the protein synthesis and genetic material replication[111].

In the cytosol, DOX is reduced to a semiquinone radical (DOX•) by reductive flavoenzymes, increasing the flux of electrons to form O_2. Many enzymes are involved in this process, such as mitochondrial NADH dehydrogenase, NADPH cytochrome P450 reductase, xanthine oxidase or endothelial nitric oxide synthase [118, 119]. Oxidative stress is generated through the re-oxidation of the DOX-semiquinone radical back to DOX, producing ROS in the form of superoxide anion ($\bullet O_2^-$) that can be further dismuted to hydrogen peroxide (H_2O_2) by superoxide dismutase (SOD)[106, 120–122]. On the other hand, semiquinone free radical may have enough stability to enter the nucleus and bind DNA with high affinity, increasing oxidative stress and inducing extensive damage to DNA [120, 123]. The increase in oxidative stress may lead to biological damage such as lipid peroxidation, protein sulfhydryl oxidation and nucleic acid damage (DNA, RNA and mtDNA) [121]. Another pathway relevant for DOX metabolism occurs through the formation of 7-deoxyaglycone but this is the less common reaction. Oxidative stress can increase because this intermediate of DOX is more soluble in lipids and intercalates the biological membrane easily, causing an increase in ROS production [100, 124].

In conclusion, the anti-cancer effect of DOX is based on induction of cell death and cycle arrest through the increase of ROS generation and inhibition of correct DNA replication and transcription.

Cardiac Toxicity

The heart is very susceptible to the effect of DOX since that organ possesses a high content in mitochondria due to the constant need of energy required for its function. In addition, the heart has a reduced pool of antioxidants when compared with other tissues. Various mechanisms are responsible for cardiotoxicity and they appear to be different from 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

and in iron metabolism and disruption, which can produce oxidative stress by several different mechanisms in cardiac cells. Furthermore, ROS can undergo cellular injuries and trigger cancer cell death. In the cytosol, NADPH-dependent oxidoreductases are able to reduce DOX into doxorubicinol by transferring electrons from NADPH resulting in $NADP^+$. It appears that the secondary alcohol formed through this reaction may be the cause for cardiotoxicity [125–128]. In fact, it has been shown that doxorubicinol metabolites and DOX *per se* can inhibit ionic pumps such as the Na^+/Ca^{2+} exchanger and the Na^+/K^+ ATPase pump in the sarcoplasmic reticulum, sarcolemma and in mitochondria, leading to a calcium homeostasis dysregulation and increasing the concentration of this signalling ion in the cell, which leads to an increase in oxidative stress [127, 129, 130].

Mitochondria have been studied regarding DOX chronic onset, as this organelle seems to be an important DOX target [131]. It has been described that DOX alters calcium homeostasis in cells and this can result in calcium-dependent depolarisation of the mitochondrial membrane [132]. Cardiolipin is a particular type of phospholipid present in the MIM and the peroxidation of this particular lipid causes bioenergetic stress in OXPHOS leading to a decrease in ATP production [132]. This occurs because cardiolipin, as previously described, provides the correct environment for mitochondrial supercomplexes activity [127][133]. The mitochondrial permeability transition (MPT) is often cause of bioenergetic failure in different organs [128].

The alteration of the redox state induced by DOX causes the oxidation of some proteins that can increase MPT induction. ANT, as was previously described, exchanges ADP for ATP in the MIM. This translocator can also be oxidized by excessive oxidative stress produced by DOX, contributing to an increased calcium released inducing MPT pore opening and decreasing mitochondrial respiration in the long term because of loss of nucleotides and cytochrome c [134]. It is known that cardiolipin plays an important role in the stabilization of ANT and the peroxidation of this phospholipid can cause ANT destabilization and an increase in Ca^{2+} release, leading to MPT sensibilization [127, 133]. Moreover, the loss of functionality of ANT can cause a decline in mitochondrial respiratory activity.

DOX-induced cardiac toxicity is associated with Ca^{2+} homeostasis dysregulation and increased MPT induction. Thus, the only solution existing to counteract these side-effects of DOX is to strictly control the dose administrated to the patient, independently of the loss of anti-cancer activity. 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 intensifies the probability of induction of MPT pore and leads to mitochondrial depolarization, energy depletion and cell death [135].

DOX-induced Lung Cancer Resistance

Cytotoxic DOX resistance is associated with multi-drug resistance (MDR). It was observed that decreases DOX accumulation can occur when compared with non-resistant cells with a specific glycoprotein (P-gp) being expressed, although this is not always the case but in some cases this does not occur [136]**.** Calcium homeostasis is also implicated in MDR. DOX treatment induce atypical drug resistance when cells are exposed to cumulative drug concentration**.**

As previously described, DOX primary toxic intervention is through the increase of oxidative stress at the mitochondrial level, in which the use of antioxidant to counteract this side-effect seems reasonable [137]. In the present study, our objective is to test whether a pre-treatment with vitamin K mitochondria-directed antioxidant derivative (MitoXT2) could enhance the effect of DOX in A549 lung cancer cells without causing toxic effect in MRC-5 lung fibroblasts cells.

1.3.1.2 Cisplatin and Cancer Treatment

Since its discovery in the mid-1960s, platinum (Pt)-containing drugs have been used in medicine to treat various diseases. Nowadays, it is the largest class of drug used against cancer both in adults and in children [138, 139]. Cisplatin has been in widespread use for many years to treat several forms of cancer, including ovarian, cervical, head and non-small-cell lung cancer [139]. However, this treatment does not come without some side effects, which include nephrotoxicity, emetogenesis and neurotoxicity [140, 141]. Although nephrotoxicity and emetogenesis might be controlled through the ingestion of liquids with diuretics and serotonin-receptor antagonists, neurotoxicity still limits the drug concentration [142]. Cisplatin can also induce cell resistance [142] and, beyond that, several factors such as disease stage, tumour histology, sex, tobacco exposure and patient age may influence chemotherapy outcomes [143]. Generally, the administration of cisplatin is intravenous at a range concentration starting on 50 to 120 mg/m^2 and needs to be repeated every 3 or 4 weeks [144]. Cisplatin is able to cross cells by passive diffusion, as shown in previous [145, 146].

The use of an analogue demonstrated that there are no alterations in CIS absorption, concluding that there is no specific CIS transporter [147]. However, recently, a close link between CTR1 cooper transporter and cisplatin efflux was shown, whereas a mutation or deletion in *CTR1* gene increased CIS resistance, decreasing CIS absorption through the cell without affecting the effectiveness of the drug in the target-site [148]. Another gene involved in CIS resistance is *ATP7B*, related to the efflux of copper in the cell, which was observed *in vitro* and in several cancer types [149].

Platinum compounds are non-specific as their means of action involve interaction with DNA and RNA, creating inter- and intra-strand crosslinks, thereby preventing cell division and growth[150, 151]. CIS creates DNA intra-strand adducts between adjacent guanines and are more widespread than cross-links between the two strands of DNA. Because of the higher rates of replication on cancer cells, more DNA replication sites are available to bind cisplatin hence increasing its antineoplastic effectiveness in tumours. In addition, cisplatin is also capable of binding to nucleophilic sites present in several molecules, which includes phospholipids, and, cytosolic, cytoskeletal, membrane and mitochondrial proteins, among others [152]. On another hand, CIS-induced ROS production can also trigger cytotoxicity, even if not directly by DNA damage signalling but instead by adduct interfering with protein transcription [153]. Moreover, there are several pathways that protect cells against CIS-induced DNA damage by trying to repair these injuries. Nucleotide excision repair (NER) is the primary pathway involved in the removal of cisplatin-nDNA adducts [154]; when those mechanisms are inhibited, it cause an inhibition in DNA replication and transcription, as well as cell cycle arrest, leading to apoptosis [139, 144, 155, 156].

Involvement of Cisplatin in ROS Production

Since the mechanisms of CIS toxicity are not yet totally understood, a large work has been done in this field. Even when considering the previous pathways, described above, it is known that the inhibition of DNA transcription or the fact that DNA adducts originate from CIS does not totally explain the high efficiency of this drug observed in malignant cells and the tissue specific toxicity [153, 157]. Furthermore, it appears that the fact that CIS binding to mitochondrial DNA causes irreversible mitochondrial damage and lead to ROS production through depletion of mtDNA-encoded proteins, leading to inhibition of respiration. On the other hand, ROS can also be a possible mechanism of cytotoxicity induced by CIS treatment in healthy cells [158]. A decrease in reduced glutathione was observed in rat kidney cortex treated with CIS, resulting in an increase in malondialdehyde

(MDA), a marker for lipid peroxidation [159]. Consequently, the oxidative status through CIS treatment seems to be dysregulated, by both the decrease of GSH levels and free radical release [160]. A consensus has been reached concerning the nephrotoxicity induced by CIS and the evidence pointed out that the oxidative stress caused by the generation of superoxide anion, hydrogen peroxide and hydroxyl radicals results from increased activity of NADPH oxidase, xanthine oxidase and adenosine deaminase[161, 162] As it was highlighted earlier in this work, antioxidant enzymes are crucial to the homeostasis of oxidative status. CIS treatment decreases the activity of several antioxidant enzymes such as catalase, superoxide dismutase and glutathione peroxidase together with the reduction of reduced and oxidized glutathione. Apparently, one important component of CIS- induced toxicity is an increase in lipid peroxidation [163]. In addition, it was also shown by Hannemann and Baumann that CIS might induce a decrease in gluconeogenesis, also contributing to nephrotoxicity [159].

Antioxidant as Protective Against CIS- Induced Toxicity

As the increase in oxidative stress has a major role in CIS induced cytotoxicity in healthy cells, the use of several antioxidants to counteract this effect has been investigated [158, 164, 165] D-methionine seems to be effective in the protection of ototoxicity induced by cisplatin at a concentration of 300 mg/kg apparently by binding to CIS, sparing GSH in the process [160]. More recently, a study evidenced that α-lipoic acid, due to its antioxidant properties, may also prevent CIS-induced kidney damage [164]. Antioxidants which target mitochondria, such as MitoQ and Mito-CP, seem to demonstrate a protective effect in nephrotoxicity induced by cisplatin but interestingly in T24 cancer carcinoma cell from human urinary bladder, these antioxidants increase CIS-induced cytotoxicity [166]. Still, it appears that the combination of some antioxidants such as vitamin A, vitamin C, genistein and quercetin increase the cytotoxicity of CIS [167]. A previous study, demonstrated that the combined treatment of vitamin C and CIS induces synergetic breast cancer cells death [87]. Despite all the controversy involving this subject, little is known about the role of ROS in CIS anti-cancer effects and how oxidative stress responses affect both cancer and healthy cells sensitivity to the drug, but it seems nonetheless that antioxidants may have a relevant functions in CIS toxicity induction.

In relation to ROS modulation in A549 lung carcinoma under CIS treatment, a recent study demonstrated that CIS causes ROS production in that cell line with a IC_{50} of 12μM [153]. The authors showed that CIS causes mitochondrial dependent ROS response, considerably increasing the rate of cell death. The generation of ROS induced by CIS occurs as

consequence of its direct effect on mtDNA, as was previously described, resulting in the impairment of the electron transport chain protein synthesis [153]. Also, it was demonstrated that mitochondrial redox homeostasis, DNA integrity and bioenergetics are key mediators of the cellular response to CIS-induced mitochondrial impairment and may be factors determining resistance to its cytotoxic effect. In conclusion, it seems acceptable to consider two major factors in CIS-induced cytotoxicity leading to cell death, namely modulation of redox homeostasis and metabolic activity [153].

1.3.1.3 Etoposide as a Cancer Treatment

Etoposide is a derivative of podophyllotoxins studied since the 19th century due to its biological activity. The antimitotic activity of that type of compounds was only discovered in the middle of the century but their high toxicity made their use prohibitive in pharmacology [168]. Several works identified antitumor properties in several aldehydes present in the root of Indian *Podphyllum* plant, leading to the synthesis of several artificial analogues including etoposide (VP-16) [169]. Etoposide was obtained by introducing a 4,6-O-ethylidene β-glucopyranoside in the podophyllotoxin structure and removing a methyl group [170]. Currently, etoposide is a widespread antineoplastic compound with a large range of antitumor activity, against neoplasms including lung cancer to several types of lymphomas [168]. Generally, when administered through oral means, doses ranging from 50 to 100 mg are administered for two to three weeks, through which the best anti-carcinogenic effect is reached, while in the case for the intravenous route through injection, the optimum dose is 20mg/mL [171]. The ADMET is an essential parameter for monitoring in each patient to maintain the effectiveness of etoposide and to limit its toxicity [171]. In fact, bioavailability is an unpredictable parameter and there is no relation between the bioavailability and the number of doses[171].

The primary mechanism for the cytotoxic effect of etoposide is its ability to stabilize the DNA double-strand, hindering the normal activity of topoisomerase II. DNA topoisomerases are nuclear enzymes which repair transient DNA strand breaks, allowing correct DNA synthesis and, by consequence, replication. Etoposide, by inhibiting DNA topoisomerase II, blocks the proper pairing of DNA breaks. The double strand DNA breaks makes DNA more sensitive and triggers the antitumor effect of the drug. Etoposide acts specifically in the late phase S and early G2 of the cell cycle, causing cell cycle arrest at the

phase G2 [169]. Moreover, the cytotoxic effect of etoposide is also mediated by the transcription factor p53, responsible for the up-regulation of Bax pro-apoptotic protein, leading to cell [172, 173]. Furthermore, another form of etoposide cytotoxicity may be triggered by the production of ROS, seen as etoposide metabolism by the cytosolic peroxidase or hepatic microsomal enzyme system undergoes the formation of highly reactive intermediates, [174, 175] including the 3-O-demethylation of etoposide. The mechanisms of etoposide resistance are mainly due to the efflux of P-glycoprotein (associated with multidrug resistance pathway), decreasing the drug intracellular concentrations [176].

For non-small-cell lung cancer, the anti-cancer activity of etoposide alone reaches only 15% [177, 178]. However, the combination of cisplatin and etoposide treatments seems to present synergetic effects in the treatment of this disease and a phase III clinical trial was performed in order to compare the efficacy of this combination in comparison with new anticancer agents [179]. Oral administration of etoposide can be a thoughtful option for palliative treatment of patient with metastatic NSCLC [169]. The toxicity of etoposide in comparison with the other two chemotherapeutic agents used in this work (DOX and CIS) is lower although neutropenia is dose limiting.

1.3.2 Antioxidant compounds

The proper regulation between antioxidant species and the pro-oxidants ones needs to be extremely well regulated. As it was previously described, ROS are essential for regulating many signal transduction pathways. In consequence, any dysregulation on this homeostatic cellular redox status, such as by increased production of free radical species or decreased oxidative protection through enzymes such as superoxide dismutase, catalase, glutathione peroxidase and other antioxidant re-generating enzymes, may lead to various human diseases, including various type of cancer [180]. Oxidative stress is caused by a multitude of different process and may be caused by biological mechanisms or by an external agent.

Antioxidants have been described as specific compounds that considerably slow down or prevent the oxidation of cell structures caused by reactive species and transition metals, when present in a lower concentration than the concentration of the oxidizable biomolecule [81, 181]. Antioxidants are capable of acting through different mechanisms, including acting as chelators of transition metals, neutralizing ROS, blocking the activity of enzymes that overproduce ROS and by increasing antioxidant gene expression [180, 182, 183].

Antioxidant properties of dietary compounds has been investigated over the years because of their ubiquity in the human diet and their numerous preventive and/or therapeutic properties in oxidative stress related diseases [81]. Several chronic degenerative diseases such as neurodegenerative disease, atherosclerosis, inflammatory damage, cardiovascular disease, diabetes and cancer have been linked with increased of the ROS production [184, 185]

The primary antioxidants in dietary intake in a normal human diet are flavonoids, accounting for about two thirds of this intake and phenolic acid accounting for the remaining one-third. Polyphenol derivatives have also been studied for their importance as preventive and/or therapeutic agents in many diseases. These compounds possess very specific properties that increase their activity as antioxidants and as free radical-scavengers. The number and the location of hydroxyl groups present in hydroxyxinnamic acids are particularly important to determine the antioxidant potential of a molecule. Phenol group are able to donate the hydrogen atom to a free-radical without losing their stability and stopping the chain reaction propagation [181].These compounds can be used as scaffolds for new drugs of the right modification is made to improve their pharmacodynamic and their phamacokynetic in the human body. With this goal in mind, several modifications have been made to caffeic acid and vitamin K by the laboratory of Dr. Fernanda Borges at the Faculty of Science of the

University of Porto. This modification allows the redirection of this antioxidant to mitochondria and allows for a higher accumulation in this organelle through the use of TPP^+ as a positive carrier.

1.3.2.1 Caffeic Acid and Derivatives

Caffeic acid belongs to the vast hydroxycinnamic acids family. These compounds are diet associated phenolic molecules with known antioxidant properties [186]. Hydroxycinnanmic acids exist naturally in plant cell walls, playing an important functional and structural role [187]. Several types of food and plants have hydroxycinnamic acid or derivatives in their constitution, including tea leaves, coffee, red wine, vegetables, whole grains and fruits, however their distribution might be unequal with a higher concentration in the outer parts of fruits [188]. As it was previously described, antioxidants have many beneficial properties in health and in the prevention of disease pathology.

Hydroxycinnamic Acid: Antioxidant Properties

Hydroxycinnamic acid has a high reduction potential related with the phenolic rings in its structure, permitting the donation of protons or electrons without losing their stability. In fact, the phenolic ring is able to stabilize and delocalize the unpaired electron resulting in a less reactive molecule protecting the cell from all the oxidant damage caused by free radicals [189, 190]. Another relevant aspect of these antioxidants is the chelating-metal transition potential, that prevents Fenton reactions from occurring and increasing potentially damaging oxidative damaging oxidative-reaction [189].

Anti-cancer properties of Hydroxicinnamic acid

Many studies have demonstrated the antioxidant properties of hydroxicinnamic acids and derivatives display relevant role in growth inhibition of several tumour cells, with their effect strongly dependent on their structural characteristics [191]. Caffeic acids is one of these examples with selective antiproliferative activity against some types of cancer cells, acting on MAP-K or AP-1 or in the transcription factor NF-kB, leading to cell cycle modulation and to apoptosis[185, 192].

1.3.2.2 Vitamin K and Derivatives

The first purified form of vitamin K, phylloquinone, was isolated from plants in 1939 and used as an anticoagulant agent in a nutritional deficiency characterized by the loss of prothrombin expression. In1943, Henrick Dam received the Nobel Prize in Physiology or Medicine for his discovery of vitamin K and his recognized study concerning the effect of this vitamin in biological function as well as its effects in human medicine. Vitamin K has been intensively investigated, more specifically regarding its particular properties in coagulation, as it is essential for the production of clotting factors II, VII, IX and X. Vitamin K family is characterized by a naphthalene ring containing two carbonyl moieties at positions 1 and 4 which confers the redox property of this molecule [193]. There are three forms of vitamin K but only Vitamin K1, also known as phylloquinone, and K2, or menaquinone, occurs naturally in plants. On the hand vitamin K3 or menadione is artificially synthesized and act as a provitamin. The primary difference between vitamin K1 and K2 resides in their prosthetic groups at position 3, with vitamin K1 having a monounsaturated poly-isoprenoid group whereas vitamin K2 presents a recurrent unsaturated trans-poly-isoprenyl group that can be repeated 1 to 13 times. Menaquinone is the most common form of vitamin K in animal , while it is produced by the intestinal microbial flora through the metabolism of exogenous naphthoquinone [193]. The structure of vitamin K3 is considerably different from the two other forms of vitamin K, since it has no aliphatic chain at position 3.

The importance of vitamin K in coagulation relates with the fact that when this vitamin is reduced to an hydroquinone form, this specific sterol acts as a cofactor of gamma-glutamyl-carboxylase, forming gamma carboxyglutamic acid by the addition of CO_2, leading to coagulation factor activation, whilst hydroquinone is oxidized to vitamin K 2,3 epoxide [194, 195]. Vitamin K epoxide is then reduced back to vitamin K by thiols such as glutathione S-transferase and epoxide reductase present in the cytosol. Thereby, a redox cycling is created linking the transformation of epoxide to quinone and then the return to hydroquinone form. The regeneration of hydroquinone is essential for another reaction of carboxylation and for regenerating prothrombin, proconvertin, plasma tromboplastin component and autoprotrombin III, amount other plasma proteins [193, 196]. All these processes undergo the formation of a redox cycle between the quinone form of vitamin K and the hydroquinone reduced vitamin K, leading to the formation of superoxide anion. Quinone can be reduced to a semiquinone radical and to hydroquinone by a one-electron reduction or a two electron reduction.

Anticancer Properties of Vitamin K

It is currently known that vitamin K displays many antioxidant properties that can be interesting for the treatment of many tumours, which mainly consist of an oxidative model and non-oxidative model [193]. As it was previously described, the redox cyclin of menadione can cause an increase in free radicals such as •OH, $•O_2^-$ and H_2O_2 [197]. The formation of hydroxyl radicals arise from the Fenton reaction where metal transitions have a significant role due to their high capacity of being reduced. This intensification of oxidative stress creates a disequilibrium between antioxidant and ROS, leading to cell death. Between the three forms of vitamin K, menadione seems to be the more cytotoxic at higher doses, which was confirmed *in vitro* in MCF-7 breast cancer cells. [198] Another study showed that catalase, SOD and glutathione, the front line of combat against free radicals, considerably decreases the effect of menadione, decreasing the cytotoxic effect of vitamin K [199]. This confirms that oxidative stress is the main factor for anticancer outcome, resulting in menadione treatment. In line with this, the oxidant activity of menadine decrease the cell viability in A549 cancer cells at a concentration of 50μM and 100μM [200]. Menadione is also capable of reacting with thiol groups present in several important antioxidant proteins such as glutathione, avoiding to act as a radical scavenger. Moreover, this reaction of arylation can also decrease the activity of other sulfhydryl enzymes as, for example, tyrosine phosphatase and p34Cdc2 protein associated with cell growth [193]. In relation to non-oxidative mechanisms of action of vitamin K in cancer treatment, two targets were identified: the cell cycle arrest and the dysregulation of transcription factor expression. Menadione undergoes cell cycle arrest in G1/S and S/G2 phases, through the hyperphosphorylation of the CDKs protein responsible for the regulation of cell cycle. Vitamin K undergoes some modification on the active site of CDC25 phosphatase, blocking its enzymatic activity leading to a cell cycle arrest in G1 phases [193].

Chapter 2
Main Goals

Main Goals

This project is based in the preliminary results obtain in our laboratory which demonstrated a significant decrease of cell viability when cancer cells were pre-treated with MitoXT1 and MitoXT2 and then incubated with anti-cancer drug.

To follow up this study, the concentration chosen for the antioxidants in test and the drugs for this work were those which demonstrated the best results in the previous work. It is important to note that all concentrations of the compounds used were non-toxic per se (toxicity less than 15% when measuring loss of cell mass mass/protein by using the sulforhodamine B method).

The present work has two tandem objectives: a) investigate whether MitoXT1 or MitoXT2 increase the cytotoxicity of classic anti-cancer agents doxorubicin (DOX), cisplatin (CIS) and etoposide on human lung cancer A549 cells and b) investigate whether the same agents protect human lung MRC-5 fibroblasts from the toxicity of the above mentioned anti-cancer agents. The study was performed with a pre-treatment with antioxidants for 24h followed by incubation for 24 or 48 h with corresponding anti-cancer agent. With this objective in mind, several experiences were performed:

1. Sulforhodamine B assay was performed to evaluate cellular density and cell viability under different treatments groups.
2. Resazurin assay was performed to measure the metabolism activity of cells under the same treatments describe above.
3. Live/Dead assay was performed to measure cellular death by flow cytometry
4. Cellular cycle was followed through flow cytometry to understand if cells treatments resulted in blockage in any phase of the cellular cycle.
5. Caspase-like activity was performed using a colorimetric assay to follow activation of the apoptotic signaling induced by the compounds in this study
6. Mitochondria membrane potential and apoptotic nuclei were followed by using epifluorescent microscopy using TMRE and Hoechst 33342 dyes.
7. Measurement of ROS production was performed using the dyes MitoSOX measured by a multi-plate based assay.

Our hypothesis is based on the fact that the antioxidants MitoXT1 and MitoXT2, used in low concentration in conjugation with the drugs can increase cancer cell sensitivity to anticancer drug effects and intensify the injuries caused.by this treatment through the increase of ROS production. And perhaps this combined treatment can increase the specificity of the drugs and reduces the lateral effects of these compounds due of the reduced doses.

Chapter 3
Material and Methods

Material and Methods

3.1 Common Reagents

Bovine serum albumin (BSA), brilliant blue G, calcium chloride ($CaCl_2$), dimethyl sulfoxide (DMSO), DL-Dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), glycerol, propidium iodide, sodium chloride (NaCl), sodium dodecyl sulfate (SDS), sulforhodamine B sodium salt (SRB), Tris pH 8.8, Tris pH 6.8, trypan-blue solution doxorubicin and cisplatin were purchased from Sigma-Aldrich Chemical Co. (Saint Louis, MO,USA). Acetic acid, ethanol, magnesium chloride ($MgCl_2$), methanol, perchloric acid, phosphoric acid, potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium hydrogencarbonate ($NaHCO_3$), sodium sulphate ($NaSO_4$) and sodium hydroxide (NaOH) were obtained from Merck (Whitehouse Station, NJ, USA). All the solutions used in cell culture application were ordered from Gibco (NY, USA) Caspase substrate 9 and 3, as well as *p*NA were acquired from Calbiochem (Darmstadt, Germany). The fluorescent probes Tetramethyl rhodaminemethylester (TMRE), Hoechst 33342, calcein-AM, ethidium homodimer-1 and MitoSOX were obtained from Molecular Probes (Life Technologies, NY, USA).

3.2 Solution preparation

3.2.1 Cell Culture Media

Cell culture medium was composed of Dulbecco's Modified Eagle Medium (DMEM) enriched with glucose (DMEM D5648) supplemented with 110mg/L of sodium pyruvate, 1.8g/L of sodium bicarbonate, 10% of FBS (foetal bovine serum) and 1% antibiotic (penicillin). Media and serum were filtered with a 0.2 porosity filter.

3.2.2 Phosphate-buffered Saline (PBS)

PBS was composed by with 15.44mM KH_2PO_4, 1.55M NaCl, and 27.09mM Na_2HPO_4, with the pH of the solution adjusted to 7.2. This solution was prepared as a 10x solution.

3.2.3 Phosphate-buffered Saline Tween (PBS-T)

PBS-T composition included 132 mM NaCl, 4mM KCl, 1.2mM NaH_2PO_4, to this solution 0.1% Tween was added.

3.2.4 Vital Microscopy Media

Vital microscopy media was used for fluorescent microscopy analyses and for flow cytometry assays. This media was composed by 12mM NaCl, 3.5mM KCl, 0.4mM KH_2PO_4, 20mM HEPES, 5mM $NaHCO_3$, 1.2mM Na_2SO_4 and 15mM Glucose Microscopy media was prepared in MiliQ water and the pH adjusted to 7.4. The medium was filtered with a 0.2 porosity filter under sterile conditions.

3.2.5 Sulforhodamine B Solution

The SRB solution was prepared in MiliQ water and composed of a 0.05% Sulforhodamine B sodium salt solution which was then maintained protected from light at 4ºC.

3.2.6 Lysis buffer

Lysis buffer was prepared with 50mM HEPES pH7.4, 100 mM NaCl, 0.1% CHAPS, 10% glycerol, and 10 mM DTT. The solution was prepared with MiliQ water. This buffer was intended in order for cell lysis in order to perform protein quantification.

3.2.7 Bradford Reagent (2X)

The Bradford reagent was prepared with 0.02% (w/v) Brilliant Blue G dissolved in 5 %(v/v) methanol and 8.5% (w/v) Phosphoric Acid. This solution was prepared in MiliQ water and paper-filtered. This reagent was stored at 4ºC and protected from the light.

3.3 Cell Lines

All cell lines were acquired from EATCC/Invitrogen (Eugene, OR, USA)

3.3.1 A549 Lung Carcinoma Cell Line

The A549 cell line is derived from human lung carcinoma tissue of a Caucasian man with 58 years old. This line was established by D.J. Giard in 1972 [201]. This cell line has a high ratio of oxidative phosphorylation and has a high replication rate (Figure 4 A). In this case, cells were used to study the therapeutic effect of two mitochondrial-directed antioxidants (MitoXT1 and MitoXT2) when in combination with the anti-cancer agents doxorubicin DOX, CIS and etoposide.

3.3.2 MRC-5 Lung Fibroblast Cell Line

The MRC-5 cell line was used as the non-tumour control. These cells are originated from fibroblasts of human lung extract from a 14 week of gestation baby and were isolated in 1966 by J.P. Jacobs [202].This cell line cannot be used over 70% confluence, otherwise it might differentiate and lead to artefacts (Figure 4 B).

3.3.3 H9c2 Cardiomyoblast Cell Line

H9c2 cells were used as a non-tumour counterpart in order to demonstrate that MitoXT1 and MitoXT2 can protect cardiac-like cells from anthracycline toxicity. H9c2 are derived from rat myocardium cells of the rat and they were obtained from an original clonal cell line isolated from embryonic BD1X rat heart tissue and exhibiting many of the properties of skeletal muscle [203].

3.3.4 Initiating a New Cell Culture

To initiate a new cell culture, a frozen cell vial was thaw to a 10 cm plate with 10 mL of culture medium. Cells were then incubated at 37 °C in a humid atmosphere with 5% of CO_2. After 24h, the medium was replaced to remove dead cells and remaining DMSO. Cells will then be ready for subsequent passages.

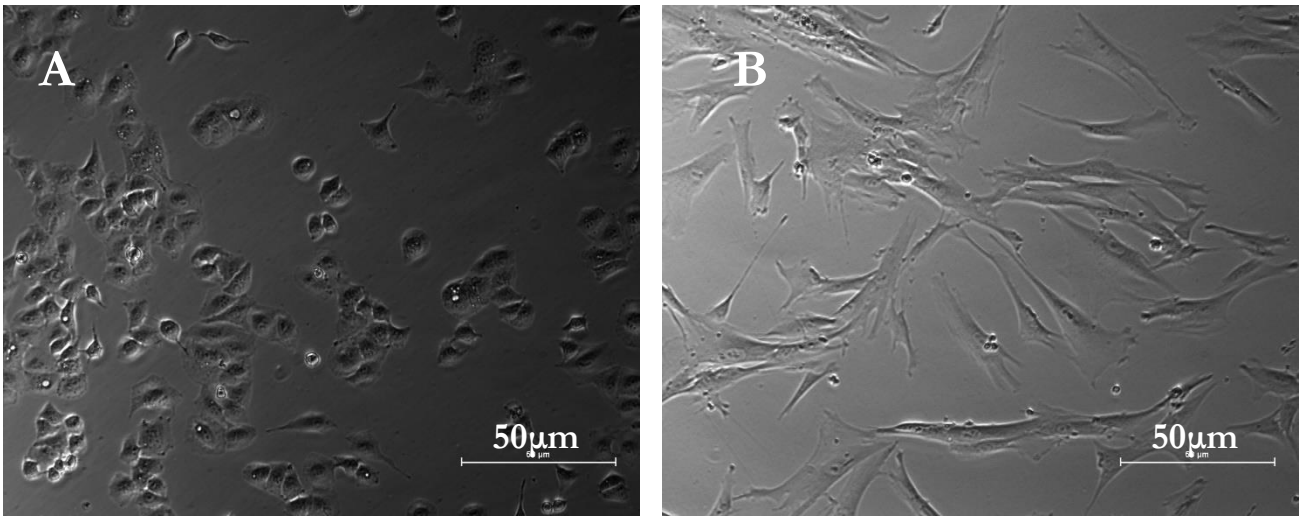

Figure 4: Morphology of A549 and MRC-5. A. A549 lung cancer cell line presents a round shape and is less spread than MRC-5 cell line. B-MRC-5 fibroblast cell line at a confluence of 70%. The shape of this cell line is elongated, having a higher surface area, which implies a smaller cell per area ratio.

3.3.5 Assessment of Cells Proliferation

A549 cells were sub-cultured two or three times per week in a 1:2 to 1:5 ratio, depending on the optimal dilution, according to the vendor. In order to sub-culture cells when approximately reached 80% confluence, the medium was removed and the cells were sensitized with PBS. The cells were then incubated with 0.05% trypsin/EDTA, in a humid atmosphere with 5% of CO_2 and at 37ºC for at least 3 minutes until cells detached. After that, trypsin activity was inhibited by fresh medium, owing to the high content in proteins. Cells were centrifuged at 800xg for three minutes, and resuspended in an adequate volume of media. After that, 1 ml of the suspension was added to a 10 cm plate and cells were incubated in a humid atmosphere with 5% CO_2, until used.

MRC-5 cells were treated in a similar fashion although it proved to be more susceptible to passage, which implied that cells may be lost to excessive manipulation. Special care is needed to avoid confluences >70% in order to avoid phenotypic alterations. Moreover, due to the fact that MRC-5 fibroblasts grow more slowly than cancer cells, they were passaged less frequently than the latter cells. MRC-5 fibroblasts were used within 15 passages.

3.3.6 Cell Counting

For the cell seeding in each experimental procedure, cells were counted previously. For this purpose, an optical microscope, a haemocytometer and trypan blue dye were used. Trypan blue is a dye that only penetrates non-viable cells, leading to blue staining of dead cells. By using this method, only viable cells were counted. Cells within the four corners of the haemocytometer were counted and the number of cells per volume was calculated by using the formula (2).

$$Cell/mL = \frac{x \times dilution\ factor}{10^{-4}\ mL} \quad (2)$$

Where x means the number of cells counted.

3.4 Assays Planning

To analyse the toxicity of the two antioxidants compounds MitoXT1 (caffeic acid derivative) and MitoXT2 (vitamin K3 derivative), synthesized by the laboratory of Professor Fernanda Borges at the School of Science of the University of Porto, jointly with anticancer drugs (DOX, CIS and etoposide), the experimental scheme shown in Figure 5 was performed. For SRB, resazurin and vital fluorescent microscopy assay, A549 cells were plated at a density of 40 000 cells/mL, MRC-5 cells at 20,000 cells/mL and H9c2 at 35,000 cells/mL for the latter only SRB and resazurin were performed. Assays with SRB and resazurin were performed in the same 48-well plate, while for fluorescent microscopy assay, a 6 multi-well plate with a glass coverslip was used. Four different experiments were performed as shown in Figure 5. After allowing cells to attach for 24h, they were pre-treated with a non-toxic concentration of antioxidants MitoXT1 and MitoXT2, 1.6 and 3.1μM for the first and 97.5 nM for the second, removing first the previous media and adding fresh media with the desired compound concentration. Twenty-four hours later, the different drugs (DOX, CIS and etoposide) were added directly to the culture media at a concentration of 0.312μM, 10μM, and 2.5μM respectively. DOX was prepared in MiliQ water, cisplatin was firstly prepared in DMSO and a dilution was then performed in PBS, while etoposide was directly prepared in DMSO at the concentration desired. Finally, after treating cells for 24h or 48h, assays were performed. MitoSOX assay was performed in a 96 multi-well plate and cells were seeded at a 10,000 cells/mL and 7500 cells/mL density for A549 and MRC-5 cells respectively. For this particular experiments, the anti-cancer agents were incubated for 3h only, after 24h of antioxidant pre-treatment to detect early effects on superoxide anion generation.

For all the other experiments such as caspase activity, Live/Dead assay and cell cycle assay, cells were cultured at a density of 50,000 cells/mL for A549 cells and at a 80,000 cells/mL for MRC-5 in 60nm plates. The experiments were performed as described before. However, in order to measure caspase-like activity, cell treatments with the anti-cancer agents were performed for 18h to avoid excessive cell death.

For H9c2 cells, two experiments were performed, namely SRB and rezasurin. In order to initially determine the concentration of MitoXT1 and MitoXT2 used in the further experiments, a dose-response curve was performed. To analyse the protective effect of these antioxidant, one concentration of each compound was chosen and tested with 0.5 and 1 μM DOX.

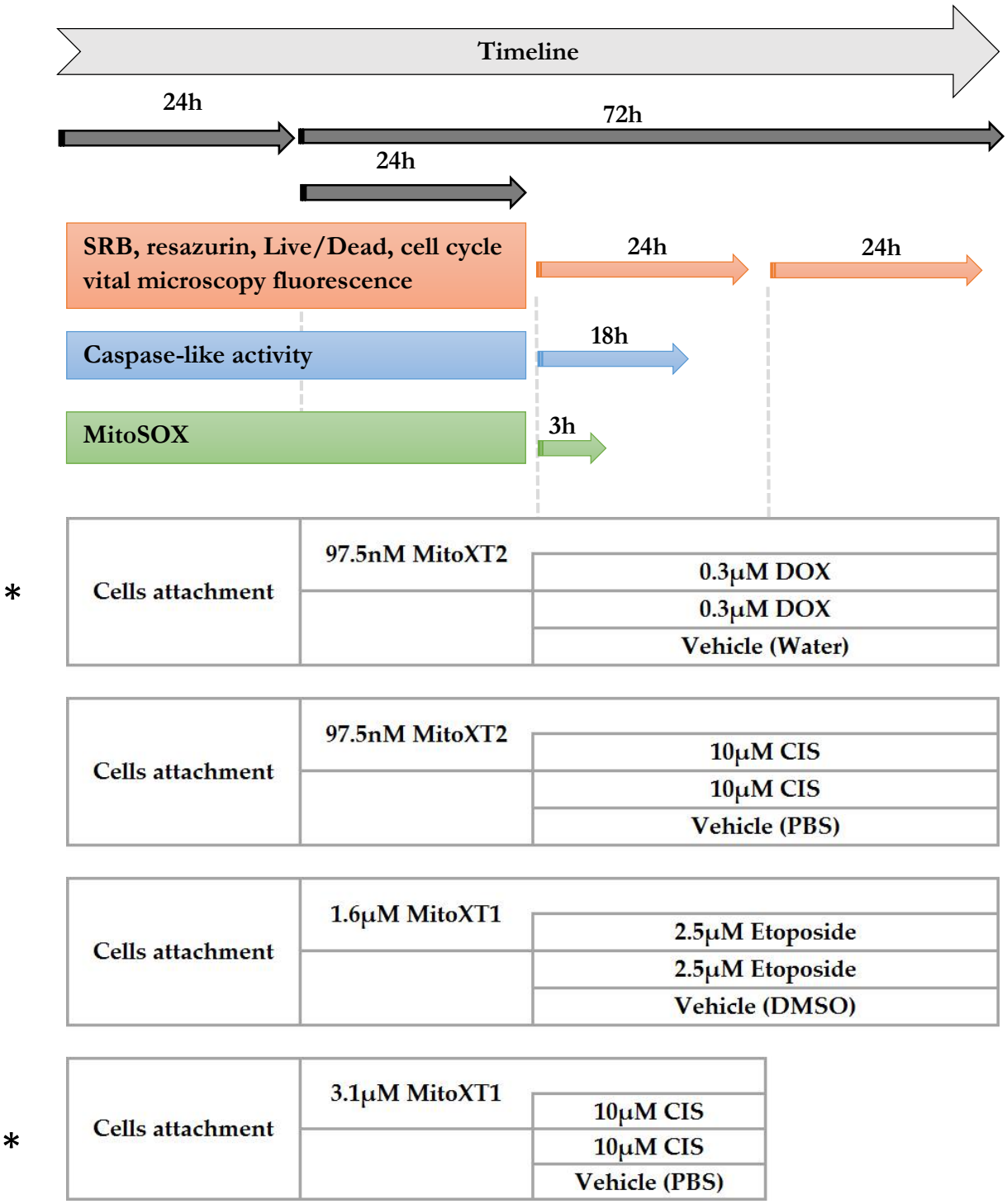

Figure 5: Timeline and experimental drug design for all the assays performed. A549 and MRC-5 cells were seeded and divided into different experimental groups depending on the treatment used. The novel mitochondrial-directed antioxidants were initially incubated with cells for 24 before adding the anti-cancer agent (doxorubicin (DOX) 48h; cisplatin (CIS) for 24 and 48h; etoposide for 48h).The effect of MitoXT2 and MitoXT1 was measured in terms of the cell viability by SRB colorimetric assay and resazurin assays, for the four experiences. The other experiments such as 3 and 9 caspases- like activities performed by colorimetric assay, Live/Dead and cellular cycle by flow cytometry, cellular morphology and mitochondrial membrane potential by vital epifluorescence microscopy, and ROS production by fluorimetry using a plate-reader, were only performed with the combination signals by *. The experiments performed with H9c2 are not described here.

3.5 Specific Methods

3.5.1 Resazurin

The resazurin assay allows to measure the cellular metabolic activity, and is based on the reduction of resazurin a blue indicator redox to resorufin (a pink compound with fluorescence) by the dehydrogenases in viable cells. Resazurin is incorporated in the phosphorylation oxidative pathway, and will act as an intermediary acceptor of electron, if cells are metabolically active. In order to perform this experiment, cells were treated exactly on the same way as explained before. Following the treatment, culture medium was removed and cells were incubated for 1 hour with 150µl of resazurin (10µg/mL) prepared in culture medium. The amount of resazurin reduced to resorufin was measured fluorometrically at an excitation wavelength of 540 nm and emission set at 590nm in a Cytation 3 reader. The data were treated considering the control as 100%. After that, the SRB assay was performed in the same wells, allowing to have two distinct protocols in the same samples.

3.5.2 Sulforhodamine B (SRB)

The sulforhodamine B assay was performed to measure the cell mass after the different treatments, according to the protocol by Vichai and Kirtikara [204]. However, some alterations were performed in the methodology. After cells were plated and treated as described before, they were fixed with 1% of acetic acid in ice-cold methanol during at least one hour at -20ºC. Posteriorly, the plate were incubated with 0.5% (w/v) of SRB in 1% acetic acid at 37ºC during 30 minutes. The excess of solution not specifically bounded to the negative charged proteins contained in cells was discarded with several washes using 1% of acetic acid in water solution, being the plate dried in an incubator at 37ºC. In the end, 500µL of Tris 10mM pH10 was added to each well to remove and dilute the SRB from proteins. To guarantee that all the SRB was removed, plates were slowly stirred during 15 minute. After that, 200 µL of each well was transferred to a 96 well plate in order to measure the optical density in a Victor X3 (Perkin Elmer) plate reader at wavelength of 540 nm. To achieve statistical analyses all the results were expressed as 100% of control. A blank sample was performed with Tris solution to subtract the background. The results were obtain using the mathematical formula in (2).

$$\frac{Treated\ cells - blanck}{Control\ cells - blanck} x\ 100 \qquad (3)$$

Sulforhodamine is a pink dye with two sulphonamide groups which specifically binds to negative charged cell protein under acidic condition. This assay give an indirect measure of the percentage of cell mass that survived to different treatments. Still, this assay does not allow to distinguish between cell death and arrest of cell cycle; two possible reason for a decrease in cell mass. A decrease in cell protein amount correlated well with cell number [204].

3.5.3 Protein Quantification

The protein quantification was achieved by the Bradford assay. This is a simple method developed in 1976 that allows to quantify protein present in different samples [205]. This assay is based on the fact that the Coomassie brilliant blue G-250 dye stains protein residues. Coomassie blue is red and when binding to basic amino acids residues it changes to blue with a maximum absorbance wavelength at 595nm. All samples pre-treated with lysis buffer, as described below, were diluted 1:2 in Bradford reagent and then 200µL of mixture was transferred to a 96 multi-well plate. A standard curve was also achieved ranging from 0,625µg/mL to 10 µg/mL using a known concentration of Bovine Serum Albumin (BSA) solution. After 15 minutes at room temperature incubation, the absorbance was read in a Cytation 3 (BioTek Instruments, Inc.) multi-plate reader.

3.5.4 Determination of Caspase 3 and 9-like Activities

To measure caspase 3 and 9-like activities in cells, treated with the test antioxidants and drugs, a spectrophotometric assay was performed. Cells were treated as explained before, collected and stored at -80ºC for the treatment groups described. Afterwards the different aliquots of cellular extracts were treated with 200µL of lysis Buffer (See lysis buffer solution). Then, 3 cycles of freezing with liquid nitrogen followed by thawing in a water bath were performed, with samples being vortexed after each cycle. The following step consisted of forcing the cells, through a 27G needle for 5-10 times. Then, the samples were centrifuged at 30,000xg for 5 minutes. Finally, the supernatant was transferred to new tubes and protein was performed. In order to perform the caspase assay, 50µg and 25µg of protein was used for caspase 9 and 3 assays respectively. Moreover, 0.1mM of caspase 3 and 9 substrates were used. After 2h incubation at 37ºC, the activity-like for caspase 3 and 9 was determined espectrofotometrically at 405 nm, by detecting the increase of the *p*-nitroanilide chromophore, after the cleavage of the respective substrates. With the purpose of determining the activity of caspase though the release of *p*NA a calibration curve was

prepared ranging 0 to 1000µM *p*NA. Values for caspase-like activity were as *p*Na released per cell protein.

3.5.5 Live/Dead Assay by Flow Cytometry

The Live/Dead assay was performed to evaluate the extension of cell death in samples. Indeed, this method involves two specific probes, calcein-AM which accumulates in viable cells and ethidium homodimer-1 (EH-1) which only permeates necrotic cells, binding to DNA. The low level of background is also an advantageous property of this assay and occurs because neither the dyes are fluorescent before cell internalization and binding to structures (for EH-1).

Calcein-AM is a green fluorescent dye which is cleaved by intracellular esterases providing that the cells are viable (Figure 6 A). On the other hand, ethidium homodimer-1 stains nucleic acid and emits red fluorescence (excitation/emission maximum ~528/61) indicating a loss of cell membrane integrity (Figure 6 B). In conjugation, this two dyes allows the research to measure the percentage of viability of cells under the treatment condition.

For this work, cells were plated as explained earlier. The experiment followed the same time point as for the SRB assay. After treatments, cells were trypsinized and then centrifuged at 500xg, during 5min, at 5ºC. The medium was discarded and the pellet was washed with 1ml of PBS, after which cells were centrifuged again at 500xg, during 3min, at 3ºC. PBS was removed and 500mL of microscopy medium (See preparation of solutions) was added. 7.2µM of calcein-AM and 18µM of ethidium homodimer-1 were added to the different samples. Vehicles for the drugs were assumed to be the control and were separated by four new tubes, the first one contained no dyes at all, the second one only contained calcein-AM dye, the third one only ethidium homodimer-1 dye and the last one had the two probes. These controls were used to optimize the settings for the flow cytometer and to avoid artefacts resulting for cell self-fluorescence. The samples were read in a Becton-Dickenson FACScalibur Flow cytometer (BD Biosciences, San Jose, CA, USA).

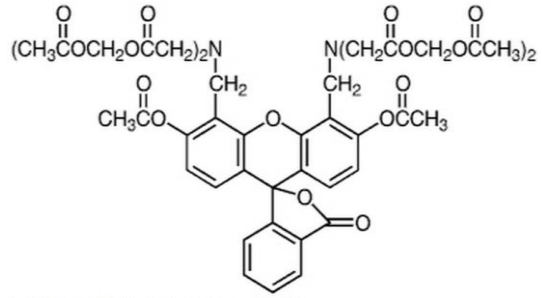

Figure 6A: **Molecular structure of calcein- AM.** Image obtained from https://www.lifetechnologies.com/order/catalog/product/C3100MP?ICID=search-product assessed in April, 24, 2015

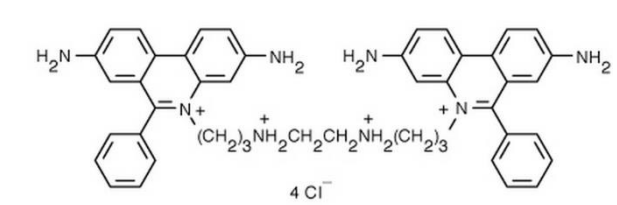

Figure 6B: Ethidium monodimer-1 molecular structure. Image obtained from https://www.lifetechnologies.com/order/catalog/product/E1169?ICID=search-product assessed in April, 24, 2015.

3.5.6 Cellular Cycle Measurements

Cell cycle progression was analysed as described by Serafim et *al.* 2008 [206]. Cells in log-phase growth were treated with different concentrations of the tested compounds as explained earlier. Cells were then detached using trypsin for 3 min. and then the same volume of growth medium was added to inhibit trypsin. Adherent and floating cells were collected and fixed with cold 70% ethanol and stored at 4ºC until the assay was performed. After washing at least 3 times the sample with PBS-T, cells were incubated with a solution containing 20µg/mL RNAse and 20µg/mL propidium iodide (PI) in PBS-T buffer for 30 minutes at 37ºC. Then the samples were analysed in a flow cytometer (Becton-Dickenson FACScalibur). The percentage of cells in the different cell cycle phases was quantified using Modfit LT software (Verity Software House, Topsham, ME, USA).

3.5.7 Vital Epifluorescence Microscopy

Mitochondrial transmembrane electric potential was detected using the tetramethyl rhodaminemethylester (TMRE) dye and the chromatin condensation. The cells were seeded and treated as described above in 6 well-plates with a glass coverslip in each well. After the respective treatment, cells were rinsed with PBS and incubated during 30min, with 50nM TMRE (tetramethylrhodamine, ethyl ester) and 10mg/mL Hoechst 33342 in microscopy medium (see Solution preparation) supplemented with 1.2mM $MgCl_2$ and 1.3mM $CaCl_2$ at

37ºC. After incubation, coverslips were removed from the wells and placed inverted on slides containing 100 µL of fresh microscopy media. All images were collected at 40X and were collected using a Nikon Eclipse TE2000U epifluorescence microscope. TMRE is a specific positive, red-orange dye for membrane mitochondria potential and it accumulates in active mitochondria due to their relative negative charge and to a great capacity to cross the membrane. Upon mitochondrial depolarization TMRE is not able to stain mitochondria anymore because of the decreased membrane potential and the fluorescence of the dye decrease. Hoechst 33342 allows the observation of the double strand DNA with a preference for sequences rich in adenine and thymine. This dye was used to label nuclei in the different cells, allowing at the same time to detected apoptotic nuclei due to a typical nuclear condensation.

3.5.8 MitoSOX-based Detection of Mitochondrial Superoxide Anion

In order to measure mitochondrial superoxide production in different treatments, cells were plated in a 96 well-plate at a concentration of 10,000 cell/well for cancer cells and 7,500/well for fibroblasts cell-line. After 24h of antioxidant pre-treatment, cells were incubated for 3h with DOX and CIS at the same concentration used for SRB at 37ºC. Next, the cells were washed with PBS and then incubated 10 min with mitoSOX prepared in microscopy media with a final concentration of 5µM. A kinetic fluorescent lecture was made over 90 min with readings each 2 minutes at 37ºC and with 510 nm excitation wavelength and 580 nm emission wavelength in a Cytation 3 (BioTek Instruments, Inc.) multi-plate reader. After this 20µM antimycin A and 20µM rotenone were added as a positive control to stimulate the maximal production of superoxide anion by mitochondria. The normalization of the results was made with SRB, and the final results were expressed in function of the cell protein (equivalent to cell number).

3.5.9 Statistical analysis

The data were processed with GraphPad Prism 6.0 Software, and all the results were expressed as means ±SEM. In order to evaluate the effect of two independent variables, such as treatment and cell lines, a two way ANOVA with a Tukey Multiple Comparison Test was adopted. To compare the effect of treatments in cells, a one-way ANOVA followed by the Tukey post-test was used. Comparisons with $p<0.05$ were considered as statistically significant.

Chapter 4
Experimental Results

Results

4.1 Effects of Tested Antioxidants and Anti-cancer Agents on A549 and MRC-5 Cell Mass and Metabolic Activity

We initially performed sulforhodamine B and resazurin assays in tandem to determine the effects of the two antioxidants[1] (MitoXT1 and MitoXT2) synthetized by the laboratory of Dr. Fernanda Borges from the Faculty of Sciences of the University of Porto. The objective was to test whether these compounds may increase the cytotoxicity of classic anti-cancer agents on lung cancer cells and, in the process, whether on the contrary they would protect healthy cells against injuries caused by the same anticancer drugs. To evaluate the effect of antioxidant and drugs in both A549 and MRC-5 cell lines, these were treated with a non-toxic concentration of these test compounds, previously determined by preliminary data from our group. Anti-cancer drugs used were DOX, CIS and etoposide.

To measure A549 and MRC-5 cell mass/protein, the sulforhodamine assay was performed. This assay does not distinguish the mechanism behind a decrease in cell mass, which can be due to the activation of cell death pathways or to the inhibition of cell cycle. Cells were pre-treated for 24h with 97.5nM MitoXT2 followed by 0.3μM DOX for 48h. DOX induced a non-significant decrease in cell mass in both cell lines. On the contrary, MitoXT2 *per se* did not affect the cell mass of any of these cell lines. However, the combination of these two treatments induced two different responses in A549 and MRC-5 cells. While in cancer cell lines a significant decrease of cell mass was observed when compared with the control, an increase in MRC-5 cell mass was observed when compared to cells treated with DOX alone (Figure 7 A). Regarding the metabolic activity assessed with the reduction of rezasurin to resofurin, it appears that DOX and MitoXT2 combination also reduces significantly the metabolic activity of A549 cells although this may also seems to occur in MRC-5 cells which appear to be also metabolically affected by this combination treatment (Figure 7 B).

[1] These compounds are described as antioxidants due to previous data from our group where they showed anti-radical activity in cell-free systems, as well activity to inhibit lipid peroxidation of mitochondrial membranes, as previously described for one lead compound [80]

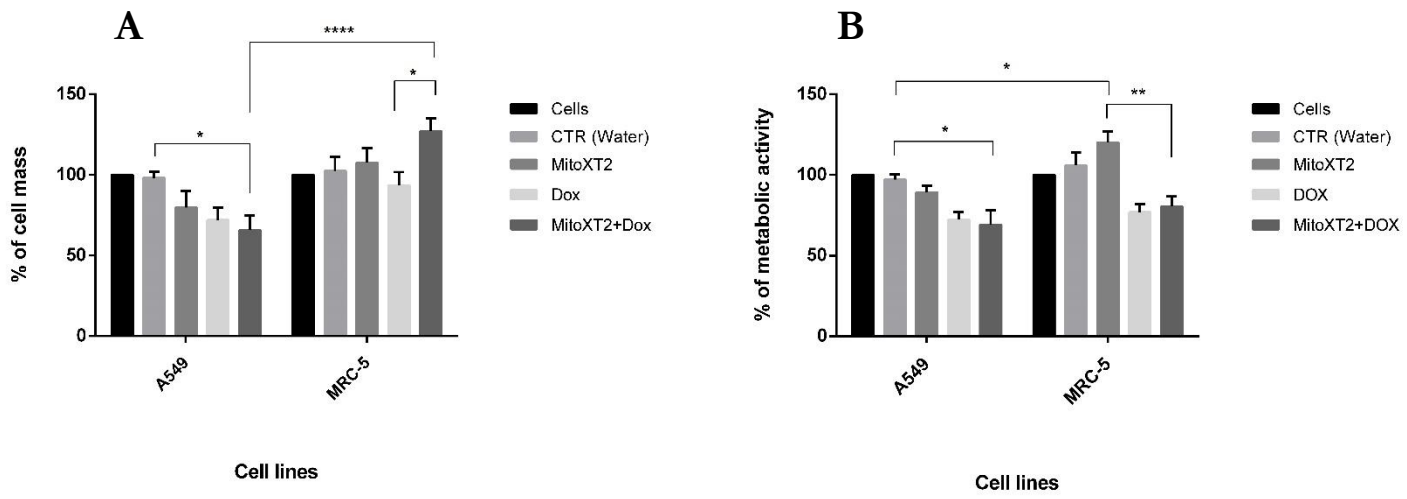

Figure 7: Effects of MitoXT2 pre-treatment followed by DOX treatment on cell mass and metabolic activity, on A549 and MRC-5 cell lines. The cells were pre-treated for 24h with 97.5nM MitoXT2 followed by an incubation period of 48h with 0.3µM DOX. **A)** Cell mass was determined using sulforhodamine B colorimetric assay as describe under the section Material and Methods. **B)** Metabolic activity was accessed by resazurin assay as described in Material and Methods section. In all experiments, the control (CTR) was considered as 100% and data are expressed as a percentage of this control ± SEM. The results derived of five independent experiments. A two way ANOVA was performed, followed by a multiple Tukey test. *($p<0.05$), ** ($p<0.01$) and **** ($p<0.0001$) represent statistical differences.

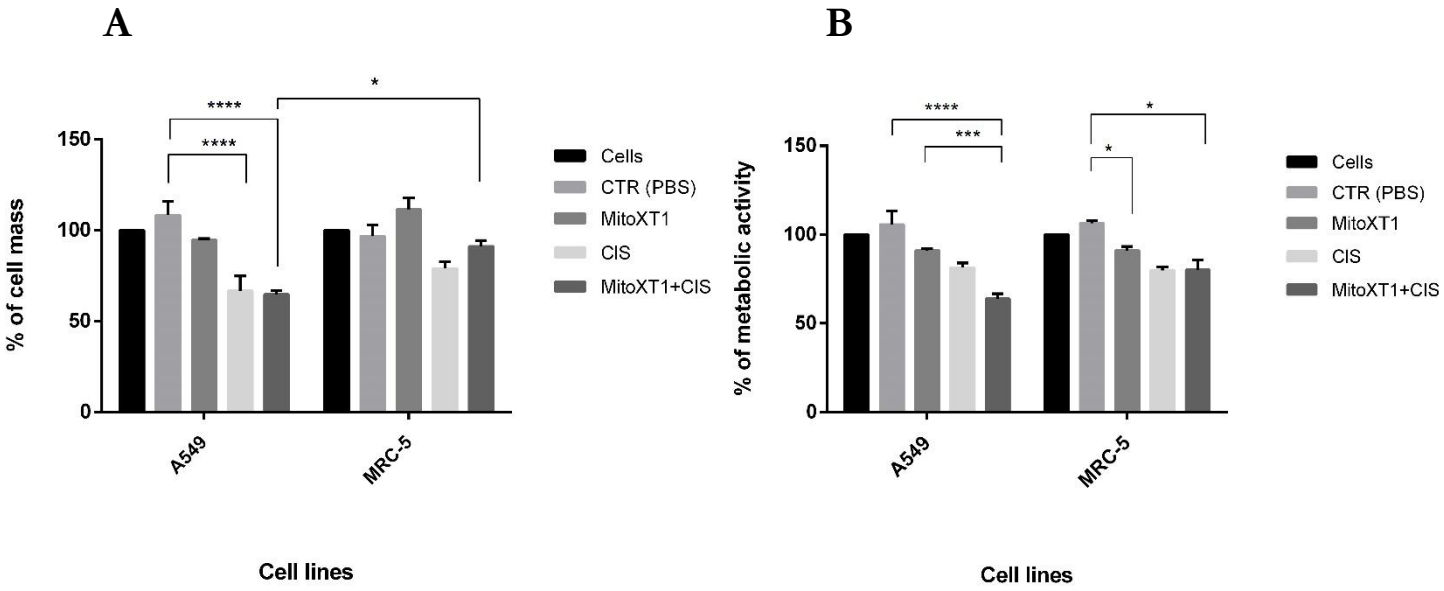

Figure 8: Effects of MitoXT1 pre-treatment followed by CIS treatment on cell mass and metabolic activity on A549 and MRC-5 cell lines. The cells were pre-treated for 24h with 3.1µM MitoXT1 followed by an incubation period of 24h with 10µM CIS. **A)** Cell mass was measured by sulforhodamine B (SRB) colorimetric assay. **B)** Metabolic activity of both A549 and MRC-5 cell lines under the different treatment conditions. Control (CTR) without any treatment was assumed to be the 100% and all the other experiments were expressed in function of this control ± SEM. Five independent experiments were realized and data were analysed by two way ANOVA followed by a multiple Tukey test.* ($p<0.05$), *** ($p<0.001$) and ****($p<0.0001$) represent statistical differences.

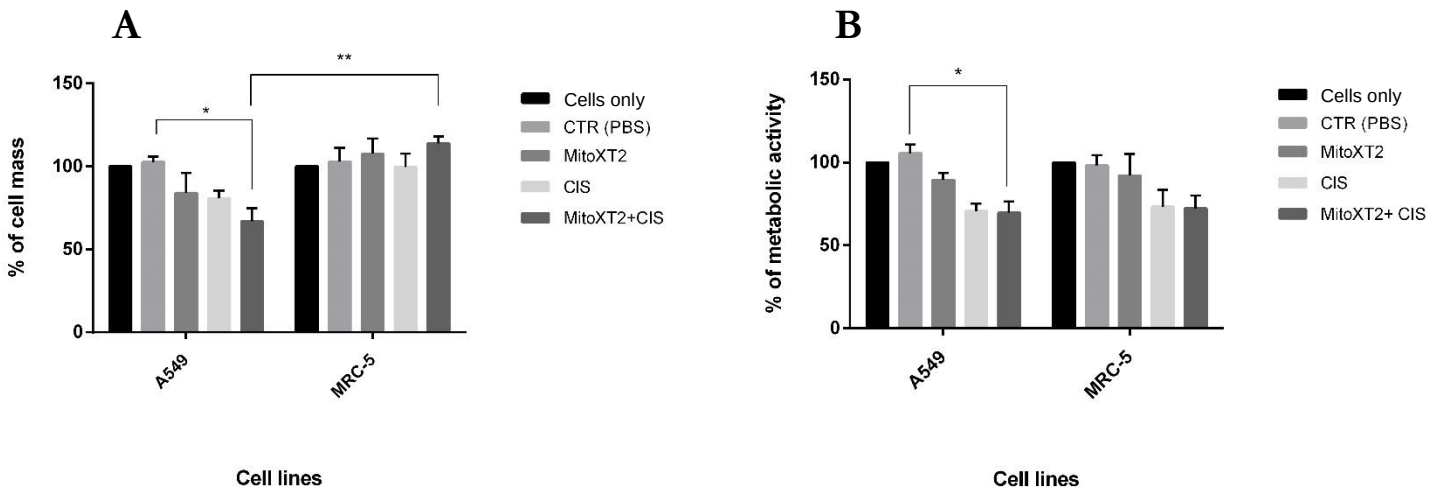

Figure 9: Effects of MitoXT2 pre-treatment followed by CIS treatment on cell mass and metabolic activity, on A549 and MRC-5 cell lines. The cells were pre-treated for 24h with 97.5nM MitoXT2 followed by an incubation period of 48h with 10µM CIS. **A)** Cell mass was determined using sulforhodamine B (SRB) colorimetric assay as describe under the section Material and Methods. **B)** Metabolic activity was accessed by resazurin assay as described in Material and Methods section. Control (CTR) without any treatment was assumed to be the 100% and all the other experiments were expressed in function of this control ± SEM. Five independent experiments were performed and data were analysed by two way ANOVA followed by a multiple Tukey test. * ($p<0.05$) and ** ($p<0.01$) represent statistical differences.

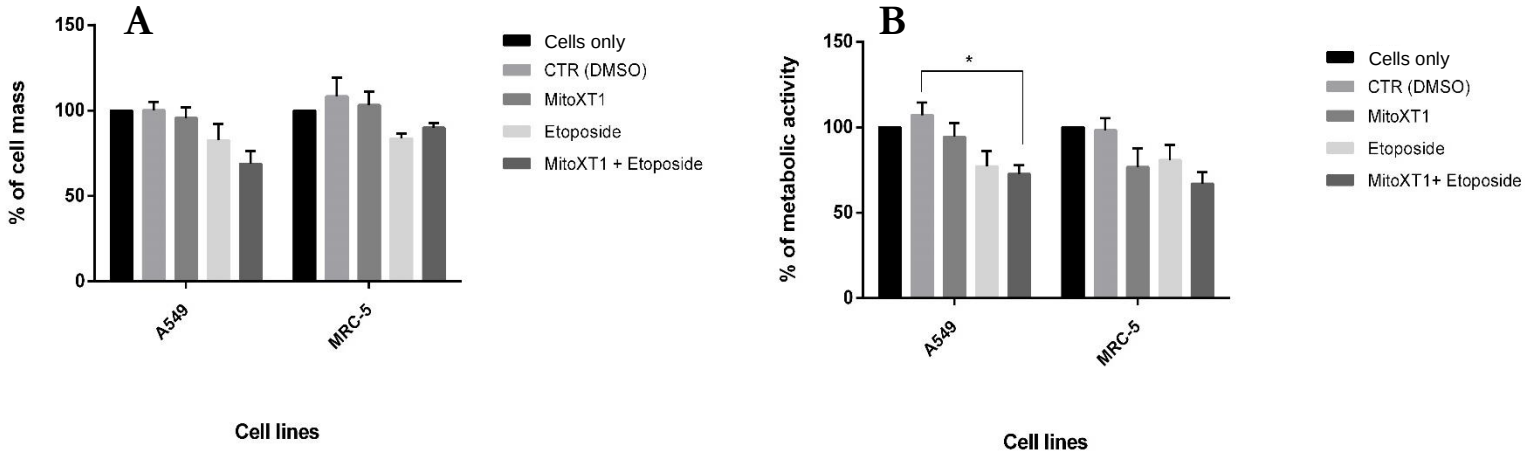

Figure 10: Effects of MitoXT1 pre-treatment followed etoposide treatment on cell mass and metabolic activity on A549 and MRC-5 cell lines. The cells were pre-treated for 24h with 1.6µM MitoXT1 followed by an incubation period of 48h with 2.5µM etoposide. **A)** Cell mass was accessed using sulforhodamine B (SRB) colorimetric assay as describe under the section Material and Methods. **B)** Metabolic activity of both A549 and MRC-5 cell lines under the different treatment condition. Control (CTR) without any treatment was assumed to be the 100% and all the other experiments were expressed in function of this control ± SEM. Five independent experiments were realized and the data were analysed by two way ANOVA followed by a multiple Tukey test. * ($p<0.05$) represent statistical difference.

Twenty-four hours of pre-treatment with 3.1μM MitoXT1 followed by 24h of 10μM CIS treatment resulted in a significant decrease of cell mass in comparison with the treatment with MitoXT1 alone on A549 cancer cells; however, this was not observed in MRC-5 cells (Figure 8 A). The results obtained by the resazurin assay are similar with those obtained through SRB, and confirmed that the metabolic activity of the cancer cell line decreased, when treated with antioxidant and drug jointly, in comparison with the vehicle (PBS) (Figure 8 B).

Another experiment was performed using 97.5 μM MitoXT2 pre-treatment for 24h followed by 10μM CIS treatment for 48h.The combined treatment of MitoXT2 and CIS showed that the combination resulted in A549 cell mass loss when compared with the vehicle control. This statistical difference was not found when MitoXT2 and CIS were used alone. MRC-5 cells presented an increase in cell mass in comparison with MitoXT2 and CIS alone (Figure 9 A). Regarding the metabolic activity, the same effect was also found (Figure 9 B).

The experiment performed with 3.1μM MitoXT1 pre-treatment followed by 2.5μM etoposide incubation did not present significant results either in cancer or in fibroblasts cells (Figure 10 A). However, the combination of these two drugs induced a significant reduction of cell viability in A549 cancer cells (Figure 10 B).

For then following assays, only two drug combinations therapies were chosen to perform further assays, including Live/Dead assay, cell cycle analysis, mitochondrial superoxide anion production and caspase-like activity experiments. The treatments chosen were MitoXT1 with CIS and MitoXT2 with DOX, as they resulted in the more promising effects.

4.2. Effects of MitoXT2 Against DOX-induced Cytotoxicity on H9c2 Cardiomyoblasts

In order to verify if the antioxidants in study would also protect another cell model against DOX toxicity, H9c2 cardiomyoblasts were selected and the SRB method performed to measure cytotoxicity. In order to initially measure toxicity, H9c2 cells were treated with several concentrations (0, 10, 25, 50, 100, 200, 500, 1000nM) of MitoXT2 and (0, 0.5, 1, 2, 2.5, 3, 3.5, 4μM) of MitoXT1, during 72h. The concentration of antioxidant that was further tested was the one that caused a minimal loss of H9c2 cell mass, i.e. 100nM for MitoXT2 and 0.5μM for MitoXT1 (See Figure 11, A e B) the results also indicated that MitoXT1 is more toxic to H9c2 cardiomyoblasts than MitoXT2. These concentrations were tested following the same experimental design as explained under the Materials and Methods section, to which 48h incubation with DOX followed. MitoXT2 did not decrease cell mass in comparison with the control. The treatment with MitoXT2 and 0.5μM DOX did not significantly decrease cell mass; however, with 1μM DOX a significant reduction in cell mass was observed, in comparison with the control. On the other hand, 1μM DOX significantly decreased cell mass when compared with the combined treatment, confirming that MitoXT2 may have afforded some protection for the highest DOX concentration tested. When performing the resazurin experiment, no significant differences in metabolic activity between the cells treated with DOX, both alone and combined were observed (Figure 12).

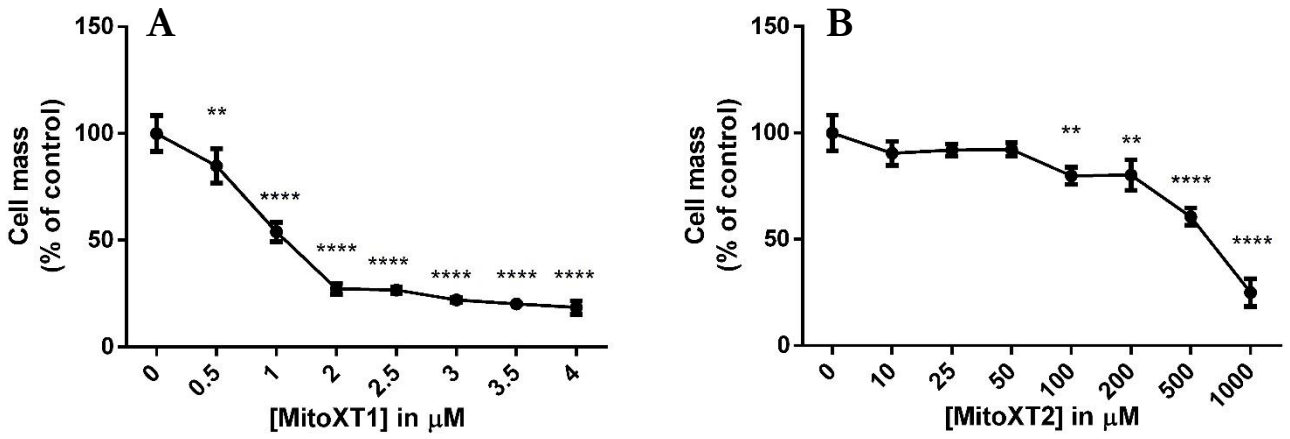

Figure 11: Effect of different concentrations of mitochondria-directed antioxidants MitoXT1 and MitoXT2 in H9c2 cells. A) H9c2 cells were incubated with MitoXT1 (0, 0.5, 1, 2, 2.5, 3, 3.5 and 4μM) for 72 hours. MitoXT1 cytotoxicity was measured by SRB colorimetric assay. **B)** H9c2 cells were incubated with MitoXT2 (0, 10, 25, 50, 100, 200, 500 and 1000nM) for 72 hours. MitoXT2 cytotoxicity was measured by SRB colorimetric assay. The control (100%) was the sample which did not receive any treatment. Data were represented in mean ± SEM of 5 independent experiments.** ($p<0.01$) and **** ($p<0.0001$) represent statistical differences with the control.

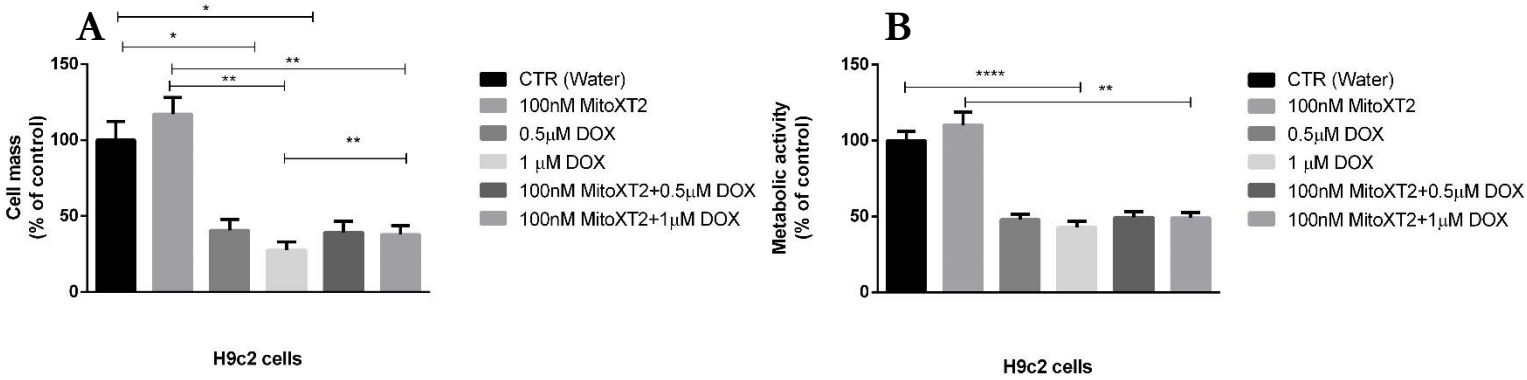

Figure 12: Protection by MitoXT2 against DOX-induced toxicity on H9c2 cells. A) Cell mass was assessed using sulforhodamine B (SRB) colorimetric assay. B) Metabolic activity of both A549 and MRC-5 cell lines under the different treatment condition. Control (CTR) with vehicle treatment was assumed to be the 100% and all the other experiments were expressed in function of this control ± SEM. Three independent experiments were performed and the data was analysed by one way ANOVA followed by a multiple Tukey test. * ($p<0.05$), ** ($p<0.01$) and **** ($p<0.0001$)) represent statistical differences .

4.3 Effects of MitoXT1 Against DOX-induced Cytotoxicity on H9c2 Cardiomyoblasts

Sulforhodamine assay was performed to compare the toxicity of DOX in the presence of MitoXT1 on H9c2 cells. MitoXT1 appears to increase slightly cell mass in comparison with the control, although not significant for the combination between MitoXT1 and both concentrations of DOX, seen as no significant difference was observed in comparison with DOX treatment alone. Moreover, it appears that the combined treatment of MitoXT1 and DOX might increase the cytotoxicity of the drug, further reducing H9c2 cell mass, at lower DOX concentration. This assay showed no protective effect of MitoXT1 against DOX induced cytotoxicity. Resazurin assay was not performed in this case (Figure 13).

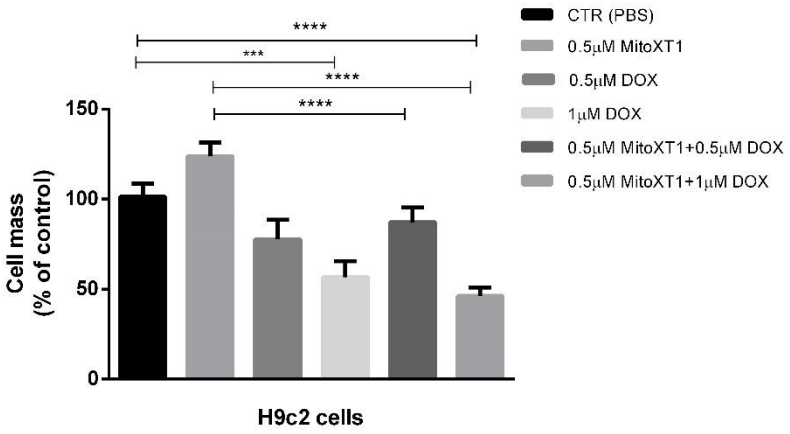

Figure 13: Protection by MitoXT1 against DOX-induced toxicity on H9c2 cells. A) Cell mass was assessed using sulforhodamine B (SRB) colorimetric assay as describe under the section Material and Methods. Control (CTR) with vehicle treatment was assumed to be the 100% and all the other experiments were expressed in function of this control ± SEM. Three independent experiments were performed and data were analysed by one way ANOVA followed by a multiple Tukey test. *** ($p<0.001$) and **** ($p<0.0001$) represent statistical differences.

4.4 Effects of MitoXT2 on Caspase 3 and 9-like Activities Induced by DOX on A549 and MRC-5 Cells

As previously described, activation of caspases 3 and 9 are involved in programmed cell death signalling. After 24h of seeding, cells were treated with antioxidants for 24h and then incubated with DOX for 18hours to measure caspase 3 and 9-like activities. When using A549 cells, both caspase 3 and 9-like activities were increased after DOX treatment, with MitoXT2 having no effect *per se*. The combination slightly increased the effects of DOX alone, with a particularly significant result for caspase-3 like activity (Figure 14, upper panel). When measuring the same end-points on MRC-5 cells, and despite DOX being again responsible for increasing caspase 3 and 9- like activities, the only result which was significant was a decrease in caspase 3-like activity when both compounds were used together (Figure

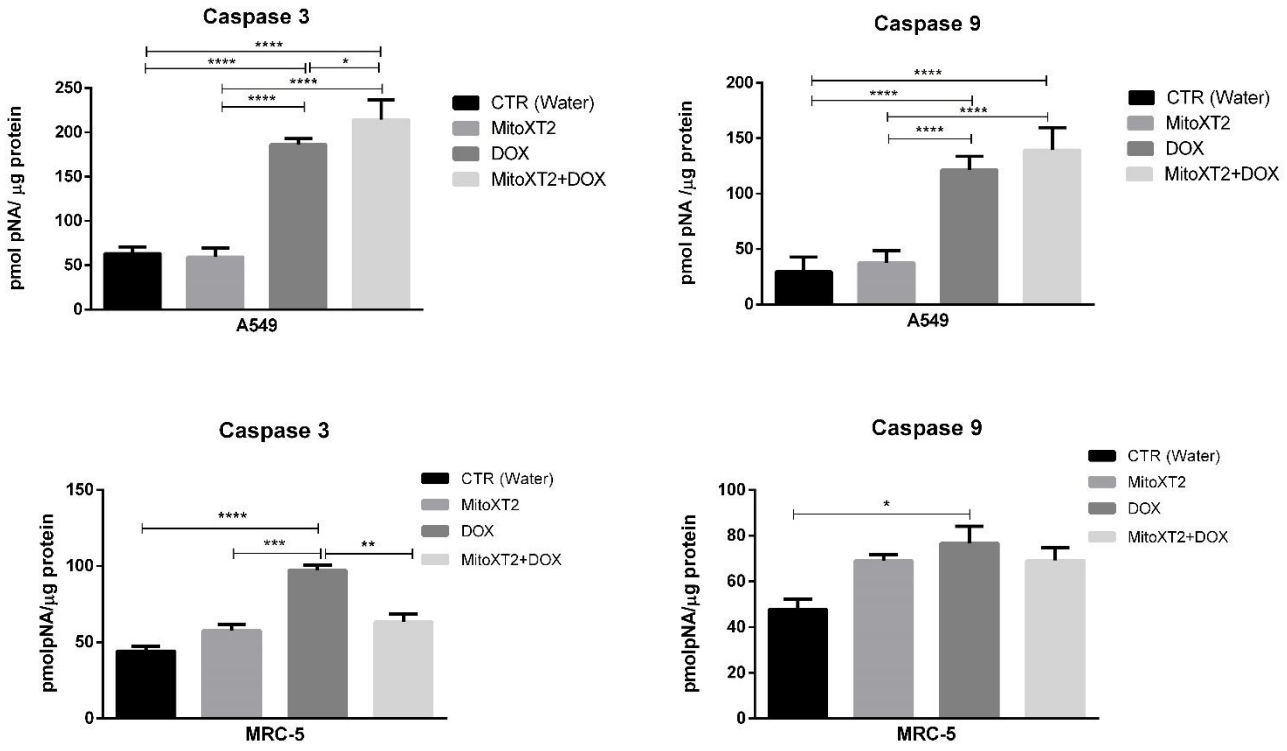

Figure 14: Effects of MitoXT2 pre-treatment on DOX induced caspase 3 and 9-like activities, assay performed on both the cell lines A549 and MRC-5. On the top left and right panels: Caspase 3 and 9-like activities respectively performed on A549 cell line. **On the bottom left and right panels**, Caspase 3 and 9 like-activity are respectively assessed on MRC-5 cell line. Caspases like activity was performed using a colorimetric assay. The control (CTR) was assumed to be the treatment with the drug vehicle in this case water. Data represents as means ± SEM of 4-5 independents experiments. A One-way ANOVA was performed followed by a multiple Tukey test. * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$) represent statistical differences.

14, lower panel).

4.5 Effects of MitoXT1 on Caspase 3 and 9-like Activities Induced by CIS on A549 and MRC-5 Cells

The same type of assay described above for caspase-like activity was performed to analyse the joint effect of MitoXT1 and CIS on both A549 and MRC-5 cells. Interestingly, in cancer cells, only the combination increased caspase 3 and 9-like activity in a significant manner (Figure 15, upper panel). Both caspase 3 and 9-like activities were increased by CIS alone, while the combination with MitoXT1 decreased the activation of both caspases on MRC-5 cell line. (Figure 15, lower panel)

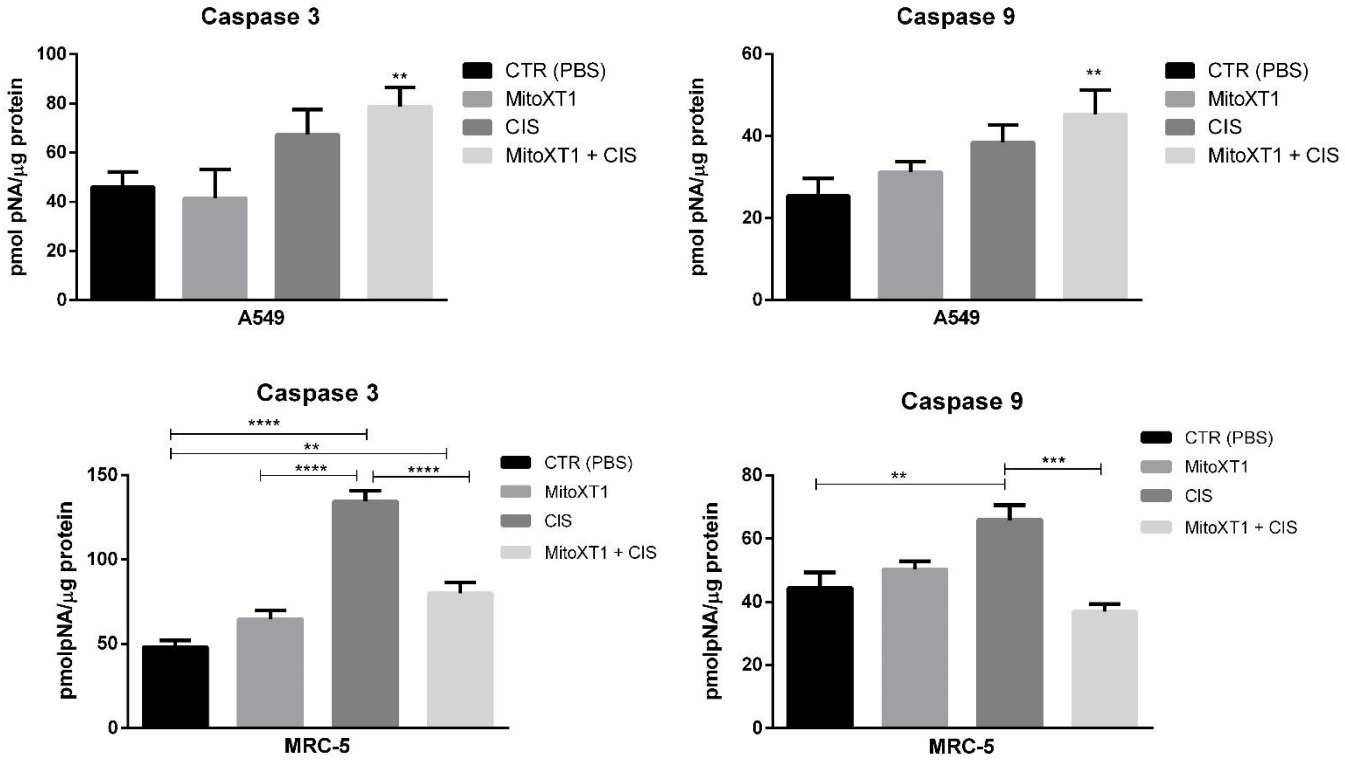

Figure 15: Effects of MitoXT1 pre-treatment on CIS induced caspase 3 and 9-like activities, assay performed on both the cell lines A549 and MRC-5. On the top left and right panels, Caspase 3 and 9 like activities respectively performed on A549 cell line. **On the bottom left and right panels**, Caspase 3 and 9 like-activity respectively accessed MRC-5 cell line.* Alone represents a comparison with control (CTR). Caspases-like activity was performed using a colorimetric assay with a specific substrate to each one of the caspase in study as describe under the Material and Methods section. The control (CTR) was assumed to be the treatment with the drug vehicle in this case PBS. Data represents as means ± SEM of 4-5 independents experiments. A one-way ANOVA was performed followed by a multiple Tukey test . * ($p<0.05$), ** ($p<0.01$), *** ($p<0.001$) and **** ($p<0.0001$) represent statistical differences.

4.6 Effect of Tested Antioxidant on Anti-cancer Agents-induced Toxicity on A549 and MRC-5 Cells

The Live/Dead assay was performed using calcein-AM and ethidium homodimer-1 to quantify cell viability and necrosis by flow cytometry. Two different treatments combinations were performed similarly to the caspase-like activity experiment, one with CIS and MitoXT1 and the other with DOX and MitoXT2.

4.6.1 MitoXT1 Pre-treatment Combined with CIS Cytotoxicity on A549 and MRC-5 Cell Death

The Live/Dead assay was used to evaluate the damage effect of MitoXT1 pre-treated cells followed by CIS incubation. Although MitoXT1 did not cause any cell death in any of the lines used, CIS decreased cell viability at a similar extension. Interestingly, the combination of both compounds decreased cell death on MRC-5 cells, while not causing cell death *per se.* (Figure 16 A and B).

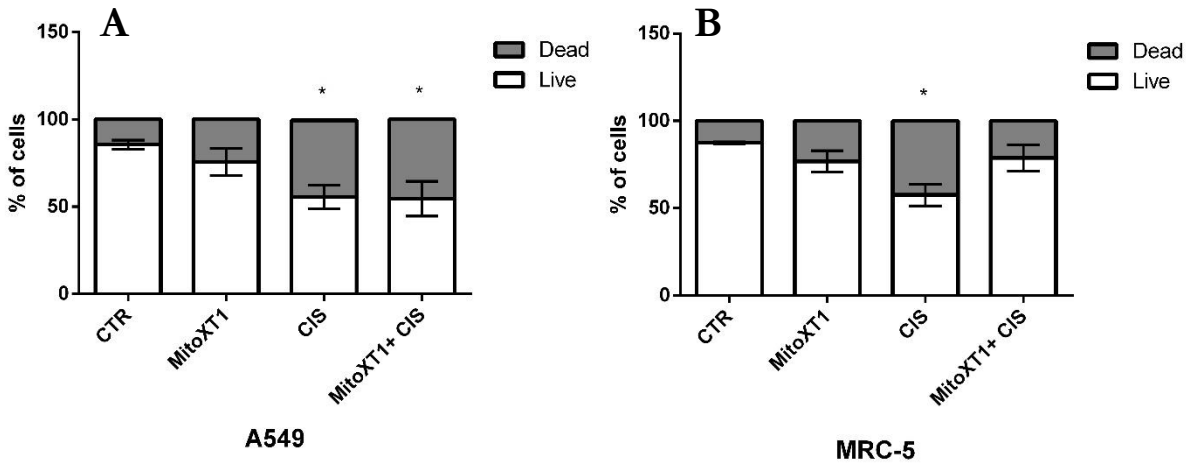

Figure16: Live/Dead assay of A549 and MRC-5 cells in the presence of CIS and MitoXT1. The experiment was performed using flow cytometry in cells labelled with calcein-AM to measure live cells and ethidium- homodimer -1 to quantify dead cells. After all the treatments were concluded, flow cytometry was performed as described under Material and Methods The assay was performed using PBS as control, MitoXT1, CIS, and 24h of MitoXT1 treatment followed by 24h CIS incubation . **A)** Experiments performed on A549 cells. **B)** Assay performed on MRC-5 cells. Data represents Mean ± SEM of 3 independent experiments. Statistical analysis was performed by using two-way ANOVA, and the symbol represents a comparison with the control (CTR). * ($p < 0.05$) represent statistical differences.

4.6.2 DOX and MitoXT2 Combined Effects on A549 and MRC-5 Cell Death

Figure 17 shows the results of the combined/single treatment with DOX or/and MitoXT2 on A549 and MRC-5 cells. From the results shown, it is possible to determine that DOX causes cytotoxicity on both cell lines, with the combined therapy treatment presenting a similar effect to that of DOX alone. MitoXT2 per se showed no effects on both the cell lines (Figure 17, A e B).

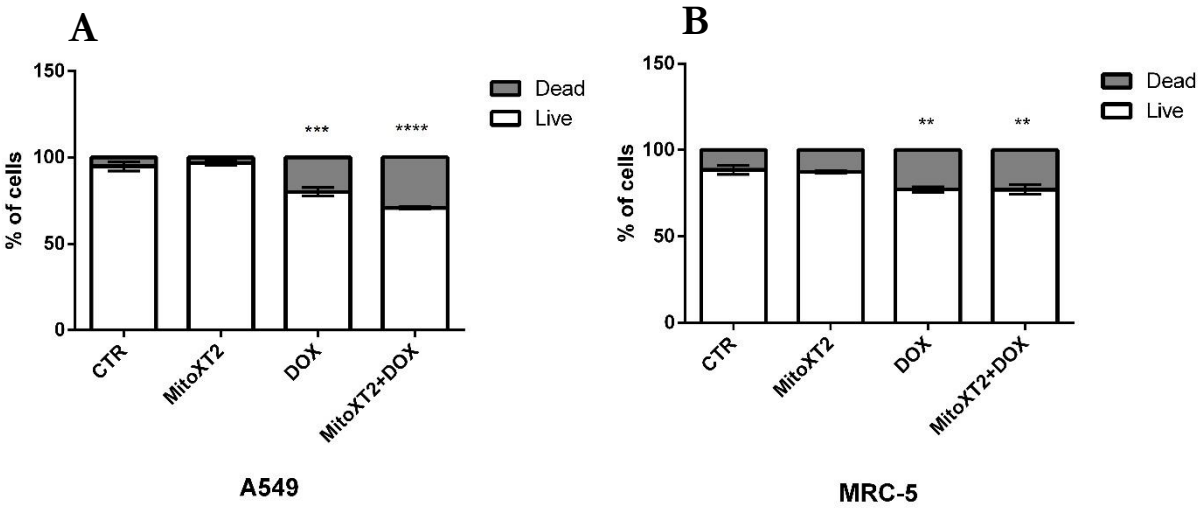

Figure 17: Live/Dead assay of A549 and MRC-5 cells in the presence of DOX and MitoXT2. The experiment was performed using flow cytometry in cells labelled with calcein-AM to measure live cells and ethidium- homodimer -1 to quantify dead cells. The assay was performed by initially incubating cells with MitoXT2 for 24hours followed by 48h of DOX incubation. **A)** Experiments performed on A549 cells. **B)** Assays performed on MRC-5 cells. Data represents Mean ± SEM of 3 independent experiments. Statistical analysis was performed by using two-way ANOVA and the symbols compare treatments with control (CTR). ** ($p< 0.01$),*** ($p<0.001$) and **** ($p<0.0001$) represent statistical differences.

4.7 Mitochondrial and Nuclear Alterations Resulting from Anti-cancer Treatments

Several morphological alterations may be indicators of programmed cell death these include nuclear condensation, loss of mitochondrial transmembrane electric potential and modification of the mitochondrial network [207].In order to complement caspase activity assays, we performed vital epifluorescence microscopy to measure nuclear and mitochondrial end-points which may suggest apoptosis.

Non-treated A549 cells presented normal cell morphology with a well-defined and filamentous mitochondrial network, representing healthy cells. MitoXT2 treated cells initially presented normal nuclear and mitochondrial morphology. However, when these antioxidant were incubated for a longer period of time a decrease in mitochondrial membrane potential was observed, suggested by as a loss of mitochondrial red TMRE fluorescence. Under DOX treatment, the mitochondrial network appears to be more condensed around the nucleus, with also evidences of chromatin condensation. The combined treatment with DOX and MitoXT2 appears to induce nuclear condensation and mitochondrial network disarray, although it does not seem higher than in DOX treatments *per se* (Figure 18).

MRC-5 cells appears to have a healthy, similar, morphology in the MitoXT1 and control groups. The intensity of the TMRE fluorescence is fainter in MRC-5 cells treated with DOX when compared with untreated cells, which represents a decrease in mitochondrial transmembrane electric potential. DOX also causes nuclear alterations in this cell line; however, the pre-treatment with MitoXT2 seems to protect cells from nuclear alterations and from the decrease of mitochondrial transmembrane electric potential (Figure 19).

When A549 cells were incubated with MitoXT1 alone, a small decrease in TMRE fluorescence appears to occur, which may result from decreased membrane potential. CIS *per se* appears to disrupt the mitochondrial structure, while the combination of both MitoXT1 and CIS appears to worsen the phenotype (Figure 20).

MitoXT1 does not appear to alter cellular morphology on MRC-5 cells compared with the control. The nucleus under CIS treatment are brighter than under the combined treatment appearing to suggest increased chromatin condensation. Interestingly, the mitochondrial transmembrane potential seems to be decreased and mitochondrial structure more fragmented in the combined treatment with MitoXT1 and CIS in comparison with CIS treatment alone (Figures 21).

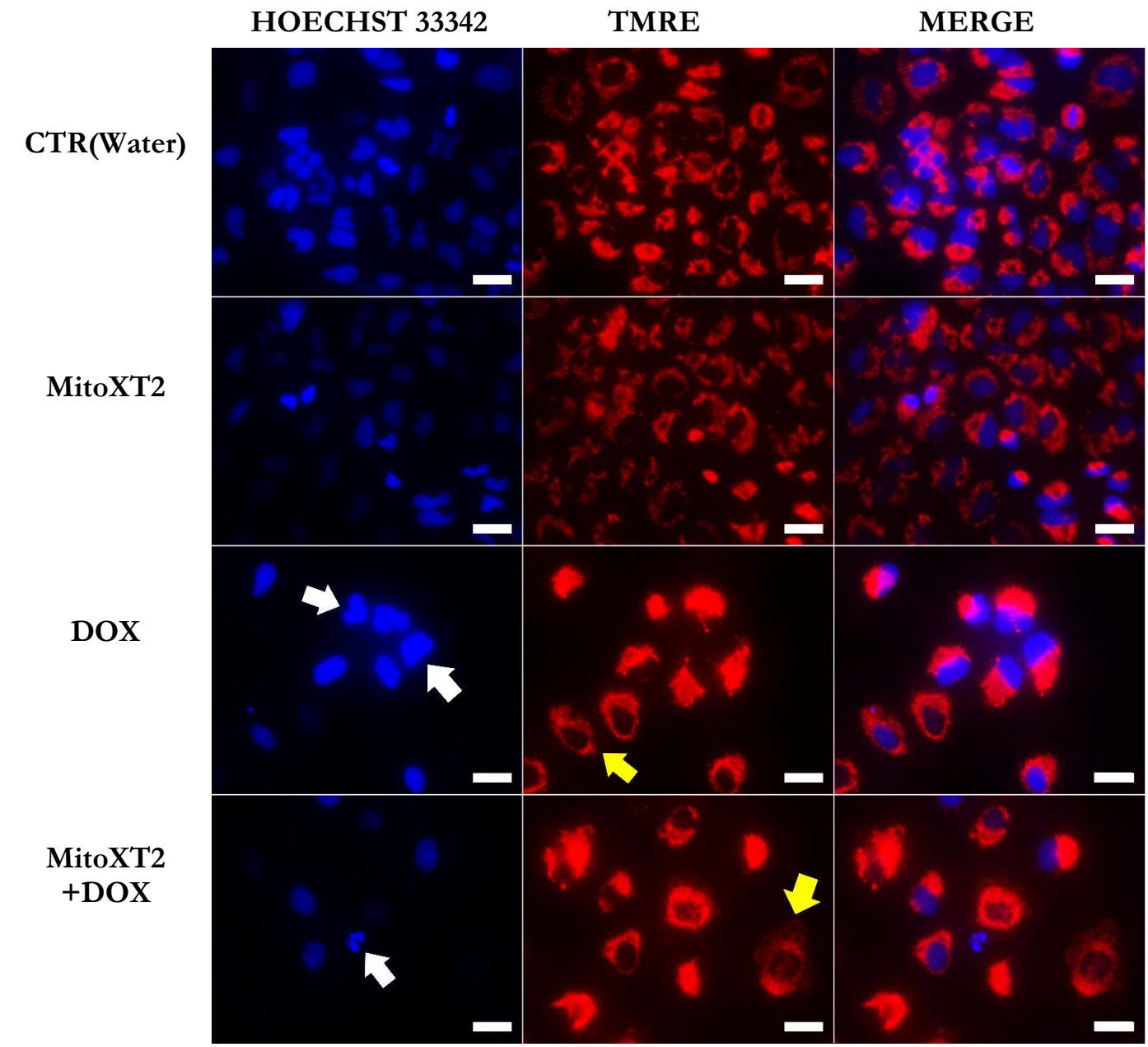

Figure 18: Nucleus and mitochondrial network morphology under MitoXT2 and DOX treatment on A549 cells. A549 cells were seeded in a 6-well multi-plate and treated as described in Material and Methods section and then incubated with 10mg/mL Hoechst and 50nM TMRE during 30 minutes. Bar represents 25µm. White arrows indicate condensed chromatin while yellow arrows point to loss of mitochondrial transmembrane electric potential.

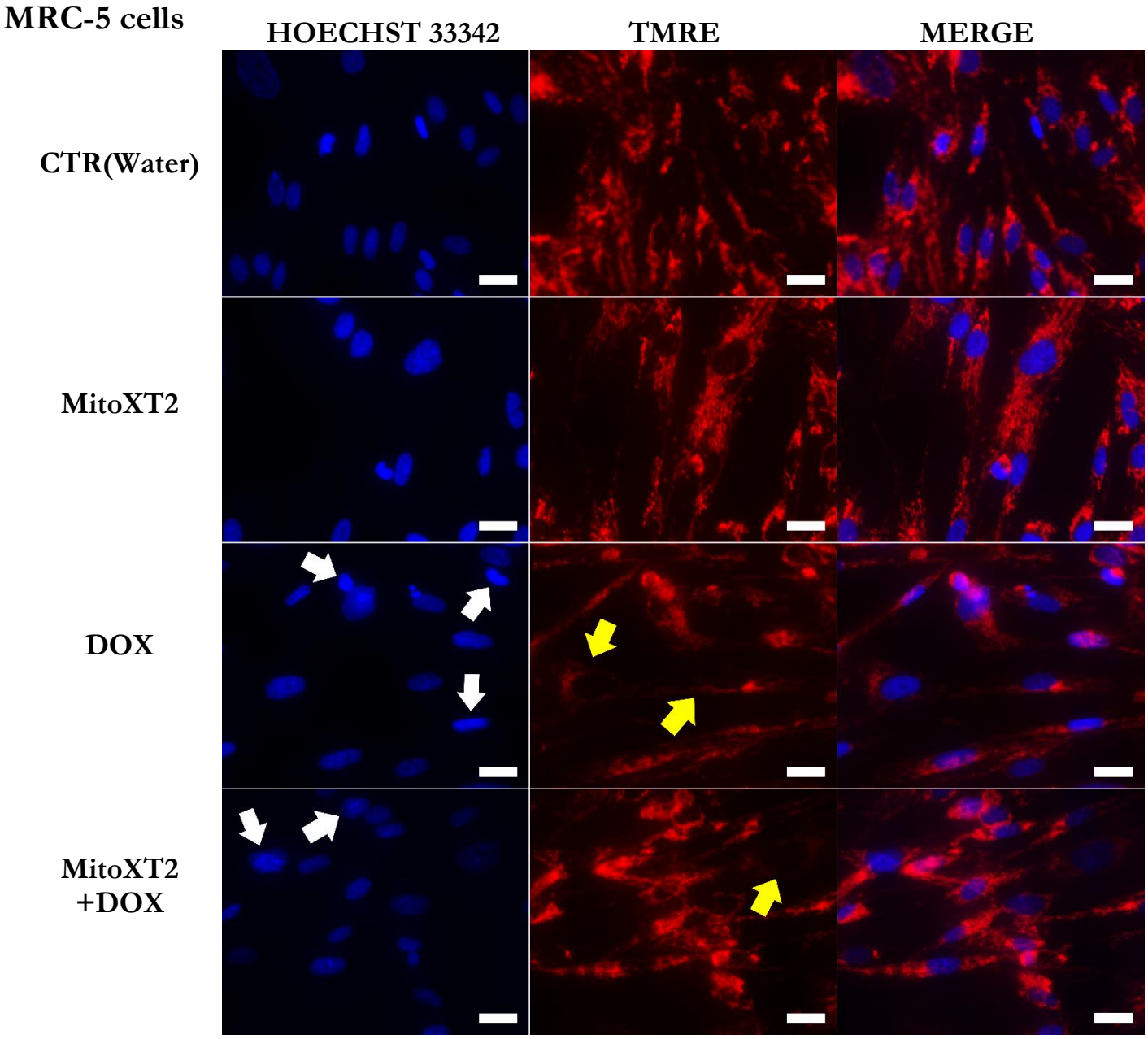

Figure 19: Nucleus and mitochondrial network morphology under MitoXT2 and DOX treatment on MRC-5 cells. MRC-5 cells were seeded in a 6-well multi-plate and treated as described in Material and Methods section and then incubated with 10mg/mL Hoechst and 50nM TMRE during 30 minutes. Bar represents 25µm. White arrows indicate condensed chromatin while yellow arrows point to loss of mitochondrial transmembrane electric potential.

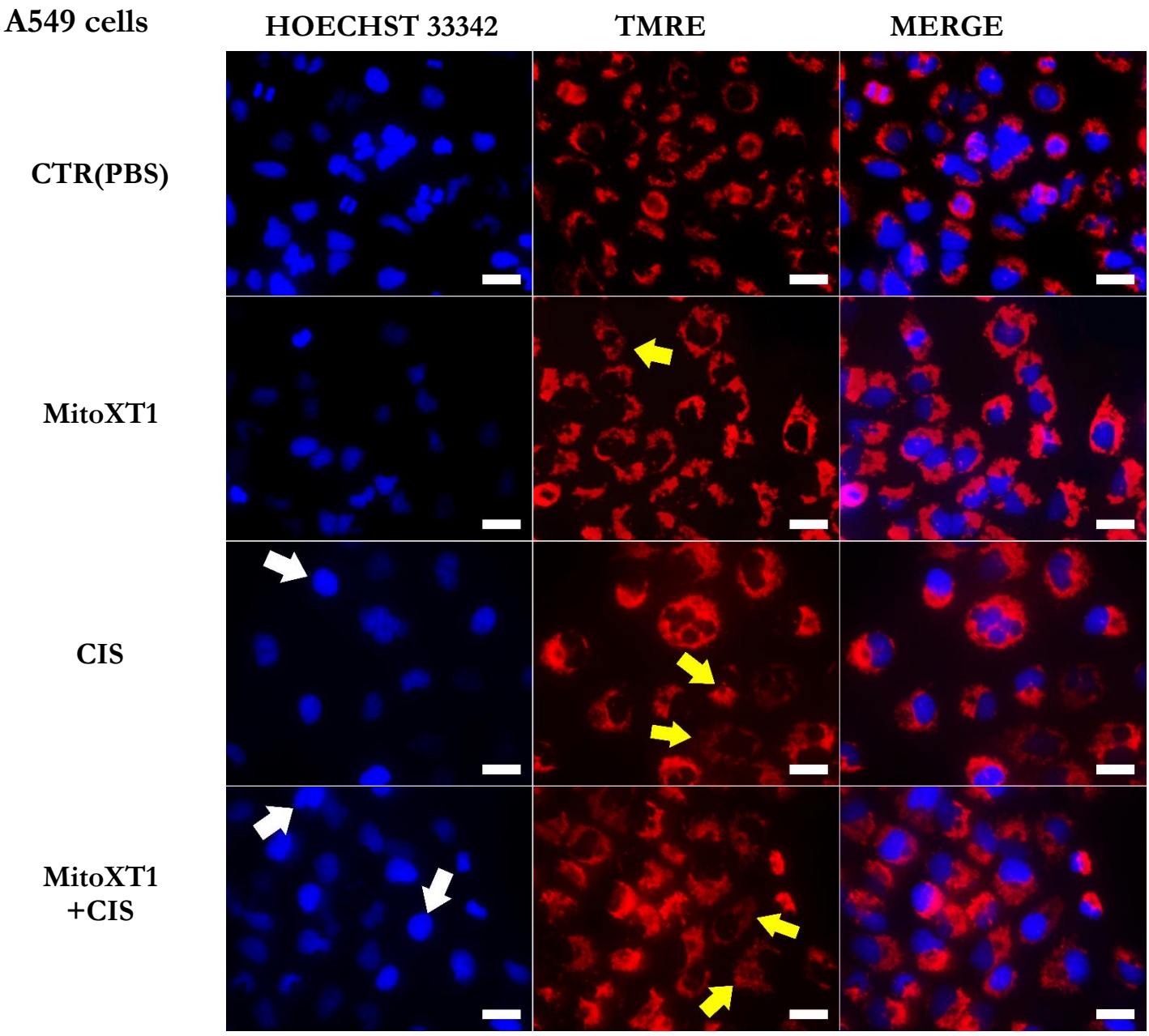

Figure 20: Nucleus and mitochondrial network co-localization under MitoXT1 and CIS treatment on A549 cells. A549 cells were seeded in a 6-well multi-plate and treated as described in Material and Methods section and then incubated with 10mg/mL Hoechst and 50nM TMRE during 30 minutes. Bar represents 25µm. White arrows indicate condensed chromatin while yellow arrows point to loss of mitochondrial transmembrane electric potential.

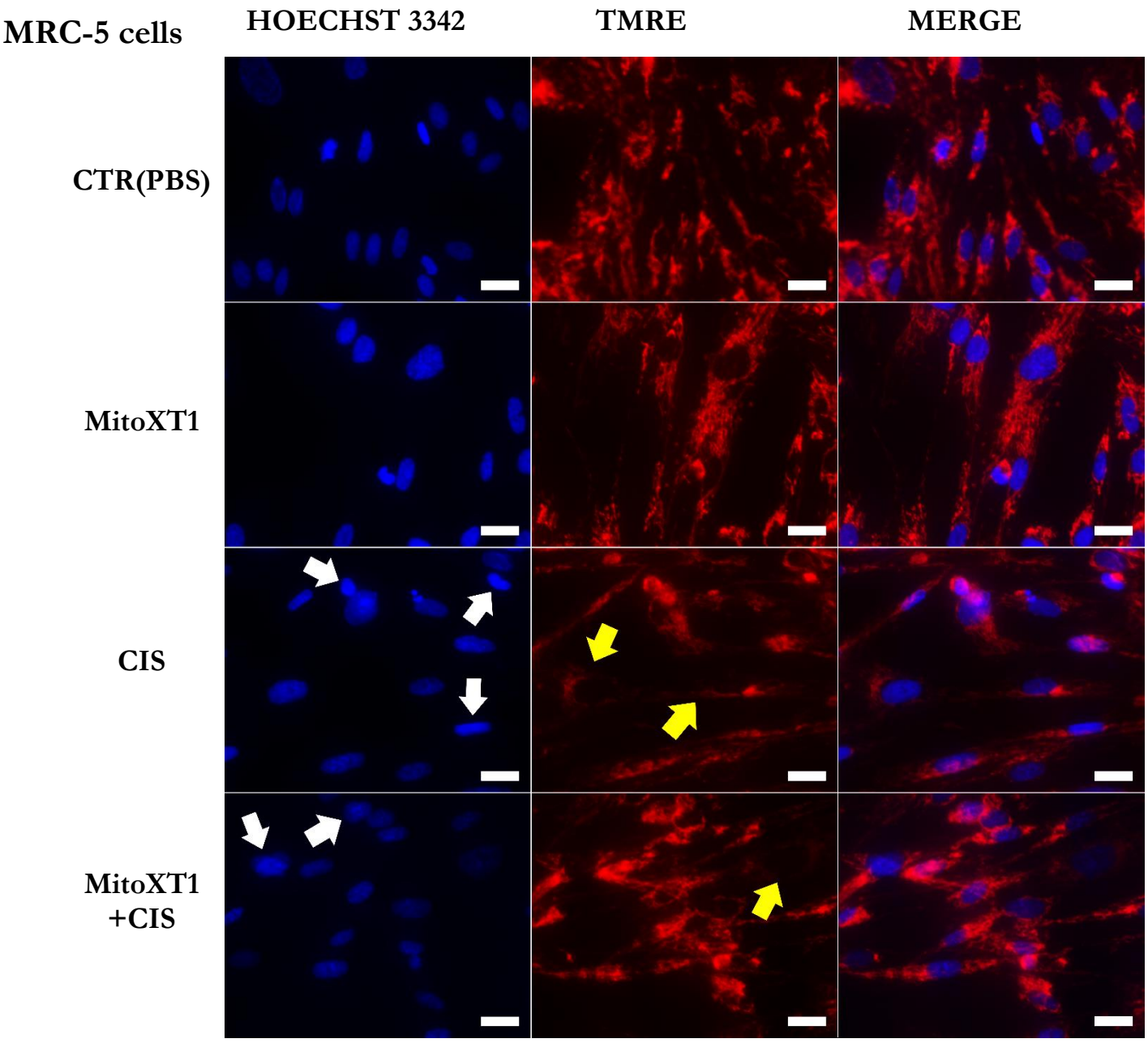

Figure 21: Nucleus and mitochondrial network co-localization under MitoXT1 and CIS treatment on MRC-5 cells. MRC-5 cells were seeded in a 6-well multi-plate and treated as described in Material and Methods section and then incubated with 10mg/mL Hoechst and 50nM TMRE during 30 minutes. Bar represents 25µm. White arrows indicate condensed chromatin while yellow arrows point to signalled loss of mitochondrial transmembrane electric potential.

4.8 Cell Cycle Measurements on A549 and MRC-5 Cells Treated with DOX and/or MitoXT2

By using propidium iodide, cell cycle was assessed on both MRC-5 fibroblasts and A549 cancer cells by flow cytometry. In order to avoid interference of the fluorescence of the different drugs used in this work on the results obtained with propidium iodide, we analysed each one of these compounds separately without any probe (data not shown). Figure 22 A shows results obtained on A549 lung cancer cells. DOX alone blocked cells on G2 phase at expense of a decrease of cells on G1 and S phase, although the differences in the letter case were smaller. MitoXT2 had no effect *per se* while the combinatory effect showed the same basic effect as for DOX alone (Figure 22 A). This can also be seen in the representative histograms (Figure 24). Results were slightly different for MRC-5 cells. While DOX also blocked cells in the G2 phase in exchange for a decrease in cells in G1 and S phase, the combination between DOX and MitoXT2 yielded different results, including a higher percentage of cells in S phase as compared with DOX alone and a decrease in cells in G2 phase, when compared with DOX alone. For the MRC-5 cell line, no effects from MitoXT2 *per se* were measured (Figure 22 B). Still, the low number of independent experiments (n=1 or 2) precluded any statistical comparison. Also, the peaks corresponding to subG1 phase, which may be indicative of apoptosis, were too small to be of any value, as observe in the representative histogram (Figure 24).

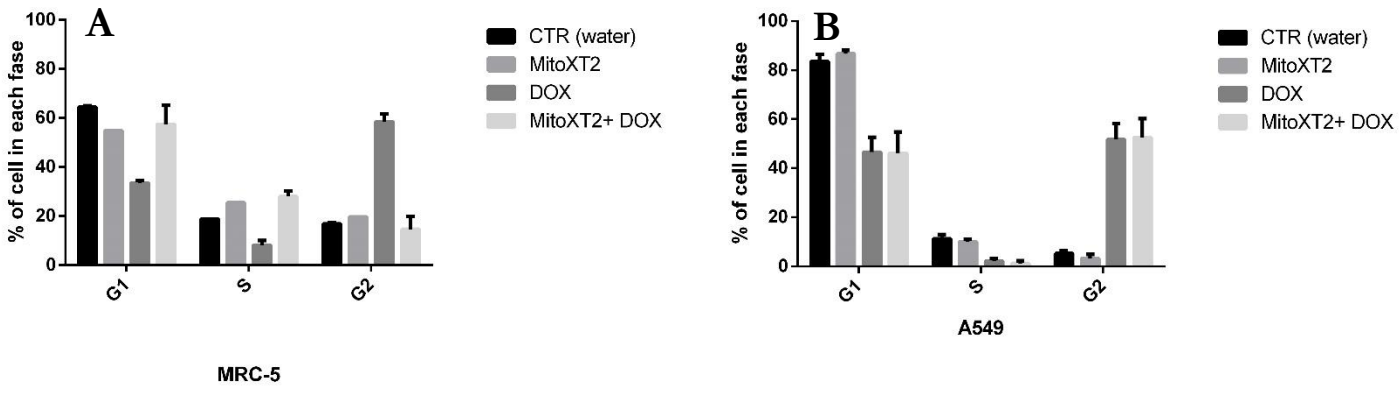

Figure 22: Cell cycle effect of DOX combined with MitoXT2 treatment on MRC and A549 cells. The cells were treated as explain under Material and Methods. This experiment was performed using propidium iodide dye incubated for 30 minutes and analysed through a cytometer **A)** A549 cells on the different states of cell cycle, under DOX, MitoXT2 and the combined treatments. **B)** MRC-**5** cells on the different cell cycle phases treated with DOX, MitoXT2 and the combined treatment. Data represents n=1 or 2 . CTR=control.

4.9 Cell Cycle Measurements on MRC-5 and A549 Cells Treated with MitoXT1 and/or CIS

When performing the same experiment on the separated and combined treatments of CIS and MitoXT1, the only visible alterations were a blockage of cells under S phase caused by CIS, in exchange for a decrease of cells under G1 phase. The combined treatment with MitoXT1 appears to protect both the cell types from this blockage induced by CIS (Figure 23 A and B). This results can be observed in the histogram obtained (Figure 25). Still, the low number of independent experiments again hindered any conclusion.

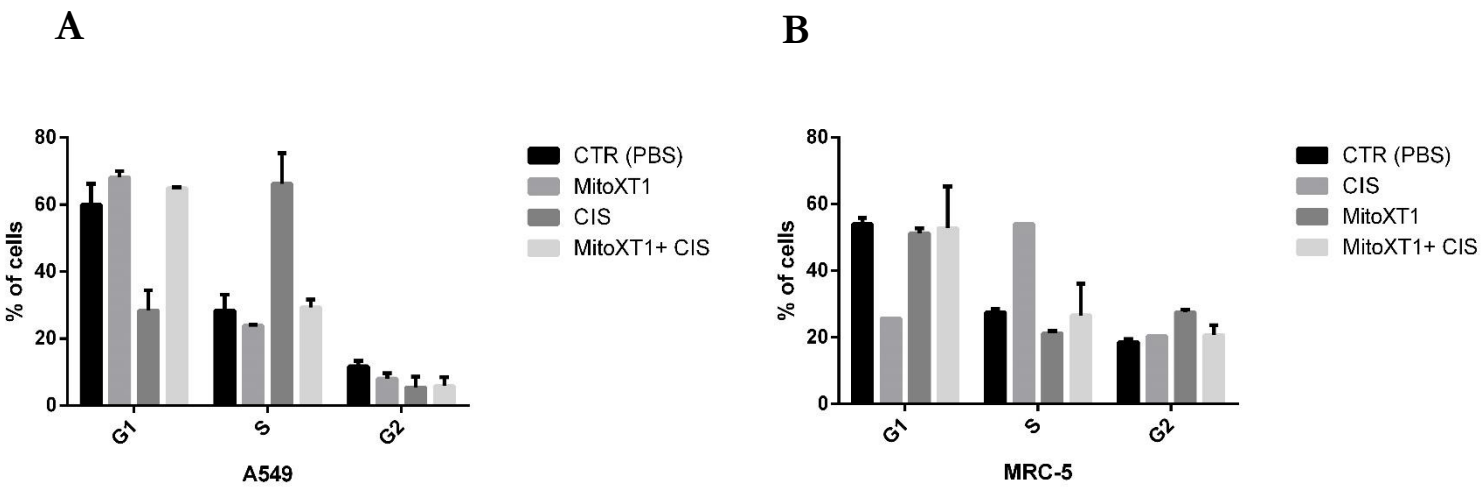

Figure 23: Cell cycle effect of CIS combined with MitoXT1 treatment on MRC and A549 cells. The cells were treated as explain under Material and Methods. This experiment was performed using propidium iodide dye incubated for 30 minutes and analysed through a cytometer **A)** A549 cells on the different states of cell cycle, under CIS, MitoXT1 and the combined treatments **B)** MRC-5 cells on the different cell cycle phases treated with CIS, MitoXT1 and the combined treatment.. Data represents n=1 or 2. CTR=control.

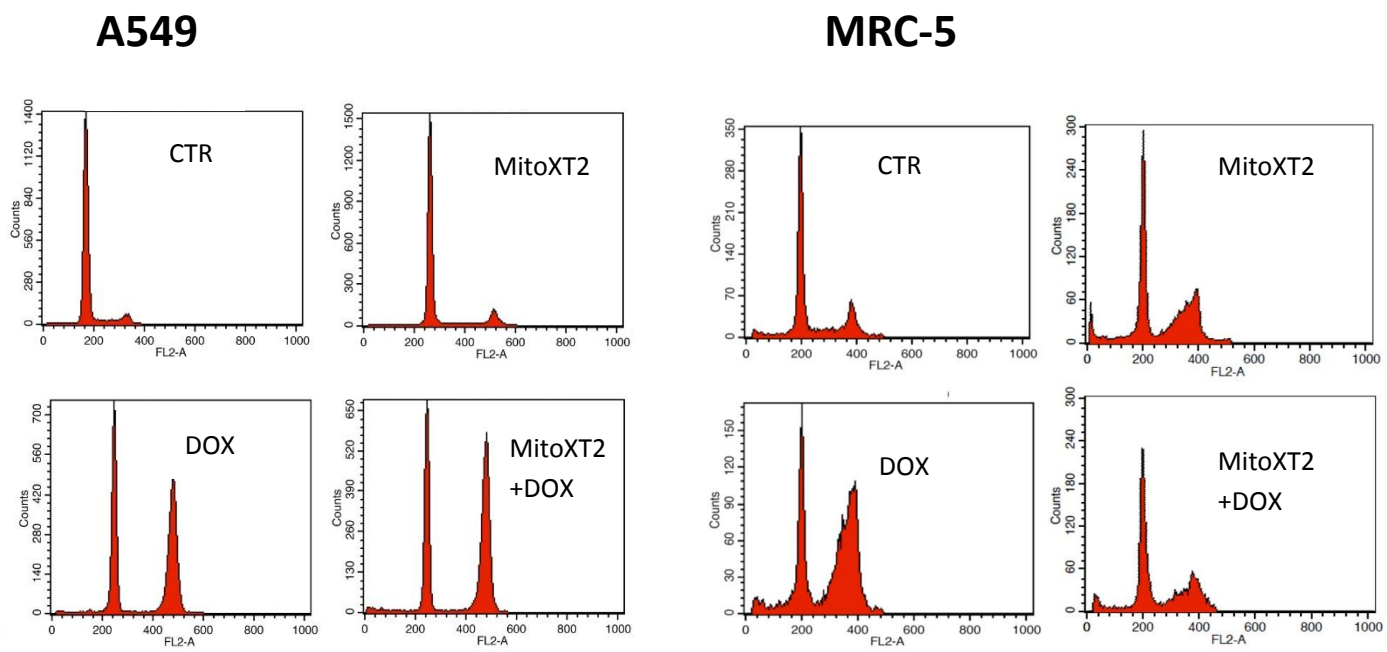

Figure 24: Histograms of cell cycle distribution of A549 and MRC-5 cells after the combined MitoXT2 and DOX treatments. The cells were pre-treated 24h with MitoXT2 followed by 24h of DOX treatment, and then were analysed with propidium iodide by flow cytometry. **Left panel:** A549 cell cycle histograms. **Right panel:** MRC-5 cell cycle histograms. Average of one or two independent experiments.

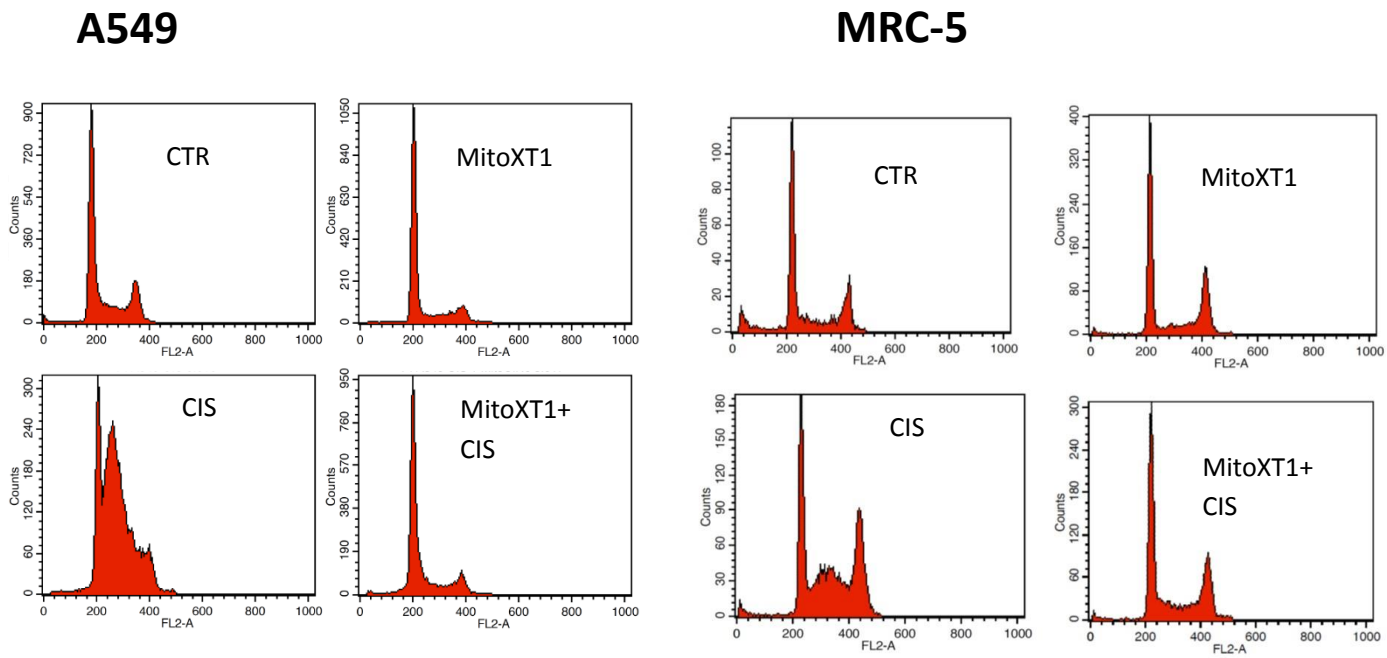

Figure 25: Histograms of cell cycle distribution of A549 and MRC-5 cells after the combined MitoXT1 and CIS treatments. The cells were pre-treated 24h with MitoXT1 followed by 24h of CIS treatment, and then were analysed with propiduim iodide by flow cytometry. **Left panel:** A549 cell cycle histograms. **Right panel:** MRC-5 cell cycle histograms. Average of one or two independent experiments.

4.10 Pre-treatment with Antioxidants and Drugs Increase Superoxide Production in Cancer Cells

To verify whether the increase of superoxide production can be involved in the observed effects when cells underwent several combined pre-treatments, the MitoSOX dye was used as described under Material and Methods. Apparently, both vehicle-treated A549 and MRC-5 cells appear to have a similar basal rate of superoxide production. Data on A549 cells shows that both DOX and MitoXT2 increase MitoSOX fluorescence when added alone to the cells, while the combination further increased the fluorescence (Figure 26 A). No differences were found for MRC-5 cells (Figure 26 A). Regarding CIS, MitoXT1 and their combination treatments, the latter showed a significant effect vs the vehicle control in A549 cells, whereas CIS or MitoXT1 did not. Again no effects were observed in MRC-5 cells when comparing the effects of the three treatments (Figure 27 A). In general, superoxide anion production in mitochondria from MRC-5 cells was lower than their tumour counterparts.

In order to have a maximal stimulation of superoxide production by the mitochondrial respiratory chain, we simultaneously added antimycin (ANT) a specific complex III inhibitor and rotenone (ROT) a complex I inhibitor. The results obtained show that under these specific inhibitors, both cell types produce more superoxide anion. DOX combined treatment with MitoXT2 in the presence of ROT and ANT showed a significant increase in comparison with DOX alone with the same inhibitors on both cell lines. Interestingly, DOX treatment with ROT and ANT does not increased significantly mitochondrial superoxide production in A549 and MRC-5 cells (Figure 26 B). CIS treatment with ANT and ROT increased by a larger amount superoxide anion content in A549, when compared with MRC-5 cells. The combination treatment between MitoXT1 and CIS, in the presence of ANT and ROT also resulted in a significant increase in mitochondrial superoxide anion (Figure 27 B). It also appeared that the effects of the two respiratory chain inhibitors in the presence of MitoXT2 were higher than with MitoXT1.

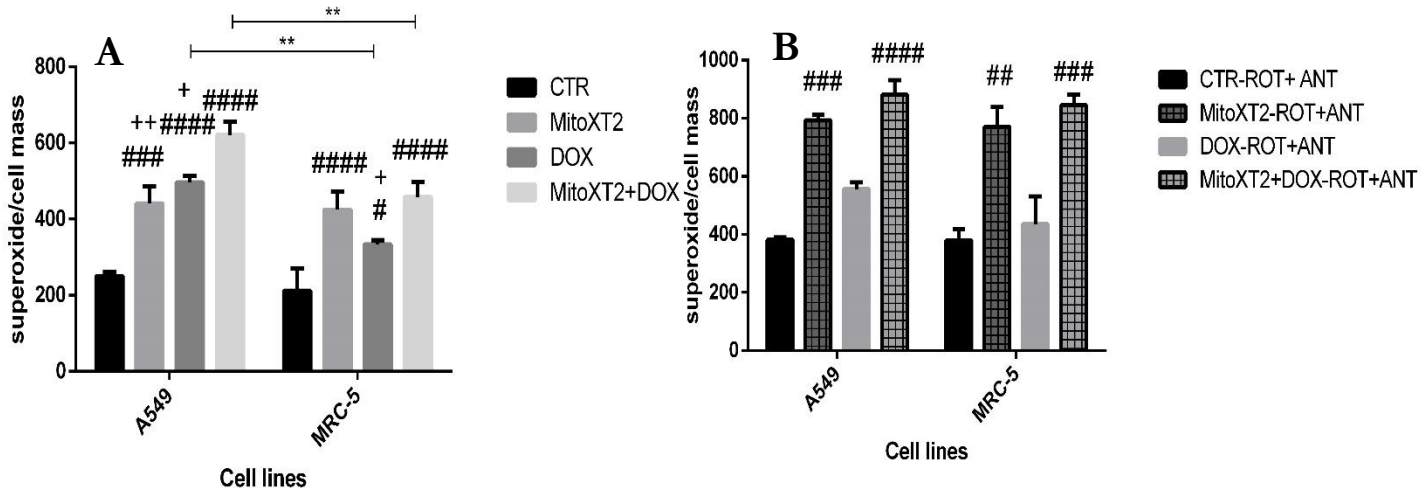

Figure 26: Superoxide production through MitoXT2 24h pre-treatment followed by a 3h DOX incubation on A549 and MRC-5 cells. Cells were pre-treated with 97.5nM MitoXT2 for 24 and then treated for 3h with 0.3µM DOX. Mitochondrial superoxide levels were quantified measuring MitoSOX dye fluorescence. Vehicle for DOX was considered as the control (CTR). **A)** Results read after 90min of incubation with MitoSOX. **B)** Positive control of superoxide production by the mitochondria respiratory chain, using two specific inhibitors of complex I and complex III, ROT and ANT. The results were normalized by cell mass quantification using SRB assay. Data represents Mean ±SEM of 4 independent experiments. Statistical analysis was performed using a two way ANOVA followed by a Tukey Test. One symbol ($p<0.05$), two symbols ($p<0.001$), three symbols ($p<0.0001$) and four symbols ($p<0.0001$). *comparison between the two cell lines, # comparison made inside the same cell line with CTR. + comparison made inside the same cell line with the combined treatment.

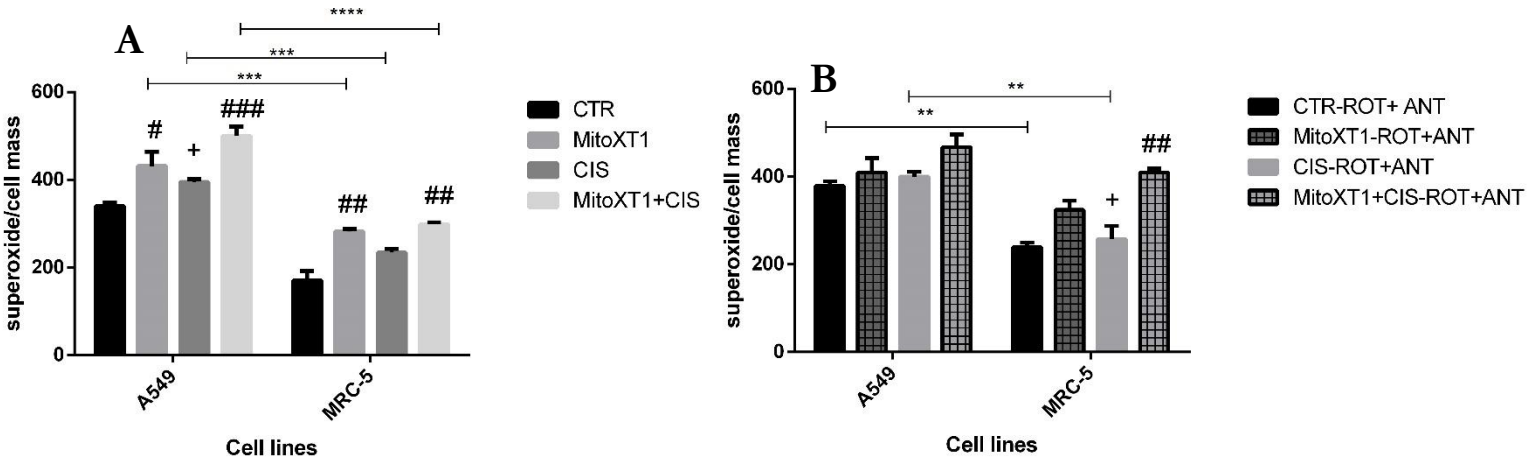

Figure 27: Superoxide production through MitoXT1 24h conditioning followed by a 3h CIS treatment on A549 and MRC-5 cells. Cells were pre-treated with 3.1µM MitoXT1 and then treated for 3h with 10µM CIS. Mitochondrial MitoSOX assay was used to measure by fluorescence the superoxide production by the cells under several treatments. Vehicle for CIS was considered as the control (CTR). **A)** Results read after 90min of incubation with MitoSOX. **B)** Positive control of superoxide production by the mitochondria respiratory chain, using two specific inhibitors of complex I and complex III ROT and ANT. The results were normalized by cell mass quantification using SRB assay. Data represents Mean ±SEM of 4 independent experiments. Statistical analysis was performed using a two way ANOVA followed by a Tukey Test. One symbol ($p<0.05$), two symbols ($p<0.001$) and three symbols ($p<0.0001$) * comparison between the two cell lines, # comparison made inside the same cell line with CTR. + comparison made inside the same cell line with the combined treatment.

Chapter 5
Discussion

Discussion

Cancer has been studied under different angles in order to discover the best therapeutic drug to treat this disease. With this objective in mind several targets have been identified, investigating possible drug-target interactions that could be useful for cancer treatment. Unfortunately this disease is multifactorial and there is no miracle drug to control the burden and mortality caused by cancer [7]. However, it has been pointed out that mitochondria are a relevant universal target for treating several maladies such as diabetes, neurodegenerative diseases and cancer [16]. As a matter of fact, mitochondria are associated with production of ROS, the regulation of programed cell death by apoptosis and with the regulation of the energy metabolism through OXPHOS and other metabolic pathways. All of these mechanisms convert mitochondria into a potential target for disease treatment [208]. Mitochondrial targeted antioxidants can be important to treat dysfunctions involving a disturbance in oxidative status, since these molecules may prevent oxidative injuries, inhibit mitochondrial membrane permeability and regulate important transcription factors involved in the apoptotic pathway such as p53 [209]. On the other hand, as most of the drugs currently in use are also cytotoxic for normal cells, antioxidants targeting mitochondria have also been studied as potential adjuvants to protect these healthy cells against oxidative injuries leading to cell death protection [81]. As it was noticed earlier in this thesis, cancer therapy currently involves a multi-factorial, multi-target approach, which is based on the heterogeneity of the disease itself. Furthermore it is also important to point out that the lack of specificity of current clinically used drug leads to a necessity to increase the concentration of drug to achieve an effective treatment, although increasing the concentration can also increase the cytotoxicity, leading to adverse effects. This is the backbone of the present research, where we wanted to use a combination of molecules to reach a more effective anti-cancer effect with minimal toxicity to non-tumour cells. Although all the effort combined to discover the "magic bullet" capable of targeting a specific sites is still not achieved, researchers have focused their energy and time to develop some new therapeutic compounds to be used in clinical trials [81].

The main goal of the present study was to understand the role of a pre-treatment with the experimental molecules MitoXT1 and MitoXT2 followed by classic anticancer agents DOX CIS and etoposide on human lung cancer cells (A549 cell line) and human lung MRC-5 fibroblasts, based on our hypothesis that these antioxidant may protects MRC-5 cells against drug-induced toxicity and on the other hand to be a potential adjunct on cancer therapy. To perform the study, we used non-toxic concentrations of compounds *per-se* so that we could test the effect of the combined incubation with cells.

Our results indicate that the test compounds, most noticeably MitoXT2, do increase the anti-cancer effects of the agents used on A549 cells, while appearing to prevent toxicity on MRC-5 cells, at least in some of the end-points measured. The fact that the results were not larger

in extension suggests that both molecules and treatment protocols still need to be optimized to increase the magnitude of effects.

The results obtained with MitoXT1 and MitoXT2 pre-treatment followed by DOX treatment were also tested on a cardiomyoblast cell line (H9c2 cells), which have been used in our laboratory to test mechanisms of DOX-induced toxicity [210, 211]. The results obtained were promising with some protection being observed for the highest DOX concentration, although a marginal significance was observed. In the contrary, Sardão et *al.* [113] noticed that a treatment with TROLOX did not induce protection against 0.5μM and 1μM DOX toxicity on H9c2 cells. In this model, only N-acetylcysteine (NAC) and p53 inhibition prevented DOX damage[113]. The fact that the antioxidants used in this work are targeted to mitochondria would suggest that these might counteract DOX-induced ROS cytotoxicity. However, the tested concentration of the test compound seems to protect H9c2 cells against higher concentration of DOX, suggesting that this particular DOX concentration is more related with an increase in ROS production which in turn makes antioxidant more efficient; however, other mechanism may be related with DOX-induced cardiotoxicity. Other assays need to be performed in order to take conclusions about this hypothesis such as the measurement of ROS content by MitoSOX and H_2DCFDA in these cardiomyoblstast to investigate the mechanisms involved in this partial protection.

Reactive oxygen species are implicated in cell cycle progression [212]. As observed in our data, the treatment with the anti-cancer drugs lead to an increase in mitochondrial superoxide in cancer cells, which can be part of the mechanism by which CIS and DOX impact the cell cycle of the cells in use. Still, both compounds also inhibit DNA replication, which is more likely to be a chief mechanism. In our data we show a significant increase in superoxide production in A549 cancer cells in comparison with MRC-5 fibroblasts cells suggesting that these cells are have more mitochondrial oxidative stress, suggesting a possible involvement in metabolic remodelling. That is in accordance with previous observations of a metabolism switch in cancer cells [50]. Still, a methodological issue ensues. ROS measurements have been a matter of discussion since it is not easy to separate mitochondrial ROS production from other ROS sources throughout the cell [213]. In our study, MitoSOX dye was used to measure mitochondrial superoxide anion. To measure total cell oxidative stress, one possibility not tested in this work is through the use of probes such as H_2DCFDA which is able to stain all types of ROS in the cytoplasm.

DOX-induced damage results in mitochondrial dysfunction at an early state, inhibiting the activity of complex I, III and IV leading to unpaired mitochondrial respiration state and increased mitochondrial oxidative stress [214] [215] .This is in accordance with the fact that under ROT and ANT condition, DOX *per se* did not significantly increase the superoxide anion production though both the cells in study. MitoXT2 increased by a large amount mitochondrial superoxide anion in the presence of the same mitochondrial inhibitors

suggesting that this antioxidant may have a different sites of action, as opposed to MitoXT1, which did not increase the effects of the mitochondrial inhibitors further.

What is interesting and surprising is the fact that our test compounds, which were supposed to act as antioxidants, did increase mitochondrial superoxide anion per se. This is a surprise since previous data from our group which served as preliminary data for this project, indicated that MitoXT1 and MitoXT2 act as antioxidants in cell-free systems and also inhibit mitochondrial lipid peroxidation (data not shown, results from José Teixeira). The protective effect of these antioxidant may also be associated with a hermesis-like effect, contributing to stimulate in intact cells mitochondrial antioxidant proteins such as MnSOD, glutathione peroxidase and glutathione-S-transferase, which may help to counteract the toxic effects of further ROS-involved stress in those cells [39]

The beneficial effects of antioxidants has been a matter of discussion over the years, pointing out to some advantages with limited side-effects, although data in humans is very controversial [39]. As previously published and in agreement with our data, ROS can also activate a mechanism of cell protection against oxidative injuries by increasing the pool of endogenous antioxidant such as SOD and catalase, increasing the cell surviving ratio. It is well known that ROS can activate different members of the MAPK family, such as ERKs, JNKs, and p38 in order to induce cell survival [216]. More work is required in the way to understand the favourable properties of antioxidant in cancer therapy and the signalling pathways there involved.

Caffeic acid and derivatives were previously investigated in the context of anti-cancer effects with different caffeic acid derivatives. The study showed that on breast cancer cells (MCF-7, MDA-MB-231 and HS 578T cell lines), caffeic acid derivatives induce toxicity in a time-dependent manner, increasing the sub-G1 apoptotic peak at 96h of incubation while, on the opposite, fibroblasts cells (BJ) which had the same treatments, showed a blockage in the S phase, suggesting that the way of action of these compounds may be dependent on the metabolism of the cells as well as in the proliferation state [191]. In our study the results obtained with SRB, resazurin and Live/Dead did not show any toxic effect of MitoXT1 (caffeic acid derivative) compound. The data on the cell cycle were very similar in both A549 and MRC-5 cell lines treated with that compound. This may be due to the reduced concentration of MitoXT1 used (only 3.1μM against 75μM in the previous study) as well as the smaller time of incubation (only 48h against 96h), especially if we take into consideration that, in the previous study, the differences on cell cycle only started appearing after the 96h period. Although the compounds used are different, we believe our results to be more relevant due to the use of smaller concentrations and incubation times. Concerning the combined treatment with CIS, a protection the cytotoxicity resulting from this drug was shown in both cells, although CIS *per se* induced a higher block in the S-phase in cancer than in fibroblasts cells. This was confirmed in others studies where it was observed that CIS-

induced cell cycle alteration was time and concentration-dependent [217]. It was also observed that 10μM CIS induced a significant increase in caspase 3 and 9 protein and activity in A549 cells, suggesting that one of the mechanisms of CIS-induced cell death is through programed cell death involving SMAC/Diablo protein [218] The same was shown in our results, however it was be interesting to observe the caspase-like activity under two caspase-specifics inhibitors to understand better the role of caspase signalling in cell death induced by the anti-cancer agents alone and in the presence of our test compounds.

Vitamin K3-induced cell death has also been investigated in the context of cell death induction. It has been described that a this vitamin shows a higher incidence of cell death than larger concentrations, which result in necrosis [193]. The IC_{50} of vitamin K3 on MRC-5 cells is 18μM which means about 180 times higher than what we used [219]. This explains the results obtained with SRB and Live/Dead assay showing no cell death induction with the concentration we use (97.5nM) in these cell lines. However, vitamin K as also been studied regarding its effect on cell cycle inhibition, and these can be explained by the inhibition of protein kinases in association with cyclin-dependents mechanisms [193]. Despite a previous study showed an inhibition of cancer cell cycle on G2 phases treated with vitamin K3, the data obtained in this study did not show any significant difference in the treatment with MitoXT2 vitamin K derivative in cancer cell line, most likely because of the small concentration used. Moreover, a recent study about vitamin K and derivatives concluded that vitamin K3 derivatives are more able to protect cell death induced by oxidative stress at nanomolar concentration than other naphthoquinones with a isoprenoid side chain [220]. In the work, a protective effect of MitoXT2 against DOX-induced toxicity in MRC-5 cells as verified by the SRB assay and by the cell cycle experiments was observed. Curiously, we see a net increase in superoxide anion production when cells were treated by the same combination plus ANT and ROT. Although this may seem a large extrapolation, it may be that the test compounds may actually "use" an increased cell ROS production to cause protection. [39]. The principal mechanisms of DOX chemotherapeutics effects are associated with the induction of apoptosis and cell cycle arrest [115] .Regarding Live/Dead assay, it is curious to notice that the combined treatment of MitoXT2 and DOX induced cell death in both cell lines. However, the effect seems to be slightly higher on the cancer cell line. Probably this is due to the reduced time of drug incubation used, and as it was observed in cell cycle assays, to the fact that DOX treatment induces a blockage in G2 phase, as previously described by Sung et *al.[221]* In fact, cell cycle arrest may lead to cell death; however to verify this hypothesis, the treatment needs to be performed for longer periods of times. Additionally, the treatment with MitoXT2 and DOX led to a decrease in caspase-like activity in MRC-5 cell line although in A549 cancer cell an increase was observed, which is a very significant and highly relevant result, showing opposed results in two cell lines with different degrees of malignancy [222]. This contributes to our suggestion that MitoXT2 presents a good selectivity towards lung cancer cells vs. their non-tumour counterparts.

Using TMRE and Hoechst 3342 as mitochondrial polarization and nuclear fluorescent dyes, polarized mitochondrial morphology and nuclear state was assessed. In our work, we identified alterations that suggest a decrease in mitochondrial transmembrane potential after CIS and DOX treatments. This is in accordance with others studies demonstrating the same with higher concentrations of DOX [222]. Cells treated with CIS showed an increase in condensed chromatin, confirming previous results [223], and indicating activation of the apoptotic program.

Although several other assays should be performed, the results of our study demonstrated that MitoXT2 has a potential protective effect against DOX induced toxicity on MRC-5 cells, also seen by the reduced cell death and by the non-alteration of cell cycle. In addition it seems that MitoXT2 could also be a potent adjunct to DOX therapy in lung cancer cells. Taken together, these results may open the door to the development of that molecule as adjunct therapy for lung cancer. Although MitoXT1 also showed toxicity on lung cancer cells when combined with anti-cancer agents, the results were not as impressive, namely with non-tumour cells. Hence more studies are necessary to establish a conclusion regarding the mechanisms involved behind the adjunct effect of MitoXT1 and MitoXT2 in drug therapy and about the relationship between drug concentrations and protection managed by these "antioxidants".

Chapter 6
Conclusion and Future Directions

Conclusion

In the present study, the effects of MitoXT1 and MitoXT2 on CIS and DOX, respectively which induce cell death and cell cycle arrest, were investigated. It was observed that MitoXT1 may induced cell death in combination with CIS in A549 cells, however it protect both cell types from cell cycle arrest induced by CIS in S phase. Nonetheless more data is required to evaluate the concentration of antioxidant required to a more efficient effect in CIS reduction toxicity in healthy cells. On the other hand MitoXT2 induced an efficient protection against cell death in fibroblast cells comparing with what occurs in cancer cells that appears to decrease the cell viability also shown in the inhibition of the cell cycle at the G2 phase.

In conclusion, MitoXT2 appears to have a protective effect on DOX-induced toxicity in MRC-5 cells, without interfering with this effect on cancer cells A549, which is an important prof of the adjunct properties conferred by this antioxidant. MitoXT1 in contrast seems to also protect cancer cells from the effect of CIS hindering the use of this antioxidant in cancer clinical therapy. Although the use of mitochondrial-targeted antioxidant seems to have a confirmed effect on adjunct cancer therapy more studies are need to found the perfect concentration and time of administration of these compounds in order to increase the quality of life of patient and also to reduce the toxicity of the clinical drug *per se*.

Future Directions

To understand completely how mitochondrial-directed antioxidants are capable of modulating cell death processes and cell cycle arrest, more work has to be done in order to elucidate these mechanisms. On one hand, we observed that MitoXT1 and MitoXT2 appeared to inhibit caspase-dependent cell death on MRC-5 cells. On another hand, we saw an increase in caspase-dependent cell death in A549 cells treated with both the antioxidant and the drug. It has been known that p53 has either the ability to induce apoptosis or cell arrest depending of its cellular location and activation status. To understand which effect p53 upregulation induces in antioxidant/drug treatments, it could be interesting to analyse p53 expression and its degree of acetylation. Adding to this, it could be interesting to measure through western blot the levels of expression of the Bcl-2 protein family in order to understand if the compounds in study modulate programmed cell death.

Moreover, to fully understand the role of mitochondria in MRC-5 and A549 cells under the different treatments, the measurement of the activity of mitochondrial complexes or global respiration might be essential. The levels of ATP may also be measured to perceive the efficiency of OXPHOS and the possible switch to glycolysis due to DOX and CIS treatment in combination with antioxidant.

As to be able to confirm the induction of mitochondrial superoxide in MitoXT2/DOX and MitoXT1/CIS treatment, it could be important to measure the ROS production using the H_2DCFDA probe. Likewise, MnSOD activity may also be quantified in order to better understand if the protective effect of the antioxidants in use in this work are due to the increase of its activity, or if it is only due to the antioxidants themselves.

Furthermore, *in vivo* studies could be essential to mimetic better the combination of mitochondrial-targeted antioxidant and anti-cancer agents and maybe lead to a clinical trial.

These results may be relevant to fully understand the way of action of mitochondrial-directed antioxidant and perhaps to the use of this compounds in adjunct therapy.

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