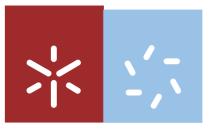
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Minho 201



**Universidade do Minho** Escola de Ciências

Luís Carlos Sá Loureiro

Functional characterization of mitochondrial suspensions by cytofluorimetric techniques towards the screening of mitochondrial target drugs



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Dissertação de Mestrado Mestrado em Bioquímica Aplicada

Trabalho efetuado sob a orientação da **Professora Doutora Maria Manuela Sansonetty Gonçalves Côrte-Real** e do **Professor Doutor Hernâni Varanda Gerós** 

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### Abstract

Besides its role in respiration, mitochondria control other key metabolic pathways, cell proliferation and regulated cell death, with a crucial role in the regulation of the intrinsic apoptotic pathway. Recent studies have focused on the screen for anti-cancer agents that induce apoptosis through permeabilization of the mitochondrial outer membrane, leading to the cytosolic release of pro-apoptotic proteins and to the activation of the caspase cascade and consequently to cell dismantling.

In the present study, staining protocols were developed and optimized aiming at the structural and functional characterization of isolated mitochondrial populations from yeast cells and rat liver by flow cytometry. Mitotracker Green and Nonyl Acridine Orange were used to measure changes in mitochondrial 3,3'mass, Dihexyloxacarbocyanine lodide and Mitotracker Red CMXROS to monitor the electrical potential of the inner membrane (Δψm), Mitotracker Red CM-H<sub>2</sub>XROS and Dihydroethidium to evaluate the accumulation of ROS, and 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein and Acetoxymethyl Ester (BCECF-AM) to measure the pH of the mitochondrial matrix. Subsequent flow cytometry studies were designed to evaluate the effect in mitochondria function of two well-known apoptosis inducers in both mammalian cell lines and yeast cells, namely bovine lactoferrin (bLf) and acetate, and to understand whether or not they act directly on mitochondria independently of upstream cellular pathways. The results showed that both bLf and acetate promoted changes in mitochondrial mass, matrix mitochondrial pH, Δψm and ROS levels of rat liver mitochondria. Moreover, the assessment of the activity of the complexes of electron transport chain (ETC) showed that bLf and acetate affect their activity and trigger mitochondrial swelling. These results are particularly interesting since the two compounds appear to induce in isolated mitochondria mainly the same alterations as those occurring in whole cells, and which have been proposed to be involved in the induction of cell death. Altogether, this study show that flow cytometry is a valuable tool to study key mitochondrial functional parameters and to screen for mitochondriatargeted drugs, including the anticancer agents, such as acetate and bLf.

#### Resumo

Para além do seu papel na respiração celular, as mitocôndrias controlam outros aspetos chaves do metabolismo, bem como a proliferação celular e a morte celular regulada, tendo um papel crucial na regulação da via apoptótica intrínseca. Estudos recentes têm-se focado na procura de agentes anticancerígenos capazes de induzir apoptose mediada pela permeabilização da membrana mitocondrial externa, conduzindo à libertação de proteínas pró-apoptóticas e à ativação da cascata de caspases e consequente desmantelamento celular. No presente estudo, foram desenvolvidos e otimizados protocolos de marcação com vista à caracterização estrutural e funcional por citometria de fluxo de populações de mitocôndrias isoladas de células de levedura e de hepatócitos de rato. As sondas Mitotracker Green e laranja de nonil-acridina foram usadas para medir variações da massa mitocondrial, o iodeto de 3,3'-Dihexiloxacarbocianina e o Mitotracker Red CMXROS para medir o potencial eléctrico da membrana mitocondrial interna (Δψm), o Mitotracker Red CM-H<sub>2</sub>XROS e dihidroetídeo para avaliar a acumulação de ROS e o acetoximetil éster de (2',7'-Bis-(2-Carboxietil)-5-(and-6)-carboxifluoresceína e(BCECF-AM) para medir o pH da matriz mitocondrial. Os estudos subsequentes de citometria de fluxo foram desenhados para avaliar o efeito de diferentes drogas na função mitocondrial, em particular da lactoferrina bovina (bLf) e do acetato, dois reconhecidos indutores de apoptose, no sentido de se compreender se atuam diretamente na mitocôndria independentemente de outras vias celulares a montante. Os resultados mostraram que tanto a bLf como o acetato promovem alterações na massa mitocondrial, pH da matriz mitocondrial, Δψm e nos níveis de ROS em mitocôndrias isoladas de fígado de rato. Além disso, a avaliação da atividade dos complexos da cadeia de transportadora de eletrões (ETC) mostrou que a bLf e o acetato afetam a sua atividade e desencadeiam a tumefação mitocondrial. Estes resultados são particularmente interessantes uma vez que os dois compostos parecem induzir, em mitocôndrias isoladas as mesmas alterações que ocorrem em células inteiras, e que têm sido propostas como estando envolvidas na indução da morte celular. No seu conjunto, este estudo mostra que a citometria de fluxo é uma ferramenta valiosa para o estudo da funcionalidade mitocondrial e para o rastreio de drogas que têm como alvo a mitocôndria, incluindo agentes anticancerígenos, tais como a bLf e o acetato.

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## **Abbreviations**

- ACD Accidental cell death
- ADP Adenosine diphosphate
- ANT Adenine nucleotide translocase
- ATP Adenosine triphosphate

BCECF-AM - 2', 7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester

- bLf Bovine lactoferrin
- COX Cytochrome c oxidase
- cyt c Cytochrome c
- DHE Carbonyl cyanide m-chlorophenyl hydrazine
- DiOC<sub>6</sub>(3) Dihexyloxacarbocyanine lodide
- DNA Deoxyribonucleic acid
- DTT Dithiothreitol
- EGTA Ethylene glycol tetraacetic acid
- ER Endoplasmic reticulum
- ETC Electron transport chain
- IMM Inner mitochondrial membrane
- IMS Intermembrane space
- Lf Lactoferrin
- MOMP Mitochondrial outer membrane permeabilization
- MPT Mitochondrial permeability transition
- mPTP Mitochondrial permeability transition pore
- mtDNA Mitochondrial DNA
- MTG MitoTracker Green
- NADH Nicotinamide adenine dinucleotide
- NAO Acridine Orange 10-Nonyl Bromide
- OMM Outer mitochondrial membrane
- PCD Programmed cell death

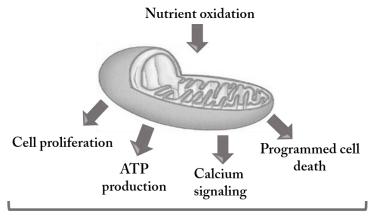
- pH<sub>IMS</sub> Intermembrane space pH
- pH<sub>mito</sub> Mitochondrial pH
- Pi Inorganic phosphate
- PTP Permeability transition pore
- RCD Regulated cell death
- ROS Reactive oxygen species
- SCFA Short chain fatty acid
- TCA Tricarboxylic acid
- VDAC Voltage-dependent anion channel
- YNB Yeast Nitrogen Base
- Δp Electrochemical gradient
- ΔpHm Mitochondrial pH gradient
- ΔΨm Mitochondrial membrane potential

## Introduction

### 1. Mitochondria in life and death

Mitochondria are organelles currently viewed as a cell dynamic compartment resulting from the equilibrium between fusion and fission processes. They play crucial roles in eukaryotic aerobic cells as the primary producers of ATP, regulators of ion homeostasis or redox state, and producers of free radicals [1]. Mitochondria are also key organelles for regulation of cell metabolism [2]. They produce the adenosine triphosphate (ATP) mainly from glucose and fatty acid oxidation, and to a lesser extend from amino acid oxidation. Nutrient oxidation within the mitochondrial matrix or the cytosol results in acetyl-CoA production, the main substrate of the Tricarboxylic Acid (TCA) cycle, which takes place in the mitochondrial matrix. These oxidative pathways are coupled to the mitochondrial electron transport chain (ETC), whose enzyme content and activity together define the mitochondrial oxidative capacity [3].

Mitochondria are involved in the maintenance of cell viability and vitality, but also play a central role in the modulation of regulated cell death (RCD) [4, 5]. There is continuous cross talk exists between cell and mitochondria, which allows the cell adaptation to physiological changes or to commit suicide [2]. Thus, mitochondria may be regarded as the guardians of the gate between life and death [4, 6].

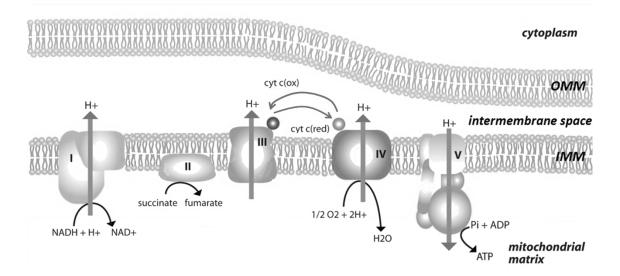


Regulation of cell metabolism

**Figure 1:** Mitochondria are responsible for ATP production through metabolism and respiration. They also play key roles in several signalling processes such as Ca<sup>2+</sup> trafficking, maintenance of cellular redox balance and apoptosis (Adapted from [3]).

#### 2. Role, features, and organization of the mitochondria

Mitochondria are double-membrane organelles. While the outer mitochondrial membrane (OMM) is rather permeable, allowing the free diffusion of small proteins and contains specialized channels for import of larger proteins, the inner mitochondrial membrane (IMM) is highly impermeable. Additionally, the IMM has a higher superficial area than the OMM and thus possesses characteristic folding morphology forming typical cristae [7]. Mitochondria contain their own mitochondrial DNA (mtDNA), which encodes a subset of proteins essential for the synthesis of adenosine triphosphate (ATP) through oxidative phosphorylation coupled with the transport of electrons by the electron transport chain (ETC) [8, 9]. The ETC consists of four large enzyme complexes (Complex I, NADH-CoQ oxidoreductase; Complex II, succinate dehydrogenase; Complex III, CoQ cytochrome c oxidoreductase; and Complex IV, cytochrome c oxidase, COX). Mobile electron carriers (e.g. coenzyme Q or ubiquinone, and cytochrome c) transport the electrons from one complex to the next with oxygen acting as the final electron acceptor allowing COX reoxidation [10]. The transfer of the high-energy electrons through the ETC results in the pumping of H<sup>+</sup> ions across the inner membrane, creating an electrochemical proton gradient that provides the energy required to drive the synthesis of ATP, thanks to a fifth complex called F<sub>0</sub>F<sub>1</sub> ATP synthase [3]. This complex consists of an  $H^+$  ion channel (F<sub>0</sub>) connected to a catalytic subunit ( $F_1$ ). The mechanism of oxidative phosphorylation consists in the utilization of the energy delivered by the flux of  $H^+$  ions through  $F_0$  to drive ATP synthesis from ADP and Pi by F<sub>1</sub> [10]. ATP production in mitochondria has to strictly match cellular needs and therefore is strongly controlled by different factors, namely the ATP/ADP-Pi ratio, the NAD<sup>+</sup>/NADH-H<sup>+</sup> ratio and local oxygen pressure. The transfer of ATP, ADP and Pi between the cytosol and the mitochondrial matrix is mediated by the Voltage Dependent Anion Channel (VDAC) and by the ATP/ADP carrier (adenine nucleotide translocase, ANT) [10].



**Figure 2:** Schematic overview about the mechanism of the mitochondrial respiratory chain. The mitochondrial electron transport chain is composed of five complexes. Electron transport between complexes I to IV is coupled to extrusion of protons from complexes I, III and IV into the intermembrane space, creating an electrochemical gradient ( $\Delta p$ ) across the inner mitochondrial membrane. Protons then flow through complex V (ATP synthase), which utilizes the energy to synthesize ATP from ADP (Adapted from [11]).

#### The mitochondrial transmembrane electrochemical potential ( $\Delta p$ )

As referred above the transfer of electrons through protein complexes I - IV in the inner mitochondria membrane, associated with the mitochondrial respiration establishes the mitochondrial transmembrane electrochemical potential or proton motive force ( $\Delta p$ ) [12, 13]. This results in a net accumulation of H<sup>+</sup> outside the membrane, constituted by a charge or electrical gradient ( $\Delta \psi m$ ) and by a chemical or concentration gradient ( $\Delta pHm$ ) [12]. Using a simplified Nernst factor for the second term,  $\Delta p$  can be represented at 37°C by the equation:  $\Delta p$  (mV) =  $\Delta \psi m$  – 60 $\Delta pHm$  [13, 14].

Together, the  $\Delta\psi$ m and  $\Delta$ pHm contribute to mitochondrial control over energy metabolism, intracellular ion homeostasis, and cell death in eukaryotic cells [12]. While  $\Delta$ p provides the bioenergetic driving force for and regulates ATP production, the  $\Delta\psi$ m provides the charge gradient required for mitochondrial Ca<sup>2+</sup> sequestration, and

regulates reactive oxygen species (ROS) production, and thus is also a central regulator of cell health [15].

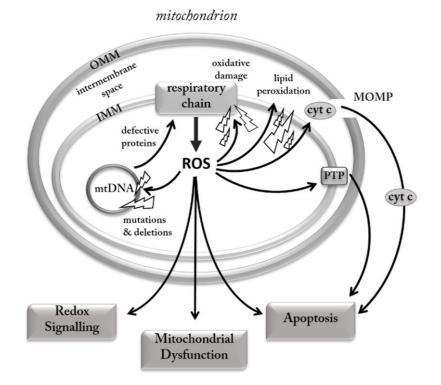
#### Accumulation of reactive oxygen species

The accumulation of ROS by mitochondria is important because it underlies oxidative damage in many pathologies and contributes to retrograde redox signalling from the organelle to the cytosol and nucleus [16]. During the electron transfer through the respiratory chain, a small percentage of electrons may prematurely reduce oxygen, forming ROS [17]. It was demonstrated that the main sites of ROS production are located at complexes I and III, the iron-sulphur centres of complex I being potentially the most important ROS generators [3].

The reduction of oxygen by one electron at a time produces relatively stable intermediates. Superoxide anion  $(O_2^{\bullet-})$  is the precursor of most ROS and a mediator in oxidative chain reactions. Dismutation of  $O_2^{\bullet-}$ , either spontaneously or through a reaction catalysed by superoxide dismutases, produces hydrogen peroxide  $(H_2O_2)$ , which in turn may be fully reduced to water or partially reduced to hydroxyl radical  $(OH^{\bullet})$ , one of the strongest oxidants in nature [17].

The rate of mitochondrial ROS accumulation is mainly determined by the membrane potential, the ratio between NADH and NAD<sup>+</sup>, the ratio between CoQH<sub>2</sub> and CoQ and the local O<sub>2</sub> concentration. It is well established that there is a strong positive correlation between  $\Delta\psi$ m and ROS production [16]. A slight mitochondrial hyperpolarization has been associated to a large stimulation of ROS accumulation [16]. Similarly, a slight mitochondrial depolarization deeply reduces ROS accumulation [18]. Thus, ROS accumulation by mitochondria can lead to oxidative damage of mitochondrial proteins, lipids and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions, including the function of TCA cycle, fatty acid oxidation, urea cycle, amino acid metabolism and FeS centre assembly that are central to the normal operation of most cells [16]. Consequently, it is predictable that mitochondrial oxidative damage contributes to a wide range of pathologies [16-18].

Mitochondrial oxidative damage can also increase the tendency of mitochondria to release IMS proteins such as cytochrome *c* (cyt *c*) to the cytosol by mitochondrial outer membrane permeabilization (MOMP) and thereby activate the cell's intrinsic apoptotic machinery [16], as discussed below.

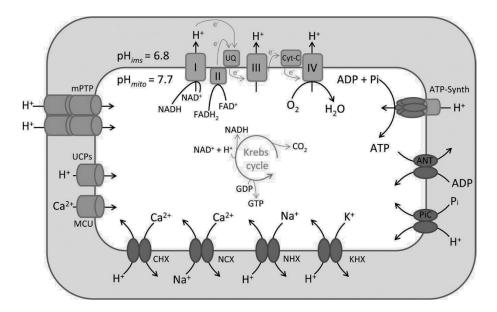


**Figure 3:** Overview of mitochondrial ROS production. ROS production by mitochondria can lead to oxidative damage to mitochondrial proteins, lipids and DNA, impairing the ability of mitochondria to synthesize ATP and to carry out their wide range of metabolic functions (Adapted from [16]).

#### Mitochondrial matrix pH

The regulation of protons and other ions is crucial for mitochondrial functionality [4]. The generation of an electrochemical proton gradient across the inner mitochondrial membrane (IMM) is an essential energy conservation event that couples the oxidation of carbohydrates and fat to the synthesis of ATP. Studies in isolated mitochondria have established that the proton chemical gradient ( $\Delta$ pHm) and the proton electrical gradient ( $\Delta$ ψm) contribute independently to the  $\Delta$ p that drives the synthesis of ATP [19].

ΔpHm drives the fluxes of metabolic substrates required for mitochondrial respiration, and the activity of electroneutral ion exchangers that maintain mitochondria osmolarity and volume. ΔpHm reflects the pH difference between the mitochondrial matrix pH (pH<sub>mito</sub>) and the pH within the IMS (pH<sub>IMS</sub>). A difference of 0.9 pH units make the pH<sub>IMS</sub> more acidic than pH<sub>mito</sub> [19]. The pH on the IMS side of mitochondria cristae might be even more acidic than the bulk IMS pH because respiratory complexes are concentrated on these invaginations, which are connected to the IMS by small tubular junctions that constrain the diffusion of solutes [20]. Recent studies indicate that the pH<sub>mito</sub> plays an important and underappreciated role in physiological and pathological situations such as apoptosis, neurotransmission, and insulin secretion [19]. From a chemiosmotic point of view, Δψm and ΔpHm are independent components that equally contribute to the Δp as H<sup>+</sup> ions return to the matrix at the level of complex V [21].



**Figure 4:** Determinants of the mitochondrial matrix pH. Protons are pumped from the matrix to the IMS by the respiratory chain complexes I, III, and IV as electrons flow from reduced substrates in the matrix to  $O_2$ . The pumping of electrically charged protons generates a  $\Delta\psi m$  and a pH gradient. (Adapted from [19]).

#### Mitochondrial calcium storage

Calcium (Ca<sup>2+</sup>) is an essential ion for cell life, acting as a key second messenger in almost all cellular functions. Cytosolic Ca<sup>2+</sup> concentration is important in signalling and regulation of many essential responses in practically all cell types [22]. To make this signalling possible, cells maintain cytosolic calcium at low levels and have different transporters that regulate the concentration of this cation in the intracellular compartments, causing the oscillations required for cell signalling [23]. Increase of intracellular [Ca<sup>2+</sup>] can be elicited through two fundamental mechanisms: i) Ca<sup>2+</sup> mobilization from intracellular stores, mainly the (ER), or ii) entry from the extracellular milieu through the opening of plasma membrane Ca<sup>2+</sup> channels [24].

Mitochondria are able to store significant amount of Ca<sup>2+</sup> bound to phosphate within their matrix. Ca<sup>2+</sup> taken up by the mitochondria regulates mitochondrial endoplasmic reticulum functioning in response to a variety of extracellular stimuli [25]. It activates three dehydrogenases of the TCA cycle, namely pyruvate dehydrogenase, isocitrate dehydrogenase, and  $\alpha$ -keto glutarate dehydrogenase. It may also regulate the ETC, the F<sub>0</sub>F<sub>1</sub> ATP synthase and ANT [26]. This induces an increased substrate uptake by mitochondria, enhanced mitochondrial NADH/NAD<sup>+</sup> ratio and increased ATP production. The high capacity to accumulate Ca<sup>2+</sup> confers to mitochondria a key role in the regulation of intracellular Ca<sup>2+</sup> signalling, which includes regulation of gene expression (including those involved in mitochondrial biogenesis and glucose uptake), cell functioning and control of protein trafficking between organelles [27].

#### 3. The role of mitochondria in regulated cell death

Cell death instances can be classified into two categories: 'accidental' and 'regulated'. Accidental cell death (ACD) is caused by severe stimulus, including physical (e.g., elevated temperatures or high pressures), chemical (e.g., potent detergents or extreme variations in pH) and mechanical (e.g., shearing). ACD is practically immediate and is insensitive to pharmacologic or genetic interventions of any kind [28]. In contrast to its accidental counterpart, regulated cell death (RCD) involves a genetically encoded molecular machinery. RCD often occurs in a relatively tardy manner and is

initiated in the context of adaptive responses that attempt to restore cellular homeostasis. Thus, while ACD is completely unpreventable, RCD can be modulated not only by inhibiting the transduction of lethal signals but also by improving the capacity of cells to mount adaptive responses to stress [28]. Importantly, RCD occurs not only as a consequence of microenvironmental perturbations but also in the context of (post-) embryonic development, tissue homeostasis and immune responses. Such completely physiologic instances of RCD are generally referred to as 'programmed cell death' (PCD) [28]. Of note, apoptosis, autophagy and programmed necrosis are the three main forms of RCD, easily distinguished by their morphological differences [29]. Apoptosis, designated before as type I cell death, is characterized by specific morphological and biochemical changes of dying cells, including cell shrinkage, nuclear condensation and fragmentation, dynamic membrane blebbing and loss of adhesion to neighbours or to extracellular matrix [30]. Biochemical changes include chromosomal DNA cleavage into inter-nucleosomal fragments, phosphatidylserine externalization and a number of intracellular substrate cleavages by specific proteolysis [31].

Autophagic cell death, designated before as type II cell death, is an evolutionarily conserved catabolic process beginning with formation of autophagosomes, double membrane-bound structures surrounding cytoplasmic macromolecules and organelles, destined for recycling [32]. Besides apoptosis and autophagic cell death, there exists a type III cell death termed programmed necrosis, which involves cell swelling, organelle dysfunction and cell lysis [33]. Since RCD processes have an important role during preservation of tissue homoeostasis and elimination of damaged cells, systems biology of cell death has a wide range of clinical applications for drug treatment of various human diseases [29]. Decrypting the labyrinth of cell death pathways has important implications in choosing the right drug targets that will prevent the death of cells in pathological conditions such as, ischemia, neurodegenerative diseases and stroke [28]. Conversely, drug treatments designed for cancer and autoimmune diseases aim at achieving maximum cell death. Because tumors carry a collection of genetic perturbations that block different functional modules within the RCD network, efficient tumor cell killing depends on activating alternative pathways and bypassing blocks in the pathways mapped individually for each tumor [29].

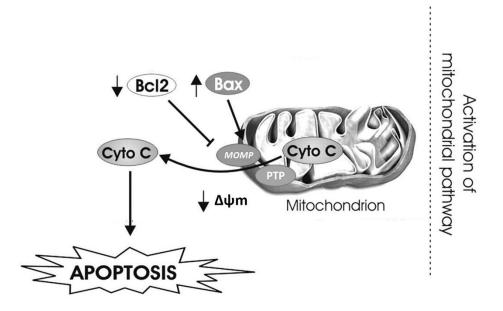
The most frequent and well-defined form of RCD is apoptosis. Apoptotic processes are of a widespread biological importance, being involved in the development, differentiation of multicellular organisms, proliferation, homeostasis, regulation and function of immune system and in the removal of defective cells [34]. Numerous extra- or intracellular factors are associated with apoptotic signaling pathways have been identified, such as attachment of death ligands to membrane death receptors, DNA damage caused by defaults in DNA repair mechanisms, treatment with cytotoxic compounds or irradiation, lack of survival signals, or development of death signals [35].

Two main signalling pathways lead to apoptosis: the extrinsic or death receptor pathway, and the intrinsic or mitochondrial pathway. The extrinsic pathway is defined as mitochondria independent, although mitochondria can be involved in the amplification of the death signal. It involves the activation of receptors in the plasma membrane through binding of ligands that trigger a proteolytic chain responsible for the characteristic morphological features of apoptosis. The mitochondrial pathway involves the permeabilization of the mitochondrial outer membrane allowing the release into the cytosol of a variety of pro-apoptotic proteins [35]. Mitochondria have a central role in this apoptotic pathway and dysfunction of this organelle induced by DNA damage and other genotoxic factors leads to apoptotic cell death [36]. Mitochondrial fragmentation during apoptosis was connected with the collapse of the mitochondrial membrane potential ( $\Delta\Psi$ m) that was considered a point of no return in the death cascade. Members of the Bcl-2 family of proteins play a key role regulate outer mitochondrial membrane (OMM) integrity and function [37].

One of the crucial events in the process linking lethal signals to controlled death of mammalian cells is the mitochondrial outer membrane permeabilization (MOMP) [38]. During early stages of apoptosis, the pro-apoptotic protein Bax translocates to the OMM and, almost instantly after translocation, concentrates into sub-mitochondrial punctate foci. In addition, Bak co-localizes with Bax in these foci.

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Bax and Bak induce cell death via MOMP that leads to the release of small proapoptotic molecules such as cytochrome *c* [39].



**Figure 5:** Schematic presentation of activation of mitochondrial pathway of apoptosis (Adapted from [41]).

Mitochondrial permeability transition (MPT) is a mitochondrial state in which the proton-motive force is disrupted. This disruption involves the mitochondrial membrane permeability transition pore (mPTP) [40]. The PTP is a transmembrane channel formed at the contact sites between the mitochondrial inner membrane (MIM) and the mitochondrial outer membrane (MOM). The components of the PTP are the voltage-dependent anion channel (VDAC) in the outer mitochondrial membrane and the adenine nucleotide translocator (ANT) in the inner mitochondrial membrane [41]. During normal mitochondrial function, the intermembrane space separates the inner and outer mitochondrial membranes and the VDAC and the ANT do not interact. When mitochondrial permeability is activated by the formation of the PTP, increase in MIM permeability to solutes up to ~1500 Da, leading to a reduction of the  $\Delta\psi$ m, swelling of the mitochondrial matrix, and OM permeabilization, presumably due to physical rupture of OM [41]. The release of mitochondrial factors is widely accepted as the initiating event of the intrinsic pathway of apoptosis, but it can also serve as an amplification mechanism of the extrinsic pathway of cell death. The proteins released can either activate a group of enzymes of the cysteine protease family known as caspases or act in a caspase-independent fashion to bring about cell death [35].

#### 4. Mitochondria as cellular target for cancer therapy

The evasion/resistance of human cancers cells to cell death is a major cause of treatment failure. The lack of efficacy of established therapeutic regimens is due, at least in part, to the oncogenic blockade of cell death pathways [42]. Thus, drugs designed to activate the cell death machinery may represent a more effective therapeutic option. This machinery is composed of catabolic hydrolases, mostly proteases and nucleases, which are held in check by specific inhibitors or by the sequestration of their activators. The permeabilization of the mitochondrial outer membrane is a potent way of unleashing such activators. Multiple apoptosis- and necrosis-inducing biochemical cascades converge on mitochondria to cause their deregulation and permeabilization [43].

Mitochondria exert both vital and lethal functions in physiological and pathological scenarios. On the one hand, mitochondria are indispensable for energy production and hence for the survival of eukaryotic cells. On the other hand, mitochondria are crucial regulators of the intrinsic pathway of apoptosis [44]. Mitochondria control the activation of apoptotic effector mechanisms by regulating the translocation of pro-apoptotic proteins from the mitochondrial inter-membrane space to the cytosol. As mitochondria are key regulators of cell death and as mitochondrial functions are often altered in neoplasia, mitochondrial-targeted compounds represent a promising approach to eradicate chemotherapy-refractory cancer cells [45].

There is ample evidence of metabolic alterations affecting the capacity of malignant cells to engage in apoptosis, necrosis and autophagy [46]. Moreover, modifications in the levels of ROS have recently been linked to the intrinsic

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chemotherapy resistance of cancer stem cells. These changes are intricately linked to the bio-energetic functions of mitochondria, making these organelles attractive drug targets [46]. Accordingly, multiple hallmarks of cancer cells, including limitless proliferative potential, insensitivity to anti-growth signals, impaired apoptosis, enhanced anabolism and decreased autophagy, have been linked to mitochondrial dysfunctions [47].

The correction of cancer-associated mitochondrial dysfunctions and the (re)activation of cell death programmes by pharmacological agents that induce or facilitate mitochondrial membrane permeabilization represent attractive strategies for cancer therapy [48].

#### 5. Drug mitochondrial targets in cancer therapy

A large number of agents with anti-cancer activity that act on mitochondria, mitocans (an acronym for "mitochondria and cancer"), hold a substantial promise to be developed into efficient anti-cancer drugs, based on their selectivity for cancer cells [48]. Thus, it is propose the study of anti-cancer agents that act via mitochondrial destabilization (mitocans) based on the site of action of the individual agents from the surface of the mitochondrial outer membrane (MOM) to the mitochondrial matrix. The selection of the sites also stems from their importance as targets for the development of drugs that hold substantial promise to be utilized in the clinical practice [65].

Acetate and lactoferrin (Lf), are two known anti-cancer agents that induce an apoptotic process mediated by the mitochondria in colorectal carcinoma cells [49, 50] and in the yeast *Candida Albicans* [51].

Lf is an 80-kDa, non-heme iron-binding glycoprotein that belongs to the transferrin family. It is found in most mucosal sites and secondary granules of neutrophils in mammals [52]. Several functions have been attributed to Lf as a key component in the host's first line of defense, contributing to a variety of physiological changes at both the cellular and organ levels. Lf is a major pleiotropic mediator that plays an important role in the development of inflammatory responses [53]. Lf also contributes to tumour growth inhibition might be related to apoptosis of these cells

induced by the activation of the Fas signaling pathway (extrinsic apoptotic pathway). Nevertheless, the exact mechanism of these functions has not been discovered so far [54].

Acetate is a short chain fatty acid (SCFA) which has been described as an endogenous metabolite of beta-oxidation of fatty acids in liver [55]. SCFAs are final product of carbohydrate's fiber fermentation by Propionibacteria that live in the human intestine [49, 50]. These natural compounds have been the subject of increased research over the past few decade. SCFAs besides mediating the ions transport for the colonic epithelium, may act as modulators of intracellular pH, cell volume, and other functions associated with ion transport, and as regulators of proliferation, differentiation and gene expression [56, 57]. For SCFAs, palmitate, propionate and acetate induced apoptosis occurred via the stereotyped biochemical events, including mitochondrial alterations, caspase activation and nuclear degradation [50]. Studies indicated that SCFAs can act as anticancer agent, through inhibition of colon cancer cell growth and differentiation, and enhancement of apoptosis in coloretal cancer cells [49]. Therefore, a diet rich in fibers has been associated with a decreased incidence and growth of colon cancers [58].

## Aims

The aim of the present thesis was to explore flow cytometric analysis to perform a functional characterization of isolated mitochondria from the yeast model system *S. cerevisiae* and rat liver, towards the screening of mitochondrial target drugs. For such purpose, a protocol for the isolation and purification of yeast and rat liver mitochondria was implemented and optimized. Then, flow cytometry analysis and western blot were used to characterize the purity and functional integrity of mitochondria. A set of staining protocols were optimised for the analysis by flow cytometry of different functional features of the isolated organelles namely, their mass, mitochondrial matrix pH,  $\Delta\psi$ m and ROS accumulation.

The effect of Lf and acetate on mitochondria function was tested in isolated mitochondria from rat liver using specific fluorescent probes combined with flow cytometry. Complementary studies were also performed to understand if the biochemical activity of the complexes of ETC and osmotic swelling were affected in response to Lf and acetate. Further investigation of the role of these drugs in mitochondria-dependent death pathways will offer new perspectives for an enhanced understanding of the effect of Lf and acetate in mammalian apoptosis.

## Materials and methods

#### Reagents

The mitochondrial markers Mitotracker Green, Mitotracker Red CMXROS; Mitotracker Red CM-H<sub>2</sub>XROS, 2', 7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF-AM), Acridine Orange 10-Nonyl Bromide (NAO) and 3,3'-Dihexyloxacarbocyanine Iodide (DiOC<sub>6</sub>(3)) were purchased from Invitrogen-Molecular Probes (Eugene, OR, USA). Yeast nitrogen base, peptone and yeast extract were purchased from Difco. Dihydroethidium (DHE), cytochrome *c*, carbonyl cyanide mchlorophenyl hydrazone (CCCP), oligomycin, succinate, adenosine 5'-diphosphate sodium salt (ADP) were purchased from Sigma-Aldrich.

#### Yeast strains and growth conditions

Saccharomyces cerevisiae wild type strain W303-1A was grown in YNB medium with 2% (w/v) lactate, pH 5.5. Frozen aliquots of yeast strain were streaked onto plates containing the adequate culture medium and incubated for 48 h at 25°C prior to each experiment. All strains were inoculated at an OD of about 0.005 and grown overnight to log phase ( $OD_{640nm} = 0.5-0.8$ ) in 500 mL flasks, in an orbital shaker, at 30 °C, 200 rpm.

#### Isolation and purification of mitochondria from yeast cells

Yeast cells were pelleted at 4°C by centrifugation at 5000 rpm for 5 min and washed with distilled water. The yeast pellet was resuspended in DTT buffer (100 mM Tris, 30 mM dithiothreitol (DTT), pH 7.0) and incubated at 30°C for 15 min. After centrifugation at 5000 rpm for 5 min at 4°C, the yeast pellet was incubated for 50-60 min at 30°C with Zymolyase buffer containing 1.35 M sorbitol, 30 mM disodium phosphate, 10 mM citric Acid, 1 mM ethylene glycol tetraacetic acid (EGTA), and 10 mg Zymolyase per g of cells wet weight, pH 5.8. After the digestion of the cell walls, cells were harvested by centrifugation at 7700g for 5 min at 4°C and the pellet was washed with Zymolyase buffer without the enzyme. Spheroplasts were centrifuged at 7700g for 5 min at 4 °C and the pellet was resuspended in ice-cold homogenization buffer containing 0.6 M mannitol, 10 mM Tris-maleate, pH 6.8, 2 mM EGTA and 0.2% (w/v)

bovine serum albumin (BSA). The lysed material was carefully maintained at low temperature to avoid proteolysis. Spheroplasts were homogenized in a final volume of 15 mL with 10 strokes using a glass-Potter homogenizer. After homogenization, the sample was diluted to 40 mL with homogenization buffer, centrifuged at 500g for 10 min at 4°C and the pellet was discarded. To isolate mitochondria, the supernatants were collected and centrifuged at 17000g for 10 min. To achieve a greater degree of purification the last two centrifugations steps were repeated. The crude mitochondrial pellet was resuspended in 2 mL of homogenization buffer. Protein quantification was determined by Lowry method [59].

#### Isolation and purification of mitochondria from rat liver

All manipulations were performed on ice with ice-cold buffers (IB; 250 mM sucrose, 1 mM EDTA, 50 mM Tris-HCl, pH 7.2). Animal experiments were performed according to the Portuguese law for animal welfare (Diário da República, Portaria 1005/92). *Rattus norvegicus*, strain Sprague-Dawley OFA (Charles-River, Spain) were housed in an accredited mouse house and treated as specified by the recommendations of the Helsinki convention (World Medical Association - ethical principles for medical research involving humans) and the Guide for the Care and Use of Laboratory Animals, published by the National Academy Press. Animals were sacrificed with 150mg/kg of pentobarbital (Eutasil®, Ceva, Algés, Portugal) and the liver was rapidly dissected out and stored in IB buffer.

The tissue was manually homogenized in IB buffer (10%, wt/vol) with 8-10 strokes using a glass-Potter homogenizer. The homogenate was diluted two-fold with IB and centrifuged at 1000g for 10 min at 4°C to pellet erythrocytes, cell debris and nuclei. Supernatants were collect and centrifuged at 10000g for 10 min at 4°C. The last two steps were repeated to increase the degree of purification. The crude mitochondrial pellet was gently resuspended in 2 mL of IB. Protein quantification was determined by Lowry method [59].

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#### Western Blot

For western blot assay, protein quantification was determined by Bradford method [60]. Fifty µg of protein was resolved in SDS-polyacrylamide gel and then electroblotted to a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Membranes were blocked in TPBS (PBS with 0.05% Tween-20) containing 5% (w/v) nonfat dry milk, washed in TPBS and incubated with primary antibody (TOM 20, GAPDH and V-ATPase) overnight. Then, after washing, membranes were incubated with secondary antibody and immunoreactive bands were detected using the Immobilon solutions (Millipore, Billerica, MA, USA) under a chemiluminescence detection system, the ChemiDoc XRS (Bio-Rad Laboratories).

#### **Fluorochrome solutions**

Mitochondria were selectively stained using 10-nonyl acridine orange (NAO), which binds to cardiolipin in the inner mitochondrial membrane. NAO is one of the most commonly used dyes for the monitoring of mitochondrial mass [61]. A concentrated stock solution of NAO (2.5 mM) was prepared in DMSO, and stored at - 20 °C.

MitoTracker Green (MTG) is a mitochondrial-selective fluorescent label commonly used in flow cytometry. It is expected that this dye selectively accumulates in the mitochondrial matrix where it covalently binds to mitochondrial proteins by reacting with free thiol groups of cysteine residues [62]. A stock solution (50  $\mu$ M) was prepared in DMSO and stored at -20°C.

Mitotracker Red CMXROS was used for monitoring the membrane potential ( $\Delta\psi$ m). Mitotracker Red CMXROS binds irreversibly to the polarized mitochondrial membrane and does not require reduction or oxidation for emission of fluorescence [63]. Mitotracker Red CMXROS was dissolved in DMSO at a concentration of 500  $\mu$ g/mL and stored at -20 °C until use.

DiOC<sub>6</sub>(3) has been also widely used as a cytofluorometric  $\Delta \psi$ m indicator. When DiOC<sub>6</sub>(3) is used at low concentrations (10–20 nM) it rapidly reaches equilibrium in the mitochondria with low quenching effects [4]. The stock solution of DiOC<sub>6</sub>(3) (0.2  $\mu$ M) was prepared in DMSO, and stored at -20<sup>o</sup>C.

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Mitochondrial ROS accumulation was evaluated with the probe Dihydroethidium (DHE). This is a fluorescent probe that up on oxidation by superoxide anion gives converted into ethydium which intercalates in the double bound nucleic acids and emits red fluorescence [64]. A stock solution of DHE (2  $\mu$ g/mL) was prepared in DMSO and stored at -20°C.

Mitotracker Red CM-H<sub>2</sub>XROS is a reduced, nonfluorescent version of MitoTracker Red (M-7512) that fluoresces upon oxidation [65]. The probe was dissolved in DMSO at a concentration of 200  $\mu$ M and stored at -20 °C until use.

BCECF (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester) was used to evaluate the mitochondrial matrix pH as it is sensitive to H<sup>+</sup>. After the hydrolytic cleavage of its ester bonds by esterases within the matrix the probe becomes entrapped. A concentrated stock solution of BCECF-AM (1.6 mM) was prepared in DMSO, and stored at -20°C.

#### Flow cytometry analysis

Sample analysis by flow cytometry was performed in an Epics<sup>®</sup> XL<sup>™</sup> (Beckman Coulter) flow cytometer, equipped with an argon-ion laser emitting a 488 nm beam at 15 mW. Monoparametric detection of red fluorescence from Mitotracker CMXROS, H<sub>2</sub>-XROS, and DHE was performed using FL-4 (525/620 nm); for the Nonyl Acridine Orange war performed using FL-3 (488/620 nm); and detection of green fluorescence from DIOC<sub>6</sub>(3), Mitotracker Green FM and BCECF-AM was performed using FL-1 (488/525 nm). For each sample, at least 20000 events were analysed at low flow rate. An acquisition protocol was defined to measure forward scatter (FS), side scatter (SS) and green fluorescence (FL1) on four decade logarithmic scales.

#### **Enzymatic assays**

#### Succinate dehydrogenase assay

Enzyme activity was measured following the reduction of the hexacyanoferrate (III). Intact mitochondria (150  $\mu$ g protein), 40 mM succinate, 0.10% BSA were added to the 100 mM potassium phosphate, pH 7.8 (final volume 3 mL). The reaction was

started by the addition of potassium hexacyanoferrate (II) (0.5 mM), and the decrease in absorbance at 420 nm was recorded.

#### NADH-cytochrome c reductase

Cytochrome c reductase activity was recorded in a spectrophotometer set at 550 nm. Samples of homogenate were diluted with cold 10 mM phosphate buffer (pH 7.5) to yield a final protein concentration of 0.25 mg/mL. Cytochrome c solution was obtained by dilution of 1% cytochrome c in 10 mM phosphate buffer (pH 7.5). To obtain a baseline, 50 mg protein were added in the absence of substrate and the reaction was measured for 0.5 min at 550 nm. The reaction was started with 0.1 mM NADH solution and the rate of cytochrome c reduction was measured for additional 1-2 min.

#### Cytochrome c oxidase

Samples of homogenate were diluted with cold 20 mM phosphate buffer (pH 7.5), to yield a final protein concentration of 0.25 mg/ml. Cytochrome *c* solution was obtained by dilution of 1% cytochrome *c* in 20 mM phosphate buffer (pH 7.5). The rate of cytochrome *c* oxidation was recorded at 550 nm and a reference of total oxidation of cytochrome *c* was obtained with addiction of a small amount of potassium ferricyanide in aliquot of cytochrome *c* solution. To start reaction of cytochrome *c* solution. The absorbance was registered at 550 nm every 15 seconds or using a recorder.

#### Mitochondrial Swelling Assay

Mitochondrial swelling was followed spectrophotometrically at 540 nm. Isolated liver rat mitochondria were resuspended in the swelling buffer containing 120 mM KCl, 10 mM Tris-HCl (pH 7.4) and 5 mM KH<sub>2</sub>PO<sub>4</sub>, to a final protein concentration of 250  $\mu$ g/ml. CaCl<sub>2</sub> (250  $\mu$ M) was used as a positive control for pore opening.

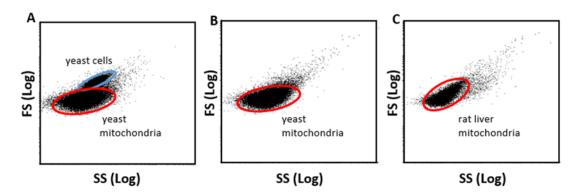
## Results

## Functional studies of mitochondrial suspensions by flow cytometry

Mitochondrial suspensions isolated from rat liver and yeast cells were prepared in order to optimise their structural and functional characterization by flow cytometry. This characterization was corroborated by biochemistry approaches including western blot as well as enzymatic assays.

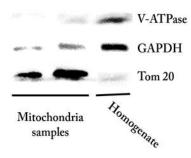
#### Characterization of the purity of mitochondrial fractions

Biparametric histograms (FS log vs SS log) of a mixture of yeast cells and isolated yeast mitochondria revealed differences in complexity and size between both biological samples. Yeast cells are far more complex and larger than isolated mitochondria (Fig. 1A). In addition, mitochondrial suspensions isolated from either yeast or rat liver formed homogeneous populations suggesting an homogeneous population which reflects a good degree of purity (Fig. 1B and C).



**Figure 1:** Flow cytometric analysis of intact yeast cells (A) and mitochondrial suspensions isolated from yeasts (A and B) and rat liver (C). Scattergrams with distinguishable populations of cells (blue) and purified mitochondrial suspensions (red). In A yeast cells and purified mitochondria were mixed prior to analysis

Western blot analysis with antibodies against proteins representative of important subcellular compartments was used to further characterize the purity of the mitochondrial population. TOM20 was used as mitochondrial marker, GAPDH as marker of the cytosol, and the subunit C of the V-ATPase as marker of the lysosome. As



**Figure 2:** Western blots of protein extracts from isolated mitochondria from rat liver and from the corresponding homogenate, using specific antibodies raised against the mitochondrial protein Tom 20, the subunit C of the V-ATPase from the lysosome, and the protein GAPDH resident in the cytosol.

can be seen in Fig. 2 both mitochondrial batches from rat liver were enriched in TOM20 and depleted in GAPDH and in the subunit C of the V-ATPase, further confirming that they were highly pure.

#### **Optimization of the staining protocols**

To perform structural and functional measurements by flow cytometry of intact mitochondria isolated from either yeast or rat liver, in a first stage of the work, several probes were tested and the corresponding staining protocols were optimized in order to monitor the mitochondrial memebrane potential ( $\Delta\psi$ m), the pH of the mitochondrial matrix, ROS accumulation, and mitochondrial mass. Both the incubation time (15, 30, 45 and 60 min), the temperature (room temperature, 30°C and 37°C), the concentration of each probe and the amount of mitochondrial protein per each assay were optimized.

#### Mitotracker Green and Nonyl Acridine Orange

Both probes were used to analyse changes in mitochondrial mass. The best staining conditions were as follows: for Mitotracker Green (MTG), 50  $\mu$ g of mitochondrial protein (in 500  $\mu$ L) incubated in the dark for 30 min at 37 °C with 0.4  $\mu$ M of the probe; for Nonyl Acridine Orange (NAO), 50  $\mu$ g of mitochondrial protein (in 500  $\mu$ L) incubated in the dark for 15 min at room temperature with 0.5  $\mu$ M of the probe. In these conditions the mitochondrial population exhibited a green positive fluorescence (FL1; 488/525 nm) with MTG staining, and an orange positive fluorescence (FL3; 488/620 nm) with NAO staining (Fig. 3).

#### 3,3'-Dihexyloxacarbocyanine Iodide and Mitotracker Red CMXROS

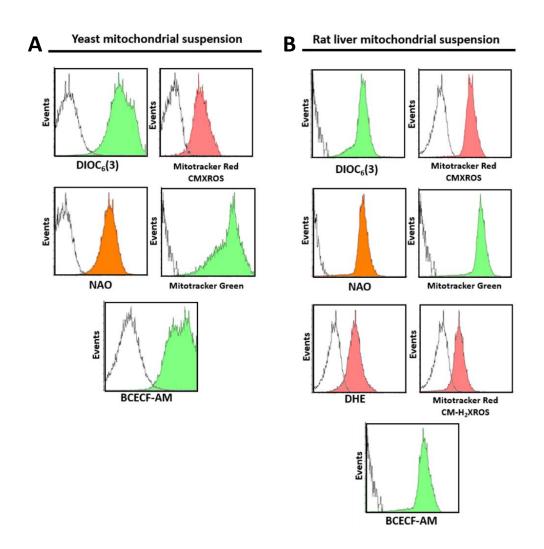
Both probes were used to assess changes in the  $\Delta \psi m$  and the best staining conditions were: for Mitotracker Red CMXROS (MTR), 50 µg of mitochondrial protein (in 500 µL) incubated in the dark for 30 min at 37 °C with 0.2 µM of the probe; for 3,3'-Dihexyloxacarbocyanine lodide (DiOC<sub>6</sub>(3)), 50 µg of mitochondrial protein (in 500 µL) incubated in the dark for 30 min at room temperature with 10 nM of the probe. As shown in Fig. 3 clear green positive fluorescence (FL1; 488/525 nm) was observed with DiOC<sub>6</sub>(3) staining and a red positive fluorescence (FL4; 525/620 nm) with Mitotracker Red CMXROS staining.

#### Mitotracker Red CM-H<sub>2</sub>XROS and dihydroethidium

Mitotracker Red CM-H<sub>2</sub>XROS and dihydroethidium (DHE) were selected to evaluate the accumulation of ROS. The best staining conditions were found when 50 µg protein of mitochondrial suspension (in 500 µL) was incubated during 30 min at 37 °C in the dark with 0.5 µM Mitotracker Red CM-H<sub>2</sub>XROS or 2ng/mL of DHE. A clear red positive fluorescence of mitochondria (FL4; 525/620 nm) can be observed in Fig. 3 after staining whith Mitotracker Red CM-H<sub>2</sub>XROS and DHE.

#### 2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester

The staining protocol with (2',7'-Bis-(2-Carboxyethyl)-5-(and-6)-Carboxyfluorescein, Acetoxymethyl Ester (BCECF-AM) was optimized to study how the pH of the mitochondrial matrix is changed after challenging isolated mitochondria with a specific substrate or drug. Results showed that the best staining condition was found when 50 µg of mitochondrial protein (in 500 µL) was incubated with 5 µM BCECF-AM during 30 min at 37°C in the dark. As shown in Fig. 3, a green positive fluorescence was clearly observed (FL1; 488/525 nm) after staining with BCECF-AM.



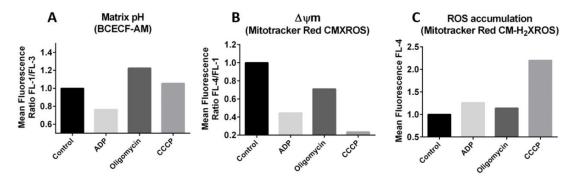
**Figure 3:** Flow cytometry analysis of isolated mitochondrial suspensions isolated from yeast cells (A) and rat liver (B) stained with  $DIOC_6(3)$  (0.4  $\mu$ M), Mitotracker Red CMXROS (0.2  $\mu$ M), NAO (0.5  $\mu$ M), Mitotracker Green BCECF-AM (1.6 mM), DHE (2ng/mL) and Mitotracker Red CM-H<sub>2</sub>XROS (0.5  $\mu$ M). Histograms in white correspond to the autofluorescence

# Response of resting mitochondria from rat liver to ADP, oligomycin and CCCP

In these assays staining with BCECF-AM, Mitotracker Red CMXROS, or Mitotracker Red CM-H<sub>2</sub>XROS was used to evaluate changes in the pH of the mitochondrial matrix, mitochondrial mass,  $\Delta\psi$ m and ROS accumulation in the mitochondrial compartment, respectively. The addition of 500 nmol of ADP to rat liver isolated resting mitochondria stained with BCECF-AM resulted in a decrease of fluorescence within 5 min, suggesting an acidification of the mitochondrial matrix due to coupling between endogenous respiration and proton driven ATP synthesis. On the contrary, the addition of 0.9  $\mu$ g/mL oligomycin promoted alkalinisation of the matrix. No significant changes in mitochondrial pH were observed upon addition of 50  $\mu$ M CCCP (Fig. 4A).

The addition of either 500 nmol ADP or 0.9  $\mu$ g/mL oligomycin, or 50  $\mu$ M CCCP to rat liver mitochondria stained with Mitotracker Red CMXROS promoted a reduction of the red fluorescence suggesting that each compound was able to induce a transient depolarization of the mitochondrial membrane. The strongest effect was observed upon addition of the protonophore CCCP (Fig. 4B).

When the effect of ADP, oligomycin, or CCCP were tested in mitochondria labelled with Mitotracker Red CM-H<sub>2</sub>XROS, only CCCP was able to promote a significant increase in the red fluorescence suggesting that the accumulation of ROS was induced following enhanced endogenous respiration due to the collapse of the transmembrane proton gradient by the protonophore (Fig. 4C).



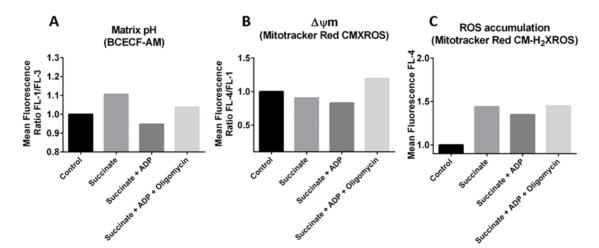
**Figure 4:** Flow cytometry analysis of resting mitochondria from rat liver after incubation with ADP (500 nmol), oligomycin (0.9  $\mu$ g/ml) and CCCP (50  $\mu$ M) for 5 min. Mitochondrial suspensions were pre-stained with BCECF-AM (A) Mitotracker Red CMXROS (B), and Mitotracker Red CM-H<sub>2</sub>XROS (C).

# Response of mitochondria from rat liver to energization with succinate

Incubation of resting mitochondria with succinate led to an increase in the pH of the matrix, which was prevented by ADP that promoted a slight acidification of the matrix. The addition of oligomycin to this mixture induced a slight alkalinisation (Fig. 5A).

Figure 5B shows that succinate induced a very small depolarization of the mitochondrial membrane within 60 min that was slightly more pronounced in the presence of 500 nmol ADP. The addition of oligomycin prevented membrane depolarization and cause a slight hyperpolarization when compared to resting mitochondria.

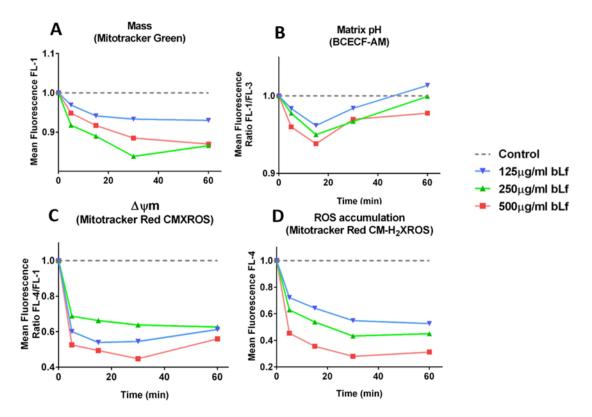
The activation of the electron transport chain with 6 mM succinate caused an increase in ROS production as shown in mitochondria suspensions labelled with Mitotracker CM-H<sub>2</sub>XROS. The presence of either 500 nmol ADP or 500 nmol ADP + 0.9  $\mu$ g/mL mM oligomycin did not affect the rate of ROS production induced by succinate (Fig. 5C).



**Figure 5:** Flow cytometry analysis of resting mitochondria from rat liver after incubation with succinate (6mM), succinate (6mM) + ADP (500 nmol) and succinate (6mM) + ADP (500 nmol) + oligomycin (0.9  $\mu$ g/ml) for 5 min. Mitochondrial suspensions were pre-incubated for 30 min with 1.6 mM of BCECF-AM (A), 0.2  $\mu$ M of Mitotracker Red CMXROS (B), and 0.5  $\mu$ M of Mitotracker Red CM-H<sub>2</sub>XROS (C).

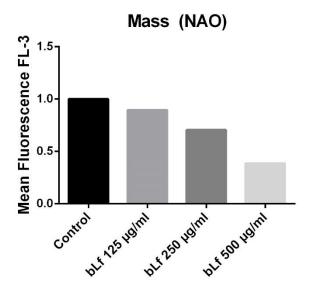
### Effect of bovine lactoferrin (bLf) on mitochondrial function

Results presented in figure 6 clearly show that 125 - 500 µg/mL bLf strongly affected rat liver mitochondrial structure/function within 60 min of incubation. In resting mitochondria labelled with Mitotracker Green a clear loss in mitochondrial mass was evident 30 min after the addition of bLf (Fig. 6A). Similar results were obtained for mitochondria stained with NAO after 60 min. incubation with bLf (Fig. 7).



**Figure 6:** Time course analysis of effect of bLf on mitochondrial function. Mitochondrial suspensions from rat liver were pre-stained with fluorescent probes to assess changes in mitochondrial mass, matrix pH,  $\Delta \psi$ m and ROS accumulation.

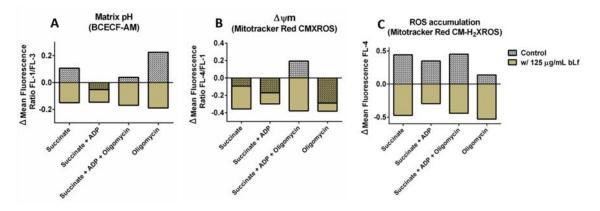
The three concentrations of bLf tested also promoted a clear acidification of the mitochondrial matrix, which was dose-dependent. However, this phenomenon was transient being more evident 15 min after incubation (Fig. 6B). As shown in Fig. 6C, the addition of 125 - 500  $\mu$ g/mL bLf caused a strong reduction of the  $\Delta\psi$ m within 5 min of incubation. ROS levels were also strongly reduced after addition of bLf being the effect more evident till 30 min of incubation and clearly dose-dependent (Fig. 6D).



**Figure 7:** Flow cytometry mass analysis of rat liver isolated mitochondria after incubation with different concentration of bLf, 125  $\mu$ g/mL, 250  $\mu$ g/mL and 500  $\mu$ g/mL for 60 min.

Subsequent experiments were performed to study how bLf affects the matrix pH,  $\Delta\psi$ m and ROS homeostasis of mitochondria energized with 6 mM succinate/500 nmol ADP. Results are depicted in Fig. 8. As can be seen, the pre-incubation of mitochondria suspensions with 125 µg/mL bLf for 60 min induced an acidification of the mitrochondrial matrix measured 5 min after the energization with succinate in all experimental conditions tested (succinate alone, succinate + ADP, succinate + ADP + oligomycin). The same result was obtained when the incubation with bLf was followed by the addition of oligomycin alone. Thus, the pre-incubation with bLf counteracted the alkalinization effect promoted by the addition of succinate alone, succinate + ADP + oligomycin or oligomycin alone (Fig. 8A).

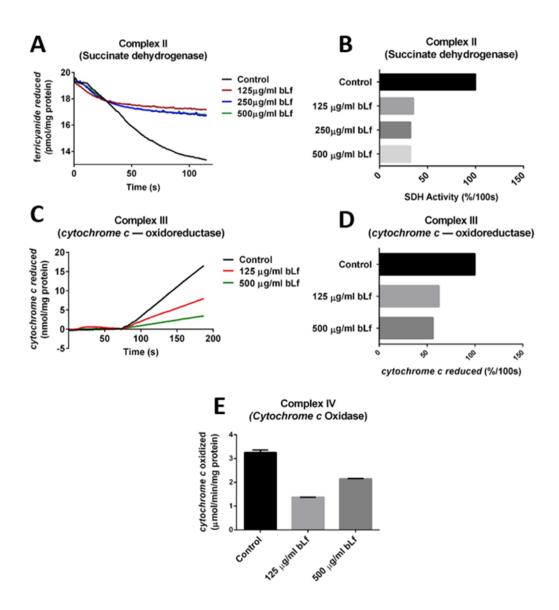
Figure 8B shows that the pre-treatment of isolated mitochondria with bLf promoted a strong depolarization of the inner membrane in all experimental conditions tested (succinate alone, succinate + ADP, succinate + ADP + oligomycin or oligomycin alone), potentiating the dissipation effect of the  $\Delta \psi$ m promoted by the addition of either succinate alone, succinate + ADP, or oligomycin alone. In what concerns ROS homeostasis, the pre-treatment of isolated mitochondria with bLf promoted a strong decrease in ROS accumulation in all energization conditions tested and in the presence of oligomycin alone, counteracting the observed increase in ROS production promoted by each condition in control samples (Fig. 8C).



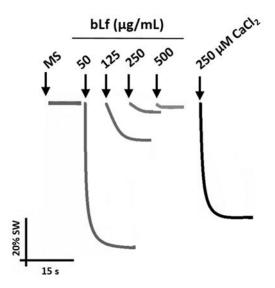
**Figure 8:** Flow cytometry analysis of mitochondrial suspensions from rat liver stained with BCECF-AM, Mitotracker Red CMXROS and Mitotracker CM-H<sub>2</sub>XROS and pre-incubated with 125 μg/mL bLf for 60 min before energization with either 6 mM of succinate or 6 mM of succinate + 500 nmol of ADP. Flow cytometry analysis was performed 5 min after energization. Concentration of oligomycin: 0.9 μg/mL of oligomycin.

As referred in material and methods, to study the effect of bLf on the activity of electron transport complexes of the inner mitochondrial membrane isolated mitochondria from rat liver were pre-incubated with 125, 250 and 500  $\mu$ g/mL of bLf for 60 min. As can be seen in figures 9A and B bLf inhibited by 65-70% the activity of the complex II, the effect being similar for all concentrations tested. The complex III was inhibited by 38% to 44% (Fig. 9C and D) and the cytochrome *c* oxidase (complex III) by 35% to 58% (Fig. 9E).

Mitochondrial swelling was followed by the decrease in absorbance of mitochondrial suspensions at 540 nm. The addition 250  $\mu$ M CaCl<sub>2</sub> (positive control) promoted a prompt mitochondrial swelling (Fig. 10). Results showed that 50 – 500  $\mu$ g/mL bLf also induced an immediate mitochondrial swelling, which was much stronger at the lowest concentration of bLf used (50  $\mu$ g/mL).



**Figure 9:** Effect bLf on the activity of Complex II (succinate dehydrogenase) (A and B), Complex III (cytochrome *c* – oxireductase) (C and D) and Complex IV (cytochrome *c* oxidase) (E) from rat liver mitochondria. Mitochondrial suspensions were pre-incubated with  $125 - 500 \mu g/ml$  bLf for 60 min before the enzymatic analysis.



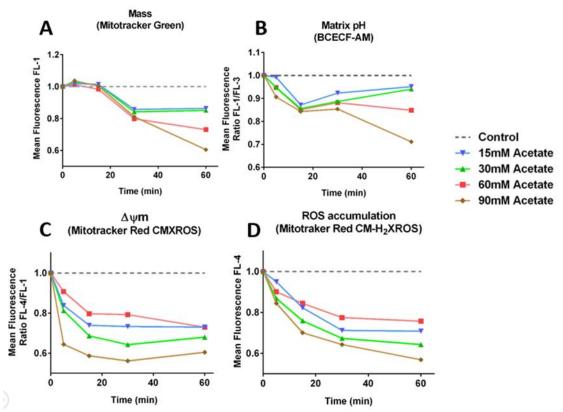
**Figure 10:** Effect of bLf on the swelling (SW) of rat liver mitochondria as assessed by the decrease in absorbance at 540 nm of the mitochondrial suspension. Positive control, 250  $\mu$ M CaCl<sub>2</sub>.

### Effect of acetate on mitochondrial function

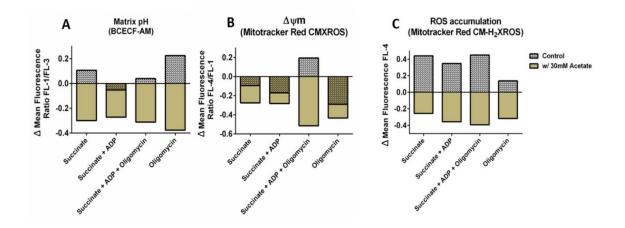
Flow cytometry experiments were also performed to evaluate mitochondrial mass, the pH of the matrix,  $\Delta \psi m$  and ROS accumulation in mitochondria in response to 15 – 90 mM acetate. As can be seen in Fig. 11, these mitochondrial parameters were very sensitive to acetate. Similarly to that observed after incubation with bLf, acetate promoted a decrease in the mitochondrial mass, the acidification of matrix, a depolarization of the inner membrane and a decrease in ROS production within 60 min of incubation in rat liver mitochondria. A clear dose-response effect was not observed in all experiments but the highest concentration of acetate used (90 mM) was always the most effective.

To study the effect of acetate in energized mitochondria suspensions from rat liver were pre-incubated with 30 mM of acetate for 60 min before the addition of 6 mM of succinate or succinate + 500 nmol of ADP, and flow cytometry analysis was performed 5 min after energization. Oligomycin alone or in combination with succinate and ATP was used to inhibit F-ATPase. As can be seen in Fig. 12, acetate induced acidification of the mitrochondrial matrix, promoted the depolarization of the inner membrane and prevented ROS accumulation in all experimental conditions tested much like the effects of Lf shown above (Fig. 8).

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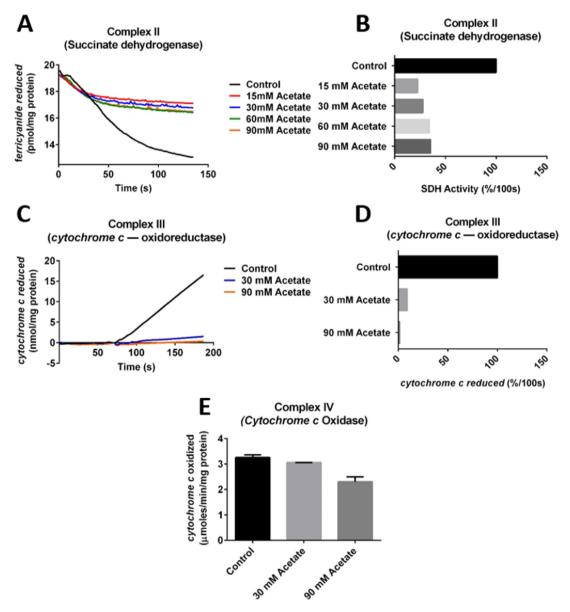


**Figure 11:** Time course analysis of effect of acetate (pH7) on mitochondrial function. Mitochondrial suspensions from rat liver were pre-stained with fluorescent probes to assess changes in mitochondrial mass, matrix pH,  $\Delta \psi$ m and ROS accumulation.



**Figure 12:** Flow cytometry analysis of mitochondrial suspensions from rat liver stained with BCECF-AM, Mitotracker Red CMXROS and Mitotracker CM-H<sub>2</sub>XROS and pre-incubated with 30mM of acetate (pH7) for 60 min before energization with either 6 mM of succinate or 6 mM of succinate + 500 nmol of ADP. Flow cytometry analysis was performed 5 min after energization. Concentration of oligomycin: 0.9  $\mu$ g/mL of oligomycin.

To study the effect of acetate on the activity of electron transport complexes of the inner mitochondrial membrane, isolated mitochondria from rat liver were preincubated with 15 - 90 mM acetate, pH 7.0, for 60 min. As can be seen in Fig. 13 over the concentration range tested acetate strongly inhibited the activity of the complex II (up to 77 %) and complex III (up to 99%). Comparatively, the inhibitory effect of acetate over the activity of cytochrome *c* oxidase was much weaker, from 7% to 26%, with 30 and 90 mM acetate, respectively.



**Figure 13:** Effect acetate (pH7) on the activity of Complex II (succinate dehydrogenase) (A and B), Complex III (cytochrome c – oxireductase) (C and D) and Complex IV (cytochrome c oxidase) (E) from rat liver mitochondria. Mitochondrial suspensions were pre-incubated with 125 – 500 µg/ml bLf for 60 min before the enzymatic analysis.

As can be seen in Fig. 14, acetate also induced a strong mitochondrial swelling over all concentrations tested. The rates and amplitude of mitochondrial swelling in response to 15- 90 mM acetate were of the same magnitude as compared with the well known mitochondrial swelling inducer CaCl<sub>2</sub>.

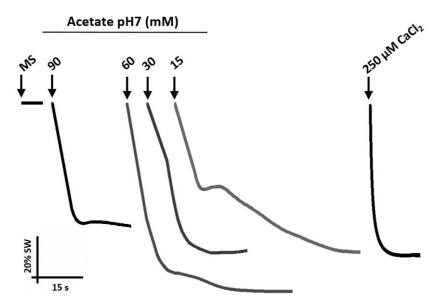


Figure 14: Effect of acetate (pH7) on the swelling of rat liver mitochondria. Positive control:  $250 \ \mu M \ CaCl_2$ .

### Discussion

## Isolated mitochondria from yeast and rat liver were structurally intact and functionally active

Due to their pivotal role in the metabolism of eukaryotic cells, in particular in the bioenergetics metabolism, mitochondria have been the focus of intensive studies through the 2<sup>nd</sup> half of last century till now. The Nobel Prize in Chemistry was awarded in 1978 to Peter Mitchell "for his contribution to the understanding of biological energy transfer through the formulation of the chemiosmotic theory" and more recently, in 1997, it was awarded (2/3) to Paul D. Boyer and John E. Walker "for their elucidation of the enzymatic mechanism underlying the synthesis of adenosine triphosphate (ATP)" (www.nobelprize.org/). Among hundreds of other studies, important ones have focused on monitoring the mitochondrial Ca<sup>2+</sup> homeostasis [66], mitochondria involvement in Programmed Cell Death induced [67, 68], and, more recently, on the mechanisms involved in the fusion and fission dynamics of mitochondria [69].

Studies of mitochondrial function in intact cells have meet some problems as the organelle is not directly accessible to substrates and potential inhibitors added to the extracellular space, and the complexity of cytosolic metabolism, together with the presence of separate pools of adenine nucleotides, nucleotides and calcium in the cytosol and mitochondrial matrix, impair accurate studies. In addition, many reagents and substrates are cell-impermeant, restricting experimental options [70]. In this context, isolated mitochondria preparations offer a number of advantages over intact cells [71].

In the present study two different batches of mitochondria suspensions were routinely isolated from yeasts and rat liver to study the effect of specific compounds on mitochondrial function. The yeast has a high degree of conservation of basic molecular and cellular mechanisms of human cells, and represents an inexpensive and simple alternative cell system. Importantly, yeast is a easily genetically tractable system for several functional studies [72]. Important work in our laboratory on mitochondria involvement in cell death [67, 73, 74] and mitochondrial dysfunction [75] have been done in yeast and mammalian cell lines and, therefore, the results of our work in intact mitochondria may complement such studies.

Rat liver is the most frequently used tissue for mitochondrial preparations because it is readily available and easy to homogenize, and contains a large number of mitochondria (1000 – 2000 per cell) [76]. In addition, the rat has become a standardized physiological and toxicological model, particularly in the pharmaceutical industry [77].

Since the present study aimed to characterise the function of isolated mitochondria the purity of the cellular fraction was evaluated by western blot. The use of TOM20, GAPDH and V-ATPase (subunit C) as a mitochondrial, cytosolic and lysosomal marker, respectively, allowed inferring that, as described in other studies [78, 79], a good degree of purity in mitochondria was achieved and that the isolation protocol was adequate to proceed with the flow cytometric and enzymatic assays.

## Flow cytometry revealed a valuable tool to study key mitochondrial functional parameters

Flow cytometric analysis requires particles in suspension either in the form of cells, isolated organelles, cell components (e.g chromosomes) or soluble cellular constituents (e.g cytocines). It allows the simultaneous quantification of multiple fluorescence emissions in the same cell or biological particle, and scattered light related to size and complexity [3]. Therefore, individual cells or sub-cellular particles from heterogeneous subpopulations can be physically isolated on the basis of their fluorescence or light scatter properties [4]. In particular, this approach has shown several advantages to study mitochondrial suspensions, as very small samples can be analyzed (5,000–10,000 mitochondria) and contaminants can be handled separately. Furthermore, it allows the analysis of different of relevant parameters and identification of mitochondrial subpopulations that can be sorted for further analysis. [80]. On contrary, when mitochondrial parameters are analyzed by flow cytometry in intact cells, the fluorescent signal is the result of the equilibrium in distribution of the probe among the different cell compartments, impairing accurate studies [81].

So far, flow cytometric studies on isolated mitochondria are very few and have been mainly focused on  $\Delta\psi$ m measured with Rhodamine-123 (Rh123) [82-84] and by 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) [85], an on ROS generation in mitochondria isolated from brain and liver mitochondria measured with 2',7'-dichlorodihydrofluorescein-diacetate (H<sub>2</sub>DCFDA) [86] or dihydrorhodamine-123 [87, 88]. However, it has been reported that some probes have a relatively high unspecific binding to mitochondria, which limits its usefulness, and at higher concentrations may inhibit mitochondrial respiration.

In the present study, flow cytometry revealed adequate to monitor important functional characteristics of the isolated mitochondrial suspensions from both yeast and rat liver. To validate the staining protocols, resting isolated mitochondria were incubated with different substrates and inhibitors. The staining protocols optimized for NAO, Mitotracker Green, Mitotracker Red CMXROS, DiOC<sub>6</sub>(3), BCECF-AM, Mitotracker Red CM-H<sub>2</sub>XROS and DHE demonstrated an effective labelling (Fig. 3), clearly supporting their effectivness to further study several aspects of mitochondrial function.

It is so far well established that oxidative phosphorylation relies on the close association of electron carriers with intrinsic protein molecules of the inner mitochondrial membrane. This transfer of electrons is coupled to oriented H<sup>+</sup> release, as well as to allosteric changes in energy-converting protein pumps. The net result is the pumping of H<sup>+</sup> across the inner membrane, from the matrix to the intermembrane space, driven by the energetically favourable flow of electrons [89]. This movement of H<sup>+</sup> has two major consequences, it generates a pH gradient across the inner membrane space and generates an inside negative  $\Delta\psi$ m [89].

In the present study, when ADP was added to isolated mitochondria from rat liver the fluorescence of pH-sensitive dye BCECF-AM decreased, suggesting that H<sup>+</sup> were pumped into the matrix through F-ATPase, promoting the acidification of mitochondrial matrix and the depolarization of the inner mitochondrial membrane, as shown with the probe Mitotracker Red CMXROS. This is in accordance with previous studies showing that the resting or State 4 respiration of freshly prepared, intact rat liver mitochondria can support energy-linked accumulation of Ca<sup>2+</sup> or Sr<sup>2+</sup> or the

oxidative phosphorylation of ADP. However, under these conditions the coupling efficiency is relatively low; about 10% maximal for Ca<sup>2+</sup>, about 20% for Sr<sup>2+</sup> accumulation, and about 12% maximal for oxidative phosphorylation of ADP [90].

Oligomycin is a well-known inhibitor of F-ATP synthase by blocking its proton channel and consequently inhibiting the translocation of protons into the matrix [91] Thus, oligomycin also reduces significantly the electron flow through the ETC [92]. It has been shown that this inhibitor causes a reduction of the endogenous respiration and supports the occurrence of an active phosphorylation III associated to it [93].

In our study oligomycin also reduced the electron flow through the ETC as suggested when alkalinization of mitochondrial matrix occurred after mitochondria labelling with BCECF-AM. It has been discussed that the electron flow is not stopped completely by oligomycin due to a process known as proton leak or mitochondrial uncoupling [92]. This can explain the depolarization observed when labelling by the probe Mitotracker Red CMXROS (Fig. 4). In agreement, Alberts B. et al describe that the proton leak or the pumping into the matrix from inner membrane space of many charged small molecules and ions, as Pi, destroy the electrochemical proton gradient [89].

In mitochondria labelled with Mitotracker Red CMXROS and BCECF-AM the protonophore CCCP caused the dissipation of the  $\Delta\psi$ m and a small increase of the pH of the matrix over the first 5 min of incubation. This result was expected since in mitochondria, the proton motive force is almost exclusively made up of the electrical component. It is well known that this protonophore allows the protons to cross back through the inner membrane by a pathway other than ATP synthase [94, 95]. The observed increase in ROS in mitochondria stained with Mitotracker Red CM-H<sub>2</sub>XROS after CCCP addition suggests that there was an increase of the flow of electrons across ETC and on oxygen consumption, which is also in agreement with previous results [21].

As shown in figure 5, the addition of 6 mM succinate to resting rat liver mitochondria caused the activation of the ETC and resulted in the accumulation of ROS, and in the alkalinisation of the mitochondrial matrix, confirming previous observations [16, 96] and validating the staining protocols with Mitotracker Red CM-H<sub>2</sub>XROS and BCECF-AM optimized in the present study.

The observed decrease of the pH of the mitochondrial matrix and of the  $\Delta\psi$ m across the inner membrane after the addition of 500 nmol ADP to mitochondria energized with 6 mM of succinate (Fig. 5) was likely due to the dissipation of the proton gradient through F-ATPase, which, in turn, was inhibited by oligomycin.

In general, the changes in the mitochondrial parameters analysed in response to the addition of the respiratory substrate succinate, of the F-ATPase inhibitor oligomycin and to the protonophore CCCP were in agreement with expected effects which successfully validated the optimized staining protocols with Mitotracker CMXROS, BCECF-AM, Mitotracker and CM-H<sub>2</sub>XROS. Afterwards these protocols were exploited to assess whether the two known inducers of mitochondria-mediated apoptosis bLF and acetate act directly on mitochondria and perturb its function. This study could reinforce the use of flow cytometric as a valuable tool to screen mitochondria-target drugs with potential clinical application, in particular in cancer therapy.

### BLf and acetate induce loss of mitochondrial mass

As reported to in Chapter 1 (General Introduction) both acetate and bLf are two known anti-cancer agents that induce an apoptotic process mediated by the mitochondria in colorectal carcinoma cells [49, 50] and in the yeast *Candida albicans* [51]. In the present study, flow cytometric analysis of mitochondrial suspensions labelled with specific fluorophores, allowed us to monitor changes in different mitochondrial functional parameters triggered by both chemicals.

Flow cytometry of mitochondrial suspensions labelled with Mitotracker Green showed that both acetate and bLf induce a consistent loss of mitochondrial mass (Fig. 6 and 11). The damage on thiol groups of cysteine residues mediated by Lf and acetate may explain, at least in part, these results. Moreover, bLf could affect cardiolipin levels because mitochondria fluorescence signal of NAO decreased after incubation with bLf (Fig. 7). Thus, bLf may disturb the permeability of the inner mitochondrial membrane because it has been suggested that cardiolipin has a special role in the inner membrane by increasing its inherent impermeability to ions [89].

#### BLf and acetate promote mitochondrial dysfunction

It has been shown that the pH of the mitochondrial matrix profoundly influences cellular functions, for instance, by direct pH modulation of enzymes [97]. The reduction of the electron flow in the inner mitochondrial membrane from astrocytes was associated with membrane depolarization and to a decrease of the pH of the matrix and O<sub>2</sub> consumption, together with a reduction in mitochondrial ROS generation [98]. The depolarization of the inner mitochondrial membrane may be regulated by modifications in the permeability of mitochondria through the formation of mitochondria permeability transition pore (mPTP) [99, 100]. Under normal physiological conditions, mPTP is closed but it can open in response to oxidative stress, mitochondrial depolarization, depletion of adenine nucleotides and increase in matrix Ca<sup>2+</sup> concentration. Pore opening causes mitochondrial permeabilization and results in mitochondrial swelling. Transient opening of the pore results in the release of cytochrome c, which activates the caspase cascade and triggers apoptosis. Sustained pore opening results in the uncoupling of oxidative phosphorylation; this limits ATP synthesis and leads to necrotic cell death. Pore opening can be a consequence of the opening of a H<sup>+</sup>-selective channel [19]. mPTP complex assumes a high-conductance state that allows the deregulated entry of small solutes into the mitochondrial matrix along their electrochemical gradient [48]. Thus, modifications of the permeability of mitochondrial inner membrane may also affect  $\Delta \psi m$  associated with osmotic swelling of the mitochondrial matrix [43]. Our results showed that both bLf and acetate promoted acidification of mitochondrial matrix, decreased Δψm, reduced ROS accumulation and induced mitochondrial swelling. Reduction in ROS accumulation may be related with the reduction of respiratory complexes activities namely of complex III that, together with complex I, are the main source of ROS production. The observed effects could be mediated, at least in part, by modifications of mPTP complex induced by acetate and bLf.

Results showed that when mitochondria are pre-treated with bLf (125  $\mu$ g/mL) the reported effects on the acidification of mitochondrial matrix, depolarization of the inner membrane potential, and on ROS accumulation were not modified by the

activation of the electron transport chain with succinate, succinate + ADP or in the presence oligomycin (succinate + ADP + oligomycin or oligomycin alone). Similar results were obtained when mitochondria were pre-incubated with acetate (30 mM) instead of bLf. The observed inhibitory effect of both acetate and bLf on the biochemical activity of the complexes of the electron transport chain, namely complexes II, III and IV may explain at least part of these results. As shown, the inhibitory effect of bLf on complex II was more pronounced than on complexes III and IV, while complex IV was less sensitive to acetate than to bLf. Previous studies showed that Lf trigger mitochondrial dysfunction in yeast cells [51] and affected the activity of complexes of the electron respiratory chain in bacteria [101].

The effect of both bLf and acetate on mitochondrial swelling was particularly evident, and of the same order of magnitude to the one promoted by 250 mM CaCl<sub>2</sub>, a well-know inducer of mitochondrial swelling [102]. As referred above mitochondrial swelling may result from uncontrolled flux of solutes in response to mPTP opening and is often associated with the loss of  $\Delta\psi$ m in intact cells [103]. Thus, the observed results corroborate that both bLf and acetate may trigger mPTP opening as reported above with flow cytometric studies. In particular, the results obtained with acetate are in accordance with previous studies reporting that acetate targets mitochondrial ANT, one of the proposed components of PTP, since it specifically permeabilises liposomes containing ANT [104]. Acetic acid, at concentrations (150 mM, pH 3.0) which induce apoptosis, was shown to induce accumulation of ROS, and a hyperpolarization followed by a depolarization, and reduction in COX acivity in yeast cells [67]. The discrepancy between these results and the ones here presented may be due to the pH and concentration studied as well as to the use of yeast whole cells instead of isolated rat liver mitochondria.

## **Concluding Remarks**

Altogether experimental evidences suggested that yeast and rat liver isolated mitochondria could be good models systems to screen drugs targeting mitochondria. Flow cytometric analysis allowed monitoring the homogeneity of mitochondrial suspension regarding relative size and complexity as well as structural and functional integrity of isolated mitochondria. These assays supported that a fast and efficient method to isolate pure and functional mithocondria from yeast cells and rat liver was optimized. Flow cytometric analysis of isolated mitochondria labelled with Mitotracker Green, NAO, Mitotracker Red CMXROS, DIOC<sub>6</sub>(3), Mitotracker Red CM-H<sub>2</sub>XROS and dihydroethidium originated populations with fluorescent intensities clearly distinct from those corresponding to mitochondrial autofluorescence. Resting rat liver isolated mitochondria labelled with these probes, responded to the addition of respiratory substrates and inhibitors of the respiratory chain complexes, as well as to protonophores, such as CCCP, confirming their structural and functional integrity.

Additionally, mitochondrial function was compromised by bLf and acetate. These novel results with isolated mitochondria from rat liver suggest that bLf and acetate are able to directly induce loss of mitochondrial mass and  $\Delta\psi$ m, matrix acidification and reduced ROS production. bLf and acetate affected the activity of the ETC complexes and induced mitochondrial swelling. These results are particularly interesting since the two compounds appear to induce in isolated mitochondria mainly the same alterations as those occurring in whole cells and which have been proposed to be involved in the induction of cell death [80, 105, 106].

The developed methodologies based on flow cytometric analysis of isolated mitochondria as well as the aforementioned results will help to further elucidate the role of bLf and acetate n mitochondrial dysfunctions, and to open new perspectives for the screening of mitochondria-targeted drugs that may be exploited to trigger apoptosis and eliminate dangerous cells, such as cancer cells.

In addition to mitochondria, other organelles, such as lysosomes, can be studied using fluorescent-based approaches. Studies with isolated lysosomes have been also performed in parallel experiments, but not shown in the present dissertation. Recent studies demonstrated that the lysosome communicates with mitochondria undergoing apoptosis [107-109]. The optimization of flow cytometric protocols to study lysosomal function will be a fundamental tool in the study of such cross-talk during apoptosis and its consequences for the fate of the cell.

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