Chapter 19

Targeting Mitophagy in Combined Therapies of Haematological Malignancies

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Abstract

Mitophagy is a selective form of autophagy that eliminates mitochondria and is part of a larger network of mitochondrial quality control processes that respond to mitochondrial damage. Treatment of haematological malignancies often involves drugs that ultimately cause cell death by mitochondrial injury and initiation of apoptosis. Thus, mitophagy is a potential cause of resistance to anticancer drugs that target the mitochondria (mitocans). Since mitophagy is integrated to mitochondrial biogenesis, mitochondrial fission and fusion, the bioenergetics profile and metabolic reprogramming of tumour cells, the blockage of mitophagy may not be sufficient to overcome resistance. In addition, the mitochondrial unfolded protein response and the outer mitochondrial membrane-associated degradation have extensive crosstalk with mitophagy, and advanced forms of neoplasms will require targeting both systems. Proteasome inhibitors and vinca alkaloids target many of the critical steps involved in resistance to mitocans, while inducers of mitochondrial turnover (biogenesis and mitophagy) like valproic acid have a variable effect depending on metabolic reprograming and the activity of oxidative phosphorylation of tumour cells. Here we discuss the mechanisms of mitophagy and its associated mechanisms, and discuss its application to the rationale of targeted combined therapies of low- and high-grade B-cell neoplasms.

Keywords: mitocans, arsenic trioxide, BNIP3, Parkin, aggresome, Proteasome inhibitors, valproic acid, vincristine, mitochondrial dynamics, mitochondrial turnover, metabolic reprogramming, lymphoma, myeloma, chronic lymphocytic leukemia (CLL)

1. Introduction

Autophagy is a cell response that aims to recycle proteins, cytoplasmic components and even organelles particularly under starvation conditions [1]. Mitochondria are one of the many organelles and cytoplasmic components that can be identified as the cargo within autophagosomes. More recently, this kind of autophagy has been referred to as bulk or non-selective autophagy, to underscore that there is no particular selection of the cellular components that may enter the autophagy process. The main driver of nutrient depletion autophagy is a catabolic response to provide amino acids and support metabolic pathways such as gluconeogenesis and ketogenesis. By contrast, mitophagy is defined as the selective autophagy of mitochondria [2]. In fact, elimination of mitochondria is particularly avoided in nutrient depletion autophagy. Since mitochondria is the source of ATP in normoxia, supports lipid biosynthesis, gluconeogenesis, ketogenesis and many other metabolic functions that take place precisely under nutrient depletion, mitochondria are spare as much as possible under catabolic conditions [3]. One of the first metabolic contexts where mitochondria were found to be eliminated with selectivity was hypoxia. Even under normal nutrient conditions, cells exposed to hypoxia will undergo increased oxidative stress. The absence of O_2 causes abnormal function of the electron transport chain (ETC) and oxidative phosphorylation (OXPHOS), and this leads to increased superoxide anion leakage and reactive oxygen species (ROS) production [4]. The main driver of response to hypoxia is the increase in mitochondrial ROS, which stabilizes the hypoxia inducing factor 1α (HIF-1α) by preventing its proteasome degradation. HIF-1α is a transcription factor that orchestrates an array of changes in mitochondrial proteins to reduce OXPHOS and particularly promotes mitophagy resulting in the reduction of mitochondrial mass. Nevertheless, mitophagy may occur under many other conditions leading to abnormal function of mitochondria, particularly if it involves increased mitochondrial ROS (mtROS). Increased mtROS can lead to collapse of mitochondrial membrane potential (MMP), mitochondrial outer membrane permeabilization (MOMP), release of cytochrome c and initiation of intrinsic apoptosis. However, elimination of mitochondria by mitophagy prior to MOMP may prevent apoptosis particularly if the production of mtROS is not massive.

We review how mitophagy is integrated to mitochondrial quality control (QC), mitochondrial turnover and mitochondrial dynamics, and we discuss the interdependency with the bioenergetics profile of the cell. This knowledge will be further considered to discuss its implication in resistance to treatment of haematological malignancies, and how combined therapies can be selected to target mitophagy, mitochondrial biogenesis and compensating mechanisms of resistance that involve the ubiquitin proteasome system (UPS), particularly in the most aggressive forms of lymphomas. We discuss the use of drugs already in clinical use that may be potentially combined based on their recognized effects on mitophagy, autophagy and mitochondrial biogenesis. We finally discuss methods that can help determine the status of resistance mechanisms based on mitophagy in lymphoma cells obtained from patients.

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2. Mitophagy and mitochondrial turnover

2.1. Mitophagy is coupled to biogenesis and both occur at perinuclear areas

Mitochondria are far from being static and frequently change their shape and size. Even in a single cell they are not equal to one another, a condition called heteroplasmy, and there is still a great variation depending on the cell type. Recently the term "mitochondrial behaviour" was proposed to embrace all these properties and states, underscoring the need to integrate the many dimensions of mitochondrial study [5]. Mitochondria spread around the cell by attaching to the microtubule network and move to the areas of high energy demands to provide OXPHOS-derived ATP. Even though mitophagy reduces the mitochondrial mass, this process is coupled to mitochondrial biogenesis through a homeostatic loop, in order to keep the mitochondrial mass in accordance with the bioenergetics demands of the cell [6]. Mitochondrial biogenesis requires duplication of mitochondrial DNA (mtDNA), the expression of new mtDNA-coded proteins, nuclear DNA-coded mitochondrial proteins and a huge work of mitochondrial protein import. This process is controlled by the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) transcription factor that regulates the expression of a large number of nuclear-encoded mitochondrial genes. Biogenesis involves elongation of existing mitochondria and further fission of the newly formed units (**Figure 1**). In fact, what is known as a mitochondrion may contain several units, each one with a single copy of mtDNA forming a nucleoid, and a completely assembled respiratory chain complex. Remarkably, the process of elongation and protein import associated with biogenesis occurs in perinuclear mitochondria [7]. The induction of biogenesis after mitophagy is often referred as the nuclear retrograde response, and is an important regulator of mitochondrial turnover because it leads to changes in the grade of heteroplasmy of the entire mitochondrial network. In cases of mutated mtDNA the grade of heteroplasmy may be critical for the severity of mitochondrial damage or malfunction.

2.2. Mitophagy is initiated after mitochondrial fission

Mitochondrial biogenesis involves elongation and fission, but fission also occurs apart from biogenesis [8]. In fact, mitochondria may be divided at positions dictated by a surrounding endoplasmic reticulum (ER) that corresponds to the joint between internal units. These sites are known as sites of ER-associated mitochondrial division (ERMD) [9]. At these sites dynamin relates protein 1 (DRP1) is recruited to constrict and further split mitochondria during fission (**Figure 1**). Fission allows the segregation of individual units with impaired or abnormal function that can no longer sustain a normal OXPHOS, the MMP, and the production of ATP [10]. Mitochondria with otherwise normal function can fuse again by the activation of Mfn1 and Mfn2, which accomplish fusion of the outer mitochondrial membrane (OMM), and OPA1 that completes fusion of internal mitochondrial membrane (IMM) [5]. In contrast, segregated mitochondria with abnormal function can no longer fuse again due to inactivation of Mfn1 and OPA1 particularly due to collapsed MMP. Thus, fission and segregation of mitochondria with collapsed MMP is the first step in the process of mitophagy (**Figure 1**) [10]. During mitophagy, the tip-ends of split mitochondria are kept sufficiently closed to prevent MOMP

and cytochrome c release [11]. However, the particular phospholipid cardiolipin (CL), which is normally concentrated in the IMM, is partially exposed at the OMM. CL at the OMM acts as an "eat me" signal to initiate mitophagy, much in the same way as phophatidylserine acts as an "eat me" signal in the cell membrane during apoptosis [12]. The collapse of MMP acts to stabilize Pink1 and recruits the E3 ubiquitin ligase Parkin, while further attachment of p62 at the OMM initiates the formation of the mitophagosome [13]. In contrast to mitophagy, during intrinsic apoptosis the entire mitochondrial network undergoes fission, and at these tip-ends occurs a massive oxidation of CL that leads to MOMP, cytochrome c oxidation and further release to the cytosol.

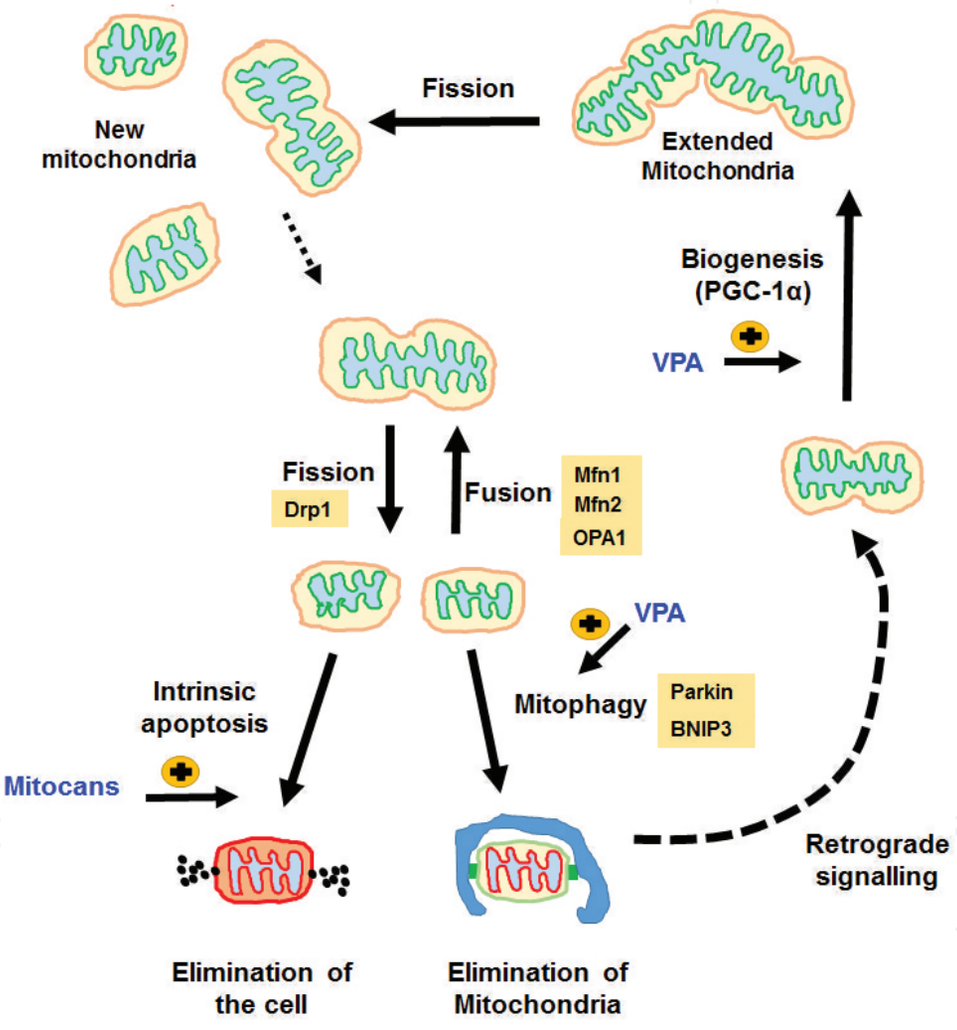

Figure 1. Transitional states of mitochondria.

Mitochondria undergo rounds of fusion and fission. Mitophagy is initiated after fission as part of mitochondrial quality control. Fission involves Drp1, while fusion requires Mfn1, Mfn2 and OPA1. Parkin and BNIP3 are the two most common mitophagy receptors. Mitophagy is coupled to biogenesis through the retrograde nuclear signalling. Mitochondrial biogenesis is initiated by mtDNA duplication and elongation, with assembly of a completely new nucleoid

and respiratory unit. The elongated mitochondria start undergoing fission ad fusion. Mitocans are anticancer drugs that target the mitochondria and induce intrinsic apoptosis after inflicting different sorts of mitochondrial damage. Mitophagy can counterbalance this damage by removing compromised mitochondria; while mitochondrial biogenesis completes a turnover cycle by maintaining mitochondrial mass. Valproic acid (VPA) induces mitophagy by upregulation of BNIP3 and mitochondrial biogenesis by upregulating PGC-1α.

2.3. Bioenergetics implications of mitochondrial fusion and fission-metabolic reprogramming

Mitophagy and mitochondrial biogenesis do not provide a complete picture of the regulation of mitochondrial network, unless it is integrated to the metabolic changes associated with mitochondrial dynamics, which is a term that is used to refer collectively to mitochondrial fusion and fission. A hyperfused network in normoxia often denotes a high respiratory rate with high OXPHOS activity, O_2 consumption, ATP production, high MMP and increased mitochondrial mass. The cycles of fission and fusion contribute to exchange mitochondrial components, dilute any defects and improve efficiency of respiration. In contrast, a network with a predominance of fission is often indicative of low OXPHOS-derived ATP, low O_2 consumption, high glycolysis rate and decreased mitochondrial mass, with low exchange of mitochondrial components. Metabolic reprogramming is a characteristic of cancer cells that enhances their ability to survive under adverse conditions such as hypoxia and nutrient deprivation [14]. Cancer cells may use either glycolysis or OXPHOS as a source of ATP, and these two alternatives may be not mutually exclusive, and can vary during the progression of the disease depending on several factors. OXPHOS redirection towards lipid and protein synthesis, truncation of Krebs's cycle and glycolysis can all be regulated to support tumour growth, meeting particular demands that appear under harsh growing conditions, such as glucose and other nutrients deprivation, lack of oxygen and the expression of particular oncogenes [15]. The oncogene c-MYC, which is characteristically overexpressed in high-grade Burkitt´s lymphoma, participates in this regulation through the expression of genes required for either OXPHOS or glycolysis [15]. We will next consider a heterogeneous group of B-cell lymphomas as an example to discuss the role of mitophagy in disease progression and response to treatment with a particular emphasis on metabolic reprogramming.

2.4. Metabolic features of low- and high-grade lymphomas

Mature B-cell neoplasms are broadly classified as low-grade (indolent) and high-grade (aggressive) considering the severity of symptoms, rate of progression and response to treatment [16]. The most frequent low-grade B-cell neoplasm is chronic lymphocytic leukaemia (CLL) also denoted as small lymphocytic lymphoma. Among high-grade B-cell lymphomas, the most frequent ones are diffuse large B cell lymphoma (DLBCL) and Burkitt´s lymphoma. CLL is a small cell lymphoma with a large number of non-dividing mature B-cells circulating in the peripheral blood. These B-cells may enter the lymph nodes where they can survive under a hypoxic environment (hypoxic niche), receive growth signals and replicate in a location often protected from the effect of anticancer drugs. However, CLL cells circulating in the peripheral

blood show activated HIF-1α, even though they are exposed to normoxic conditions. Progression of CLL often leads to a transformation into high-grade lymphomas such as DLBCL, a process designated as Ritcher´s transformation [17]. Metabolic reprogramming parallels to some extent the grading and aggressiveness of these lymphomas [18]. In one extreme, CLL cells appear as small mature lymphocytes with metabolic features much similar to normal B-cells. This includes an efficient use of OXPHOS, low rates of glycolysis with a peripheral arrangement of predominantly fused mitochondria. By contrast, high-grade lymphoma cells appear as large immature cells with very high rates of glycolysis, low use of O_2 and OXPHOS, high production of lactic acid and an extensive use of glucose and glutamine. High-grade lymphomas may show high rates of mitophagy and autophagy with a network distribution that is often more perinuclear than peripheral [19]. When metabolically reprogrammed cells do not derive ATP from OXPHOS, they are less vulnerable to mtROS under hypoxia.

2.5. Mitophagy and metabolic reprogramming cause resistance to mitocans

Mitocans are a heterogeneous group of anticancer drugs that target the mitochondria and initiate apoptosis [20]. Mitocans may enhance OXPHOS (hexoquinase II blockers, sodium dichloroacetate, 2-bromopyruvate), block the ETC (tamoxifen, adaphostin), oxidize thiol groups and deplete mitochondrial glutathione (arsenic trioxide) or destabilize VDAC. Eventually, mitocans increase mtROS and initiate apoptosis by inducing MOMP. Mitocans like arsenic trioxide (ATO) are used to treat promyelocytic leukaemia but other haematological malignancies are often resistant [21]. ATO targets the ETC and oxidizes thiol groups by increasing mtROS. Metabolic reprogramming and mitophagy can have a great influence in resistance to the induction of apoptosis by ATO or other mitocans. In low-grade lymphomas mitocans like ATO that target the ETC and alter OXPHOS, induce massive mtROS and trigger apoptosis. However, the cytotoxicity of ATO is significantly reduced in high-grade lymphomas because of their low OXPHOS-dependency and high rates of mitophagy with increased mitochondrial turnover [19]. ATO as other mitocans can hardly inflict mitochondrial damage in these OXPHOS-independent cells, and in addition, damaged mitochondria are effectively replaced by a high mitochondrial turnover. A high rate of mitochondrial biogenesis is also required to assist rapid proliferation, which is a characteristic feature of these high-grade lymphoma cells.

2.6. Transport of mitophagosomes over the microtubule network

Mitophagy is initiated after fission and segregation of mitochondria with collapsed MMP. The first step involves the role of the E3 ubiquitin ligase Parkin [13]. Many proteins of OMM become ubiquitinated by Parkin. Among these Mfn1, Mfn2, Miro and Paris help to highlight the interaction of mitophagy with mitochondrial turnover and dynamics. Ubiquitination of Mfn1 precludes further fusion, ubiquitination of Miro immobilizes mitochondria and fixes it to the microtubule network, and ubiquitination of Paris causes an increase in the expression of PGC-1α, initiating the nuclear retrograde signalling that leads to biogenesis [22, 23]. Interestingly, Parkin is able to carry on K48 and K63 linked polyubiquitination, but recent studies support a preferential K63, K11 and K6 links for Parkin [24]. While K48 is a polyubiquitin

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modification known to target proteasome degradation, K63-linked polyubiquitin has been more specifically related to mitophagy, and even the mitochondrial deubiquitinase (DUB) USP30 was shown to block Parkin-mediated mitophagy by selectively eliminating this kind of polyubiquitins [25]. The ubiquitination of OMM proteins recruits p62, a linker molecule that has a ubiquitin binding domain (UBD) and a LC3 interaction region (LIR) domain, and recruits LC3 to initiate the formation of the mitophagosome by sequestering the ubiquitinated organelle (**Figure 2**) [26]. This initial phase has extensive opportunities for crosstalk between the UPS and mitophagy. A mitophagy receptor is defined as a molecule that is shuttled to the OMM, where it interacts with LC3 or gamma-aminobutyric acid receptor-associated protein (GABARAP) to initiate the mitophagosome formation [27]. BNIP3 and NIX are two mitophagy receptors that respond to increase in mtROS by inserting into the OMM [28]. These BH3-only molecules of the Bcl2 family have an LIR domain to initiate the formation of the LC3-decorated mitophagosome, even in the absence of p62 (**Figure 2**). BNIP3 can even cause the recruitment of Parkin to the OMM facilitating also the p62-dependent mitophagy pathway. Nix in particular is responsible for mitophagy during the normal maturation of erythrocytes [29]. The maturation of mitophagosomes ultimately leads to the fusion with lysosomes. This requires the transport of mitophagosomes along the microtubule network and is critical to complete the mitophagy process. The encounter of mitophagosomes and lysosomes often occurs at the perinuclear area at the minus end of the microtubule network. This is often quite evident in high-grade lymphoma cells as well as other aggressive tumour cells. In fact, the term mitoaggresome has been used to describe the occurrence of this end stage of mitophagy at the perinuclear area [30]. Mitochondrial elongation and biogenesis occur in mitochondria located at the perinuclear area. Thus, mitophagy and the nuclear retrograde response appear to occur at perinuclear location, having significance to interpret the occurrence of perinuclear mitochondrial clusters (PNMC) in high-grade B-cell neoplasms as suggestive of high basal mitochondrial turnover [19].

2.7. Mitophagy and the mitochondrial unfolded protein response (mtUPR)

The term aggresome is used to describe aggregates of ubiquitinated proteins located at perinuclear areas. These perinuclear areas close to the microtubule organizing centre (MTOC) are enriched with proteasomes and function as proteolytic centres. Aggresomes are formed when these proteolytic centres are no longer able to cope with an overload of misfolded ubiquitinated proteins [31, 32]. The transport of ubiquitinated proteins to the proteolytic centres depends on dynein motor-proteins that move along the microtubule network towards the minus end at the MTOC. Elimination of misfolded proteins and aggresome formation are part of the unfolded protein response (UPR) that occurs upon ER-stress [33]. Impaired proteolysis by inhibition of proteasomes increases the occurrence of aggresomes. Once aggresomes are formed, they cannot be cleared by proteasomes and in fact, persistence of aggresomes leads to proteasome inhibition and apoptosis. However, aggresomes may be cleared by autophagy. This involves a modification of K48-linked polyubiquitination to K63-linked polyubiquitination and involves the histone deacetylase HDAC6 [34]. Mitochondria have their own protein QC system that includes the proteases Lon and ClpX in the matrix, and i-AAA in the inter-membrane space. The mitochondrial unfolded protein response (mtUPR)

is regulated by the transcription factor CHOP and involves upregulation of proteases and chaperones [35]. When this system is overloaded, misfolded mitochondrial proteins are exposed at the OMM where they become ubiquitinated and shuttled for proteasome degradation. Because of the similarity with ER-associated degradation (ERAD), this mitochondrial process has recently been designated as OMM-associated degradation (OMMAD) [2]. This stress pathway functions as a mitochondrial protein QC independently of mitophagy and may be similar to the ER-stress associated UPR, involving K48 ubiquitination of OMM misfolded proteins [35]. Mitochondrial UPR and mitophagy work in an integrated manner. If the amount of mitochondrial damage is low, mtUPR and OMMAD may be enough to achieve the required mitochondrial protein quality control. However, a more severe damage would require mitophagy as a higher level of mitochondrial quality control leading to recycling of the whole organelle.

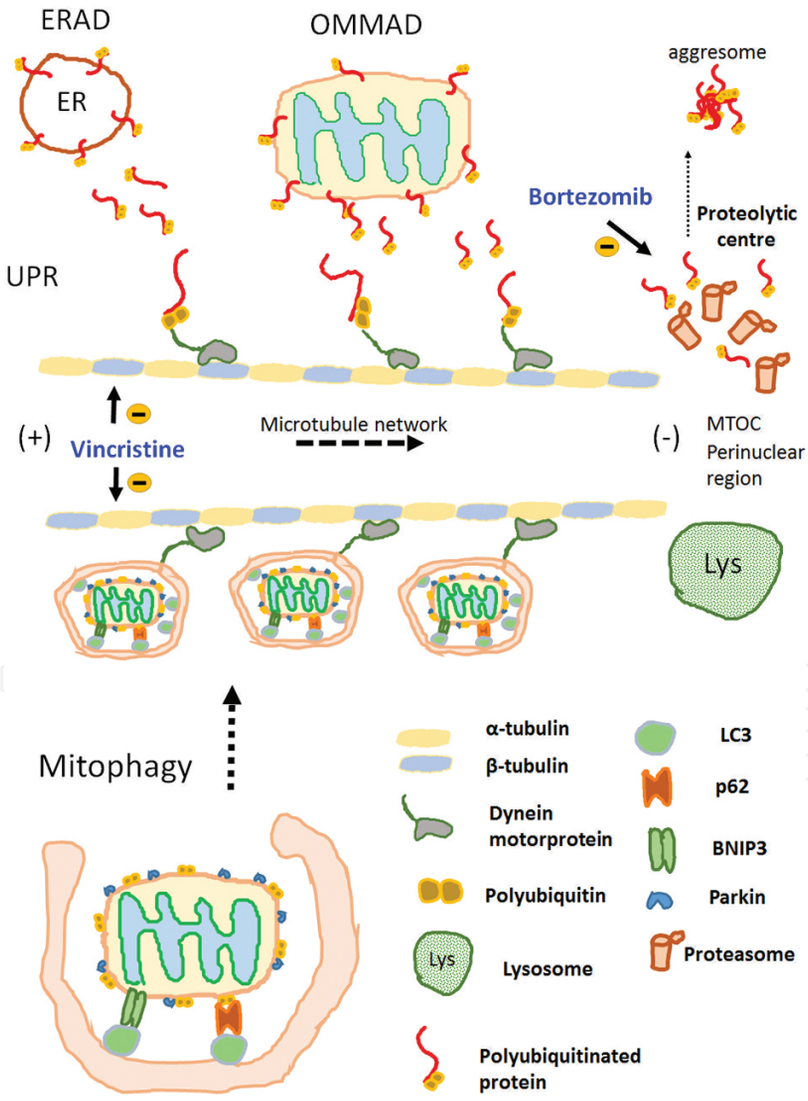

Figure 2. Mitophagy, OMMAD and ERAD require oriented transport through microtubules.

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2.8. The transport to the perinuclear region in UPR and mitophagy

The transport of mitophagosomes and autophagosomes along the microtubule is required for fusion with lysosomes [36]. In fact, not only mitophagosomes and autophagosomes but also the entire mitochondrial network moves along microtubules. Mitochondrial biogenesis, fusion, fission and mitophagy also occur over the microtubule network, and drugs affecting the microtubule network have a profound impact on all of these processes. Microtubules metaphorically represent the railways over which cargo-carrying motor-proteins move along. Kinesins are motor-proteins that carry cargo towards the positive end of microtubules that is at the cell periphery. In contrast, dyneins carry cargo towards the minus end at the perinuclear area. In a pioneer study, Lee et al. used nocodazole and overexpression of dynamitin to cause inhibition of the motor-protein dynein, and prove that QC mitophagy involved the transport of mitophagosomes through motor-proteins towards the perinuclear area, to fuse with lysosomes at the PNMC (**Figure 2**) [30]. Even though mitophagy can be blocked very specifically by knocking down the motor-protein dynein, blocking the dynamics of the microtubule has a profound impact on all motor-proteins moving along the network. Vincristine, as other vinca alkaloids, binds to α tubulin and prevents further polymerization, leading to a complete destabilization of the network. These drugs compromise the entire dynamics of the microtubule network and have profound impact on all cargo-carrying motor-proteins (**Figure 2**). This explains why cells are arrested in metaphase during mitosis, but also explains why mitophagy and UPR are blocked, and why the PNMC and proteolytic centres are disrupted.

The UPR is a protein quality control mechanism that upregulates proteases and chaperones in response to misfolded and damaged proteins. The OMMAD functions in a similar way as the ERAD. In ERAD and OMMAD, ubiquitinated proteins are transported through microtubules to the proteolytic centres where proteasomes are clustered. Similarly, mitophagosomes are transported to the perinuclear region to fuse with lysosomes. Dynein motor-proteins transport cargo towards the minus end close to the microtubule-organizing centre (MTOC). Vincristine as other drugs affecting microtubule dynamics block transport and halt ERAD, OMMAD and mitophagy. Bortezomib may create an overload of toxic misfolded ubiquitinated proteins at the ER and mitochondria.

2.9. Cancer cells may be addicted to UPR and mitophagy

For several reasons, cancer cells may be adapted to an abnormally high load of misfolded proteins. One of the best examples is that of multiple myeloma cells [33]. These are malignant post-germinal centre B lymphocytes that actively produce and secrete immunoglobulins. Due to the huge amount of protein synthesis, myeloma cells are liable to ER-stress and therefore require activation of the UPR, induction of chaperones and autophagy for survival [37]. A significant number of genes involved in the UPR are frequently mutated in patients with multiple myeloma, and the UPR is highly active and increases in advanced disease stages [37]. Multiple myeloma cells may be addicted to the UPR for survival, and drugs that target protein homeostasis, such as proteasome inhibitors, shift the balance of the UPR from prosurvival to proapoptotic. However, myeloma cells further evolve alternative mechanisms to deal with ER-stress such as autophagy, and the disease remains incurable mainly due to therapy resistance.

Remarkably, the mitophagy receptor BNIP3 is also upregulated in advanced forms of multiple myeloma [38]. Most myeloma cells reside in a bone marrow hypoxic niche, and hypoxia increases ER-stress and upregulates UPR to deal with an overload of misfolded proteins at the ER and the mitochondria. Therefore, the UPR and mitophagy may be crucial to avoid the potential joint toxicity of undegraded misfolded proteins and damaged mitochondria.

2.10. Targeting mitophagy and the UPR with microtubule stabilizing drugs

Several mechanisms of resistance to proteasome inhibitors have been elucidated. The therapeutic targeting of UPS and UPR has similarities to the therapeutic targeting of mitophagy, and the potential resistance mechanisms may be comparable. Unfolded protein response and mitophagy are flows that may be potentially toxic after being halted. When proteasome is inhibited, the halted flow of proteolysis becomes lethal to the cells, due to accumulation of misfolded proteins. Similarly, when mitophagy is inhibited, the halted flow of elimination of damaged mitochondria becomes potentially lethal to the cell. However, as discussed before, mitochondria have an internal protein QC mechanism represented by the mtUPR that involves chaperones and proteases. In addition, it can target misfolded proteins to the OMM for further ubiquitination and shuttling to the proteasome (OMMAD). This means that blocking mitophagy does not warrant an accumulation of potentially lethal organelles that trigger apoptosis. Remarkably, by destabilizing the microtubule network, both the UPR and mitophagy become halted. Therefore, the microtubule network is a target where mitophagy, OMMAD and ERAD are convergent and lead to accumulation of ubiquitinated misfolded proteins and mitochondria (**Figure 2**). However, achieving a misfolded protein accumulation capable of causing mitochondrial toxicity and an increase in ER-stress, requires the concurrent inhibition of proteasomes. In a similar way, lethality of mitochondria accumulation depends much on the quality of mitochondria and factors such as mtROS level. The latter is much influenced by metabolic reprogramming and the rate of OXPHOS dependency. As commented above, aggressive neoplasm often have low OXPHOS activity, and damage inflicted to the ETC may have a low impact in increasing mtROS. This situation accounts for resistance to mitocans and tolerance to accumulation of damaged mitochondria. However, there are mitocan drugs that make tumour cells shift to OXPHOS, and thus they can become vulnerable to increased mtROS. This is the case of 2-deoxyglucose (2DG) and 3-bromopyruvate (3BP) that block mitochondrial-bound hexokinase and of dichloroacetate (DCA) that blocks pyruvate-dehydrogenase-kinase (PDK) [39]

2.11. The pro-death role of mitophagy receptor BNIP3

BNIP3 is a "BH3-only member" of the Bcl2 family that was originally described as a pro-death molecule that often triggers a caspase-independent mode of apoptosis. More recently, BNIP3 was also characterized as a mitophagy receptor, and created a controversy about whether it was a pro-death or pro-survival molecule [28]. Supporting its pro-death role, the knock- down of autophagy genes or the chemical inhibition of autophagy, enhanced apoptosis of cells where BNIP3 was overexpressed. However, BNIP3 upregulation enhances mitophagy and may help to eliminate potentially harmful damaged mitochondria [40]. The reconciliation of this dual

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role controversy requires the consideration of mitophagy as a flowing system. If accumulation of mitochondrial BNIP3 triggers cell death, then the rate of mitophagic flow must be sufficiently high to eliminate these BNIP3-bearing mitochondria. Otherwise, if mitophagy is blocked downstream, the result is that BNIP3 is accumulated at the mitochondria initiating cell death. In normal cells, BNIP3 is under transcriptional control of HIF-1α, and the most active inducer of BNIP3 is hypoxia and the increase of intracellular ROS [41]. BNIP3 targets to damaged mitochondria particularly under increase of intracellular ROS to initiate mitophagy. If mitophagic flow is not high enough to eliminate mitochondria, BNIP3 will induce cell death, otherwise mitophagy eliminates damaged mitochondria contributing to cell survival and tolerance to hypoxia. Thus, the pro-death role of BNIP3 is dependent on a balance between mitochondrial damage and mitophagic flow [19].

2.12. Valproic acid upregulates BNIP3 but also induces mitochondrial biogenesis

Downregulation of BNIP3 may result in failure of tumour cells to undergo cell death, and is associated with a chemo-resistant phenotype and decreased patient survival [42]. Samples from patients with multiple myeloma were found methylated at the BNIP3 promoter, and methylation was significantly correlated with poor patient survival rates [43]. The finding that many haematological and other tumour cells have epigenetic silencing of BNIP3, led to the hypothesis that epigenetic drugs that restore expression of BNIP3 could cause tumour cell death. In fact, this was confirmed in some kinds of lymphoma, leukaemia and epithelial tumours. Burkitt´s lymphoma cells have epigenetic silencing of BNIP3 and VPA is a histone deacetylase (HDAC) inhibitor that upregulates expression of BNIP3. However, upregulation of VPA improves growth and resistance to death in these cells, and even antagonizes the effect of mitocans like ATO and improves tolerance to hypoxia [19]. Even though VPA increases the expression of BNIP3 in high-grade lymphoma, microarray data from studies conducted in low-grade lymphoma cells (CLL) showed that VPA upregulates BNIP3 and many other mitochondria-related genes, including PGC-1α that encodes a master regulator of mitochondrial biogenesis [44]. Upregulation of PGC1α by VPA was also confirmed in SH-SY5Y neuroblastoma cells, and even in fibroblasts from patients with mutation in mtDNA polymerase (POLG) as well as normal controls [45, 46]. Thus, VPA induces mitochondrial biogenesis through PGC-1 α and upregulates mitophagy through BNIP3, leading to an increase in mitochondrial mass and an enhanced mitochondrial turnover [44]. This results in an enhanced metabolic rate and increased OXPHOS. Patients with POLG mutation are extremely sensitive to VPA toxicity because, in contrast to normal patients, POLG-deficient cells cannot tolerate the increased function of mitochondrial respiratory chain [46]. This exemplifies the fact that defective mitochondria cannot tolerate increased OXPHOS derived from increased biogenesis. It also underscores the importance of metabolic reprogramming and the bioenergetics profile of cancer cells on the outcome of increased mitochondrial biogenesis. Metabolically reprogrammed cells of high-grade lymphomas that have a low rate of OXPHOS and a high rate of glycolysis can tolerate increased biogenesis. In contrast, low-grade lymphoma cells that are OXPHOS-dependent with low levels of glycolysis will increase OXPHOS upon induction of mitochondrial biogenesis by drugs like VPA, leading to increased mtROS and apoptosis. This was confirmed recently in studies conducted in peripheral blood circulating cells obtained

from CLL patients, where VPA treatment is correlated with the upregulation of several genes involved in apoptosis [47]. In addition, VPA was synergic with fludarabine on the induction of apoptosis, and CLL patients treated with VPA plus fludarabine had a better outcome than patients treated with fludarabine alone. In contrast, the effect of VPA in high-grade B-cell neoplasms such as Burkitt´s lymphoma is quite the opposite, providing survival advantage even under hypoxia and antagonizing the apoptotic effect of ATO that is a mitocan known to increase mtROS [19].

2.13. Mitophagy and bioenergetics profile in the cytotoxic response to mitocans

From the above discussion, it is clear that the pro-death effect of mitocans is dependent on several interacting factors such as the bioenergetics profile and the induction of increased mtROS, mitochondrial mass, mitochondrial dynamics and mitophagic flow. VPA influences many of these factors, but the bioenergetics profile appears critical in determining the sensitizing or resistance effect in combination with mitocans. The initiation of intrinsic apoptosis by mitocans demands achieving a critical threshold of damage across the mitochondrial network. If the mitochondrial damage inflicted by a mitocan is persistent, an increase in mitochondrial biogenesis may result in an increased amount of damaged mitochondria and an overload of mitophagic flow. However, mitophagic flow coupled to biogenesis may be high enough as to keep the cells below the apoptotic threshold. Thus, the efficacy of mitocans will increase if mitophagic flow is blocked, and conversely the anticancer efficacy of blocking mitophagy will be enhanced with the addition of mitocans. However, blocking mitophagy may still leave an overloaded mtUPR, and in the more aggressive forms, the addition of proteasome inhibitors will contribute to increase damage inflicted by mitocans and blockage of mitophagy [19]. Finally, mitocans such as 3BMP, 2DG and DCA may sensitize to mtROS production in OXPHOS-independent aggressive forms of neoplasms. The induction of mitochondrial biogenesis and blockage of mitophagy present a certain analogy with therapeutic strategies that induce ER-stress to create an overload of the UPS, while at the same time a proteasome inhibitor makes the overload even larger and more toxic. However, in the case of mitochondria, there is a further need to assure that treatment makes them a real source of toxicity to the cells, and this involves mitocans and evaluation of the bioenergetics profile of the target cells.

3. Assessing basal mitophagic flow in lymphoma cells

Lymphoma cells can be obtained from peripheral blood or lymph node biopsies of patients to be analysed for biological features involved in drug sensitivity, including mitophagic flow and mitochondrial biogenesis. Mitophagy and biogenesis can be considered in a steady state that determines the actual mitochondrial mass. As discussed above, this turnover can have a profound impact on the threshold of mitochondrial damage that initiates apoptosis. This is a reason why assessing the basal rate of mitophagy provides a first approach to predict sensitivity to mitocans, and provides a preliminary rationale for combined therapies. Since mitophagy has to be interpreted as a flow, inhibitors provide an indication of the magnitude

of this flow. Accumulation of mitochondria occurs after halting mitophagy, while biogenesis is still active. Thus, the change in mitochondrial mass after halting mitophagy is an indicator of mitophagic flow [48]. Colocalization between autophagosomes and mitochondria can also provide an indicator of mitophagy. When mitophagosome formation is blocked upstream, such as by knocking down ATG genes or using chemical inhibitors of the initiation of autophagy, colocalization of mitochondria and autophagosomes decreases. By contrast, when mitophagy is blocked downstream, as for example with vincristine or other inhibitors of autophagosome-lysosome fusion, colocalization between autophagosomes and mitochondria will increase. The morphology and dynamics of the mitochondrial network also provide interesting information. Since fission is the first step in mitophagy and is required after elongation during biogenesis, a high rate of basal mitophagy will often show a fragmented network particularly at the perinuclear area. A fragmented PNMC is characteristic of high rates of basal mitophagy and biogenesis. The use of vincristine or other microtubule stabilizing drugs will show a disruption of the PNMC and a transition to a peripheral distribution of the fragmented network.

3.1. Methods to assess mitochondrial mass and mitophagosomes

Changes of mitochondrial mass can be traced by quantifying mtDNA using QPCR, by immunochemical methods such as western blot using antibodies directed against proteins of the OMM like TOM20, or at the single cell level using flow cytometry, with probes such as mitotracker or nonyl-acridine-orange (NAO) that binds to mitochondrial CL (**Figure 3**). Colocalization between mitochondria and autophagosomes requires a probe for each structure and microscopic analysis. One alternative is immunofluorescence with anti LC3 antibodies and the fixable series of mitotrackers [49]. A second alternative is transient transfection with a plasmid for expression of GFP-LC3 together with mitotracker. A third alternative is the use of mono-dancylcadaverine (with UV excitation and collecting a narrow band of blue fluorescent emission) and mitotracker [50, 51]. These are just some reference alternatives and many more can be found elsewhere [49, 50]. Some less frequently used, although more robust alternatives in quantitative aspects, are image flow cytometry and subcellular flow cytometry (**Figure 3**). The former relies on colocalization metrics derived from thousands of low magnification images obtained in flowing cells, while the latter refers to the analysis by flow cytometry of subcellular particles obtained from cells that have fluorescently labelled autophagosomes and mitochondria, in order to quantify those particles that have dual fluorescence as an indicator of colocalization [52, 53]. Although assessment of LC3-I and LC3-II by western blot can give a measure of bulk autophagy (which is often present in lymphoma cells), it does not provide the confidence that mitochondria are being part of that autophagic flow. Therefore, assessment of mitochondrial mass and colocalization is necessary. Another alternative to derive a measure of mitophagic flow using western blot, is the use of antibodies against mitophagy receptors such as BNIP3 or Parkin. The accumulation after the addition of flow inhibitors can also provide a measurement of mitophagic flow. This alternative is particularly interesting for clinical samples since no transfection is required, although caveats such as epigenetic silencing of BNIP3 should be considered in each case. The expression of tandem proteins combining monomeric red fluorescence protein and green fluorescence protein

(mRFP-GFP) facilitates the monitoring of formation and maturation of autophagosomes, and the late stage after fusion with lysosomes. Because GFP but not mRFP fluorescence is quenched by low pH, the autophagosomes are "green-red" double fluorescent but autophagolysosomes are red-only fluorescent. This allows using the ratio single red versus double fluorescence as an indicator of autophagic flux without the need of inhibitors [54]. This measure should be complemented with colocalization between autophagosomes and mitochondria. A new alternative is a similar construction that includes the coding of mitochondria targeting domain, and allows tracking of mitophagosomes until fusion with lysosomes and a direct measurement of mitophagic flux [55]. Each of these methods has its strengths and limitations to measure mitophagic flux, and the use of more than one method has been widely recommended [50]. The measurement of turnover as a result of biogenesis and mitophagy has been recently facilitated with tandem probes that exploit the spectral change of the protein DsRed1-E5 over time (from green to red fluorescent). This probe is called mitotimer, includes a mitochondrial targeting sequence and its expression is under the control of doxycycline [56]

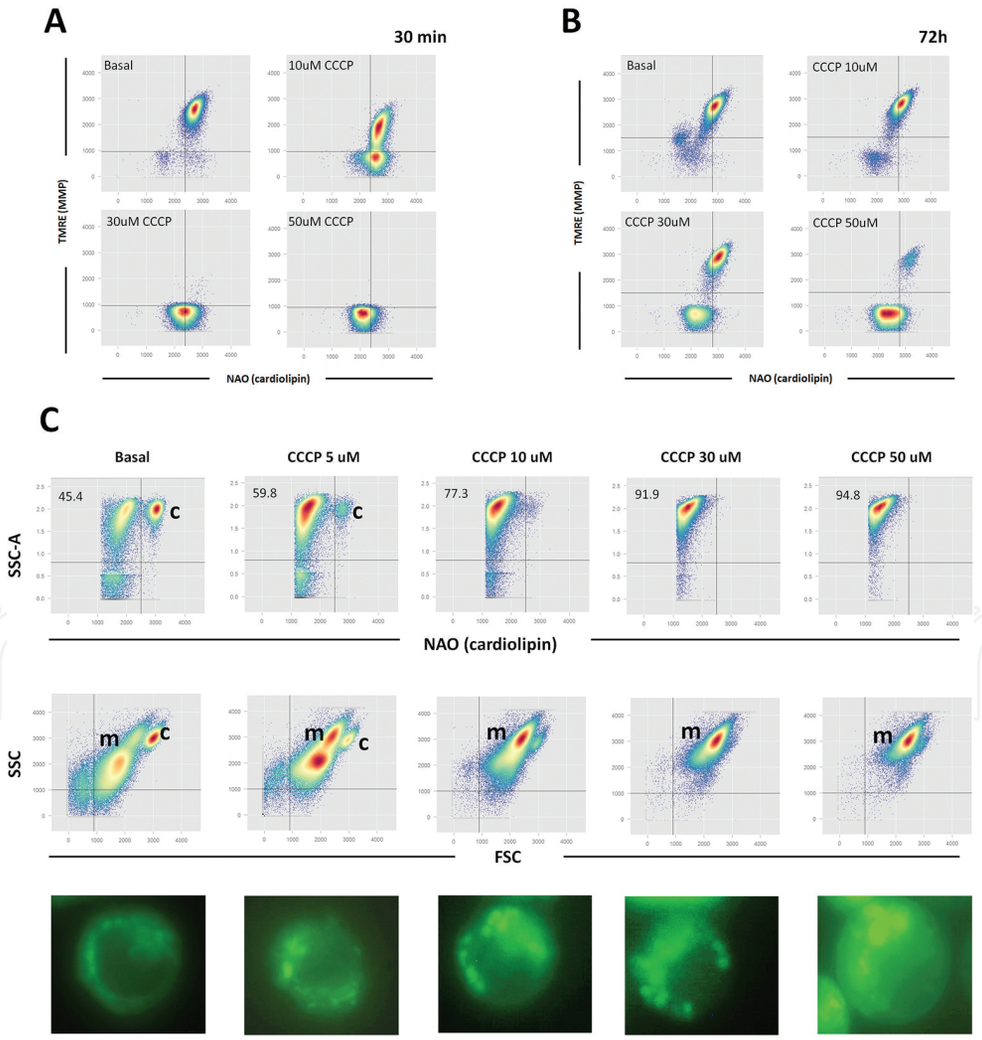

Figure 3. Fission is the first step of mitophagy.

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Gamma-aminobutyric acid receptor-associated protein (CCCP) is an uncoupler of OXPHOS that causes massive depolarization and increases mtROS due to leakage of superoxide anion from the ETC. This generalized mitochondrial damage triggers mitophagy as a compensating response, and CCCP is thus commonly used as a positive control of bulk mitophagy. In panel A, Burkitt´s lymphoma cells were exposed to increasing doses of CCCP and analysed by flow cytometry. CL content was assessed by the NAO probe (x-axis) and MMP was assessed by tetramethylrhodamine ethyl ester (TMRE) probe (y-axis). Although CCCP caused collapse of MMP as evaluated at 30 min, panel B shows that by 72 h MMP was completely recovered in cells treated with 10 and 30 μM CCCP. An evaluation of mitochondrial fission by subcellular flow cytometry is shown in panel C. Labelled cells were ruptured and immediately run in a flow cytometer, acquiring only particles having NAO fluorescence that corresponded to isolated mitochondria (m) or entire cells that were not ruptured (c). Numbers indicate percentage of mitochondria having a high SSC-A signal. The morphological changes, caused by massive fission of the mitochondrial network and initiation of mitophagy, correlate with an increase of the pulse area of the side scatter light signal (SSC-A). The fluorescence images of matching samples of non-ruptured NAO-labelled cells are shown at the bottom of panel C to illustrate the morphological counterpart of the SSC-A signal increase.

4. Conclusion

Mitophagy is a critical mechanism in the progression of B-cell neoplasms and other haematological malignancies. It is also critical in drug-resistance, particularly in advanced forms of myeloma and high-grade lymphomas. However, mitophagy is at the centre of an integrated system of resistance that involves mitochondrial biogenesis, dynamics, the UPS and metabolic reprogramming. Thus, even though mitophagy is critical, there is no single mechanism of resistance to drug treatment of advanced forms of B-cell neoplasms. Even though some particular mechanisms such as UPR and ERAD may prevail at some stages (as occurs in multiple myeloma), other mechanisms such as mitophagy, autophagy and metabolic reprogramming evolve with disease progression, and these neoplasms remain incurable. However, when these mechanisms are considered together as a system, and its occurrence is demonstrated in a particular case, a combined therapy can be designed to tackle one or all of them. For example, OXPHOS-dependent circulating CLL cells are quite sensitive to increased mitochondrial biogenesis and blockage of mitophagy. The more advanced forms become less OXPHOS-dependent, are more tolerant to increased mitochondrial biogenesis and have increased levels of basal mitophagy. These features make these neoplasm resistant to mitocans. In addition, a cross talk with the proteasome may compensate for blockage of mitophagy through OMMAD, and conversely mitophagy and autophagy may compensate for defective UPR under treatment with proteasome inhibitors. By characterizing this resistance system in particular patients at a particular disease stage, the combination of drugs that better tackle the biological behaviour can be defined.

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