HHS Public Access
Author manuscript
J Med Chem. Author manuscript; available in PMC 2019 September 16.

Published in final edited form as:
J Med Chem. 2019 July 25; 62(14): 6469–6481. doi:10.1021/acs.jmedchem.9b00101.

Proteasome Activation as a New Therapeutic Approach To Target Proteotoxic Disorders

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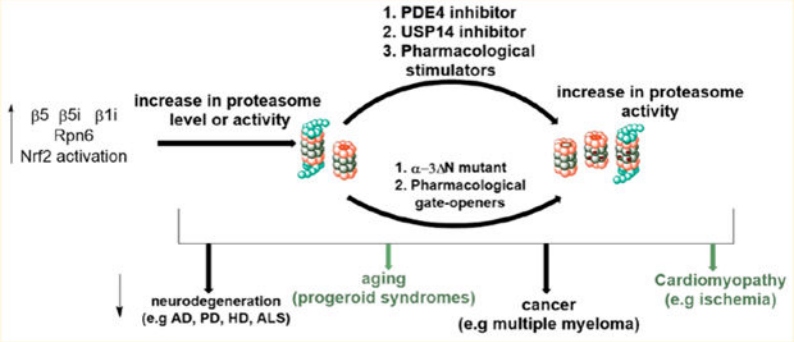
Abstract

Proteasomes are multienzyme complexes that maintain protein homeostasis (proteostasis) and important cellular functions through the degradation of misfolded, redundant, and damaged proteins. It is well established that aging is associated with the accumulation of damaged and misfolded proteins. This phenomenon is paralleled by declined proteasome activity. When the accumulation of redundant proteins exceed degradation, undesirable signaling and/or aggregation occurs and are the hallmarks of neurodegenerative diseases and many cancers. Thus, increasing proteasome activity has been recognized as a new approach to delay the onset or ameliorate the symptoms of neurodegenerative and other proteotoxic disorders. Enhancement of proteasome activity has many therapeutic potentials but is still a relatively unexplored field. In this perspective, we review current approaches, genetic manipulation, posttranslational modification, and small molecule proteasome agonists used to increase proteasome activity, challenges facing the field, and applications beyond aging and neurodegenerative diseases.

Graphical Abstract



1. INTRODUCTION

Precise and accurate regulation of important biological activities for self-maintenance requires the cellular protein pool to be in a continuous flux.[1] This protein quality control is

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Author Contributions
The manuscript was written through contributions of both authors.
The authors declare no competing financial interest.

maintained by the protein homeostasis (proteostasis) network, which consists of the protein synthesis machinery (the ribosomes), protein folding complexes (the chaperones), and two proteolytic systems: the proteasomal and the lysosomal (autophagy) systems.[2]

Molecular chaperones assist in cotranslational folding of nascent polypeptides attached to the ribosome, thereby preventing them from nonproductive interactions and aggregation.[3] Furthermore, chaperones recognize unfolded or aberrant proteins and assist them in regaining stability. Proteins with damages beyond repair are eliminated through the proteolytic pathways to avoid proteotoxicity due to aggregation or undesirable interactions.[4,5]

The plasticity and crosstalk among the protein synthesis, protein folding, and protein degradation systems sustain proteostasis under different cellular and environmental conditions such as xenobiotic and oxidative stress, cellular growth, and differentiation. The activities of the different proteostasis systems are functionally connected, and compensatory strategies are in place to elude proteostasis failure if the activity of one or more of the network components deteriorates.[6–8] However, as we age, malfunctioning of the protein homeostasis network is inevitable and interferes with crucial signaling pathways and is often associated with multiple human diseases.[9,10] Modulation of intracellular protein concentration via regulation of the proteolytic machineries has long been validated as promising milieu for the development of treatments for different human diseases such as neurodegeneration, cancer, and autoimmunity.[9,11–15]

The proteasome is the cell's first defense mechanism against accumulating proteotoxic stresses induced by oxidative damage. The 20S proteasome complex is capable of directly targeting oxidatively damaged proteins to detoxify the cell.[16–18] Reactive oxygen species, from exogenous sources, the mitochondrial respiratory chain, and other cellular metabolism, accumulate as we age, causing substantial damage to proteins and other macromolecules.[19,20] Upon oxidative damage, proteins unfold and expose hydrophobic regions which makes them prone to aggregation. Thus, to combat increasing levels of oxidatively damaged proteins, an increase in the 20S proteasome complex is generated by disassembly of the 26S proteasome.[21,22] However, when accumulation of damaged proteins exceed degradation, deregulation of proteostasis and proteotoxic stress occurs, which are the hallmarks of several neurological disorders, including Parkinson's disease (PD), Alzheimer's disease (AD), Huntington's disease (HD), and amyotrophic lateral sclerosis (ALS, also called Lou Gehrig's disease).[9,10,23–27] Furthermore, decline in 26S proteasome activity and accumulation of ubiquitinylated proteins have been observed in post-mortem brains of AD patients.[28] This suggests that both 20S and 26S are essential for the degradation of these aggregation-prone proteins.

Genetic manipulation of the proteasome proteolytic systems in animal models of different neurodegenerative disorders suggest that stimulating the activities of the proteolytic systems could be an effective strategy to treat these disorders.[29,30] In aged individuals, who are the major victim of this disease class, the proteasome exists mainly as the latent 20S,[19,31–33] thus making 20S a better target for these diseases.[34,35] Here, we discuss approaches that

have been implemented in increasing both 20S and 26S proteasome-mediated proteolysis, challenges facing the field, and applications beyond aging and neurodegenerations.

1.1 Structure of the Human Proteasome.

In eukaryotic cells, the ubiquitin proteasome system (UPS) is the major selective proteolytic system that regulates the concentration of proteins involved in numerous cellular processes.[12] At the center of the UPS is the proteasome. The human 26S proteasome consist of a barrel-shaped 20S core particle (CP) capped by one or two 19S regulatory particles (RPs) also called PA700.[36–38] The 20S CP is a threonine protease that consists of four stacked rings. The two inner β-rings contain three catalytic subunits (β5, β2, and β1) that display chymotrypsin-like (CT-L), trypsin-like (Tryp-L), and caspase-like (Casp-L) activity, respectively.[39–42] The outer α-rings serve as gated channels that regulate substrate entry and product exit from the inner catalytic chambers. These outer rings also act as docking surfaces for the 19S RP. The α and β-rings are each composed of heteroheptameric subunits; α1–α7 and β1–β7, respectively. The 26S proteasome is formed when the 28-subunit CP is docked on one or both ends by the ATPase 19S cap (PA700). The 19S RP is responsible for 20S gate opening, substrate recognition and binding, unfolding, and threading of ubiquitinated substrates into the 20S CP.[38,43–46] A number of other non-ATPase regulatory particles such as the 11S complex (PA28) and PA200 (Blm10 in yeast) also reversibly associate with the 20S CP by docking onto the α-rings.[47–50]

The 19S RP consist of two subcomplexes, the base that interacts directly with the 20S and a peripheral lid.[51,52] The base is comprised of hexameric AAA–ATPase subunits, Rpt1– Rpt6, and tetrameric non-ATPase subunits, Rpn1, Rpn2, Rpn10, and Rpn13. The ATPase activity in the base is essential for protein substrate unfolding, gate opening, and translocation of substrate into the 20S core. The lid is made of nine non-ATPase subunits; Rpn3, Rpn5–Rpn9, Rpn11, Rpn12, and Sem1. The lid, specifically the Rpn11 subunit, functions as a deubiquitinase.[53] Rpn13, Rpn10, and other reversibly associated proteins, such as radiation sensitivity abnormal 23 (Rad23) and dual-specificity protein kinase 2 (Dsk2) serve as ubiquitin receptors that direct polyubiquitinated proteins to the proteasome.[54,55] The Rpn subunits also create docking site(s) for other proteins including the proteasome associated deubiquitinating enzymes, ubiquitin specific peptidase 14 (USP14), and ubiquitin C-terminal hydrolase 37 (UCH37).[56,57]

In immune cells and during an immune response, or in response to treatments with cytokines, such as interferon-γ (IFN-γ) or tumor necrosis factor-α (TNF-α), the constitutive catalytic 20S proteasome subunits β1, β2, and β5 are swapped with the inducible subunits LMP2 (β1i), MECL-1 (β2i), and LMP7 (β5i), respectively, forming the immunoproteasome (i20S).[58] The immunoproteasome has an altered substrate binding pocket which results in cleavage pattern optimized for generating peptides for presentation on the major histocompatibility complex (MHC) class I molecules.[59–62] The i20S can also associate with the IFN-γ-inducible 11S (PA28) regulatory complex on one end and 19S on the other end to form a hybrid proteasome, or it can associate with the 11S on both ends.[63] The so-called thymoproteasome, with β1i, β2i, and β5t catalytic subunits has also been

identified in cortical epithelial cells of the thymus.[64] The t20S is believed to play critical role in the positive selection of CD8+ T cells.

1.2 The 26S Proteasome and Ubiquitin-ATP-Dependent Proteolysis.

The 26S proteasome mainly targets structured proteins for degradation, although a certain fraction of misfolded and intrinsically disordered proteins (IDPs) are also degraded by the 26S in an ubiquitin-independent manner (Figure 1).[65–68] Structured proteins due for degradation are tagged with chains of polyubiquitin which serve as a degron for their turnover.[69] Polyubiquitinated proteins are recognized by the ubiquitin receptors, Rpn10 and Rpn13.[54,55] The ubiquitin tag is then removed by deubiquitinases such as the Rpn11, USP14, and UCH37.[57] The ATPase activities of the 19S base then unfolds and directs the protein into the catalytic chamber for degradation.[65,70,71]

Ubiquitin is a small protein (~8 kDa) of 76 amino-acids, consisting of seven lysine (K) residues at positions 6, 11, 27, 29, 33, 48, and 63 through which it can be attached to protein substrates (Figure 1).[72,73] Although polyubiquitination of substrate proteins at K-48 of ubiquitin serves as the primary degron for degradation via the 26S proteasome, ubiquitin ligation at other lysine residues, such as K-63, have been shown to direct proteins toward autophagy-mediated proteolysis.[74] A well-defined series of enzymes, ubiquitin ligases (E1, E2, and E3) coordinate the attachment of mono- and polyubiquitin to proteins. Ubiquitin is first activated in an ATP-dependent reaction by an E1 ubiquitin-activating enzyme, to which it becomes attached by a thioester bond. Subsequently, the activated ubiquitin is transferred to the active site cysteine of the E2 ubiquitin-conjugating enzyme. Ubiquitin-protein ligase (E3), together with E2 catalyze the transfer of ubiquitin onto the protein that is destined for degradation (Figure 1, left panel).[75–78]

1.3 The 20S Proteasome and Ubiquitin-ATP-Independent Proteolysis.

Unlike the 26S proteasome which primarily degrades polyubiquitinated proteins,[39] the 20S directly degrades misfolded, oxidatively damaged, and intrinsically disordered proteins and does not require the unfoldase activity of the 19S base. Furthermore, 20S-mediated proteolysis does not require polyubiquitination of its substrates (Figure 1 right panel).[67,68,79] The 20S proteasome exist mainly in the closed-gate/latent conformation in which access to the catalytic chamber is occluded by converging N-terminal residues of the alpha subunits.[39,80] The 20S-mediated proteolysis is thought to involve a direct interaction of the protein substrate with the alpha-3 subunit, which brings about a conformational change and subsequent degradation of the target protein.[81] A number of non-ATPase regulatory particles such as the 11S complex (PA28) and Blm10 (PA200) also reversibly associate with the 20S CP by docking onto the α-rings. This interaction induces an open-gate conformation that promotes ubiquitin- and ATP-independent proteolysis.[48–50] Therefore, small molecules that can induce open-gate conformations of the 20S proteasome can thus mimic ATP-and ubiquitin-independent proteolysis seen with non-ATPase caps.

2. MECHANISMS OF INCREASING PROTEASOME-MEDIATED PROTEOLYSIS

2.1. Proteasome Activation by Agonist Induced Conformational Alteration.

2.1.1. Peptide-Based Protea-some Agonists.—Endogenous protein activators such as the 19S, PA200, and PAN (from archaeal species) contain a conserved C-terminal hydrophobic-tyrosine-any amino acid (Hb-Y-X) motif that triggers 20S gate opening upon ATP binding.[43] These Hb-Y-X motifs are inserted into intersubunit pockets created by neighboring α subunits. In these pockets, interactions with conserved residues are believed to result in a rotation in the α subunits and a displacement of a reverse-turn loop that maintains the open-gate conformer. Conceivably, C-terminal peptides derived from the Hb-X-Y motifs of Rpt2 and Rpt5 subunits of the 19S proteasome were found to enhance 20S-mediated peptide and protein degradation in vitro.[44,82] More recently, proline- and arginine-rich (PR) peptides previously reported as allosteric proteasome inhibitors[83,84] were modified with C-terminal Hb-X-Y residues to achieve activating properties.[85] PA26 and PA28 lacking the Hb-Y-X motif activate the 20S proteasome through a mechanism distinct from that of ATPase activators and does not involve α subunit rotation.[43] Thus, the diversity in structure and mechanism of proteasome activation by pharmacological agents and endogenous activators suggest the presence of different allosteric pockets that can be targeted for modulation of proteasome activities.

Synthetic peptide called proteasome-activating peptide 1 (PAP1) has also been reported to increase the CT-L proteasome activity via a gate opening mechanism in the α-ring of the 20S CP.[86] This peptide protected fibroblasts from hydrogen peroxide-induced oxidative stress. Most importantly, PAP1 prevented the aggregation of superoxide dismutase 1 (SOD1) in a cellular model of amyotrophic lateral sclerosis (ALS). Similarly, Kisselev et al.[87] reported evidence of hydrophobic peptide-induced gate opening in the α-ring of the 20S CP.

Although clinically used proteasome inhibitors are peptide-based,[88] peptide drugs and peptide-based proteasome activators are limited by intrinsic peptide properties such as poor membrane permeability and metabolic instability.[89] Furthermore, peptide activators baring the Hb-Y-X motifs may also compete with endogenous proteasome activators (19S, 11S), with possibilities of intricate cellular outcomes.

2.1.2. Small Molecule Proteasome Agonist.—Tanaka et al.[90] in 1988 demonstrated that the eukaryotic 20S proteasome existed mainly in the latent state and could be activated in biochemical assays with low concentrations of sodium dodecyl sulfate (0.04–0.08% SDS) or with poly-lysine. SDS is believed to act via partial denaturation of the 20S and is characterized by inhibition at concentrations greater than 0.08%.[80] SDS-like proteasome activation has also been observed with lipids,[91] fatty acids,[92] and the natural product oleuropein.[93] Although the activity-response of these molecules preclude their use in physiologically relevance systems, they are invaluable in vitro tools.

Progress toward the discovery of drug-like proteasome agonist has been slow, but a few have been identified. Molecules that interact with either the 20S or both the 20S and 26S

proteasome, function through two main mechanisms, namely: (1) *Gate-openers*: Molecules that promote substrate entry into the catalytic pocket through allosteric interactions that induce gate-opening in the alpha ring of the 20S proteasome. (2) *Stimulators*: Molecules that promote 20S and/or 26S-mediated degradation through allosteric interactions that enhance substrate binding and/or degradation in one or more catalytic sites. Among the first reported 20S agonists is the triterpenoid, betulinic acid (Figure 2, compound 1),[94] which was reported to specifically enhance the CT-L activity of the 20S proteasome. Unfortunately, chemical modifications to improve activity resulted in proteasome inhibitors with complicated structure activity relationships (SAR).

In a search for novel activators, Jones et al.[95] screened the NIH clinical collection (NCC) and Prestwick library and identified chlorpromazine (Figure 2, compound 2) and other phenothiazines as 20S agonists that specifically enhanced 20S CT-L activity while promoting the degradation of intrinsically disordered proteins (IDPs), such as α-synuclein and tau in biochemical and cellular assays. Chemical modifications of chlorpromazine abolished its dopamine D2 activity while maintaining a significant proteasome stimulating ability. Coincidentally, methylene blue, a structural analogue of chlorpromazine has been demonstrated to reduce the levels of amyloid beta (Aβ) and rescues early cognitive deficit by increasing proteasome activity in a mouse model of AD.[96] This further supports the robustness of phenothiazines as proteasome activators in AD therapy.

In a more mechanistic study, Njomen et al.[97] identified the imidazoline TCH-165 (Figure 2, compound 3) and its analogues as 20S activators that induce gate-opening in the alpha ring of the 20S proteasome, as observed by atomic force microscopy (AFM). TCH-165 was found to enhance the degradation of IDPs in biochemical and cellular assays. In cells, TCH-165 also shifted the 20S–26S equilibrium toward the 20S, presumably by competing with the 19S cap for 20S binding. Interestingly, degradation of ubiquitinated proteins was largely maintained by single capped 20S.

In an effort to establish secondary assays for validation of “true” activators, Trader et al. screened a small library of the NCC and discovered MK-886 (Figure 2, compound 4)[98] and AM-404 as new classes of 20S activators capable of enhancing the degradation of α-synuclein in cell culture. In a parallel effort, Coleman and Trader[99] identified two 20S activators and a cytisine derivative (Figure 2, compound 5) as stimulator of both 20S and 26S. Among the 20S activators was the natural product, ursolic acid (Figure 2, compound 6), another triterpenoid and an analogue of betulinic acid. The mechanism of proteasome activation by ursolic acid[99] appears to be distinct from that of betulinic acid,[94] further supporting the complexity associated with these class of molecules and proteasome activators, in general.

Although some of these molecules have already been classified in literature[35] as stimulators and gate-openers, it should be noted that imidazolines[97] are the only class of 20S agonist with biophysical data (AFM)[97] supporting their gate-opening activity.

2.2. Proteasome Activation by Modulation of Posttranslational Modification.

2.2.1. Small Molecule Kinase Modulators.—Proteasome activation has also been realized through upstream modulation of kinases, with ensuing post-translational modification of proteasome subunits. The human 26S proteasome undergoes reversible phosphorylation in response to the ever-changing pathophysiological state of the cells.[100] More than 455 phosphorylation sites have been identified in the human 26S proteasome, and they play different roles ranging from modulation of 26S assembly, stability, and activity. For example, osmotic stress inhibits proteasome activity via p38-MAPK-dependent phosphorylation of Rpn2.[101]

Phosphorylation of the proteasome Rpt6 subunit by cAMP-dependent protein kinase A (PKA) has been reported to upregulate 26S assembly and proteasome activity in vitro and in vivo.[102,103] Rolipram (Figure 3, compound 8), a small molecule inhibitor of phosphodiesterase type 4 (PDE4),[104] was found to raise the level of cAMP in the brain of mice, activated PKA and presumably increased proteasome activity through subunit (Rpt6) phosphorylation. Most importantly, rolipram promoted the clearance of abnormal tau and improved cognition in mouse model of Alzheimer's disease.[105]

PKA-mediated phosphorylation of the non-ATPase 19S subunit Rpn6 has also been shown to enhance the degradation of ubiquitinated and aggregation-prone proteins such as tau in different cell lines.[106] These studies validated the involvement of the 26S proteasome in the degradation of partially unfolded proteins (or intrinsically disordered proteins, IDPs), further supporting the significance of targeting either the 20S and/or 26S in the quest for increasing proteasome-mediated IDP clearance.

2.2.2. Small Molecule Deubiquitinase Inhibitors.—The 19S proteasome subunit Rpn11 has deubiquitinase activity that recycles polyubiquitin by removing them en bloc from protein substrates that are committed for degradation.[53] Additional deubiquitinases USP14 and UCH37 reversibly interact with the proteasome and can trim ubiquitin chains independent of substrate commitment to degradation.[56,57,107]

Lee et al.[108] demonstrated that ubiquitin chain trimming by USP14 inhibits substrate degradation by the proteasome. In the same study, small molecule inhibitor of USP14, IU1 (Figure 3, compound 7), was found to enhance the degradation of different proteasome substrates, including tau. IU1 also promoted the degradation of oxidized proteins, thereby conferring a cytoprotective effect toward oxidative stress in HEK cells.

Interestingly, inhibition of USP14 by siRNA knockdown or use of its small molecule inhibitor led to accumulation of polyubiquitinylated proteins with increased apoptosis of multiple myeloma cells.[109] This translated to extended survival in xenograft models of multiple myeloma. In a parallel study, the small molecule inhibitor of USP14 and UCH37, b-AP15, also promoted the accumulation of polyubiquitin and delayed tumor progression in a mouse model of multiple myeloma[110] as well as four different models of solid tumors.[111] These intricate outcomes seen with USP14 inhibition might be a result of its many diverse cellular functions. Thus, targeting USP14 represents an exciting approach that may effectively translate in combating multiple types of cancers.

Furthermore, increasing proteasome-mediated proteolysis by inhibition of USP14 blocks autophagy flux.[112] The mechanism of autophagy inhibition appears to involve enhanced proteasomal degradation of the autophagosome–lysosome fusion protein, UV radiation resistance-associated gene (UVRAG). This result is consistent with Choi et al.'s[113] observation that proteasome activation via generation of open-gate mutant inhibited autophagy flux. Given the interplay between the two proteolytic systems, one is tempted to hypothesize that the antitumor effcacy of deubiquitinase inhibitors is directly related to autophagy inhibition and secondary to proteasome activation. However, genetic and pharmacological inhibition of the same USP14 was found to correct an in vivo model of impaired mitophagy[114] and also increased autophagy flux through suppression of K-63 ubiquitination of Beclin 1.[115] These complex observations further suggest the need to address the effect of increased proteasome activity at the level of molecules used, mechanism of increasing proteasome activity, and other targets of the small molecule that may be involved. For example, gate opening induced by deletion of α−3 N-terminal and by small molecule gate openers are anticipated to generate different conformationally active states of the proteasome, with ensuing disparity in protein degradation profile.

2.3. Proteasome Activation by Genetic Manipulation.

2.3.1. Upregulation of Proteasome Activity through Subunit Overexpression.

—Pioneer work involving upregulation of proteasome activity through genetic manipulation were first illustrated by Gaczynska et al.[116,117] In these studies, overexpression of β5i (LMP7) subunit in lymphoblasts and HeLa cells resulted in increased CT-L and Tryp-L activities, while overexpression of β1i (LMP2) only elevated the Tryp-L like activity. Therefore, increasing the activity of the immunoproteasome could also increase antigen processing thereby boosting immune response. In 2005, Chondogianni and co-workers[118] published a more in-depth study on proteasome activation via subunit upregulation: Stable over-expression of β5 catalytic subunit in two established (WI38/T and HL60) and primary (IMR90) human fibroblast cell lines resulted in elevated levels of other β-subunits and increased level of assembled proteasome, with a concomitant increase in all three proteasome proteolytic activities. Consistent with the role of the proteasome, β5-overexpressing cell lines conferred protection against several oxidation-induced proteotoxic stresses, via enhanced degradation of oxidized proteins. A few parallel studies have also found β5-overexpression to protect against oxidative stress in human lens epithelial cells[119] and to prevent replicative senescence of human bone marrow stromal cells.[120] These findings further corroborate the role of the proteasome in combating diverse classes of proteotoxic disorders.

2.3.2. Proteasome Upregulation through Nrf2 Activation.—

The transcription factor (nuclear factor (erythroid-derived 2)-like 2 (Nrf2), is a basic leucine zipper[121] that controls the expression of antioxidant enzymes including glutathione S-transferase, NADPH quinone oxidoreductase 1[122] as well as proteasome subunits.[123] Accordingly, overexpression of the proteasome has also been achieved through small molecule activators of Nrf2. Kwak et al.[123] demonstrated that the indirect antioxidant 3H-1,2-dithiole-3-thione (D3T), (Figure 4, compound 9) upregulated both 20S and 19S proteasome subunits as well as proteasome activity only in Nrf2 positive fibroblast. A more recent article showed that activation of Nrf2

with D3T significantly improved cognitive deficits in a mouse model of Alzheimer's disease and dramatically reduced the level of insoluble amyloid beta (Aβ), as well as oxidative stress.[124] The triterpenoid, 18α- glycyrrhetinic acid (18α-GA), has also been shown to delay replicative senescence in human fibroblast through Nrf2-mediated upregulation of proteasome subunit and activity.[125] In *Caenorhabditis elegans*, 18α-GA also decreased Aβ deposition and delayed the progress of AD while promoting proteasome-dependent life span extension.[126] Increased proteasome activity has also been attained by activating Nrf2 with the food additive *tert*-butylhydroquinone (t-BHQ) and food supplement sulforaphane. The increase in proteasome activity with t-BHQ and sulforaphane was accompanied by delayed differentiation, increased self-renewal and pluripotency in human embryonic stem cells (hESCs), and in induced pluripotent stem cell (iPS-IMR90).[127] Similar activities have been observed by others in neuroblastoma cells, where sulforaphane (Figure 4, compound 10) - Nrf2-mediated upregulation of proteasome activity protected against oxidative stress.[128]

2.3.3. Cap-Upregulation and Generation of Open-Gate Mutants.—

The 20S proteasome exists mainly in its latent state conformation in which entrance into the catalytic chamber is occluded by converging N-terminal of the α-subunits.[41,90,129] Proteins must access these gated channels to be degraded by the 20S core particle. Sensibly, one genetic approach to increase proteasome activity has been aimed at increasing accessibility through these gated channels. Certainly, over-expression of 19S Rpn6 subunit led to enrichment of assembled 26S and increased proteasome activity in embryonic stem cells.[130] Meanwhile, enhancement of proteasome activity via overexpression of PA28α was found to protect against proteinopathy and ischemia/reperfusion injury in mice.[131] These findings further highlight the importance of proteasome activation beyond neurodegenerative diseases and aging.

In a more recent study, Choi et al.[113] took a unique approach toward genetic upregulation of proteasome activity. Choi et al. showed that deleting the N-terminal of α−3 subunit generates an α−3ΔN mutant that is incorporated into fully assembled 20S and 26S proteasomes. These α−3ΔN mutant proteasomes showed increased degradation of about two hundred 20S and 26S protein substrates, including tau and α-synuclein. Furthermore, the enhanced degradation of α-synuclein significantly delayed the formation of α-synuclein aggregates. Significant resistance against oxidative stress was also observed with cells carrying the open-gate mutant.

Most of these genetic approaches have only been explored at the in vitro levels (Figure 4), with little or no supporting animal data. However, one could envision the delivery of proteasome β5 subunit, 19S Rpn6, and/or α−3ΔN as gene therapy for the treatment of several diseases.

2.4. Proteasome Activation via Unnknown Mechanisms.

Pyrazolones have previously been reported to have neuroprotective function in a cellular model of ALS, with significant increase in the median survival time of an ALS transgenic mouse model.[132–134] More recently, the activity of pyrazolone in the ALS model has been linked to proteasome activation.[135] This conclusion was based on enhanced degradation of

model proteasome substrates in cells, as well as affnity pull-down of Rpt2 and Rpt3 as interacting partners of pyrazolone. However, given that the activities of these compounds were not assayed with purified proteasome, it is not clear if they are direct proteasome agonists, modulators of endogenous proteasome activators (based on the pull-down assay with 19S subunits) or upstream modulators of proteasome posttranslational modifiers. Thus, in this review, and based on our classification, we categorize pyrazolones as enhancers of proteasome activity with unknown mechanism of action.

In another study by Leestemaker et al.,[136] a chemical genetic screen was used to identify PD169316, a p38 MAPK inhibitor, as enhancer of 26S proteasome activity. Although the exact mechanism behind 26S activation by p38 MAPK inhibitor could not be deciphered, the authors also demonstrated a decrease in the level of α-synuclein as well as protection against toxic α-synuclein in primary mouse neurons. Most importantly, the authors also developed activity-based probes that bind to the proteasome catalytic sites in an activity-dependent manner, thereby allowing for the screening of proteasome modulators.[136]

3. CHALLENGES AND CONSIDERATIONS

3.1. Crosstalk with Autophagy and Intricate Outcomes.

The proteolytic arm of the protein homeostasis network is sustained by both the proteasome and the autophagy pathways. At the protein level, autophagy seems to be instigated to compensate for cellular stresses not addressed by the proteasomal clearance system.[137] A well-balanced crosstalk between the two catabolic pathways ensures efficient maintenance of energy. In support of this, several studies have demonstrated that proteasome inhibition activates the autophagy pathway.[8,138–140] Conversely, acute inhibition of autophagy also activates the proteasome, although chronic autophagy inhibition hinders proteasome-mediated proteolysis.[7,141] Proteasome activation is therefore anticipated to reduce (the need for) autophagic clearance of proteins. Indeed, such phenomena were observed with the hyperactive open-gate 20S proteasome[113] as well as when proteasome activity was enhanced by targeting USP14[112] or with imidazolinebased 20S agonist (unpublished data from our lab). These observations could be specific to the type of agonist, mechanism of proteasome activation, and cell type, making the outcome of this crosstalk more difficult to predict. This is further illustrated by the case of USP14, where activation of 26S proteasome through genetic and pharmacological manipulation triggers conflicting outcomes in autophagic flux.[112,114,115]

3.2. Drug Repurposing Challenges.

Current paradigm in identifying small molecule proteasome activators has been to screen clinical collections of drugs.[95,98,99,136] Hits from these libraries would typically be optimized to eliminate activity at their previous targets while maintaining activity at the new target (proteasome).[95,142] This optimization and repurposing of hits from clinical library is challenging and has in part contributed to the slow progress in identifying small molecule proteasome agonist. Perhaps, identifying the “true” binding pockets of current 20S agonists through proteomic or crystallographic studies would allow for a streamline, computer-aided, structure-based drug design that could overcome this problem. While this may sound easy

on paper, it could be quite a daunting task given the numerous binding sites and mechanisms of activation of this humongous 2.5 MDa complex. Nonetheless, this knowledge is anticipated to be invaluable in driving the field forward.

3.3. Lack of Standard Assays for Efficacy Studies.

Canonical proteasome activity assays use peptide substrates specific for each proteasome catalytic sites (CT-L, Suc-LLVYAMC; Tryp-L, Boc-LRR-AMC; Casp-L, Z-LLE-AMC). These peptides are conjugated with a C-terminal fluorophore that is activated upon proteasomal cleavage.[40,143] The small size of these peptides allows them to navigate the narrow gate of latent 20S proteasome, resulting in high and varying baseline 20S proteasome activity. Furthermore, even this so-called standard assay is not standard as different laboratories use different substrate to enzyme ratios as well as different buffer systems. Moreover, detergent-like molecules that can relax this narrow 20S gate just enough to increase peptide substrate accessibility to the catalytic core appear as positive hit in these peptide assays. These non-bona fide agonists fail in assays that utilize more physiologically relevant protein substrates.[98] These limitations result in ambiguous comparison of potency for proteasome agonists, from batch to batch and from lab to lab. Furthermore, these primary screens employ purified 20S and fluorogenic peptides specific to just one 20S catalytic site, thereby ignoring the allosteric interactions between the three catalytic sites that drive protein degradation.[144] As such, stimulators of the other catalytic sites as well as molecules that act upstream of the proteasome are also missed in this screen.

The quest for proteasome agonists has been paralleled by the development of new assays to address these deficiencies and validate this new approach of targeting proteotoxic disorders. Among these assays are: (1) The IDPs based biochemical assays of Njomen et al.[97] and Jones et al.,[95] which address the need for physiologically relevant substrates. (2) The larger peptide mass spectrometry-based assay of Trader et al.,[98] which eliminates non-bona fide activators. (3) The peptide-FRET reporter assay of Coleman and Trader,[99] which addresses the problem of high background activity, and (4) the proteasome activity-based probe of Leestemaker et al.,[136] which addresses the need for a high-throughput cellular system. Each of these methods described has one or more of the following limitations, namely, the use of peptides or small molecule probes instead of protein substrates of interest, tedious, and not high-throughput compatible, and/or measures the state of activeness of the proteasome as opposed to its ability to degrade specific proteotoxic proteins. Therefore, there is still the need for a cell-based and protein-based assay that is amenable to high-throughput screening.

3.4. Anticipated Toxicity of Proteasome Activators.

Enhancing proteasome activity reduces the proteotoxic burden cells experience upon aging. In humans,[145,146] rodents,[96,147,148] and cells,[125,149–154] increased proteasome activity delays aging[150,155–157] and results in longer lifespan[158–160] by reducing proteotoxic pathologies. Supporting this idea, cells from human centenarians exhibit enhanced proteasome activity compared to cells from adults of different ages.[145]

Cellular studies using short-term exposure to proteasome agonists have thus far not illustrated inherent toxicity, however, no studies have yet defined any long-term effects.[85]

The lack of initial toxicity may be due to the protective roles of chaperones or other protein complexes, which greatly limit the access of the 20S proteasome to its targets, thus yielding proteolytic selectivity.[34,68,161,162] In addition, the rate of proteasomal degradation of disordered regions is also dependent on the composition and length of specific disordered initiation sequences.[163] Stimulation of proteasome activity is therefore anticipated to have a differential effect on the clearance of different proteins.

Proteasome inhibition is the mechanism of action of most chemotherapeutics targeting blood cancer such as multiple myeloma.[164] Thus, a question that has become of concern is whether proteasome activators can cause cancer. Indeed, enhanced degradation of misfolded proteins through Nrf2-mediated upregulation of proteasome activity has been demonstrated to promote tumorigenesis.[165] Other studies demonstrated that deletion of Keap1, a negative regulator of Nrf2, and Pten drive the progress of non-small-cell lung cancer.[166,167] It should, however, be noted that Nrf2 activation drives the expression of many genes, including cyclin B and cyclin-dependent kinase 1(CDK1),[168] which are involve in driving mitotic division.[169] Thus, the effect of proteasome activation on tumorigenesis must be examined on a case by case basis.

4. CONCLUSION

Herein, we discussed different strategies that have been developed to increase proteasome activity while also reflecting on some challenges associated with the field. We further touched on the potential of proteasome activation being applicable to the reduction of proteotoxic stresses seen in aging, neurodegeneration, and other disorders. This means the appropriate mechanism of increasing proteasome activity will likely be contingent on the disease under consideration. Furthermore, just like any new field, the concept of proteasome activation has also prompted a lot of apprehensions such as tumor causing potential and effect on other proteolytic pathways. It is, however, important to keep in mind that the physiological outcome of proteasome activation will depend largely on the mechanism employed and how “specific” it is to the proteasome network. Most of all, this field is still at its infant stage with positive outcomes so far. Thus, the next few years should shine some light on some of these concerns as we move from basic proof of concept to effcacy validation in different animal models of diseases under consideration.

ACKNOWLEDGMENTS

Financial support for this work was provided by the National Institute of Allergy and Infectious Diseases (1R21AI117018-01A1) and the National Institute of General Medical Sciences (T32GM092715) of the National Institutes of Health. The authors also gratefully acknowledge financial support from Michigan State University.

Biography

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ABBREVIATIONS USED

AD	Alzheimer’s Disease
AFM	atomic force microscopy
ALS	amyotrophic lateral sclerosis
Blm 10	bleomycin-sensitive
Boc-LRR-AMC	tert-butoxyl-leucyl-arginyl-arginyl-7-amino-4-methylcoumarin
Cdk1	cyclin dependent kinase 1
CP	core particle
D3T	3H-1,2-dithiole-3-thione
Dsk2	dual-specificity protein kinase 2
Hb-Y-X	hydrophobic-tyrosine-any amino acid
HD	Huntington’s disease
HL60	peripheral blood promyeloblast
IDP	intrinsically disordered proteins
IMR90	lung fibroblast
LMP2	low molecular mass polypeptide 2
LMP7	low molecular mass polypeptide 7
MAPK	mitogen-activated protein kinase

MECL-1	multicatalytic endopeptidase complex subunit 1
MHC	major histocompatibility complex
NCC	NIH (National Institutes of health) Clinical Collection
Nrf2	nuclear factor (erythroid-derived 2)-like 2
PA28	proteasome activator 28 subunit

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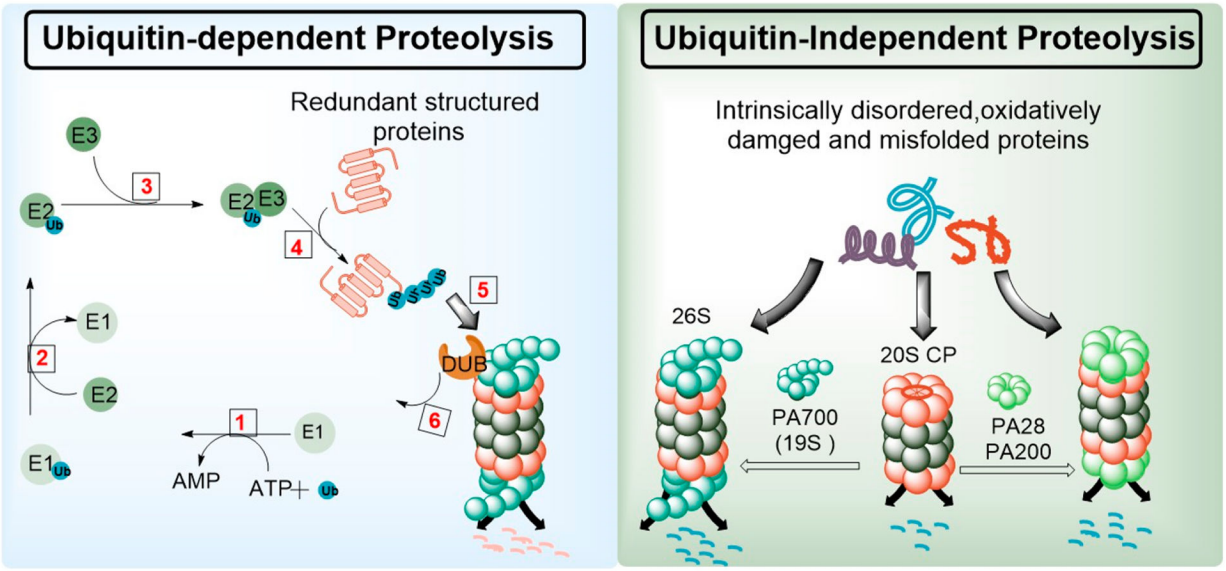

Figure 1.

The proteasome degradation system. Structured proteins with polyubiquitin tags are degraded by the 26S proteasome in an ATP dependent manner (left). Partially unfolded, intrinsically disordered, and oxidatively damaged proteins are directly degraded by the 26S, free 20S, or 20S capped by non-ATPase caps such as PA28 and PA200, without the need for polyubiquitination (right).

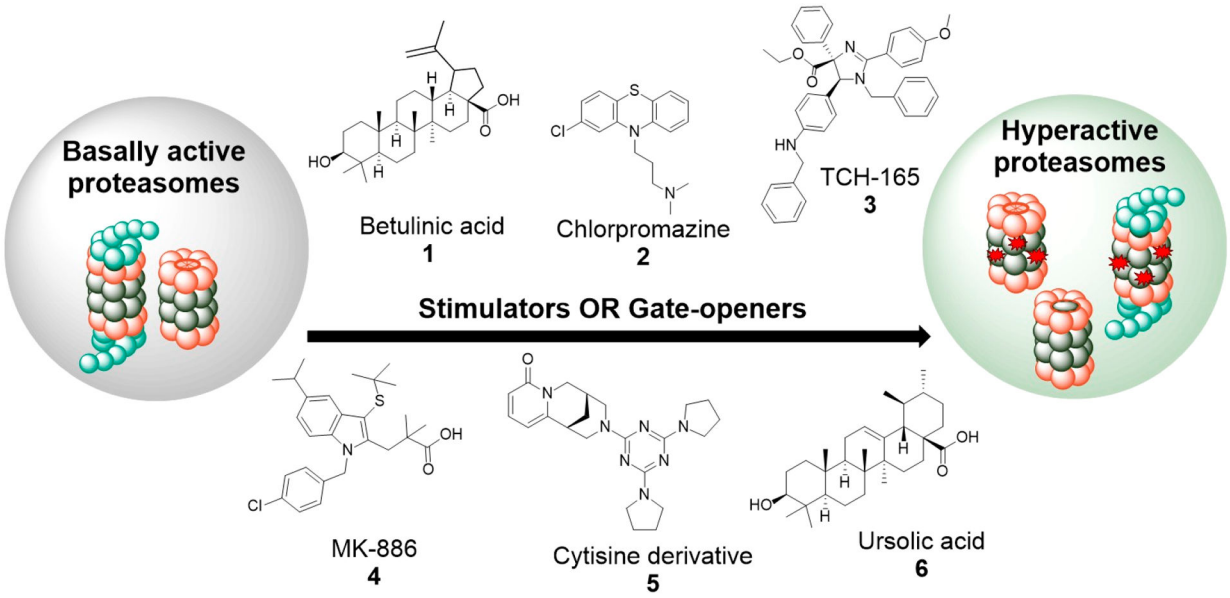

Figure 2.

Small molecule proteasome agonists: Small molecules that directly interact with the proteasome increase the activity of the enzyme either though gate-opening or through allosteric interaction that increase substrate binding and/or processing.

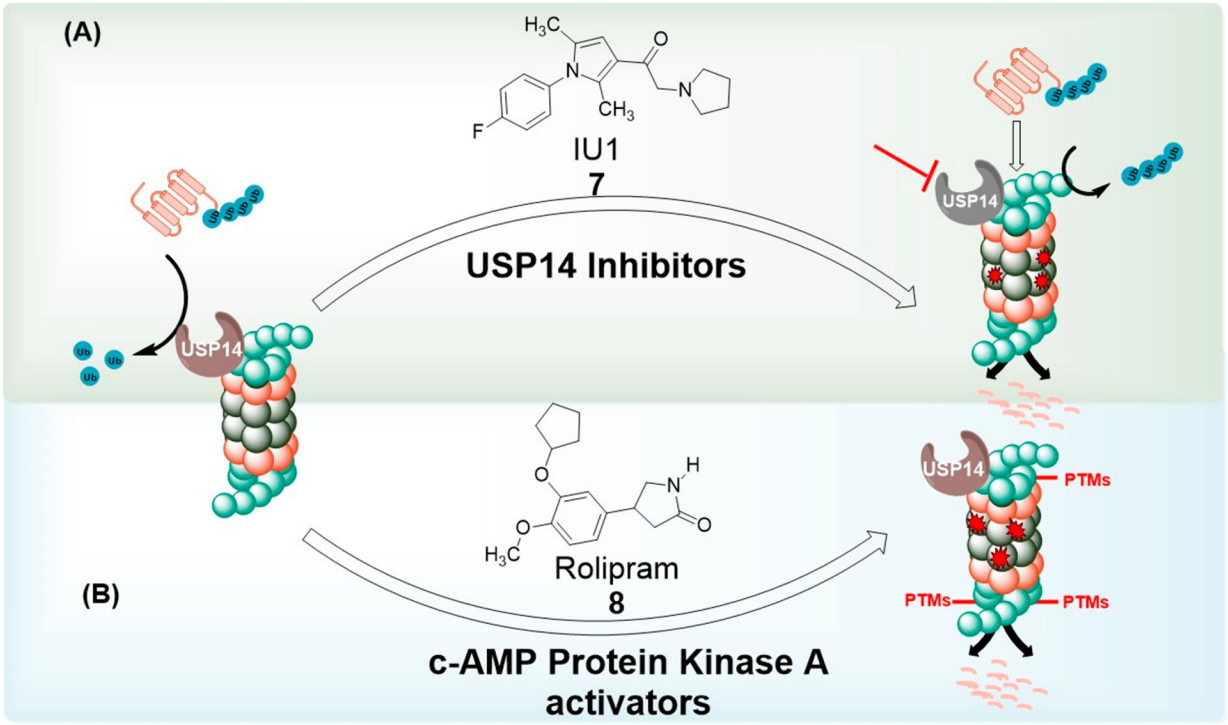

Figure 3.

Enhancement of proteasome by modulation of posttranslational modifications (PTMs): (A) Inhibition of USP14 prevents ubiquitin chain trimming, thereby promoting 26S-mediated protein degradation. (B) Phosphorylation of 19S Rpn6 subunit through activation of c-AMP PKA enhances 26S-mediated proteolysis.

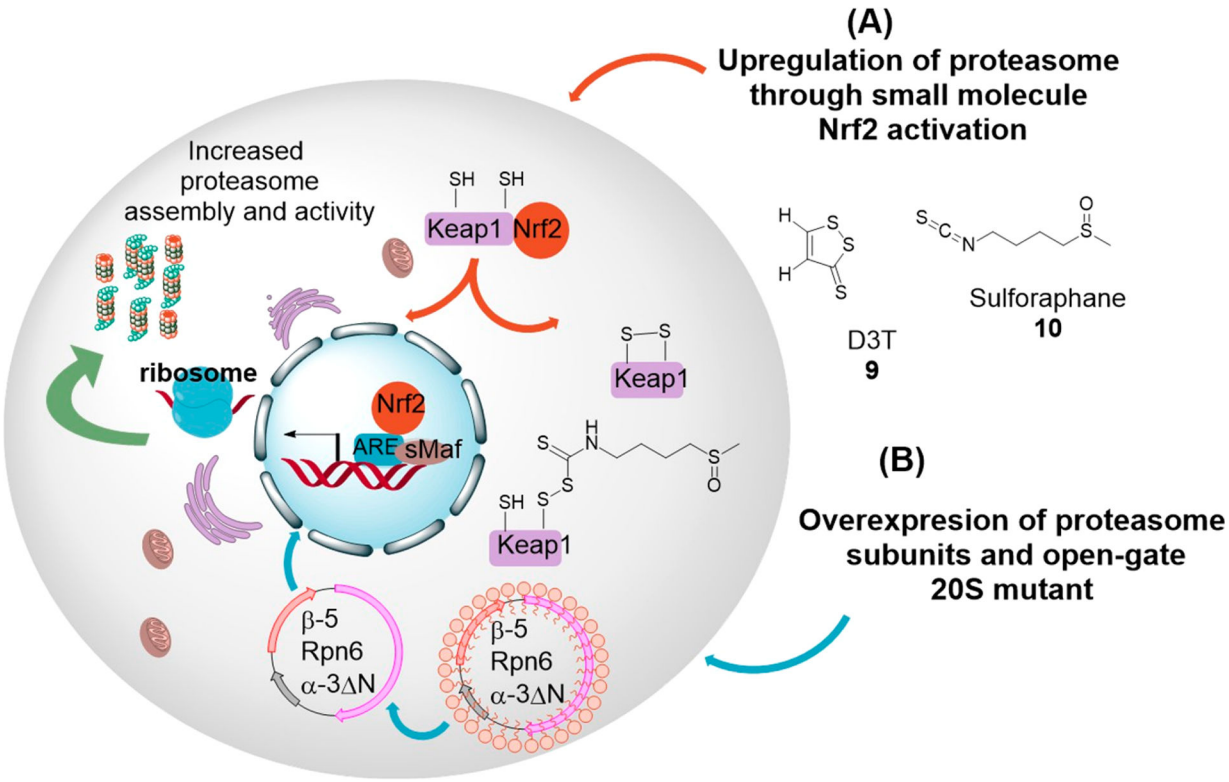

Figure 4.

Genetic upregulation of proteasome activity: (A) Activation of Nrf2 through small molecule oxidation and/or conjugation of keap1 cysteine residues promotes Nrf2-driven proteasome expression and activity. (B) Overexpression of proteasome β−5 and Rpn6 subunits increases 26S assembly and activity, while overexpression of N-terminal truncated α−3 subunit results in hyperactive open-gate 20S and 26S proteasomes.