



**Universiteit
Leiden**
The Netherlands

The use of activity based protein profiling to study proteasome biology
Paniagua Soriano, Guillem

Citation

Paniagua Soriano, G. (2016, February 11). *The use of activity based protein profiling to study proteasome biology*. Retrieved from <https://hdl.handle.net/1887/37766>

Version: Corrected Publisher's Version

License: [Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden](#)

Downloaded from: <https://hdl.handle.net/1887/37766>

Note: To cite this publication please use the final published version (if applicable).

Cover Page



Universiteit Leiden



The handle <http://hdl.handle.net/1887/37766> holds various files of this Leiden University dissertation.

Author: Paniagua Soriano, Guillem

Title: The use of activity based protein profiling to study proteasome biology

Issue Date: 2016-02-11

Chapter 2: Towards Understanding Induction of Oxidative Stress and Apoptosis by Proteasome Inhibitors*

Introduction

The ubiquitin-proteasome system (UPS) is the major cytosolic and nuclear protein turnover machinery [1, 2]. Ubiquitylated proteins are recognized and processed to produce small- and medium size oligopeptides that are further processed by aminopeptidases to deliver amino acids for reuse in protein synthesis. The UPS ensures controlled protein turnover by the time-dependent targeting and degradation of its substrates and in this fashion determines the half-life of each cytosolic and nuclear protein. The UPS also partakes in the degradation of misfolded and dislocated proteins from the ER and therefore plays a major role in the cellular response to ER stress, and is responsible for the removal of proteins damaged by oxidative stress [3, 4]. Part of the peptide pool produced by proteasomes and further trimmed by downstream aminopeptidases are transported to the luminal side of the endoplasmic reticulum, where they are loaded onto major histocompatibility complex class I (MHCI) molecules for presentation at the outer cell surface to the immune system [5-8]. CD4⁺ cytotoxic T-cells discriminate between self peptides and foreign peptides presented in this fashion and by processing virally encoded proteins for MHCI mediated antigen presentation proteasomes contribute to the detection and eradication of virally infected cells.

Proteasomes are expressed almost ubiquitously throughout the kingdoms of life (Eubacteria generally do not contain proteasomes except some actinomycetes and mycobacteria). Although proteasomes have evolved over time, the overall layout of the inner proteolytic assemblies, called 20S core particles (CP), has remained remarkably conserved. 20S proteasomes are C2-symmetric barrel-like structures that consist of four rings of seven protein subunits each, arranged in an $\alpha\beta\beta\alpha$ fashion with two outer α rings and two inner β rings. In 1995, the crystal structure of the archaeal 20S proteasome was solved [9]. In prokaryotes the α -subunits are identical and the same holds true for the β -subunits. In 1997 the crystal structure of the yeast 20S proteasome was solved [10] and in 2002 the structure for mammalian 20S was determined [11]. In eukaryotes both α -subunits and β -subunits have diverged such that, though the overall C2-symmetrical geometry is maintained, the seven α -subunits in each α ring are unique, as is the case for the seven β -subunits. In prokaryotes each β -subunit is catalytically active. In yeast and all

* Antioxid. Redox Signal., 2014 Dec; **21**(17): p. 2419-2443

other eukaryotes however, each β ring contains only three β -subunits with enzymatic activity (β 1, β 2 and β 5). Thus eukaryotes lack enzymatic activity of β 3, β 4, β 6 and β 7 but this loss is offset by a diverged substrate specificity of the remaining subunits. Of these, β 1 is also known as 'caspase-like' because it recognises and processes substrates having acidic residues at position 1 (P1 – the amino acid occupying in the proteasome active site the position containing the scissile amide bond). The β 2-subunit cuts preferentially C-terminal of basic amino acids and is therefore also referred to as 'trypsin-like', whereas β 5 prefers hydrophobic residues and is referred to as 'chymotrypsin-like'.

Proteasome subunits are only catalytically active when part of a 20S core particle. The assembly of 20S particles have been subject to intensive studies leading to detailed insight into the various consecutive steps by which these superstructures are formed [12, 13]. The proteasome-assembling chaperones (PAC) 1-4 form heterodimers that direct the α ring assembly. Once seven α -subunits are assembled into one ring, the β -subunits are incorporated. UMP1 is essential for correct assembly of the β -subunits. Their precursor peptides (β -propeptides) are essential for proper β ring formation. The β 7-subunit is the last subunit incorporated into the ring, forming one half of a 20S proteasome. Assembly of a core 20S particle from two halves is guided by the C-terminal tail of the β 7-subunits, which acts as a chaperone. Finally, CP maturation is accomplished by intramolecular cleavage of the propeptide of the inactive subunits to generate the active site [12-14]. Experimental data suggests that the N-terminus of the α -subunits in the α -rings form a gate that closes the pore of the catalytic chamber, restricting the access of substrates. As a consequence, the 20S core particle alone shows a basal catalytic activity, which is enhanced when bound to regulatory particles (RP) [14].

In vertebrates, specific tissues express the interferon-gamma-inducible immunoproteasomes. In these particles the catalytic β subunits of the constitutive proteasome are replaced by β 1i, β 2i and β 5i respectively [15, 16]. The immunoproteasome 20S core particle are assembled *de novo* and cannot derive from subunit exchange starting from constitutive proteasome 20S particles, as proposed earlier [17, 18]. Immunoproteasomes have a slightly different substrate preference compared to constitutive proteasomes and this difference in cleavage correlates with MHC Class I peptide bonding specificity, which is a very important feature in immunology [6]. Recently the crystal structure of the mouse immunoproteasome at 2.9 Å resolution was solved this structure revealed some differences between the immunoproteasome and constitutive proteasome active sites, thus underscoring that the substrate specificity is slightly different [19].

In 2007, Murata *et al.* discovered a new protein with an overall sequence highly similar to $\beta 5$ and $\beta 5i$, suggesting that this protein may belong to the same protein family although of a larger size [20]. This protein, named $\beta 5t$, is expressed specifically in thymic cortical epithelial cells, where it substitutes $\beta 5i$, in immunoproteasomes [21]. This resulting 20S core particle has been dubbed the thymoproteasome and ensuing studies suggested a specific role for thymoproteasomes in positive T-cell selection. In 2010, our group showed by means of activity-based protein profiling that $\beta 5t$ is catalytically active. The inhibitor profile resulting from a competitive activity-based protein profiling assay performed on thymoproteasomes moreover suggests that the $\beta 5t$ active site pocket is more hydrophilic than $\beta 5$ and $\beta 5i$ [22]. This altered inhibitor preference may reflect an altered substrate preference as well, which in turn may help explaining the role of $\beta 5t$ in positive T cell selection.

Next to the three distinct 20S proteasome core particles (constitutive proteasome, immunoproteasome and thymoproteasome), a number of hybrid or ‘intermediate’ 20S particles have been discovered in the past decade [23-28]. These particles may contain mixtures of constitutive proteasome and immunoproteasome active sites. Although to date only intermediate proteasomes have been identified that contain one ($\beta 5i$) or two ($\beta 1i$ and $\beta 5i$) of the three inducible catalytic subunits of the immunoproteasome, it may well be that more, and more complex intermediate proteasomes exist, adding to the complexity of the 20S core particle (20S-CP) family and its contribution to protein turnover and antigen presentation.

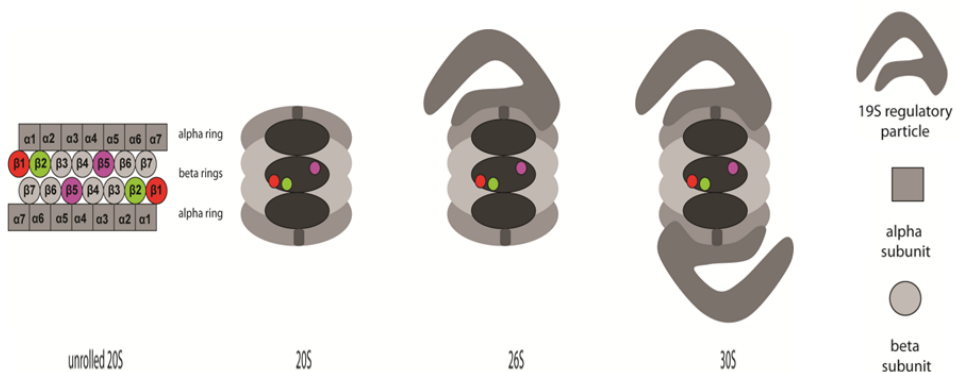


Figure 1. Schematic representation of the eukaryotic constitutive proteasome. Cross section of the 20S core particle containing the $\beta 1$, $\beta 2$ and $\beta 5$ active subunits that confer the caspase-like, tryptic-like and chymotryptic-like activity. Attachment of one 19S regulatory particle to the 20S core yields the 26S proteasome and two 19S caps with one 20S yields the 30S proteasome particle. In immune

competent tissues the active subunits can be replaced by their immunologic counter parts $\beta 1i$, $\beta 2i$ and $\beta 5i$ forming the immunoproteasome. Replacement of $\beta 5i$ by the thymus specific $\beta 5t$ makes the thymoproteasome.

20S core particles are capable of degrading peptides and small, or unfolded proteins but their physiological role is limited. To become fully functional, 20S particles associate with one or two of a number of regulatory caps [29]. Of these, the 19S cap is the most studied and the most important complex to associate with constitutive proteasome 20S core particles. 19S caps bind to the α -rings of a mature 20S thus giving rise to 26S proteasomes (one 19S cap associated) or 30S proteasomes (one 19S cap at both ends of the 20S barrel, Figure 1). 19S caps regulate 20S mediated protein turnover in an ATP-dependent fashion by identifying and binding polyubiquitinated proteins, unfolding the substrates, and translocating these into the 20S catalytic chamber. 19S caps are assembled from 19 subunits, which can be divided in two subcomplexes: the lid and the base. The base is composed of 10 different proteins, 4 non ATPases and 6 AAA+ ATPases that form a hetero-hexameric ring, which in presence of ATP, binds the α -rings of the 20S facilitating the opening of the gate [30]. The base promotes the unfolding of the substrate, opens the pore to permit the entrance of the targeted substrates into the 20S inner chamber and translocates these. Of the 4 non-ATPases proteins, two are ubiquitin receptors and the other two can bind to the ubiquitin shuttle proteins Rad23, Ddi1 and Dsk2 [30]. The lid is situated on top of the base and contains 9 non-ATPases proteins. Its main function is to recognize and bind polyubiquitinated substrates and deubiquitylate these. The lid subunit Rpn 11 is the only deubiquitinating enzyme (DUB) incorporated into the 26S proteasome. Two additional DUBs, Usp14 and Uch37, are described as proteasome-associated proteins, however their precise binding position to the 26S is unknown [31].

Apart from the 19S caps, other proteasome activators have been found such as the PA28 protein family and PA200. These regulatory particles activate the proteasome in an ATP-independent manner in contrast to the 19S cap. The PA28 complex, also known as 11S, has 3 isoforms in higher eukaryotes, called PA28 α , β and γ . PA28 α and PA28 β form a heteroheptamer, while the PA28 γ , which is mainly found in the nucleus forms a homoheptamer [32]. Both complexes bind to the α rings and promote gate opening. Some studies have been reported that reveal the involvement of 11S activators in the production of peptides for antigen presentation through MHC class I complexes. However some cells and tissues which are not involved in the immune system, do express the 11S regulatory particles. 11S particles may also be part of hybrid proteasomes, with a 19S cap on one end and an 11S activator on the other [32]. The monomeric activator PA200 (Blm10 in yeast) can partially open the gate of the 20S-CP, thus helping substrate entry in the proteolytic chamber. Although the 20S-CP is expressed in all eukaryotes, plants and

yeasts only contain PA200/Blm10 and do not have any of the PA28 isoforms. The function of the PA200 is poorly understood, but some studies point towards its involvement in the degradation of specific substrates [14].

Proteasome inhibitors

Many different proteasome inhibitors (PIs) have been described over the past decades. PIs are derived both from natural sources and through organic synthesis. Both covalent reversible, covalent irreversible and non-covalent inhibitors are known. PIs have been reviewed extensively before [33-35], thus we will here focus mainly on site-selective inhibitors, for which we provide both a qualitative (different types of inhibitors) and a quantitative (potency and subunit selectivity) analysis. Figure 2 shows five classes of covalent inhibitors and their inhibition mechanisms. The first class is represented by the peptide aldehydes, with **MG-132** as its most widely used member. Aldehydes form covalent, reversible bonds within proteasome active sites, and inactivate catalytic activities by hemiacetal formation with the N-terminal threonine of the proteasome subunits. A major drawback of aldehydes is their cross reactivity towards cysteine and serine proteases [35]. A well-known class of electrophilic traps is the family of epoxyketones. Inhibitors containing the epoxyketone moiety are highly selective for the proteasome, and no off targets have been found to date [36]. The structure of **epoxomicin** co-crystallized in yeast proteasomes reveals the molecular basis for this specificity. A morpholine ring is formed between the active site threonine and the epoxyketone, in which both the γ -hydroxyl and the free amine of the N-terminal threonine participate [37]. Another class of PIs are the boronic acids, with **Bortezomib** as the most renowned example [38]. Bortezomib (Velcade, PS-341) has been approved for the treatment of multiple myeloma (MM) patients [39]. Boronates form tetrahedral adducts with active site threonines, which are stabilized by hydrogen bonding [40]. Vinyl sulfones were initially used as cysteine protease inhibitors, but were also found to be potent PI's [41, 42]. Vinyl sulfones are more readily synthesised than epoxyketones and have been used in many peptide inhibitors and activity based probes. Vinyl sulfones form a covalent adduct by conjugate addition of the hydroxyl-group of the active site threonine [43]. The last class of PIs discussed here are β -lactones, which form a covalent and relatively stable adduct to the proteasome by the attack of the catalytic threonine to the lactone, thereby forming a ester bond. In case of **Marizomib** (salinosporamide A, NPI-0052), the nucleophilic displacement of the chloride by the hydroxyl results in the formation of a tetrahydrofuran ring, which further stabilizes the adduct [44].

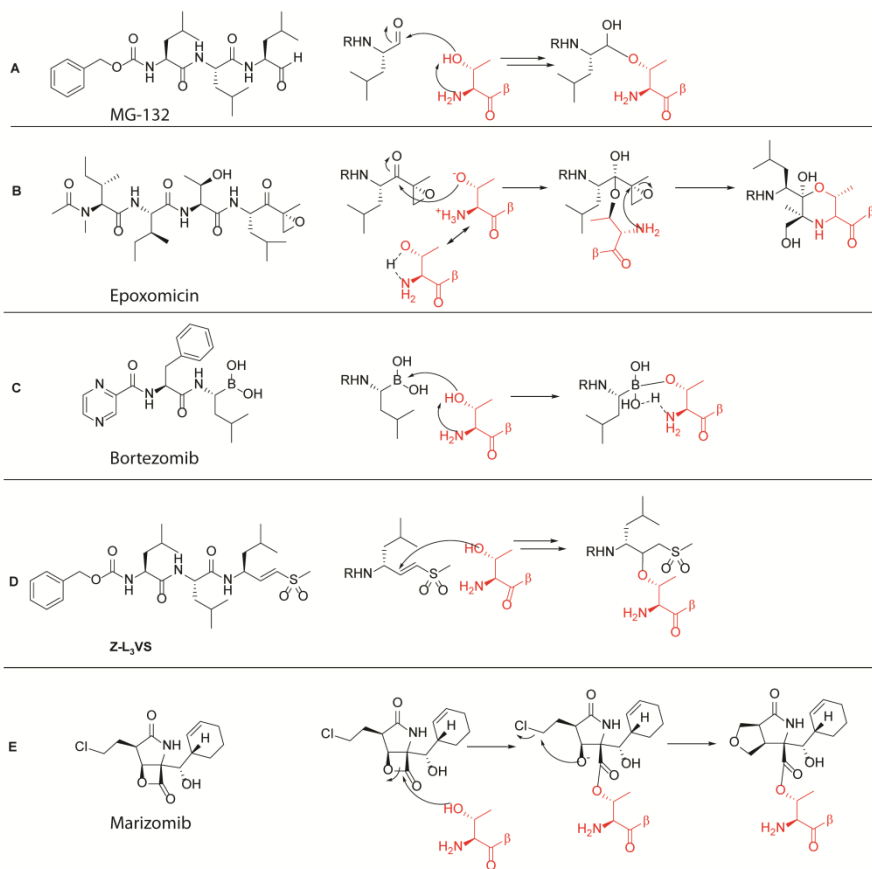


Figure 2. Chemical structures of covalently binding proteasome inhibitors and the reaction mechanism of the electrophilic trap with the N-terminal active site threonine of the proteasome. MG132 is an example of an aldehyde warhead, Epoxomicin contains an epoxyketone, Bortezomib bears a boronic acid, ZL3VS holds a vinylsulfone and Marizomib is an example of β -lactone warhead.

Proteasome inhibitors as drugs and clinical candidates

Figure 3 shows the molecular structure of several PIs currently used in the clinic or that are studied as clinical candidates. Bortezomib was the first PI approved by the FDA for the treatment of multiple myeloma (MM) and refractory mantle cell lymphoma (MCL). Based on the great success of Bortezomib [45], other proteasome inhibitors have entered clinical trials, and **Carfilzomib** was recently approved for the treatment of MM. Unfortunately,

patients treated with Bortezomib often develop resistance against the drug. The mechanisms behind Bortezomib resistance are poorly understood, but recent studies in cell lines indicated three main pathways by which cells can acquire resistance to PIs: 1) by upregulation of proteasome subunits, 2) by mutations in the $\beta 5$ subunit or 3) by upregulation of efflux pumps [46], although other mechanisms such as PI-resistant NF- κ B activity, upregulation of chaperones such as Hsp27, Gp78 and Hsp90, overexpression of anti-apoptotic proteins like Bcl2 and XIAP or activation of autophagy can confer resistance to Bortezomib [47].

Two other boronates are currently under clinical investigation. These are **Delanzomib** (CEP-18770) and **Ixazomib citrate** (MLN-9708). Delanzomib was developed as an orally available analogue of Bortezomib (administered intravenously [48]). Delanzomib showed promising results in toxicity studies and is currently under Phase I-II clinical investigations [49]. MLN-9708, another orally bioavailable boronate currently in Phase III trials (<http://clinicaltrials.gov>, Newly Diagnosed Multiple Myeloma NCT01850524; Relapsed and/or Refractory Multiple Myeloma NCT01564537; Relapsed or Refractory Systemic Light Chain (AL) Amyloidosis NCT01659658), has improved properties compared to Bortezomib, such as slower off rate, large volume of distribution, improved pharmacodynamics and pharmacokinetics and antitumor activity. Importantly, MLN-9708 also shows activity in solid tumors [50]. Peptide epoxyketones have entered clinical trials as well and Carfilzomib was recently approved for the treatment of MM. Carfilzomib causes higher inhibition of chymotrypsin-like activity (88%) at maximal tolerated dose than Bortezomib (70%) and also higher partial response rates than Bortezomib [51]. In addition, side effects are reduced upon treatment with Carfilzomib compared to Bortezomib, which may be due to a lower number of off targets thanks to the proteasome-specific electrophilic trap represented by the epoxyketone [52, 53]. Extensive medicinal chemistry studies led to the development of the orally bioavailable epoxyketone **ONX-0912**, which is currently in Phase I clinical studies for the treatment of MM [54]. Finally, the β -lactone, Marizomib, is under clinical investigation for the treatment of MM, leukemia, lymphomas and solid tumors. Marizomib is the most potent of all PIs under clinical investigations and inhibits not only $\beta 5$ but also $\beta 1$ and $\beta 2$ [55].

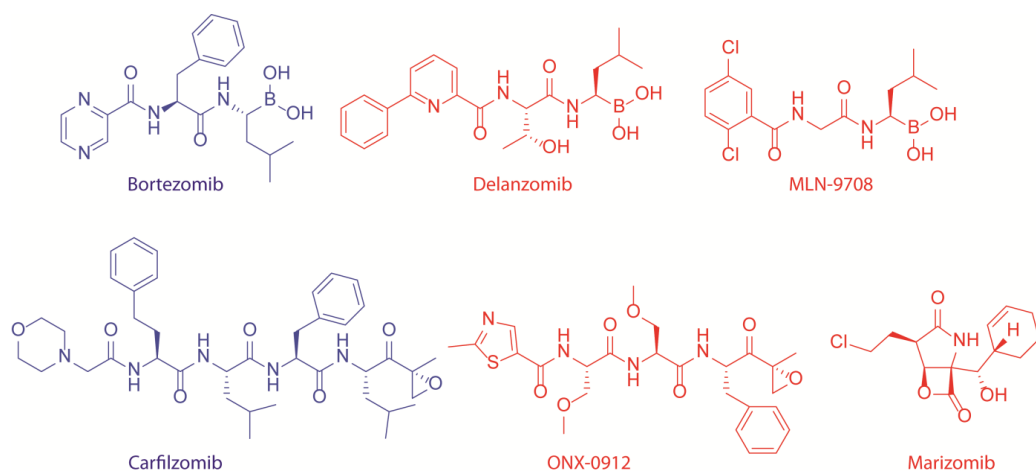


Figure 3. Chemical structures of drugs (blue) or drug candidates (red) based on proteasome inhibitors.

Current research aims at novel therapeutic applications of inhibitors/modulators of the ubiquitin proteasome system in cancer and other diseases which emphasizes the increasing importance of these compounds for the clinic. In this issue some of these topics will be discussed such as the design of small-molecule noncompetitive regulators that target proteasome function by allostery and dynamics [56], the design of small-molecule noncompetitive neddylation regulators for targeted anti-cancer therapy with less anticipated cytotoxicity compared to PIs [57], how impairment of the UPS is implicated in the pathogenesis of a wide variety of neurodegenerative disorders [58], the impact of proteasome inhibition and the potential prognostic value of proteasome activities in heart diseases [59] and in atherosclerosis [60] and why the proteasome is a promising therapeutic target to combat malignant tumour growth in the lung [61].

Activity based probes

In the past decades, various activity based probes (ABPs) for the proteasome β -subunits have been developed. Generally, ABPs consist out of three parts: 1. The electrophilic trap ('warhead'), which covalently modifies the active site threonine of the β -subunit; 2. A recognition element, providing recognition by β -subunits; and 3. A reporter group, such as a radiolabel, biotin or fluorophore. The first activity-based proteasome probe reported is [^3H]-lactacystin [62, 63], which was used to establish binding of lactacystin to all

proteasome β -subunits in a 2D-gel electrophoresis experiment. The natural product epoxomicin, which was found to exhibit antitumor effects, was found to target the proteasome by using **biotin-epoxomicin** as a probe, followed by detection of luminescence upon treatment with avidin-horseradish peroxidase [64, 65]. $^{125}\text{I-NIP-L}_3\text{VS}$ was used to prove that vinyl sulfones not only target cysteine proteases, but also covalently bind to proteasome β -subunits [41]. All β -subunit were visualized by NLVS in a 2D-gel electrophoresis experiment using autoradiography detection of ^{125}I . More recently, $^{125}\text{I-NIP-L}_3\text{VS}$ was used to screen for inhibitors of the proteasome by incubation of cellular extracts with a potential inhibitor, followed by labelling of residual proteasome activity by $^{125}\text{I-NIP-L}_3\text{VS}$. Next, the samples were resolved on SDS-PAGE and inhibition of a proteasome β -subunit is reflected by a decrease in intensity of the corresponding band [66].

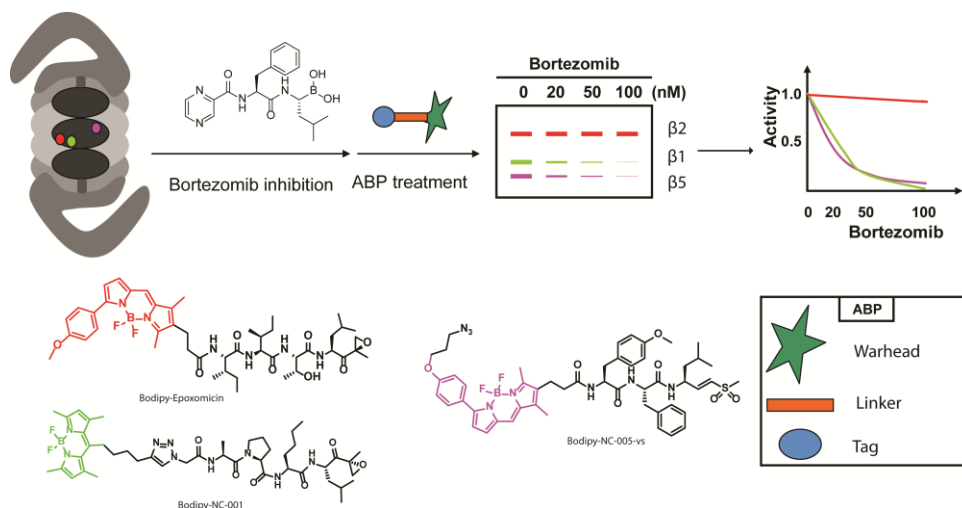


Figure 4. Schematic workflow of a competitive activity-based proteasome profiling (ABPP) experiment. After exposure to Bortezomib the residual proteasome activity was determined with two subunit specific ABPs for $\beta 1$ (BODIPY-NC-001) and $\beta 5$ (BODIPY-NC-005-vs), while the pan-reactive ABP (BODIPY-epoxomicin) will label residual $\beta 2$ subunit activity. Proteins were resolved by SDS-PAGE and band intensity can be quantified after scanning the gel. In this example a relatively insensitive cell line was tested hence the high Bortezomib concentrations used.

In the last years, various fluorescent ABPs for the proteasome subunits have been developed. The first such probe developed is the weakly fluorescent **dansyl-Ahx₃-L₃-VS** [67], which was followed by the BODIPY TMR containing **MV151** [68] and **Bodipy-FL-Ahx₃-L₃-VS** [69]. Using pan-reactive fluorescent probes MV151 and **BODIPY-epoxomicin** [70], all

subunits of both the constitutive and immunoproteasome can be visualized by fluorescent scanning, directly after resolving cellular extracts that have been incubated with the probes on SDS-PAGE. Figure 4 shows a schematic overview of the work-flow used for activity-based proteasome profiling. Visualization of proteasome activity by fluorescent ABP is straight forward, time efficient and provides higher resolution compared to either biotinylated or radiolabelled probes. MV151 and BODIPY-epoxomicin can be used both in living cells and in cell extracts [68, 70]. Next to ABPs that target all subunits, β -subunit specific ABPs have been developed. Based on NC-001, **BODIPY-NC-001** shows highly specific labelling of both β 1c and β 1i, without labelling of the other subunits. The same applies to **BODIPY-NC-005-vs**, which is based on NC-005-mvs, which only labels β 5c and β 5i subunits [70]. However, generating a fluorescent ABP for β 2 proved to be more difficult: attachment of a BODIPY fluorophore to **LU112** yielded a compound that also labels β 5 [71]. Therefore a two steps labelling using **N₃-NC-002** has to be used to specifically label β 2 subunits [72]. **UK101-B660** and **UK101-Fluor**, both based on the β 1i selective inhibitor UK101, are used to selectively label β 1i, both in cell extracts and in living cells [73]. Interestingly, the fluorophore is not attached to the N-terminus of the inhibitor, but to the P2 substituent, since the S2 pocket is rather large and solvent exposed, allowing for the introduction of bulky substituents. In general the proteasome ABPs can be used to quantify relative proteasome activity, to perform competitive activity based protein profiling (ABPP) to test the inhibition profile of potentially new inhibitors and as imaging probes according to the scheme in figure 4 [74].

Molecular mechanisms of proteasome inhibitors-induced apoptosis

Review of clinical, preclinical and biochemical literature on the use of PIs in organisms, tissues and cells shows several corroborative observations: PIs induce cell cycle arrest and caspase mediated apoptosis that somehow affects oncogenically transformed cells more than healthy tissues [75]. This suggests that proteasome inhibition impacts stronger on fast proliferating tissues [76] and that PIs are remarkably “clean” in their mode of action by specifically targeting only the active subunits of the proteasome [36, 77]. Having said so, a plethora of exceptions to this dogma have become known. For instance, PIs do show adverse effects in the clinic, indicating that healthy tissues are affected, the anti-neoplastic effects are limited to the treatment of several fast proliferative myeloma types of cancers but is less effective against quiescent cells or solid tumours and transformed cells evolve resistance to PI treatments [78]. Work in cell cultures showed that prerequisite of PIs for apoptosis induction is that they should deactivate at least two out of the three active proteasome subunits with potencies that eliminate >50% of the

subunits activity [79, 80]. It has been observed that multiple myeloma cells showing increased proteasome stress (balance between poly- and free ubiquitin) with the proteasome workload exceeding the proteasome capacity to process substrates are more sensitive to PI induced apoptosis [81]. Next to this, tremendous scientific efforts have been undertaken in the past decade to unravel the molecular mechanisms of PI-induced apoptosis. It is remarkable that proteasomes – major factors in protein homeostasis in every cell type – are in fact valid therapeutic targets and a detailed understanding in the mode of action of clinical proteasome drugs in effecting apoptosis may give invaluable information for designing future drugs. What makes understanding the mechanisms of apoptosis induction by PIs even more complicated is that the knowledge of the cell biological basis evolved alongside the development of more specific PIs leading to several controversies in the literature. On top of this, the above mentioned cellular effects of PIs may induce both cell protective and cell death pathways simultaneously, stressing the need to understand the kinetics and the cross-talk between the different effects.

Besides its role of guarding the cellular amino acid homeostasis by degrading damaged or misfolded proteins, the UPS is instrumental for defining the repertoire of peptides used for antigen presentation of every cell and is vital for regulating signal transduction molecules that decide between cell survival and cell death both in the cytoplasm and the nucleus [36, 82]. Proteasome inhibition has been reported to have numerous effects on cells [82, 83], including the following: 1) Cell cycle arrest by activation of G2/M checkpoints, 2) Perturbation of cyto-protective and pro-death signaling transduction pathways, 3) ER stress and Ca^{2+} release, 4) Oxidative stress by Reactive Oxygen Species (ROS) production, 5) Depolarization of mitochondrial potential, and as a consequence of these effects, apoptosis. Also in patients, cell death is caused by non-cell autonomous mechanisms such as inhibition of IL6 secretion, inhibition of VEGF secretion and angiogenesis [77, 78].

In the mid 90's, the discovery that the UPS regulates cell cycle progression [84] and NF κ B signalling [85] combined with early observations that PIs induce apoptosis [76], sparked the idea that the proteasome might be a suitable anti-neoplastic target that should be targeted with specific inhibitors [86]. Cell cycle progression is driven by oscillations in the activity of cyclin-dependent kinase (CDK) complexes with cyclins. CDK is activated by cyclins (short-lived regulatory proteins that undergo fast degradation at exit from cell cycle) and inhibited by p21 (WAF1/CIP1) or p27 (KIP1) proteins at the G2/M and G1/S transitions, respectively [84]. Cell cycle dependent phosphatases (CDC25A-C) antagonize the CDK complex kinase activity to ensure strict control of the cell cycle and fidelity of proliferation. Levels of all proteins engaged in this pathway is tightly controlled by the UPS and intervention via PIs disrupts the cell cycle accompanied by observations of p53

stabilization, decrease in NFkB level and accumulation of CDK complex activators and inhibitors, in different cell types both in cell culture as in animal models [36, 86-88]. It is not clear why cells arrest at the G2/M and not at the G1/S checkpoint but this might be explained by cell cycle dependent life-time of the p27 protein [89] or by the inability of the UPS to degrade cohesion complexes that hold together sister chromatids prior to mitosis [90]. It is also not clear how cells induce apoptosis under prolonged G2/M arrest. Investigations focused on the elevated levels of the tumour suppressor protein p53 as signalling molecule in this. The p53 protein is a short-lived sensor of DNA damage and oncogene activation, and in non-stressed p53 levels are maintained at low concentration via ubiquitination by the specific E3 ligase MDM2 [91]. Stress and DNA damage sensing kinase pathways (MAPK and ATM) mediated p53 phosphorylation [92] prevents its degradation and activates the transcription factor function of p53 that drives the expression of pro-apoptotic genes like Bax [93]. Early investigations showed a controversial role for the tumour suppressor gene p53 upon PI exposure where apoptosis was p53 dependent [94], p53 independent [95, 96] or showed mixed effects [87] indicating that forced accumulation of p53 might not be an universal pathway for PI induced apoptosis.

The nuclear factor-kB (NFkB) family is an ubiquitously expressed group of transcription factors essential for leukocyte differentiation that drive a strong pro-survival program encompassing the synthesis of growth factors such as interleukin-6 (IL-6), cell adhesion molecules (E-selectin), detoxifiers (COX2 cyclooxygenase-2, NOS nitric oxide synthase) and anti-apoptotic factors (Bcl-2) in response to noxious stimuli including (oxidative) stress, bacterial/viral antigens, inflammation and UV radiation [36]. NFkB is sequestered in the cytoplasm by its inhibitory binding partner (Ikb), which after receptor activation is phosphorylated, poly-ubiquitinated and degraded via the UPS allowing the free NFkB to translocate to the nucleus and activate transcription [85, 97]. Reports from the Anderson lab [87, 98, 99] revealed the elevated NFkB activity in hematopoietic cancers, which justifies the rationale of using PIs to inhibit this pathway for malignant cell survival. Of particular interest is their analysis of the gene expression profile in a MM cell line exposed to Bortezomib at concentrations that induce cell cycle arrest and apoptosis [98]. Data showed the expected down regulation of survival pathways and anti-apoptotic proteins as well as up-regulation of cell death signals via the canonical mitochondrial release of cytochrome C and caspase 9 activation but also via the Jun kinase and death receptor/caspase 8 dependent apoptotic pathway. Expression of the 26S proteasome complex genes was found elevated and surprisingly protein folding chaperones like heat shock protein 70 went up indicating activation of a stress response. It was reported

previously that NFκB inhibition might not be enough to induce cell death in MM cells [100], a finding supported by studies on carcinoma cells [101] and myeloma cells [102].

In 2003, three studies, with partially overlapping observations, of PI induced apoptosis appeared. The combined results suggest that a) disruption of the unfolded protein response leads to apoptosis [103], b) generation of reactive oxygen species (ROS) and mitochondrial dysfunction triggers apoptosis [104] and c) induction of the pro-apoptotic Jun kinase pathway together with ROS kills leukemic cells [105]. Accumulation of polyubiquitinated and improperly folded proteins is an undisputed result of proteasome activity inhibition that imposes an unfolded protein burden on the ER [102, 106]. The ER is the cell organelle that serves functions in lipid metabolism, regulated Ca²⁺ storage and chiefly, the assembly, folding and post-translational modification of newly synthesized proteins [107]. Misfolded proteins are retained in the ER and retrotranslocated into the cytosol for proteasome based degradation, a process called ER-associated degradation (ERAD) [8]. PIs can block ERAD, leading to protein accumulation in the ER, which activates the cytoprotective phase of the Unfolded Protein Response (UPR), but also cause cytosolic accumulation of misfolded proteins in the nucleus and cytosol and the heat-shock response in the cytosol. The UPR consists of three branches activated by distinct sensors: the rapidly induced PERK (double-stranded RNA-activated protein kinase (PKR)-like ER kinase), the evolutionarily conserved IRE-1 (inositol requiring enzyme 1) and the ATF6 (activating transcription factor 6), recently reviewed by Hetz [108]. The three sensors are transmembrane proteins that contain a luminal ER domain that interacts and senses unfolded proteins and a cytosolic part that conveys the signal to the nucleus in order to modulate gene expression programs. The initial signals from the UPR as conveyed by PERK result in a general slowdown in protein synthesis for immediate alleviation of the ER protein burden. IRE-1 in turn induces the synthesis of lipids, ERAD proteins and chaperones to increase the ER protein processing capacity and ATF6 induces the synthesis of ER-resident protein folding chaperones such as BiP (member of the heat shock protein HSP70 family [109]). Interestingly, the UPR seems to be activated in two waves: a first acute signalling through PERK, IRE-1 and an autophagy signal aimed mainly at repressing protein synthesis, followed by a second wave of IRE-1, ATF6 and PERK signalling to accommodate and equip the ER for facing up to a stress situation [108]. However, in the case of sustained ERAD block and protein burden, the IRE-1 and ATF6 signals decline while the PERK signalling persists and eventually leads to apoptosis induction via the eIF2α/ATF4/CHOP pathway [110].

The question how a basically cytoprotective pathway like the UPR, can also drive a cell's commitment to apoptosis has just recently been elucidated [111] and it might be physiologically relevant for host defence against the intracellular organisms

Mycobacterium tuberculosis [112]. PERK-mediated phosphorylation of the ubiquitous translation initiating factor eIF2 α leads to its inactivation and thereby to global inhibition of mRNA translation but specifically induces ATF4 (activating transcription factor 4) translation [113]. ATF4 drives the expression of another transcription factor, CHOP (C/EBP-homologous protein), which has pro-apoptotic effects by repressing transcription of the anti-apoptotic Bcl-2 protein, induction of TRAIL-R2 death receptors that activate caspase 8 mediated apoptosis, cytotoxic Jun kinase activation and elevation in ROS by upregulating the ERO1 α (ER oxidase 1 α) that promotes disulfide bond formation in newly translated proteins. [110] The recent mechanism proposed by the Kaufman lab [111] states that immediately after an insult eIF2 α phosphorylation slows down translation and subsequent induction of ATF4/CHOP and their downstream gene targets function to restore protein synthesis. In case protein synthesis increases before proteostasis equilibrium is achieved, ERO1 α activity continues to increase the ROS levels driving the cell in a pro-apoptotic state that will lead to cell death.

Dissection of the timing of activation, the sequence of events and the magnitude of the signal induced by the three UPR branches discussed above has been performed both in cell lines [114] and in tissues of UPR gene defective mice [113] using ER specific inhibitors of protein folding and trafficking like thapsigargin (Tg) and tunicamycin (Tm). Although ER stress induction by PIs is undisputed in the literature, the nature of a PI effect on the three UPR branches is less clear. At one end of the spectrum Bortezomib induced apoptosis by disrupting the IRE1 signalling in myeloma cells [103] or by inhibiting PERK and eIF2 α phosphorylation but activating the ER resident caspase 4 mediated apoptosis in pancreatic cells [115]. These observations can be explained from the mechanism detailed above: although IRE1 signalling seemed disrupted [103], the paper showed a clear accumulation of CHOP that can drive apoptosis. In pancreatic cells CHOP and eIF2 α activities were found [115] which according to the Kauffmann model [111] of ER-stress-induced transcription regulation increase protein synthesis leading to apoptosis. At the other side of the spectrum, PIs were found to induce apoptosis via the PERK/ATF4/CHOP terminal UPR in multiple myeloma [102] and head and neck squamous cell carcinoma cells [101]. Interestingly, PI induced UPR via PERK can also activate the expression of cytoprotective elements like the anti-apoptotic Mcl-1 protein [116] and the Nrf2 (nuclear factor-erythroid 2-related factor 2) transcription factor. Moreover, accumulating evidence points towards the existence of an ER-mediated apoptotic cascade proceeding via the ER-resident caspase 4 activation [117-119] besides the two canonical apoptosis pathways regulated by death receptors via caspase 8 and mitochondrial damage in conjunction with caspase 9.

A second mechanism of PI induced apoptosis that has been the subject of intense scrutiny comes from the observation that PIs cause intracellular ROS levels to steadily rise inducing

an oxidative state that pushes the cell towards cell death. Anti-oxidants like vitamin C, *N*-acetylcysteine and glutathione are able to quench the ROS molecules and prevent apoptotic death [120] except in one study that found that vitamin C can complex to Bortezomib preventing it from inhibiting the proteasome [121]. Although studies with PIs equipped with structurally unrelated warheads to Bortezomib [122, 123] show cytoprotective effects upon antioxidant treatment, the results of antioxidant studies should be interpreted carefully.

Cells robustly maintain their reduction/oxidation (redox) homeostasis in a reducing state to prevent oxidative damage or degradation of vital bio-molecules [124]. This “reductive field” regulates levels of ROS molecules providing them a physiological function as signalling molecules for differentiation, cell cycle progression, growth arrest and apoptosis. ROS molecules such as the superoxide anion ($O_2^{\bullet-}$) hydroxyl radical (OH^{\bullet}), hydrogen peroxide (H_2O_2) and several other organic radicals are either side products of electron transport chains in the mitochondrial respiration cycle, of enzymatic metabolism (for instance, p450 cytochrome) or function as signalling molecules produced by the NADPH oxidases (NOX) family [125]. From the atoms necessary for life, sulphur is easily oxidized and sulphur containing amino acids like methionine and cysteine are prone to react with ROS. Cysteine is the main nucleophile in the active site of most phosphatases [126], ubiquitin chain E1,E2,E3 ubiquitin ligases and their antagonists deubiquitinating enzymes [127] which are essential enzymes for the post-translational control of vital pathways in the cell. Evidence accumulates that ROS can exert both physiological and pathological effects by oxidizing active cysteines and that a plethora of regulatory proteins (NFkB, p53, pyruvate kinase, ATM, amongst others) have evolved ROS sensing propensities by strategically incorporating cysteine residues that upon reacting with ROS influence the activity of the protein [128].

ROS are continuously produced by leakage of electrons in the mitochondrial respiratory chain, in the ER by the activity of the ERO1 α flavoenzyme needed for disulfide bond formation of newly translated proteins, in phagosomes for host defense against microorganisms and by NOXs at the cytosol side of the plasma membrane upon recruitment by major signalling receptors to participate via ROS production to the amplification of their signalling cascades in growth and proliferation (e.g. neuronal growth factor, NGF), immune response (e.g. toll like receptors, TLR) and apoptosis (e.g. tumor necrosis factor α , TNF α) [129]. Although the first evaluations that PI induced ROS is necessary for apoptosis were performed in solid-tumour model systems [101, 104], the PI effects in hematopoietic malignancies like mantle cell lymphoma [96], leukemia cells [123] and MM [130, 131] showed to be more robust. Mitochondria, and ER-stress induced ROS [101], were indicated as source of ROS generation because all studies found a decrease of

the mitochondrial membrane potential ($\Delta\Psi_m$) that is indicative of mitochondrial damage leading to leakage of ROS in the cytoplasm. Mitochondria received most attention because they are both a ROS producer and a convergence point for ROS induced apoptosis which upon damage release cytochrome C that together with Apaf-1 and pro-caspase 9 form the canonical apoptosome system that activates executioner caspases to induce cell death [120]. Treatment with anti-oxidants reduced the ROS levels and the apoptotic events, indicating that ROS play an essential role in PI induced apoptosis. However, the use of organelle specific ROS reporters would be advisable for the future for more precise determination of the ROS source.

Interestingly, differences in apoptosis induction pathways were found between different PIs with Bortezomib mainly functioning through the mitochondria/caspase 9 pathway [101, 104, 130] and NPI-0052 mainly through the FADD (Fas associated death domain)/caspase 8 pathway [123, 130]. Bortezomib has been shown to repress the cytoprotective Bcl-2 protein [130] leading to release of the pro-apoptotic Bax protein which together with Bak injures the mitochondria [132]. Activation of the FADD is more difficult to explain but it might proceed via JNK signalling or terminal UPR response to ER stress [110]. It should be mentioned that the proapoptotic Bak and Bax proteins also reside in the ER and are suggested to regulate Ca^{2+} storage and apoptotic events [133]. Release of Ca^{2+} in the cytoplasm can trigger apoptosis by activating the Ca^{2+} dependent CaMKII that signals to downstream apoptotic pathways [134]. Alternative mechanisms of Bortezomib induced apoptosis are the stabilization of the pro-apoptotic protein NOXA in medulloblastoma independently of p53 activity [135] or p53 dependent in mantle cell lymphoma [96]. Both were ROS dependent and function because NOXA binds to and displaces the anti-apoptotic Mcl-1 from a complex with Bak [136], which upon release binds to Bax promoting mitochondrial injury. As an exception, PI induced but ROS independent apoptosis was found in colon cancer models to proceed by p53 stabilization, driving the expression of pro-apoptotic PUMA (p53 up-regulated modulator of apoptosis) that in turn promoted Bax activated apoptosis [83]. Evidence is accumulating that the ER plays a central role in PI mediated apoptosis by release of Ca^{2+} in the cytoplasm, UPR signalling and via an ER resident caspase 4 pathway [117-119] that gets activated upon ER stress. Recent work shows that caspase 4 is recruited to the ER by transmembrane protein 214 (TMEM214), which was essential for ER stress-induced pro-caspase-4 activation and apoptosis [119]. Taken together, a logic interpretation is that ROS report upon the stress state and integrity of an organelle like the ER or the mitochondrion and in some cases, excessive ROS production might be a symptom of their injury.

The onset of apoptosis by PIs has been linked to activation of intracellular stress sensing kinase cascades like the MAPK (mitogen-activated protein kinase) pathways, which

physiologically govern cell proliferation, stress response and survival [137]. Of the three MAPK modules, the Jun-N-terminal kinase (JNK) and p38 MAPK branches are associated with induction of apoptosis while the extracellular signal-regulating kinase (ERK) is cytoprotective [138]. PIs appear to induce apoptosis in MM cells in part by suppressing ERK and activating JNK [105, 137, 139], which was accompanied with increased ROS production. It remains unclear whether ROS production led to JNK activation and ERK repression or merely a symptom of damaged mitochondria after action of Bax-Bak membrane destabilizing complexes. Interestingly, caspase 8 activation was found to take place [105, 137] indicating that the extracellular death receptor pathway was activated. Studies of ER stress and JNK activation showed that the IRE1 branch of UPR binds to TRAF2 (TNF receptor associated factor 2) an adaptor protein of the TNF α receptor and via a kinase signalling cascade can activate JNK [140]. Recent studies, reviewed by David Ron [110], show that both IRE1 and the PERK branches of the UPR can activate the pro-apoptotic JNK pathway or directly interact with mitochondrial membrane permeabilizing factors which link the ER stress effects of PIs with the four known pathways of apoptosis induction via the extrinsic caspase 8, the mitochondrial caspase 9, the ER resident caspase 4 and the Ca²⁺ dependent CaMKII. Lately, Bortezomib has been used in clinical experiments with organ transplantation as an agent to deplete healthy plasma cells that produce donor-specific anti-human leukocyte antigen antibodies (DSAs), responsible for graft rejection (reviewed by the Woodle group [141]). Although the mechanism of cell death is not known, this work suggests that some of the mechanisms discussed above are also at play in healthy tissues.

Resistance to proteasome inhibitors

PIs ability to overcome the resistance to classic anticancer therapies brought about a wave of initial enthusiasm [87]. However, PI resistant tumour clones appeared that employ various mechanisms of protection including upregulating proteasome synthesis (80), drug efflux pumps such as Pgp [142], and PI metabolizing enzymes [78]. Interestingly, PI induced UPR via PERK can also activate the expression of cytoprotective elements such as the anti-apoptotic Mcl-1 protein [116] and the Nrf2 (nuclear factor-erythroid 2-related factor 2) transcription factor. Nrf2 phosphorylation liberates it from its inhibitor Kelch-like ECH-associated protein 1 (KEAP1), driving the expression of some 200 genes involved in oxidative stress/redox signalling [143] which can ensue resistance to PI treatment.

Constitutive activation of Nrf2 is emerging as a prominent molecular feature in many tumour types [144] and Nrf2 phosphorylation likely restores the redox balance in

oxidatively stressed tumours and clears electrophilic xenobiotics. Elevated Nrf2 levels in acute myeloma leukaemia [145] correlated with reduced ROS levels and sensitivity for Bortezomib treatment and it has been shown that Nrf2 and Nrf1 [146, 147] also upregulate the expression of proteasome genes that increases the capacity to remove damaged proteins and scavenge PIs [148]. Other mechanism of PI resistance in human myelomonocytic THP1 cells were an Ala49Thr mutation in the highly conserved binding pocket of the $\beta 5$ subunit accompanied by overexpression of the PSMB5 gene [149]. Another study of induced Bortezomib adaptation in leukaemia and myeloma cells showed increased expression of functional $\beta 5$, $\beta 2$ and $\beta 1$ subunits, 11S activator caps, alongside reduced protein biosynthesis and transcription of chaperones [150].

Aggresome formation [115], upregulation of chaperones HSP27 and HSP70 [115] and autophagy are also pathways that convey resistance to PIs. Autophagy mediates the breakdown of insoluble protein aggregates and aggresomes in the cytosol through encasing it in a double-layered membrane that are later fused with the lysosome for degradation into its constitutive components [151]. Autophagy can take over the processing of proteasome substrates, mitigating cellular stress and ultimately apoptosis and cell death. The link between autophagy and the proteasome is still uncertain but evidence is pointing towards ER-stress mediated autophagy [152, 153]. However, the role of autophagy in PI induced apoptosis is controversial as some studies suggest that autophagy is a mechanism of resistance to PIs [152, 153] while others suggest that autophagy might enhance PI lethality perhaps depending on the cell type and the cell state being either normal or oncogenically transformed [154-156]. An intriguing study in yeast proteasomes showed that S-glutathiolation, a post-translational modification, controls the 20S gate opening. The 20S CP itself might be under redox control as the activity of S-glutathiolation on two discrete cysteine residues of the $\alpha 5$ subunits that control 20S gate opening proved to open the gate, increasing the protein processing power of the proteasome [157].

It should be noted that the mechanistic knowledge discussed here comes from studies in different cell culture models, primary cell cultures and animals. Immortal cell cultures often have disturbed genetic patterns that might not reflect the defects encountered during oncogenesis *in vivo*, so they might react differently to PI stress. The un-physiologically high glucose concentrations in cell culture media might be taken into account as well; it affects the cell metabolism and may influence the PI response. There are clear differences in PI response between tissues, as hematopoietic cells show fast activation of terminal UPR, intensified ROS production, mitochondrial damage and onset of apoptosis. In contrary, adherent growing neuronal, epithelial or fibroblast cell models or the ones closer to normal tissues like MEFs (mouse embryonic fibroblasts) show a

higher tolerance to PI requiring higher PI dosing, more delayed apoptotic responses, less ROS induction and apoptosis onset via other pathways than the mitochondrial. This might be in part explained by the composition of the proteasome in the cell, either constitutive or immune proteasome which is often overlooked in studies but it is not unimportant because PIs have different affinities for constitutive and immune proteasome. Physiological details like the total proteasome concentration in the cell, the presence of efflux pumps and perhaps the shape of the cell might matter. In a spherical cell mitochondria might be closer to the ER thus ROS species emanating from the stressed ER [109] might reach other organelles faster by diffusion and affect a larger area than in the case of a polarized and elongated cell where the signalling gradient might be more diffuse giving the cell more time to take counteractive actions by activating defence mechanisms like anti-oxidant or anti-apoptotic factors production. It has been postulated that secretory cells like B-cell that produce large quantities of antibodies or β -cells from pancreatic islets endowed with the production of insulin poses an intrinsically stressed ER that activates a terminal UPR earlier than other cell types [78]. Poor oxygen transport into solid tumours might render the cells hypoxic, which activates the HIF1 α (hypoxia induced factor) that in turn can activate Nrf2 to drive ROS quenching genes [128] making these cells less vulnerable to PI induced ROS.

In conclusion, this review illustrates that many genes and cellular events are involved in PI-induced apoptosis. Global systems biology approaches may be used to identify the gene partners, their regulation, post-translational modification and kinetics in order to establish which pathway is chiefly responsible for induction of apoptosis. A cell's decision to commit to apoptosis might be a convergence of signals from several pathways underscoring the need for system wide analysis. Moreover, ROS and Ca²⁺ levels should also be determined because these factors have important regulatory and signalling function. First of all, the concentration, constitution and activity of the proteasome in a given cell population should be determined [74]. Second, global mass spectrometry driven proteomics studies can be used to determine the protein concentrations of as much as possible proteins in order to see which pathways are up or down regulated. Third, analysis of the transcriptome is necessary to determine the response at the level of gene transcription. Fourth, these measurements should be performed at several time points after PI treatment to determine the kinetics of different pathway responses. With the advent of superior MS methods and machines, determination of PTM status of proteins has become increasingly feasible as in the case of kinases [158] and phosphatases [126] activities. An interesting method to probe the reactivity of cysteine side chains has been recently launched [159] which might be instrumental for determination of the oxidation state of proteins, an important PTM to be scored when dealing with ROS induced phenomena.

This technique uses a smart adaptation of the general alkylation principle of cysteines by iodoacetamide combined with global activity based profiling, to determine the hyper reactive cysteines in the proteome suggested to play a role in the catalytic site of enzymes or function as ROS sensors (see Figure 5). Combination of these techniques might provide us with a clearer picture of the course of events during PI induced apoptosis and will surely afford novel start points for therapy.

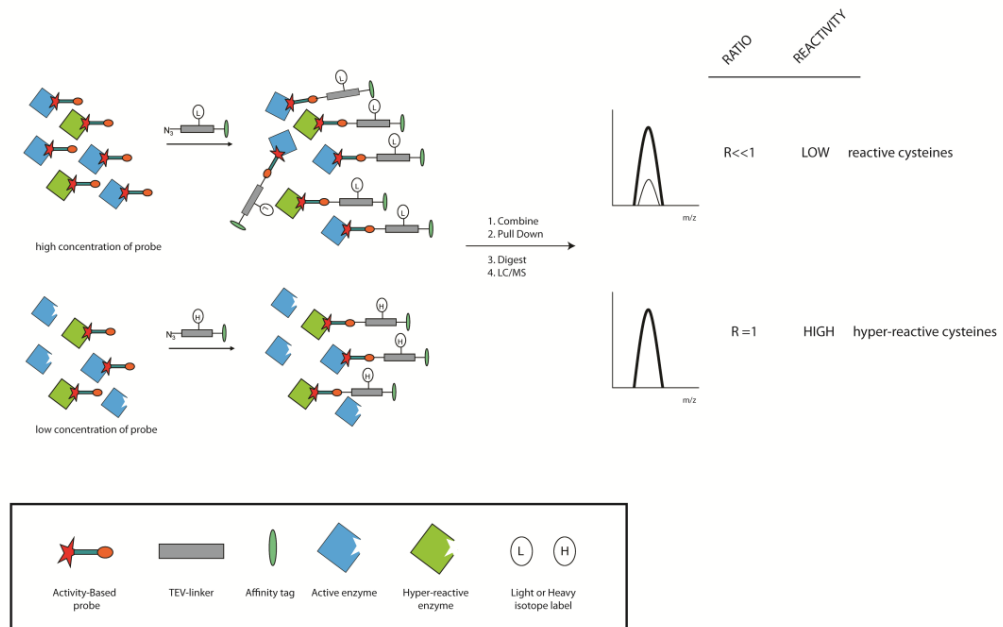


Figure 5. Basic scheme of two-step ABPP coupled with isotope labeling which allows quantification of the cysteine reactivity status.

The global picture emerges that under physiological conditions, the cell is kept in a reductive state in order to prevent oxidative damage of essential bio-molecules like DNA, RNA, proteins and lipids. Blocking the proteasome in the nucleus, cytoplasm and in the ERAD leads to arrest of NF κ B signalling, increased p53 levels, cell cycle arrest, ER stress inducing some form of UPR signalling, possible ER-resident caspase 4 activation and elevated production of ROS. Release of Ca²⁺ from the ER, depletion of glutathione pools and signalling via the JNK pathway injure the mitochondria impairing the oxidative phosphorylation energy pathway which further increases ROS production pushing the cell in an oxidative phase. If protein synthesis continues, ROS production will further damage the mitochondria induce cytochrome C release and activation of caspase 9 that, in

conjunction with caspase 8 activation via upregulation of death receptor signalling, mediate the onset of cell death by apoptosis (Figure 6).

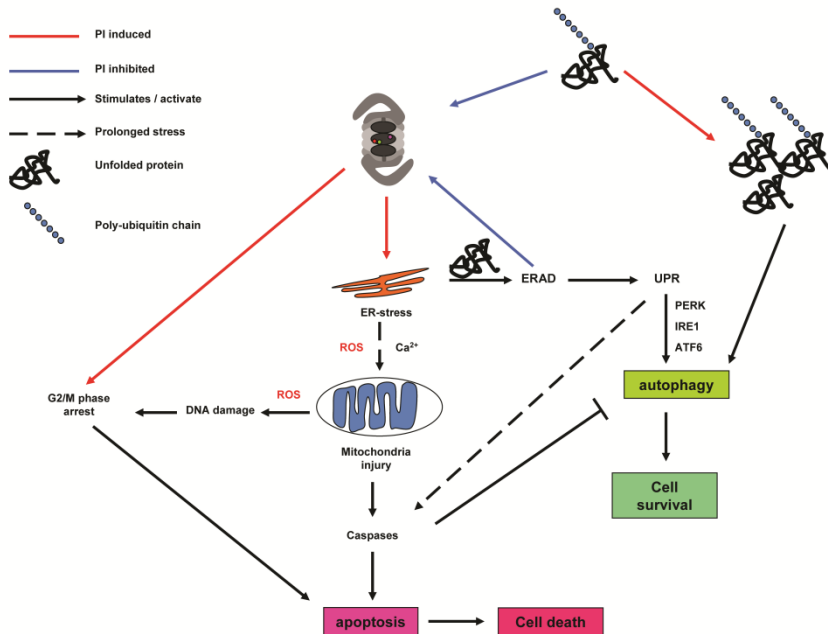


Figure 6. Global picture of our view of the cell behavior after PI exposure. Under physiological conditions, proteins assigned for degradation are cleared by the ubiquitin proteasome system. Acute proteasome inhibition leads to adverse effects on cells: cell cycle arrest in the G2/M phase checkpoint, ER stress, activation of the UPR system and accumulation of polyubiquitinated proteins that in some cases are cleared via autophagy. Sustained proteasome inhibition leads to release of ROS, mitochondrial injury, DNA damage, activation of cell death pathways and onset of apoptosis.

References

1. Baumeister, W., et al., *The proteasome: paradigm of a self-compartmentalizing protease*. Cell, 1998. **92**(3): p. 367-380.
2. Hershko, A. and A. Ciechanover, *The ubiquitin system*. Annu. Rev. Biochem., 1998. **67**: p. 425-479.
3. Hershko, A., *The ubiquitin pathway for protein degradation*. Trends Biochem. Sci., 1991. **16**(7): p. 265-268.
4. Grune, T., T. Reinheckel, and K.J. Davies, *Degradation of oxidized proteins in mammalian cells*. FASEB, 1997. **11**(7): p. 526-534.
5. Groettrup, M., C.J. Kirk, and M. Basler, *Proteasomes in immune cells: more than peptide producers?* Nat. Rev. Immun., 2010. **10**(1): p. 73-78.
6. Kloetzel, P.M., *Antigen processing by the proteasome*. Nat. Rev. Mol. Cell Bio., 2001. **2**(3): p. 179-187.

7. Kloetzel, P.M. and F. Ossendorp, *Proteasome and peptidase function in MHC-class-I-mediated antigen presentation*. *Curr. Op. Immu.*, 2004. **16**(1): p. 76-81.
8. Smith, M.H., H.L. Ploegh, and J.S. Weissman, *Road to Ruin: Targeting Proteins for Degradation in the Endoplasmic Reticulum*. *Science*, 2011. **334**(6059): p. 1086-1090.
9. Lowe, J., et al., *Crystal structure of the 20S proteasome from the archaeon T. acidophilum at 3.4 Å resolution*. *Science*, 1995. **268**(5210): p. 533-539.
10. Groll, M., et al., *Structure of 20S proteasome from yeast at 2.4 Å resolution*. *Nature*, 1997. **386**(6624): p. 463-471.
11. Unno, M., et al., *Structure determination of the constitutive 20S proteasome from bovine liver at 2.75 Å resolution*. *J. Biochem.*, 2002. **131**(2): p. 171-173.
12. Gallastegui, N. and M. Groll, *The 26S proteasome: assembly and function of a destructive machine*. *Trends Biochem. Sci.*, 2010. **35**(11): p. 634-642.
13. Tomko, R.J., Jr. and M. Hochstrasser, *Order of the proteasomal ATPases and eukaryotic proteasome assembly*. *Cell Biochem. and Bioph.*, 2011. **60**(1-2): p. 13-20.
14. Tanaka, K., T. Mizushima, and Y. Saeki, *The proteasome: molecular machinery and pathophysiological roles*. *Biol. Chem.*, 2012. **393**(4): p. 217-234.
15. Tanaka, K., *Role of proteasomes modified by interferon-gamma in antigen processing*. *J. Leuko. Biol.*, 1994. **56**(5): p. 571-575.
16. Akiyama, K., et al., *cDNA cloning and interferon gamma down-regulation of proteasomal subunits X and Y*. *Science*, 1994. **265**(5176): p. 1231-1234.
17. Griffin, T.A., et al., *Immunoproteasome assembly: cooperative incorporation of interferon gamma (IFN-gamma)-inducible subunits*. *J. Exp. Med.*, 1998. **187**(1): p. 97-104.
18. Groettrup, M., et al., *The subunits MECL-1 and LMP2 are mutually required for incorporation into the 20S proteasome*. *PNAS*, 1997. **94**(17): p. 8970-8975.
19. Huber, Eva M., et al., *Immuno- and Constitutive Proteasome Crystal Structures Reveal Differences in Substrate and Inhibitor Specificity*. *Cell*, 2012. **148**(4): p. 727-738.
20. Murata, S., et al., *Regulation of CD8+ T cell development by thymus-specific proteasomes*. *Science*, 2007. **316**(5829): p. 1349-1353.
21. Tomaru, U., et al., *Exclusive expression of proteasome subunit {beta}5t in the human thymic cortex*. *Blood*, 2009. **113**(21): p. 5186-5191.
22. Florea, B.I., et al., *Activity-based profiling reveals reactivity of the murine thymoproteasome-specific subunit beta5t*. *Chem. & Biol.*, 2010. **17**(8): p. 795-801.
23. Drews, O., et al., *Mammalian proteasome subpopulations with distinct molecular compositions and proteolytic activities*. *MCP*, 2007. **6**(11): p. 2021-2031.
24. Guillaume, B., et al., *Two abundant proteasome subtypes that uniquely process some antigens presented by HLA class I molecules*. *PNAS*, 2010. **107**(43): p. 18599-18604.
25. Klare, N., et al., *Intermediate-type 20 S proteasomes in HeLa cells: "asymmetric" subunit composition, diversity and adaptation*. *J. Mol. Biol.*, 2007. **373**(1): p. 1-10.
26. Vigneron, N. and B.J. Van den Eynde, *Proteasome subtypes and the processing of tumor antigens: increasing antigenic diversity*. *Curr. Op. Immu.*, 2012. **24**(1): p. 84-91.
27. De, M., et al., *Beta 2 subunit propeptides influence cooperative proteasome assembly*. *J. Biol. Chem.*, 2003. **278**(8): p. 6153-6159.
28. Gomes, A.V., et al., *Contrasting proteome biology and functional heterogeneity of the 20 S proteasome complexes in mammalian tissues*. *Molecular & cellular proteomics : MCP*, 2009. **8**(2): p. 302-315.
29. Glickman, M.H., et al., *A subcomplex of the proteasome regulatory particle required for ubiquitin-conjugate degradation and related to the COP9-signalosome and eIF3*. *Cell*, 1998. **94**(5): p. 615-623.
30. Lander, G.C., et al., *Complete subunit architecture of the proteasome regulatory particle*. *Nature*, 2012. **482**(7384): p. 186-191.
31. Liu, C.W. and A.D. Jacobson, *Functions of the 19S complex in proteasomal degradation*. *Trends Biochem. Sci.*, 2013. **38**(2): p. 103-110.
32. Stadtmueller, B.M. and C.P. Hill, *Proteasome activators*. *Mol. Cell*, 2011. **41**(1): p. 8-19.
33. Beck, P., C. Dubiella, and M. Groll, *Covalent and non-covalent reversible proteasome inhibition*. *Biol. Chem.*, 2012. **393**(10): p. 1101-1120.

34. Kisselev, Alexei F., W.A. van der Linden, and Herman S. Overkleeft, *Proteasome Inhibitors: An Expanding Army Attacking a Unique Target*. Chem. & Bio., 2012. **19**(1): p. 99-115.
35. Rentsch, A., et al., *Synthesis and Pharmacology of Proteasome Inhibitors*. Ang. Chem. Int. Ed., 2013. **52**(21): p. 5450-5488.
36. Adams, J., *The proteasome: a suitable antineoplastic target*. Nat Rev Cancer, 2004. **4**(5): p. 349-360.
37. Groll, M., et al., *Crystal Structure of Epoxomicin:20S Proteasome Reveals a Molecular Basis for Selectivity of α' , β' -Epoxyketone Proteasome Inhibitors*. JACS, 2000. **122**(6): p. 1237-1238.
38. Adams, J., et al., *Potent and selective inhibitors of the proteasome: Dipeptidyl boronic acids*. Bioorg. & Med. Chem. Let., 1998. **8**(4): p. 333-338.
39. San Miguel, J.F., H. van de Velde, and P.G. Richardson, *Bortezomib plus Melphalan and Prednisone for Multiple Myeloma Reply*. New Eng. J. Med., 2008. **359**(24): p. 2613-2614.
40. Groll, M., et al., *Crystal Structure of the Boronic Acid-Based Proteasome Inhibitor Bortezomib in Complex with the Yeast 20S Proteasome*. Structure, 2006. **14**(3): p. 451-456.
41. Bogoy, M., et al., *Covalent modification of the active site threonine of proteasomal β subunits and the Escherichia coli homolog HslIV by a new class of inhibitors*. PNAS, 1997. **94**(13): p. 6629-6634.
42. Nazif, T. and M. Bogoy, *Global analysis of proteasomal substrate specificity using positional-scanning libraries of covalent inhibitors*. PNAS, 2001. **98**(6): p. 2967-2972.
43. Groll, M., et al., *Probing Structural Determinants Distal to the Site of Hydrolysis that Control Substrate Specificity of the 20S Proteasome*. Chem. & Biol., 2002. **9**(5): p. 655-662.
44. Groll, M., R. Huber, and B.C.M. Potts, *Crystal Structures of Salinosporamide A (NPI-0052) and B (NPI-0047) in Complex with the 20S Proteasome Reveal Important Consequences of β -Lactone Ring Opening and a Mechanism for Irreversible Binding*. JACS, 2006. **128**(15): p. 5136-5141.
45. Goldberg, A.L., *Development of proteasome inhibitors as research tools and cancer drugs*. J. Cell Biol., 2012. **199**(4): p. 583-588.
46. Kale, A.J. and B.S. Moore, *Molecular mechanisms of acquired proteasome inhibitor resistance*. J. Med. Chem., 2012. **55**(23): p. 10317-10327.
47. Chauhan, D., et al., *The bortezomib/proteasome inhibitor PS-341 and triterpenoid CDDO-Im induce synergistic anti-multiple myeloma (MM) activity and overcome bortezomib resistance*. Blood, 2004. **103**(8): p. 3158-3166.
48. Dorsey, B.D., et al., *Discovery of a Potent, Selective, and Orally Active Proteasome Inhibitor for the Treatment of Cancer*. J. Med. Chem., 2008. **51**(4): p. 1068-1072.
49. Piva, R., et al., *CEP-18770: A novel, orally active proteasome inhibitor with a tumor-selective pharmacologic profile competitive with bortezomib*. Blood, 2008. **111**(5): p. 2765-2775.
50. Kupperman, E., et al., *Evaluation of the Proteasome Inhibitor MLN9708 in Preclinical Models of Human Cancer*. Cancer Res., 2010. **70**(5): p. 1970-1980.
51. O'Connor, O.A., et al., *A Phase 1 Dose Escalation Study of the Safety and Pharmacokinetics of the Novel Proteasome Inhibitor Carfilzomib (PR-171) in Patients with Hematologic Malignancies*. Clinical Cancer Res., 2009. **15**(22): p. 7085-7091.
52. Arastu-Kapur, S., et al., *Nonproteasomal Targets of the Proteasome Inhibitors Bortezomib and Carfilzomib: a Link to Clinical Adverse Events*. Clinical Cancer Research, 2011. **17**(9): p. 2734-2743.
53. Molineaux, S.M., *Molecular Pathways: Targeting Proteasomal Protein Degradation in Cancer*. Clin. Cancer Res., 2012. **18**(1): p. 15-20.
54. Zhou, H.-J., et al., *Design and Synthesis of an Orally Bioavailable and Selective Peptide Epoxyketone Proteasome Inhibitor (PR-047)*. J. Med. Chem., 2009. **52**(9): p. 3028-3038.
55. C. Potts, B., et al., *Marizomib, a Proteasome Inhibitor for All Seasons: Preclinical Profile and a Framework for Clinical Trials*. Curr. Cancer Drug Targ., 2011. **11**(3): p. 254-284.
56. Gaczynska, M. and P.A. Osmulski, *Harnessing proteasome dynamics and allostery in drug design*. Antioxid. Redox Signal., 2013.
57. Zhao, Y., et al., *Targeting neddylation pathways to inactivate Cullin-RING ligases for anti-cancer therapy*. Antioxid. Redox Signal., 2013.
58. McKinnon, C. and S.J. Tabrizi, *The Ubiquitin-Proteasome System in Neurodegeneration*. Antioxid. Redox Signal., 2013.
59. Drews, O. and H. Taegtmeyer, *Regulation of the Ubiquitin-Proteasome System in Heart Disease: The Basis for New Therapeutic Strategies*. Antioxid. Redox Signal., 2013.

60. Wilck, N. and A. Ludwig, *Targeting the Ubiquitin-Proteasome System in Atherosclerosis: Status quo, challenges and perspectives*. Antioxid. Redox Signal., 2013.
61. Meiners, S., et al., *Regulation of the Proteasome: Evaluating the Lung Proteasome as a New Therapeutic Target*. Antioxid. Redox Signal., 2013.
62. Craiu, A., et al., *Lactacystin and clasto-Lactacystin β -Lactone Modify Multiple Proteasome β -Subunits and Inhibit Intracellular Protein Degradation and Major Histocompatibility Complex Class I Antigen Presentation*. J. Biol. Chem., 1997. **272**(20): p. 13437-13445.
63. Fenteany, G., et al., *Inhibition of proteasome activities and subunit-specific amino-terminal threonine modification by lactacystin*. Science, 1995. **268**(5211): p. 726-731.
64. Sin, N., et al., *Total synthesis of the-potent proteasome inhibitor epoxomicin: a useful tool for understanding proteasome biology*. Bioorg. & Med. Chem. Lett., 1999. **9**(15): p. 2283-2288.
65. Meng, L., et al., *Epoxomicin, a potent and selective proteasome inhibitor, exhibits in vivo antiinflammatory activity*. PNAS, 1999. **96**(18): p. 10403-10408.
66. Bogyo, M., *Screening for selective small molecule inhibitors of the proteasome using activity-based probes*. Ubi. Prot. Degra., Pt B, 2005. **399**: p. 609+.
67. Berkers, C.R., et al., *Activity probe for in vivo profiling of the specificity of proteasome inhibitor bortezomib*. Nat. Methods, 2005. **2**(5): p. 357-362.
68. Verdoes, M., et al., *A Fluorescent Broad-Spectrum Proteasome Inhibitor for Labeling Proteasomes In Vitro and In Vivo*. Chem. & Biol., 2006. **13**(11): p. 1217-1226.
69. Verdoes, M., et al., *Chemical proteomics profiling of proteasome activity*. Methods Mol. Biol., 2006. **328**: p. 51-69.
70. Verdoes, M., et al., *A panel of subunit-selective activity-based proteasome probes*. Org. & Biomol. Chem., 2010. **8**(12): p. 2719-2727.
71. Geurink, P.P., et al., *Incorporation of Non-natural Amino Acids Improves Cell Permeability and Potency of Specific Inhibitors of Proteasome Trypsin-like Sites*. J. Med. Chem., 2013.
72. Mirabella, Anne C., et al., *Specific Cell-Permeable Inhibitor of Proteasome Trypsin-like Sites Selectively Sensitizes Myeloma Cells to Bortezomib and Carfilzomib*. Chem. & Biol., 2011. **18**(5): p. 608-618.
73. Carmony, K.C., et al., *A bright approach to the immunoproteasome: Development of LMP2/ β 1i-specific imaging probes*. Bioorg. & Med. Chem., 2012. **20**(2): p. 607-613.
74. Li, N., et al., *Relative quantification of proteasome activity by activity-based protein profiling and LC-MS/MS*. Nat. Protoc., 2013. **8**(6): p. 1155-1168.
75. An, B., et al., *Novel dipeptidyl proteasome inhibitors overcome Bcl-2 protective function and selectively accumulate the cyclin-dependent kinase inhibitor p27 and induce apoptosis in transformed, but not normal, human fibroblasts*. Cell Death Differ., 1998. **5**(12): p. 1062-1075.
76. Drexler, H.C., *Activation of the cell death program by inhibition of proteasome function*. PNAS, 1997. **94**(3): p. 855-860.
77. Adams, J. and R. Stein, *Novel inhibitors of the proteasome and their therapeutic use in inflammation*. Annu.Rep. Med. Chem., 1996. **31**: p. 279-288.
78. Orłowski, R.Z. and D.J. Kuhn, *Proteasome inhibitors in cancer therapy: lessons from the first decade*. Clin. Cancer Res., 2008. **14**(6): p. 1649-1657.
79. Kisselev, A.F., A. Callard, and A.L. Goldberg, *Importance of the different proteolytic sites of the proteasome and the efficacy of inhibitors varies with the protein substrate*. J. Biol. Chem., 2006. **281**(13): p. 8582-8590.
80. Miller, C.P., et al., *Specific and prolonged proteasome inhibition dictates apoptosis induction by marizomib and its analogs*. Chemico-Biol. Interac., 2011. **194**(1): p. 58-68.
81. Bianchi, G., et al., *The proteasome load versus capacity balance determines apoptotic sensitivity of multiple myeloma cells to proteasome inhibition*. Blood, 2009. **113**(13): p. 3040-3049.
82. Nencioni, A., et al., *Proteasome inhibitors: antitumor effects and beyond*. Leukemia, 2007. **21**(1): p. 30-36.
83. Ding, W.X., et al., *A coordinated action of Bax, PUMA, and p53 promotes MG132-induced mitochondria activation and apoptosis in colon cancer cells*. Mol. Cancer Ther., 2007. **6**(3): p. 1062-1069.
84. King, R.W., et al., *How proteolysis drives the cell cycle*. Science, 1996. **274**(5293): p. 1652-1659.
85. Palombella, V.J., et al., *The Ubiquitin-Proteasome Pathway Is Required for Processing the Nf-Kappa-B1 Precursor Protein and the Activation of Nf-Kappa-B*. Cell, 1994. **78**(5): p. 773-785.

86. Adams, J., et al., *Proteasome inhibitors: A novel class of potent and effective antitumor agents*. *Cancer Res.*, 1999. **59**(11): p. 2615-2622.
87. Hideshima, T., et al., *The proteasome inhibitor PS-341 inhibits growth, induces apoptosis, and overcomes drug resistance in human multiple myeloma cells*. *Cancer Res.*, 2001. **61**(7): p. 3071-3076.
88. Ling, Y.H., et al., *Mechanisms of proteasome inhibitor PS-341-induced G(2)-M-phase arrest and apoptosis in human non-small cell lung cancer cell lines*. *Clin. Cancer Res.*, 2003. **9**(3): p. 1145-1154.
89. Pagano, M., et al., *Role of the Ubiquitin-Proteasome Pathway in Regulating Abundance of the Cyclin-Dependent Kinase Inhibitor P27*. *Science*, 1995. **269**(5224): p. 682-685.
90. Rao, H., et al., *Degradation of a cohesin subunit by the N-end rule pathway is essential for chromosome stability*. *Nature*, 2001. **410**(6831): p. 955-959.
91. Haupt, Y., et al., *Mdm2 promotes the rapid degradation of p53*. *Nature*, 1997. **387**(6630): p. 296-299.
92. Wu, G.S., *The functional interactions between the p53 and MAPK signaling pathways*. *Cancer Biol. & Ther.*, 2004. **3**(2): p. 156-161.
93. Miyashita, T. and J.C. Reed, *Tumor-Suppressor P53 Is a Direct Transcriptional Activator of the Human Bax Gene*. *Cell*, 1995. **80**(2): p. 293-299.
94. Lopes, U.G., et al., *p53-dependent induction of apoptosis by proteasome inhibitors*. *J Biol. Chem.*, 1997. **272**(20): p. 12893-12896.
95. Herrmann, J.L., et al., *Prostate carcinoma cell death resulting from inhibition of proteasome activity is independent of functional Bcl-2 and p53*. *Oncogene*, 1998. **17**(22): p. 2889-2899.
96. Perez-Galan, P., et al., *The proteasome inhibitor bortezomib induces apoptosis in mantle-cell lymphoma through generation of ROS and Noxa activation independent of p53 status*. *Blood*, 2006. **107**(1): p. 257-264.
97. Ghosh, S. and M. Karin, *Missing pieces in the NF-kappa B puzzle*. *Cell*, 2002. **109**: p. S81-S96.
98. Mitsiades, N., et al., *Molecular sequelae of proteasome inhibition in human multiple myeloma cells*. *PNAS*, 2002. **99**(22): p. 14374-14379.
99. Mitsiades, N., et al., *The proteasome inhibitor PS-341 potentiates sensitivity of multiple myeloma cells to conventional chemotherapeutic agents: therapeutic applications*. *Blood*, 2003. **101**(6): p. 2377-2380.
100. Hideshima, T., et al., *NF-kappa B as a therapeutic target in multiple myeloma*. *J. Biol. Chem.*, 2002. **277**(19): p. 16639-16647.
101. Fribley, A., Q.H. Zeng, and C.Y. Wang, *Proteasome inhibitor PS-341 induces a apoptosis through induction of endoplasmic reticulum stress-reactive oxygen species in head and neck squamous cell carcinoma cells*. *Mol. Cell. Biol.*, 2004. **24**(22): p. 9695-9704.
102. Obeng, E.A., et al., *Proteasome inhibitors induce a terminal unfolded protein response in multiple myeloma cells*. *Blood*, 2006. **107**(12): p. 4907-4916.
103. Lee, A.H., et al., *Proteasome inhibitors disrupt the unfolded protein response in myeloma cells*. *PNAS*, 2003. **100**(17): p. 9946-9951.
104. Ling, Y.H., et al., *Reactive oxygen species generation and mitochondrial dysfunction in the apoptotic response to bortezomib, a novel proteasome inhibitor, in human H460 non-small cell lung cancer cells*. *J. Biol. Chem.*, 2003. **278**(36): p. 33714-33723.
105. Yu, C.R., et al., *The proteasome inhibitor bortezomib interacts synergistically with histone deacetylase inhibitors to induce apoptosis in Bcr/Abl(+) cells sensitive and resistant to ST1571*. *Blood*, 2003. **102**(10): p. 3765-3774.
106. Werner, E.D., J.L. Brodsky, and A.A. McCracken, *Proteasome-dependent endoplasmic reticulum-associated protein degradation: an unconventional route to a familiar fate*. *PNAS*, 1996. **93**(24): p. 13797-13801.
107. Ellgaard, L. and A. Helenius, *Quality control in the endoplasmic reticulum*. *Nature Rev. Mol. Cell Biol.*, 2003. **4**(3): p. 181-191.
108. Hetz, C., *The unfolded protein response: controlling cell fate decisions under ER stress and beyond*. *Nature Rev. Mol. Cell Biol.*, 2012. **13**(2): p. 89-102.
109. Walter, P. and D. Ron, *The unfolded protein response: from stress pathway to homeostatic regulation*. *Science*, 2011. **334**(6059): p. 1081-1086.
110. Tabas, I. and D. Ron, *Integrating the mechanisms of apoptosis induced by endoplasmic reticulum stress*. *Nature Cell Biol.*, 2011. **13**(3): p. 184-190.

111. Han, J., *et al.*, *ER-stress-induced transcriptional regulation increases protein synthesis leading to cell death*. *Nature Cell Biol.*, 2013. **15**(5): p. 481-+.
112. Seimon, T.A., *et al.*, *Induction of ER stress in macrophages of tuberculosis granulomas*. *PLoS One*, 2010. **5**(9): p. e12772.
113. Harding, H.P., *et al.*, *Regulated translation initiation controls stress-induced gene expression in mammalian cells*. *Mol. Cell*, 2000. **6**(5): p. 1099-1108.
114. Lin, J.H., *et al.*, *IRE1 signaling affects cell fate during the unfolded protein response*. *Science*, 2007. **318**(5852): p. 944-949.
115. Nawrocki, S.T., *et al.*, *Bortezomib inhibits PKR-like endoplasmic reticulum (ER) kinase and induces apoptosis via ER stress in human pancreatic cancer cells*. *Cancer Res.*, 2005. **65**(24): p. 11510-11519.
116. Hu, J.S., *et al.*, *Activation of ATF4 mediates unwanted Mcl-1 accumulation by proteasome inhibition*. *Blood*, 2012. **119**(3): p. 826-837.
117. Binet, F., S. Chiasson, and D. Girard, *Evidence that endoplasmic reticulum (ER) stress and caspase-4 activation occur in human neutrophils*. *Biochem. Biophys. Res. Commu.*, 2010. **391**(1): p. 18-23.
118. Yamamuro, A., *et al.*, *Caspase-4 Directly Activates Caspase-9 in Endoplasmic Reticulum Stress-Induced Apoptosis in SH-SY5Y Cells*. *J. Pharm.I Sci.*, 2011. **115**(2): p. 239-243.
119. Li, C., *et al.*, *Transmembrane Protein 214 (TMEM214) Mediates Endoplasmic Reticulum Stress-induced Caspase 4 Enzyme Activation and Apoptosis*. *J. Biol. Chem.*, 2013. **288**(24): p. 17908-17917.
120. Chandra, J., *Oxidative Stress by Targeted Agents Promotes Cytotoxicity in Hematologic Malignancies*. *Antioxid. Redox Signal.*, 2009. **11**(5): p. 1123-1137.
121. Zou, W., *et al.*, *Vitamin C inactivates the proteasome inhibitor PS-341 in human cancer cells*. *Clin. Cancer Res.*, 2006. **12**(1): p. 273-280.
122. Papa, L., E. Gomes, and P. Rockwell, *Reactive oxygen species induced by proteasome inhibition in neuronal cells mediate mitochondrial dysfunction and a caspase-independent cell death*. *Apoptosis*, 2007. **12**(8): p. 1389-1405.
123. Miller, C.P., *et al.*, *NPI-0052, a novel proteasome inhibitor, induces caspase-8 and ROS-dependent apoptosis alone and in combination with HDAC inhibitors in leukemia cells*. *Blood*, 2007. **110**(1): p. 267-277.
124. Goel, A., D.R. Spitz, and G.J. Weiner, *Manipulation of cellular redox parameters for improving therapeutic responses in B-cell lymphoma and multiple myeloma*. *J. Cell. Biochem.*, 2012. **113**(2): p. 419-425.
125. Brieger, K., *et al.*, *Reactive oxygen species: from health to disease*. *Swiss Medical Weekly*, 2012. **142**.
126. Karisch, R., *et al.*, *Global Proteomic Assessment of the Classical Protein-Tyrosine Phosphatome and "Redoxome"*. *Cell*, 2011. **146**(5): p. 826-840.
127. Bedford, L., *et al.*, *Ubiquitin-like protein conjugation and the ubiquitin-proteasome system as drug targets*. *Nat. Rev. Drug Discov.*, 2011. **10**(1): p. 29-46.
128. Nathan, C. and A. Cunningham-Bussel, *Beyond oxidative stress: an immunologist's guide to reactive oxygen species*. *Nat. Rev. Immu.*, 2013. **13**(5): p. 349-361.
129. Bae, Y.S., *et al.*, *Regulation of reactive oxygen species generation in cell signaling*. *Mol. Cells*, 2011. **32**(6): p. 491-509.
130. Chauhan, D., *et al.*, *A novel orally active proteasome inhibitor induces apoptosis in multiple myeloma cells with mechanisms distinct from Bortezomib*. *Cancer Cell*, 2005. **8**(5): p. 407-419.
131. Nerini-Molteni, S., *et al.*, *Redox homeostasis modulates the sensitivity of myeloma cells to bortezomib*. *Redox homeostasis modulates the sensitivity of myeloma cells to bortezomib*. *Brit. J. Haem.*, 2008. **141**(4): p. 494-503.
132. Soriano, M.E. and L. Scorrano, *The interplay between BCL-2 family proteins and mitochondrial morphology in the regulation of apoptosis*. *Adv. Exp. Med. Biol.*, 2010. **687**: p. 97-114.
133. Scorrano, L., *et al.*, *BAX and BAK regulation of endoplasmic reticulum Ca²⁺: A control point for apoptosis*. *Science*, 2003. **300**(5616): p. 135-139.
134. Timmins, J.M., *et al.*, *Calcium/calmodulin-dependent protein kinase II links ER stress with Fas and mitochondrial apoptosis pathways*. *J. Clin. Inv.*, 2009. **119**(10): p. 2925-2941.
135. Ohshima-Hosoyama, S., *et al.*, *Bortezomib stabilizes NOXA and triggers ROS-associated apoptosis in medulloblastoma*. *J. Neuro-Onc.*, 2011. **105**(3): p. 475-483.
136. Willis, S.N., *et al.*, *Proapoptotic Bak is sequestered by Mcl-1 and Bcl-x(L), but not Bcl-2, until displaced by BH3-only proteins*. *Genes & Dev.*, 2005. **19**(11): p. 1294-1305.

137. Yu, C.R., *et al.*, *The hierarchical relationship between MAPK signaling and ROS generation in human leukemia cells undergoing apoptosis in response to the proteasome inhibitor Bortezomib*. *Exp. Cell Res.*, 2004. **295**(2): p. 555-566.
138. Ichijo, H., *et al.*, *Induction of apoptosis by ASK1, a mammalian MAPKKK that activates SAPK/JNK and p38 signaling pathways*. *Science*, 1997. **275**(5296): p. 90-94.
139. Hideshima, T., *et al.*, *Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341*. *Blood*, 2003. **101**(4): p. 1530-1534.
140. Urano, F., *et al.*, *Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1*. *Science*, 2000. **287**(5453): p. 664-666.
141. Walsh, R.C., *et al.*, *Proteasome inhibitor-based therapy for antibody-mediated rejection*. *Kid. Int.*, 2012. **81**(11): p. 1067-1074.
142. Rumpold, H., *et al.*, *Knockdown of PgP resensitizes leukemic cells to proteasome inhibitors*. *Biochem. Biophys. Res. Com.*, 2007. **361**(2): p. 549-554.
143. Cullinan, S.B., *et al.*, *Nrf2 is a direct PERK substrate and effector of PERK-dependent cell survival*. *Mol. Cell. Biol.*, 2003. **23**(20): p. 7198-7209.
144. Hast, B.E., *et al.*, *Proteomic Analysis of Ubiquitin Ligase KEAP1 Reveals Associated Proteins That Inhibit NRF2 Ubiquitination*. *Cancer Res.*, 2013. **73**(7): p. 2199-2210.
145. Rushworth, S.A., K.M. Bowles, and D.J. MacEwan, *High Basal Nuclear Levels of Nrf2 in Acute Myeloid Leukemia Reduces Sensitivity to Proteasome Inhibitors*. *Cancer Res.*, 2011. **71**(5): p. 1999-2009.
146. Radhakrishnan, S.K., *et al.*, *Transcription Factor Nrf1 Mediates the Proteasome Recovery Pathway after Proteasome Inhibition in Mammalian Cells*. *Mol. Cell*, 2010. **38**(1): p. 17-28.
147. Steffen, J., *et al.*, *Proteasomal Degradation Is Transcriptionally Controlled by TCF11 via an ERAD-Dependent Feedback Loop*. *Mol. Cell*, 2010. **40**(1): p. 147-158.
148. Sykietis, G.P. and D. Bohmann, *Stress-Activated Cap'n'collar Transcription Factors in Aging and Human Disease*. *Sci. Signal.*, 2010. **3**(112).
149. Oerlemans, R., *et al.*, *Molecular basis of bortezomib resistance: proteasome subunit beta 5 (PSMB5) gene mutation and overexpression of PSMB5 protein*. *Blood*, 2008. **112**(6): p. 2489-2499.
150. Ruckrich, T., *et al.*, *Characterization of the ubiquitin-proteasome system in bortezomib-adapted cells*. *Leukemia*, 2009. **23**(6): p. 1098-1105.
151. Rapino, F., M. Jung, and S. Fulda, *BAG3 induction is required to mitigate proteotoxicity via selective autophagy following inhibition of constitutive protein degradation pathways*. *Oncogene*, 2013.
152. Aronson, L.I. and F.E. Davies, *DangER: protein ovERload. Targeting protein degradation to treat myeloma*. *Haematologica*, 2012. **97**(8): p. 1119-30.
153. Rosati, A., *et al.*, *BAG3: a multifaceted protein that regulates major cell pathways*. *Cell death & Disease*, 2011. **2**: p. e141.
154. Ding, W.X., *et al.*, *Oncogenic transformation confers a selective susceptibility to the combined suppression of the proteasome and autophagy*. *Mol. Cancer Ther.*, 2009. **8**(7): p. 2036-2045.
155. Hoang, B., *et al.*, *Effect of autophagy on multiple myeloma cell viability*. *Mol. Cancer Ther.*, 2009. **8**(7): p. 1974-1984.
156. Myeku, N. and M.E. Figueiredo-Pereira, *Dynamics of the Degradation of Ubiquitinated Proteins by Proteasomes and Autophagy ASSOCIATION WITH SEQUESTOSOME 1/p62*. *J. Biol. Chem.*, 2011. **286**(25): p. 22426-22440.
157. Silva, G.M., *et al.*, *Redox control of 20S proteasome gating*. *Antioxid. Redox Signal.*, 2012. **16**(11): p. 1183-1194.
158. Ge, F., *et al.*, *Quantitative phosphoproteomics of proteasome inhibition in multiple myeloma cells*. *PLoS One*, 2010. **5**(9).
159. Weerapana, E., *et al.*, *Quantitative reactivity profiling predicts functional cysteines in proteomes*. *Nature*, 2010. **468**(7325): p. 790-U79.

