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Autophagy: In the cROSshairs of cancer

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Abstract

Two prominent features of tumors that contribute to oncogenic survival signaling are redox disruption, or oxidative stress phenotype, and high autophagy signaling, making both phenomena ideal therapeutic targets. However, the relationship between redox disruption and autophagy signaling is not well characterized and the clinical impact of reactive oxygen species (ROS)-generating chemotherapeutics on autophagy merits immediate attention as autophagy largely contributes to chemotherapeutic resistance. In this commentary we focus on melanoma, using it as an example to provide clarity to current literature regarding the roles of autophagy and redox signaling which can be applicable to initiation and maintenance of most tumor types. Further, we address the crosstalk between ROS and autophagy signaling during pharmacological intervention and cell fate decisions. We attempt to elucidate the role of autophagy in regulating cell fate following treatment with ROS-generating agents in preclinical and clinical settings and discuss the emerging role of autophagy in cell fate decisions and as a cell death mechanism. We also address technical aspects of redox and autophagy evaluation in experimental design and data interpretation. Lastly, we present a provocative view of the clinical relevance, emerging challenges in dual targeting of redox and autophagy pathways for therapy, and the future directions to be addressed in order to advance both basic and translational aspects of this field.

Graphical abstract

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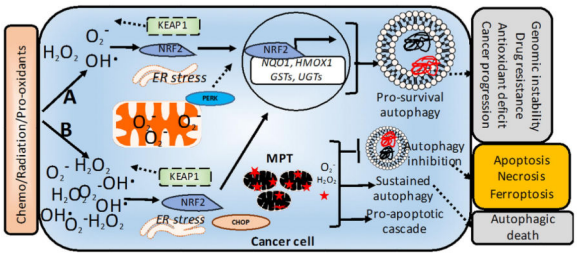

Keywords

cancer; autophagy; cell fate; reactive oxygen species; therapy

1. Introduction

Melanoma arises from the neoplastic transformation of normal, pigment-producing melanocytes, which reside in the basement layer of the epidermis, middle layer of the eye (uvea), and mucous membranes such as the gastrointestinal tract or oral cavity [1]. Recent studies in melanoma biology highlight the role of two critical cellular mechanisms as contributors to disease initiation and tumor maintenance; redox state and autophagy. In melanocytes, reactive oxygen species (ROS) serve as critical signaling mediators while autophagy serves as a cellular ‘quality control’, recycling mechanism. However, in melanoma cells, there exists a redox imbalance, or oxidative stress phenotype, where the accumulation of ROS is attributable to mitochondrial uncoupling, oncogenic mutations (i.e., $BRAF^{V600E}$) and switch to glycolytic phenotype [2]. Compared to melanocytes, melanomas also display increased autophagic activity, which aids in tumor cell survival through sustained proliferative signaling, apoptosis evasion, and chemotherapeutic resistance [3-5]. Both redox signaling and autophagy have thus become attractive therapeutic targets to shut down pro-survival signaling features selectively in melanoma. However, there is a dearth of studies that examine the biological significance of the mechanistic relationship between ROS and autophagy in melanoma, feedback mechanisms, and the potential for pharmacological targeting of both pro-survival pathways as a therapeutic avenue. The purpose of this commentary is to address the known mechanisms that regulate the crosstalk between ROS and autophagy in melanoma cell fate outcomes and to present relevant research studies that contribute to our understaning of this signaling relationship. We provide clarification to current classifications of autophagy with regard to cell fate, particularly during pharmacological induction of oxidative damage, the technical challenges of measuring ROS and autophagy experimentally as well as the clinical relevance of dual targeting of redox and autophagy pathways for therapy. Finally, the preclinical and clinical challenges, and future directions in the field of redox-regulated autophagy as a therapeutic target are presented.

2. Role of ROS in melanoma

2.1 Pro-oxidant state potentiates melanomagenesis

Melanocytes are dendritic cells of neural crest origin, and synthesize melanin in specialized organelles called melanosomes. Biologically, melanin functions in the epidermal layer to absorb light as a UV-protective mechanism, which results in a high pro-oxidant state in melanocytes [6]. Melanocytes have an intrinsic antioxidant defense network in order to maintain redox homeostasis [7]. However, prolonged disruption of redox balance can result in rapid hydrogen peroxide (H_2O_2) generation and impaired antioxidant activity (catalase, heme oxygenase 1), culminating in an oxidative stress state, due to an imbalance of ROS and antioxidant capacity [8]. In addition to the pro-oxidant state of melanocytes, genetic polymorphisms in genes encoding antioxidant repair enzymes, specifically GPX1, have been associated with a significantly increased risk of breast cancer and melanoma [9, 10]. As such, elevated ROS and impaired antioxidant activity results in oxidative stress and melanomagenesis. Appropriately, melanoma has been termed a “ROS-driven tumor” [8]. Many preclinical studies have demonstrated that melanoma cells are particularly susceptible to increases in ROS compared with melanocytes [11-14]. This observed redox sensitivity is likely attributable to *i)* elevated antioxidant capacity in melanocytes, able to efficiently counteract oxidative insult compared with melanoma cells (which may have antioxidant gene mutations [15-17]) and *ii)* the metabolic requirement *for* and sensitivity *to* chronically elevated ROS levels for maintenance of oncogenic signaling [18]. A proposed model for the basis of this selective sensitivity of melanoma cells to ROS-generating therapeutic agents is summarized in Figure 1.

2.2 ROS modulation as a therapeutic strategy

A variety of ROS molecules cause irreversible macromolecular damage. Chronically high levels of ROS in cancer cells promotes genomic instability and uncontrolled proliferation. The ‘threshold concept’ of ROS as a therapeutic target has emerged as a viable anti-cancer strategy that takes advantage of the selective vulnerability of cancer cells to disruptions in redox homeostasis [19]. We define ROS threshold as a window of redox cycling which a cell can sustain, without affecting or triggering cell death mechanisms. We have recently demonstrated that human melanoma cells have an elevated oxidative stress phenotype compared with melanocytes, and that disrupting the melanoma cell oxidative stress ‘threshold’ perturbs PI3K/AKT/mTOR oncogenic signaling required for survival, and culminates in cell death [20]. Clinical efforts that seek to modulate redox disruption in melanoma patients are reinforced by multiple studies showing increased serum levels of oxidative stress markers (malondialdehyde; MDA, oxidized protein products, SOD activity etc.) in metastatic melanoma patients compared with either healthy volunteers or non-metastatic patients [21, 22]. Figure 1 depicts the nature of selective ROS generation resulting in different cell fates between normal melanocytes and melanoma cells.

In the way of pro-oxidant therapeutics, we present the small molecule STA-4783 (Elesclomol) as a case study to highlight the utility of a pro-oxidant therapy, in which elevation of intracellular ROS results selectively in melanoma cell apoptosis. Elesclomol showed exquisite selectively in tumor reduction *in vivo*, that resulted in a significant

doubling of mean progression free survival (PFS) when combined with paclitaxel for patients with metastatic melanoma [23-25]. Elesclomol complexes with (Cu)II to disrupt mitochondrial electron transport chain, rapidly depolarize mitochondrial membranes, and induce apoptosis [23]. The success of Elesclomol against aggressive, late stage melanoma in the clinic highlights the significance of developing ROS-inducing drugs as anti-melanoma agents. Further, Elesclomol treatment resulted in apoptosis in Vemurafenib-resistant melanoma cells, the result of a chronically elevated mitochondrial respiration and oxidative stress [14]. Elesclomol has also been evaluated in clinical trials for efficacy against solid tumors, lung, breast, and prostate cancers, and most recently acute myeloid leukemia [26-28]. At the time of submission of this commentary, Elesclomol is under evaluation for combined efficacy with Paclitaxel in a Phase II clinical trial for ovarian, fallopian tube, and primary peritoneal cancers, thus laying the foundation for the clinical relevance of pro-oxidant therapy. In addition to Elesclomol, there is overwhelming support for pharmacological redox modulation as a viable therapeutic target across cancer subtypes. Specifically, we highlight recent studies in which pharmacological redox modulation results in melanoma cell apoptosis, summarized in Table 1 [20, 29-33].

2.3 Antioxidant modulation as a therapeutic strategy

In contrast to a pro-oxidant approach, disrupting the redox balance through modulation of the antioxidant defense system has proved to be much less efficacious as an anti-melanoma strategy [34]. We discuss two approaches and their cell death efficacy: 1) increasing cellular antioxidants and 2) depleting cellular antioxidants.

Historically, antioxidant supplementation has been proposed as a valid anticancer strategy, more often than depletion of cellular antioxidants. Preclinical studies have demonstrated that antioxidant supplementation leads to selective melanoma cell death through increased ROS-scavenging activity mediated by elevated SOD and catalase activity [35-37]. By contrast, dietary polyphenol antioxidants such as like resveratrol, tocopherol, and quercetin exert direct ROS-scavenging activity and have produced highly variable preclinical and clinical outcomes [38]. Clinical efforts have consistently failed to demonstrate antioxidant supplementation as an efficacious anti-cancer strategy, offering either negative or conflicting outcomes. A clinical trial showed that the incidence of melanoma increased in women receiving a combination of antioxidant supplementation [39]. Additionally, a pilot trial showed that alpha-tocopherol supplementation failed to provide protection against chemotherapy-induced DNA damage in melanoma patients [40]. The lack of clinical translation of early preclinical data had been perplexing, although newer preclinical data is pointing towards a lack of benefit of this modality. The utility of increasing the cellular antioxidant pool likely holds the greatest anti-cancer potential in prevention. The potential of antioxidnats to protect tumor cells when used as a therapeutic, may explanation the observed lack of clinical efficacy. As an alternative approach to antioxidant supplementation, the employment of antioxidant-depleting strategies has resulted in considerable higher efficacy in melanoma through excessive ROS generation and cell death [41, 42]. For example, downregulation of the NRF2 antioxidant defense pathway has shown marked anti-melanoma activity [43]. Additionally, depletion of the glutathione system in melanoma appears to have marked success in potentiating a pro-apoptotic response, as the accumulation of ROS leads

to melanoma inhibition both *in vitro* and *in vivo* [44, 45]. Inhibition of SOD activity also shows selective anti-melanoma activity and represents an attractive therapeutic target [45, 46].

One consideration for antioxidant-modulating strategies is the finding that many antioxidant enzymes are found to be mutated in cancer, including SOD2, a mitochondrial matrix enzyme responsible for superoxide ($O2^{\cdot -}$) detoxification [41]. However, emerging evidence points to molecular redundancies in which SOD1 is overexpressed in cancer cells to cope with the genetic or functional loss of SOD2 [41]. Further, literature suggests that a concomitant antioxidant capacity is required to cope with increased oxidative stress and metabolic demands of cancer cells, implicating antioxidant modulation in tumorigenesis [47]. Therefore, future studies that evaluate the role of antioxidant gene mutations and loss of function will be necessary before the clinical implementation of an antioxidant-modulating therapy, which may promote melanoma development, as was found in prostate cancer prevention trial with selenium and vitamin E (SELECT) and the Nutritional Prevention of Cancer Trial (NPCT) [48].

2.4 Considerations for ROS evaluation

Experimental set up and data analyses to evaluate ROS can be quite complex. An exhaustive review of such methods is beyond the scope of this commentary, but a few basic guidelines are suggested. The fluctuating O_2 tension in cell culture environment, and supplements contained in culture media can be sources of artifacts regarding measures of ROS and antioxidants, and must be taken into consideration [49]. In tissue culture assessments of ROS, there are many useful tools for generation and detection of total and specific ROS molecules. Further, the use of Electron Spin Resonance (ESR) spectroscopy is a robust methodology for qualitative and quantitative assessment of specific ROS molecules. The use of fluorescent-scanning microplate readers and fluorescence microscopy should be utilized judiciously, with careful considerations to minimize photobleaching and well-to-well scatter with use of black, clear-bottom culture plates. Chemiluminescent and chromogenic reagents are practical alternatives to validate ROS. The dynamics of ROS is an important criterion, as intracellular ROS are rapidly produced and detoxified. Therefore, kinetic assessment of ROS with controls (inducers and quenchers) is recommended. In animal models or patients, the modulation and detection of ROS is more complex than cell culture models, and as such, the detection of oxidative stress biomarkers from serum can provide clues to the overall basal redox status and after pharmacological modulation. Various biomarkers of oxidative stress including serum MDA, lipid peroxidation, reduced glutathione, catalase, glutathione peroxidase, and immunostaining for 8-hydroxy-2′-deoxyguanosine may be used. Therapy-naïve melanoma patients had an elevated oxidative stress profile, as assessed by increased plasma levels of Malondialdehyde (MDA) and decreased erythrocyte levels of SOD, compared with healthy control volunteers [22]. Interestingly, after complete surgical removal of melanoma tissues, patients showed decreased oxidative stress (serum MDA), which was elevated again after chemotherapy intervention with 5-(3,3-dimethyltriazene-1-yl)-imidazole-4-carboxamide (DTIC) and nitrosourea 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU) [22]. Clinical evaluation of melanoma patient serum for thiols, catalase and SOD activity levels, and protein oxidation products has also been demonstrated as valid

means to conclude overall level of oxidative stress [21]. For detailed information regarding the measurement of redox status, the reader is encouraged to read an excellent review by Liu-Smith *et al* [50].

3. Role of autophagy

3.1 Autophagy in melanoma development

Autophagy is an evolutionarily conserved catabolic process by which damaged proteins and organelles are encapsulated in the double-membraned autophagosome and degraded after delivery to the lysosome in a highly orchestrated series of molecular events. Although there are 3 subtypes of autophagy, the scope of this commentary focuses on macroautophagy, which will be referred to hereafter as “autophagy”. Under basal cellular conditions, autophagy serves to maintain homeostasis by recycling of cytosolic components, but is induced in response to a multitude of stimuli including starvation, viral invasion, hypoxia, increased intracellular ROS, and toxin/drug exposure [51]. Stress signaling kinases like JNK1 stimulate autophagy by phosphorylation of BCL2, which promotes the interaction of Beclin-1 and Vps34. Low ATP levels and hypoxia also stimulate autophagy by impairing mTOR kinase activity through reduced RHEB GTPase activity. Autophagy is generally recognized as a pro-survival mechanism, although classifications exist where autophagy is considered a type of programmed type-II cell death [52, 53]. The role of autophagy in melanoma, like other cancers, although complex, can be simplified as follows: protective against tumor initiation (pro-survival for normal melanocytes), and protective against chemotherapeutic agents, immune surveillance, and apoptotic signals (pro-survival for melanoma cells). So, while the published literature can appear overwhelmingly complex with regard to the function of autophagy in cell fate decisions, we present the viewpoint that the role of autophagy is exquisitely simple: cell survival at all costs.

A key autophagy-related protein 5 (ATG5; required for autophagosome formation), functions to induce senescence in melanocytes to prevent transformation [54, 55]. Melanocytes can undergo senescence due to increased polyploidy and subsequent autophagy activation during treatment with chemotherapeutic agents [56]. In contrast, loss of ATG7 results in melanocyte senescence and prevents melanoma development in $BRAF^{V600E}$/PTEN-null mice, supporting a pro-tumorigenic role for autophagy [57]. Further, loss of ATG7 resulted in decreased melanocyte proliferation owed to increased *p16Ink4a* activity that triggered senescence. Perhaps in the background of BRAF and PTEN mutation, autophagy is required for continued tumor growth, but in non-transformed melanocytes it contributes to senescence. However, since autophagy is executed independently of ATG7, perhaps it is dispensable contextually, and evaluation of autophagy proteins like tumor suppressor Beclin-1 might provide a more complete picture of the significance of autophagy during melanocyte transformation. Mechanistically, Beclin-1 is a negative regulator of Myeloid Cell Leukemia 1 (Mcl-1) and promotes proteasomal degradation of Mcl-1 to restrain melanocyte transformation [58]. The autophagic protein, Beclin-1 constitutively suppressed melanocyte transformation, and loss of the protein in patients contributed to increased Mcl-1 and melanoma progression [58]. Further, autophagy is linked to melanogenesis and in melanocytes, LC3 has been shown to drive MITF expression and

increase melanin content [59]. As such, autophagy-related proteins like LC3 would be detrimental when lost in normal melanocytes, as it is critical for melanogenesis and senescence activation.

In contrast to the anti-transformative role of autophagy in melanocytes, the role of autophagy in melanoma tumor development and progression is complex [5, 60, 61]. Specifically, we found several studies that conclude conflicting roles for autophagy in melanoma development and as prognostic markers. Mirraco *et al* found that Beclin-1 and LC3B messenger RNA levels had significant inverse correlation with melanoma progression, with the lowest expression seen in metastatic lesions, suggesting that loss of autophagy drives melanoma progression [62]. In addition, Liu *et al* demonstrated that ATG5 and LC3B protein levels decreased in metastatic melanoma patient samples compared with benign nevi, indicating that a loss of autophagy may promote tumor formation [63]. However, these two patient-based studies have limitations in that they cannot address the dynamic process of protein turnover during autophagy, as lower protein level could represent a high turnover and thus high autophagic activity. In contrast, several studies report high levels of autophagy proteins as predictors of melanoma, supporting the pro-survival role of autophagy in melanoma development and progression. Specifically, elevated LC3B expression, p62 and high autophagosome number in melanoma tumors are associated with aggressive disease and poor patient survival [5, 60, 61]. One possible explanation for these divergent conclusions of the role of autophagy may be owed to genetic mutations which modulate the autophagy process. Specifically, *PIK3CA and PTEN* mutations in the PI3K/AKT/mTOR pathway have been shown to drive high autophagic activity [64-67]. The clinical relevance of AKT-driven autophagic activity in melanoma is underscored by the clinical response of melanoma patients after combined inhibition of PI3K/AKT/mTOR activity and autophagy [4, 68]. A summary of studies that examine the effect of pharmacological modulation of autophagy on melanoma cell fate is included in Table 2 [69-74].

3.2 Autophagy as a programmed death mechanism

With regard to autophagic cell death, there have been very few melanoma studies that show an exclusive role for autophagy as a cell death mechanism. Opinion in the field is that features of autophagy occur during cell death induction, and what really exists is cell death '*with*' autophagy, not cell death '*by*' autophagy [75]. The published literature shows that some studies conclude that apoptosis is the consequence of autophagy induction, while others argue that the two are independent cell death mechanisms. These differences can be corrected when examining the nature of external stimulus applied as was done in some studies [76-78].

Another consideration for evaluation of autophagic cell death is the contribution of the signaling pathways. For example, many natural compounds used in chemoprevention, have demonstrated anti-inflammatory, ant-oxidant and pro-apoptotic activities. Therefore, caution is warranted in the interpretation of studies with regard to autophagic cell death, due to the multiple signaling cascades being affected simultaneously. We must clarify that some components of the autophagic machinery have been shown to be involved in the "switch"

from either pro-survival autophagy to pro-apoptotic, or from necrosis to apoptosis, which are mediated through the multifunctional, scaffolding protein SQSTM1/p62. These mechanisms can include p62 serving as a scaffold for caspase 8 oligomerization and subsequent processing (switch from autophagy to apoptosis) and failure of recruitment of Receptor-interacting serine/threonine-protein kinase 1 (RIPK1) to p62 (resulting in switch from apoptosis to necrosis) [79-82]. The contribution of autophagy proteins to cell death, either apoptotic or necrotic has been demonstrated, but the exclusive, upstream initiation of autophagy by ATG/Beclin-1-dependent mechanisms that lead to autophagosome formation and subsequent programmed cell death are lacking, particularly in melanocytes and melanoma biology [83]. We acknowledge that autophagic cell death, and autophagy induction can exist concurrently with the induction of apoptosis. Additionally, the emergence of ferroptosis- which is iron and ROS-dependent programmed cell death- certainly warrants further investigation, as a recent study shows that ferroptosis is an autophagic cell death process [84]. Future studies will be required to determine whether inducing autophagy actually results in autophagy-regulated programmed cell death, and the clinical relevance of inducing autophagy for melanoma therapy. Overall, preclinical and clinical studies highlight the therapeutic significance of pharmacological inhibition of autophagy with either chloroquine or hydroxychloroquine (CQ; HCQ) with melanoma regression and increased overall patient survival [5, 68, 85].

3.3. Challenges in autophagy measurement

Although *in vivo* measurements of autophagy can be more reliable than *in vitro* culture models, it is important to be aware of the limitations and challenges when interpreting data. While the ease of modulating autophagy dynamics, throughput nature and ability to use multiple cell lines and assays makes cell culture a good model system; it is essential to overcome the challenges of data interpretation. Caution is warranted in the measurement of mTOR-mediated autophagy, as both autophagic stimulation and inhibition can occur contextually. For example, under serum-starved conditions, mTOR-induced autophagy will be significantly higher than in serum-replete conditions, and surrogate markers of mTOR inhibition (phosphorylation of P70S6K targets phospho-rpS6, phospho-4EBP1), which might suggest autophagy induction, can actually conflict with other readouts of autophagy inhibition. Additionally, one must be cautious in interpretation of data after pharmacological inhibition of mTOR (i.e., rapamycin, rapalogs) compared with starvation-induced autophagy, as the result will often be different in magnitude. This difference can be attributed to measuring the “basal autophagic flux” during pharmacological inhibition, versus measuring the “maximal autophagic capacity” during serum-starved conditions. A further complication of mTOR-mediated autophagy is the use of autophagy inhibitors such as 3-methyladenine (3-MA), which block autophagosome formation by inhibition of class III PI3Ks. The caution of using 3-MA or wortmannin (similar mechanism of PI3K inhibition) as autophagy inhibitors comes from multiple reports that use of 3-MA (in combination with autophagy modulator of interest) actually stimulates autophagy due to the temporal-specific inhibition of class III PI3Ks (transient), and *prolonged* inhibition of class I PI3Ks, which in turn activates autophagic flux [86].

One particularly challenging aspect of measuring autophagy markers in clinical samples is the inability to capture the dynamic process of "autophagic flux". In contrast to *in vitro* studies, obtaining end point measures of either LC3B-II, ATGs, Beclin-1, or selective autophagy substrates such as p62, in human samples are not conclusive measures of autophagy. There are many conflicting reports of autophagy protein levels being predictive of overall patient survival, or protein (IHC) staining correlating with disease state or prognosis [60-62, 87-89]. Presumably, these protein outputs can be increased in patients with *high* autophagy tumors, or increased in patients with *low* autophagy tumors, reflected by a lack of protein degradation. In addition to measuring autophagy protein levels by staining intensity, the evaluation of autophagosome formation by transmission electron microscopy (TEM) is considered the "gold standard" for autophagosome measurement. However, technical challenges of TEM are rampant in that clinical samples are often not preserved for subsequent TEM evaluation and pathology expertise is crucial for proper interpretation of TEM images, as melanosomes can be mistaken for double-membraned autophagosomes [90]. Further, melanosomes are degraded by autophagy and are present in the cell as macromelanosomes and autophagic giant melanosome complexes, which can add a significant complication to evaluation of autophagosome formation not owed to melanosome degradation [91]. Other barriers arise in TEM from the melanosome content in some but not all melanomas, and thus, one has to be cautious regarding the interpretation of autophagy by autophagosome number if melanosomes are highly enriched in a sample. However, the combination of proper experimental controls, sample processing, and pathology expertise can be implemented to take advantage of TEM as an excellent tool to evaluate autophagosome formation in experimental conditions.

Given the divergent conclusions of published studies regarding autophagy protein markers and autophagosome formation, the utility of autophagy inhibitors (lysosomotropic agents) such as chloroquine (CQ) or hydroxychloroquine (HCQ) in clinical trials for melanoma (either alone or as a combination therapy) supports conclusions in the literature that autophagy is a pro-survival feature of melanomas, and so inhibiting the process pharmacologically is not only of clinical relevance, but a priority [5, 68, 85]. A recent study by Kraya *et al* demonstrates the utility of assessing secreted proteins in serum from patients with metastatic melanoma as a way to evaluate the intracellular dynamics of autophagy in patients, and to stratify patients as having low versus high autophagy melanoma tumors, which could point to therapeutic response [92]. This study signifies the transition to validation and acceptance of more relevant approaches to evaluating patient autophagy levels as a prognostic factor or as a guide to a specific therapeutic approach. Overall, the clinical implementation of autophagy inhibitors seems to be most relevant in conjunction with current melanoma chemotherapies, presumably due to the role of autophagy in drug resistance and as a pro-survival mechanism in melanoma tumor cells. Specifically, autophagy inhibition has been shown to potentiate the anti-melanoma effect of multiple preclinical and clinical drugs, including mTOR inhibition by temsirolimus, BRAF inhibition with Vemurafenib, and DNA alkylation by temozolomide [85, 93].

4. ROS-autophagy crosstalk and therapeutic targeting

Reactive oxygen species are generated in response to chemotherapeutic agents [22]. Excessive ROS production results in disruption of the electron transport chain and production of $O2^{\cdot -}$, leading to mitochondrial membrane depolarization and initiation of mitochondria-induced apoptosis. It is generally accepted that ROS generation precedes downstream cellular cascades, including those that determine cell fate either survival (autophagy) or death (apoptosis, necrosis). However, ROS generation has also been shown to occur following apoptotic stimulation (TRAIL-induced), or autophagy inhibition, which places ROS downstream of cell fate cascades [94, 95]. As expected, ROS and autophagy can regulate each other depending on the stimulus, and so there exists much complexity in dissecting the interplay between ROS and autophagy in cell fate. Based on our review of the literature, ROS regulates autophagy and subsequent pro-survival versus pro-death cell fate contextually. Cell fate outcomes are largely dependent on the amount of ROS generated and the cell's antioxidant response. A summary of studies that examine the effect of dual modulation of ROS and autophagy on melanoma preclinical and clinical outcomes is presented in Table 3 [43, 76, 85, 96-100].

During starvation, H_2O_2 is produced as a result of class III PI3K activation that stimulates autophagy through oxidation of ATG4. This inhibits the de-lipidation potential of ATG8, ultimately increasing the formation of lipidated LC3-rich autophagosomes [101]. Both $O2^{\cdot -}$ and H_2O_2 can also induce autophagy through AMPK activation and subsequent mTOR inhibition, and by transcriptional regulation of autophagy genes such as *SQSTM1* (p62) and *BECN1* [102-104]. ROS may also activate KEAP1/NRF2 in which case, transcriptional upregulation of selective autophagy substrate SQSTM1 (p62) occurs [105]. Several studies have shown a similar trend where exogenously applied ROS leads to autophagy induction, and in a majority of studies the cell fate is apoptosis. A summary of the role of ROS and autophagy and resulting cell fate in melanocytes and melanoma can be found in Tables 1-3. A more critical evaluation of the published literature, specifically with reference to those studies that induce ROS with pharmacological agent and measure autophagy and apoptosis, reveals a lack of thorough evaluation of autophagy with proper experimental controls (see also *Challenges in autophagy measurement*). In melanoma, the lack of studies that evaluate a dose-response effect of ROS on autophagy (stimulation or inhibition) in a mechanistic fashion is very concerning from a therapeutic standpoint. Perhaps the role of autophagy is dispensable if the ultimate output is cell death? In the aforementioned published literature in which autophagy outputs are properly assessed and still found to be stimulated during redox disruption, the role of autophagy as a survival feature seems to be validated, even though ROS can stimulate autophagic and apoptotic signaling pathways concurrently. In our view, the cell's ability to induce protective autophagy can co-exist with apoptosis, but at reaching a certain 'threshold', pro-survival autophagy cannot rescue the induction of a cell death cascade. Studies that cite induction of autophagy and apoptosis are essentially providing the viewpoint of what we might deem as cells that "die trying". It must also be noted that the experimental endpoints of apoptosis and autophagic cell death in response to external stimulus cannot capture the potential cell-to-cell intracellular signaling variability (i.e. some cells induce apoptosis while others induce pro-survival autophagy). However, bonafide

mechanistic measures of autophagy actually promoting apoptosis, as opposed to parallel intracellular assessments, might provide a more accurate account of a cell fate “switch” during exogenous ROS generation. Further, it cannot be overstated that conflicting measures of autophagy inhibition and induction can occur due to the pharmacological approach utilized. In multiple other cancers, the inhibition of autophagy during high ROS is better characterized, and the molecular mechanisms specifically, caspase activity point to a “switch” in the cellular signaling that blunts pro-survival autophagy and instead induces a pro-death apoptotic cascade [106]. In particular, cleavage of Beclin-1 by caspase-8 during high oxidative damage leads to failure of Beclin-1-regulated autophagy and subsequent autophagy inhibition [107]. Further, there is evidence of oxidative damage of autophagy gene promoter regions, including p62, which is significant enough to inhibit autophagy altogether [108]. There is also evidence that ROS induction inhibits autophagy through decreased expression of autophagy initiator ULK1 [109]. Additionally, autophagy proteins may be subject to redox-specific post-translational modifications at thiol residues, which may determine their role as pro-survival versus pro-death [110]. Specifically, phosphorylation of p62 at Ser. 349 during oxidative stress leads to increased NRF2 activity by competitive binding to KEAP1 [111].

Clearly, there is a contextual role of ROS on inhibition versus stimulation of autophagy and the resultant cell fate. We present a graphical illustration of the divergent cell fate outcomes with relation to ROS generation in Figure 2. Preclinical evaluation of chemotherapy-induced ROS by the anthracycline, Mitoxantrone further demonstrates that functional autophagy is a requisite for efficacious melanoma therapy, highlighting the clinical significance of ROS-autophagy considerations in translational approaches [112]. Future mechanistic studies of these autophagy inhibition mechanisms during ROS generation will ultimately unveil the potential impact of pro-oxidants in melanoma. Moreover, pro-oxidant chemotherapeutics that stimulate pro-survival autophagy may have enhanced efficacy when combined with autophagy inhibitors.

5. Future directions

There are several key issues that hamper the current understanding of the interplay between ROS and autophagy dynamics. Firstly, there is a lack of studies that evaluate the dynamic interplay between autophagy and redox signaling. *In vivo* studies that evaluate the relationship between ROS production and autophagy in melanoma are limited, and clinical studies are absent which evaluate autophagy measurements after traditional ROS-inducing chemotherapies. Evidence supporting ROS and autophagy, as individual targets are promising, but we urge the push for mechanistic evaluation of ROS-autophagy relationship, using proper pharmacological and genetic approaches, in panels of cell lines or human tissues, and use of multiple methodological assessments of autophagy. Another key issue is regarding the understanding of the role of autophagy as a programmed cell death mechanism. In future studies, there is a need to clarify the role of autophagy as a cell death mechanism using preclinical and clinical models. Molecular cross talk and genetic overlap between autophagy, apoptosis and necrosis mechanisms does exist but bonafide autophagic cell death mechanisms and molecular markers are needed.

One particular challenge to understanding ROS-autophagy dynamics is first in recognizing the significance of targeting two pro-survival mechanisms simultaneously. In many published studies we found that pro-oxidant treatment (inclding test compounds, chemotherapeutics) resulted in ROS-induced apoptosis and autophagy stimulation. Overall, the literature suggests that autophagy is induced as a cell survival mechanism, which can be concurrent with activation of an apoptotic response. It is essential to evaluate the utility of prooxidant *and* autophagy inhibitors in cases where autophagy induction is observed. As such, the efficacy of compounds may improve, as the pro-survival autophagy response is diminished. One challenge moving forward would be to judiciously evaluate published studies while determining the relevance of combination therapies. In our opinion, determining the role of autophagy in cell death during use of chemotherapeutic agents is an important challenge. While we suggest that autophagy is primarily a pro-survival mechanism in response to redox disruption, there may be cross-talk with cell death programming that remains to be elucidated. Evaluation of autophagy dynamics in relationship to treatment outcomes, survival curves, tumor burden, and progression-free survival in preclinical and clinical studies is needed. Specifically, determining if autophagy is a valid target for drug-resistant tumors, holds much promise. Furthermore, it is crucial to determine whether current chemotherapy regimens induce ROS, but also stimulate pro-survival tumor cell autophagy and thus contribute to drug-resistant populations.

In conjunction with the molecular interplay, determinants of oxidative stress and autophagy markers in patient serum can be used to evaluate the ROS-autophagy interplay during chemotherapy regimens. Such markers could also be an useful avenue for stratification to drug response, as was demonstrated with Elesclomol [113]. A future challenge and consideration is the reliability of autophagy proteins as *actual* autophagy determinants when evaluating therapy-naïve versus treated patients, as therapeutic modalities may modulate the overall tumor microenvironment and autophagy status [114, 115]. Retrospective studies, which evaluate tumor autophagy markers and correlate with a ROS-induced chemotherapy agent, may provide useful evidence about the ROS-autophagy interplay in patient outcomes. Assessment of immunotherapy-ineligible, or chemotherapy-resistant patients' response to dual ROS-autophagy modulating agents would open a therapeutic route for these patients. These challenges present opportunities for future research in the emerging field of redox-autophagy dynamics.

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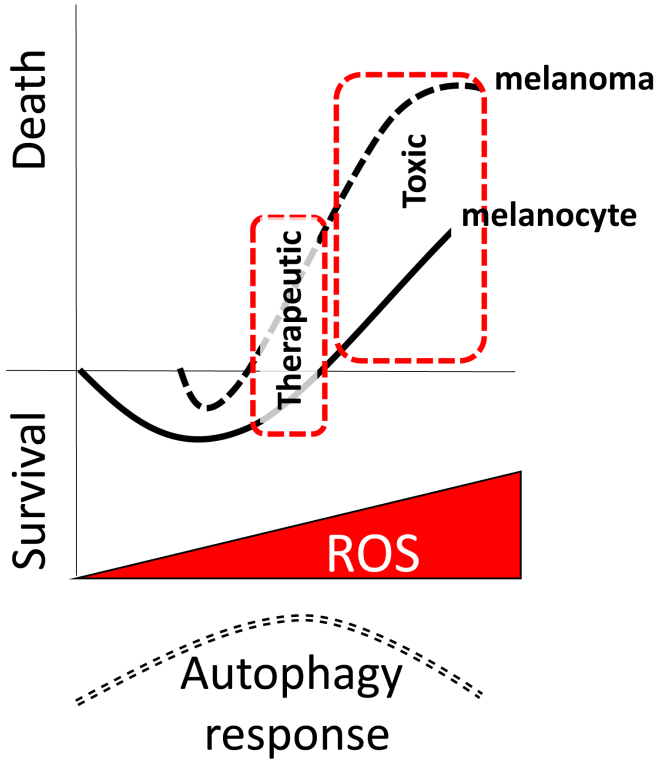

Figure 1. Hormetic effect of ROS increase on melanoma versus melanocyte cell fate: harnessing for therapeutic potential

The proposed model is based on the threshold premise of reactive oxygen species having differential effects on cell response. The hormetic principle proposed here speculates that different levels of ROS are stimulatory for melanoma and melanocyte survival and provoke survival responses such as autophagy in attempt to rid the cell of oxidized/damaged contents. However, as ROS increases beyond the cell's coping threshold, cell fate switches to death as apoptotic mechanisms ensue. The autophagy response (dashed curve, bottom) is induction as ROS increases, but eventual shut down/decline of the pro-survival autophagic mechanisms. We propose that the hormetic curve is altered in melanoma cells (dashed line) so that at the same amount of exogenous ROS generation, the melanocyte's response (solid line) will be survival and the melanoma cell response will be death. This represents a therapeutic window for ROS-generating anti-cancer agents, and past a certain threshold, high enough ROS will lead to toxicity in both cell types. Contributing factors to this proposed hormetic alteration is based on literature showing decreased antioxidant capacity in melanoma cells owed to mutations in SOD1/2, and the existing basal oxidative stress state in melanoma cells that render them particularly susceptible to further ROS escalation.

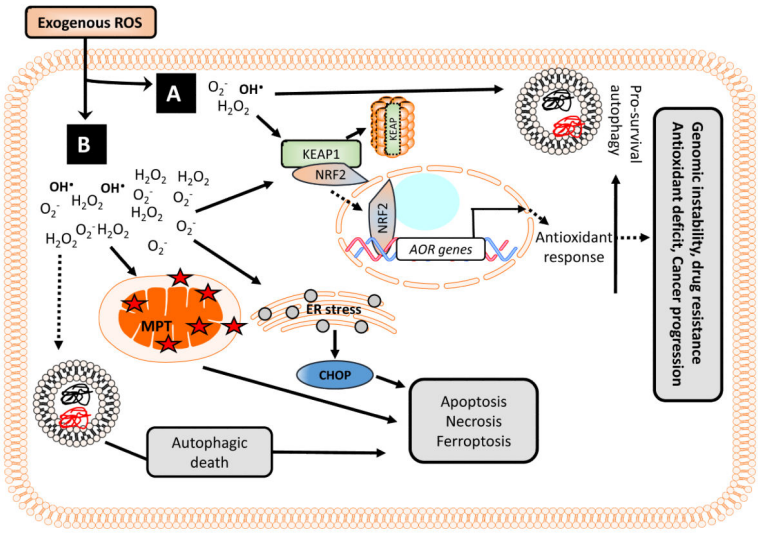

Figure 2. Role of ROS-mediated autophagy during melanoma cell fate determination

(A) Cancer cells respond to low or transient exogenous ROS generation (chemotherapy, radiation) by KEAP1/NRF2-mediated oxidative stress response, and ER-mediated unfolded protein response to rid the cell of damaged lipids and proteins. Autophagy is initiated and sustained, leading to cell survival. The result is a cell with increased baseline autophagic activity, and continued exogenous ROS-induced stress leads to increased redox stress, genomic instability and therapeutic resistance over time. (B) Cancer cells respond to high or sustained exogenous ROS generation by an attempted KEAP1/NRF2-mediated oxidative stress response and initial autophagy activation. However, sustained ER stress, mitochondrial membrane depolarization, and activation of the caspase cascade results in the a pro-death response, by which time the autophagy response has been inhibited because the cell has committed to die. The molecular switch by which autophagy can no longer serve as a survival mechanism occurs when autophagy proteins become dysfunctional, participate in pro-apoptotic signaling, are post-translationally modified or the cell's pro-apoptotic, necrotic, or ferroptotic signals have hierarchy above the proautophagic response. In some instances, a sustained autophagic response may result in autophagic cell death in the absence of pro-apoptotic signaling.

Table 1

Effect of pharmacological modulators of ROS on melanoma cell fate.

20Modulator	Model	ROS effect	Techniques	Cell fate	Overall conclusion	Ref
Nexrutine	Melanoma cells & melanocytes	Induction	H_2DCFDA, mitoSOX; Oxidative stress markers	Apoptosis	ROS, PI3K/mTOR inhibition & apoptosis induction	[20]
Benzofuroxan N-Br & N-I derivatives	B16F10-Nex2 cells; syngeneic mouse model	Induction	Dihydroethidium; fluorescent microscopy;	Apoptosis	Mitochondrial ROS & reduced tumor burden	[29]
Luteolin	Melanoma cells	Induction	H_2DCFDA	Apoptosis	ER stress/ROS with apoptosis	[30]
Isoliquiritigenin	B16F10 cells	Induction	H_2DCFDA	Apoptosis	Mitochondrial ROS & apoptosis	[31]
DDSD	B16F10 cells & syngeneic mouse model	Induction	H_2DCFDA	Apoptosis	Mitochondrial apoptosis & reduced tumor burden	[32]
4-DACL	Melanoma cells, spheroids, melanocytes	Induction	CellROX Green & Deep Red Reagent	Apoptosis	Mitochondrial ROS, inhibition of proliferation and cell cycle	[33]

4-DACL: (±)-4-deoxyaustrocortilutein; DDSD: (5(R), 19-diacetoxy-15,18(R and S), dihydro spata-13, 16(E)-diene

Table 2

Effect of pharmacological modulators of autophagy on melanoma cell fate.

Modulator	Model	Autophagy effect	Measures of autophagy	Cell fate	Overall conclusion	Ref
CQ	Melanoma cells	Inhibition	LC3B-I/II, ATG5, ATG7; autophagic flux	Cell death	Sensitivity to autophagy is independent of BRAF	[69]
α-Mangostin, Sorafenib	Melanoma cells	Inhibition	LC3B-I/II and ATG5	Apoptosis	Inhibition of AKT/ERK, ER stress, autophagy inhibition	[70]
AJ-5	Melanoma cell lines	Induction	TEM; GFP-LC3 puncta; autophagic flux by LC3B-I/II and Beclin-1 proteins	Autophagic cell death; apoptosis	ATM-CHK2 activation, apoptosis & autophagic cell death	[71]
Pentoxifylline	Melanoma cells	Induction	LC3B-I/II, p62, ATG5, Beclin-1; TEM; p62 co-localization with LC3B	Apoptosis	Ca^{2+} overload, ER stress, mTOR inhibition & autophagy activation through ATG5	[72]
Mibefradil, Pimozide	Melanoma cell lines	Inhibition	LC3B-I/II, Beclin-1, ATG5-12, p62	Apoptosis	ER stress, autophagy inhibition, apoptosis induction	[73]
Ursolic acid Resveratrol	Melanoma cells	Inhibition	Acridine orange; Beclin-1, LC3B-I/II & p62 protein	Decreased viability	Reduced cell viability through autophagy inhibition	[74]

AJ-5: binuclear cyclometalated Pd(II) complex; CQ: chloroquine

Table 3

Effect of pharmacological induction of ROS and subsequent effect on autophagy on melanoma models

Modulator	Model system	Effect on ROS	Effect on autophagy	Outcome	Conclusion of ROS-autophagy relationship	Ref
PFT-μ + NVP-AUY922	Melanoma cells & xenograft model	Induction	Inhibition	Apoptosis; decreased tumor growth	ER stress, ROS induction and autophagy inhibition	[43]
Graveoline	Melanoma cells	Induction	Induction	Apoptosis	ROS generation upstream of autophagy	[76]
Hydroxychloroquine (HCQ) + temozolomide (TMZ)	Metastatic melanoma patients	TMZ induce ROS	Inhibition	Stabilized disease and response	Therapeutic potential of combination compared to TMZ alone	[85]
Polygonatum cyrtonema lectin (PCL)	Melanocytes Melanoma cells	Induction	Induction	Apoptosis	Glutathione depletion, mitochondrial ROS and induction of autophagy	[96]
Cisplatin	Human melanoma cells	Induction	Inhibition	Apoptosis	ROS generation and inhibition of Beclin1/LC3B-mediated autophagic response	[97]
Physalin A	Melanoma cells	Induction	Induction	Apoptosis	ROS generation, autophagy induction	[98]
Fisetin	Melanoma cells	Induction	Transient induction	Apoptosis	ER stress and ROS generation, autophagy induction	[99]
Usnea barbata	B16 mouse melanoma	Induction	Induction	Apoptosis	Oxidative stress induces autophagosomes and apoptosis	[100]