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Targeting endoplasmic reticulum stress for cancer therapy

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1. ABSTRACT

The endoplasmic reticulum (ER) stress response, in combination with autophagy, represents an adaptive mechanism to support cellular survival in response to a great variety of detrimental conditions, such as low nutrient levels, hypoxia, calcium imbalance, or accumulation of misfolded proteins. However, when stress conditions become too severe and excessive, this cellular stress response system turns on its pro-apoptotic module, which then gains dominance and triggers cell death. In tumor cells, the cell-protective features of the ER stress response appear to be chronically activated and thus provide support for continuous proliferation and survival even under adverse microenvironmental conditions, which may include chemotherapy. However, persistent activity of these pro-survival pathways primarily in tumor cells may provide a window of opportunity for therapeutic intervention that is principally aimed at these tumor-specific conditions. Appropriate therapeutic regimens would seek to further aggravate this already engaged system in tumor cells in order to exhaust its protective features and instead trigger its pro-apoptotic module. There is accumulating evidence that this can indeed be accomplished, and that tumor-specific ER stress can be exploited by treatment with select pharmacological agents. The principles of this promising new approach to cancer therapy, as well as representative ER stress-aggravating compounds, will be presented in this review.

2. INTRODUCTION

The endoplasmic reticulum (ER), an organelle of all eukaryotic cells, presents as a membranous labyrinth of branching tubules and flattened sacs that extend from the perinuclear space throughout the cytoplasm. Typically, its membrane constitutes more than half of the entire membrane mass of an average animal cell, and its lumen oftentimes comprises more than 10% of the total cell volume. This extensive network provides several critical functions, which include lipid and protein biosynthesis, assembly of lipid bilayers, regulation of calcium homeostasis and storage, and transport of newly synthesized molecules to various subcellular destinations or the cell surface (1).

A most critical aspect of protein synthesis in the ER is the accomplishment of proper protein folding, which involves N-linked glycosylation and the help of several chaperone proteins, such as calnexin, calreticulin, and members of the family of heat shock proteins (HSPs), such as GRP78 (glucose regulated protein of molecular weight 78, also called BiP). Yet despite this concerted effort, many protein molecules fail to achieve their properly folded state and consequently are removed via a process called ERAD (ER associated degradation). ERAD involves retro-translocation of irreparably misfolded proteins from the ER back into the cytosol, where they are ubiquitinated and then subjected to degradation via the proteasome (1). In

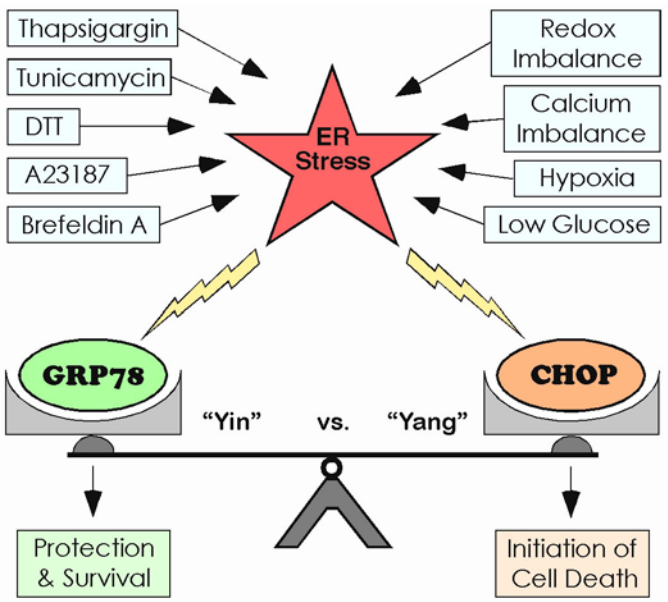

Figure 1. Simplified depiction of the yin-yang principle of ER stress. A variety of stimuli disturb ER homeostasis and trigger ER stress. In response, there is increased expression of GRP78 and CHOP (and several other proteins), which struggle for dominance in order to ensure protection or survival (in the case of GRP78) or to initiate cell death (in the case of CHOP). See text for further details. DTT: dithiothreitol.

addition, misfolded and denatured proteins may aggregate and be assembled into aggresomes, which are proteinaceous inclusion bodies that may form in instances when ERAD is impaired or overwhelmed by a high load of damaged proteins. This process sequesters the potentially cytotoxic components and delivers this compacted body for autophagic removal and recycling (2).

Overall, the various functions of the ER are central to cellular survival, and tightly regulated control mechanisms are in place to maintain proper ER homeostasis. However, numerous microenvironmental or intracellular changes can disrupt this fine-tuned balance and create a condition commonly called ER stress. In response, the cell musters substantial efforts to mount an adaptive reaction, called the ER stress response (also called the unfolded protein response, UPR, when the primary trigger is based on the accumulation of misfolded/unfolded proteins), which primarily serves to restore proper ER homeostasis (3-6). Tumor cells in particular have mastered the art of employing the ER stress response, inclusive of ERAD and autophagy, for their survival benefit and towards increased chemoresistance (7,8). As a result, the baseline activity level of their ER stress response system is different from that in normal cells and thus may provide a therapeutic window for cancer therapy (9,10). Below, I will introduce the concept of ER stress as a potential Achilles' heel of cancer cells and discuss emerging approaches to exploit this feature for cancer therapeutic purposes.

3. THE YIN-YANG PRINCIPLE OF ER STRESS

A broad spectrum of insults can cause ER stress and trigger the ER stress response. These include nutrient deprivation (in particular low glucose levels), changes in calcium concentration, alterations in the oxidation-reduction balance, hypoxia, acidification, and others (Figure 1). Additionally, several pharmacological agents are commonly used as experimental inducers of ER stress (Figure 1), and these have been most valuable in studying this process in the laboratory. Traditional members of this group of agents are the sesquiterpene lactone thapsigargin and the ionophore A23187, both of which interfere with calcium homeostasis (11); the antibiotic tunicamycin, which blocks protein glycosylation (12); the reducing agent dithiothreitol (DTT), which prevents the formation of disulfide bonds between cysteine residues of proteins (13); the antiviral antibiotic brefeldin A, which inhibits transport of proteins from the ER to the Golgi apparatus (14); and 2-deoxy-D-glucose (2-DG), which primarily inhibits glycolysis and thus mimics conditions of hypoglycemia (15).

In response to such insults, the ER stress response activates a set of adaptive pathways with the ultimate goal to alleviate the stressful disturbance, to restore proper ER homeostasis, and to ensure cellular functioning and survival. However, if ER stress is too extensive or excessively prolonged, this same system will turn on an opposing, pro-apoptotic module, which will trigger cell death and as a result will eliminate the cell. In this sense, the ER stress response follows a yin-yang principle, where moderate stress levels trigger its pro-survival mechanism ("yin"), but where severe stress dominantly activates its cell death-inducing module ("yang").

A number of cellular proteins critically contribute to these events and channel the response through three distinct

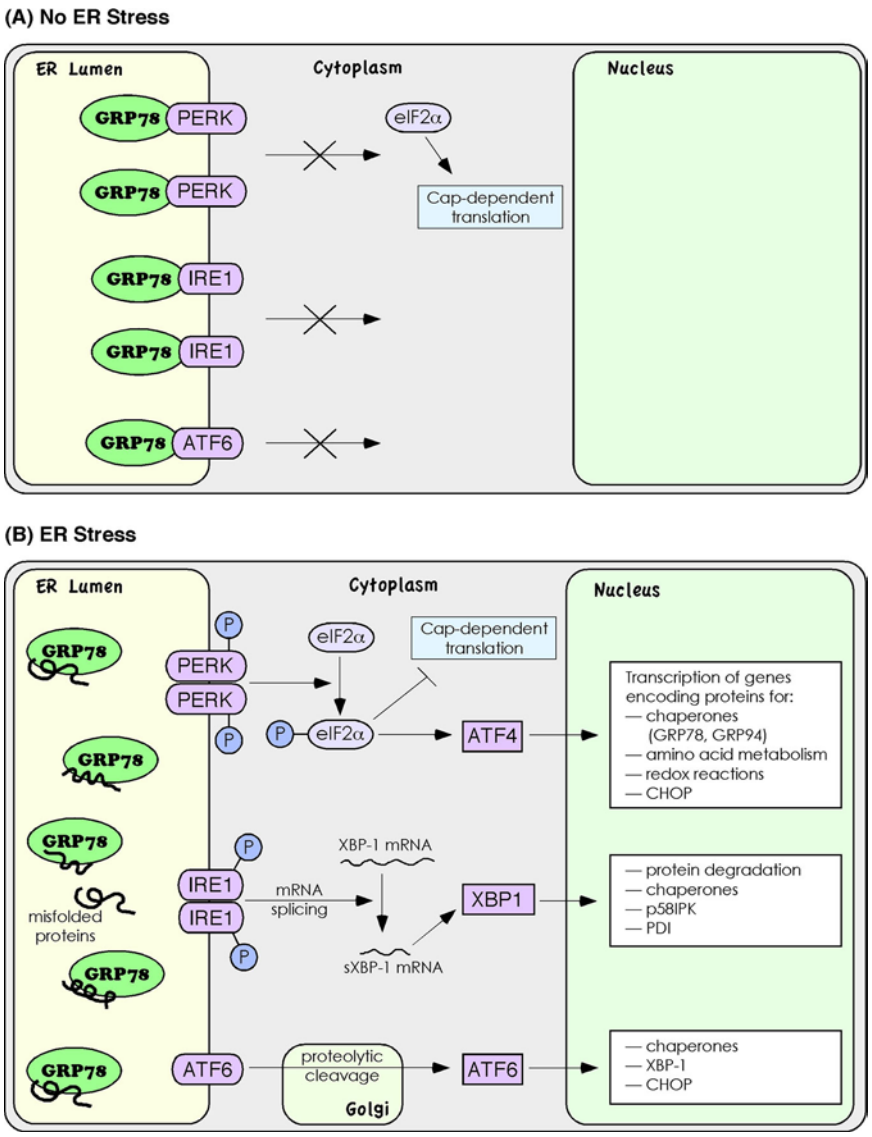

Figure 2. Simplified depiction of ER stress (UPR signaling). (A) In the absence of ER stress, GRP78 binds to and inhibits the activities of three major ER transmembrane proteins, pancreatic ER kinase (PKR)-like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6), which act as sensors and initiators of ER stress signaling. (B) The accumulation of misfolded proteins in the lumen of the ER causes GRP78 to dissociate from PERK, IRE1, and ATF6, which leads to homodimerization and autophosphorylation of PERK and IRE1, respectively, and proteolytic cleavage of ATF6 (via migration to the Golgi apparatus), altogether activating all three signaling pathways and mounting the unfolded protein response (UPR). The kinase activity of PERK leads to phosphorylation of eukaryotic initiation factor 2 alpha (eIF2a), which terminates global cap-dependent translation, but exempts selected ER stress-associated proteins, such as activating transcription factor 4 (ATF4). IRE1 is a dual-activity enzyme with serine-threonine kinase function and endoribonuclease activity; its activation removes an intron from the mRNA encoding X-box binding protein 1 (XBP1) to generate a splice variant (sXBP1) encoding the active XBP1 transcription factor. ATF6 translocates to the Golgi apparatus, where it undergoes proteolytic cleavage that results in its active form. All three transcription factors, ATF4, ATF6, and XBP1 translocate into the nucleus where they stimulate the expression of a variety of gene products collectively involved in managing and coping with ER stress. For further details regarding these processes, see excellent recent reviews (16-18).

signaling pathways governed by pancreatic ER kinase (PERK), inositol-requiring enzyme (IRE1), and activating transcription factor 6 (ATF6), respectively (Figure 2). Recent reviews (16-18) have provided comprehensive descriptions of these pathways and their various components, and therefore these interactions will not be presented in greater detail here. Instead, this current discourse will focus on selected representatives to illustrate those parts of the ER stress response that appear exploitable for improved cancer therapies. For this purpose, the yin-

yang principle of ER stress can be reduced to and illustrated by the expression levels and balanced activities of two key ER stress regulators, GRP78 and CCAAT/enhancer binding protein homologous protein (CHOP, also called GADD153) (Figure 1).

As its name implies, GRP78 was originally identified as a protein strongly induced by lowered levels of glucose. It has important roles in protein folding and assembly, in ER calcium binding, and in targeting irreversibly misfolded proteins for degradation. In addition, it is the master regulator of the pro-survival "yin" module of the ER stress response by virtue of its ability to control the activity of the three signaling pathways linked to PERK, IRE1, and ATF6 (6,8) (Figure 2). On the flipside, CHOP represents a critical executor of the pro-apoptotic "yang" arm of the ER stress response (19,20). The increased activity of this transcription factor suppresses anti-apoptotic Bcl-2, stimulates death receptor 5 (DR5) expression, activates caspases, and triggers mitochondrial events that function to integrate and amplify the death pathway (see detailed refs. in (20)).

In essence, the ER stress response can be viewed as a balance of interdependent "yin-yang" modules, where elevated levels of GRP78 attempt to restore ER homeostasis and thus are cell protective, whereas unrestrictedly high levels of CHOP may gain dominance and tip the balance towards apoptosis in those cases where stress is too severe and cannot be resolved (21) (Figure 1). Altogether, this system musters substantial protective efforts in order to support cellular survival, y*et al*so ensures controlled destruction of the cell when excessive cellular damage threatens the organism as a whole.

More recently, autophagy has been recognized as an important player in the life-and-death decisions of the ER stress response (22-24). This particular mechanism helps cells endure periods of low nutrient supply and some other detrimental conditions, and appears to function primarily by generating energy via the breakdown of the cell's own components (25,26). Several recent reports have shown that ER stress can stimulate autophagy, and reciprocally, that blocking autophagy can aggravate ER stress (10,22,27-29).

Similar to ER stress, the process of autophagy appears to follow a yin-yang principle as well (30,31). On one hand, autophagy is cell protective and provides energy via the recycling of cellular components under starvation conditions (26,32); as well, it prevents the accumulation of potentially cytotoxic aggresomes, which otherwise cannot be removed via ERAD (33-35). On the other hand, however, excessive autophagy may proceed to the point of complete cellular depletion and self-destruction. Initially, these dual functions have generated some confusion as to whether autophagy may represent a cell survival or a cell death mechanism, and it is not yet entirely clear how to exploit this process for therapeutic benefit. However, due to autophagy's interrelated connection to ER stress, it appears that simultaneously targeting both, autophagy and ER stress, may hold promise for enhanced therapeutic outcomes (see below).

4. ER STRESS AS AN ACHILLES' HEEL OF CANCER

Under regular *in vivo* conditions, most normal cells generally do not experience ER stress and therefore express only very limited amounts of GRP78, if any, and negligible levels of CHOP (Figure 3A). Similarly, when put into culture *in vitro*, such cells require intentional exposure to ER stress-inducing conditions, such as experimental hypoglycemia or pharmacological agents like thapsigargin or tunicamycin, in order to trigger GRP78 and CHOP expression. The length and severity of exposure determines the magnitude of CHOP induction, which is decisive for the struggle between the yin-yang modules and the decision whether CHOP-controlled events dominate and apoptosis will take place (36). In fact, because of their relatively short-lived attempt for control, CHOP expression levels can be used as a convenient readout to reveal the acute phase of ER stress (20,36).

As prolonged exposure of cells to elevated CHOP levels results in cytotoxicity (36), one of the pro-survival functions of GRP78 is to subdue CHOP transcription, which is achieved via GRP78's binding to and inactivation of the ER transmembrane signaling components PERK, IRE1, and ATF6 (3,37). However, during conditions of prolonged and excessive stress, GRP78 remains bound to and occupied with the repair of misfolded proteins in the lumen of the ER, and therefore stays dissociated from those transmembrane proteins that continue to stimulate CHOP expression (Figure 2B); as a consequence, CHOP expression remains high under these conditions and cell death ensues.

In contrast to normal cells, most cancer cells display signs of chronically elevated baseline ER stress levels, as indicated by permanently increased expression of the yin component GRP78 (38) (Figure 3B). Overexpression of this protein enables tumor cell growth and survival within sub-optimal microenvironments of hypoglycemia, acidity, or hypoxia, and also supports the increased cellular demands on protein folding due to revved up protein synthesis. For instance, the unrestricted growth of tumors may expose cells at the frontline of expansion to regions with insufficient blood supply and therefore low oxygen and glucose availability (39). The latter condition is further exacerbated by the general metabolic phenotype of tumor cells that shifts the emphasis of sugar breakdown from oxidative phosphorylation to aerobic glycolysis (Warburg effect), necessitating the need for further increased sugar consumption, possibly resulting in local hypoglycemia and acidosis (40) and representing the classical trigger for the expression of GRP78 and related proteins.

The protective yin function of GRP78 also provides for the suppression of pro-apoptotic pathways, as exemplified above for the restraint of pro-apoptotic CHOP. As a consequence, many tumor cells display increased resistance towards various forms of chemotherapy, and not

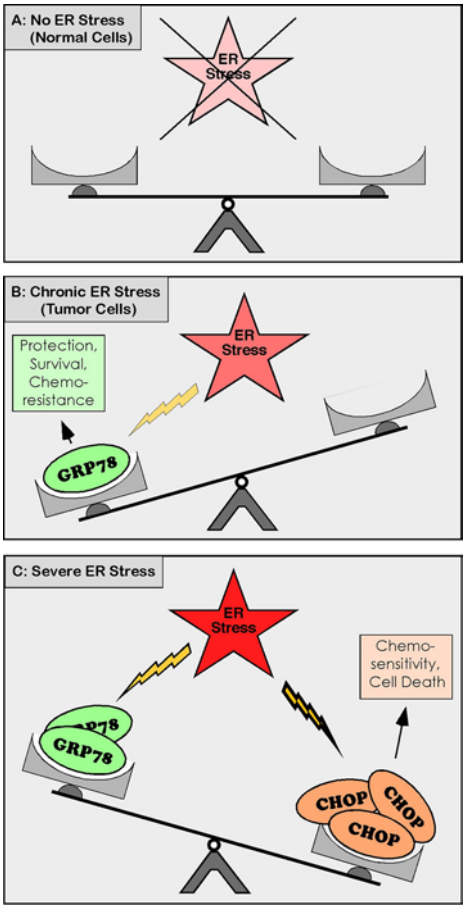

Figure 3. Differential intensity of ER stress levels. (A) This panel depicts the absence of ER stress, which represents the situation in most normal cells under normal physiologic conditions. (B) Most tumor cells display elevated levels of GRP78 (but not CHOP), which indicates low-level, chronic activation of the protective component of the ER stress response system that is supportive of cellular survival and chemoresistance. (C) Severe stress results in greatly increased CHOP expression, which dominates the ER stress response and triggers cell death, despite continued protective efforts of GRP78. See text for further details.

coincidentally this situation may explain the poor prognosis of cancer patients presenting with tumors that stain strongly positive for GRP78 (41-44). However, while the chronic ER stress condition appears to provide a definitive advantage to tumor cells, this differential to normal cells may at the same time present an opportunity for therapeutic intervention specifically aimed at this stress system.

Permanently elevated levels of GRP78 in tumor cells reveal low-level, chronic activation of the ER stress response, which is required as an adaptive defense strategy of these cells (7,38,45). This "low/chronic ER stress" condition (Figure 3B) sets most tumor cells apart from normal cells, which generally display a "no ER stress" condition (Figure 3A). Thus, the presence of chronic ER stress may constitute an Achilles' heel specifically found in tumor cells, i.e., these differential baseline conditions may provide a therapeutic target for pharmacologic intervention. Recent examples in the literature (see below) indicate that controlled pharmacologic aggravation of pre-existing ER stress in tumor cells can "overload" this already engaged system, i.e., it will overwhelm and incapacitate the protective components and will activate the pro-apoptotic module (i.e., CHOP), which then gains dominance and initiates cell death (Figure 3C). In comparison, normal cells are expected to be relatively protected, because their ER stress system harbors greater reserves to accommodate the increased stress levels; here, defensive components will dominate and will resist stress-induced toxicity.

In essence, because the defensive yin module of ER stress already is engaged to combat and neutralize chronic stress, a smaller margin is left for tumor cells to accommodate additional ER stress; consequently, treatment of such cells with drugs that are able to specifically trigger further ER stress would be expected to result in two desirable anticancer outcomes: (i) such drugs by themselves might result in increased antitumor effects, and (ii) the overload and subsequent breakdown of the ER stress defense system might increase the tumor cells' sensitivity towards conventional chemotherapeutic agents. Examples to illustrate the reality of both scenarios will be presented below.

In summary, the tumor-specific therapeutic exploitation of the ER stress response would entail the targeted aggravation of the pre-existing ER stress condition in tumor cells, i.e., a shift from "low/chronic ER stress" (Figure 3B) to "severe ER stress" conditions (Figure 3C), which would establish dominance of the pro-apoptotic yang module and resultant cell death. At the same time, normal cells would initiate their ER stress response from its inactive state (Figure 3A), and therefore enjoy more leeway to unfold the protective yin components.

The veracity of this model has been indicated by *in vivo* studies. For example, after treatment of tumor-bearing animals with drugs that specifically trigger ER stress, the key marker of the pro-apoptotic ER stress mode (i.e., CHOP) can be detected in tumor tissues of these animals, but not in their normal tissues (46,47). Concurrently, increased CHOP levels are closely aligned with more widespread apoptosis in tumor tissues and overall reduced tumor growth. Analyzing this relationship *in vitro* revealed that knockdown of CHOP greatly reduced drug toxicities in tumor cells, verifying that this pro-apoptotic ER stress protein indeed is central to mediating the antitumor effects of ER stress-targeted agents (48-51).

Notably, in order to maintain the tumor-selective cytotoxic outcome of this strategy, a moderate-intensity approach should be applied, which would sufficiently aggravate ER stress in tumor cells, but at the same time, would only modestly trigger ER stress in normal cells. Therefore, exceptionally potent pharmacologic triggers of ER stress might not be ideal for this type of therapeutic intervention; rather, those compounds with only moderate potency might display superior therapeutic efficacy. The tumor-specific aggravation of ER stress by such

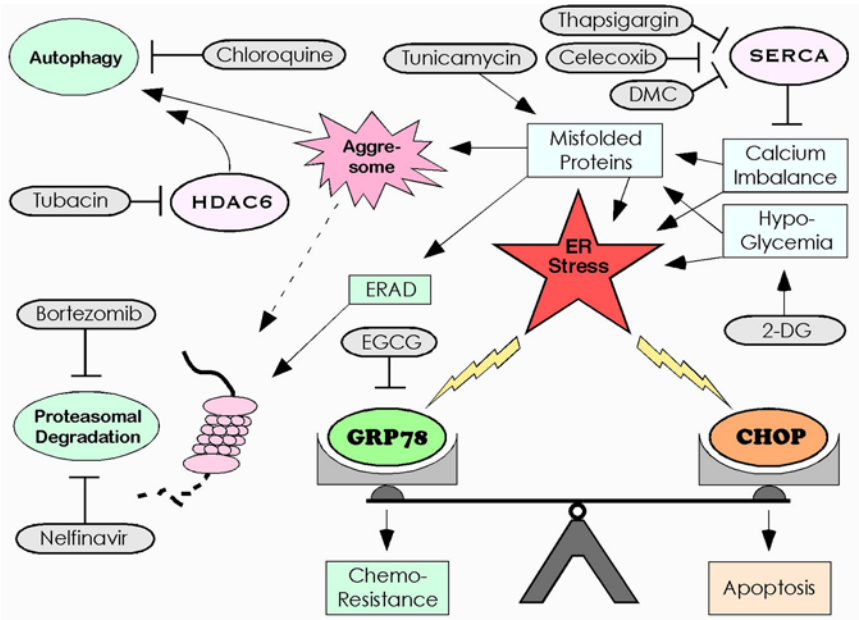

Figure 4. Scheme of proposed interactions between ER stress and associated protein disposal mechanisms, and specific targets for pharmacological intervention. See text for details. 2-DG: 2-deoxyglucose; DMC: 2,5-dimethyl-celecoxib; EGCG: epigallocatechin gallate; ERAD: ER-associated degradation; HDAC6: histone deacetylase 6; SERCA: sarcoplasmic/endoplasmic reticulum calcium ATPase.

compounds could conceivably be further enhanced via the combined application of agents that aggravate ER stress by different mechanisms, and via simultaneous inclusion of drugs that might act via the suppression of overly active defensive yin components, such as GRP78. Indeed, an increasing number of studies indicate the feasibility of this strategy, and representative examples will be presented below.

5. PHARMACOLOGICAL TARGETING OF ER STRESS

ER stress can be triggered by diverse mechanisms, and a variety of distinct pharmacologic agents have been characterized as being able to cause ER stress (representative examples are shown in Figure 4 and presented below). Some of these compounds are known to exert additional biological activities, which must be taken into consideration when cancer therapeutic applications are being considered.

5.1. Thapsigargin and tunicamycin

Thapsigargin and tunicamycin represent classical inducers of ER stress (Figure 4), and they have been used extensively to study this process in the laboratory for the past two decades. Thapsigargin acts via potent inhibition of an ER transmembrane calcium pump, the sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), which maintains the steep calcium gradient between the cytosol and the ER (52). Inhibition of SERCA leads to massive leakage of calcium out of its ER storage compartment and represents a powerful trigger for ER stress (53). Tunicamycin is a nucleoside antibiotic that inhibits N-linked glycosylation and blocks the formation of N-glycosidic protein-carbohydrate linkages (12). As glycosylation constitutes a critical step to ensure proper folding of many proteins, its blockage by tunicamycin leads to the accumulation of unfolded/misfolded proteins, resulting in ER stress; as well, the antibiotic prevents the general synthesis of all N-linked glycoproteins.

Both thapsigargin and tunicamycin, besides serving as valuable tools to study ER stress mechanisms in the laboratory, are being investigated for their potential cancer therapeutic potential. The development of thapsigargin as a potential anticancer agent faces several challenges, in particular since it has been classified as a potent tumor promoter and overall is not well tolerated by experimental animals (54). In addition, it stimulates arachidonic acid metabolism and, independently, causes histamine release (55). While these characteristics of thapsigargin represent prohibitive drawbacks in the context of systemic chemotherapy, its exceptionally potent cytotoxicity could be exploited in alternative approaches that may be based on tumor-targeting mechanisms. For instance, a pro-drug version of thapsigargin that is specifically activated by tumor cells has shown promising antitumor efficacy in preclinical animal models (56,57).

Tunicamycin displays a broad toxicity profile, which also limits its suitability for systemic cancer therapeutic approaches. Nonetheless, in the laboratory it has shown promising results, in particular as a chemosensitizing agent. For instance, tunicamycin was able to restore cisplatin sensitivity of a cisplatin-resistant head-and-neck carcinoma cell line *in vitro* and enhanced the antitumor effects of cisplatin in a mouse model of squamous-cell carcinoma (58). However, in a later study

(59), this same combination resulted in antagonistic effects on cell death in several cancer cell lines *in vitro*. The reasons for this discrepancy are unclear, although it is noted that different cell types, different concentrations of tunicamycin, and different pre-incubation times were used in these two studies: synergistic outcome in the first study was achieved by 24 hours of pre-incubation with tunicamycin concentrations up to 0.5 µg/mL, whereas the second study applied 1.25 µg/mL for only 8 hours of pre-incubation in all experiments. Unfortunately, the 2009 report did not refer to the closely related 1999 study, and therefore sensible comparisons are difficult. Otherwise, variable profiles of drug efflux transporters may also play a role in differential outcomes of such drug combination experiments (60,61). As well, tunicamycin-induced effects on partner drugs may depend on the particular mechanism of partner drug function: for example, in side-by-side cytotoxicity assays, tunicamycin antagonized the topoisomerase I and II inhibitors camptothecin and etoposide, respectively, but did not reveal such effects on microtubule-targeting drugs paclitaxel or vincristine (62).

In other studies, tunicamycin has been shown to sensitize various tumor cell lines to apoptosis induced by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), which involved the transcriptional activation of death receptor 5 (DR5; also called TRAIL receptor 2, TRAIL-R2) by ER stress-induced CHOP (63,64). In a related study, sensitization towards TRAIL was shown to also involve inhibition of the cell cycle regulator cyclin D (65), and it is interesting to note that cyclin D downregulation represents a well-established consequence of ER stress (66,67). Moreover, besides acting through its immediate ER stress-inducing effects, tunicamycin may also affect tumor cells via its ability to block protein glycosylation; for instance, the compound was shown to prevent N-glycosylation of epidermal growth factor receptor (EGFR), and this facet, combined with ER stress, appeared to further sensitize EGFR-overexpressing non-small cell lung cancer (NSCLC) cells to killing by the small-molecule EGFR inhibitor erlotinib (Tarceva®) (68).

5.2. Proteasome and protease inhibitors

The specific turnover, removal, and destruction of surplus and damaged proteins are critical for proper cellular functioning, and this task is controlled by the 26S proteasome (Figure 4). Inhibition of this process is thought to block the final step of ERAD and thus cause an accumulation of misfolded and other superfluous protein, which represents a trigger for ER stress (69-74). As a compensatory mechanism, autophagic clearance is increased (74-76), although it seems that autophagy is unable to fully compensate for complete elimination of proteasome activity, and as a result ER stress-induced apoptosis ensues.

The first proteasome inhibitor to reach clinical use was bortezomib (PS-341; Velcade®), which has been approved for the treatment of multiple myeloma (MM) and mantle cell lymphoma (77,78). Due to its antibody-secreting phenotype, which places high demands on a well-functioning ER, MM appears to represent a particularly sensitive tumor type for ER-targeted therapy. Indeed, the rate of antibody production and proteasome load has been closely correlated with these cells' response to killing by bortezomib (71,73,79), and this observation fits well with the above presented model that tumor cells are more sensitive to the aggravation of ER stress because their baseline ER stress system is less capable to accommodate additional insults.

Treatment of MM, as well as cells of other tumor types, with bortezomib *in vitro* and in mouse models *in vivo* was shown to trigger ER stress, as indicated by increased expression of GRP78, CHOP, and other markers (69,72,73,80). In addition, other mechanisms besides ER stress have been presented to explain bortezomib's cytotoxicity. For example, proteasome inhibition by bortezomib induces caspase-mediated apoptosis via the intrinsic mitochondrial pathway, as well as via the extrinsic death receptor-initiated pathway (81,82). However, in this context it is interesting to note that ER stress has been shown to activate both of these pathways as well. For example, the master regulator of the pro-apoptotic ER stress response module, CHOP, has been shown to transcriptionally activate the expression of death receptor 5, leading to increased cellular sensitivity to TRAIL and caspase 8 activation (20,83,84). As well, CHOP down-regulates anti-apoptotic Bcl-2 and favors activation of mitochondrially controlled apoptosis (20,85-87). Thus, altogether, it is conceivable that activation of these intrinsic and extrinsic pathways by bortezomib may be orchestrated secondary to the aggravation of ER stress.

Noteworthy as well is the proposition early on of a critical role for nuclear factor (NF)-kappaB in mediating the cytotoxic outcome of bortezomib (88). It was suggested that proteasome inhibition by bortezomib may prevent the degradation of IkappaB, an inhibitor of NF-kappaB, and thus may block NF-kappaB function, which appears to be required for MM survival (89). However, the balance of a large number of important regulatory proteins is affected as a result of proteasome inhibition, and it became debatable whether the antitumor effect of bortezomib should be ascribed to its impact on a single protein (90). Here as well, several studies have indicated a link between ER stress and NF-kappaB, which seems to indicate that bortezomib's effect on NF-kappaB might be a consequence of ER stress (73,91,92).

A different class of proteasome inhibitors is represented by drugs that initially were developed as inhibitors of human immunodeficiency virus (HIV) protease. These compounds, such as nelfinavir (Viracept®) and atazanavir (Reyataz®), are widely prescribed antivirals and currently are under investigation for potential repositioning as anticancer agents. Due to their protease inhibitory activity, they also block proteasome function and elicit pro-apoptotic ER stress responses similar to bortezomib (Figure 4), including the accumulation of polyubiquitinated proteins and aggresome formation, and increased expression of ER stress response markers GRP78 and CHOP (70,74,93,94). The cancer therapeutic potential of nelfinavir has been established in mouse models of

glioblastoma and prostate cancer (70,74), and several clinical trials are currently ongoing to verify its benefit in monotherapy fashion or as sensitizer for conventional chemotherapeutic agents and radiation therapy.

5.3. Celecoxib and its analogs

Celecoxib (Celebrex®) had been developed as a selective inhibitor of cyclooxygenase-2 (COX-2) and, besides its medical use for inflammatory conditions and pain, has been approved as an adjunct for the therapy of familial adenomatous polyposis (FAP) (95). However, over the years additional pharmacological activities and targets of this drug emerged (96-98). For instance, it was discovered that celecoxib is able to inhibit certain members of the carbonic anhydrase family of enzymes more potently than it inhibits its original target, COX-2 (99,100). Yet another target of celecoxib, and possibly the most relevant with regards to the potential treatment of advanced types of cancers, is the transmembrane ER calcium pump SERCA (Figure 4). Inhibition of SERCA by celecoxib and the resulting increase of cytosolic calcium levels was first reported by Johnson *et al.* (101). As such drastic alterations in calcium homeostasis are well known triggers of ER stress, it was not surprising that subsequent studies clearly demonstrated activation of the ER stress response (e.g., induction of GRP78 and CHOP) by celecoxib *in vitro* and in animal tumor models *in vivo* (see detailed refs. in (97)).

The above cited studies, and a large number of related ones, added fuel to the long-ranging and at times controversial debate as to the relevance of celecoxib's COX-2 independent functions for its anticancer effects. In short, it appears that COX-2 inhibition is critically important for celecoxib's well-established chemopreventive properties in the case of colorectal cancer; however, with regards to its potentially therapeutic effects on already established and advanced cancers, it seems that COX-2 inhibition may be negligible and other pharmacological activities may be more relevant (96,97).

Additional insight into the dualism between COX-2 dependent versus COX-2 independent effects of celecoxib was provided by structure-function analysis of closely related analogs of this compound, where specific biological properties were either enhanced or minimized (102,103). For example, the analog 2,5-dimethyl-celecoxib (DMC) has lost COX-2 inhibitory function, yet maintains the ability to inhibit SERCA (Figure 4) and severely aggravates ER stress (104,105). Conversely, unmethylated-celecoxib (UMC) exerts even more potent COX-2 inhibitory function than the parental celecoxib molecule itself, yet this compound triggers ER stress only marginally (106,107). When compared side by side *in vitro*, the cytotoxic potency of these compounds was DMC>celecoxib>UMC, which was congruent with their ability to trigger ER stress, but did not at all relate to their COX-2 inhibitory potency (47,80,106,107). Beyond mere correlation, a cause-and-effect relationship between drug-induced ER stress and cytotoxic outcome was established via knockdown experiments: blocking GRP78 expression with siRNA approaches led to increased tumor cell killing by celecoxib and DMC, whereas reduction of CHOP expression protected cells from the cytotoxic activity of these compounds (47,48,80,108,109).

The ability of celecoxib and, even more so DMC, to aggravate ER stress and enhance tumor cell death was also verified in mouse tumor models, as indicated by increased GRP78 and CHOP immunoreactivity, in parallel with elevated TUNEL positivity revealing extensive cell death in tumor tissues (47,80,108). Intriguingly, ER stress-mediated antitumor effects of DMC are not restricted to tumor cells, but also appear to involve cells of the tumor vasculature. In this regard, it was demonstrated that DMC triggered pro-apoptotic ER stress specifically in endothelial cells derived from human brain tumor (glioblastoma) specimens, but had no such effect on endothelial cells isolated from normal brain (110). Earlier studies had shown that glioblastoma-derived endothelial cells display signs of chronic ER stress, as indicated by continuously elevated levels of GRP78 (111), which appears to provide protection from conventional chemotherapy such as temozolomide, the current standard of care for patients with glioblastoma (112). The finding that such chemoresistant cells are sensitive to killing by DMC (110), an ER stress-targeting agent, provides additional support for the above stated idea that pre-existing ER stress might be an Achilles' heel—not only of tumor cells but also of tumor-associated endothelial cells—and may be exploitable by agents that specifically aggravate such pre-existing ER stress conditions.

5.4. Other ER stress aggravators

In view of the great variety of impacts that are able to trigger ER stress, it is not surprising that there are numerous approaches to manipulate ER stress experimentally with the ultimate goal to exploit this cellular system for cancer therapy. In addition to the above detailed methods, several others are at various stages of preclinical development. A few select examples will be presented here.

Among the various histone deacetylases (HDACs), HDAC6 in particular has been shown to play a role in the regulation of ER stress (2). This particular enzyme is critical for the recruitment of irreparably misfolded proteins into the aggresome, and cells deficient in this function cannot form aggresomes properly and become hypersensitive to misfolded proteins (113,114). Inhibition of HDAC6 by specific inhibitors, such as the small-molecule inhibitors tubacin or LBH589 (115,116), is thought to block aggresome assembly and result in increased cellular loads of unwanted proteins, creating a backlog and thus aggravating ER stress (Figure 4).

Besides GRP78, several other proteins perform chaperone function and thus participate in the ER stress response. As such, they too are potential targets for pharmacological intervention. The best-studied example is cytosolic heat shock protein 90 (HSP90), which binds to a large number of client proteins and thereby influences a variety of intracellular processes, and its ER homologue glucose regulated protein 94 (GRP94) (117). Both of these proteins are targets of the natural product geldanamycin and its modified derivative, 17-allylamino-17-demethoxygeldanamycin (17AAG) (118,119). A large

number of preclinical studies have established the anticancer properties of these compounds in a broad variety of cancers, and several clinical trials are exploring the efficacy of 17AAG and several other novel HSP90 inhibitors in different types of tumors (see reviews (120-122)).

Although the induction of ER stress by geldanamycin and 17AAG has been well documented (123-126), the extent of contribution of these pathways to the antitumor outcomes of HSP90 inhibitors has not been established. Rather, in view of the large number of cellular proteins known to interact with HSP90, it is quite likely that other cellular processes may be as important, or even more important, than ER stress-regulated mechanisms. Quite fittingly, HSP90 has been considered a "superchaperone" complex (127,128), as it is part of a large composite that interacts with a variety of client proteins involved in cell-specific oncogenic processes.

Autophagy is closely interconnected to ER stress (31,129-131), and manipulation of this process may feed back on ER stress as well. For example, chloroquine, the traditional antimalarial drug, has been widely used to block autophagy, and this inhibition is believed to lead to the accumulation of aggresomes, which triggers ER stress (Figure 4). In keeping with the general model that aggravated ER stress may overwhelm the protective features of the ER stress response system, chloroquine has been demonstrated to augment the chemosensitivity of tumor cells (45,132,133). Moreover, there are promising results from clinical trials with glioblastoma patients, where this compound has displayed chemosensitizing effects when used as an adjuvant to the standard glioblastoma chemotherapeutic agent temozolomide (134).

5.5. Inhibitors of GRP78

Based on the yin-yang principle of the ER stress response, manipulation of these pathways for therapeutic purposes may consist of the enhancement of the pro-apoptotic yang module (e.g., prolonged CHOP expression), or conversely on the suppression of the pro-survival yin components, in particular GRP78. In this regard, means to block GRP78 function are therapeutically attractive and are being pursued by different types of approaches, including anti-sense and siRNA-mediated knockdown of gene expression (38) and pharmacological targeting. In view of GRP78's well-established function to suppress apoptosis and provide for chemoresistance (42,44,111,135,136), blockage of this tumor cell-protective protein is of particular interest.

5.5.1. Genistein

Several naturally occurring compounds have been found to inhibit GRP78 expression or activity. For example, the isoflavone and soy ingredient genistein was shown to block the binding of a specific transcription factor to the promoter region of the GRP78 gene, thereby preventing induced GRP78 transcription in response to ER stress (137-139). This result suggested that the known anticancer effects of genistein might be related to its ability to reduce the expression of this pro-survival ER stress regulator. In contrast, two other studies using different experimental systems demonstrated that treatment with genistein caused a time- and dose-dependent increase in GRP78 expression in different human carcinoma cell lines (140,141). In these latter cases, pro-apoptotic CHOP was greatly increased as well, and the overall outcome displayed significantly reduced tumor cell survival, despite the increased amounts GRP78. The *in vivo* relevance of some of these *in vitro* results is unclear, as very high concentrations (up to 100 μM) of genistein are sometimes used, whereas in comparison, blood concentrations reported in humans are in the range of 0.5 to 5 μM (142). It is therefore unlikely that dietary isoflavone consumption will result in plasma concentrations of genistein that are necessary to achieve the antiproliferative or pro-apoptotic outcomes generally reported from studies *in vitro*, although more long-lived and stable synthetic analogs and conjugates may reveal improved *in vivo* efficacy (143).

Overall, the cellular effects of genistein are complex and also involve components other than the ER stress response. For example, the compound has been recognized to act as a general inhibitor of tyrosine kinases, to block topoisomerase II function, and to downregulate the activity of matrix metalloproteinase 9 (MMP9) (144). In addition, it is structurally similar to 17beta-estradiol and thus exerts antiestrogenic effects in cells that are positive for estrogen receptor (145). Altogether, it might not be possible to ascribe the anticancer effects of this isoflavone to just one individual target protein, but rather to a drug-induced multifactorial process where different targets combine to achieve therapeutic benefit.

5.5.2. EGCG

Similar multi-target considerations as above also apply to another GRP78 inhibitor, the major polyphenolic green tea component (–)-epigallocatechin-3-gallate (EGCG), which is being investigated intensely as a possible adjunct to current cancer therapeutic regimens. Among its many recognized molecular effects is its ability to bind to and inhibit the ATPase activity of GRP78 (146), which may provide a reasonable explanation for green tea's noted ability to sensitize tumor cells to chemotherapeutic treatment (44,146,147) (Figure 4). However, numerous other biological effects and cellular targets of EGCG have been recognized (148). For example, EGCG has also been found to inhibit the function of HSP90 (149), to block proteasome activity (150), and to bind to the tumor metastasis-associated cell surface laminin receptor (151), to name but a few. These multifaceted properties greatly complicate the attempts to unequivocally link EGCG effects to ER stress, and for this reason additional studies are needed to fully characterize the role of the EGCG-GRP78 interaction for potential chemosensitizing applications.

5.5.3. Microbial metabolites

Several other natural products, most of them microbial metabolites, have been found to interfere with GRP78 expression or function, although many of them have not been well characterized. To identify inhibitors of GRP78 expression, several groups used a reporter system

where the gene for luciferase was cloned downstream of the GRP78 promoter. Cells transfected with this GRP78-luciferase construct were exposed to ER stress-inducing conditions, such as low glucose concentrations or to the glycolytic inhibitor 2-DG, either of which triggered ER stress and consequently increased expression of luciferase. This system was then used to screen for novel compounds able to block hypoglycemia-induced luciferase expression, i.e., GRP78 promoter activity in response to ER stress. This type of approach led to the discovery of versipelostatin (152) and some of its more potent glycosylated derivatives (153), prunustatin A (154), efrapeptin J (155), verrucosidin (156), deoxyverrucosidin (157), piericidin A (158), as well as the plant product artigenin (159) and the cyanine dye pyrvinium (160). Several of these agents were shown to be non-toxic when added to regular euglycemic medium, but caused massive cell death under hypoglycemic conditions, which was ascribed to the lack of protection when induction of GRP78 was blocked under conditions of metabolic stress.

5.5.4. Biguanides

Intriguingly, preferential cytotoxicity under conditions of lowered glucose, in combination with prevention of GRP78 increase, was also demonstrated for the widely prescribed anti-diabetic drug metformin and other members of the biguanide class, such as phenformin and buformin (161). This outcome is remarkable in view of epidemiological studies showing a decrease in cancer incidence in metformin-treated patients (162). As with many other compounds, several additional biological functions of metformin have been described, and it has been suggested that its proposed anticancer effects may be the product of its combined individual activities targeted at cancer cell metabolism (163).

5.5.5. Subtilase Cytotoxin

A very different mechanism of GRP78 inhibition is displayed by the bacterial AB5 subtilase cytotoxin, a member of the AB5 toxins that are important virulence factors for several major bacterial pathogens, such as *Bordetella pertussis*, *Vibrio cholerae*, *Shigella dysenteriae*, and certain pathotypes of *Escherichia coli* (164). Subtilase toxin consists of a catalytic A subunit (SubA) and five B subunits, where SubA harbors protease function that is able to specifically cleave GRP78 at a di-leucine motif (position 417 and 418 in mouse GRP78) (165). Intriguingly, the resulting shorter protein is able to preferentially sequester newly synthesized light chains in activated B cells, resulting in the blockade of antibody secretion and thus providing immune evasion and survival advantage to toxin-producing bacteria (166). In order to evaluate the cancer therapeutic potential of this remarkably selective cleavage of GRP78, SubA was fused to epidermal growth factor (EGF) in order to target GRP78 in tumor cells overexpressing EGF receptor (EGFR). Amazingly, the engineered EGF-SubA fusion protein proved cytotoxic to different EGFR-positive cancer cell lines at low picomolar concentrations *in vitro*, and significantly inhibited tumor growth in xenograft mouse tumor models *in vivo* (167).

As no other intracellular targets besides GRP78 are known to be proteolytically cleaved by SubA, it was somewhat surprising that EGF-SubA by itself was highly effective at inducing tumor cell death, as it suggested that GRP78 might be essential for tumor cell viability (rather than "merely" for cytoprotection). In contrast, other studies showed that the knockdown of GRP78 by antisense or RNA interference methods generally was not cytotoxic to tumor cells (44,111,131,135,168), although cell type specific responses are possible (37). However, it is conceivable that commonly used knockdown methods less effectively remove GRP78 as compared to EGF-SubA, and that small amounts of residual GRP78 suffice for cell survival. In any case, in keeping with the above presented yin-yang model of ER stress, treatment with EGF-SubA was also shown to greatly enhance tumor cell killing by the ER stressor thapsigargin *in vitro* (167).

5.5.6. Extra-ER GRP78

The evaluation and characterization of GRP78 inhibitors as specific modulators of the ER stress response system has been impeded by other biological effects that are exerted by many of these agents, which makes it difficult to ascribe ER stress as the main target mediating their potential anticancer activity. Further complicating this issue are new findings describing novel GRP78 functions outside of the ER stress response system. For example, besides its traditional ER luminal location, this protein has also been detected in the cytosol (169), in the nucleus (170), in mitochondria (171), and at the cell surface in particular in tumor cells (172-176). Although the physiological function of cell surface GRP78 is still emerging, recent evidence has revealed its presence in cell surface complexes with specific proteins that play important roles in signal transduction and the regulation of cell growth (177,178). Thus, although increased ER stress can actively promote cell surface localization of GRP78 (175), it appears that the protein's location at the cell surface serves other processes than the control of ER stress. For this reason, the use of any or all of the above described inhibitors of this multifaceted protein is likely to affect these additional GRP78 functions as well, and thereby may impinge on tumor cell growth and survival, as well as chemosensitization, by means other than the immediate effects on the ER stress response system. However, very little insight is available in this regard.

5.6. Combinations of ER stressors

Some of the above-presented ER stress-aggravating agents have revealed promising anticancer activity in preclinical models. There are indications, however, that these outcomes can be further optimized when specifically selected compounds are mixed for combination treatments. The rationale for this approach is based on the assumption that agents that effect ER stress by different molecular mechanisms would create synergy when combined. As a result, lower drug concentrations would suffice to trigger pro-apoptotic ER stress in tumor cells, yet would keep systemic side effects at a minimum. Results from several studies appear to support this expectation and have provided evidence that certain combinations of ER stress-targeting drugs indeed are able to achieve desirable antitumor outcomes.

For example, combining proteasome inhibitors (such as bortezomib, NPI-0052, or MG132) with inhibitors of SERCA (such as thapsigargin, celecoxib, or the non-coxib celecoxib analog DMC) severely aggravated ER stress and generated greatly increased tumor cell death (46,72,179-181). Similarly, the combination of celecoxib or DMC with the proteasome inhibitor nelfinavir resulted in synergistically increased ER stress and concomitant tumor cell death, and this outcome could also be achieved in highly multidrug-resistant tumor cell variants (48). Other groups provided evidence that combining the proteasome inhibitor bortezomib with HDAC6 inhibitors resulted in synergistic antitumor activity *in vitro* and *in vivo* (116,182-186). Similarly, the combination of bortezomib with the HSP90 inhibitor geldanamycin, or with the classical ER trigger brefeldin A, superinduced the ER stress response and caused greatly enhanced antitumor activity as well (181,187).

Synergistic aggravation of ER stress and subsequently enhanced tumor cell death in response to the combination of two different pharmacological ER stressors could also be documented in animal tumor models (72,80,182,185). In these cases, ER stress and concomitant cell death was greatly increased in tumor tissue from drug-treated animals, but was absent in normal organs; as a result, inhibition of tumor growth could be accomplished without obvious toxicity to the drug-treated animals. Therefore, proof-of-principle of therapeutic efficacy of rationally selected dual drug combinations aimed at the ER stress response has been established in appropriate pre-clinical models.

Many more dual or triple combinations aimed at the ER stress response are possible, and it will be important to identify the most effective ones and subsequently establish their therapeutic efficacy in clinical trials. Obviously, not all combinations will reveal similar promise, and unexpected outcomes are possible. One such surprising result was recently published by Hoang *et al.* (188). These authors demonstrated that treatment of multiple myeloma cells individually with either the proteasome inhibitor bortezomib or the autophagy inhibitor chloroquine resulted in ER stress and subsequent cell death, as expected. Based on the rationale that autophagy represents an alternative survival mechanism in case of proteasome inhibition, the authors then combined both drugs, with the expectation that blocking both processes simultaneously should enhance the cytotoxic outcome of drug treatment. However, surprisingly, the addition of chloroquine resulted in an antagonistic effect, i.e., chloroquine reduced the extent of cell killing by bortezomib (188). Intriguingly, however, inhibition of autophagy did enhance the cytotoxic response to the SERCA inhibitor thapsigargin (188). Thus, these types of results indicate that each combination of pharmacological ER stressors needs to be carefully investigated in appropriate models, in order to identify and verify the most therapeutically useful ones.

6. PERSPECTIVE

Tumor-specific ER stress is being recognized as a potential target for cancer therapy. A number of pharmacological agents have been identified as aggravators of ER stress and triggers of the pro-apoptotic module of this cellular system. However, additional studies are required to identify those ER stress aggravators—and their combinations—that optimally effect antitumor outcomes without prohibitive toxicity and side effects. While some of these combinations may display promising anticancer effects on their own, additional efforts are needed to also define their potential sensitizing properties in support of conventional chemotherapies that do not target the ER stress response system. Because very many combinations are possible, with some of them perhaps displaying highly tumor type-specific efficacy, a lot more work lies ahead towards the optimized exploitation of chronic ER stress for cancer therapeutic purposes.

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