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The role of cell signaling in the crosstalk between autophagy and apoptosis in the regulation of tumor cell survival in response to sorafenib and neratinib

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Abstract

The molecular mechanisms by which tumor cells survive or die following therapeutic interventions are complex. There are three broadly defined categories of cell death processes: apoptosis (Type I), autophagic cell death (Type II), and necrosis (Type III). In hematopoietic tumor cells, the majority of toxic stimuli cause these cells to und ergo a death process called apoptosis; apoptosis specifically involves the cleavage of DNA into large defined pieces and their subsequent localization in vesicles. Thus, 'pure' apoptosis largely lacks inflammatory potential. In carcinomas, however, the mechanisms by which tumor cells ultimately die are considerably more complex. Although the machinery of apoptosis is engaged by toxic stimuli, other processes such as autophagy ("self-eating") and replicative cell death can lead to observations that do not simplistically correspond to any of the individual Type I-III formalized death categories. The 'hybrid' forms of cell death observed in carcinoma cells result in cellular materials being released into the extracellular space without packaging, which promotes inflammation, potentially leading to the accelerated re-growth of surviving tumor cells by macrophages. Drugs as single agents or in combinations can simultaneously initiate signaling via both apoptotic and autophagic pathways. Based on the tumor type and its oncogene drivers, as well as the drug(s) being used and the duration and intensity of the autophagosome signal, apoptosis and autophagy have the potential to act in concert to kill or alternatively that the actions of either pathway can act to suppress signaling by the other pathway. And, there also is evidence that autophagic flux, by causing lysosomal protease activation, with their subsequent release into the cytosol, can directly mediate killing. This review will discuss the interactive biology between apoptosis and autophagy in carcinoma cells. Finally, the molecular actions of the FDA-approved drugs neratinib and sorafenib, and how

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Conflict of Interest

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they enhance both apoptotic and toxic autophagic processes, alone or in combination with other agents, is discussed in a bench-to-bedside manner.

This review is intended to provide a basic understanding of autophagy and apoptosis and how the two processes can be interconnected and how they are regulated by cellular signaling processes including the actions of protein and lipid kinases and p53. The review in its final sections will present evidence of how novel combinations of anti-cancer. therapeutic agents, particularly those utilizing the multi-kinase inhibitors sorafenib and neratinib, can manipulate autophagy to kill cancer cells and to permanently alter transcription via autophagic destruction of HDAC proteins.

Autophagy.

Autophagy (self-eating) is an evolutionary conserved process that was originally, in yeasts, solely a mechanism for recycling intracellular components thereby maintaining the viability of cells undergoing nutrient. depravation stress [1]. In higher eukaryotes, however, the roles of autophagy in the regulation of survival or cell death, particularly in cancer cells, are considerably more complicated [2]. In mammalian cells, three protein kinases play important roles in the regulation of autophagy; mammalian target of rapamycin (mTOR), Unc-51 like autophagy activating kinase (ULK1) and the AMP-dependent protein kinase (AMPK) [3]. The AMPK and mTOR are regulated by diverse upstream signals including reduced levels of growth factors and nutrients, lower ATP levels and by reactive oxygen species and DNA damage [4]. The AMPK and mTOR both regulate the kinase ULK1. Phosphorylation of ULK1 by mTOR reduces its catalytic activity whereas phosphorylation by the AMPK activates ULK1, i.e. inactivation of mTOR and a parallel activation of the AMPK will generate a near-maximal activation of ULK1 [5]. The direct target of ULK1 and the “gate-keeper” of autophagosome formation is ATG13. Phosphorylation of ATG13 begins a process of protein complex formation, including proteins such as ATG5-ATG12, Beclin1 (ATG6) together with a Class III PI3K Vps34, that ultimately leads to the formation of double-membrane autophagosomes; autophagosomes sequester misfolded proteins as well as dysfunctional organelles such as damaged mitochondria [6–10]. Additional proteins that are often studied in autophagy research and who associate with autophagosomes include LC3 (ATG8) and p62 SQSTM1 [6–10]. LC3 is a ubiquitin-like protein that when lipidated by ATG4 associates with the additional ATG proteins and with the phagophore assembly site of the nacsent autophagosome where it pays an essential role in causing membrane elongation and vesicle formation. P62 is a ubiquitin binding protein and is a cargo protein within the autophagosome; thus, it can bind LC3. Autophagosomes fuse with acidic endosomes / lysosomes, forming autolysosomes. This transitioning process is termed “autophagic flux [11].” Hence, in a system where autophagosome formation is progressing faster than autophagic flux, or in a system where flux has been abolished entirely, the expression levels of LC3 and p62 will be observed to rise. In the acidic autolysosome the proteins and organelles within the double-membrane vesicle are digested by proteases, e.g. of the cathepsin family, and the breakdown products are then recycled to maintain cell viability [12].

Apoptosis.

Apoptosis is mediated via specific activation(s) of proteases called caspases. Caspases are expressed as inactive pro-forms, with each activated caspase catalyzing the cleavage and activation of its downstream pro-caspase or B cell interacting killer (BH3) domain protein target [13]. Two ‘classic’ apoptosis pathways have been identified, termed the intrinsic pathway and the extrinsic pathway [14]. The intrinsic pathway is initiated by activation of the toxic BH3 domain proteins such as BAX and BAK which form pores in the outer mitochondrial membrane [15]. This pore formation causes mitochondrial dysfunction, i.e. reduced ATP and NADPH production, concomitant with the release of cytochrome c and apoptosis inducing factor (AIF) into the cytoplasm. Cytochrome c together with Apaf-1 and ATP facilitates the activation of pro-caspase 9, leading to the cleavage and activation of pro-caspase 3. Caspase 3 then acts to execute the cell, with portions of DNA and cytosol forming discrete vesicles [16]. Cytosolic AIF enters the nucleus where it causes DNA fragmentation processes usually viewed as being more similar to cells undergoing a necrotic death process. The extrinsic apoptosis pathway is initiated by death receptor signaling, e.g. CD95 and FAS-Ligand; DR4/DR5 with TNF-related apoptosis inducing ligand (TRAIL) [17]. Death receptor activation permits the docking protein FADD to associate with the receptors, and FADD via conformational alterations to cause the processing and activation of pro-caspase 8. The death receptor – FADD – pro-caspase 8 complex is known as the Death-Inducing Signaling Complex (DISC) [18]. The ability of caspase 8 to become activated is prevented by the anti-apoptotic protein FLICE like inhibitor protein short (FLIP-s) [17, 18]. Caspase 8 has well-recognized two routes to cause tumor cell death. One route involves caspase 8 cleaving and activating the toxic BH3 domain protein BID; cleaved BID associates with protective BH3 domain proteins, e.g. BCL-2, BCL-XL and MCL1, that in turn dislocates the proteins BAX and BAK from the protective BH3 domain proteins into their unbound state in the outer mitochondrial membrane where they aggregate to form pores [19]. Cytochrome c is released through these pores which goes on the mediate the activations of pro-caspase 9 and pro-caspase 3 and apoptosis. The second route for caspase 8 is to act as a direct activator of pro-caspase 3, missing out the mitochondria / cytochrome c step [20]. This can also occur as part of a secondary amplification loop. Generally, based on our ~20-year research experience with chemotherapies in carcinoma cells, the first route, via BID and the mitochondrion, is the most prevalent.

The interfaces between autophagy and apoptosis; autophagy regulating apoptosis and apoptosis regulating autophagy.

Autophagy is observed in cells that are under “stress,” e.g. those deprived of nutrients or exposed to chemotherapy [21–23]. This can be an attempt by the cell to alleviate any given toxic stress and restore homeostatic balance. Alternatively, the induction of autophagosome formation may reflect a crosstalk between the processes of autophagy and apoptosis and thus could be controlling an execution process. If the stress persists and autophagy is no longer able to restore homeostasis and survival; cells can respond by activating apoptotic processes in order to ensure their controlled and efficient elimination, without triggering local inflammation / immune cell activation [23]. Therefore, regulatory ties between autophagy

and apoptosis (and vice versa) may represent an evolutionary advantage to cells, allowing for a more controlled response to any given stress signal. Thus, although autophagy and apoptosis are distinct cellular processes, with different biochemical and morphological appearances, the actual protein networks that control their respective regulation and execution are highly interconnected.

Regulation of autophagy by apoptosis:

One of the issues face d by scientists not within the “autophagy cognoscenti” is understanding the complexity of cross talk between apoptosis and autophagy with data often having the potential to appear contradictory [21–26]. Determining which interactions are “important” in the regulation of cell death by autophagy and of apoptosis is of essential as we try to either protect the cells from cell death, as in neurodegenerative diseases, or induce cell death, as in cancer treatment. Thus, cell death processes cannot be simplistically, neatly and discretely categorized and the myriad of different types of cell death overlap. One of the first studies to explore the interaction s between autophagy and apoptosis demonstrated that TNFα-induced apoptosis was reduced by autophagy [27]. Similar data were observed in immortal epithelial cells where autophagy acted to degrade expression of the death receptor linker protein FADD [28]. Other studies linked autophagy to facilitating the mitochondrial permeability transition, with the concept that both apoptosis and autophagy could enact this process to cause cell death [29]. In part, this may be because cathepsin proteases, released from autolysosomes, can cleave BID, thereby displacing BAX and BAK from protective BH3 domain proteins such as BCL-2, BCL-XL and MCL-1 [30, 31]. Data from neurons argued that autophagy was induced downstream of caspase activation, also as part of the killing process [32]. Others have observed the reverse situation or that inhibition of autophagy causes activation of the apoptosis machinery [33–36].

Beclin1 and BCL-2:

Probably the most important autophagy-regulatory protein whose functions were initially tied to apoptosis-autophagy was Beclin1. Loss of Beclin1, and autophagy, was demonstrated to be important in the process of tumorigenesis [37–39]. Significantly, Beclin1 contains a BH3 domain and was shown to interact with BCL-2 family proteins, and that molecular knock down of these proteins or the use of inhibitory drugs, caused Beclin1 to be freed and for it to associate with autophagosome formation protein complexes [39–41]. The confluence of autophagy and cell death or cell viability is also of prime importance in cells infected with lytic viruses. For example, enhanced autophagy prevents parvovirus from causing cell death [42]. In our own studies, we have shown drugs that inhibit chaperone proteins, including AR12, sorafenib and pazopanib, can elicit endoplasmic reticulum (ER) stress responses via eIF2α as well as inactivating mTOR which collectively result in the protection of infected cells, via autophagosome formation, from virus-mediated lysis [43, 44]. As our findings were reliant on dysregulating host chaperone / ER / autophagy biology, these approaches could, for example, prevent the replication of proteasome inhibitor drug-resistant strains of HIV [45].

In addition to BCL-2 family anti-apoptotic proteins, the caspase 8 inhibitor FLIP-s has also been shown to inhibit autophagy by interfering with the function of ATG3 in the LC3

lipidation / conjugation system, by competing with LC3 for the binding of ATG3, thus attenuating LC3 lipidation [46]. FLIP-s is a phospho-protein and can also be rapidly dephosphorylated and/or down regulated in response to exposure to many chemotherapeutic agents, resulting in caspase independent cell death. [47]. When autophagosome formation is induced, the interaction between FLIP-s and ATG3 is substantially decreased. Separate regions within the FLIP-s protein itself have been shown to control its anti-autophagic and anti-apoptotic activities [46]. Inhibition of apoptosis by FLIP-s takes place at the plasma membrane / DISC, and inhibition of autophagy most likely occurs at sites of autophagosome formation liminal to the plasma membrane / death receptors / caspase 8. Hence, compartmentalization of FLIP-s into different pools may provide a way to achieve independent regulation of FLIP-s mediated autophagy and apoptosis. Additional routes for caspase 8 recruitment to the autophagosomes have been described [48–50].

Ubiquitinated pro-caspase 8 can bind to p62 through the ubiquitin binding domain o, p62 and is subsequently recruited to the autophagosome through a direct interaction between p62 and LC3. Additionally, p62 is also required for activation of caspase 8, by facilitating its self-oligomerization [51, 52]. The second route of caspase 8 recruitment to the autophagosomes has been shown to take place through an interaction between the docking protein FADD and the autophagy-regulatory protein ATG5 [53]. Although ATG5 has been shown to interact with FADD through its death domain, a deficiency of FADD within a cell does not significantly influence autophagic formation. In contrast, the loss of FADD expression recovered caspase dependent killing induced by overexpression of ATG5, that provides evidence for the important role of l e ATG5-FADD interaction in regulating apoptosis rather than autophagy. Autophagy can also attenuate cell death by selectively reducing the abundance of pro-apoptotic proteins [26, 48–53]. Colon cancer cells lacking the pro-apoptotic BH3 domain protein BAX are resistant to TRAIL-induced cell death, unless autophagy is inhibited. In cells lacking BAX, DISC processing of pro-caspase 8 still occurs as per in a wild type cell. However, death receptor activation also, in parallel, can stimulate autophagosome formation. Activated caspase 8 can then be sequestered in.to the autophagosomes where after fusion with lysosomes, it is degraded. If cells cannot readily form autophagosomes, activated caspase 8 proceeds to kill cells via apoptosis.

Published data has suggested that autophagosome mediated activation of caspases requires the formation of autophagosomes, or at the very least autophagosomal membranes with associated protein complexes, but not autophagic flux or autolysosomal activity per se [54, 55]. Hence, depletion of autophagosome regulatory gene products can inhibit caspase 8 function whereas pharmacological inhibition of autophagosome-lysosomal fusion can enhance caspase 8 activation. Upon the inhibition of protein degradation pathways, e.g. ubiquitination, cells can upregulate their autophagic response to facilitate autophagolysosomal protein turnover, which is dependent upon the lipidation and autophagosome membrane localization of LC3. This leads to the enrichment of LC3 on these membranes that can serve as a molecular hub to recruit the ubiquitin binding protein p62 and one of its binding partners, caspase 8. The induced oligomerization and activation of caspase 8 subsequently initiates the downstream extrinsic apoptosis cascade [54, 55]. In agreement with these results, more recent studies have shown that over expression of wild type p62 in glioma cells was able to activate caspase 8 and then promote HAMLET (a

complex of oleic acids and decalcified α-lactalbumin that was discovered to selectively kill tumor cells both *in vitro* and *in vivo*), induced apoptosis, whereas knockdown of p62 manifested the opposite effect [56].

Regulation of apoptosis by autophagy:

In contrast to information presented in prior paragraphs, the regulation of apoptosis by autophagy can also be observed. Specific autophagy proteins can regulate apoptosis via mechanisms which are not be related to their canonical role in autophagic signaling. The apoptotic-faciliatory function of these proteins does not need to activate autophagic flux. Inhibition or genetic alteration of these specific autophagy genes may affect apoptosis, since usually neither autophagosme formation nor autolysosomal function are required for their pro-apoptotic function [57]. Covalent conjugation of the autophagy-related proteins ATG5 and ATG12 involves in an ubiquitylation like process that involves ATG7 and ATG10 and is essential to autophagosome formation. Therefore, ATG5 and ATG12 are integral parts of the autophagic machinery and are required for the induction of autophagy [58].

The autophagosome regulatory protein ATG5 plays a dual role in the regulation of autophagy and apoptosis. Studies have indicated that over-expression of ATG5 can sensitize tumor cells to chemotherapy. Whilst silencing the ATG5 gene with short interfering RNA, induced partially resistant of tumor cells to chemotherapy. This study suggested that ATG5 was cleaved by calpains, a family of Ca^{2+} dependent cysteine proteases, during apoptosis, producing an amino-terminal cleavage product. Calpain induction and subsequent ATG5 cleavage appear to be universal phenomena in apoptotic cells [59, 60]. Calpains have been reported to cleave ATG5 and the cleaved product is able to induce apoptotic cell death [58–60]. The cleavage product, an NH_2-terminal ATG5 fragment, translocates from the cytosol to the mitochondria. Full length ATG5 and truncated ATG5 are found in cells undergoing apoptosis. However, only the truncated form of ATG5 has been shown to co-immunoprecipitate with the anti-apoptotic protein BCL-XL, triggering cytochrome c release and caspase activation. These results indicated that truncated ATG5 loses its autophagy inducing function and now functions as a pro-apoptotic protein that inhibits anti-apoptotic BCL-2 homologs, resulting in the activation of BAX, BAX and mitochondrial-dependent apoptosis [58–60]. A number of groups have also observed a calpain / cathepsin induced site specific Cleavage of Beclin1 following induction of apoptosis [61–64]. The COOH-terminal fragment of Beclin1 can translocate to mitochondria and cause the release of cytochrome c in response to additional apoptotic signals [61–64]. Conversely, the terminal effector caspase, caspase 3, can inhibit autophagy and promote apoptosis by the cleavage and *inactivation* of Beclin1. This process may thus represent an amplifying loop for inducing greater, and greater, levels of apoptotic cell death. Thus, with respect to Beclin1 and ATG5, activation of apoptosis can induce site specific cleavage of these autophagic proteins, which may serve to suppress protective autophagy and at the same time, generate mitochondria targeted cleavage products which may function as positive feedback loops leading to an enhanced apoptotic response.

Similarly, the autophagy regulatory protein ATG12 also has also been shown to have a dual function, participating in both autophagy and apoptosis and is necessary for caspase

activation in response to a range of apoptotic stress inducers. Non-conjugated ATG12 can bind to and inhibit MCL-1 and BCL-2 by a BH3-like motif, which can induce mitochondrial-dependent apoptosis [65]. Knockout of ATG12 in apoptotic cell has also been shown to prevent BAX activation and cytochrome c release. Knockdown of several other essential autophagic genes did not show any significant effect upon apoptosis, supporting a specific role for ATG12 in apoptosis, one that does not require true autophagosome formation [65]. Further studies have indicated that 'free' unconjugated ATG12 is has a short half-life and could be broken down in a ubiquitination / proteasome dependent manner. Free ATG12 could thus potentially facilitate proteasome inhibitor-dependent apoptosis, arguing that proteasome inhibitors in part act in this manner as anti-cancer agents [66].

ATG3 and ATG12:

The autophagy regulatory protein ATG3 is a non-canonical ubiquitination E2 enzyme regulating the conjugation of ubiquitin-like ATG8 to phosphatidylethanolamine in the autophagy process [67]. A conjugate comprised of ATG12 and ATG3 can regulate mitochondrial homeostasis and cell death via apoptosis, mediated by pathways downstream of mitochondria. The ATG3-ATG12 complex localizes in the mitochondrial outer membrane, leading to expansion in mitochondrial mass and inhibition of cell death mediated by mitochondrial pathways, but that had no effect on death receptor-mediated apoptosis [68]. These results reveal a role for the ATG12-ATG3 conjugate in mitochondrial homeostasis and implicate the ATG12 conjugation system in cellular functions distinct from the initial phase of auophagosome formation. Disturbing the complex formation significantly reduce s selective mitochondrial autophagy, called mitophagy, but has no effect upon non-selective autophagy [69].

Beclin1, protective BH3 domain proteins and the regulation of autophagy.

The regulation of apoptosis by other proteins essential for the recruitment of autophagy-regulatory proteins to sites of autophagosome formation include the class III phosphatidylinositol 3 kinase (PIK3C3, Vps34)-Beclin1-ATG13 complex, which serves as a central regulator of autophagy downstream of the AMPK, mTOR and ULK1 [70]. The most studied relationship between autophagic and apoptotic proteins is the relationship between Beclin1, the anti-apoptotic BH3 domain proteins, BCL-2, including the family members MCL-1 and BCL-XL, and the pro-death BH3 only domain proteins BAX and BAK [71, 72]. There are at least two distinct cellular pools of BCL-2, one at the ER where it is bound to Beclin1 and the other at mitochondria where it is bound to BAX and BAK [72, 73]. Binding of BCL-2 to the BH3 domain of Beclin1 has been shown to inhibit autophagy, i.e. Beclin1 is sequestered and cannot facilitate autophagosome formation [73, 74]. Following autophagosome formation, a BH3 only protein, either BIK, BAD, or NOXA, can competitively bind to BCL-2, displacing Beclin1. The displacement of Beclin1 can be augmented by c-Jun NH_2-terminal kinase (JNK1) phosphorylation of BCL-2 and this event is required for complete release of Beclin1, whence it can interact with and activate Vps34. This promotes double membrane nucleation and the subsequent promotion of autophagosome formation [75]. BCL-2 has a higher affinity for BAX or BAK than it does for Beclin1, and as a result, the low basal levels of BCL-2 phosphorylation may be

insufficient for its dissociation from mitochondrial BAX or BAK [76]. In parallel, the interaction between BCL-2 and Beclin1 may function as a rheostat that maintains autophagy at levels that are compatible with cell survival rather than cell death [22]. Mutations of either the BH3 only domain within Beclin1, or the BH3 receptor domain within BCL-2 or BCL-XL, disrupt the Beclin1-BCL-2 complex, resulting in the cellular context of enhanced autophagosome formation [74–76]. The inducible BH3 domain protein BIM can function as an inhibitor of autophagy but has been shown to lead to induction of apoptosis in a number of studies and effect possibly mediated via release of BIM from MCL-1 after down regulation of the latter protein [77]. It would be expected that binding of Beclin1 to BCL-2 would induce apoptosis through neutralizing the anti-apoptotic function of BCL-2. However, apoptosis was not shown to be induced [1]. In addition, BCL-2 retained its full anti-apoptotic activity [77].

The autophagy-regulatory protein Beclin1 can play an anti-apoptotic role against TRAIL, traditional chemotherapy, ionizing radiation, checkpoint immunotherapy, nutrient deprivation, angiogenesis inhibitors and hypoxia [78–80]. The precise mechanism(s) of Beclin1 mediated inhibition of apoptosis are not yet clear but may be related to unregulated autophagosome formation as an adaptive or anti-injury mechanism, leading to the clearing of apoptotic cells [80]. In addition, ER localized BCL-2, but not mitochondria localized BCL-2, can inhibit autophagy, consistent with the initial concept that ER associated class III PI3K activity was crucial in the nucleation step of autophagosome formation [80]. Beclin1 can co-localize with BCL-XL within mitochondria via its BH3 domain suggesting a differential role of BCL-XL in Beclin1 complex when compared with BCL-2 [81]. Similar to the regulation of apoptotic pathways by the actions of autophagic proteins, so components of the apoptotic machinery can regulate autophagosome formation via their molecular interactions with autophagy proteins. The most extensively studied example for this type of regulation is the role played by bcl-2 family members and the potential to inhibit both pathways. In resting cells, it has been proposed that BCL-2 is constitutively bound to Beclin1; under these circumstances, this permits low levels of autophagy. Under an autophagic inducing cell stress, however, BCL-2 and Beclin1. dissociate from each other, resulting in an increased availability of free Beclin1 that can then act to promote autophagosome formation. A combination of BCL-2 dependent regulation and feedback loops between Beclin1 and caspases thus robustly enforces a sequential activation of cellular responses depending upon the intensity and duration of stress levels [82, 83].

Regulatory phosphorylation of BCL-2 and autophagy.

As previously stated, regulatory phosphorylation of Beclin1 and BCL-2 constitutes another mechanism controlling dissociation rate of BCL-2 from Beclin1. Phosphorylation of BCL-2 at multiple sites by JNK1 and also by extracellular signal related kinases (ERK1/2) can reduce the disassociation constant of BCL-2 for Beclin1, leading to the autophagosome formation [84–86]. Furthermore, phosphorylation of Beclin1 within its BH3 domain by death associated protein kinase (DAPK) has been shown to induce autophagy by promoting its dissociation from BCL-XL [87]. Phosphorylation of Beclin1 by the MAP4K MST1 can increase the affinity of Beclin1 for BCL-2, thereby suppressing autophagy but also importantly inducing apoptosis [88]. These findings argue that pro-survival BCL-2 family

members inhibit autophagy by restraining their apoptotic interaction partners BAX and BAK, the effectors of intrinsic apoptosis, [89, 90]. Consistent with this model, overexpression of BAX alone has been shown to stimulate autophagy [91]. The death receptor docking protein FADD can regulate autophagy by binding to ATG5-ATG12 [92, 93]. And, caspases can inhibit autophagy; for example, the pan-caspase inhibitor z-VAD and direct caspase 8 inhibition using IETD have both been proposed to inhibit RIP-dependent autophagy and cell death in L929 cells [93]. n.b. this data could be that this is actually necroptotic cell death with accompanying autophagy [94].

Autophagy and sphingolipids.

Ceramide is generally associated with growth arrest and tumor cell death, particularly in hematopoietic cancer cells. Ceramide is not only a potent j, duce r of apoptosis, but it can also trigger autophagic cell death in malignant glioma cells via activation of the pro-apoptotic member of the BCL-2 family, BNIP3 [95]. Further apoptotic-pathway regulation of autophagosome formation is found in leukemic cells. where down-regulation of BCL-2 induces autophagy, leading to tumor cell death [96]. In neural precursor cells, deprivation of growth factors also leads to an autophagic cell death, which can be blocked by over-expressing anti-apoptotic BCL-2, an involvement that has also been recognized in autophagic cell death induced by HSP1, a molecule first identified as interacting with BCL-2/BCL-XL [97, 98].

Autophagy and different cellular compartments.

The spatial separation of proteins into different cellular compartments may represent an additional mechanism to achieve the independent regulation of autophagy and apoptosis. At least two cellular pools of BCL-2 exist, one at the ER and the other at the mitochondria, and both appear competent to regulate both autophagy and apoptosis [73]. The regulation of autophagy by the binding of BCL-2 to Beclin1 at the ER membrane is facilitated by nutrient deprivation autophagy factor 1 and inhibited by the toxic BH3 only protein BIK [99]. Moreover, as mentioned previously, ERK1/2 and JNK1 specifically phosphorylate the regulatory loop of BCL-2, leading to disruption of its association with Beclin1 [84, 85]. Thus, cells with either mutations in the phosphorylation sites of the regulatory loop of BCL-2 or even deficiency in JNK pathway activation appear to be unable to undergo starvation-induced autophagosome formation [100]. In addition, the disruption of BCL-2-Beclin1 complex is thought to be mediated by pro-apoptotic BH3-only proteins such as BAD and BAX [101, 102].

Thus, compartmentalization of BCL-2 together with organelle specific sets of associated interacting proteins, may result in a dynamic and independent regulation of autophagy and apoptosis. BIM-Long can interact directly with Beclin1, this interaction occurs at a site different from the BCL-2-binding region on Beclin1 [73]. BIM mediated regulation of autophagy is Beclin1 dependent and is disrupted by starvation. BIM-L is sequestered by dynein in healthy cells and dissociated following apoptotic stimulus [103]. The interaction of BIM-L with dynein facilitates the loading and potential fusion and positioning of lysosomes, arguing that the absence of BIM may lea d to impairment of the later degradative

phase of autophagy [104]. The anti-apoptotic protein MCL-1 has also been shown to regulate autophagy. MCL-1 degradation is an early event not only following induction of apoptosis, but also under nutrient deprivation conditions where MCL-1 levels regulate activation of autophagy. Furthermore, deletion of MCL-1 in cortical neurons of transgenic mice activates a strong autophagic response. This response has been demonstrated to be converted to apoptosis by either reducing the levels of the autophagy regulator Beclin 1, or by a concomitant activation of BAX [105].

Autophagy and p53.

The “guardian of the genome” p53, a signal transduction integrator, can have its expression enhanced by diverse stimuli, including hypoxia, DNA damage, nutrient stress, and ischemia-reperfusion [106–109]. P53 can regulate apoptosis via both the intrinsic and extrinsic pathways. In the nucleus, p53 acting as a transcription factor promotes the expression of the pro-apoptotic proteins BAX, BID, PUMA, and NOXA, and simultaneously acts to inhibit BCL-2 expression, facilitating mitochondrial and ER dysfunction. In the cytoplasm, p53 can act in a non-canonical fashion to promote activation of the TRAIL receptor and FAS (CD95) receptor [110]. In addition, p53 has been proposed to activate Apaf-1; Apaf-1 is a component of the apoptosome containing cytochrome c, leading to the ATP-dependent activation of pro-caspase 9 [111].

P53 is well-recognized as a potent inducer of apoptosis and multiple p53 target genes can also stimulate autophagy. This effect is often seen following inhibition of the signaling axis converging on mTOR [112]. Other studies have shown autophagy to be induced through increased expression of a direct p53 target gene called damage regulated autophagy modulator (DRAM), a lysosomal protein that functions at the crossroads between p53 induced autophagy and cell death [113]. dram is essential for the network of signals regulating p53-regulated apoptosis and autophagy [114]. In HCT116 human colorectal cancer cells exposed to prolonged nutrient deprivation, endogenous wild type p53 in a post-transcriptional fashion to down regulate expression of LC3. This effect was shown to reduce autophagic flux. Loss of p53 impaired this flux and caused excessive LC3 accumulation upon starvation, culminating in apoptosis [115]. Moreover, inhibition of p53 function triggers autophagy mainly in the G1 phase cells and less in S phase cells; p53 mediated autophagy is not seen in the G2/M phases, indicating the p53 mediated autophagy is cell cycle dependent [116].

Although nuclear p53 functions as a pro-autophagic transcription factor, in the cytoplasmic pool of p53, published data argues that / acts co suppress autophagy [116]. Cytosolic p53 can repress autophagy by interacting with the autophagy protein FIP200 (FAK family kinase-interacting protein; ATG17 in yeast), blocking the activation of the ULK1–FIP200–ATG13 complex and inhibiting autophagosome formation [117, 118]. In human, mouse as well as nematode cells, ablation of p53 function resulted in enhanced autophagosome levels which relied upon the canonical autophagy-activation pathway converging on mTOR inhibition [119, 120]. Human p53 null cancer cells also have increased their baseline levels of autophagy that can be reduced by expression of wild type p53. Inhibition of autophagy by p53 is maximal when the p53 nuclear localization sequence is deleted. This data argues that

the cytoplasmic pool of p53 acts to inhibit autophagy, and that autophagy inhibition by p53 can be uncoupled from the pro-apoptotic role of p53.

Autophagy and DNA damage.

Autophagosome formation can be enhaced following DNA damage, notably by activation of ataxia telangiectasia mutated (ATM) kinase or from Poly (ADP-Ribose) Polymerase 1 (PARP1) [121]. From a translational perspective in cancer, DNA damage can occur following exposure of tumor cells to ionizing radiation as well as to agents which da mage DNA such as platinum containing drugs, doxorubicin and vinca alkaloids. In studies describing the role of autophagy after exposure to ionizing radiation, the initial radiation-induced production of autophagosomes acts to protect cells, with inhibition of autophagy enhancing radiosensitivity [122, 123]. However, it is also known that the inclusion of drugs that by themselves also stimulate autophagy, e.g. vitamin D and analogues, when combined with ionizing radiation collectively enhance autophagosome formation and under these conditions, autophagy mediates tumor cell killing [124, 125]. This implies low levels of autophagy protect and prolonged autophagy kills. This concept in signaling has been previously observed several decades ago in the ERK1/2 pathway, wherein a modest short-lived activation of ERK1/2 causes cell growth whereas prolonged high activation of the pathway causes growth arrest and eventually cell death.

Our own studies examining DNA damage signaling have focused on activation of ATM, leading to enhanced phosphorylation o the AMPK with subsequent autophagosome formation, but also examining the role of PARP1 inhibitors as regulators of autophagic flux [126, 127]. Our studies in ovarian cancer cells demonstrated that the PARP1 inhibitor niraparib as a single agent could activate ATM which caused inhibition of mTOR and enhanced ATG13 phosphorylation, resulting in autophagosome formation. Inhibition of autophagy by genetic knock down of Beclin1 or ATG5 reduced the lethality of the PARP1 inhibitor niraparib as a single agent.

Autophagy and Caspases.

Recent seminal studies by The Thorburn laboratory at The University of Colorado who have performed some of the most important work in dissecting the interactions between apoptosis and autophagy have shown that the autophagy-regulatory machinery can control the mechanism of programmed cell death by serving as a scaffold rather than by degrading autophagosome cargo [25]. The switch between death receptor signaling and ATP-dependent apoptosis versus a largely ATP-independent form of killing called necroptosis can be defined by the functionality of the MAP3K7 gene (TAK1). MAP3K7 is a tumor suppressor and loss of its expression enhances the lethality of TRAIL. In the absence of MAP3K7, the receptor / FADD / pro-caspase 8 DISC does not form, and instead, the autophagy regulatory protein p62/SQSTM1 facilitates a different complex of proteins to form on the outer membrane of autophagosomes. The p62 protein localizes RIPK1, a kinase that regulates necrosis via phosphorylation of mixed-lineage kinase-like (MLKL), to this complex, which ultimately results in the cell dying via a combination of autophagic and more specifically MLKL-dependent plasma membrane pore formation processes leading to necrosis. If, however, both

p62 and MAP3K7 levels are low, RIPK1 does not enter the complex and the DISC, sans death receptor, catalyzes the activation of pro-caspase 8 with an apoptotic execution of the cell.

Caspases are the initiators and effector, participating in apoptotic cascades. However, caspases also play a role in regulating the crosstalk between autophagy and apoptosis [128]. A number of autophagic proteins have been identified as targets of caspase mediated cleavage, which will ultimately lead to an inactivation of their autophagic function. Proteins include p62, Beclin1, VPS34, ATG3, ATG4D and ARBRA1. Studies have shown that caspase 6 and 8 cleavage of p62 can inhibit autophagy, including the formation of LC3 punctae and p62 degradation were evident, indicating that caspase cleavage of autophagy related proteins can affect the autophagic process, specifically p62 [129]. Other studies demonstrated that apoptosis blocks Beclin1 dependent autophagosome synthesis. After its cleavage, both the NH_2-and COOH terminal Beclin1 fragments change their subcellular localizations; these fragments do not interact with the Class III PI3K Vps34, which is required for autophagosome formation. As a result, the cleavage of Beclin1 has been shown to be a critical event whereby caspases inhibit autophagy [130].

As noted previously, caspase 8 is an essential trigger involved in death receptor–induced apoptosis [131] Multiple autophagy-regulatory proteins are caspase 8 substrates. Caspase 8 over expression can induce ATG3 degradation which was depended upon caspase 8 enzymatic functionality. Mutation of the caspase 8 cleavage site on ATG3 abolished its cleavage both *in vitro* and *in vivo,* demonstrating that ATG3 was a direct target of caspase 8. Autophagy was seen to be inactive during apoptosis and blockage of caspases or over expression of a non-cleavable ATG3 protein re-established autophagic activity [132]. ATG4D over expression induces apoptosis, which is preceded by the caspase independent recruitment of ATG4D to mitochondria and is facilitated by a putative COOH-terminal BCL-2 BH3 domain. ATG4D also acquires affinity for damaged mitochondria in cells treated with hydrogen peroxide. These data suggest that ATG4 D is an autophagy regulator that links mitochondrial dysfunction with apoptosis. [133].

Pagliarini et al have demonstrated that apoptotic stimuli induce a rapid decrease in the level of the autophagic factor Activating Molecule in Beclin1 Regulated Autophagy (AMBRA1), whose degradation was shown to be prevented by concomitant inhibition of caspases and calpains [134]. Furthermore, whereas caspases were responsible for AMBRA1 cleavage at position D482, calpains were involved in complete AMBRA1 degradation. These studies also demonstrated that AMBRA1 levels are critical for the rate of apoptosis induction. RNA interference mediated AMBRA1 down regulation further sensitized cells to apoptotic stimuli, while AMBRA1 over expression antagonizes cell death by prolonging the induction of autophagosomes. Collectively, this argues AMBRA1 is an important target of apoptotic proteases resulting in the dismantling of the autophagic machinery and the accomplishment of the cell death program. This phenomenon may be explained in that inhibition of autophagy, which can often precede apoptosis, serves to prevent the simultaneous activation of contradicting pro survival and pro death processes in the cell, enabling a transition in the direction of cell death. In agreement with this, expression of a mutant form of the autophagic protein AMBRA1 that cannot be cleaved by caspases, has been shown to confer partial

protection from apoptotic cell death and a corresponding increase in autophagy [134]. As we have already mentioned, in some cases of caspase cleavage of autophagic proteins, an unexpected phenomenon exists, such that the cleaved product of Beclinl acquires a pro-apoptotic function, conferring an additional stimulus for cells that are undergoing cell death. Starvation induced autophagy in fruit-fly nurse cells require the insect caspase Dcp1 and was prevented by the apoptosis inhibitor and E2/E3 ligase Baculoviral inhibitor of apoptosis repeat-containing (Birc) 6 gene/BIRC6 (BRUCE) [135, 136].

Autophagy, Apoptosis and Cancer Experimental Therapeutics; sorafenib.

Several laboratories have reported that molecules previously defined as intermediaries in the activation of apoptosis pathways also function as intermediaries in the activation of autophagosome formation, further calling into question the individualized specific roles of both apo ptosis and autophagic cell death, in addition to our ability to distinguish these processes. The anti-cancer drug sorafenib, originally developed as an inhibitor of RAF family kinases but subsequently discovered to be a potent Class III receptor tyrosine kinase and a chaperone ATPase inhibitor is currently approved for the treatment of refractory renal cell and hepatocellular carcinoma highlights this issue [137]. Sorafenib as a single agent can cause activation of caspase s 9 and 3 and in parallel, via inhibition of the chaperone GRP78, cause an endoplasmic reticulum stress response which acts to increase the expression of Beclin1 and ATG5 resulting in autophagosome formation [137–140]. We have more recently shown in our laboratory that sorafenib can enhance the cytotoxicity of the anti-folate and non-small cell lung cancer therapeutic pemetrexed through an autophagy dependent mechanism. Pemetrexed causes DNA damage and a build-up of the AMP-like metabolite ZMP [139]. DNA damage signaling via ATM causes phosphorylation and activation of the AMPK, and ZMP causes the allosteric activation of the AMPK, collectively leading to mTOR inactivation, ULK1 activation and the phosphorylation of ATG13, with concomitant autophagosome formation. As we subsequently discovered in other projects, drug combinations that strongly enhance autophagosome formation also can rapidly reduce the protein levels of multiple histone deacetylase (HDAC) proteins via this mechanism, particularly the cytoplasmic HDAC6 and its client chaperone HSP90 [127,141–144]. This finding also explains, in part, why HDAC inhibitors cause an ER stress response as a primary effect of their biology; inhibition of HDAC6 results in elevated acetylation of HSP90, an effect that reduces the ability of HSP90 to act as a chaperone. Other chaperones are regulated in a similar manner. These events collectively lead to elevated levels of mis-folded and denatured proteins which in turn promote an ER stress response.

The secondary degradation effect upon HDAC6 and HSP90, after the initial induction of autophagosome formation caused by activation of the AMPK and ULK1 and inactivation of mTOR, induces greater levels of misfolded proteins, more endoplasmic reticulum stress and still further and greater levels of autophagosome formation. Similarly, other work has indicated that sorafenib induced autophagy in hepatocellular carcinoma mediated through Beclin1 [145]. Conversely, recent data has suggested that the anti-cancer effects of sorafenib are mediated by the induction of apoptotic cell death and that the simultaneous activation of autophagy via IRE1, led to an inhibition of apoptosis and thus limited the efficacy of t e dmg [146, 147]. These conflicting findings are an issue when trying to understand the

mechanisms of action of any therapeutic agent; i.e. at what drug concentration should the drug be used for in vitro studies? A first step is to determine the safe maximal plasma cancer ration of the drug in patients from its initial phase I trials. The safe peak plasma level of sorafenib 400 mg BID in plasma is approximately 13 μM, but due to ~90% of the drug being protein bound in the plasma, in vitro studies using greater than 2 μM of the drug are unlikely to represent the key physiologic mechanisms of drug action.

Due to its chaperone inhibitory properties, sorafenib via inhibition of GRP78 can cause an ER stress response, which whilst increasing the levels of Beclin1 and ATG5, also leads to an overall reduction in protein translation, with a rapid reduction in the levels of short half-lived proteins such as MCL1 and to a lesser extent BCL-XL; the half-life of BCL-2 is ~48h [148]. This results in greater levels of unbound BAX and BAK and primes the cell for being killed via additional negative effects on mitochondrial function. For example, sorafenib, via inhibition of ERK1/2 signaling, results in enhanced BIM expression in turn that reduces the abilities of BCL-2, BCL-XL, and MCL-1 to sequester the BH3 only proteins BAX and BAK [148, 149]. In a number of hepatic carcinoma cell lines, chemotherapeutic sensitivity has been associated with BAD expression. Stable cell lines derived from HuH7 cells over expressing BAD were found to exhibit a striking five-fold increase in their chemotherapy sensitivity [150]. Sorafenib treatment has also been shown to induce PUMA in a variety of cancer cells, irrespective of their p53 status. In these studies, deficiency in PUMA abrogated sorafenib induced apoptosis and caspase activation and rendered sorafenib resistance in colony formation and xenograft tumor assays [151]. Conversely, however, another pro apoptotic protein, NOXA, has been shown to sharply down regulated by sorafenib [152].

Autophagy, Apoptosis and Cancer Experimental Therapeutics; neratinib.

The drug neratinib was developed as an irreversible inhibitor of ERBB1, ERBB2 and ERBB4 (the EGF receptor family) [153]. Our interest in neratinib came about due to our inability to translate to drug combinations that included the irreversible inhibitor of ERBB1, ERBB2 and ERBB4, afatinib [154, 155]. In performing our studies with afatinib, we had generated in vivo multiple independent clones of the H1975 NSCLC line; a line that egresses a mutant active ERBB1. Afatinib resistant cells maintained their viability by enhancing signaling through ERBB3, c-MET and c-KIT [155]. In side-by-side in vitro analyses of these “resistant” cells when exposed to afatinib or neratinib, we discovered that as predicted, they were resistant to afatinib. However, in these afatinib-resistant NSCLC cells neratinib lethality actually was increased. This posits the question, what are the different targets and mechanisms of action comparing afatinib and neratinib [144]?

In 2011, Davis et al published an in-depth study of kinase inhibitor selectivity against 440 different protein kinases [156]. In this manuscript it was shown that in addition to inhibiting ERBB1/2/4, neratinib was also an inhibitor of multiple MAP4Ks. In contrast, afatinib only inhibited ERBB1/2/4 at or below its physiologic clinically relevant range. As noted previously, phosphorylation of Beclin1 by the MAP4K MST1 can increase the affinity of Beclin1 for BCL-2, thereby suppressing autophagy [88]. MAP4Ks inhibited by neratinib below its plasma C max included STK10, YSK1, YSK4, MAP3K4, MAP4K3, MAP4K5, MST2, MST3 and MST4. In the course of our studies we had demonstrated that neratinib as

a single agent, and to a greater extent when combined with HDAC inhibitors, rapidly increased autophagosome and later autolysosome level, arguing that autophagic flux was being stimulated [144]. In parallel, performing control (total protein level) control studies, we discovered that ERBB1/2/3/4 expression was reduced by neratinib; the reduction in RTK levels required autophagosome formation and was reduced when the expression of cathepsin proteases was knocked down.

Mechanistic analyses went on to illustrate how cancer therapeutic drug combinations are complicated and highly interactive; the elevated levels of autophagosome formation cause by [neratinib + HDAC inhibitor] required multiple independent and cooperative signals [144,157,158]. The initial driving signals behind this drug combination were: (1) a DNA damage and elevated reactive oxygen species response which, via ATM activation, enhanced AMPK phosphorylation which was responsible for ULK1 S317 phosphorylation (a kinase activating site); (2) a plasma membrane phagocytosis response, termed LC3-associated phagocytosis (LAP), which could occur in the absence of EGF receptor family proteins that reduced receptor and RAS expression leading to mTOR inactivation and reduced phosphorylation of ULK1 S757 (a kinase inhibitory site) [159–161]. These two events resulted in a complete activation of ULK1 which, via ATG13, resulted in the formation of autophagosomes. The formation of autophagosomes, and subsequently autolysosomes, was then responsible for: (3) the autophagic degradation of chaperone proteins and HDAC proteins. The loss of HDAC6 and reduced levels of HSP90, HSP70 and GRP78 will result in the accumulation of unfolded/denatured proteins that will cause activation of PKR-like endoplasmic reticulum kinase (PERK) and the phosphorylation of its substrate eIF2α. The enhanced eIF2α phosphorylation was causal in reduced expression of MCL-1 and BCL-XL, and in the enhanced expression of Beclin1 and ATG5. Thus, two integrated signals impacting upon ULK1 set in motion autophagosome formation which through autophagic degradation results in greater levels of autophagy-facilitating proteins and in parallel with reduced expression of proteins that maintain mitochondrial homeostasis. Over-expression of BCL-XL, bu not of dominant negative caspase 9, protected the drug treated cells. Knock down of BAX and BAK together, cathepsin B or AIF, however, did significantly reduce killing by the drug combination. These findings collectively suggest that killing required mitochondrial dysfunction but that killing was not purely apoptotic. Perhaps the most unexpected series of data sets to come out of this research concerned the actions of autophagy against HDAC protein levels; not only was HDAC6 expression reduced by many of the drug combinations we have developed by autophagy, but in a cell type fashion, the cytosolic stores of all HDACs could be degraded via autophagy [144, 158]. Thus, a drug combination that does not contain a recognized HDAC inhibitor, can de facto have HDAC inhibitory properties via elevating the numbers of autophagosomes / autolysosomes. And with respect to HDAC expression and the regulation of transcription, many investigators today are working to understand the biology of genes that can regulate the immunogenicity of cancer cells and the immunological environment in tumors [162, 163]. The recently developed checkpoint inhibitory antibodies for immunotherapy are approved for many tumor types, frequently termed as “hot,” but nevertheless, many tumor types such as breast cancer remain refractory to immunotherapy and are considered “cold.” In breast cancer cells [neratinib + HDAC inhibitor] can rapidly reduce the protein levels of HDAC1, HDAC2 and

HDAC3 [144, 158]. These three HDACs were already known to play important roles in maintaining or suppressing the expression of checkpoint immunotherapy targets such as PD-L1, as well as regulating the expression of Class I HLA proteins. In our work, we directly linked stimulated autophagy to the reduced expression of HDACs1–3, and using siRNA tools, linked the expression of HDACs1–3 to the elevated expression of PD-L1 and to the diminished expression of Class I HLA proteins. In vivo studies demonstrated that in addition to [neratinib + HDAC inhibitor] exposure significantly reducing tumor growth, the drug combination also enhanced the efficacy of an anti-PD1 checkpoint inhibitory antibody [158]. similar data have been obtained when combining neratinib with PARP1 inhibitors and pemetrexed with sildenafil [126, 127]. At present, based on our work, there is an open phase I clinical study combining neratinib with the HDAC inhibitor sodium valproate (NCT03919292). Should this combination prove safe in patients, one obvious extension to the study would be the infusion of immunotherapy.

Conclusions.

The interface between autophagy and apoptosis is multi-factorial and crosstalk occurs at different stages of each process. Although simplistically autophagy is an evolutionary conserved survival mechanism, as the prior paragraphs have discussed, autophagy in mammalian cells is considerably more complicated and can act to protect cells from toxic stresses as well as to act alone or in concert with apoptotic signaling processes to kill tumor cells. Autophagy and apoptosis can act together to cause mitochondrial dysfunction with release into the cytosol of cytochrome c and AIF. Cytochrome c activates caspase 9/3 to cause apoptosis whilst AIF causes a necrotic DNA cleavage pattern. In the absence of caspase activation, lysosomal cathepsin proteases can cleave BID, in a manner similar to caspase 8, leading to activation of BAX and BAK resulting in tumor cell death. Autophagy for many years has been linked to preventing death receptor-induced tumor cell killing, in part by reducing the levels of FADD. More recent studies have shown that signaling by RIPK family enzymes can cause tumor cell killing downstream of the receptors when apoptosis is blocked.

The role of autophagy in controlling the responses to chemotherapeutic agents is also complex. For some traditional cytotoxic chemotherapeutic agents, an inability to stimulate autophagic flux results in greater levels of cell killing. With other more recently developed “targeted” therapeutic agents, autophagic flux may be essential for tumor cells to be killed. In particular, as protective BH3 domain proteins can inhibit autophagy by sequestering Beclin1, approaches to enhance “toxic” autophagy signals appear to be warranted [37, 163]. Perhaps the most novel component of autophagic actions is that autophagy can reduce the protein levels of multiple HDAC proteins. By reducing the protein levels of HDACs autophagy has the potential to act in an epigenetic fashion, regulating the transcription of multiple genes independently of whether the drug or drug combination includes an HDAC inhibitor. For example, in 4T1 mammary tumors, previously exposed 14 days prior to [neratinib + HDAC inhibitor], still exhibited reduced expression of ERBB1, K-RAS and PD-L1, and increased expression of Class I MHCA [158]. Another important conclusion to draw from autophagy in tumor cell responses to therapeutic agents concerns the evolution of tumor cells under a therapeutic stress. Matched parental and fulvestrant-resistant MCF7

mammary carcinoma cells illustrate this point. It is known that the transformation and tumorigenic evolution of mammary ductal epithelial cells requires a reduction and/or loss in Beclin1 expression [37]. And, wild type MCF7 cells express lo w levels of Beclin1. However, matched fulvestrant-resistant MCF7 cells express over 3-fold greater levels of Beclin1 and are more capable of forming autophagosomes and autolysosomes [139]. Thus, for a drug combination that utilizes autophagy as a component of its killing mechanism, e.g. the combination of sorafenib and pemetrexed, the fulvestrant-resistant MCF7 cells are more effectively killed than the wild type parental cells. Collectively, all of these findings imply that a patient's tumor may initially respond to chemotherapy wherein autophagy is 'protective' only to evolve under the therapeutic stress into a tumor where the role of autophagy is reversed, and autophagy mediates chemotherapy-induced killing. To fully understand these processes in the clinic will require levels of personalized medicine and proteomic screening at present beyond the capabilities of almost all NCI-designated cancer centers.

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