DangER: protein ovERload. Targeting protein degradation to treat myeloma

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ABSTRACT

Myeloma is a malignancy of the antibody-producing plasma cells and, as such, these cells synthesize large quantities of unfolded or misfolded immunoglobulin. The build-up of excess protein triggers a number of downstream signal transduction cascades, including endoplasmic reticulum stress and autophagy. As a result, myeloma cells are uniquely reliant on these and other protein handling pathways for their survival. Strategies aimed at targeting this vulnerability have proved successful with the proteasome inhibitor, bortezomib, already licensed for clinical use. In addition to the proteasome, various other points within the protein handling pathways are also the subject of drug discovery projects, with some already progressing into clinical trials. These include compounds directed against heat shock proteins, the unfolded protein response and pathways both upstream and downstream of the proteasome. More recently, the role of autophagy has been recognized in myeloma. In this review, we discuss the various pathways used by myeloma cells for survival, with particular emphasis on the emerging role and conundrum of autophagy, as well as highlighting pre-clinical research on novel inhibitors targeting protein handling pathways.

Key words: autophagy, proteasome, ER stress, heat-shock chaperones, unfolded protein response.

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Introduction

Myeloma is a genetically heterogeneous clonal B-cell malignancy, characterized by the accumulation of abnormal antibody-producing plasma cells in the bone marrow. It is the second most common hematologic malignancy and affects about 3,500 people in the UK each year. It tends to be a cancer of the elderly with a slightly increased incidence in men compared to woman, as well as having a higher incidence in African American populations and first degree relatives.[1]

Current treatment strategies include the use of immunomodulatory drugs such as thalidomide and its derivatives, proteasome inhibitors such as bortezomib, synthetic steroids like dexamethasone and prednisolone, alkylating agents and anthracycline, as well as autologous stem cell transplantation whenever possible. Despite a plethora of drugs currently used to treat myeloma, the disease remains incurable, with median survival ranging from three to six years depending on the stage of the disease.[2,3]

The introduction of targeted treatments such as thalidomide, bortezomib and lenalidomide has improved the median survival of myeloma patients. However, patients continue to relapse. This is in part due to the tumor cells developing resistance due to interactions with the bone marrow microenvironment.[2] There is, therefore, an urgent need for novel therapies to treat this disease and this will be achieved by developing a solid understanding of tumor biology.

Although myeloma is a complex malignancy, there are underlying common characteristics that are currently being exploited to treat this disease. As a result of the large quantities of unfolded or misfolded immunoglobulin that these cells produce, they are integrally reliant on their core protein handling machinery. This idea is exemplified by the success of the proteasome inhibitor, bortezomib, which was licensed by the Food and Drug Administration in 2003 for the treatment of refractory myeloma.[4] However, in addition to removal by the ubiquitin proteasome pathway, removal of excess proteins is also achieved by the autophagy pathway. This is a catabolic process whereby cells degrade portions of cytoplasm, including organelles. It has been shown to be an important part of normal development as well as playing a role in neurodegenerative diseases and cancer.[5] Inhibitors of the autophagy pathway are currently being tested in myeloma in two phase I/II trials in combination with bortezomib (*www.clinicaltrials.gov*) underscoring the importance of these protein handling pathways in treating this malignancy.

A third pathway for removal of excess proteins is the aggresome pathway which is activated if proteasome activity is compromised. Misfolded, ubiquitinated protein aggregates are recognized by histone deacetylase 6 (HDAC6) and transported by the microtubule network to aggresomes for clearance. Recent results demonstrate synergy between an HDAC6 inhibitor and bortezomib[6] and clinical trials testing this combination have begun. The role of aggresomes and HDACs has

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been extensively reviewed and so will not be covered further here.[7,8] We aim to provide an overview of the protein handling and stress response pathways. We highlight the potential points in these pathways that can be targeted therapeutically, and review the supporting pre-clinical data.

Protein handling pathways

Under normal conditions, the synthesis, folding and degradation of cellular proteins are balanced processes. In myeloma cells, however, the protein folding capacity of the endoplasmic reticulum (ER) is overloaded by large quantities of immunoglobulin and cells must adapt to this continual stress.[9] A close relationship with common signaling nodes therefore exists between the ubiquitin proteasome pathway, cellular stress pathways such as the unfolded protein response (UPR), heat shock response and autophagy.

Ubiquitin proteasome pathway

The main cellular pathway for the removal and destruction of proteins is the ubiquitin proteasome pathway. This involves the sequential enzymatic transfer of ubiquitin monomers onto an elongating polyubiquitin chain bound at the lysine 11 or lysine 48 residues of target proteins.[10] In the first step of this cascade, an E1 activating enzyme, typically ubiquitin activating enzyme (UAE, also known as UBA1), binds ubiquitin. A second ubiquitin monomer binds to a different site on the E1 enzyme and the first ubiquitin is transferred to an E2 ubiquitin-conjugating enzyme. The final step involves the transfer of ubiquitin from the E2 enzyme to the lysine 48 of the target protein in a process catalyzed by an E3 ubiquitin ligase. This process is then repeated to produce a polyubiquitin chain.[11] While there are only eight E1 or E1-like enzymes that have been described so far, there are around forty E2 enzymes and even more E3 enzymes.[12] Therefore, specificity of target protein degradation is achieved by the action of the E2 and E3 enzymes. Tagging proteins in this manner targets them for degradation via the proteasome; proteins that are monoubiquitinated or ubiquitinated at other residues will not be degraded by this process.

The 26S proteasome consists of a 20S catalytic core particle and either one or two 19S regulatory subunits. The 20S core particle contains the active site and is responsible for the hydrolysis of peptide bonds. On the other hand, the 19S particle is important for recognizing the substrate, deubiquitination, unfolding and translocation of the substrate into the catalytic core.[13] The 20S core contains four heptameric rings, the two alpha rings located at either end with the two beta rings in the middle. These rings are stacked above each other thus forming a hollow cylinder. The alpha rings act as gates that are opened upon binding of the 19S subunits whereas the beta rings contain the active sites. Of the seven subunits of the beta rings, only three are catalytically active: β1 has caspase-like activity, β2 exhibits trypsin-like activity and β5 has chymotrypsin-like activity.[14] Substrate proteins are degraded into peptides that vary in length from 3 to 25 amino acids. Each substrate is cleaved at multiple locations ensuring no partially hydrolyzed proteins exit the proteasome, only short amino acid chains. These amino acid chains are then further hydrolyzed by the aminopeptidase enzymes to release free amino acids.

Another form of the proteasome, the immunoproteasome, can also be found at elevated levels in myeloma cells.[15] The immunoproteasome is induced in response to interferon-gamma and contains three catalytically active β subunits together with an 11S regulatory subunit. The function of the immunoproteasome is to produce peptides for presentation to major histocompatability complex class I molecules.[16] These antigenic peptides are derived from defective ribosomal products which constitute a large percentage of all newly synthesized proteins.[17]

The multiple enzymatic steps involved in ubiquitination, 26S proteasome activity and immunoproteasome activity result in many sites within the UPP that can be therapeutically inhibited. Inhibition of this pathway results in a build up of polyubiquitinated proteins, ER stress and resulting cell death.[18,19] Importantly, targeting this pathway exploits the myeloma cells' critical dependence on the UPP, revealing a therapeutic window between the myeloma cells and normal cells. This therapeutic potential is discussed in more detail below.

ER stress and the unfolded protein response

ER stress results from an imbalance between the amount of unfolded or misfolded protein in the ER lumen and the capacity of the ER machinery to refold these proteins. Activation of the ensuing signal transduction pathway is termed the UPR, the main functions of which are to reduce the amount of protein that enters the ER and, secondly, to increase the folding capacity of the ER. If both of these strategies fail, apoptosis is triggered.[20] Additionally, if proteins cannot be folded correctly in the ER, they are retrotranslocated to the cytoplasm for degradation by the proteasome, a process termed ER-associated degradation (ERAD).[21] Thus, therapeutically manipulating this pathway can interfere with the cells' ability to deal with high protein loads, cellular stress and result in tumor cell death. Importantly, the ER stress pathway overlaps with protein removal via the proteasome, as co-administration of bortezomib with compounds that induce ER stress enhances myeloma cell apoptosis.[22]

The three arms of the UPR are regulated by an ER-resident transmembrane protein that detects unfolded proteins in the ER lumen and transmits the signal to the cytoplasm. These are inositol requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and activating transcription factor 6 (ATF6) which are held in an inactive state by glucose-regulated protein, 78kDa (GRP78/BiP). In the presence of unfolded proteins, GRP78 dissociates from these proteins allowing their activation.[23]

IRE1 is an unusual protein in that it contains both a kinase and endoribonuclease domain, the latter of which is important for the splicing of the transcription factor X-box binding protein 1 (XBP1). Spliced XBP1 regulates the expression of genes involved in ER expansion as well as those coding for ER chaperone proteins.[24] It can also activate Akt to mediate anti-apoptotic signals.[25] On the other hand, the kinase domain of IRE1 is capable of autophosphorylation, with subsequent downstream activation of c-Jun N-terminal kinase (JNK).[26] It is this activity of IRE1 that links it to other protein degradation pathways within the cell, such as the autophagy pathway, as JNK phosphorylates Bcl2, allowing the essential autophagy gene, *Beclin1*, to associate with phosphatidylinositol-3-kinase (PI3K) Class III to trigger autophagy (Figure 1).[27] The role of autophagy in protein degradation and ameliorating ER stress is discussed below.

The PERK branch of the UPR is important for the shut-

down of global protein translation. This is achieved by PERK phosphorylation of the α subunit of eukaryotic initiation factor 2 (eIF2α) and subsequent inhibition of the guanine nucleotide exchange factor eIF2B, blocking eIF2 activation.[28] Despite global inhibition of translation, there is selective translation of specific mRNAs; one such example is ATF4. Downstream of ATF4, CHOP activates GADD34 which acts via PP1 to dephosphorylate eIF2α, thus acting as a negative feedback loop.[29] Important links to the autophagy pathway are provided by ATF4 upregulation of the autophagy genes LC3B and Atg12[30,31] and CHOP upregulation of Atg5.[31] In addition, indirect links to autophagy are provided by inhibition of molecules in the PI3K Class I pathway that negatively regulate autophagy; ATF4 inhibits mammalian target of rapamycin (mTOR) via Redd1[32] and CHOP inhibits Akt via Tribbles Homolog 3 (Trb3) (Figure 1).[29]

ATF6 exists as 2 isoforms, α and β, but it is the α isoform that appears to be essential in the UPR.[33] Full activation requires a 2-step process involving transportation to the golgi followed by cleavage to reveal a nuclear localization signal.[34] In the nucleus, ATF6α transcriptionally regulates genes coding for ER chaperones and has been shown to be involved in ER expansion, enabling the ER to correct the stress and handle more protein.[33] Association with XBP1 regulates the induction of ERAD components.[29,35] ATF6 also plays a role in quiescence which is mediated by Akt-independent activation of Rheb and mTOR,[36] further linking the UPR and autophagy pathways.

Heat shock chaperone proteins

Heat shock proteins (HSP) are a group of ubiquitously expressed chaperone proteins that are up-regulated in times of stress and aid in efficient protein folding. They exist as part of multi-protein complexes together with co-chaperone proteins, such as HSP70 and HSP40. In addition to playing important roles in ensuring the correct folding of their 'client' proteins, they also prevent their aggregation.[37] A number of these 'client' proteins play vital roles in the survival of cancer cells and thus the HSP family has received a lot of attention as a potential drug target. The HSP90 family consists of the cytosolic HSP90α and HSP90β, the ER-resident Grp94 and the mitochondrial Trap-1, whereas the HSP70 family cytosolic proteins are HSP70 and heat shock

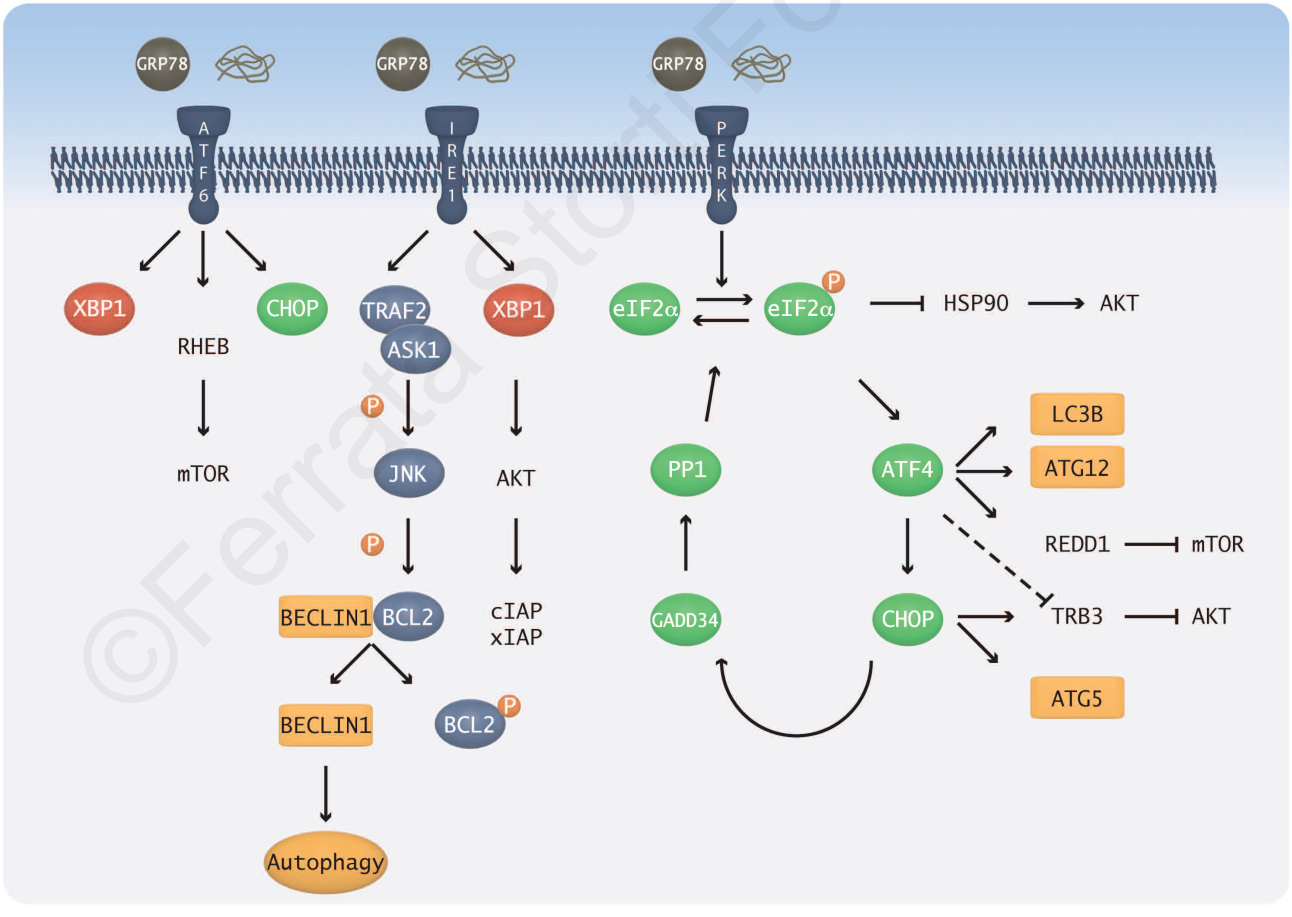

Figure 1. Relationship between the ER stress and autophagy pathways. The three ER stress sensor molecules, PERK, IRE1 and ATF6, are held in an inactive state by GRP78. The presence of unfolded protein results in their release and subsequent activation. The PERK and IRE1 branches are the most important for ER stress-induced autophagy: in the IRE1 branch, Bcl2 is phosphorylated by JNK, resulting in the release of Bcl2 from a complex with Beclin1 thus allowing autophagy to be activated. In the PERK branch, ATF4 and CHOP both lead to the upregulation of key autophagy genes. CHOP can also inhibit Akt via Trb3, thereby allowing autophagy activation.

cognate 70 (HSC70), the ER protein GRP78 and the mitochondrial protein mortalin.[38] The interest in HSP90 is based on the finding that its 'client' proteins include many oncogenic proteins, including receptor tyrosine kinases, cell cycle proteins and cell surface receptors such as FGFR3, Akt, TERT and p53.[39] In addition, the expression of HSP90 is regulated by pathways known to be abberantly activated in a variety of cancers. Despite a large number of client proteins regulated by HSP90 (*http://www.picard.ch/downloads/Hsp90interactors.pdf*), the specificity for each is guided by the association of HSP90 with numerous co-chaperones, such as Cdc37.[37]

The function of HSPs are integrally linked to the UPP such that proteins that cannot assume their biologically active conformation are targeted for degradation. We have also shown that inhibition of HSP90 induces the UPR[40] and HSP90 is able to stabilize IRE1.[41] This suggests that the chaperone systems that exist in the cytoplasm and ER are able to co-ordinate their response to alleviate ER stress, and when therapeutically targeting one pathway, there may be a paradoxical upregulation of another.

Autophagy

In addition to the UPP, the autophagy pathway also acts to remove excess protein from the cell and there seems to be a relationship between these two pathways. The last ten years has seen an explosion in the field of autophagy research and many of the genes, processes and signaling pathways have been elucidated. Three distinct types of autophagy have been recognized based on the manner in which cargo is delivered to lysosomes, but we will focus on macroautophagy, known more simply as autophagy. At basal levels, autophagy plays an important homeostatic role, maintaining a balance between the catabolism and synthesis of macromolecules and organelles.[42] It is up-regulated in response to conditions of stress, such as nutrient deprivation and the presence of aggregated proteins and hypoxia, ensuring cell survival.[5,42] In addition, it is also

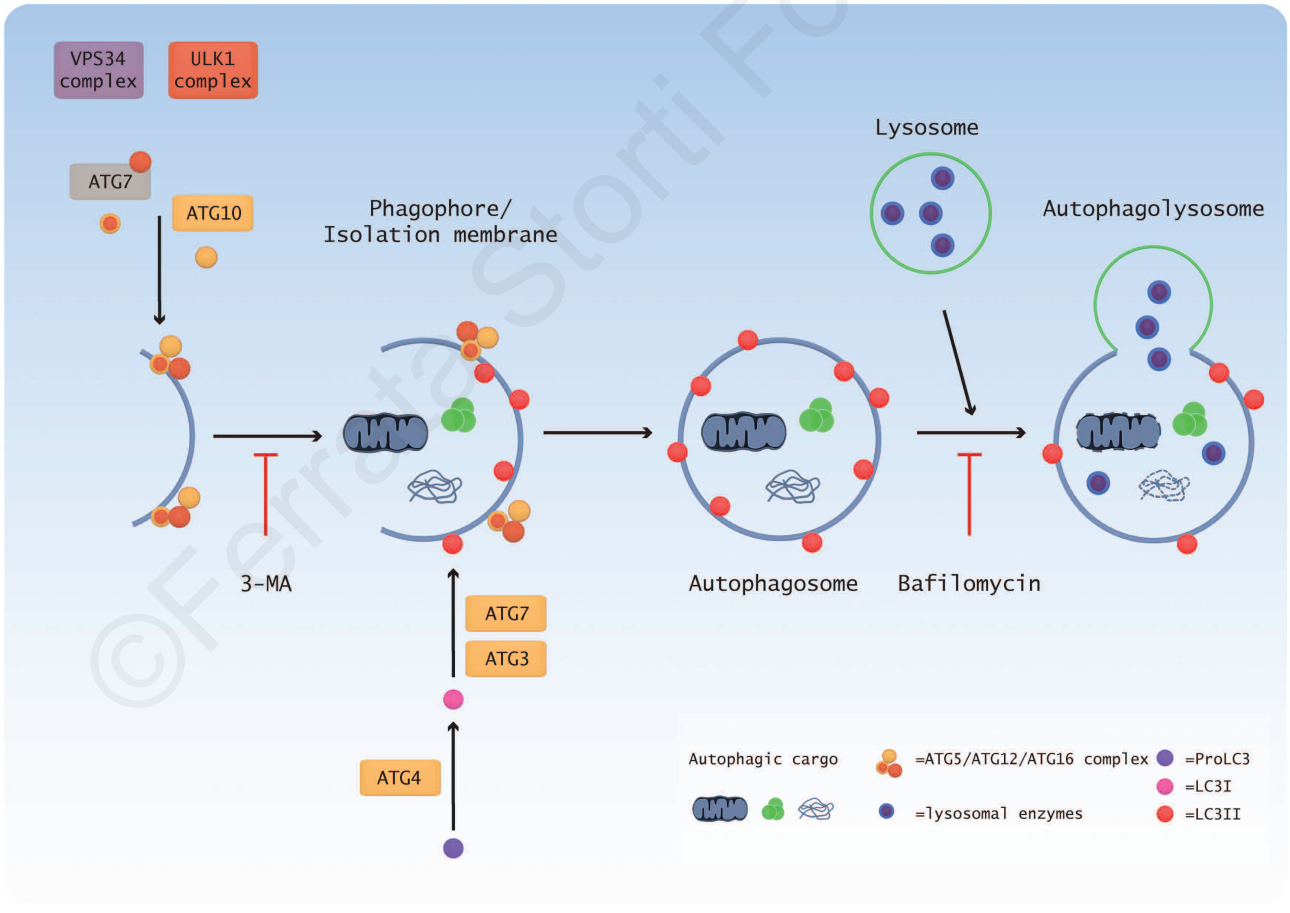

Figure 2. Schematic overview of the autophagy pathway. Initiation of autophagy results in the formation of the ULK1 complex, consisting of ULK1, FIP200, Atg13 and Atg17, which is negatively regulated by mTOR. Atg1 also acts to recruit additional Atg proteins to the phagophore. Vps34 forms a core complex with Beclin1 and p150 and produces PI3P for membrane formation. The Atg5-Atg12-Atg16 complex is formed by the combined action of Atg7 and Atg10 while processing of LC3 is accomplished by Atg4, Atg3 and Atg7. The completed autophagosome contains cytosolic proteins and/or organelles and subsequently fuses with lysosmes. The enzymes present in lysosomes degrade the autophagosome contents and this material is then recycled back to the cell. 3-MA: 3-methyladenine.

implicated in development, tissue remodeling and a number of human diseases.[5] Although initially studied as a response to nutrient starvation, its role in cancer has received much attention as a potential therapeutic target. However, at the same time, its precise role is controversial as it appears to have a dual function: either inhibiting oncogenic transformation or potentially promoting cancer growth and survival. Therefore, there is uncertainty about whether to inhibit or promote autophagy for cancer treatment. The process of autophagy can be divided into four steps: induction, nucleation, vesicle expansion and closure, and autolysosome formation. Each step is regulated by the co-ordinated action of a number of autophagy related (Atg) proteins (Figure 2).

Autophagy mechanism and processes

Autophagy induction requires the activity of ULK1, the mammalian homolog of the yeast Atg1. It forms a complex with Atg13, FIP200 (yeast Atg17) and Atg101 and is negatively regulated by mTOR. Under autophagy permissive conditions, mTOR is inactive and, therefore, Atg13 is no longer hyperphosphorylated, relieving its inhibitory block on ULK and allowing its activation.[43] Nucleation then occurs with the formation of a phagophore which envelopes cytosolic proteins and organelles. The main requirement for vesicle nucleation is the activity of PI3K Class III (hVps34), which exists in a core complex with Beclin1 (yeast Atg6) and p150 (yeast Vps15), and interacts with a number of proteins to either inhibit or promote autophagy.[44-46] Additionally, the binding of Bcl-2 or Bcl-xL to Beclin1 can inhibit autophagy by preventing Beclin1 binding to hVps34.[27]

The phagophore expands and closes to completely sequester the material in an autophagosome, a double membrane-bound vesicle which is the characteristic feature of autophagy. The expansion of the developing autophagosome is mediated by two ubiquitin-like conjugation reactions. In the first, Atg7 and Atg10 link Atg12 to Atg5, which subsequently interacts with Atg16L and oligomerizes to form the Atg16L complex. In the second reaction, Atg8/LC3 is cleaved by Atg4 and joined to a lipid moiety, phosphatidylethanolamine (PE), by Atg3 and Atg7 to produce LC3II, which is located on the surface of autophagosomes.[47,48] In addition, LC3 controls the expansion of the phagophore[49] and its lipidation is required for closure of the autophagosome.[50,51]

The final steps in the autophagy process involve fusion between the autophagosome and lysosmes and the degradation of the autophagosome contents. While the exact details of how this happens are not completely understood, a number of proteins have been shown to be essential in this process, including LAMP1, LAMP2, UVRAG and Rab7, which are required for membrane fusion.[52-54] Degradation of the autophagosome contents is carried out by numerous acid hydrolases, the major group being the cathepsins. Release of the autolysosome contents back to the cell is mediated by the action of Atg22 in yeast[55] but the mammalian ortholog has yet to be identified (Figure 2).

Two sides to every story

The role of autophagy in cancer is unclear, with some lines of evidence indicating a tumor suppressor role whereas others suggest it may promote tumors. In support of a tumor suppressive role, a number of key autophagy genes have been demonstrated to be tumor suppressor genes. For example, *Beclin-1*, the mammalian homolog of the yeast autophagy-related gene 6 (Atg6), has been shown to be monoallelically deleted in 40-75% of cancers of the breast, ovaries and prostate,[56] and UVRAG is monoallelically deleted in colon cancer cells.[57] In addition, mice lacking Beclin1 or another autophagy protein, Bif-1, have a higher incidence of spontaneous tumors.[56,58] Autophagy has also been shown to be essential for establishing senescence,[59] a state of cell cycle arrest, preventing tumor establishment and/or growth.

On the other hand, the finding that autophagy is up-regulated in certain cancers argues for a role in promoting tumors. For instance, at the centre of solid tumors, where the nutrient supply is limited and cells are often faced with a hypoxic environment, autophagy acts as a survival mechanism by supplying energy and essential nutrients, and allowing cells to adapt to the low oxygen conditions. Autophagy is also up-regulated in response to radiation and certain anti-cancer drugs, such as temozolomide and etoposide, and is the likely cause of resistance to such treatments.[60] Finally, autophagy may play a role in cancer metastasis by promoting the survival of cells detached from the extracellular matrix that would normally undergo apoptosis.[61]

Therefore, the role autophagy plays in cancer is the subject of some controversy. Is it a protective mechanism leading to tumor cell survival, or does activation of this pathway result in tumor cell death? The reality is that autophagy probably plays a role in both processes. Its precise function may depend on the nature of the activating signal, the impact of other signaling pathways, the stage of the disease, as well as the genetic make-up of the cell and the extent of cell damage. However, the prevailing view is one of autophagy being important early on to suppress tumor formation. But later, once the tumor is established, it functions to promote tumor survival.[62] In the case of myeloma, high levels of autophagy have been noted in cell lines and patient samples[63,64] and this was associated with shorter overall and progression-free survival.[63]

The relationship between autophagy and apoptosis further confounds the situation. Does autophagy activate or repress apoptosis, or can both processes be activated independently and simultaneously?[65] The nature of the interplay between these pathways is crucial for us to further our knowledge of cell death in relation to cancer therapy. Clearly, many questions remain unanswered but if targeting autophagy is to be a viable option for cancer, and more specifically myeloma treatment, these questions to be addressed. The development of more specific autophagy inhibitors, and unraveling of pathways regulating autophagy will go far in helping tackle these issues.

Targeting protein handling pathways in myeloma

Although the development of proteasome inhibitors has led the way in targeting protein handling pathways in myeloma, there are many other potential targets both within the UPP as well as in the stress response and autophagy pathways. Some of these targets are early *in vitro* proof-of-principle whereas other examples have progressed to phase III clinical trials.

Proteasome inhibitors

Bortezomib is currently the only proteasome inhibitor licensed for clinical use. It binds reversibly to the chymotrypsin-like β5 subunit of the 20S proteasome core par-

ticle and inhibits its function.[66] A number of other reversible inhibitors are under development, including MLN9708 and CEP-18770. MLN9708 has been demonstrated to have good pharmacokinetic and pharmacodynamic properties and, importantly, showed activity in xenograft models.[67] CEP-18770 has shown activity in myeloma cell lines and primary patient cells and resulted in complete tumor regression in mouse models.[68] In addition, combinations with melphalan and bortezomib prevented, or at the very least, delayed tumor progression *in vivo*.[69] Interestingly, bortezomib, CEP-18770 and MLN9708 were also shown to inhibit the caspase-like activity of the proteasome, and inhibitors of the trypsin-like activity are also under development.[70]

The development of resistance to bortezomib has prompted the need for inhibitors with properties distinct from that of bortezomib. This has led to the development of irreversible inhibitors such as carfilzomib, NPI-0052 and ONX0912, which target both the proteasome and immunoproteasome. Carfilzomib was found to be a strong inhibitor of the chymotrypsin-like activity of the proteasome both *in vitro* and *in vivo*[71] and, importantly, demonstrated activity in cells resistant to bortezomib, melphalan and dexamethasone.[72] NPI-0052, in contrast to bortezomib and carfilzomib, appears to have activity against all three enzymatic activities of the proteasome.[73] In addition, its non-peptidic structure ensures it is not degraded by intracellular proteases to which bortezomib and carfilzomib would be subject.[14] It is able to induce apoptosis in primary myeloma cells refractory to treatment with conventional myeloma therapies[73] and combines synergistically with bortezomib and lenalidomide *in vivo*.[74-76] ONX0912 is the only irreversible proteasome inhibitor that is orally bioavailable. It too has shown activity in myeloma cells resistant to conventional therapy and enhances tumor regression *in vivo*. Importantly, it combines either synergistically or additively with lenalidomide, bortezomib, dexamethasone and a histone deacetylase inhibitor.[75]

In addition to the competitive inhibitors described above, some work has been carried out looking into allosteric proteasome inhibitors in the hope that these may be used to overcome resistance to the competitive inhibitors.[77] Allosteric inhibitors work by interacting with the regulatory α subunits of the 20S proteasome, thereby blocking proteasome function non-competitively.[77-79] Some of these are structurally similar to the anti-malarial drug chloroquine and their prior approval for clinical use will hopefully speed up their testing. Finally, while most research has concentrated on targeting the 20S proteasome core, the 19S regulatory subunit has recently emerged as a potential drug target[80] but is yet to be tested in myeloma.

Clearly, the aim behind developing reversible/irreversible and competitive/allosteric inhibitors is to increase the anti-proteasome effect and induce more potent myeloma cell apoptosis while decreasing the clinical side-effect profile but maintaining patient tolerance and ease of administration. Pre-clinical data look promising for all the above mentioned approaches, but further clinical trials will be required to determine which approach is the best.

Inhibitors of the ubiquitin proteasome pathway

In addition to directly targeting the proteasome, it may be possible to target pathways both upstream and downstream of it. Ubiquitin E3 ligases are responsible for the ubiquitination of a variety of substrate molecules, leading to their degradation by the 26S proteasome. Skp1-Cullin-F box protein (SCF) constitutes the largest family of E3 ligases and acts as a scaffold ensuring the correct positioning of the substrate and E2 enzyme for ubiquitin transfer. An inhibitor of SCF^{Skp2} identified by a high-throughput screen and referred to as Compound A has been tested *in vitro* against myeloma.[81] Compound A was able to overcome resistance to bortezomib, dexamethasone, doxorubicin and melphalan, and was synergistic with bortezomib. It also induced autophagy.[81] HDM2, the human homolog of murine double minute 2 (MDM2), acts as the E3 ligase for p53 and is often over-expressed in a variety of cancers. It is a direct downstream target of p53 and marks it for degradation. Nutlin-3, which disrupts the interaction between p53 and HDM2, was tested in combination with bortezomib and was shown to have additive cytotoxicity. But in co-culture experiments with bone marrow stromal cells, the effect of Nutlin-3 on apoptosis was reduced.[82] In addition to the proteasome, cullin-ring ligases are also involved in protein degradation, and NEDD8- activating enzymes (NAE) catalyze the initial step in the NEDDylation cascade in a similar manner to ubiquitin E1 enzymes.[83] The NAE inhibitor, MLN4924, was recently tested in myeloma and showed activity against cells both sensitive and resistant to bortezomib, as well as primary patient cells. Importantly, it showed activity *in vivo* with significant inhibition of tumor growth and a reduction in tumor burden in two different models.[84]

Additionally, it is possible to target processes that occur after proteins exit the proteasome. Aminopeptidases catalyze the hydrolysis of amino acids from the N terminal of proteins. Tosedostat (CHR-2797), a novel metalloenzyme inhibitor, was demonstrated to induce cell cycle arrest and apoptosis of myeloma cell lines. Synergy with dexamethasone was observed while it was additive with bortezomib and melphalan. In addition, tosedostat up-regulated the UPR and induced autophagy.[85]

Heat-shock chaperone inhibitors

To date, most research has concentrated on developing inhibitors for HSP90, given that it sits at the intersection of numerous pathways that are aberrantly activated in cancer. Initial drug development efforts were centered around two natural products, radicicol and geldanamycin; research into geldanamycin is more pronounced. The geldanamycin-based inhibitors have undergone a number of iterations to develop drugs with improved toxicology and solubility. These are now being tested in the clinic.

17-AAG was the first to be developed and showed single agent activity *in vitro* against myeloma cell lines and primary patient samples. It sensitized primary cells to treatment with conventional and novel therapies, including doxorubicin and bortezomib.[86,87] This was related to the loss of cell surface expression of IGF-1R and IL-6R, with subsequent inhibition of numerous signaling pathways. Activity was mirrored *in vivo* where, in addition, it significantly prolonged survival.[87] However, 17-AAG also induces the UPR,[40] and HSP70 is up-regulated in response to HSP90 inhibition,[88] suggesting that in order to achieve an optimal clinical response with HSP90 inhibitors, they should be used in a combination approach. To this end, 17-AAG or its analog 17-DMAG, in combination with either of the PI3K pathway inhibitors, perifosine or rapamycin, demonstrated synergistic cytotoxicity in myeloma cells, inhibited angiogenesis, and was able to overcome the protective effects of the bone marrow microenvironment.[89,90] Similar effects on

myeloma cell death were demonstrated by the combination of 17-DMAG and the autophagy inhibitor 3-MA.[91] The next iteration of 17-AAG is IPI-504. This compound has improved solubility and tissue retention compared to 17-AAG, and showed similar synergy with bortezomib.[92] However, in contrast to 17-AAG, IPI-504 was shown to block the UPR,[93] not activate it.[40] However, the reason for this discrepancy is not clear.

A number of synthetic small molecule HSP90 inhibitors with various molecular scaffolds are also available. One of the first to be described was NVP-AUY922 which showed activity at sub-micromolar concentrations in a range of cell lines and, importantly, activity *in vivo*.[94] When tested in myeloma, NVP-AUY922 showed low nanomolar sensitivity in both cell lines and primary patient samples, even in the presence of bone marrow stromal cells. This was associated with strong induction of apoptosis and downregulation of important survival pathways.[95] It also demonstrated synergy with histone deacetylase inhibitors, melphalan and doxorubicin in primary patient samples refractory to conventional therapies.[96] Similarly, the orally available NVP-BEP800 was able to induce apoptosis in myeloma cell lines and primary patient samples with pronounced inhibition of the STAT3, ERK and Akt pathways. Weak synergy was demonstrated with melphalan whereas combinations with bortezomib, doxorubicin or SAHA were weakly antagonistic.[97]

Two additional oral HSP90 inhibitors have been tested in myeloma. NVP-HSP990 induces apoptosis and cell cycle arrest in myeloma cell lines and primary samples. This was associated with downregulation of 'client' proteins (IL-6R, MEK and Akt) and subsequent upregulation of HSP70 and HSP27.[98] SNX-2112, which exists as a pro-drug SNX-5422, was able to induce cytotoxicity and cell cycle arrest in myeloma cell lines and patient samples. Downregulation of Akt and ERK pathways was demonstrated and, importantly, maintained in the presence of extracellular cytokines. SNX-2112 inhibited angiogenesis and osteoclastogenesis and, when tested *in vivo*, significantly prolonged survival. In addition, it was able to induce apoptosis, down-regulate ERK and block angiogenesis *in vivo*.[99]

The novel purine scaffold HSP90 inhibitor, PU-H71, demonstrated activity in myeloma cells both sensitive and resistant to conventional therapies, and was synergistic with thalidomide, bortezomib, dexamethasone and melphalan. It induced cell cycle arrest, apoptosis and the UPR. Interestingly, PU-H71 appeared to work by targeting HSP90 and the ER HSP90 paralog, GRP94.[100] The non-ansamycin, non-purine inhibitor, KW-2478, also down-regulated levels of HSP90 'client' proteins and IgH translocation products (FGFR3, c-Maf and cyclin D1). These effects on 'client' proteins were mirrored *in vivo* where, in addition, it significantly inhibited tumor growth.[101]

More recently, there has been a move to developing inhibitors of HSP70, given that HSP90 inhibitors induce HSP70 expression. The aim would, therefore, be combination treatment with both inhibitors.[88] MAL3-101 is a non-ATP site inhibitor of HSP70 that induced apoptosis and cell cycle arrest in myeloma cell lines, and demonstrated synergy with both proteasome and HSP90 inhibitors. Synergy with proteasome inhibitors was confirmed *in vivo*.[102] Finally, in addition to directly targeting HSP90 or HSP70, it is possible to inhibit heat shock factor 1 (HSF-1), the transcription factor that induces their expression. While many of the compounds currently used as HSF-1 inhibitors are non-specific, and their mechanism of action is not fully understood, efforts are underway to design more potent and specific inhibitors of this transcription factor.[103]

Unfolded protein response modulators

The potential for targeting the UPR to achieve cell death has been recognized, with the three UPR sensors, IRE1, PERK and ATF6, being the obvious candidates: they are all kinases, with two crystal structures being solved[104,105] and siRNA knockdown of each kinase resulting in apoptosis in myeloma cells.[106] Recently, specific inhibitors of the endoribonuclease domain of IRE1 that inhibit the splicing of XBP1 have been described[107,108] and have shown activity *in vivo* in combination with bortezomib.[107] Inhibition of the kinase domain of IRE1 has also been shown to be effective[104] and more specific inhibitors are under clinical development (Walter *et al.*, patent n. WO 2010/031056 A2, 2010; Korennykh *et al.*, patent n. WO 2011/047384 A2, 2011), as are inhibitors of PERK (Cardozo *et al.*, patent n.WO 2011-146748 A1, 2011). Although the cleavage of ATF6 has been targeted, the compound tested was a general serine protease inhibitor[109] and, to date, no clinical grade inhibitors have been reported.

In a similar manner, many inhibitors of GRP78, the protein which holds IRE1, PERK and ATF6 in their inactive form, are available. Most are natural products but lack true specificity. The one most commonly used as a chemical tool is versipelostatin, which inhibits GRP78 transcription, but it only appears to inhibit the UPR under conditions of glucose deprivation.[110] Drugs that target protein transport have also been tested in myeloma. Brefeldin A inhibits the transport of proteins from the ER to the Golgi[111] while Eeyarestatin I blocks ERAD by interfering with p97, an AAA-ATPase that transfers proteins from the ER to the cytosol.[112] Protein disulfide isomerases (PDI), enzymes responsible for oxidative protein folding in the ER, have also been examined as

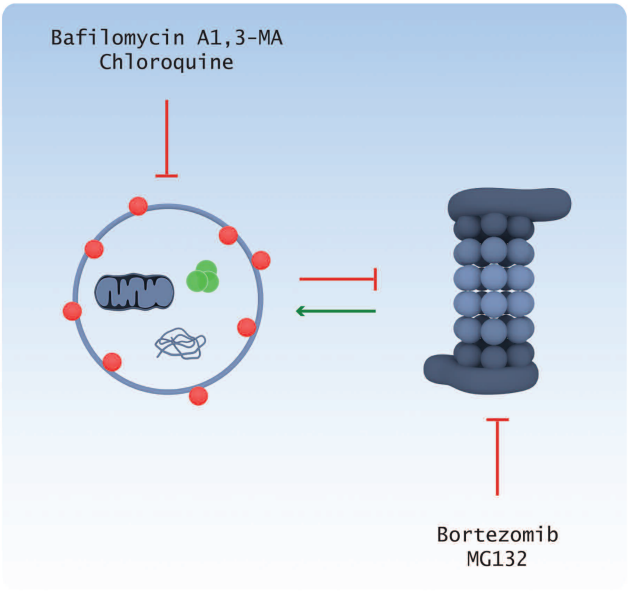

Figure 3. Relationship between the autophagy and proteasome pathways. Proteasome inhibition by bortezomib or MG132 results in the compensatory upregulation of the autophagy pathway, whereas inhibition of the autophagy pathway by bafilomycin A1, 3-MA or chloroquine inhibits the proteasome.

Table 1. Studies in myeloma that demonstrate the upregulation of autophagy by a particular compound or treatment.

Compound/treatment	Target	Reference
Compound A	SCF^{SKP2} via p27 degradation	81
Diquinoline	Unknown	120
CHR-2979	Aminopeptidase	85
HYD-1	D amino peptide via ROS	121
Tetraspanin	CD81 & CD82	122
Syrbactins	20S proteasome	123
Rapamycin + Perifosine	mTOR & Akt	124
CAL-101	PI3K p110δ	125
Clarithromycin	50S ribosome	126
Tipifarnib + Bortezomib	Ras & 26S proteasome	127
Gene knockdown	PERK, IRE1 or ATF6	106

Table 2. Studies in myeloma examining the effect of a compound or treatment that induces autophagy, in combination with autophagy inhibitors.

Compound/treatment	Autophagy inhibitor	Effect	Reference
Bortezomib	3-MA /CHQ/Beclin1 siRNA	Antagonistic	64
Thapsigargin	3-MA	Synergistic	64
8-Amino-adenosine	3-MA or CHQ	Synergistic	129
17-DMAG	3-MA	Synergistic	91
PI-103	Baf	Synergistic	63
Bortezomib	Baf	Synergistic	118
Bortezomib	3-MA or LC3B siRNA	Antagonistic	118
Doxorubicin/ Melphalan	3-MA/CHQ/siRNA (Atg5 orBeclin1)	Synergistic	130
Nutrient starvation	3-MA	Synergistic	131

3-MA: 3-methyladenine; CHQ: chloroquine; Baf: Bafilomycin A1.

potential drug targets. Compounds targeting PDI are metabolized by glutathione S-transferases, releasing nitric oxide in cells.[113] In myeloma cells, they were able to enhance cytotoxicity mediated by bortezomib and prolong survival *in vivo*.[114] As this is a relatively new area of research, to date all inhibitors are tool compounds for use in *in vitro* models, although clinical grade inhibitors are under development for use in future clinical trials.

Autophagy modulators

Although much is known about the regulation of autophagy by the PI3K pathway, studies exploring the role of other autophagy regulatory pathways are in their infancy.[115-117] Importantly, we know that UPR activation, removal of proteins by the proteasome and autophagy are interlinked. While inhibition of the proteasome leads to a compensatory upregulation of autophagy,[64,118] blocking autophagy inhibits the proteasome (Figure 3).[119] It is clear, therefore, that these interactions need to be better understood if we are to target these intracellular protein handling pathways effectively.

A number of researchers have begun to look at the role of autophagy in myeloma. Most of these papers have shown that a particular drug or drug combination induces autophagy (Table 1). But, as can be seen from the varied list of drug targets, no clear pattern has emerged. However, there are some familiar faces among those drugs that up-regulate autophagy. These include the proteasome inhibitor bortezomib, the PI3K/Akt/mTOR inhibitors rapamycin and perifosine, and inhibitors of the three UPR sensor molecules, PERK, IRE1 and ATF6. As mentioned above, bortezomib is already in clinical use but agents targeting Akt and mTOR are still in clinical trials. However, as a result of a negative feedback loop within the PI3K/Akt/mTOR pathway, they have had limited success, and combinations of rapamycin and bortezomib were shown to be antagonistic.[128] There has, therefore, been a move to develop dual PI3K/mTOR inhibitors in order to circumvent this problem.

Perhaps of greater interest are the papers that look at a drug that induces autophagy in combination with autophagy inhibitors as a potential mechanism for eliciting apoptosis (Table 2). While most of the combinations are synergistic, there are two striking exceptions. In the work of Hoang *et al.*[64] and Kawaguchi *et al.*[118] the combination of bortezomib with 3-methyladenine (3-MA), or siRNA against LC3B or Beclin1, was antagonistic. However, Kawaguchi *et al.* also demonstrate that bortezomib in combination with bafilomycin A1 (Baf) is synergistic, whereas Hoang *et al.* show that combining bortezomib and chloroquine (CHQ) is antagonistic. 3-MA inhibits autophagy at the level of PI3K Class III, therefore, acting early on in the pathway. Bafilomycin and CHQ inhibit the fusion between the autophagosome and lysosome, therefore acting as late stage inhibitors (Figure 2). So, is the stage at which the autophagy inhibitor acts important? And why is there a difference in cells treated with bortezomib/Baf *versus* bortezomib/CHQ? In trying to understand these results, it is important to note that 3-MA has been shown to be a more specific inhibitor of PI3K Class I and not PI3K Class III[132] and chloroquine, in addition to its effect on autophagy, is known to inhibit the proteasome.[79] This is further complicated by the cross-talk between these two protein degradation systems. The main effect of proteasome inhibition seems to be induction of ER-stress,[133] which in turn up-regulates autophagy.[29] But we still do not know exactly how the reciprocol regulation of these pathways occurs in response to drug treatment. The different responses observed with the combination of bortezomib/Baf *versus* bortezomib/CHQ really underscores the urgent need for more specific autophagy inhibitors to delineate autophagy on-target, as opposed to off-target, effects.

Conclusions

Significant progress has been made in the development of targeted molecules for the treatment of myeloma. By understanding the basic biology of this tumor, particularly its dependence on protein handling pathways such as the ubiquitin proteasome pathway, ER stress and the unfolded protein response, heat shock proteins and autophagy, scientists have been able to exploit its weaknesses to promote its destruction. This, together with intuitive drug design, will lead to the development of more specific inhibitors with, hopefully, fewer side-effects for patients. This will be particularly important in the case of autophagy, given its important role during development. The initial excitement with bortezomib has been superseded by the development of more specific irreversible

proteasome inhibitors, and inhibitors of other aspects of protein degradation are beginning to emerge. While some of these drugs are still only in early pre-clinical testing stages, especially modulators of the UPR, testing of other compounds has reached clinical trials and the initial results seem promising. Additionally, the field of autophagy research holds great promise for the identification of further therapeutic targets. However, the complex interactions between pathways and the possible upregulation of redundant pathways mean that further study is needed. As has been shown with HSP90 inhibitors, for example, there is upregulation of other HSPs and induction of the UPR.[40,87,88] Indeed, HSP90 inhibitors have, in general, performed poorly as single agents for the treatment of multiple myeloma in the clinic.[134] highlighting the need to look at these compensatory mechanisms in order to enhance apoptosis of myeloma cells. Additionally, the bone marrow microenvironment plays an important role in protecting cells from apoptosis, both by the direct binding of myeloma and stromal cells, and due to the secretion of numerous cytokines that influence cell growth.[135] Testing of new drugs and drug combinations in the laboratory should, therefore, always be tested in a situation that mimics this environment.

The key questions now will be how best to combine these agents to achieve even better responses. For example, should a proteasome inhibitor be combined with an autophagy inhibitor, and can this be enhanced by adding an ER stress or heat shock protein inhibitor? This research needs to be accompanied by studies examining the interactions between the various protein handling pathways highlighted in this review. By understanding how these various pathways intersect and overlap, we will be able to achieve the greatest clinical benefit for our patients.

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