



# Endoplasmic Reticulum Stress and Autophagy in Cancer

# 18

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## 18.1 Introduction

The endoplasmic reticulum (ER) is an important organelle responsible for protein folding and modification and disturbances in the ER environment will lead to ER stress and subsequently causes accumulation of unfolded or misfolded proteins. Although ER stress activates the unfolded protein response (UPR) mechanism to reestablish ER homeostasis, unresolved ER stress can lead to cellular processes such as apoptosis or autophagy. In cancer, tumor cells are dependent on these processes to combat and neutralize the chronic stress and harsh conditions of the tumor microenvironment, leading to tumor survival and tumor expansion; hence, the ER stress response is thought to be cytoprotective. It is now known that ER stress, apoptosis, and autophagy share overlapping molecular pathways and can occur in parallel under similar conditions. Fundamental knowledge in these processes has also generated a great deal of insight into the pathophysiological aspects of cancer, and has provided important considerations in strategizing cancer pharmacotherapy. A number of drugs targeting these processes have been developed and were proven to be promising in both preclinical and clinical studies.

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## 18.2 Endoplasmic Reticulum Stress (ER Stress)

The ER is an intracellular organelle that provides crucial biosynthetic, stress-sensing, and signaling functions in eukaryotic cells [1, 2]. It is the main subcellular compartment for the synthesis, folding, modification, and transport of proteins which are destined to be secreted or embedded in the plasma membrane [3, 4]. The ER is also the major site for the biosynthesis of steroid, cholesterol, and lipid. It is the major intracellular calcium ( $\text{Ca}^{2+}$ ) storage organelle in the cell, and thus plays an important role in calcium homeostasis and calcium-mediated signaling pathways [5]. Nascent proteins are folded and modified correctly in the ER before being transported via the Golgi apparatus to the cell surface or other desti-

nation. It is an orchestrated process involving folding, assembly, modification, quality control, and recycling of proteins in a highly oxidizing and calcium-rich ER environment. Proteins translocated into the ER lumen are folded into their proper three-dimensional shapes and modified and assisted by ER-resident enzymes, such as chaperones, glycosylating enzymes, and oxidoreductases [6–8]. Incomplete or misfolded forms are eliminated by quality control systems, including the ER-associated degradation (ERAD) pathway and autophagy [7, 9, 10].

Physiological and pathological conditions such as hypoxia, nutrient fluctuations, altered ER-calcium levels, oxidative injury, inflammation, and viral infections may disrupt the protein folding environment in the ER, causing the accumulation of unfolded or misfolded proteins in the ER lumen [3]. This cellular condition is known as ER stress. ER stress leads to a complex intracellular signal transduction pathway, known as unfolded protein response (UPR), an adaptive mechanism to reestablish ER homeostasis [5, 11]. The UPR primarily aims at reestablishing ER homeostasis by coordinating temporal shut down in protein translation, upregulating ER chaperone genes to increase protein-folding capacity in the ER, and promoting ERAD pathway to remove misfolded proteins [4, 5]. However, when the initial cellular responses fail to restore ER homeostasis, persistent ER stress will elicit an alternative response called the “terminal UPR,” which actively promotes cell death to eliminate the damaged cells [7, 12, 13]. Activation of the UPR represents the defining criterion of ER stress, although the terms UPR and ER stress are often used interchangeably [8].

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## 18.3 Unfolded Protein Response (UPR)

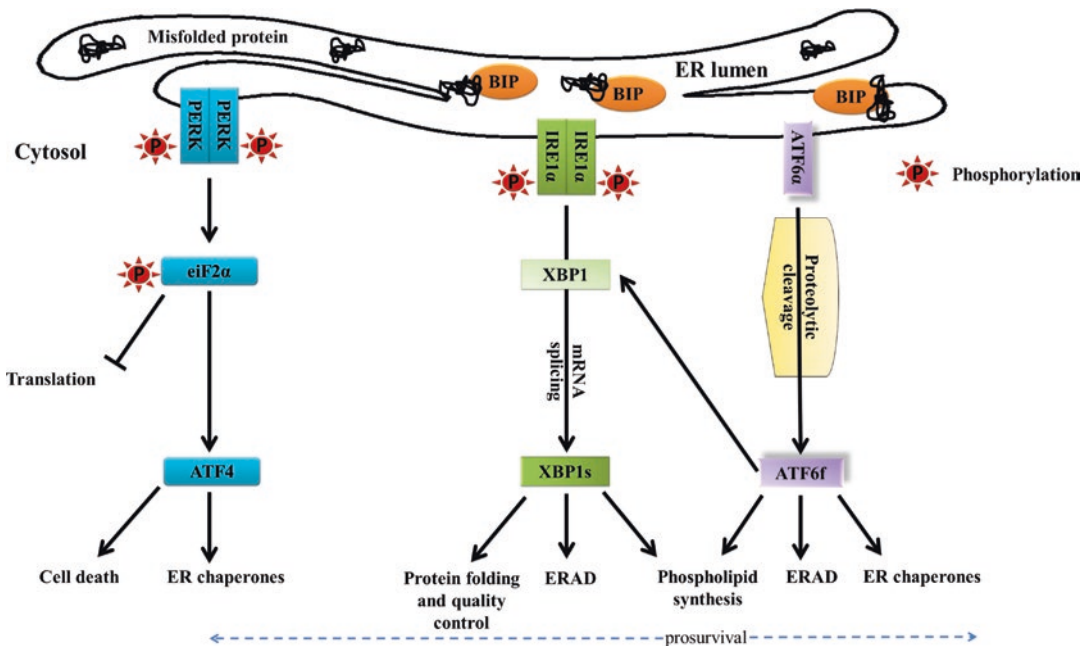
The UPR in mammalian cells is governed by three transmembrane ER stress sensors, namely PERK (protein kinase RNA-like ER kinase), IRE1 $\alpha$  (inositol-requiring enzyme 1 $\alpha$ ), and ATF6 $\alpha$  (activating transcription factor 6 $\alpha$ ) [3]. In the absence of ER stress, the ER luminal domains of PERK,

IRE1 $\alpha$  and ATF6 $\alpha$  are associated with immunoglobulin heavy chain binding protein known as BIP (also known as GRP78), where this interaction maintains all three transmembrane proteins in their inactive state. BIP, a 78-kDa glucose-regulated protein, is well established as an ER chaperone that participates in protein folding and assembly and has been widely used as a marker for ER stress [14]. During ER stress, the accumulating misfolded or unfolded proteins cause BIP to dissociate from the three transmembrane ER stress sensors, and subsequently bind to these misfolded or unfolded proteins. This is due to higher natural affinity of BIP to unfolded proteins compared with the ER stress sensor luminal domains [4]. The release of BIP causes the homodimerization, trans-auto-phosphorylation, and activation of both IRE1 $\alpha$  and PERK and translocation of ATF6 $\alpha$  to the Golgi apparatus and subsequent activation [8, 12, 15].

Activated PERK phosphorylates eukaryotic translation initiator factor 2 $\alpha$  (eIF2 $\alpha$ ) and attenuates general protein translation, thereby relieving the protein burden on the stressed ER by reducing new protein synthesis and preventing further accumulation of unfolded proteins.

Phosphorylation of eIF2 $\alpha$  also regulates translation via inhibition of rRNA synthesis [5, 8]. Paradoxically, eIF2 $\alpha$  phosphorylation allows selective translation of activating transcription factor 4 (ATF4), a transcription factor that controls the expression of genes encoding ER chaperones (e.g., BIP and GRP94), autophagy, and apoptosis [16, 17]. ATF4 favors the expression of antioxidant response, amino acid biosynthesis, and transport genes to sustain cell survival [4]. Depending on the severity and duration of stress, PERK activation can lead to either survival or cell death [18, 19]. Figure 18.1 illustrates the UPR pathway upon exposure to moderate ER stress.

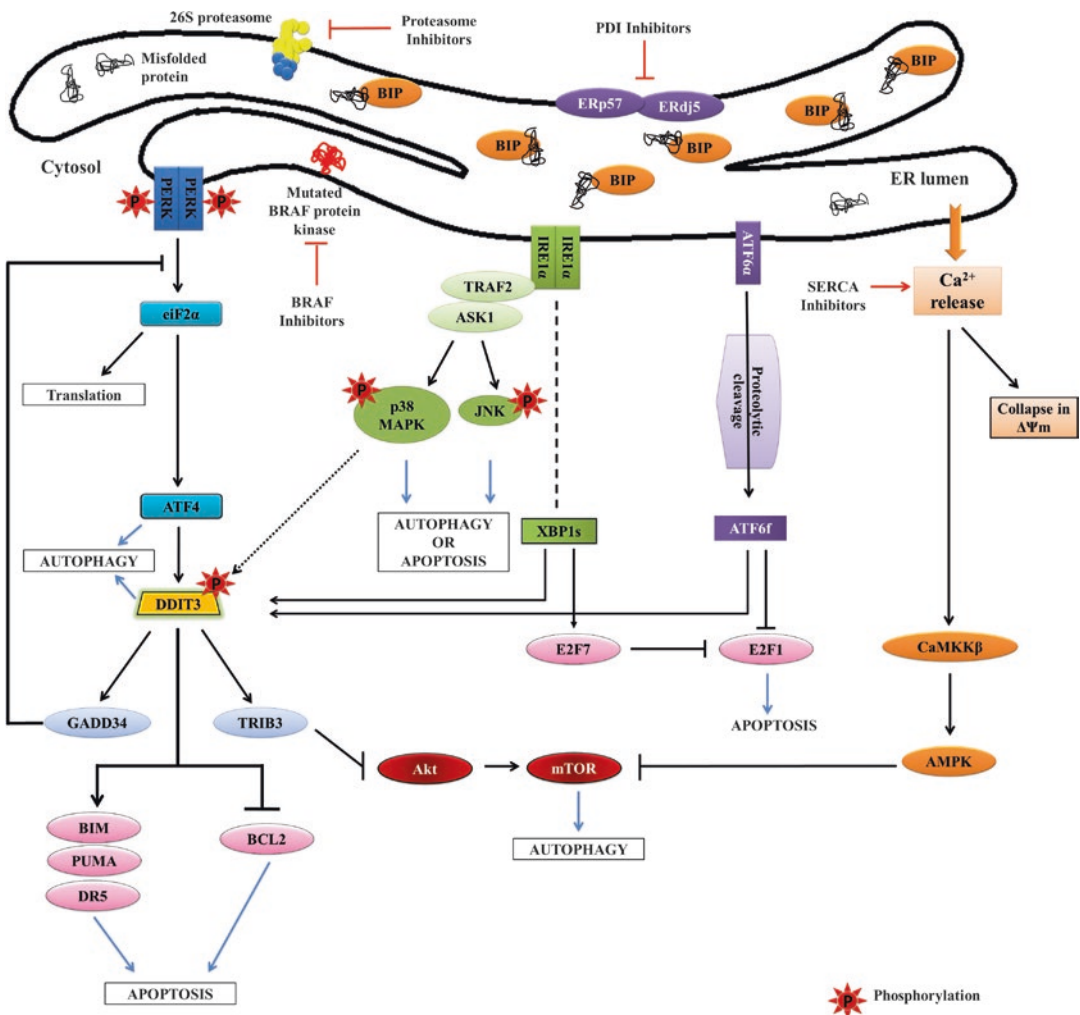
During prolonged ER stress, ATF4 stimulates the transcription of DNA-damage-inducible transcript 3 (DDIT3; also known as CHOP [CCAAT/enhancer binding protein homologous transcription factor] or GADD153 [growth arrest and DNA damage-inducible gene 153]), a transcription factor that is activated by all three arms of the UPR [5]. DDIT3 itself is a transcription factor that is critical in supporting the ER stress-induced apoptotic program [20]. In addition to its prodeath functions, DDIT3 participates in relieving the general block on translation via induction of



**Fig. 18.1** The UPR pathway upon exposure to moderate ER stress

growth arrest and DNA damage-inducible protein 34 (GADD34). GADD34 activates protein phosphatase 1 alpha (PP1A) to dephosphorylate eIF2 $\alpha$  and dephosphorylated eIF2 $\alpha$  resumes its function in general translation. If the protein folding capacity of the ER has not been reestablished, a premature restoration of protein synthesis will increase protein load in the stressed ER, thus amplifying the damage [5, 8]. Although eIF2 $\alpha$  phosphorylation is downregulated during prolonged ER stress, PERK signaling is sustained, possibly to sensitize cells to cell death via DDIT3 induction [17]. Figure 18.2 illustrates the UPR pathway during severe and prolonged ER stress.

Similar to PERK, the release of BIP allows IRE1 $\alpha$  to undergo dimerization and autophosphorylation. IRE1 $\alpha$  is a bifunctional molecule with serine/threonine protein kinase and endoribonuclease (RNase) activity in its cytosolic domain [8]. Hence, this process leads to the activation of its cytosolic RNase domain, which removes a 26-nucleotide intron from the mRNA encoding the transcription factor X box-binding protein 1 (XBP1), producing mature spliced XBP1 mRNA. The spliced XBP1 mRNA is subsequently translated into an active and stable transcription factor, termed spliced XBP1 (XBP1s). XBP1s regulates the transcription of



**Fig. 18.2** The UPR pathway and ER stress-Ca<sup>2+</sup> signaling during severe and prolonged ER stress and antitumor targets

several genes involved in protein folding and quality control, ERAD, and phospholipid synthesis [21, 22]. ERAD is a process where misfolded proteins are retro-translocated from ER to the cytosol to be degraded by the 26S proteasome. Meanwhile, phospholipid synthesis is required for ER membrane expansion during ER stress [5, 11]. Through a process known as regulated IRE1-dependent decay (RIDD) of mRNA, IRE1 RNase domain degrades a subset of mRNAs encoding certain proteins of the secretory pathways and proteins located in the ER [11, 16].

Upon severe ER stress, XBP1s upregulates the expression of DDIT3 [5]. On the other hand, prolonged activation of IRE1 $\alpha$  recruits the adaptor molecule TNF receptor-associated factor 2 (TRAF2), which further recruits apoptosis signal regulating kinase 1 (ASK1). This leads to a mitogen-activated protein (MAP) kinase activation cascade that activates c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (p38 MAPK) which further activates BIM and cause the inactivation of BCL-2 [3, 5, 23]. However, IRE1 $\alpha$  is turned off upon prolonged ER stress, leading to ablation of the prosurvival XBP1s expression. Attenuation of IRE1 $\alpha$  signaling is one possible mechanism to explain the transition from the adaptive UPR to prodeath events [11, 17] (Fig. 18.2).

ATF6 $\alpha$ , a type II transmembrane protein, translocates to the Golgi apparatus once released from BIP, where it is proteolytically cleaved to generate a transcriptionally active fragment, termed ATF6f transcription factor. ATF6f mediates the adaptive response to ER protein misfolding by increasing the transcription of genes that increase ER capacity and the expression of *Xbp1* [24, 25]. The transcription target of ATF6f includes genes involved in ERAD, phospholipid synthesis, and ER chaperones, thereby enhancing cellular folding and degradation capacity [8, 16, 17] (Fig. 18.1). ATF6f also contributes to upregulation of DDIT3 during prolonged ER stress [5] (Fig. 18.2).

Taken together, the three UPR transcription factors, ATF4, XBP1s and ATF6f, regulate a large set of partially overlapping UPR target genes during ER stress which modulates adaptation to

stress or the induction of cell death under severe conditions [11]. The mechanisms underlying the switch from adaptive phase to prodeath events are still unclear, although it could be possibly through programs that sense the duration of the ER stress condition [17]. If the UPR is successful to increase the protein folding capacity and reduce the amount of misfolded proteins in the ER, BIP reassociates with PERK, IRE1 $\alpha$ , and ATF6 $\alpha$ , thereby inactivating these signaling modules. However, in case of excessive or prolonged ER stress, signaling pathways leading to cell death, either as apoptosis or autophagy, would be initiated [5, 8]. In certain situations, UPR may upregulate the autophagy machinery to eliminate damaged ER and abnormal protein aggregates [11]. In this context, autophagy is activated as an adaptive mechanism to reestablish ER homeostasis. However, if autophagy reaches a point of no return, cell death will be triggered. Therefore, just like in the case of UPR, persistent ER stress switches the cytoprotective functions of autophagy to cell death-promoting mechanisms [5, 26].

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## 18.4 ER Stress and Cell Death

Several signaling pathways leading to apoptosis and autophagy would be initiated if ER stress is too severe to be relieved [27]. DDIT3 plays an important role in ER stress-induced cellular death, as this factor is a target gene common to all three apical ER stress sensors/executioners [1]. Duration and/or strength of PERK signaling may determine whether prosurvival or prodeath outcome predominates. Transient PERK signaling protects cells by temporarily reducing protein synthesis and thus reducing misfolded protein levels in the ER, but may be insufficient to induce DDIT3 to threshold level, given DDIT3's inherent mRNA and protein instability. Since DDIT3 mRNA and protein have short half-lives, a strong and chronic activation of PERK is necessary to increase steady-state level of DDIT3 to promote cell death [28]. Persistent PERK signaling during prolonged ER stress is known to impair cell proliferation and promotes apoptosis via DDIT3 [29]. DDIT3 represses BCL-2 expression, upregulates BCL-2-interacting

mediator of cell death (BIM) transcription, and promotes translocation of BAX to mitochondria [30–32]. It is also known to bind and induce the promoters of p53 upregulated modulator of apoptosis (*PUMA*), lipocalin 2 (*LCN2*), tribbles homologue 3 (*TRIB3*), and death receptor 5 (*DR5*) [33–37].

As a mediator of the mitochondrial apoptotic pathway, PUMA is known to result in the displacement and activation of BAX/BAK through its binding to antiapoptotic BCL-2 proteins, leading to mitochondrial dysfunction and caspase activation, hence initiating apoptosis [38, 39]. LCN2 is known to exacerbate hypoxia-induced cytochrome c release from mitochondria and caspase-3 activation [40]. Meanwhile, TRIB3 induces both apoptosis and autophagy. It indirectly activates unc-51-like autophagy-activating kinase1 (ULK1) which augments autophagosome formation and reduces autophagy flux. TRIB3 levels inhibit the activity of the kinase Akt by interacting with it and activating forkhead box O1 (FoxO1), a transcription factor that is negatively regulated by Akt, where it is translocated to the nucleus, and induces the proapoptotic gene, *BIM* [41]. It is also noted that DDIT3-mediated DR5 induction is responsible for ER stress apoptosis via caspase 8 [42]. PERK-dependent activation of ATF4 and DDIT3 has been demonstrated to upregulate the transcription of a set of autophagy genes, which are implicated in the formation, elongation, and function of the autophagosome [43].

In addition, IRE1 $\alpha$  promotes cell death by recruiting a TRAF2-ASK1 complex, leading to the activation of JNK and p38 MAPK cascades upon prolonged ER stress. JNK promotes apoptosis through the phosphorylation-mediated regulation of Bcl-2 family members [5, 31, 44]. JNK exerts its proapoptotic effect by activating proapoptotic BH3-only protein BIM and by suppressing the antiapoptotic BCL-2 [5]. The p38 MAPK also phosphorylates and suppresses the antiapoptotic BCL-2 protein [45]. BCL-2 not only functions as an antiapoptotic protein, but also acts as an antiautophagy protein via its inhibitory interaction with BECN1. Both JNK and p38 MAPK have been proposed to induce

autophagy by promoting dissociation of BECN1 from BCL-2. BECN1 is an essential autophagy regulator that participates in autophagosome formation [5, 45, 46]. In addition, p38 MAPK is known to phosphorylate DDIT3 and enhances DDIT3's ability to function as a transcriptional activator [5, 47] (Fig. 18.2). The apoptosis-inducing activity of the third arm of UPR, ATF6 $\alpha$ , has not been widely recognized. This is at least partly due to the fact that ATF6 $\alpha$  does not induce apoptosis in cell lines commonly used in research. However, it has been shown that ATF6f mediates apoptosis via suppression of antiapoptotic protein, myeloid cell leukemia sequence 1 (Mcl-1) [48].

The mechanisms underlying the switch from adaptive phase to prodeath events remain elusive, although several hypotheses were suggested. The expression of the transcription factor DDIT3 is thought to be a decisive effector of the switch between adaptive UPR to cell death and the duration and amount of elevated DDIT3 level were hypothesized to be the decisive factor in determining the cell's fate [26]. Upon severe ER stress, ATF4, XBP1s, and ATF6f transcription factors induce the transcription of DDIT3. On the other hand, PERK/eIF2 $\alpha$ /ATF4 branch is essential to upregulate DDIT3 protein expression. The transcriptional activity of DDIT3 is then enhanced through the phosphorylation by p38 MAPK [5, 31]. Prolonged high level of DDIT3 protein expression is considered an indicator of the switch to proapoptotic module [8]. DDIT3 alters the balance between prosurvival and proapoptotic Bcl-2 family members and thus promotes apoptosis through the mitochondrial pathway. In addition, a molecular switch to cell death events could also involve TRIB3, a downstream transcriptional target of DDIT3. TRIB3 binds directly to prosurvival Akt kinase, thereby preventing its phosphorylation and reducing its kinase activity. During severe or persistent ER stress, induction of TRIB3 would be more robust, leading to autophagy and apoptosis through TRIB3-mediated inhibition of Akt/mTOR axis [5, 31, 49, 50] (Fig. 18.2).

In fact, IRE1 $\alpha$  activities, namely (1) XBP1 mRNA splicing, (2) regulated IRE1-dependent

decay of mRNAs, and (3) JNK/p38 MAPK activation, are also thought to be responsible for the life/death switch under prolonged ER stress conditions [51, 52]. Recently, the role of E2F1 has been described as a potential mechanistic survival/death switch under ER stress conditions [4, 53]. E2F1 is a member of the E2F family of transcription factors involved in several cellular functions such as proliferation, differentiation, and cell death [54, 55]. Upon ER stress induction, E2F7 as one of XBP1 target gene has been demonstrated to be positively regulated and the combined activity of E2F7 and activated ATF6 results in a specific but timely downregulation of E2F1 expression. This results in the removal of E2F1-dependent basal inhibition of both PUMA and NOXA that will induce the apoptotic program [4]. Timely and coordinated expression levels of E2F1 are crucial for determining the survival/death cell fate under ER stress conditions [4].

In addition to the three UPR branches, ER stress- $\text{Ca}^{2+}$  signaling also leads to cell death during severe and prolonged ER stress. As ER is the major intracellular calcium storage organelle in the cell, ER stress activation is frequently accompanied by calcium release into the cytosol, causing an increase in cytosolic free calcium ions. Increases in cytosolic calcium concentration upon treatment with different ER stress inducers lead to calcium/calmodulin-dependent kinase kinase- $\beta$  (CaMKK $\beta$ )-dependent activation of AMPK, that ultimately leads to inhibition of mTOR and stimulation of autophagy [5, 56]. In addition, mitochondrial intake of calcium ions following its release into the cytosol from the ER causes a collapse in the inner mitochondrial transmembrane potential ( $\Delta\Psi_m$ ). A long-lasting or permanent  $\Delta\Psi_m$  dissipation is often associated with cell death [57, 58].

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## 18.5 ER Stress in Cancer and Therapeutic Strategies

Tumor cells are often present within a hostile microenvironment and are confronted with chronic metabolic stress conditions. Following initiation of malignancy, poor vascularization of

the tumor mass leads to stressful conditions in the tumor microenvironment, including low oxygen supply, nutrient deprivation, and pH changes. Therefore, many tumor types are thought to be dependent on an adaptive UPR to combat and neutralize the chronic stress and harsh conditions of the tumor microenvironment [5, 26, 44]. On the other hand, most normal cells are not subjected to stress and their UPR pathways are in an inactive state [44].

Both UPR activation and upregulation of BIP represent hallmark of several human cancers. UPR activation enables cancer cells to survive, adapts to adverse environmental conditions, and leads to growth arrest driving dormancy, which promotes resistance to conventional chemotherapy [59–62]. In addition, there are emerging evidences that linked mutations in three sensor genes such as ATF6 $\alpha$ , IRE1 $\alpha$ , and PERK in tumorigenesis [63–66]. The presence of missense, nonsense, and silent mutations in these genes seems to have tumor- or tissue-specific significance.

While BIP is generally too low to be detected in normal cells, many tumor cell lines display permanently elevated levels of BIP, which reflects the cancer cells' ongoing effort to neutralize the chronic stress within the cells [26]. Elevated BIP is among the critical prosurvival mechanisms of tumor cells to withstand and thrive under detrimental microenvironmental conditions [8]. Similar to BIP, IRE1 $\alpha$ /XBP1 signaling pathway is important for tumor growth and survival under stress conditions. An increase in XBP1 expression and splicing has been demonstrated in various human cancers, including breast cancer. Moreover, sustained IRE1 $\alpha$  signaling was shown to enhance cell survival and proliferation [44, 67]. PERK/eif2 $\alpha$ /ATF4 pathway also plays a role in cancer progression during stress condition. Hypoxia induces activation of the PERK pathway in tumor cells as an adaptive response to promote survival under hypoxic conditions. ATF4 is overexpressed in many solid tumors and is involved in promoting proliferation and survival during nutrient deprivation and severe hypoxia [44, 67].

In addition, several ER stress-associated markers are specifically upregulated in both neuroblastoma and melanoma cells under ER stress

conditions [68]. DDIT3 and four other genes associated with ER stress were induced greater than twofold, namely ERdj5 (PDIA19; an ER-resident protein containing DnaJ and thioredoxin domains), ERp57 (GRP58; PDIA3; an ER-resident protein disulfide isomerase), calreticulin, and calnexin (both ER-resident chaperones) [68]. Protein disulfide isomerase (PDI) family members such as ERdj5 and ERp57 are consistently upregulated in neuroectodermal tumors and a generalized inhibition of PDI activity revealed a significant sensitization of tumor cells to ER-stress apoptosis. PDIs are endoplasmic reticulum chaperone proteins, catalyze disulfide bond breakage, formation, and rearrangement, and are required for protein folding in the endoplasmic reticulum (ER). The observation that knockdown of ERdj5 or ERp57 enhanced the extent of cell death induced by chemotherapeutic drugs suggests that downregulating ER stress responses may be therapeutically valuable; the ER resident proteins ERdj5 and ERp57 may thus be anticancer targets and PDI inhibition in general appears to be a novel therapeutic strategy [68–70]. Recently, there are a few synthetic small molecule PDI inhibitors such as PACMA31, 16F16, and CCF642 which have proven efficacy in cancer models, but have yet to progress to clinical studies [69, 71–73].

Since tumor cells engage adaptive UPR, only a small margin is left for the tumor cells to accommodate additional ER stress. Drugs that aggravate the preexisting ER stress condition in tumor cells may cause a shift from adaptive UPR to severe ER stress, leading to cell death. At the same time, exposure to ER stress-inducing agents causes activation of adaptive UPR in normal cells. Thus, moderate intensity ER stress inducers would be required to sufficiently aggravate ER stress in tumor cells, but at the same time, only modestly trigger ER stress in normal cells, in order to produce tumor-selective cytotoxic outcome. It was hypothesized that exceptionally potent pharmacologic triggers of ER stress might not be ideal in this situation [26].

A variety of distinct pharmacologic agents have been identified to trigger ER stress by different mechanisms. These agents include protea-

some inhibitors and sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) inhibitors, among others [26]. Although these compounds affect the UPR pathway, UPR may not be the primary mechanism of action of these drugs [44]. In the context of cancer research, thapsigargin (an inhibitor of SERCA), tunicamycin (an inhibitor of protein glycosylation), and brefeldin A (an inhibitor of protein transport from ER to Golgi) are frequently used in experiments as ER stress inducers to investigate the details of ER stress response [8].

The degradation of the majority of misfolded proteins is mediated by the 26S proteasome through the ERAD pathway [44]. Inactivation of the proteasome by proteasome inhibitors causes accumulation of misfolded proteins bound for the ERAD pathway, thereby triggering the UPR [26]. Bortezomib is a proteasome inhibitor and was approved by the US FDA in 2003 to treat multiple myeloma and mantle cell lymphoma [8]. Treatment of multiple myeloma cells with bortezomib causes rapid upregulation of the components in the UPR, including PERK, ATF4, and DDIT3, resulting in cell death. On the other hand, bortezomib sensitized pancreatic cancer cells to ER stress-induced apoptosis by induction of DDIT3, GADD34 and JNK, while PERK activation and eIF2 $\alpha$  phosphorylation were not detected [44]. Several mechanisms have been proposed to explain the cytotoxicity of bortezomib, including effects on NF- $\kappa$ B, cell cycle proteins, apoptosis-regulatory proteins and caspases, as well as ER stress. Although ER stress represents only one of several processes associated with bortezomib-induced cell death, it is conceivable that it might indeed represent the key component, whereas other observed events might be orchestrated secondary to the aggravation of ER stress [8]. Bortezomib is further discussed in Chap. 17.

Inhibitors of human immunodeficiency virus (HIV) protease are known to inhibit the proteasome [26]. Two widely prescribed HIV protease inhibitors, namely nelfinavir and atazanavir, cause the accumulation of polyubiquitinated proteins, aggresome formation, and an increase in BIP and DDIT3 expression [74, 75]. In addition,



nelfinavir has been shown to induce ER stress, autophagy, and apoptosis *in vitro* and *in vivo* in nonsmall-cell lung carcinoma [76]. Nelfinavir is currently in clinical trials for repositioning as an anticancer agent [26]. A Phase II trial of nelfinavir in combination with chemoradiation for locally advanced inoperable pancreatic cancer (LAPC) revealed that nelfinavir showed acceptable toxicity and promising survival in pancreatic cancer [77]. The study reports the clinical outcome in 23 patients with LAPC treated with chemoradiotherapy plus nelfinavir which shows moderate median and 1-year overall survival at 17.4 months and 73.4%, respectively [77].

In another Phase II trial of nelfinavir in combination with the proteasome inhibitor bortezomib in 12 patients with advanced hematologic malignancies, promising activity in advanced, bortezomib-refractory multiple myeloma was noted [78]. Nelfinavir alone significantly upregulated the expression of proteins related to UPR in peripheral blood mononuclear cells and inhibited proteasome activity. Of ten evaluable patients in the dose escalation cohort, three achieved a partial response, four stable disease for two cycles or more, while three had progressive disease as best response [78]. Of nine patients given oral nelfinavir before and during radiation therapy for advanced rectal cancer, five patients exhibited good tumor regression on MRI assessed by tumor regression grade (mrTRG) [79]. Unfortunately, nelfinavir monotherapy does not result in a meaningful improvement in clinical outcomes among patients with recurrent adenoid cystic carcinoma [80]. Nelfinavir is currently in clinical trials for various cancers such as cervical intraepithelial neoplasia and advanced renal cancers (Table 18.1

). However, atazanavir is not on any clinical trials involving cancer at this moment.

The SERCA is a transmembrane protein that actively imports calcium ions from the cytosol into the ER lumen, thereby establishing a steep calcium gradient between the ER lumen and cytosol. Inhibition of SERCA results in massive leakage of calcium ions from ER to the cytosol and thus efficiently triggers ER stress. Thapsigargin, a naturally occurring sesquiterpene lactone, is an exceptionally potent inhibitor

of SERCA. However, its clinical usage is fraught with several challenges; it is quite toxic and not well tolerated by experimental animals. A pro-drug of thapsigargin, also known as mipsagargin or G202, has been synthesized and was found to produce substantial tumor regression against a panel of human cancer xenografts *in vivo* at doses that were minimally toxic to the host [148]. Interestingly, mipsagargin demonstrated an acceptable tolerability and favorable pharmacokinetic profile in a phase I clinical trial in patients with refractory, advanced, or metastatic solid tumors [81].

Certain diaryl-substituted pyrazoles, for example, celecoxib, are another class of compound that has emerged as SERCA inhibitors [26]. Nevertheless, celecoxib might not attain sufficient level of ER stress in tumor tissues because it was initially developed as COX-2 inhibitor. However, celecoxib analogues with minimized COX-2 inhibitory function, but significantly increased ER stress-inducing ability have been developed [8]. AR-12/OSU-03012 is an antitumor celecoxib-derivative that has progressed to Phase I clinical trial as an anticancer agent and has activity against a number of infectious agents including fungi, bacteria, and viruses [149]. It has been shown to suppress tumor cell viability through multiple mechanisms including activation of endoplasmic reticulum stress, inhibition of PDK-1/Akt signaling and the induction of autophagy [150–152]. Although a Phase I clinical trial of AR-12 in adult patients with advanced or recurrent solid tumors or lymphoma has been completed, its overall outcome remain unpublished.

In both oncogenic BRAF melanoma cell lines and in patients who failed clinical treatment for skin melanomas, the presence of oncogenic BRAF was responsible for ER stress induction and cell survival [153, 154]. In particular, human skin melanoma is characterized by oncogenic BRAF mutations, such as BRAF<sup>V600E</sup>. In addition, approximately 8–14% of colorectal cancers (CRC) in early and advanced stages exhibit the BRAF<sup>V600E</sup> mutation [155–158]. The BRAF serine/threonine protein kinase is a downstream signaling protein in the epidermal growth factor receptor-mediated

**Table 18.1** Current therapeutic target in the ER stress and autophagy signaling pathways and clinical trial stages

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
UPR pathway	SERCA	Mipsagargin	Phase I: Advanced metastatic solid tumors Phase II: Hepatocellular carcinoma (completed/unpublished)	Sorafenib	[81] <a href="https://clinicaltrials.gov/ct2/show/NCT01777594">https://clinicaltrials.gov/ct2/show/NCT01777594</a>
		AR-12	Phase II: Recurrent or progressive glioblastoma (completed/unpublished)		<a href="https://clinicaltrials.gov/ct2/show/NCT02067156">https://clinicaltrials.gov/ct2/show/NCT02067156</a>
ERAD	26S proteasome	Bortezomib	Phase I: Advanced or recurrent solid tumors or lymphomas (completed/unpublished) Approved by FDA for multiple myeloma (2005) Approved by FDA for mantle cell lymphoma (2006)		<a href="https://clinicaltrials.gov/ct2/show/NCT00978523">https://clinicaltrials.gov/ct2/show/NCT00978523</a> The U. S. Food and Drug Administration <sup>a</sup>
			Phase III: Multiple myeloma (recruiting)	Lenalidomide; dexamehasone	<a href="https://clinicaltrials.gov/ct2/show/NCT01863550">https://clinicaltrials.gov/ct2/show/NCT01863550</a>
			Phase III: Stem cell transplant in multiple myeloma (recruiting)	Lenalidomide; dexamehasone	<a href="https://clinicaltrials.gov/ct2/show/record/NCT01208662">https://clinicaltrials.gov/ct2/show/record/NCT01208662</a>
			Phase III: T-cell acute lymphoblastic leukemia or stage II–IV T-cell lymphoblastic lymphoma (recruiting)	Combination chemotherapy	<a href="https://clinicaltrials.gov/ct2/show/NCT02112916">https://clinicaltrials.gov/ct2/show/NCT02112916</a>
			Phase III: Multiple myeloma (recruiting)	Selinexor; dexamehasone	<a href="https://clinicaltrials.gov/ct2/show/NCT03110562">https://clinicaltrials.gov/ct2/show/NCT03110562</a>
			Phase III: Untreated multiple myeloma (recruiting)	Daratumumab	<a href="https://clinicaltrials.gov/ct2/show/NCT02541383">https://clinicaltrials.gov/ct2/show/NCT02541383</a>
			Phase III: Relapsed and refractory multiple myeloma (recruiting)	Dexamehasone	<a href="https://clinicaltrials.gov/ct2/show/NCT02811978">https://clinicaltrials.gov/ct2/show/NCT02811978</a>
			Phase III: Newly diagnosed myeloma (recruiting)	Elotuzumab	<a href="https://clinicaltrials.gov/ct2/show/NCT02495922">https://clinicaltrials.gov/ct2/show/NCT02495922</a>
		Nelfinavir	Approved by FDA for HIV (1997)		The U. S. Food and drug Administration <sup>a</sup>
			Phase II: Pancreatic cancer	Chemoradiation	[77]
			Phase II: Multiple myeloma	Bortezomib	[78]
			Phase II: Advanced rectal cancer		[79]
			Phase II: Advanced melanoma, lung, or kidney cancer (recruiting)	Nivolumab, radiation therapy	<a href="https://clinicaltrials.gov/ct2/show/NCT03050060">https://clinicaltrials.gov/ct2/show/NCT03050060</a>
			Phase II: Cervical intraepithelial neoplasia (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT01925378">https://clinicaltrials.gov/ct2/show/NCT01925378</a>

			Phase II: Advanced pancreatic cancer (recruiting)	Oregovomab	<a href="https://clinicaltrials.gov/ct2/show/NCT01959672">https://clinicaltrials.gov/ct2/show/NCT01959672</a>
			Phase II: Localized pancreatic cancer (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT02024009">https://clinicaltrials.gov/ct2/show/NCT02024009</a>
			Phase II: Kaposi sarcoma (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT0307745">https://clinicaltrials.gov/ct2/show/NCT0307745</a>
			Phase II: Squamous cell carcinoma larynx (recruiting)	Chemoradiotherapy	<a href="https://clinicaltrials.gov/ct2/show/NCT02207439">https://clinicaltrials.gov/ct2/show/NCT02207439</a>
			Approved by FDA for unresectable or metastatic melanoma with the BRAF <sup>V600E</sup> mutation (2011)		The U. S. Food and Drug Administration <sup>a</sup>
			Phase II: Colorectal cancer		[82]
			Phase III: Metastatic melanoma		[83]
			Phase II: Relapsed or refractory advanced solid tumors, non-Hodgkin lymphoma (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT03220035">https://clinicaltrials.gov/ct2/show/NCT03220035</a>
			Phase II: Tumors harboring BRAF genomic alterations (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT02304809">https://clinicaltrials.gov/ct2/show/NCT02304809</a>
			Phase II: Metastatic melanoma (recruiting)	Cobimetinib	<a href="https://clinicaltrials.gov/ct2/show/NCT02414750">https://clinicaltrials.gov/ct2/show/NCT02414750</a>
			Phase II: Metastatic melanoma (recruiting)	Adoptive T-cell therapy with tumor-infiltrating lymphocytes	<a href="https://clinicaltrials.gov/ct2/show/NCT02354690">https://clinicaltrials.gov/ct2/show/NCT02354690</a>
			Phase II: BRAF <sup>V600E</sup> mutation positive craniopharyngioma (recruiting)	Cobimetinib	<a href="https://clinicaltrials.gov/ct2/show/NCT03224767">https://clinicaltrials.gov/ct2/show/NCT03224767</a>
			Phase II: BRAF mutated melanoma with brain metastasis (recruiting)	Cobimetinib	<a href="https://clinicaltrials.gov/ct2/show/NCT02537600">https://clinicaltrials.gov/ct2/show/NCT02537600</a>
			Phase III: Metastatic or unresectable locally advanced melanoma (recruiting)	Atezolizumab; cobimetinib	<a href="https://clinicaltrials.gov/ct2/show/NCT02908672">https://clinicaltrials.gov/ct2/show/NCT02908672</a>
			Phase IV: BRAF <sup>V600E</sup> mutation-positive malignancies (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT01739764">https://clinicaltrials.gov/ct2/show/NCT01739764</a>
			Approved by FDA for unresectable or metastatic melanoma with the BRAF <sup>V600E</sup> mutation (2014)		The U. S. Food and Drug Administration <sup>a</sup>
			Phase II: Melanoma, untreated brain metastases		[84]
			Phase III: BRAF-mutated metastatic melanoma		[85]
			Phase II: BRAF <sup>V600E</sup> -mutant metastatic NSCLC	Trametinib	[86]
			Phase II: BRAF <sup>V600E</sup> -mutant melanoma brain metastases	Trametinib	[87]

(continued)

**Table 18.1** (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
			Phase III: Metastatic BRAF <sup>V600E/K</sup> -mutant melanoma	Trametinib; monotherapy	[88]
			Phase III: Metastatic BRAF <sup>N600E/K</sup> -mutant melanoma	Trametinib; monotherapy	[89]
			Phase II: BRAF <sup>V600E</sup> -mutated rare cancers (recruiting)	Trametinib	<a href="https://clinicaltrials.gov/ct2/show/NCT02034110">https://clinicaltrials.gov/ct2/show/NCT02034110</a>
			Phase II: BRAF-mutant melanoma or solid tumors (recruiting)	Trametinib; navitoclax	<a href="https://clinicaltrials.gov/ct2/show/NCT01989585">https://clinicaltrials.gov/ct2/show/NCT01989585</a>
			Phase II: BRAF-mutated ameloblastoma (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT02367859">https://clinicaltrials.gov/ct2/show/NCT02367859</a>
			Phase II: Stage III melanoma (recruiting)	Trametinib	<a href="https://clinicaltrials.gov/ct2/show/NCT02231775">https://clinicaltrials.gov/ct2/show/NCT02231775</a>
			Phase III: Stage III-IV BRAF <sup>V600E</sup> melanoma (recruiting)	Trametinib; Ipilimumab; nivolumab	<a href="https://clinicaltrials.gov/ct2/show/NCT02224781">https://clinicaltrials.gov/ct2/show/NCT02224781</a>
		Encorafenib	Phase I: Metastatic BRAF-mutant melanoma		[90]
			Phase I: Metastatic BRAF-mutant colorectal cancer	Cetuximab; alpelisib	[91]
			Phase II: Relapsed/refractory multiple myeloma	Bimimetinib	<a href="https://clinicaltrials.gov/ct2/show/NCT02834364">https://clinicaltrials.gov/ct2/show/NCT02834364</a>
			Phase III: BRAF V600E-mutant metastatic colorectal cancer (recruiting)	Chemotherapy	<a href="https://clinicaltrials.gov/ct2/show/NCT02928224">https://clinicaltrials.gov/ct2/show/NCT02928224</a>
mTOR signaling pathway	mTOR	Rapamycin (Sirolimus)	Phase I: Relapsed and refractory solid tumors	Cyclophosphamide; topotecan	[92]
			Phase I: Refractory solid malignancies	Sunitinib	[93]
			Phase I: Invasive bladder cancer	Intravesical BCG	<a href="https://clinicaltrials.gov/ct2/show/NCT02753309">https://clinicaltrials.gov/ct2/show/NCT02753309</a>
			Phase II: Metastatic, RAI-refractory, differentiated thyroid cancer (recruiting)	Cyclophosphamide	<a href="https://clinicaltrials.gov/ct2/show/NCT03099356">https://clinicaltrials.gov/ct2/show/NCT03099356</a>
			Phase II: Metastatic hormone-resistant prostate cancer (recruiting)	Docetaxel; carboplatin	<a href="https://clinicaltrials.gov/ct2/show/NCT02565901">https://clinicaltrials.gov/ct2/show/NCT02565901</a>
			Phase II: Recurrent/refractory germ cell tumors (recruiting)	Erlotinib	<a href="https://clinicaltrials.gov/ct2/show/NCT01962896">https://clinicaltrials.gov/ct2/show/NCT01962896</a>
			Phase II: Advanced, radioactive iodine refractory Hurthle cell thyroid cancer (recruiting)	Sorafenib	<a href="https://clinicaltrials.gov/ct2/show/NCT02143726">https://clinicaltrials.gov/ct2/show/NCT02143726</a>

			Phase II: Recurrent/refractory solid and CNS tumors (recruiting) Approved by FDA for advanced RCC (2007)	Metronomic chemotherapy	<a href="https://clinicaltrials.gov/ct2/show/NCT02574728">https://clinicaltrials.gov/ct2/show/NCT02574728</a> The U. S. Food and Drug Administration <sup>a</sup>
	Temsirolimus (CCI-779)		Phase I: Advanced solid tumors	Cetuximab	[94]
			Phase I: Recurrent pediatric solid tumors	Perifosine	[95]
			Phase I: Advanced NSCLC	Pemetrexed	[96]
			Phase II: Advanced endometrial cancer	Temsirolimus and alternating megestrol acetate; tamoxifen	[97]
			Phase II: Relapsed/refractory primary CNS lymphoma		[98]
			Phase II: Resistant ovarian cancer/advanced/recurrent endometrial carcinoma		[99]
			Phase I: Advanced cancers (recruiting)	Metformin	<a href="https://clinicaltrials.gov/ct2/show/NCT01529593">https://clinicaltrials.gov/ct2/show/NCT01529593</a>
			Phase I: Advanced cancers (recruiting)	Bevacizumab; valproic acid; cetuximab	<a href="https://clinicaltrials.gov/ct2/show/NCT01552434">https://clinicaltrials.gov/ct2/show/NCT01552434</a>
			Phase I: Advanced or metastatic solid tumors (recruiting)	Cetuximab	<a href="https://clinicaltrials.gov/ct2/show/NCT02215720">https://clinicaltrials.gov/ct2/show/NCT02215720</a>
			Phase I: Relapsed acute lymphoblastic leukemia and non-Hodgkin's lymphoma (recruiting)	Etoposide; cyclophosphamide	<a href="https://clinicaltrials.gov/ct2/show/NCT01614197">https://clinicaltrials.gov/ct2/show/NCT01614197</a>
	Everolimus (RAD001)		Approved by FDA for advanced RCC (2009), advanced pancreatic neuroendocrine tumor (2011), renal angiomyolipoma (2012) and hormone receptor-positive, HER2-negative breast cancer (2012)		The U. S. Food and Drug Administration <sup>a</sup>
			Phase I: Prostate cancer	Radiation	[100]
			Phase I: Triple-negative breast cancer	Doxorubicin; bevacizumab	[101]
			Phase I: Diffuse large B-cell lymphoma	R-CHOP-21	[102]
			Phase Ib: Metastatic gastroesophageal adenocarcinoma	mFOLFOX6	[103]
			Phase II: Elapsed estrogen receptor-positive high-grade ovarian cancers	Letrozole	[104]
			Phase II: Hormone receptor-positive, HER2-negative metastatic breast cancer	Dexamethasone	[105]

(continued)

Table 18.1 (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
			Phase II: Relapsed or refractory indolent lymphoma		[106]
			Phase II: Metastatic clear cell renal cell cancer	Pazopanib	[107]
			Phase II: Advanced follicular-derived thyroid cancer		[108]
			Phase II: Castration-resistant prostate cancer	Bicalutamide	[109]
			Phase II: Peripheral T-cell lymphoma	CHOP	[110]
			Phase II: Metastatic non-clear cell renal cell carcinoma	Sunitinib	[111]
			Phase III: Estrogen receptor-positive metastatic breast		[112]
			Phase III: Hormone receptor positive metastatic breast cancer (recruiting)	Aromatase inhibitors	<a href="https://clinicaltrials.gov/ct2/show/NCT02511639">https://clinicaltrials.gov/ct2/show/NCT02511639</a>
			Phase III: High risk of relapse, ER+ and HER2—primary breast cancer (recruiting)	Adjuvant hormone therapy	<a href="https://clinicaltrials.gov/ct2/show/NCT01805271">https://clinicaltrials.gov/ct2/show/NCT01805271</a>
			Phase III: Metastatic breast cancer (recruiting)	Fulvestrant; exemestane	<a href="https://clinicaltrials.gov/ct2/show/NCT02404051">https://clinicaltrials.gov/ct2/show/NCT02404051</a>
			Phase IV: Progressive pancreatic neuroendocrine tumors (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT02842749">https://clinicaltrials.gov/ct2/show/NCT02842749</a>
			Phase IV: Liver cancer (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT02081755">https://clinicaltrials.gov/ct2/show/NCT02081755</a>
			Phase I: Solid tumor cancers	Paclitaxel; carboplatin	[113]
	Ridaforolimus (Deforolimus; AP23573)		Phase I: Advanced solid tumors		[114]
			Phase II: Advanced breast cancer	Dalotuzumab; exemestane	[115]
			Phase II: Estrogen receptor-positive breast cancer	Dalotuzumab; exemestane	[116]
	PI3K/mTOR	BEZ235	Phase II: Advanced pancreatic neuroendocrine tumors		[117]
			Phase II: Locally advanced or metastatic transitional cell carcinoma		[118]

			Phase I: Advanced solid tumors (completed/unpublished)			<a href="https://clinicaltrials.gov/ct2/show/NCT01195376">https://clinicaltrials.gov/ct2/show/NCT01195376</a>
			Phase I: Advanced solid tumors (completed/unpublished)			<a href="https://clinicaltrials.gov/ct2/show/NCT01343498">https://clinicaltrials.gov/ct2/show/NCT01343498</a>
			Phase Ib: Castration-resistant prostate cancer (completed/unpublished)	BKM120		<a href="https://clinicaltrials.gov/ct2/show/NCT01634061">https://clinicaltrials.gov/ct2/show/NCT01634061</a>
			Phase Ib/II: HER2 negative, locally advanced or metastatic breast cancer (completed/unpublished)	Paclitaxel		<a href="https://clinicaltrials.gov/ct2/show/NCT01495247">https://clinicaltrials.gov/ct2/show/NCT01495247</a>
			Phase III: Advanced breast cancer (completed/unpublished)			<a href="https://clinicaltrials.gov/ct2/show/NCT00620594">https://clinicaltrials.gov/ct2/show/NCT00620594</a>
Proautophagics	Temozolomide		Approved by FDA for glioblastoma (2005)	Radiotherapy		[119]
			Phase I: Recurrent malignant gliomas	Metronomic temozolomide		[120]
			Phase I/II: Glioblastoma	Immunotherapy		[121]
			Phase I/II: Recurrent malignant gliomas	Nimustine		[122]
			Phase III: Primary CNS lymphoma	Induction chemotherapy; whole-brain radiotherapy		[123]
			Phase II: Glioblastoma	Bevacizumab; Irinotecan		[124]
			Phase II: High-grade glioma	Radiotherapy; lomustine		[125]
			Phase II: NSCLC with brain metastases	Pemetrexed		[126]
			Phase II: Metastatic melanoma	Ipilimumab		[127]
			Phase II: Refractory or relapsed neuroblastoma	Irinotecan-temozolomide with temsirolimus or dinutuximab		[128]
			Phase II: Metastatic colorectal cancer			[129]
			Phase II: Refractory or relapsed neuroblastoma	Bevacizumab, irinotecan		[130]

(continued)

**Table 18.1** (continued)

Pathway	Therapeutic targets	Current drugs	Clinical trial stages/type of cancer	Combined with/versus	References
			Phase II: Glioblastoma	Neoadjuvant temozolomide; hypofractionated accelerated radiation therapy	[131]
			Phase III: High-risk low-grade glioma	Radiotherapy	[132]
			Phase III: Glioblastoma (recruiting)	Radiotherapy; nivolumab	<a href="https://clinicaltrials.gov/ct2/show/NCT02667587">https://clinicaltrials.gov/ct2/show/NCT02667587</a>
			Phase III: Glioblastoma (recruiting)	Nivolumab	<a href="https://clinicaltrials.gov/ct2/show/NCT02617589">https://clinicaltrials.gov/ct2/show/NCT02617589</a>
			Phase III: Extensive stage small-cell lung cancer (recruiting)		<a href="https://clinicaltrials.gov/ct2/show/NCT02772107">https://clinicaltrials.gov/ct2/show/NCT02772107</a>
			Phase III: Recurrent glioblastoma (recruiting)	Bevacizumab	<a href="https://clinicaltrials.gov/ct2/show/NCT03149575">https://clinicaltrials.gov/ct2/show/NCT03149575</a>
			Phase III: High-grade gliomas (recruiting)	Interferon-alpha	<a href="https://clinicaltrials.gov/ct2/show/NCT01765088">https://clinicaltrials.gov/ct2/show/NCT01765088</a>
		Arsenic trioxide	Approved by FDA for acute promyelocytic leukemia (2000)		The U. S. Food and drug Administration I
			Phase II: Relapsed or refractory malignant lymphoma		[133]
			Phase II: Hepatocellular carcinoma	Locoregional therapy	[134]
			Phase II: Recurrent or refractory stage 4 neuroblastoma or metastatic paraganglioma/pheochromocytoma	(131)I-MIBG	[135]
			Phase II: Acute promyelocytic leukemia	All-trans retinoic acid; chemotherapy	[136]
			Phase II: Relapsed small-cell lung cancer		[137]
			Phase III: Acute promyelocytic leukemia	All-trans retinoic acid	[138]
			Phase III: Non-high-risk acute promyelocytic leukemia	All-trans retinoic acid	[139]
			Phase III: Acute promyelocytic leukemia	Anthracyclines	[140]
			Phase III: Untreated acute promyelocytic leukemia (recruiting)	Tretinoin; chemotherapy	<a href="https://clinicaltrials.gov/ct2/show/NCT02339740">https://clinicaltrials.gov/ct2/show/NCT02339740</a>
			Phase III: High-risk acute promyelocytic leukemia (recruiting)	Tretinoin; chemotherapy	<a href="https://clinicaltrials.gov/ct2/show/NCT02688140">https://clinicaltrials.gov/ct2/show/NCT02688140</a>



Autophagy inhibitors	Chloroquine	Phase I: Metastatic or unresectable pancreatic cancer Phase I: Stage IV small-cell lung cancer (completed/unpublished) Phase I/II: Breast ductal carcinoma in situ (completed/unpublished) Phase I: Advanced solid tumors (recruiting) Phase I/II: IDH1/2-mutated solid tumors (recruiting) Phase II: Breast cancer (recruiting)	Gemcitabine	[141] <a href="https://clinicaltrials.gov/ct2/show/NCT00969306">https://clinicaltrials.gov/ct2/show/NCT00969306</a> <a href="https://clinicaltrials.gov/ct2/show/NCT01023477">https://clinicaltrials.gov/ct2/show/NCT01023477</a> <a href="https://clinicaltrials.gov/ct2/show/NCT02071153">https://clinicaltrials.gov/ct2/show/NCT02071153</a> <a href="https://clinicaltrials.gov/ct2/show/NCT02496741">https://clinicaltrials.gov/ct2/show/NCT02496741</a> <a href="https://clinicaltrials.gov/ct2/show/NCT02333890">https://clinicaltrials.gov/ct2/show/NCT02333890</a> <a href="https://clinicaltrials.gov/ct2/show/NCT01446016">https://clinicaltrials.gov/ct2/show/NCT01446016</a>
		Phase II: Advanced or metastatic breast cancer (recruiting) Phase I: Advanced solid tumors and melanoma Phase I: Advanced solid tumors Phase I: Relapsed/refractory myeloma Phase I/II: Glioblastoma	Taxane chemotherapy Temozolomide Vorinostat Bortezomib Radiation; temozolomide Gemcitabine	[142] [143] [144] [145] [146] [147]
		Phase I/II: Pancreatic adenocarcinoma Phase II: Metastatic pancreatic adenocarcinoma Phase II: Colorectal cancer (recruiting) Phase II: Recurrent breast cancer (recruiting)	Vorinostat; Regorafenib Everolimus	<a href="https://clinicaltrials.gov/ct2/show/NCT02316340">https://clinicaltrials.gov/ct2/show/NCT02316340</a> <a href="https://clinicaltrials.gov/ct2/show/NCT03032406">https://clinicaltrials.gov/ct2/show/NCT03032406</a>
		Phase I/II: Pancreatic cancer Phase I/II: Advanced BRAF mutant melanoma Phase I/II: Renal cell carcinoma	Gemcitabine; abraxane Dabrafenib; trametinib Aldesleukin	<a href="https://clinicaltrials.gov/ct2/show/NCT01506973">https://clinicaltrials.gov/ct2/show/NCT01506973</a> <a href="https://clinicaltrials.gov/ct2/show/NCT02257424">https://clinicaltrials.gov/ct2/show/NCT02257424</a> <a href="https://clinicaltrials.gov/ct2/show/NCT01550367">https://clinicaltrials.gov/ct2/show/NCT01550367</a>

<sup>a</sup><https://www.accessdata.fda.gov/scripts/cder/daf/index.cfm>

MAP kinase pathway, which activates MEK through its phosphorylation. BRAF<sup>V600E</sup> mutation leads to constitutive BRAF kinase activity, which sustains the MAP kinase signaling pathway. BRAF<sup>V600E</sup>-mediated p38 MAPK activation stimulates both the IRE1 $\alpha$ /ASK1/JNK and TRIB3 pathways. BCL-XL/BCL-2 phosphorylation by active JNK releases BECN1, whereas TRIB3 inhibits the Akt/mTOR axes, resulting in an increase in basal autophagy [154].

Vemurafenib and dabrafenib are BRAF inhibitors which have been approved by the USA FDA and EMA for the treatment of BRAF-mutated metastatic melanoma. In an open-label, multicenter 2-year follow-up of vemurafenib in 3219 patients with BRAF<sup>V600</sup> mutation-positive metastatic melanoma, data suggest that long-term vemurafenib treatment is effective and tolerable [83]. Although vemurafenib and dabrafenib demonstrated impressive antitumor activity in advanced melanoma with objective response rates around 50% [85, 159], disappointing results were seen for patients with BRAF<sup>V600E</sup>-mutated colorectal cancer. In the Phase II study evaluating vemurafenib in patients with metastatic BRAF<sup>V600E</sup>-mutated colorectal cancer, of 21 patients, only one patient had confirmed partial response (5%) and the median progression-free survival (PFS) was 2.1 months [82]. Dabrafenib monotherapy did not show meaningful clinical activity with only one confirmed partial response among the 11 patients with BRAF<sup>V600E</sup>-mutated colorectal cancer included in the Phase I trial [84]. Encorafenib, another potent and selective oral BRAF inhibitor, showed signs of efficacy in patients with BRAF-mutant advanced melanoma but lack of objective response in patients with colorectal cancer [160]. All three drugs are currently in several clinical trials for other tumors (Table 18.1).

Treatment of tumor cells with drugs that trigger further ER stress might result in two desirable anticancer outcomes. First, the drugs by themselves might result in increased antitumor effects. Second, the overload and subsequent breakdown of the UPR adaptive system might increase the tumor cells' sensitivity toward conventional chemotherapeutic agents [26]. Targeting of alterna-

tive pathways is an attractive strategy to improve antitumor therapy in apoptosis-resistant cancer. In view of the fact that ER stress is basally activated in many cancers, aggravation of the preexisting ER stress condition and the subsequent activation of autophagy represent an alternative therapeutic target to improve cancer therapy [27].

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## 18.6 Autophagy

The ubiquitin-proteasome system (UPS) and lysosomes are two primary intracellular protein degradation pathways recognized in eukaryotic cells. Differences between these two major protein degradation systems depend on their functional significance and the type of substrates taken in for degradation [161]. The UPS catalyzes the rapid degradation of abnormal proteins and short-lived regulatory proteins, leading to a control of a diversity of essential cellular processes [162]. In the lysosomal protein degradation pathway, degradation of extracellular materials is mediated by endocytosis, whereas degradation of intracellular long-lived cytoplasmic proteins and damaged organelles is mediated by three types of autophagy, macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA), which are classified based on their transport of cytoplasmic materials into the lysosome for degradation [163, 164].

Autophagy literally means self-digestion in Greek [165]. Macroautophagy, usually refers to autophagy, is responsible for the turnover of unnecessary or dysfunctional organelles and proteins, such as damaged mitochondria [166]. These processes are important to maintain a well-controlled balance between anabolism and catabolism to facilitate normal cell growth and development. It is also a survival pathway, required during starvation or growth factor deprivation, as it provides an alternative energy source [167, 168]. Autophagy process provides catabolic intermediates for intracellular production of ATP when energy supplies are limited. It plays an essential role during starvation, cellular differentiation, cell death, cell survival, aging, and tumor prevention [164, 166, 169].

Autophagy pathway is a multistep process characterized by induction, vesicle nucleation, extension, and completion of an isolation membrane to form an organelle called autophagosome [170]. Briefly, the autophagy process begins with the formation of a preautophagosomal structure known as isolation membrane or phagophore [171]. The isolation membrane engulfs and elongates to form the autophagosome, surrounding the components destined to be recycled. The autophagosome, which is a double membrane-bounded structure, undergoes maturation, and fuses with both endosomal and lysosomal vesicles to form autolysosome [171–173]. The sequestered contents are subsequently degraded by lysosomal hydrolases and are recycled. Based on morphological features, the term “autophagic cell death” has been described in instances of cell death that are accompanied by massive cytoplasmic vacuolization.

The core autophagy machinery is composed of four major functional groups: (1) the unc-51-like kinases (ULKs) (ATG1-ATG13-ATG17 kinase complex), (2) the Class III phosphatidylinositol-3-kinase catalytic subunit type 3 (PI3KC3) complexes, including Class III PI3K (the mammalian orthologue of vascular protein sorting 34; VPS34), p150/VPS15 (the mammalian orthologue of Vps15), BECN-1 (the mammalian orthologue of ATG6/Vps30) and ATG14L (ATG14), (3) two ubiquitin-like conjugation systems: ATG12 and ATG8, and (4) ATG9 and its cycling system [174]. The ULKs (the mammalian orthologues of ATG1, which exist in a large complex with mammalian ATG13), focal adhesion kinase family interacting protein of 200 kDa (FIP200; the mammalian homologue of ATG17), and the recently identified ATG101 play a crucial role in autophagy induction [175–179]. ULK1 is part of a family of kinases in humans (ULK1–4). Isoform ULK1 is the most important component in autophagy and in some cells lines, blocking both ULK1 and ULK2 is necessary to completely shut down autophagy [180].

The ULK1 kinase regulates proautophagic signals by phosphorylating many substrate proteins [181]. The numerous substrates of ULK1 include itself and other subunits of the ULK1

complex; other elements of the core autophagy machinery, including PI3KC3–C1 subunits such as BECN1 and ATG9; and other autophagy-related proteins such as AMBRA1 [180, 181]. Autophosphorylation of the kinase domain’s activation loop at Thr180 of ULK1 is essential for activation upon autophagy induction [182, 183]. Subsequently, phosphorylation of these downstream molecules by ULK1 is an important step in the initiation of autophagy.

The early stages of the phagophore membrane nucleation are dependent on the Class III PI3KC3 complex which consists of the Class III PI3KC3 protein, its regulatory protein kinase p150/VPS15, and BECN1 [184]. BECN1 is a 60-kDa tumor suppressor protein and is identified from a yeast two-hybrid screen as a BCL-2 interacting protein [185]. Several studies have demonstrated that several binding molecules positively regulate BECN1 activity and autophagosome formation and maturation. For example, ultraviolet radiation resistance-associated gene (UVRAG), ATG14L, and activated molecule in BECN1 regulated autophagy protein (AMBRA1) associate with BECN1 to activate autophagy [186–190].

The Class III PI3KC3 phosphorylates phosphatidylinositol to generate PI(3)P which is an essential early event in autophagy initiation, downstream of ULK1 [187, 191, 192]. PI3KC3 forms two distinct complexes, known as complexes I and II (PI3KC3–C1 and PI3KC3–C2) which contain the catalytic subunit VPS34/Vps34, the putative protein kinase VPS15/Vps15 and BECN1/ATG6 [187, 192]. PI3KC3–C1 contains ATG14L/ATG14, which directs the complex to phagophore initiation sites [186, 187, 193–196]. PI3KC3–C1 facilitates elongation meanwhile PI3KC3–C2, which contains UVRAG, directs endosome and autophagosome maturation [180].

The next stage of phagophore membrane elongation (expansion and closure of the autophagosome) requires two ubiquitin-like systems [197]. The ubiquitin-like protein ATG12 conjugates with ATG5 in an ATG7- and ATG10-dependent manner [161]. The ATG5–ATG12 complex interacts with ATG16 to form a stable and large multimeric complex called the ATG16L complex,

which localizes on the outer surface of the extending autophagosomal membrane [170]. This complex is important in the stimulation and localization of the microtubule-associated protein 1 light chain 3 (LC3) conjugation reactions. LC3 is first cleaved by ATG4 to expose a C-terminal glycine residue required for subsequent activation and conjugation reactions [198]. It is then conjugated to the lipid phosphatidylethanolamine (PE), also via ATG7 and E2-like ATG3, and is subsequently recruited to both outer and inner surfaces of the autophagosomal membrane [197, 199]. Actually, two forms of LC3 are produced posttranslationally in various cells; the unconjugated form (LC3-I) is in the cytosol, while the conjugated form (LC3-II) targets the autophagosomal membrane with the assistance of the ATG16L complex [199, 200]. ATG16L complex is a ubiquitin-protein ligase (E3)-like enzyme that functions as a scaffold for LC3-II lipidation by localizing to the source membranes during autophagosome formation [200, 201]. The association of LC3-II to the autophagosome is crucial for membrane elongation of the autophagosome and the final limitation of the membrane to form the vacuoles [161]. The ATG5–ATG12–ATG16 complex is recycled, while the LC3 complex stays on the membrane until it is degraded by the lysosome [161]. In mammalian autophagy, LC3-II protein is used as an index of autophagosome formation or as an autophagosomal marker [202]. These conjugation systems are considered to be uniquely important to the autophagosome formation and have been identified as possible drug targets in cancer [203].

ATG9 system is required for phagophore expansion. It is the only transmembrane protein in the autophagy core machinery and has been proposed to play a key role in directing membrane from donor organelles for autophagosome formation [204]. ATG9 trafficking from the plasma membrane and trans-Golgi network involves two conserved sorting signals for proper function in autophagy, namely ATG9 interaction with the AP1/2 clathrin adaptor complex and phosphorylation of ATG9 at Tyr8 by SRC kinase and at Ser14 by ULK1. SRC kinase directly

phosphorylates Tyr8 of ATG9 and promotes the interaction of ATG9 with the AP1/2 complex and leads to the movement of ATG9 away from the juxtannuclear region [205]. As with Tyr8, phosphorylation at Ser14 enhances the binding of ATG9 with the AP2 complex and promotes ATG9-AP1 interaction. Zhou and co-workers showed that phosphorylation of ATG9 at both the Tyr8 and Ser14 sites is required for maintaining proper autophagy under both basal conditions and in response to starvation-induced stress [205]. Finally, ATG9 binds the small Rab GTPases (RABGAP) protein TBC1D5, and both TBC1D5 and the AP2 complex contribute to the correct sorting of ATG9-containing vesicles during the initiation of autophagy [206].

The completed autophagosome membrane subsequently fuses with lysosome via the actions of the lysosomal proteins including the lysosomal-associated membrane protein 1 (LAMP1), LAMP2, member of RAS oncogene family (Rab7), and UVRAG [207]. The eventual autolysosome is a single membrane-bound acidic vesicle where the contents are digested and recycled by lysosomal hydrolases such as cathepsins (CTS), and its nutrient and energy are recycled [208]. These single membrane autolysosomes filled with degraded cytoplasmic materials can be easily observed using transmission electron microscopy (TEM) [170]. In addition, the adapter protein sequestosome 1 (SQSTM1/p62), which targets specific substrates to autophagosomes and LC3II are degraded along with other cargo proteins and are used as a measure of autophagy flux [209]. The autophagy cargo receptor p62/SQSTM1 binds ubiquitin on cargo to deliver cargo proteins to autophagosomes by docking onto LC3 on autophagosomes. P62 itself is an autophagy substrate that accumulates when autophagy is inhibited [210].

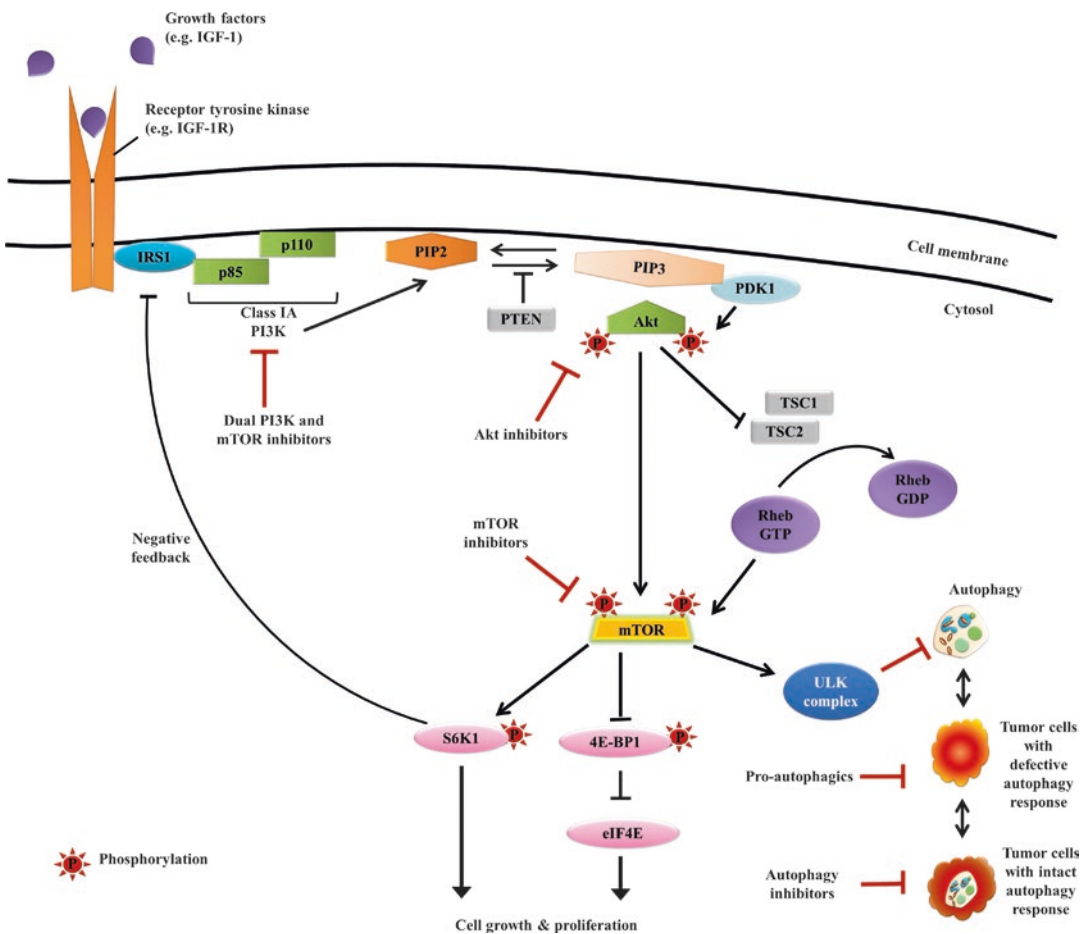
The Nomenclature Committee on Cell Death (NCCD) recommends that the term “autophagic cell death” be used based on some biochemical and functional considerations, before indicating that a cell death is mediated by autophagy. Some of the considerations include making sure that the investigated cell death can be suppressed by the inhibition of the autophagic pathway using

chemicals and/or genetic means (e.g., gene knock-out or RNAi silencing of essential autophagy modulators such as AMBRA1, ATG5, ATG12, or BECN1) [211].

One of the most-studied and important pathways involved in autophagy regulation is the PI3K-Akt-mTOR signaling pathway. The mammalian target of rapamycin, commonly known as mTOR, is a serine/threonine kinase which belongs to the family of phosphatidylinositol 3-kinase-related kinases. It regulates translation and cell growth by its ability to phosphorylate both binding protein of eukaryotic translation inhibition factor eIF4E (4E-BP1) and p70 ribosomal S6 kinase (p70S6k). Upon stimulation by a variety of signals including cytokines, growth factors, cellular stress such as heat shock,

hypoxia, and oxidative stress, PI3K is recruited to the inner cell membrane via phosphorylated receptor tyrosine kinases and catalyzes the phosphorylation of phosphatidylinositol-3,4-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-triphosphate (PIP3). The recruitment of inactive Akt from the cytosol to the plasma membrane requires that the pleckstrin homology (PH) domain of Akt binds to PIP3 synthesized at the plasma membrane by PI3K. Akt is then phosphorylated at Thr308 by phosphatidylinositol-dependent kinase 1 (PDK1) [212, 213]. PTEN phosphatase antagonizes PI3K-Akt signaling by converting PIP3 back to PIP2 [212]. (Fig. 18.3).

Upstream PI3K and Akt activation by growth factors leads to the activation of mTOR and sub-



**Fig. 18.3** Autophagy signaling pathway and antitumor targets

sequently phosphorylation of downstream substrates. Phosphorylation of p70S6k promotes ribosome biogenesis, and increases the capacity of the translational machinery for protein synthesis [214]. Phosphorylation of 4E-BP1 initiates the transcription of a subset of mRNAs important for cell growth and proliferation [214–216]. The mTOR kinase is a key regulatory component that controls the induction of autophagy [217]. Inhibition of mTOR (by nutrient depletion, starvation, or rapamycin) leads to cell cycle arrest, inhibition of cell proliferation, immunosuppression, and induction of autophagy. Increased levels of the mTOR kinase are found to inhibit the autophagy process, resulting in an increased in cell growth and tumor development [173]. Rapamycin, a specific mTOR inhibitor, complexes with the cytosolic receptor FK506-binding protein (FKBP12), and subsequently binds to a distinct region of mTOR upstream of the catalytic domain [218]. It induces autophagy and inhibits the proliferation of a variety of cells [219].

In eukaryotic cells, mTOR exists in two different complexes: mTORC1; a rapamycin-sensitive complex defined by its interaction with the supplementary protein Raptor (regulatory-associated protein of mTOR) and mTORC2; a rapamycin-insensitive complex defined by its interaction with Rictor (rapamycin-insensitive companion of mTOR) [220–222]. mTORC1 and mTORC2 accessory complexes consist of mTOR, mammalian lethal with SEC13 protein 8 (mLST8) (also known as GβL) and DEP domain-containing mTOR-interacting protein (Deptor) [223]. mLST8 binds to the kinase domain of mTOR, and stabilizes the interaction of Raptor with mTOR in a rapamycin-sensitive pathway [224]. Raptor is the first protein shown to bind directly to mTOR that is required to mediate mTOR regulation of p70S6k and 4E-BP1 activities [221, 225]. On the other hand, PRAS40 and Deptor play roles as distinct negative regulators of mTORC1 [226, 227].

In a rapamycin-sensitive mTOR signaling pathway, much of the knowledge about mTORC1 function comes from the use of rapamycin, a bacterial macrolide antibiotic [228]. Upon entering

the cell, rapamycin binds FK506-binding protein (FKBP12), its intracellular receptor, which subsequently binds to the FKBP12-rapamycin binding domain (FRB) of mTOR, thus inhibiting the mTORC1 functions [229, 230]. Rapamycin weakens the interaction between mTOR and Raptor [231]. However, the exact mechanism of how rapamycin and several rapamycin derivatives bind to FKBP12 to inhibit mTORC1 signaling is not completely understood [232]. Various conditions including starvation or lack of nutrients such as amino acids and/or glucose mimic rapamycin treatment, hence inhibit mTOR function in cultured cells, as indicated by rapid inactivation of p70S6k and hypophosphorylation of the 4E-BP1 [233].

Studies have shown that mTORC1 controls autophagy through the regulation of a protein complex consisting of ULK1, mAtg13, and FIP200 [176, 178, 234]. ULK complex is directly controlled by mTOR, leading to maintenance of the mAtg13 hyperphosphorylation state and suppression of autophagy induction [235]. A study has demonstrated that inhibition of mTOR by rapamycin leads to dephosphorylation of ULK1, ULK2, and mATG13, and activates ULKs to phosphorylate FIP200. These results suggested that the ULK-ATG13-FIP200 complexes are direct targets of mTOR and important regulators of autophagy in response to mTOR signaling [178]. One of the most important proteins involved in the regulation of mTORC1 activity is the tuberous sclerosis complex (TSC), which is a heterodimer of two proteins, TSC1 (also known as hamartin) and TSC2 (also known as tuberin) [230]. TSC1 and TSC2 function as a GAP (GTPase-activating protein) that negatively regulates a small GTPase called Rheb (Ras homologue-enriched in brain). TSC1 and TSC2 inhibit mTORC1 signaling by transforming Rheb into its inactive GDP-bound state [236, 237].

On the other hand, mTORC2 consists of mTOR, mLST8, Rictor, Deptor, mammalian stress-activated map kinase-interacting protein 1 (mSIN1; also known as MAPKAP1), and the recently identified protein observed with Rictor (PROTOR) [223, 238]. Rictor is defined as a novel mTOR-interacting protein defining a sec-

ond raptor-independent mTOR complex [220, 239]. Unlike mTOR-Raptor, the mTOR-Rictor complex does not bind to FKBP12-rapamycin, and is insensitive to rapamycin treatment [220, 222]. Therefore, rapamycin treatment does not represent a complete inhibition of mTOR function [240]. mTORC2 stimulates cell signaling through activation and phosphorylation of the proproliferative and prosurvival kinase Akt [241]. Akt regulates cellular processes such as metabolism, survival, apoptosis, growth, and proliferation by phosphorylating various effectors. mTORC2 activates Akt directly by phosphorylation at Ser473, which is a site needed for its maximal activation [242, 243].

In addition, mTORC2 controls various members of the AGC subfamily of kinases which includes serum and glucocorticoid-induced protein kinase 1 (SGK1) and several members of PKC family including PKC $\alpha$  [220], PKC $\epsilon$  [244], PKC $\delta$  [245], and PKC $\zeta$  [246]. mTORC2 is also known to phosphorylate mammalian Ste20-like kinases 1 (MST1) which is a core component kinase in the Hippo signaling pathway [247]. The Hippo pathway is composed of a group of evolutionarily conserved protein kinases that inhibit cellular growth and promote apoptosis [248, 249]. MST1 phosphorylates and activates large tumor suppressor (LATS) kinases, which in turn phosphorylate and inhibit Yes-associated protein 1 (YAP1), a co-transcription factor that promotes proliferation and survival [250]. mTORC2 is reported to be involved in the regulation of cytoskeletal organization through Rho GTPases and PKC $\alpha$  [220, 239]. Inhibitors of mTOR kinase domain have been developed to suppress the activity of both mTOR complexes (mTORC1 and mTORC2) [251, 252]. Figure 18.3 illustrates the simplified autophagy signaling pathways.

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## 18.7 Autophagy and Cancer

The role of autophagy in cancer is rather perplexing. It is widely known that the autophagic pathway is deregulated in tumor cells. Several proteins and pathways related to autophagy signaling are deregulated during cancer develop-

ment [189, 253]. Cell lines derived from hepatic, pancreatic, and breast carcinoma exhibit low autophagic activity, as compared with normal cells from the same origin [189, 254]. Autophagic capacity is known to increase during premalignant stages of pancreatic carcinogenesis, and then decreases during the transition of pancreatic adenoma into adenocarcinoma, suggesting that a decreased autophagic activity possibly contributes to the malignancy of pancreatic cancer [255, 256]. A decrease in autophagic capacity is also observed during animal experimental carcinogenesis, where cells from preneoplastic liver nodules or primary hepatocellular carcinomas induced by chemical carcinogens showed a decreased autophagic capacity as compared to normal liver cells [256, 257]. In addition, BECN1 is found to be mono-allelically deleted in a high percentage of ovarian, breast, and prostate cancers (based on the 17q21 and gene mapping studies). However, BECN1 is adjacent to the known tumor suppressor gene breast cancer 1 (BRCA1) on chromosome 17. Genomic analysis of BECN1 in The Cancer Genome Atlas (TCGA) demonstrated that allelic loss of BECN1 does not occur independently of codeletion with BRCA1, suggesting instead that BRCA1 loss is the driver mutation in hereditary and sporadic breast cancer [258–260].

There is a direct link between tumorigenesis and the disruption of the autophagy signaling pathways. PTEN deletions as well as the amplifications of both Class III PI3K and Akt are found in several cancers [261, 262]. The mTOR signaling pathway is constitutively activated in many tumor types. For example, the mTOR pathway is frequently found to be hyperactive in cancers such as breast cancer, suggesting that mTOR is an attractive target for cancer drug development and therapy [263–265]. The mTOR signaling network contains a number of tumor suppressor genes which includes *PTEN*, *LKB1* (liver kinase B1), *TSC1/2*, and a number of proto-oncogenes such as *PI3K*, *Akt*, and *eIF4E* genes [266]. Several alterations in genes such as *KRAS*, *EGFR*, *LKB1*, *PTEN*, *PIK3CA* (encoding the p110 catalytic subunit of PI3K), as well as *Akt1* mutations, *EGFR* and *PIK3CA* amplification, and *PTEN*

deletion have been described in NSCLC, which lead to uncontrolled mTOR pathway signaling [267]. In addition, dysregulation of the mTOR pathway appears to be more common in squamous lung carcinoma than adenocarcinoma [267, 268].

Cancer-related changes in pathways at the downstream of mTOR such as p70S6k and eIF4E are reported in breast carcinoma [269, 270]. In addition, malignant cell types undergo massive autophagosomes and eventually cell death when responding to anticancer agents and traditional herbs indicate the potential utility of autophagic cell death induction in cancer therapy [173, 271, 272]. Autophagic cell death characterized by an increase in the number of autophagic vacuoles in the cytoplasm, followed by cell demise has been observed in various diseases such as Alzheimer's disease [273], Huntington's disease [274–277], and Parkinson's disease [278]. Thus, manipulation of autophagy is considered an attractive strategy to increase the efficacy of cancer treatments, prevent cancer development, and limit tumor progression.

However, autophagy is divergent in nature in both tumor suppression and tumor progression [279]. Although the argument supports that if cells cannot activate autophagy, protein synthesis will predominate over protein degradation and cellular growth continues (typical characteristic of tumor cells), that was not the case for most. For example, a study in human epidermoid lung carcinoma cells revealed that the autophagic pathway in response to nutrient deprivation is not downregulated when compared to their normal counterparts [280]. Human colon cancer cells which are able to survive for long period of time in the absence of nutrients have a high rate of autophagic activity [281]. Studies in colorectal cancer cells revealed that these cancerous cells harbor functional autophagic machinery to prolong cell survival during shortages of nutrients [282]. A study by Fuji and coworkers has also shown that strong LC3 expression in the peripheral area of pancreatic cancer tissue is correlated with poor outcome and short disease-free period [283]. Activated autophagy observed in pancreatic cancer cells is thought to be a response to

factors in the cancer microenvironment, such as hypoxia and poor nutrient supply. In addition, autophagy was found to be upregulated in RAS-transformed cancer cells to promote cancer cells growth, survival, tumorigenesis, invasion, and metastases [284–286]. Upregulation of autophagy in cancer cells is caused by direct activation of the transcription factors of the microphthalmia-associated transcription factor (MiTF)/TFE family that control autophagy and lysosomal biogenesis or by removal of a repressive phosphorylation on the autophagy initiation machinery [286–288].

In lung cancer, deletion of *Atg7* dramatically alters tumor pathology from carcinomas to that of benign oncocytomas [289, 290]. ATG7-deficient tumors accumulate dysfunctional mitochondria and prematurely induce p53 and proliferative arrest. As defective mitochondria is a major autophagy substrate, this indicates that benign human tumors manifest a phenotype of defective autophagy, perhaps explaining their benign status [286]. Autophagy has been identified as the key mechanism of cell survival in estrogen receptor-positive (ER+) breast cancer cells undergoing treatment with 4-hydroxytamoxifen (4-OHT) [291]. Antiestrogen therapy is the standard treatment for ER+ breast cancers which improves overall survival and provides chemoprevention [292, 293]. Unfortunately, approximately half of the women treated with antiestrogen therapy either do not respond or their breast cancer ultimately acquires resistance during treatment [294, 295]. Studies have shown that autophagic activity reduces the efficacy of chemotherapy and tamoxifen therapy in ER+ breast cancer cells [291, 296, 297], supporting the thesis that blocking autophagy signaling pathways may provide a new mechanism of anticancer therapy for resistant tumors.

In another example, electron microscopy examination of autophagic vesicles in melanoma tumors from 12 patients enrolled in a Phase II clinical trial of temozolomide and sorafenib therapy revealed that autophagic index (mean number of autophagic vacuoles per cell) is significantly higher in patients who derived little or no clinical



benefit from the combination of temozolomide and sorafenib treatment. Patients who had stable disease or responded to therapy had low levels of autophagy in their tumors. These findings further validate the preclinical evidence that autophagy plays a critical role in resistance to chemotherapy. Results of this study indicate that pretreatment levels of autophagy can predict resistance to therapy. Patients with aggressive melanoma are more likely to have higher levels of autophagy in their tumor and therefore may respond to autophagy inhibition as a therapeutic strategy [298]. Hence, the divergent nature of autophagy has resulted in strategies for using proautophagics or autophagy inhibitors depending on the inherent nature of the cancer involved.

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## 18.8 Autophagy Signaling Pathways and Therapeutic Strategies in Cancer

### 18.8.1 mTOR Signaling Pathway Inhibitors

Rapamycin (Sirolimus) as the first prototype of an mTOR inhibitor has poor aqueous solubility and strong immunosuppressive properties. Therefore, its utilization at doses capable of exerting anticancer effects is rather limited [299]. Nevertheless, trials utilizing rapamycin as a single agent or combination therapy are still being carried out. In a Phase I study of rapamycin and sunitinib in patients with advanced NSCLC, combination of rapamycin and sunitinib is reported to be well tolerated and has warranted further investigation in Phase II trials [300]. However, the same was not observed in another recent study. Combination of sunitinib and rapamycin was observed to be quite toxic in all cohorts of patients with refractory solid malignancies [93]. The addition of rapamycin was thought to be able to decrease the sunitinib-induced VEGF production, but on the contrary, VEGF levels went further up along with sunitinib and rapamycin administration; it only came down during the sunitinib-off weeks [93]. However, in another recent Phase I trial, combination of oral

rapamycin, topotecan, and cyclophosphamide was well tolerated in patients with relapsed/refractory solid tumors. Biomarker studies demonstrated modulation of angiogenic pathways with reduction of thrombospondin-1 and soluble vascular endothelial growth factor receptor-2 levels, respectively [92]. Several Phase II trials with rapamycin in combination therapy are currently recruiting patients with bladder, thyroid, prostate, and central nervous system (CNS) tumors (Table 18.1).

Various rapamycin analogues have since been developed. Temsirolimus (CCI-779) is the first mTOR inhibitor approved by the US FDA for cancer treatment, and is considered a first-line treatment for patients with advanced renal cell carcinoma (RCC) with poor prognostic features [301]. A number of clinical trials were carried out for this drug, mainly as combination therapy with other chemotherapy drugs. Moderate clinical activity was observed in patients with bone and soft-tissue sarcoma given a combination of temsirolimus and cixutumumab in a Phase II trial [302] and in patients with metastatic adrenocortical carcinoma, the same combination therapy resulted in 40% of patients achieving prolonged stable disease [303]. Similarly, in a recent Phase I study of temsirolimus in combination with cetuximab in patients with advanced solid tumours, both the median PFS and overall survival (OS) were <1 year and less than half of the patients had stable disease at the end of the trial, indicating modest clinical activity [94].

In another recent Phase I study combining perifosine (an Akt inhibitor) and temsirolimus, although stable disease was seen in 9 of 11 subjects with high-grade gliomas, no partial or complete responses were achieved [95]. However, the combination of these Akt and mTOR inhibitors was considered safe and feasible in patients with recurrent/refractory pediatric solid tumors [95]. When temsirolimus was tested as a single therapy in patients with relapsed or refractory primary CNS lymphoma in a Phase II trial, complete response was seen in five patients (13.5%), partial response in 12 patients (32.4%), and an overall response rate of just 54% [98]. In platinum-refractory/resistant ovarian cancer or

advanced/recurrent endometrial carcinoma, although temsirolimus treatment was well tolerated, it did not meet the predefined efficacy criteria [99]. Phase I and Phase II clinical trials with temsirolimus and sorafenib carried out in patients with metastatic melanoma did not produce sufficient activity to justify further use [304, 305]. Similarly, in a Phase II trial for metastatic colorectal cancer, temsirolimus had limited efficacy in chemotherapy-resistant KRAS mutant disease [306].

Everolimus is another rapamycin analogue which was already approved as an anticancer agent. Everolimus (RAD001; rapamycin derivative 001) is a hydroxyethyl ether derivative of rapamycin that has been developed for oral administration [307]. This drug was approved by FDA for use in a variety of cancers, including advanced renal cell carcinoma, advanced pancreatic neuroendocrine tumors, renal angiomyolipoma, and HER2-negative breast cancer. Everolimus is structurally similar to temsirolimus, binds to an intracellular protein, FKBP12, forming a complex that inhibits the mTOR kinase. In a recent Phase I trial to assess safety and efficacy of everolimus in combination with liposomal doxorubicin and bevacizumab in patients with advanced metaplastic triple negative breast cancer, only patients with the presence of PI3K pathway aberration were associated with a significant improvement in objective response rate, but not the clinical benefit rate [101]. A randomized Phase II study indicated that combination therapy of everolimus with tamoxifen increased the clinical benefit rate (defined as the percentage of all patients with complete or partial response or stable disease at 6 months), time to progression (TTP), and overall survival compared with tamoxifen alone in postmenopausal women with aromatase inhibitor-resistant metastatic breast cancer [308]. Further Phase III trials in combination therapy with aromatase inhibitors and adjuvant hormone therapy in hormone receptor positive metastatic cancer are currently underway.

Everolimus given for 14 days in combination with R-CHOP-21 (rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone

delivered in a 21-day cycle) in patients with diffuse large B-cell lymphoma was proven to be safe. A total 23 of 24 patients achieved an overall response, and all 23 attained a complete metabolic response by PET, suggesting that drugs that target the PI3K-mTORC pathway added benefit when combined with standard R-CHOP [102]. The combination of everolimus plus CHOP was also effective in patients who are newly diagnosed with peripheral T-cell lymphomas, with objective response rate up to 90% [110]. The combination of mFOLFOX6 and everolimus in patients with metastatic gastroesophageal adenocarcinoma was also considered to be an active regimen with 83% of the patients experiencing a partial response [103]. Everolimus as a single therapy has demonstrated clinically relevant anti-tumor activity in patients with advanced differentiated thyroid cancer; median PFS and OS were 9 and 18 months, respectively [108].

Ridaforolimus (deforolimus or AP23573) has been tested in Phase I and Phase II clinical trials, and has shown promising results in several tumor types including sarcoma [299, 309]. Ridaforolimus received fast track and orphan drug status from the US FDA, as well as orphan status from the European Medicines Agency. Latest Phase I trials indicate that ridaforolimus as single therapy or in combination with other chemotherapy drugs was safe and well-tolerated [113, 114]. However, in a previous Phase II trial study on the efficacy and safety of single-agent ridaforolimus in patients with relapsed or refractory hematologic malignancies, results were unremarkable. Of the 52 patients evaluated, partial responses were noted in five subjects, while hematologic improvement and stable disease were observed in less than half of the patients [310]. In addition, the combination of ridaforolimus and dalotuzumab was no more effective than exemestane in patients with advanced ER-positive breast cancer, and the incidence of adverse events was higher [116]. Thus, the combination was not further pursued.

PI3K/Akt/mTOR pathway is often constitutively activated in human tumor cells and thus has been considered as a promising drug target. BEZ235 is a potent imidazo (4,5-c) quinoline

derivative that inhibits PI3K and mTOR kinase activities by binding to the ATP-binding cleft of these enzymes, and induces G1 arrest [311]. Preclinical studies have suggested that BEZ235 is a potent dual PI3K/mTOR modulator with favorable pharmaceutical properties. For example, it inhibits VEGF-induced HUVEC cell proliferation and survival in vitro and VEGF-induced angiogenesis in vivo [312]. The compound also inhibits microvessel permeability in BN472 mammary carcinoma grown orthotopically in syngeneic rats, suggesting that this compound is potentially antiangiogenic [312]. Deregulated angiogenesis and high tumor vasculature permeability are known VEGF-mediated characteristics of human tumors. In addition, BEZ235 is found to produce significant tumor growth inhibition in xenograft models of pancreatic cancers and breast cancer cells [313, 314]. However, in a Phase II trial of the BEZ235 in patients with everolimus-resistant pancreatic neuroendocrine tumours, BEZ235 was poorly tolerated by patients. Although evidence of disease stability was observed, the study did not proceed to stage two [117]. Similarly, BEZ235 showed modest clinical activity and an unfavorable toxicity profile in patients with advanced and pretreated transitional cell carcinoma, with just a minority of patients experienced a clinical benefit [118]. Several Phase I/II clinical trials of BEZ235 in patients with advanced solid malignancies such as prostate and breast cancer were completed, but reports on the safety and efficacy of this drug have yet to be published.

### 18.8.2 Proautophagics

Temozolomide is the first proautophagic cytotoxic drug used to overcome apoptosis resistance in cancer cells, and was approved for use in glioblastoma multiforme (GBM) [119]. It has demonstrated therapeutic benefits in patients with glioblastoma, and has been evaluated for several types of apoptosis-resistant cancers [315]. Temozolomide is a prodrug, a monofunctional alkylating agent, and is chemically related to dacarbazine. It is the 3-methyl derivative of the

experimental anticancer drug, mitozolomide. The ability of temozolomide in inducing autophagic cell death was reported in various preclinical studies [316–319]. In addition, temozolomide has demonstrated proapoptotic activities in malignant melanoma cells [320]. In a systematic assessment of three randomized controlled trials addressing whether temozolomide holds any advantage over conventional therapy for high-grade gliomas, it was shown that temozolomide is an effective therapy for GBM. The drug prolongs survival, delays disease progression, and has a low incidence of early adverse events [321]. Similar outcomes were observed in a Phase II study involving erlotinib in combination with radiation therapy and temozolomide to treat GBM and gliosarcoma. Patients treated with the combination of erlotinib and temozolomide during and following radiotherapy had better survival than historical controls [322].

In a later Phase II trial, patients with unresectable or multifocal glioblastoma, an upfront regimen of temozolomide and bevacizumab was well tolerated, and provided a significant level of disease stabilization [323]. In patients with recurrent glioblastoma, either used as a single agent in a dose-intense schedule or in combination with other chemotherapeutic agents, temozolomide was proven to be well tolerated and safe [324–326]. In pediatric patients with recurrent solid tumors or brain tumors, low-dose temozolomide improved tolerability and was convenient as outpatient therapy [327]. However, in a recent Phase II trial, bevacizumab plus irinotecan combination resulted in a superior PFS-6 rate and median PFS compared with temozolomide in patients with glioblastoma that harbors a nonmethylated O(6)-methylguanine DNA methyltransferase promoter [124]. Patients with an O(6)-methylguanine–DNA methyltransferase (MGMT) nonmethylated (nmMGMT) glioblastoma (GBM) have a particularly short median survival of 12.6 months and do not substantially benefit from temozolomide chemotherapy [124, 328].

The combination of adjuvant temozolomide and lomustine, an alkylating agent, was associated with a significant improvement in OS and event-free survival (EFS) compared with adju-

vant temozolomide alone in the Children's Oncology Group ACNS0126 study [125]. This effect was most apparent in patients whose tumors had MGMT overexpression, as well as those who did not undergo gross-total resection and in those with glioblastomas. In a current Phase II study, neoadjuvant temozolomide was associated with an encouraging favorable long-term survival with acceptable toxicity in patients with glioblastoma [131]. Temozolomide in combination with vorinostat was also well tolerated in children with recurrent CNS malignancies with myelosuppression [329]. Vorinostat is a broad inhibitor of histone deacetylase (HDAC) activity which induces apoptosis, inhibits angiogenesis, and downregulates immunosuppressive interleukins. Several Phase III trials using temozolomide in combination with targeted monoclonal antibodies or interferon-alpha in glioblastomas and high-grade gliomas are currently recruiting patients (Table 18.1).

However, poor therapeutic effects were observed in patients with NSCLC. In a current efficacy and safety study of temozolomide in a total of 31 pretreated patients with NSCLC, only two patients achieved partial response and three had stable disease [330]. Moreover, the researchers pointed out that prolonged low daily doses of temozolomide produce minimal activity in patients with advanced NSCLC. In a recent Phase II study, combination therapy of pemetrexed and temozolomide group achieved the same efficacy in PFS and OS as the pemetrexed and cisplatin group, but with less toxicity. High-dose pemetrexed plus temozolomide may be a better regimen for treating NSCLC with brain metastasis due to its better safety profile [126]. A further Phase III study in patients with extensive small-cell lung cancer is currently underway.

Arsenic trioxide (ATO) has recently been introduced as part of a regimen in the therapy and management of acute promyelocytic leukemia (APL) [331]. It is now considered to be "the most biologically active single drug in APL" by a panel of International Leukemia Experts for the European Leukemia Net. The North American Intergroup Study Cancer and Leukemia Group B (CALGB) 9710 demonstrated that adults with

APL receiving two cycles of ATO consolidation had significantly improved OS and decreased relapse risk (RR) [332]. It also achieves great success as a single agent and in combination with all-trans retinoic acid (ATRA) in the treatment of APL.

Arsenic trioxide (ATO) is known to induce both autophagy and apoptosis depending on cell types; therefore, its role as an autophagy inducer remains largely uncertain. In some preclinical trials, ATO induces the autophagy pathway in ovarian carcinoma cells, and synergizes with everolimus to induce the cytotoxicity of ovarian cancer cells. The enhanced cytotoxicity is accompanied by the upregulation of ATG5-ATG12 conjugate and LC3-II, a hallmark of autophagy [333]. In another recent study, ATO induces the autophagic degradation of the BCR-ABL1 oncoprotein, known to cause chronic myeloid leukemia (CML) and Ph+ acute lymphoblastic leukemia (ALL) [334]. However in other studies, in the presence or absence of ionizing radiation and in specific low concentrations, ATO induces apoptosis in MTLn3 cells, known to be highly malignant and resistant to both radio- and chemotherapy [335]. Interestingly, in human glioma cells, ATO induces both autophagy and apoptosis in vitro and in vivo, mediated by the inhibition of PI3K/Akt and activation of MAPK signaling pathway [336].

In a Phase I clinical study, ATO given concomitantly with radiation therapy in children with newly diagnosed anaplastic astrocytoma, glioblastoma, or diffuse intrinsic pontine glioma, was safe and well tolerated by patients throughout the entire dose escalation [337]. ATO was also reported to be well tolerated when used in combination with temozolomide and radiotherapy in malignant gliomas [338], or when used in combination with bortezomib, high-dose melphalan, and ascorbic acid in multiple myeloma (MM) patients [339]. A Phase II study to evaluate the efficacy and feasibility of a sequential treatment consisting of induction and consolidation with ATO followed by autologous hematopoietic cell transplantation for relapsed APL revealed that ATO demonstrates outstanding efficacy. Of the 23 patients who underwent autologous hema-

topoietic cell transplantation with PML-RAR $\alpha$ -negative PBSC graft, posttransplant relapse occurred only in three patients, and there was no transplant-related mortality. The 5-year event-free and overall survival rates were 65% and 77%, respectively [340].

A recent study showed that the combination of ATO and ATRA exerts at least equal and probably superior antileukemic efficacy compared with ATRA and standard chemotherapy in low- and intermediate-risk APL [136]. In a Phase III study in which a chemotherapy-free ATRA and ATO treatment regimen was compared with the standard chemotherapy-based regimen (ATRA and idarubicin) in both high-risk and low-risk patients with APL, ATRA and ATO have a high cure rate and less relapse and a lower incidence of liver toxicity [138]. Similarly, a recent Phase III trial showed that ATRA-ATO had an edge over ATRA-chemotherapy over time and that there was significantly greater and more sustained antileukemic efficacy in low- and intermediate-risk APL [139]. ATO consolidation cycles are well tolerated in pediatric patients with APL and allow significant reduction in cumulative anthracycline doses while maintaining excellent survival and a low relapse risk for both standard and high-risk patients with APL [140]. Other Phase III clinical trials using ATO as combination therapy with other chemotherapy drugs and/or tretinoin are currently ongoing for APL.

### 18.8.3 Autophagy Inhibitors

The knowledge that autophagy plays a role as a cell survival pathway in response to therapeutic and cellular stresses in the tumor microenvironment (which is highly acidic and hypoxic) implies that autophagy may work in favor of cancer cells. Therefore, inhibition of protective autophagy may break the resistance mechanism for survival of the harsh tumor microenvironment and lead to cell death [341]. Since autophagy activities are known to differ according to stages of cancer, modulation of autophagy is postulated to enhance efficacy of anticancer therapy. In a preclinical study, effects of imatinib, with or without differ-

ent types of autophagy inhibitors, on human malignant glioma cells were investigated [342]. It was demonstrated that suppression of imatinib-induced autophagy by 3-methyladenine (3-MA) or siRNA against ATG5 (which inhibits autophagy at an early stage) attenuates the imatinib-induced cytotoxicity. On the other hand, inhibition of autophagy at a late stage by bafilomycin A1 or RTA 203 enhances imatinib-induced cytotoxicity through the induction of apoptosis [342]. The therapeutic efficiency of imatinib may be augmented by inhibition of autophagy at a late stage, which could help sensitize the glioma cells to anticancer therapy [342].

The current autophagy inhibitors used in trials for human cancer are chloroquine (CQ) and hydroxychloroquine (HCQ). Both drugs are widely used as antimalarial agents and have gained much attention as potential chemosensitizers in treating tumors when used in combination with cytotoxic chemotherapeutic agents [343–345]. CQ inhibits lysosomal acidification and prevents autophagy by blocking autophagosome fusion and degradation [344, 346, 347]. CQ also sensitizes cancer cells to chemotherapeutic agents through autophagy-independent mechanisms and has other anticancer effects that are independent of its effects on autophagy [348].

A number of clinical trials have revealed the promising role of CQ, an autophagy inhibitor, as a novel antitumor drug. In an early glioblastoma study, where patients were treated with CQ in conjunction with radiation and temozolomide, the results showed a significantly prolonged median survival compared with controls [349]. Addition of CQ to conventional treatment for GBM also improves mid-term survival of patients [350]. Gemcitabine–CQ combination as a first- or late-line treatment in patients with metastatic or unresectable pancreatic cancer is well tolerated and shows promising effects on the clinical response [141]. A number of Phase I/II trials in solid tumors such as breast cancer are currently recruiting patients.

Although initial glioblastoma studies that used CQ in combination with chemotherapy and radiation therapy revealed median survival greater than control, there was no significant

improvement in survival of patients with glioblastoma treated with HCQ [145]. A Phase II trial of HCQ as a single agent in patients with previously treated metastatic pancreatic cancer demonstrated no clinical benefit and provided inconsistent evidence of autophagy inhibition [147]. Since this study was carried out in patients with advanced disease, thus, there was a limitation for HCQ to improve end-stage disease outcome [348]. The results appear to be similar to an earlier Phase I study involving patients with advanced NSCLC. Although HCQ, with or without erlotinib, was found to be safe and well tolerated, the overall response rate was as low as 5% [351]. In other Phase I/II trials, HCQ in combination therapy with other drugs such as temozolomide, vorinostat, bortezomib, and gemcitabine are proven to be safe and tolerable among patients with advanced solid tumors and myeloma [142–144, 146]. So far, clinical trials of CQ and HCQ as autophagy inhibitors have demonstrated the safety of targeting autophagy for cancer therapy. More potent and autophagy-specific inhibitors such as Lys05 and drugs that target ULK1, VPS34, and ATG4B are in development and early preclinical stage [348]. Table 18.1 summarizes the various drugs targeting the autophagy pathways and clinical trial stages based on published reports as well as other trials listed in the NIH [ClinicalTrials.gov](http://ClinicalTrials.gov) website.

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## 18.9 Crosstalk in ER Stress, Autophagy, and Apoptosis

Many cellular processes including apoptosis, autophagy, translation, and energy metabolism are controlled by the ER stress and mTOR signaling pathway. However, the crosstalk among these three signaling pathways has been identified only recently. It has been shown that Akt inactivation mediates ER stress-induced cell death. Long-term exposure to ER stress dephosphorylates Akt, induces DDIT3 expression, and causes cell death. Treatment with PI3K inhibitor alone also decreases phosphorylation of Akt, upregulates DDIT3 expression, and causes cell death, suggesting that PI3K/Akt inhibition specifically

induces DDIT3 expression. Thus, Akt inactivation is important in ER stress-induced DDIT3 expression and cell death [352]. In addition, ER stress-induced apoptosis has been reported to be partly mediated by reduced insulin signaling through reduced Akt phosphorylation and increased glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ) activity. GSK3 $\beta$  is a proapoptotic Akt substrate whose activity is inhibited by Akt phosphorylation [353]. Prolonged ER stress has been shown to inhibit Akt/TSC/mTOR pathway, induce DDIT3 expression, and trigger apoptosis cell death [354]. On the other hand, ER stress negatively regulates Akt/TSC/mTOR pathway to enhance autophagy-mediated cell death [355].

It has been suggested that ER stress promotes autophagy and/or apoptosis via TRIB3-dependent inhibition of Akt/mTOR pathway [49, 50]. It was also proposed that ATF4 negatively regulates mTOR via DNA damage inducible transcript 4 (DDIT4, also known as Redd1) expression in response to ER stress. DDIT4 is a cellular stress responsive gene that has been shown to inhibit mTOR activity [356, 357]. ER stress also leads to CaMKK $\beta$ -dependent activation of AMPK, which ultimately leads to inhibition of mTOR and stimulation of autophagy [358]. In addition, it has been demonstrated that ER stress induces BIP expression and promotes an interaction between BIP and Akt. The physical interaction between BIP and Akt at the plasma membrane of cells following induction of ER stress prevents Akt phosphorylation [359]. To sum up, these observations suggest that ER stress may negatively regulate Akt and/or mTOR activity via various pathways, and ultimately leads to cell death.

It is also widely accepted that reactive oxygen species (ROS) generation precedes downstream cellular cascades, including those that determine cell fate either survival (autophagy) or death (apoptosis). Excessive ROS production disrupts the electron transport chain and produces reactive oxygen molecules, leading to depolarization of the mitochondrial membrane and initiation of mitochondria-induced apoptosis. However, ROS generation has also been shown to occur downstream after apoptotic stimulation (TRAIL-induced), or autophagy inhibition [360–362]. However, cell

fate outcomes are largely dependent on the amount of ROS generated and the cell's antioxidant response. During starvation, reactive oxygen molecules are produced as a result of Class III PI3K activation that stimulates autophagy through oxidation of ATG4, ultimately increasing the formation of lipidated LC3-rich autophagosomes [363]. Both  $O_2^-$  and  $H_2O_2^-$  induce autophagy through AMPK activation and subsequent mTOR inhibition, and by transcriptional regulation of autophagy genes such as SQSTM1 (p62) and BECN1 [364–366]. A number of studies have similarly demonstrated that exogenously applied ROS leads to autophagy induction or apoptosis.

Functional relationships between apoptosis and autophagy are gaining much interest, as both cell deaths are not mutually exclusive. Perturbations in the apoptotic machinery, such as caspase inhibition, have been reported to induce both autophagic cell death and necroptosis [367, 368]. Inhibition of autophagy in cancer cells results in an accelerated cell death that manifests the hallmarks of apoptosis including chromatin condensation, MOMP, and activation of caspases [369]. In some cases, mixed phenotypes of both autophagy and apoptosis are detected in response to common stimuli [346, 369]. Studies in a variety of experimental systems indicate that autophagic cell death is likely to be context- and cell type-dependent. Autophagy can delay the onset of apoptosis following starvation, DNA damage, and hemodynamic stress [173]. For example, 1-day fasting causes liver autophagy in rats, but when starvation is prolonged for a few days, hepatocytes succumb to apoptosis [370]. Similarly, hematopoietic cell lines withdrawn from growth factor first activate autophagy, and eventually apoptosis [167]. Studies have also demonstrated that certain compounds have the ability to trigger both apoptosis and autophagic cell deaths simultaneously in cancer cells [371, 372]. Blocking of one pathway will trigger the activation of another [373]. Researchers have also hypothesized that there are factors (either external or internal) that may affect the preferential shunting into either biochemical cascades that will ultimately result in either apoptosis or autophagic cell death [374].

Crosstalks between autophagy and apoptosis exist at multiple levels because both pathways share mediators and pathway regulators. Several signals and pathways involved in autophagy are in common with apoptosis. Starvation and oxidative stress can trigger both apoptosis and autophagy. BCL-2 proteins function to inhibit both apoptosis and autophagy, providing another clue to the interplay between both processes. BECN1, the essential autophagy protein and haploinsufficient tumor suppressor, interacts with several cofactors such as AMBRA1, BIF-1, and UVRAG to activate the lipid kinase Class III PI3K, and induce autophagy [375]. In normal conditions, BECN1 is bound to and inhibited by BCL-2 or the BCL-2 homologue BCL-X<sub>L</sub>, well-characterized apoptosis regulators, which involve an interaction between the BH3 domain in BECN1 and the BH3 binding groove of BCL-2/BCL-X<sub>L</sub>. BH3-only proteins can competitively disrupt the interaction between BECN1 and BCL-2/BCL-X<sub>L</sub> to induce autophagy. Nutrient starvation can stimulate the dissociation of BECN1 from its inhibitors, either by activating BH3-only proteins (such as BAD) or by post-translational modifications of BCL-2 (such as phosphorylation) that may reduce its affinity for BECN1 and BH3-only proteins [375]. Antiapoptotic BCL-2 family members participate in the inhibition of autophagy, whereas the proapoptotic BH3-only proteins participate in the induction of autophagy.

A recent finding suggests a link between autophagy and the extrinsic apoptotic pathway mediated by p62/SQSTM1. Autophagy is recently known to be responsible in selective degradation of polyubiquitinated proteins via SQSTM1, which encodes for p62 protein. P62 interacts with LC3 via its LC3 interacting region (LIR). Recent studies indicate that p62 is recruited to damaged mitochondria via binding to ubiquitinated outer mitochondrial membrane proteins, suggesting that p62 may serve as an autophagy receptor for ubiquitinated proteins and damaged mitochondria [376–378]. In addition to its role in autophagy, p62 mediates a cell's decision to undergo apoptosis or survival through its organization of signaling complexes in the cyto-

plasm [377, 379, 380]. Upon cytokine stimulation, p62 activates the NF- $\kappa$ B pathway, which subsequently induces the prosurvival genes, such as antiapoptotic and cell proliferation genes, and induces the expression of inflammatory genes such as cytokines, chemokines, and adhesion molecules [380–383]. However, p62 is also found to activate caspase-8 in the extrinsic apoptosis pathway, resulting in the initiation of apoptosis and cell death [379].

The expression of Patched (Ptc) induces apoptosis, but this activity is suppressed by its ligand, sonic hedgehog (SHH). Interestingly, hedgehog inhibition is found to induce autophagy through upregulation of BNIP3, and is also found to increase apoptosis in hepatocellular carcinoma cells at the same time [384]. In a recent study, apoptosis suppressed by the knocking down of PP2A can be reversed by the administration of 3-MA, a known autophagy inhibitor. The elevated accumulation of LC3-II and the decline of the autophagy substrate p62 are also observed in PP2Ac-small interfering RNA transfected cells. However, overexpression of PP2Ac suppresses the accumulation of LC3-II and restores p62 [385]. Interestingly, 3-MA increases cell death induced by diamindichloridoplatin (DDP), which suggests the protective function of autophagy in DDP-induced cell death [385].

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## 18.10 Future Directions

There are increasing evidences that three major processes, i.e., apoptosis, ER stress, and autophagy, share overlapping molecular pathways and can occur in parallel under similar conditions. Fundamental knowledge in apoptosis, ER stress, and autophagy has also generated a great deal of insight into the pathogenesis of cancer, and has provided important considerations in strategizing cancer pharmacotherapy. Much effort and investment have been devoted to experimental drugs modulating apoptosis, ER stress, and autophagy. A number of drugs have proven to be promising during preclinical and clinical studies, but these drugs appear to be effective in one type of cancer and not in other. The percentage of patients who

totally responded or partially responded to these treatments, either as single agent or in combination therapies, is relatively low, even though the outcome of these trials suggests some potential. These unforeseen effects are probably due to the specific-targeted nature of the therapy, in addition to the interconnected relationships between these cell death pathways. The contradictory role of autophagy and the status of autophagy in the human tumors concerned remain speculative, and further complicate the response to conventional anticancer treatment.

Currently, modulating apoptosis, ER stress, and autophagy by various means may be an important strategy to fight against the disease. Cancers which are resistant to the apoptotic effects of certain chemotherapy drugs may be sensitive to drugs that evoke ER stress or autophagic cell deaths. An intact autophagy pathway has a role in promoting carcinogenesis as well as in suppressing it. It also has a role in the development of resistance to treatment. Therefore, if autophagy response and activity are normal in tumors, combining standard chemotherapy drugs with autophagy inhibitors may sensitize tumor cells to anticancer agents. Cancer cells which present defects in the autophagy pathway may be managed by replacement of autophagy-inducing signals, e.g., proautophagics, or by inhibiting mTOR kinase. In some other cases, utilizing both autophagy and apoptosis inducers may present a deadly strategy against highly resistant tumors. Thus, devising personalized pharmacotherapeutic strategy based on the autophagy status of the tumors has become an attractive option and offers significant potential to be translated into the clinic.

Combination of anticancer drugs of many different classes with autophagy inhibitors and inducers is underway but with little rationale for deciding or selecting patients who are most likely to benefit from these therapies. So far, targeted drugs like oblimersen, bortezomib, and mTOR inhibitors such as everolimus and ridaforolimus have shown to be useful in some clinical trials. These novel classes of drugs appear to work synergistically in combination with other chemotherapeutics, and have also shown specific



activities against certain cancers. Clinical trials of CQ or HCQ as autophagy inhibitors have also demonstrated the safety of targeting autophagy for cancer therapy. Since these drugs are specifically targeted against certain molecules or receptors in the pathway, further unveiling of the tumor's characteristics such as receptor or protein status may be critical in assessing patient's response and clinical trial success. Furthermore, a number of known genes that play a role in these cell death pathways are either activated or inactivated in several cancers. This will certainly affect not only the promotion and progression of cancer, but also their response to treatment. Therefore, to optimize and personalize treatment strategies, the genetic profile of the tumors is important.

For example, RAS- and BRAF-mutant tumours are often associated with high levels of autophagy and exhibit autophagy dependency. These would be good markers to select patients in which autophagy can be inhibited therapeutically [285, 348, 386–388]. Other markers include signal transducer and activator of transcription 3 (STAT3) and IL6 in breast cancer cells [389], JNK1 in colon cancer [390], and EGFR-mutated or amplified tumors [391]. Some clinical trials have already used these markers to evaluate efficacy and for validation. This may provide information on the optimal point in the pathway to be targeted, and can also be identified as prognostic markers. At the same time, the development of both robust tissue markers and relevant techniques that can be used in the clinical context needs to occur along with novel treatments, which will be another challenge.

Although recent studies have incorporated some predictive biomarkers by examining tumor status, the utility of such practice remains non-conclusive. For example, the expression of peptidyl O-glycosyltransferase GaLNT14 has been proposed to be a potential marker of dulanermin or Apo2L/TRAIL activity in NSCLC as high GaLNT14 mRNA and protein expression in tumor cell lines are associated with Apo2L/TRAIL sensitivity [392]. An increase in PFS and OS was observed in GaLNT14-positive patients with advanced NSCLC in the dulanermin arm,

indicating the potential predictive response biomarker for Apo2L/TRAIL-based cancer therapy [393]. On the other hand, in a Phase Ib/II trial on mapatumumab, a humanized mAb against TRAIL-R1, strong expression of TRAIL-R1 (indicated by immunohistochemical staining) did not appear to be a prerequisite for the effectiveness of mapatumumab in patients with relapsed or refractory follicular lymphoma [394]. Noteworthy, in the two patients who experienced a partial or complete response, the TRAIL-R1 staining was either undetected or weak [394]. However, this could be an isolated case, and trials with bigger sample size should be carried out. Tumor profiling would remain as a good strategy to identify patients who may respond to the relevant treatment.

Fundamental knowledge of cell death pathways remains an area of major interest among scientists in the field of cancer. More studies to characterize these pathways and identify potential targets, and further evaluation of the efficacy of the current drugs in various cancers are certainly warranted.

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