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Review

# Killing of tumor cells: A drama in two acts

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ABSTRACT

Cancer still represents a major health problem worldwide, which urges the development of more effective strategies. Resistance to chemotherapy, a major obstacle for cancer eradication, is mainly related to an intrinsic failure to activate the apoptotic pathways. However, a protective effect of autophagy toward cancer cells has been recently observed, thus adding further complexity to the development of an effective approach counteracting cancer cell growth and improving the response to therapy.



**Contents**



## 1. Prelude to the drama "Killing of tumor cells"

Cancer is a complex multistep disorder resulting from genetic and epigenetic abnormalities leading to the transformation of normal into malignant cells. Despite enormous progress in the understanding of cancer biology, to date resistance of cancer cells to standard protocols, including radiotherapy and chemotherapy, is recognized as a major problem, given that therapy could block tumorigenesis only temporary, and select a cell population carrying advantageous mutations, making tumors no longer responsive [1].

It is widely agreed that the growth ability of tumor cells could be related not only to an abnormal signaling leading to uncontrolled cell proliferation, but also to the inability of cancer cells to activate apoptosis [2]. In fact, the apoptotic pathway(s) may be significantly altered in cancer cells compared to untransformed cells [3], and this feature might be exploited for anti-cancer drug development [4].

More recently, it has been widely recognized that tumorigenesis and cancer cell survival could be favored by autophagy. This process is physiologically relevant under stress conditions given that it is in charge for the elimination of misfolded proteins and damaged organelles, and is coupled to the production of energy to be re-used for basic metabolic reactions [5]. Micro-environmental hypoxia as well as nutrient deprivation accompanying cancer development mimic the emergency conditions triggering autophagy; moreover, DNA and cellular damage induced by anti-cancer therapy stimulate autophagy that eliminates intracellular damaged components, thus ensuring survival of cancer cells (reviewed by [6–10]). The impact of either apoptosis or autophagy on cancer cell survival/death is illustrated in Fig. 1.

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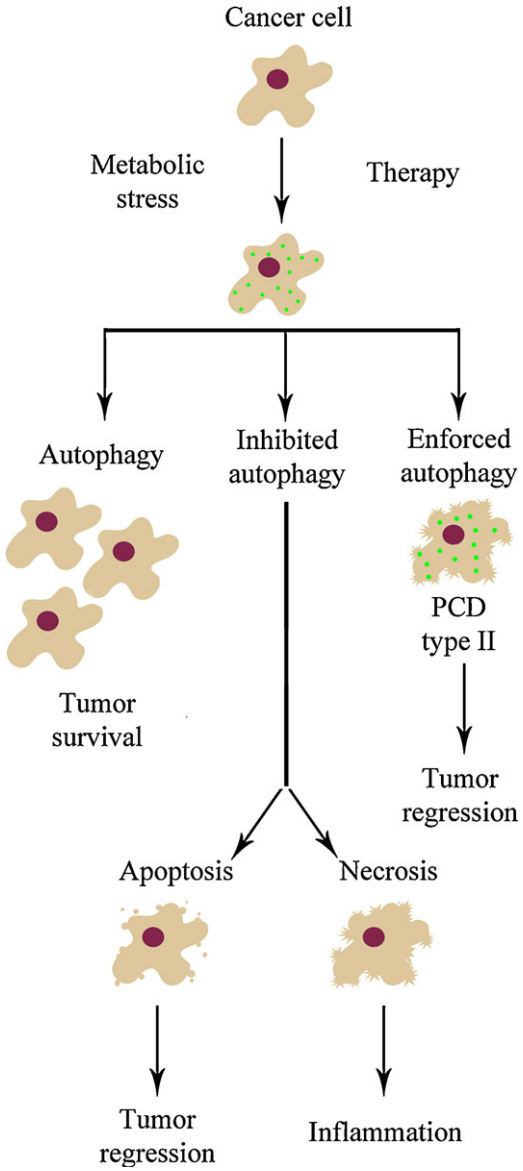


**Fig. 1.** Impact of apoptosis and autophagy on cancer cells. Cancer cells suffering from intrinsic metabolic or therapy-induced stress activate autophagy that protects them and allows their survival (left). Under conditions of autophagy inhibition, stress triggers cell death through the activation of apoptosis or necrosis, causing tumor regression or inflammation, respectively (center). Enforced autophagy (right) turns the physiological protective effect into type II programmed cell death (PCD) leading to tumor regression.

## 2. Act I: the leading role of apoptosis

DNA damaging agents and stress conditions such as heath shock, oxidants and free radicals, are able to activate the apoptotic pathway(s). Also cancer therapy-associated agents, *i.e.* chemotherapeutic drugs and radiation, which play a leading role in the killing of neoplastic cells, could be potent inducers of apoptosis. An impressive body of evidence points to the correlation between drug efficacy and ability to induce apoptosis in cancer cells. Accordingly, defects in the apoptotic machinery are currently investigated in drug-refractory cancer cells.

By consequence, a number of experimental strategies have been developed, aiming at manipulating specific apoptotic regulators or players. Given that tumor cell survival could be promoted by inactivation of pro-apoptotic signaling or activation of anti-apoptotic factors, two main classes of regulators are generally considered: (i) crucial factors essential for the execution of apoptosis, *e.g.* caspases, a family of cysteine proteases with unique substrate specificities; (ii) negative regulators of apoptosis, *e.g.* survivin. A detailed description of these categories is presented below.

(i) Caspases are synthesized as zymogens, whose activation is triggered by extrinsic and intrinsic signals [11]. From initiator caspases (caspase-2, -8, -9, and -10) a proteolytic wave reaches effector caspases (caspase-3, -6, and -7), which, in the active status, cleave many target proteins (detailed in the CASBAH online database http://bioinf.gen.tcd.i.e./casbah/), leading to the dismantling of the dying cell [12].

Mutations in different pro-caspase genes and/or caspase protein modifications leading to reduced expression level/activity, were detected in some cancer cell lines as well as in tumors (reviewed in [13,14]). Thus the search for overcoming this inactivation, therefore activating caspases, has become important in the development of new anti-cancer drugs (Fig. 2).

Modulation of caspase activity for therapeutic purposes has been approached experimentally. Inducible caspases were designed by fusion with synthetic dimerization domains; the delivery of these chimeric, controllable, caspases by adenoviral gene transfer triggered apoptosis in drug-treated tumor cells [15]. Accordingly, Jurkat cells transfected with a plasmid encoding caspase-3 fused with an antibody against the antigen HER2 (human epidermal growth factor receptor 2), which is widely expressed on the surface of some tumor cells, were able to produce and secrete the immunocaspase-3 fusion protein. This product selectively proved to bind the antigen over-expressing cancer cells, be internalized, subjected to lysosomal cleavage and released into the cytosol, thus promoting cell death. This fusion protein also causes suppression of HER2-over-expressing tumors *in vivo* [16]. Moreover, adenoviral constructs expressing caspase-3 have been developed, whose enforced expression in cancer cells was effective in inducing apoptosis [17].

A different approach was based on a small-molecule activator of pro-caspase-3 (PAC-1), which promotes direct and immediate activation of pro-caspase-3 both in cancer cell lines and animals [18]. High-throughput drug screening succeeded in the identification of small molecules acting as caspase activators, which have been shown to induce apoptosis in several cell lines deriving from prostate, breast, colorectal and lung cancers [19]. This approach is especially suitable against cancers in which pro-caspase-3 is present at high concentration but not converted to the active form.

Caspase inactivation could be also related to genetic, epigenetic and post-translational alterations; for caspase-8, this condition was bypassed by the use of demethylating agents, interferon or retinoic acid [20]. However, this approach does not target exclusively caspases and could have uncontrolled side effects.

(ii) To avoid undesired protein cleavage under physiological conditions, caspase activity is controlled by a family of IAPs (inhibitors of apoptotic proteins), originally described as physical inhibitors of caspases [21]. By consequence, the release of their negative regulatory function could have a positive effect on caspase activity. IAPs are characterized essentially by a BIR (baculovirus IAP repeat) domain at the N-terminus, eventually in multiple copies, essential for protein recognition and interaction, and by other structural domains at the C-terminus, including RING (really interesting new gene) and CARD (caspase activation and recruitment domain) [22].

Survivin, the smallest member of IAP family, displays a single BIR domain flanked by a long C-terminal α-helix. It is over-expressed in most cancers; the highest is its expression, the more unfavorable the prognosis [23]. On the basis of its exclusive over-expression in cancer cells, survivin is an optimal target for new

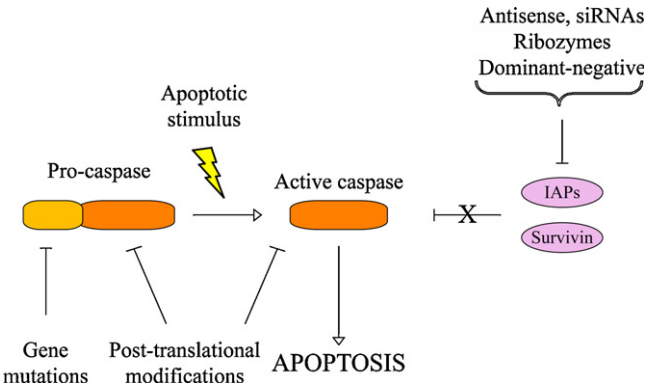


**Fig. 2.** Modulation of caspases and IAPs. Mutations in pro-caspase gene and/or post-translational modifications of either pro-caspase or caspases could affect caspase activity. Normally, caspase activity is controlled by IAP (inhibitor of apoptotic proteins) family, of which survivin is the most important member. Several strategies, based on antisense molecules, siRNAs, ribozymes and dominant-negative proteins, have been developed to avoid undesired IAPs activity and therefore, to allow caspase activation. The final goal of these approaches is the activation of the apoptotic machinery, thus committing cells to death.

anti-cancer drugs; in fact, various approaches have been used to down-regulate or block survivin in cancer cells [24–27].

Survivin epitopes present in a number of malignancies can be exploited in vaccination therapies based on therapeutic cytotoxic T-lymphocyte (CTL) response. *In vitro* induced survivin-specific CTLs recognized HLA-A2-matched tumor cell lines and primary survivin-expressing malignant cells from patients with leukemia [28]. In a preclinical model for survivin-directed vaccination, a reduction in tumor cells as well as blood vessels was reported [29]. To improve the delivery of a survivin DNA vaccine, intradermal electroporation was developed, with promising results [30].

IAP molecular antagonists such as antisense, siRNAs, ribozymes, and dominant-negative mutants resulted in sensitization of cancer cells to chemotherapy [23]. However, as recently reviewed [31–34], IAPs have other functions than caspase regulation, being involved in signaling pathways governing cell division, inflammation, survival; thus, their manipulation in cancer cells might have deleterious consequences for normal cells. The rationale of the strategy based on the manipulation of IAPs is schematized in Fig. 2.

## 3. Change of scenography: autophagy takes the scene

Autophagy is an evolutionarily conserved catabolic process that involves sequestration, transport and degradation of organelles and macromolecules to lysosomes, and further generation of energy and catabolic products to be recycled [5,35–37].

From a molecular point of view, autophagy is a very complex process being promoted by a number of factors and counteracted by many negative regulators [38,39]. While apoptotic events concern mainly nuclear compartments, autophagy occurs essentially in the cytoplasm, and implies typical formation of autophagosomes and autolysosomes [40] (Fig. 3). A feature that combines both processes is the notion that they have to be maintained inactive under normal cell growth conditions and activated "on demand".

The molecular mechanisms governing autophagy have been intensively studied during the last decade, providing a good understanding of why, when and how autophagy can be activated (reviewed in [41]). Having decrypted the molecular machinery of this process (even if additional investigation is needed to bring to light all the autophagy actors), the next step is to identify autophagy regulators. In this respect, it has been shown that signaling and execution of autophagy are mediated by the so-called "Three Musketeers", *i.e.* phosphorylation, acetylation and ubiquitylation [42].

Phosphorylation is a post-translational modification normally used to switch on/off critical proteins that are regulators of basic cellular pathways, including autophagy. In fact, the best-characterized autophagy regulator, mTOR (mammalian target of rapamycin), inhibits autophagy through the phosphorylation of its downstream targets ULK1 (Unk51-like kinase) and ATG13 [43,44]. The phosphorylation of these two proteins abolishes their possible interaction, thus blocking autophagy. On the contrary, when mTOR is inhibited, ULK1 phosphorylates FIP200 (family-interacting protein of 200 kDa), allowing the formation of the ULK1/ATG13/FIP200 complex, which is indispensable for autophagy [43]. Moreover, other protein kinases, like JNK (c-Jun N-terminal kinase) and DAPK (death-associated protein kinase), induce autophagy through the phosphorylation of their targets, *i.e.* Bcl-2 [45] and Beclin-1 [46], respectively. Finally, it has been observed that also LC3 (microtubule-associated protein light chain 3), which is a specific marker for autophagy (being incorporated into the autophagosomal membrane), can be phosphorylated by PKA and PKC. The modification occurs within the domain conferring to LC3 the ability to interact with LIR (LC3 interaction region)-containing proteins [47] such as p62 and NBR1 (neighbour of BRCA1 gene 1), thus impeding LC3 to go inside the autophagosomal membranes [48].

Acetylation is another type of post-translational modification that modulates acceptor protein features. As recently demonstrated by Morselli et al. [49], the so-called "acetylproteome" is fundamental for a correct execution of autophagy. Indeed, these authors demonstrated that two different pro-autophagy agents, *i.e.* resveratrol and spermidine, induce autophagy through the deacetylation of several pro-autophagic proteins such as ATG5, ATG7 and LC3. Interestingly, resveratrol is an activator of Sirtuin-1, a cytosolic

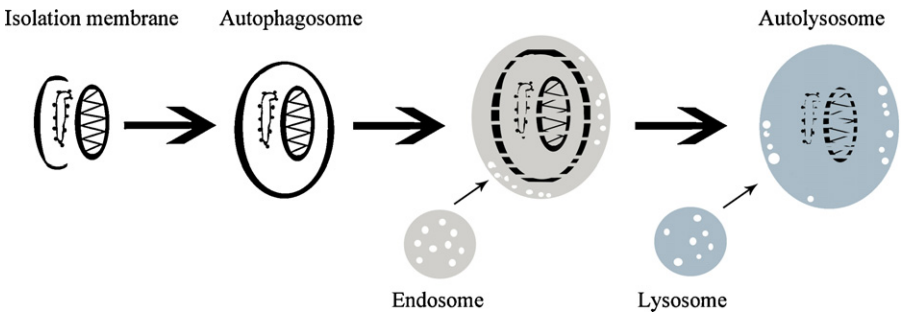


**Fig. 3.** Different steps in the generation of autolysosome. After the formation of the isolation membrane, autophagosome is assembled and further fused with endosomes and lysosomes to originate the autolysosome.

deacetylase, while spermidine is an acetylase inhibitor; however, these two compounds induce autophagy converging on the acetylproteome and, in some cases, on the same targets. Notably, both compounds activate autophagy in an mTOR-independent way [49]. Another acetylproteome-modifying protein involved in autophagy control is HDAC6 (histone deacetylase 6), which regulates the mobility of aggregate-containing bodies that have to be degraded by autophagy [50], as well as the maturation and fusion of autophagosome with lysosomes [51].

While the two processes above mentioned regulate the autophagic pathway, the last Musketeer, *i.e.* ubiquitylation, is mainly involved in the recognition of proteins that have to be degraded through autophagy. Indeed, two LIR-containing proteins, p62/SQSTM1 and NBR1, bind poly-ubiquitylated proteins to target them for autophagic degradation [52]. Another ubiquitin-binding protein, named NDP52 (nuclear dot protein 52), has been recently described to recognize and bind selectively ubiquitylated bacteria to avoid their proliferation and promote their disruption [53].

However, as Alexandre Dumas *docet*, although three Musketeers are main actors in the autophagic process, they require the presence of D'Artagnan, *i.e.* phosphatidylinositol 3-phosphate (PI3P). PI3P is formed through the phosphorylation of PI on position 3 of its inositol ring. This event is triggered by class III PI3 kinases, which are the orthologues of yeast vesicular protein-sorting (Vps); they add a phosphate group only to the position 3, so being very specific [54]. The importance of PI3P during autophagy is highlighted by the key role played by Vps34 within this process; in fact, without this kinase, the autophagic yeast pathway is blocked [55–57].

Although further data have to be collected to define PI3P role during autophagy, it is likely that autophagosomes form, at least in part, within PI3P-enriched structures called omegasomes; interestingly, upon starvation, Vps34 colocalizes with omegasomes, possibly producing the PI3P found in these structures [54]. To better underline the importance of PI3P, it is interesting to note that one of the most important player during autophagy, Beclin-1, participates in the formation of PI3-kinase (PI3-K) multiproteic complex, which comprises also Vps34, Vps15 and other proteins, whose role is to enhance (or to inhibit in some circumstances) the activity of Vps34 itself [58]. Finally, few years ago, the PI3P phosphatase Jumpy has been discovered and correlated to autophagy, given that in different cell types the knockdown of Jumpy gene resulted in high levels of basal autophagy [59].

## 4. Act II: one, no one, and one hundred thousand roles of autophagy

The microenvironment in which cancer cells live is often acid, hypoxic and poor of nutrients; moreover, cancer cells are characterized by a high metabolic rate, thus producing more ROS (reactive oxygen species) than the normal counterpart. Increased oxidative stress, leading to lipid peroxidation as well as DNA and protein damage, could be a "danger" signal possibly perceived as a trigger of cell death. In this case, cancer cells could be killed mainly by apoptosis but also by necrosis. On the other hand, ROS trigger autophagy and stimulate its protective removal activity toward damaged cellular components. Moreover, autophagy is often related to cancer cell response to chemotherapeutic agents because it could rescue cancer cells from death by providing them with amino acids, monosaccharides and lipids; these molecules can be further re-used to fuel cancer cell metabolism with energy and novel components useful to counteract the nutrient-poor condition of tumor microenvironment.

It has to be reminded that, beside its physiological pro-survival function, autophagy is also referred as Type II PCD [60], a caspase-independent paradigm of cell death. In fact, it has been widely reported that drug treatment of cancer cell lines could activate autophagy, which is characterized by large autophagic cytoplasmic vacuoles [61]. The cellular reactions modulated in cancer cells by metabolic stress and anti-cancer therapies are illustrated in Fig. 1.

Given that autophagy is crucial for cancer cell response to chemotherapeutic drugs, a novel goal in clinical research is represented by its manipulation to enhance the efficacy of anti-tumor treatments. The best example of selective targets is mTOR, a serine/threonine protein kinase belonging to the PI3K/Akt family [62]. The mTOR inhibitor rapamycin and its analogues (rapalogs) are currently employed in clinical trials (http://clinicaltrials.gov/ct2/results?term=rapamycin). Generally, rapalogs are active against mTORC1 but ineffective against mTORC2, thus having a limited potential [63]. Yang and co-workers recently demonstrated that rapamycin reduces the metastatic potential of melanoma cells and renders them sensible to apoptotic stimuli by increasing Bax and Bid protein levels in cancer cells [64]. Using another biological model, *i.e.* glioma-initiating cells (glioma cells with stem-like properties), Zhuang et al. also demonstrated that rapamycin sensitizes cancer cells to radiotherapy [65].

The continuous search for novel compounds able to modulate autophagy leads to the discovery of several new mTOR inhibitors, like RAD001, WYE-125132 and PI-103. The rapalog RAD001 has been reported to induce autophagy in several cell lines and, more interestingly, to act as chemo- and radio-sensitizer [66,67]. WYE-125132, another rapalog, has been successfully used on different human tumor models, *in vitro* and *in vivo*, and found to act as anti-proliferative agent through the inhibition of both mTORC1 and mTORC2 [68]. Furthermore, PI-103 is a dual inhibitor of PI3K/mTOR and has been reported to induce autophagy without the involvement of the Akt pathway. This feature renders PI-103 very promising because the Akt pathway is known to be involved in different mechanisms of survival to chemotherapy. Using this novel compound in combination with the lysosomal agent monensin, Fan et al. observed that glioma cells undergo apoptosis through the intrinsic apoptotic pathway, thus demonstrating the fundamental role of autophagy in cancer cell response to toxic agents [69]. Finally, the compound NVP-BEZ235, another dual PI3K/mTOR inhibitor, has been reported to induce autophagy possibly acting as type II PCD, reducing the proliferation of glioma cells *in vitro* and *in vivo*, suggesting that this novel compound could be useful in cancer treatment [70].

Promising results have been obtained using acetylproteome-modulating agents in combination with drugs normally used in chemotherapy. Indeed, the histone deacetylase inhibitor valproic acid, in combination with tamoxifen, enhanced the efficacy of the second drug redirecting estrogen receptor-positive breast cancer cells to apoptosis. Moreover, cells surviving the combined treatment showed clear signs of autophagy that can be counteracted by inhibitors of autophagy or siRNAs directed against Beclin-1 mRNA. Thus, also in this case, autophagy acts as a survival mechanism against chemotherapy [71]. On the other hand, autophagy can kill by itself cancer cells, as demonstrated by Puissant et al., who showed that resveratrol is able to induce type II PCD in chronic myelogenous leukemia cells [72].

To resolve the autophagy paradox, according to which autophagy may play opposite roles in cancer biology [73], Martinez-Outschoorn et al. recently proposed the "battery-operated tumor growth" hypothesis that points out the feeder role of autophagy in cancer cells [74]. This model could explain the apparent opposite effects of autophagy against chemotherapeutic drugs. In this scenario, autophagy could be a survival mechanism but, by inhibiting or enhancing it, it would be possible to impede cancer cells to use autophagy to promote their own survival. Based

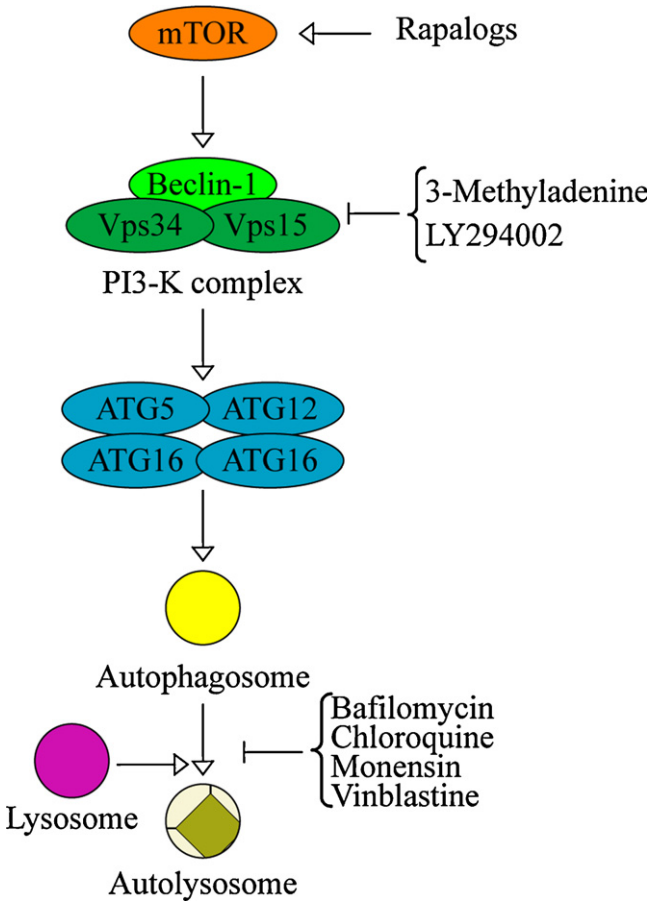


**Fig. 4.** Chemical manipulation of the autophagic machinery. The upstream autophagy regulator, mTOR, is targeted and inhibited by rapamycin and its derivatives, called rapalogs, therefore activating autophagy. Downstream, PI3-K complex could be negatively regulated by kinase inhibitors, *e.g.* 3-methyladenine and LY294002, which ultimately block the autophagic process. Finally, autophagosome fusion with the lysosome to form the autolysosome could be abolished by the so-called lysosomal antagonists, including bafilomycin, chloroquine, monensin and vinblastine.

on this idea, manipulating the autophagic machinery either by its inhibition or potentiation, could be useful to bypass the beneficial effect of autophagy after chemo- or radio-therapy [74].

For the above considerations, the inhibition of autophagy may be therapeutically beneficial in some circumstances, given that it can sensitize cancer cells to different agents, including DNA-damaging drugs, anti-hormones and radiation. In this view, autophagy inhibitors/activators combined with anti-cancer agents can negatively influence cancer cell survival and increase cell death, providing a therapeutic advantage against cancer. The most used inhibitors of the sequential complexes of the autophagy machinery are shown in Fig. 4.

## 5. Backstage

Few cautionary considerations have to be kept in mind when speculating about the balance between death and survival (Fig. 5).

(1) Apoptosis and autophagy are not governed by hyper-selective functions. Indeed, common regulators supporting a cross-talk between these processes have been described, including ATG5, Bcl-2, Beclin-1, DAPK, FLIP, JNK, LC3, p62 and mTOR [41]. This intercorrelation adds further complexity to the yet intricate signaling pathways driving drug response of cancer cells.

(2) The subcellular location of autophagy regulating factors could discriminate between pro- and anti-autophagy functions. This is the case of p53 [75]: nuclear p53 acts as an autophagy-inducing transcription factor, while within the cytoplasm it has an autophagy-inhibitory function [76]. These effects could be mediated by the downstream factor p21, which is described as a negative regulator of autophagy [77]; when accumulated in the cytoplasm, p21 promotes autophagy even under nutrient-rich conditions [78]. In this respect, it has been recently reported that the mitochondrial pool of Bcl-2 (a negative regulator of autophagy) could bind and sequester the positive regulator AMBRA-1 (activating molecule in Beclin-1-regulated autophagy), thus blocking autophagy. However, under nutrient deprivation, AMBRA-1 dissociates from Bcl-2 and binds Beclin-1 in the ER to favor autophagy [79].

(3) Beyond the role of autophagy in cancer, recent lines of evidence suggest that treatment of cancer cells with different anti-proliferating agents like 2-methoxyestradiol [80], GX15-070 [81], oridonin [82] and berberines [83] can activate both autophagy and apoptosis. These results suggest that cytotoxic drugs may induce both processes.

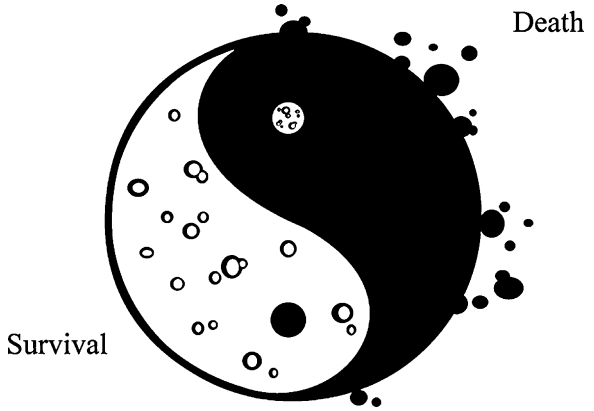


**Fig. 5.** Balance between death and survival. The dark side of cell metabolism, that is death, is represented by apoptosis with the typical membrane blebs; the white circle with autophagic vesicles denotes the possible contribution of autophagy to death. Conversely, the survival white side is characterized by autophagy hallmarks (vesicles) and by a black spot meaning unsuccessful apoptosis.

In conclusion, it is clear that the characterization of the molecular bases of death/proliferation processes is instrumental to the development of drugs effective against cancer [84,85]. In this respect, many questions are still unresolved and need a further effort by the scientific community to understand the nature of the signals discriminating between opposite functions of the same factor and to identify the regulators of the interconnection of different processes [86].

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