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Minireview

Mitochondria-driven elimination of cancer and senescent cells

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Abstract

Mitochondria and oxidative phosphorylation (OXPHOS) are emerging as intriguing targets for efficient elimination of cancer cells. The specificity of this approach is aided by the capacity of non-proliferating non-cancerous cells to withstand oxidative insult induced by OXPHOS inhibition. Recently we discovered that mitochondrial targeting can also be employed to eliminate senescent cells, where it breaks the interplay between OXPHOS and ATP transporters that appear important for the maintenance of mitochondrial morphology and viability in the senescent setting. Hence, mitochondria/OXPHOS directed pharmacological interventions show promise in several clinically-relevant scenarios that call for selective removal of cancer and senescent cells.

Keywords: adenosine nucleotide translocase; cancer; mitochondria, oxidative phosphorylation, quiescence; senescence.

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Introduction: mitochondria and oxidative phosphorylation

Mitochondria play a key role in multiple cellular processes such as energy generation, biosynthesis, reactive oxygen species (ROS) production as well as cell death. Similar to other essential cellular functions like cytoskeleton remodeling or DNA replication, mitochondrial respiration can be targeted to induce cell death in specific situations, such as in cancer. Respiration is driven by the oxidative phosphorylation (OXPHOS) system, which resides in the mitochondrial inner membrane (MIM) and consists of the electron transport chain (ETC) and mitochondrial ATP synthase. The ETC uses electrons provided by the tricarboxylic acid cycle (TCA) in the form of NADH and succinate to reduce oxygen in a multi-step process of mitochondrial respiration, which involves respiratory complexes I-IV (CI-IV). During electron transfer to oxygen, these complexes (with the exception of CII) generate an electrostatic and proton gradient across the MIM referred to as the mitochondrial membrane potential ($\Delta\Psi_{m,i}$). $\Delta\Psi_{m,i}$ propels ATP synthase to produce ATP from ADP and inorganic phosphate. This requires ATP/ADP and phosphate transport across the MIM, carried out by adenosine nucleotide translocases (ANT1-3 in mammals) and the phosphate carrier. Hence, OXPHOS and ADP/ATP transporters are functionally interlinked, and disruption of this link can significantly contribute to cell death.

Oxidative phosphorylation and ADP/ATP transport in proliferating and non-proliferating cells

The relative importance of individual OXPHOS functions, and their relevance to cell death upon OXPHOS disruption, depends strongly on the state of cellular proliferation (Figure 1). In non-proliferating cells, such as in post-mitotic somatic cells, OXPHOS is needed to produce ATP (Figure 1B). Indeed, non-proliferative cells feature higher rates of respiration compared to cells that proliferate and have active TCA cycle, which is accompanied by efficient antioxidant defense to counter OXPHOS-derived ROS (Blecha *et al.*, 2017; Lemons *et al.*, 2010). On the other hand, in proliferating cells such as cancer cells (Figure 1A) the role of OXPHOS in ATP generation is less critical, as sufficient amounts of ATP are gained from glycolysis that is needed in these cells to supply metabolic intermediates and reducing equivalents for biosynthesis (Vander Heiden *et al.*, 2009), and the level of respiration is often, but not always, reduced. Functional OXPHOS in proliferating cells is still important to drive

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synthesis of essential metabolites such as pyrimidine nucleotides required for DNA replication in proliferating cells, and to provide ETC-derived ROS that in physiological concentrations have a pro-growth signaling function (Loffler *et al.*, 2005; Sullivan *et al.*, 2015; Weinberg *et al.*, 2010). In fact proliferating cancer cells probably aim for a respiratory ‘sweet spot’. With too little respiration, they cannot form tumors (Tan *et al.*, 2015; Weinberg *et al.*, 2010), while a certain reduction in respiration might be beneficial to modulate levels of TCA metabolites that function as signaling molecules and promote invasiveness and other typical aspects of cancer phenotype (Sciacovelli *et al.*, 2016).

The different contribution of OXPHOS to ATP production in proliferating and non-proliferating cells impacts on the ratio of ANT transporters. ANTs are abundant in the MIM and localize close to ATP synthase. ANTs play an important role in cellular energy metabolism by catalyzing the exchange of mitochondrial ATP for cytosolic ADP, thus controlling mitochondrial bioenergetics. Hence, decrease of ANT activity/expression can result in uncoupled respiration and lower efficacy of ATP synthesis (Schonfeld *et al.*, 1996). Human ANT has three isoforms (ANT1, ANT2, ANT3), whose relative expression depends on the developmental stage, proliferation status and cell or tissue types. ANT1 is highly expressed in oxidative cells where it provides ADP, the substrate for mitochondrial ATP synthase. ANT1 is the predominant isoform in the heart and skeletal muscle, and its deficiency is implicated in various pathological states (Levy *et al.*, 2000). ANT2 is preferentially expressed in proliferative and undifferentiated cells characterized by high rates of glycolysis (Barath *et al.*, 1999). Unlike ANT1 and ANT3, ANT2 is able to provide ATP for hydrolysis by the F1 complex of ATP synthase running in reverse, which maintains $\Delta\Psi$m,i and mitochondria integrity when the ETC function is impaired or its contribution to ATP generation is minor (Chevrollier *et al.*, 2011). Therefore, ANT2 induction under glycolytic metabolism may reflect cellular response to preserve mitochondrial function needed for survival. Besides $\Delta\Psi$m,i maintenance, inverse ATP/ADP exchange is also essential to provide energy for the intra-mitochondrial metabolic pathways and for the mtDNA replication and expression. ANT2 is therefore expressed prominently in cancer cells which, unlike healthy cells, intensively employ glycolysis to support proliferation and adaptation to the intra-tumoral hypoxic conditions (Jang *et al.*, 2008). Finally, unlike the ubiquitous ANT1/2, ANT3 is expressed in the liver in a tissue-specific manner.

To summarize, OXPHOS is important in both proliferating and non-proliferating cells, but each situation will emphasize its unique functional aspects. This is mirrored by the repertoire

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of the ANT isoforms that regulate ATP/ADP exchange across the MIM, depending on the metabolic needs of the cell.

OXPHOS inhibition and cell death

Given the many functions of OXPHOS, it is logical that its suppression may result in cell death, which will depend on the particular circumstances of the cell and on environmental factors such as nutrient availability. When discounting extrinsic, OXPHOS-unrelated factors, the susceptibility to OXPHOS disruption may be defined by its aggregate effect on ATP and ROS production, and on the biosynthesis of essential metabolites. Accordingly, in proliferating and cancer cells that are not deprived of nutrients, ETC-derived ROS, produced from the highly reduced redox centers upstream of the ETC/OXPHOS blockade, will be the most important factor in cell death induction, which is contributed to by the less efficient antioxidant defense in these proliferating and cancer cells (Blecha *et al.*, 2017; Kluckova *et al.*, 2015) (Figure 2A). If nutrients such as aspartate or pyruvate become limiting, OXPHOS inhibition may also interfere with anabolic pathways such as nucleotide synthesis (Garcia-Bermudez *et al.*, 2018; Molina *et al.*, 2018; Sullivan *et al.*, 2018). OXPHOS dysfunction results in the failure to produce aspartate, a nucleotide precursor, and to convert dihydroorotate into orotate in the *de novo* pyrimidine synthesis pathway (Gregoire *et al.*, 1984; Sullivan *et al.*, 2015). Importantly, while the increase in ROS, when sufficiently robust, will induce cell death in most proliferating cells, interference with the OXPHOS-linked biosynthetic pathways may result in most circumstances in the temporary arrest of proliferation rather than in cell death (Gui *et al.*, 2016; Loffler, 1980).

When properly supplied with nutrients, such as in normal perfused tissues, non-proliferating somatic cells are resistant to ETC inhibition-induced ROS, as these are efficiently neutralized by the elevated antioxidant defense (Blecha *et al.*, 2017) (Figure 2B). However, non-proliferating cells in metabolically compromised environments, such as poorly perfused areas of tumors, are highly susceptible to OXPHOS inhibition-induced ATP depletion, because they cannot compensate the deficit in mitochondrial ATP generation by glycolysis. They fail to scavenge glucose from the extracellular milieu as they are unable to increase glucose uptake by efficient upregulation of glucose transporters in the plasma membrane (Blecha *et al.*, 2017). This is also true for quiescent-like tumor-initiating cells (Viale *et al.*, 2014), and has been shown clearly for multiple OXPHOS inhibitors in the spheroid model (Missios *et al.*,

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2014; Yan *et al.*, 2015). Hence, OXPHOS inhibitors that produce sufficient amounts of ROS will induce cell death as single agents in both proliferating cells and in non-proliferating cells in metabolically-compromised environments. On the other hand, OXPHOS inhibitors that do not produce sufficient ROS should be combined with agents such as tyrosine kinase or topoisomerase inhibitors for maximal effectivity against proliferating cancer cells, to ensure elimination of both proliferating cancer cells as well persistent quiescent-like tumor-initiating cells that mediate disease relapse (Senkowski *et al.*, 2015).

Quiescence *versus* senescence

In contrast to cancer cells that are wired for proliferation, most cells in human body are post-mitotic and quiescent. They are not actively dividing, but they do not lose the ability to re-enter the cell cycle upon appropriate stimuli. Indeed, the latent proliferative potential of post-mitotic quiescent cells plays a key role in normal physiology and tissue homeostasis. Unlike quiescence, cellular senescence represents well-established anti-cancer defense mechanism forcing potentially oncogenic cells into a cell cycle arrest (Munoz-Espin and Serrano, 2014). Tumor cells may become senescent for example in response to conventional chemotherapy due to development of chronic DNA damage, because of oncogene activation, or spontaneously as a result of oxygen or nutrient deprivation. Senescent cells also accumulate in many normal tissues naturally with age as a consequence of depletion of the proliferative potential of the cell, which limits their uncontrolled proliferation and decreases risk of tumorigenesis. To enter senescence the $p53/p21^{waf1}$ and/or $p16^{INK4a}$/Rb pathways need to be engaged, followed by inhibition of cyclin dependent kinases (CDKs) via activated checkpoints. While cellular senescence was originally described as an irreversible cell cycle arrest, several lines of evidence now indicate that senescence may actually be reversible. For example, acute inactivation of Rb protein, but not the germline loss of Rb function, in senescent cells results in the reversal of the senescent phenotype leading to cell cycle re-entry (Sage *et al.*, 2003). Furthermore, while induction of $p16^{INK4a}$ prevents phosphorylation of Rb protein and leads to G1 arrest in senescent cells, its inhibition on the background of inactivated p53 resumed rapid growth of senescent cells, indicating crucial relationship between Rb and p53 proteins in senescence induction and maintenance (Beausejour *et al.*, 2003). Recently it was also found that cells released from senescence and re-enter the cell

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cycle strongly enhanced Wnt-dependent clonogenic growth potential presenting cancer stem-cell like markers (Milanovic *et al.*, 2018).

Senescent cells produce various cytokines, chemokines, proteases and growth factors (collectively referred to as 'senescence-associated secretory phenotype, SASP), affecting their immediate microenvironment as well as distant sites in a paracrine manner (Sabin and Anderson, 2011). Cytokine production by pre-malignant cells turned senescent promotes recruitment of immune cells, and immune-mediated clearance of these cells helps to prevent tumor initiation (Kang *et al.*, 2011). However, prolonged existence of senescent cells due to inefficient clearance results in pro-tumor effects on neighboring cells, such as induction of proliferation via pro-inflammatory cytokines and growth factors (Eggert *et al.*, 2016). This paracrine SASP-mediated pro-inflammatory signaling could therefore explain how relatively low numbers of senescent cells can promote a systemic failure, since production of SASP contributes to chronic inflammation, loss of tissue function and its regeneration (Hubackova *et al.*, 2016). Therefore, even though in the beginning senescence represents an anti-cancer barrier because of the proliferation arrest and elimination of potential oncogenic cells, persistence of senescent cells in the organism for example due to faulty clearance by immune cells is detrimental. It results in exacerbation of tissue damage and development of age-related diseases (Minamino *et al.*, 2009; Sone and Kagawa, 2005), and promotes adverse effects of chemotherapy and cancer relapse by production of pro-inflammatory cytokines (Demaria *et al.*, 2017).

Previous studies on prematurely aged transgenic mice with forced induction of apoptosis in $p16^{Ink4a}$ expressing cells showed correlation between senescent cell removal and delay of the 'aged' (senescent) phenotype (Baker *et al.*, 2011). Furthermore, late-life clearance of senescent cells attenuates the progression of already established age-related disorders. It was also observed that accumulation of senescent cells negatively influences the life-span (Baker *et al.*, 2016) as well as adaptive thermogenesis (Berry *et al.*, 2017). Since senescent cells are resistant to apoptotic stimuli, targeting of anti-apoptotic Bcl-2 protein by specific inhibitors leads to elimination of senescent cells, being beneficial for "rejuvenation" of aged hematopoietic stem cells and restoration of tissue homeostasis (Chang *et al.*, 2016). Similarly, removing of senescent cells using combination of SRC kinase inhibitor dasatinib and antioxidant quercetin reduced hepatic steatosis in aged, obese and diabetic mice (Ogrodnik *et al.*, 2017), and prolonged their life span (Xu *et al.*, 2018).

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Senescent cells remain metabolically highly active, perhaps even more so than quiescent cells (Figure 1C). Increased oxygen consumption, energy production, lipid catabolism and levels of ROS due to hyperactive mitochondrial respiration indicate a robust metabolic shift (Dorr *et al.*, 2013). Kaplon and colleagues have recently shown that increased OXPHOS is crucial for the induction of oncogene-induced senescence (Kaplon *et al.*, 2013), accompanied by upregulation of PGC1α, a 'master regulator' of mitochondrial biogenesis; this yields increased respiration, antioxidant defense and fatty acid oxidation (St-Pierre *et al.*, 2006). They also found a role of the mitochondrial 'gate-keeper' pyruvate dehydrogenase (PDH) as a crucial mediator of senescence induced by the BRAFV600E mutation. Suppression of the PDH inhibitory enzyme pyruvate dehydrogenase kinase 1 (PDK1) and induction of the PDH-activating enzyme pyruvate dehydrogenase phosphatase 2 (PDP2) resulted in enhanced use of pyruvate in the tricarboxylic acid (TCA) cycle, causing increased respiration, redox stress and development of senescence. It was shown that high production of SASP factors and senescence-related ROS generate the endoplasmic reticulum (ER) stress. ER stress promotes formation of misfolded or toxic proteins, which initiates energy-intensive repair mechanisms. Therefore, blocking ATP synthesis or glucose utilization leads to elimination of senescent cells through caspase-12- and caspase-3-dependent apoptosis (Hutter *et al.*, 2004).

Mitochondria may link age-related accumulation of oxidative damage caused by ROS produced due to declining ETC function to physiologic alterations associated with aging. ROS, such as the superoxide radical anion, H_2O_2, and the hydroxyl radical, can attack multiple cell components and enhance senescent phenotype with cytokine production, which could result in spreading of senescence. To maintain homeostasis in aged cells, mitochondria import calcium to preserve the ETC function, but as a consequence this also depolarizes mitochondria, resulting in decreased overall ATP production and increased ADP/ATP ratio. AMPK in response to this elevation activates a series of compensatory responses including fatty acid oxidation, inhibition of fatty acid synthesis and stimulation of glucose uptake. Another way for senescent cells to maintain mitochondrial homeostasis after ETC dysfunction is upregulation of mitochondrial fusion, which allows mitochondria to maintain cristae, stimulate more ATP synthase activity, prevent mitochondrial membrane depolarization and escape autophagic/mitophagic degradation (Palikaras and Tavernarakis, 2014).

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Mitochondrial targeting in senescent cells

Altered mitochondrial function is an important determinant of the senescent phenotype. Targeting mitochondria in senescent cells is therefore a logical way to eliminate senescent cells in the context of pathological senescence as well as senescence-associated diseases. Hubackova *et al.* recently reported the role for mitochondria in specific elimination of senescent cells using mitochondria-targeted tamoxifen (MitoTam) (Hubackova *et al.*, 2018). MitoTam, tamoxifen tagged with a triphenyl phosphonium-group (Zielonka *et al.*, 2017), was originally developed as an anti-cancer agent targeting CI in the MIM (Rohlenova *et al.*, 2017). MitoTam interferes with the electron transfer from the catalytic NDUFV1 subunit to ubiquione at the Q module of CI. Unlike other CI inhibitors such as IACS-010759, BAY 87-2243 or metformin considered for clinical use (Chae *et al.*, 2016; Ellinghaus *et al.*, 2013; Molina *et al.*, 2018), MitoTam also dissipates $\Delta\Psi_{m,i}$, which contributes to its cytotoxicity in senescent cells (Figure 2C). In cancer cells, MitoTam induced cell death most efficiently when the rate of OXPHOS and its assembly state into respiratory supercomplexes were high. In this situation, OXPHOS inhibition at CI led to efficient ROS-mediated cell death enhanced by disruption of respiratory supercomplexes, which further increased ROS (Rohlenova *et al.*, 2017). While MitoTam was highly efficient in the elimination of senescent cells, unrelated ETC inhibitors, directed against CI or other respiratory complexes, were rather inefficient in cell death induction in the senescent settings. This disproves the notion that OXPHOS inhibition by MitoTam or the other ETC inhibitors is sufficient for the elimination of senescent cells. Unlike other ETC inhibitors, MitoTam rapidly disrupts mitochondrial morphology in a manner consistent with the simultaneous inhibition of OXPHOS and dissipation of $\Delta\Psi_{m,i}$ (Rohlenova *et al.*, 2017). When $\Delta\Psi_{m,i}$ is compromised, ATP synthase switches to a reverse mode (i.e. cleaving ATP to ADP+P_i) to maintain residual $\Delta\Psi_{m,i}$. In this scenario, ANT2 supplies ATP from glycolysis that is limiting for the reverse function of ATP synthase. ANT2 levels decrease in senescent cells (Kretova *et al.*, 2014). It is possible that ATP synthase activity is required to maintain the structural integrity of mitochondria in senescent cells under conditions of stress for example when $\Delta\Psi_{m,i}$ is reduced and OXPHOS is compromised, and ANT2 is needed to provide ATP to maintain ATP synthase active. This could explain why the ATP synthase inhibitor oligomycin A induced cell death in senescent cells selectively similarly to MitoTam, while other ETC inhibitors were ineffectual. Importantly, the adverse effect of MitoTam on mitochondrial integrity in senescent cells was reversed by the overexpression of ANT2 in these cells. Therefore, the mechanism of cell

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death induction by MitoTam in senescent cells most likely involves the combined effect of uncoupling and CI inhibition, and is linked to the loss of mitochondrial integrity associated with the lower expression of ANT2 in senescent cells.

Conclusions

Mitochondrial/OXPHOS targeting induces cell death in cancer cells, non-proliferating cells and senescent cells by different mechanisms. In cancer cells, ROS resulting from OXPHOS inhibition play the major role, as cancer cells cannot withstand the oxidative insult due to the limitation of available antioxidant capacity. OXPHOS inhibitors that do not produce ROS will still interfere with the biosynthetic functions, resulting in growth suppression but not cell death in most proliferating cells. In contrast, quiescent and senescent cells are protected from ROS-induced cell death by their increased antioxidant defense and do not depend on OXPHOS-linked biosynthesis. Therefore, the failure to compensate for the loss of OXPHOS-derived ATP seems the major OXPHOS-linked sensitivity in quiescent cells, while senescent cells are exclusively susceptible to the perturbation of mitochondrial integrity. OXPHOS-directed mitochondrially targeted agents, while developed against proliferating cancer cells, can be repurposed towards elimination of drug-resistant, non-proliferating tumor-initiating cells as well as spontaneous or chemotherapy-induced, tumor-derived and non-tumor senescent cells. In this context, mitochondria-directed anti-cancer agents such as MitoTam, now entering a phase I clinical trial in cancer, might provide a new therapeutic modality to treat senescence-associated pathologies.

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Figure legends

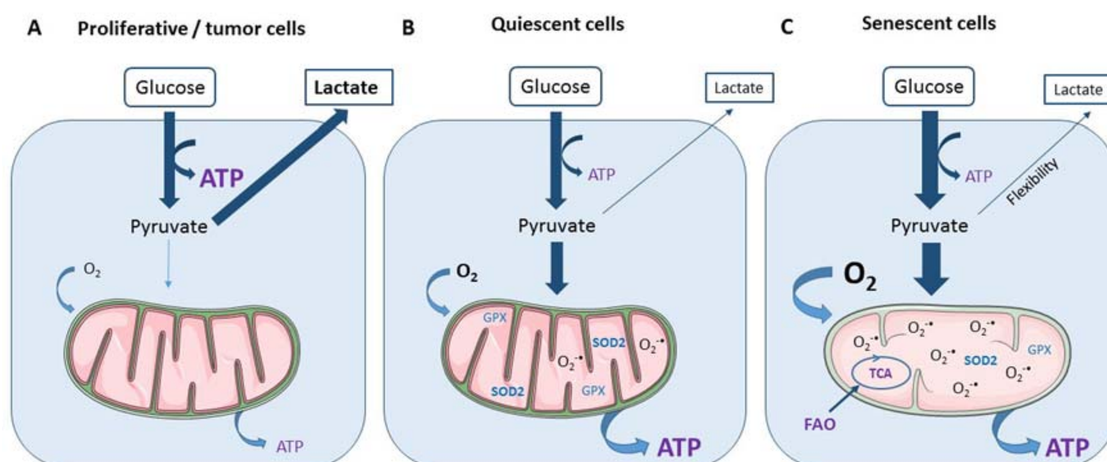

Figure 1 Energy production in cancer, quiescent and senescent cells.

(A) In cancer cells and proliferating cells, a large fraction of cellular ATP is produced by glycolysis, which is upregulated to satisfy the requirements of biosynthesis. (B) In quiescent cells most ATP is produced by OXPHOS. The glycolytic contribution to ATP production is minor and cannot be easily increased. (C) Senescent cells, like quiescent cells, produce most ATP in OXPHOS, but their glycolytic ATP supply can be readily upregulated when required. SOD2 – superoxide dismutase 2, GPX1 – glutathione peroxidase 1, FAO – fatty acid oxidation.

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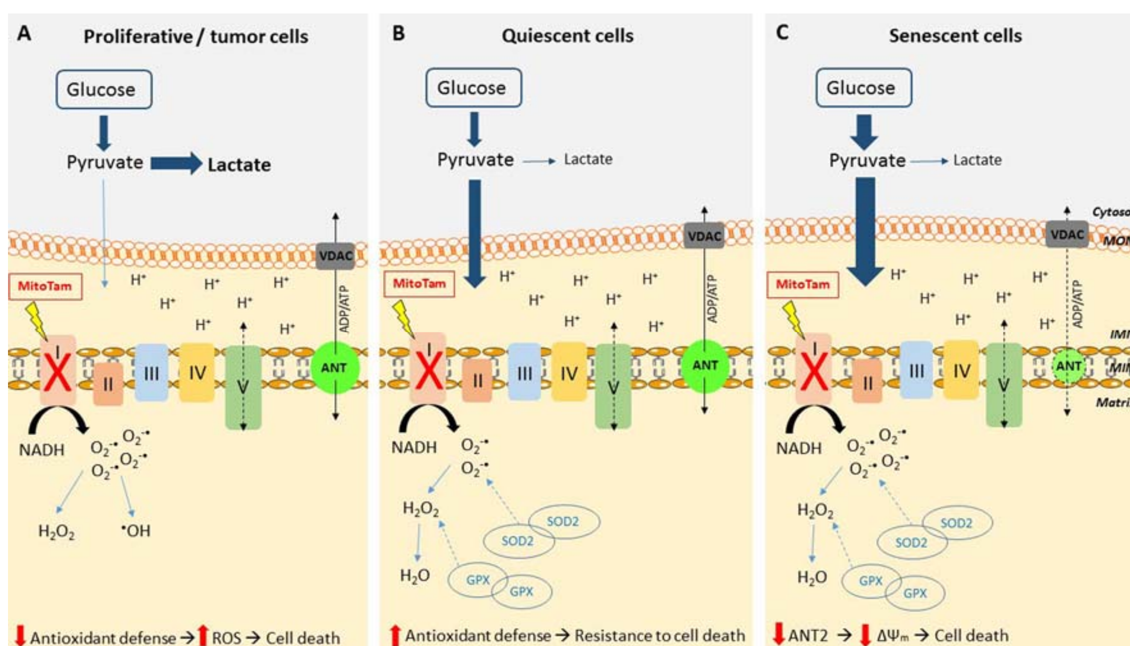

Figure 2 Cell death induction by MitoTam in cancer, quiescent and senescent cells. (A) Glycolysis is the main source of energy in normal proliferating and cancer cells, OXPHOS function is secondary. These cells therefore do not feature high mitochondrial antioxidant defense capability. Blockade of CI by MitoTam leads to increase generation of ROS, which are not properly cleared and result in cell death. (B) OXPHOS is key for ATP production and the antioxidant defense is strong in non-proliferating quiescence cells. The ROS induced by MitoTam upon CI inhibition are therefore efficiently cleared when glucose is in sufficient supply. It should be noted that when glucose supply is limiting (unusual in normal perfused tissues but quite frequent in tumors) these cells cannot efficiently switch to ATP production by glycolysis and are also killed MitoTam. (C) In senescent cells, ATP production in OXPHOS is also important and antioxidant defense is high. Therefore, ROS induction after CI inhibition by MitoTam will not induce cell death, and senescent cells are flexible in ATP production that can be switched to glycolysis even in low glucose conditions. However, these cells are exquisitely sensitive to the combined effect of MitoTam on CI respiration and the loss of $\Delta\Psi_{m,i}$. This is due to the faulty ATP/ADP exchange across the MIM, resulting from low ANT2 expression in senescent cells, which prevents maintenance of mitochondrial integrity by mitochondrial ATP synthase under such stress conditions. SOD2 – superoxide dismutase 2, GPX1 – glutathione peroxidase 1, I – complex I, II – complex II, III – complex III, IV – complex IV, V – complex V (ATP synthase).

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