Proteasome regulators: activators and inhibitors

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Abstract

This mini review covers the drug discovery aspect of both proteasome activators and inhibitors. The proteasome is involved in many essential cellular functions, such as regulation of cell cycle, cell differentiation, signal transduction pathways, antigen processing for appropriate immune responses, stress signaling, inflammatory responses, and apoptosis. Due to the importance of the proteasome in cellular functions, inhibition or activation of the proteasome could become a useful therapeutic strategy for a variety of diseases. Many proteasome inhibitors have been identified and can be classified into two groups according to their source: chemically synthesized small molecules and compounds derived from natural products. A successful case of developing a proteasome inhibitor as a clinically useful drug is that the peptide boronate, PS341 (Bortezomib), was approved for the treatment of multiple myeloma. In contrast to proteasome inhibitors, small molecules that can activate or enhance proteasome activity are rare and are not well studied. The fact that over-expression of the cellular proteasome activator PA28 exhibited beneficial effects on the Huntington's disease neuronal model cells raised the prospect that small molecule proteasome activators could become useful therapeutics. The beneficial effect of oleuropein, a small molecule proteasome activator, on senescence of human fibroblasts also suggested that proteasome activators might have the potential to be developed into anti-aging agents.

Keywords

Proteasome; activator; inhibitor; natural products; betulinic acids; peptides; anti-cancer; bortezomib

Introduction

There are two major cellular proteolysis systems: lysosomes and proteasomes. While lysosomes are critical in breaking down proteins taken into cells through endocytosis, proteasomes are the main machinery for intracellular protein degradation in eukaryotes [1, 2]. In addition to degradation of damaged or misfolded proteins, evidences suggest that proteasomes are involved in many essential cellular functions, such as cell cycle control, cell differentiation, signal transduction, antigen processing in immune responses, stress signaling, inflammatory responses, and apoptosis. Proteins undergo polyubiquitination before proteasomal degradation [3–5]. Overall, the ubiquitin-proteasome pathway is essential for maintaining optimal cellular functions.

Structure of proteasomes

A model of the 20S proteasome is illustrated in Fig. (1). The proteasome (20S) has a cylindrical structure that contains four rings stacked on top of each other. Each ring is

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composed of seven subunits. The two outer rings contain α subunits that do not have
enzymatic activity. The two inner rings are comprised of β subunits, where the proteolytic
activities reside. The β subunits possess three major proteolytic activities: a chymotrypsin-
like (β5), a trypsin-like (β2), and a caspase-like (β1) activity. These proteolytic activities
enable the proteasome to cleave proteins into small peptides, usually 8–12 amino acids in
length. The 20S proteasome is associated with 19S components to form the 26S
proteasomes. The 19S components regulate the entry of polyubiquitin marked proteins into
the 20S proteasome [6]. The 19S complex, also termed PA700, activates the proteasome for
protein degradation. In addition to PA700, there are at least two other intracellular protein
complexes, PA28 and PA200 that can also activate the 20S proteasome using certain types
of peptide substrate as models [7, 8]. The ability to activate the proteasome and the
evolutionary conservation of these two activators imply that they may play a key role in
intracellular protein degradation and normal cellular functions. In addition, an isoform of the
proteasome, immunoproteasome, has been described in cells under influence of cytokines,
such as interferon γ [9]. A key role of the immunoproteasome is to generate peptide
fragments that bind to MHC class I molecules in antigen presentation.

Due to the importance of the proteasome in the regulation of cellular function, targeting the
proteasome has been used as strategies in developing new treatments against cancer,
inflammatory disease, and ischemic stroke. Besides the potential clinical usefulness, specific
proteasome inhibitors provide interesting and important tools for cell biologists to dissect
cellular processes that involve the proteasome.

Proteasome Inhibitors

Many proteasome inhibitors were synthesized or discovered during the last two decades.
Major progresses have been made in identifying potent and specific inhibitors as well as
understanding the mechanisms of action. Reviews and perspectives providing insightful
information on proteasome inhibitors had been previously published [10–13]. The current
known proteasome inhibitors can be classified into two groups according to their sources:
chemically synthesized small molecules and compounds derived from nature products.

a) Peptide derivatives

Majority of small molecule proteasome inhibitors are peptide derivatives that bind to the
catalytic site of β1, β2, and β5 subunits of the proteasome. Based on chemical structures,
synthetic peptidyl inhibitors include peptide aldehydes, peptide boronates, and peptide
sulfones. The chemical structure of some peptide derivatives are shown in Fig. (2). The first
generation of synthetic tri-peptide aldehydes, such as MG132, was widely used as biological
probes to study the structure and function of the proteasome [14]. MG132 inhibits the β5
catalytic subunit of proteasome through interaction with threonine in the catalytic site to
form a reversible hemiacetal intermediate. In addition to tri-peptide aldehydes, di-peptide
aldehydes were also shown to inhibit the proteasome. CEP1612 is a dipeptide aldehyde
proteasome inhibitor that was highly selective for the chymotrypsin-like proteolytic activity
of the proteasome [15].

Other reactive functional groups such as boronate and vinyl sulfones were used instead of
the aldehyde in hope of obtaining compounds with enhanced potency and selectivity (Fig.
(2)). The ZLVS and NLVS are examples of vinyl sulfones that can preferentially and
irreversibly inhibit the chymotrypsin-like (β5) proteasome activity. Recently, peptide vinyl
sulfones with phenolic substituents attached to the sulfone instead of a methyl group, were
reported to be selective for the caspase-like (β1) proteasome activity [16]. Peptide boronates
were shown to have significantly improved inhibitory activity over the aldehyde or vinyl

sulfones on proteasome inhibition. MG262, a boronate analog of MG132, exhibited a 100-fold increase in anti-proteasome activity [17].

Due to involvement of the proteasome in many cellular functions, inhibition of the proteasome is predicted to cause severe side effects in potential clinical usage. Nevertheless, the peptide boronate, PS341 (Bortezomib), was successfully developed into an anti-cancer drug for the treatment of multiple myeloma [18]. Bortezomib is not as toxic as many people had predicted. It appeared that only the chymotrypsin-like activity of the proteasome was affected at therapeutic doses [19]. With the other two main proteolytic activities remaining, normal cells seem to suffer little consequence in the presence of the drug. Bortezomib is currently undergoing a number of clinical trials, including combinations with other anti-cancer drugs, for the treatment of hematologic malignancies other than multiple myeloma. Many mechanism of action studies indicated that bortezomib altered the level of cellular proteins associated with cell growth. Bortezomib stabilized IκB [20–25], activated c-Jun-N-terminal kinase [26–28], stabilized the CDK inhibitors p21 and p27 [20, 28, 29], stabilized the tumor suppressor p53, and stabilized pro-apoptotic proteins [30–32]. Stabilization of these proteins in cancer cells can result in apoptosis or inhibition of cancer cell proliferation.

b) Natural Products

Many natural product-derived proteasome inhibitors also contain peptidyl structures (Fig. (3)). Tyropeptin A is a tripeptide aldehyde natural product isolated from *Kitasatospora* sp. MK993-dF2. Like MG132, tyropeptin A preferentially inhibits the chymotrypsin-like proteasome activity through binding to the β5 subunit of the proteasome [33, 34]. Peptide epoxyketones, isolated from various microbials, are small peptides with a ketone epoxide functional group. For example, epoxomycin was derived from *Streptomyces hygroscopicus* [35]. TMC-86 and TMC-89 were isolated from *Streptomyces sp.* [36, 37] (Fig. (3)). Peptide epoxyketones inhibit the proteasome by covalently modifying the catalytic sites of the β subunits. Carfilzomib (PR-171), an epoxyketone peptide structurally related to epoxomicin, is in Phase 2 clinical trials for patients with relapsed solid tumors including non-small cell lung cancer, small cell lung cancer, ovarian cancer, and renal cancer [38]. It is also in a phase 2 single-agent trial for patients with multiple myeloma and in a phase 1 study for lymphoma patients. Some peptide epoxyketone derivatives, such as dihydroeponemycin analogs, were shown to preferentially target the immunoproteasome [39].

PR39 is a natural occurring antibacterial peptide containing 39 amino acid residues isolated from pig intestine. R39 was shown to inhibit the proteasome. Unlike small tripeptide proteasome inhibitors that bind to the proteolytic active site located at β5 subunit, PR39 binds to the non-proteolytic α7 subunit of the 20S proteasome [40]. It was also reported that PR11 (first 11 residues of PR39 sequence: RRRPRPPYLPR) and its analogs exhibited similar activity to that of PR39. Inhibition of the proteasome by PR11 and PR39 resulted in accumulation of IκB, a factor that regulates the NF-κB-dependent gene expression pathways [41].

Other natural product proteasome inhibitors include lactacystin and belactosins isolated from *Streptomyces sp.* [42–50]. Salinosporamide A was isolated from the marine actinomyces, *Salinospora tropica* [51, 52]. These peptide proteasome inhibitors contain a reactive β-lactone or β-lactone precursors that can form an ester complex with amino acids containing a hydroxyl group, such as threonine. For example, lactacystin was proposed to be converted non-enzymatically to omuralide that reacts with the threonine in the catalytic sites of the proteasome. Salinosporamide A (NPI-0052) is undergoing clinical Phase 1b trials in drug combination therapy for patients with non-small cell lung cancer, pancreatic cancer, or melanoma. TMC-95A and its diastereoisomers are cyclic polypeptides derived from *Apiospora montagnei* Sacc [53]. This class of compounds is very potent proteasome

inhibitors with IC_{50} at low nanomolar range. Unlike the previously described proteasome inhibitors that contain a reactive functional group and form covalent adducts at catalytic sites, TMC-95A and related compounds do not carry any specific catalytic site reactive group. Instead, TMC-95A binds to the catalytic site through hydrophobic and multiple H-bond interaction. Recently, another cyclic peptide, argyrin A, derived from the myxobacterium *Archangium gephyra* was reported to be a potent proteasome inhibitor. Structurally, argyrin A is not related to TMC-95A (Fig. (3)). In comparison with bortezomib, argyrin A displayed similar proteasomal inhibition profiles on the caspase-, trypsin-, and chymotrypsin-like proteolytic activities of the proteasome [54].

Natural products derived from plant sources, such as celastrol isolated from the traditional herbal medicine "Thunder-god vine" and withaferin A isolated from Indian winter cherry, were shown to inhibit the proteasome at low micromolar concentrations [55, 56]. Celastrol is a triterpene and withaferin A is structurally related to steroids (Fig. (4)). Withaferin A was reported to induce apoptosis and inhibit the growth of human breast cancer cells [57]. Gliotoxin is a fungal metabolite structurally related to the epipolythiodioxo-piperazines with a broad range of biological activities and found to be particularly toxic to rodents. It was reported that gliotoxin inhibited the 20S protesaome [58, 59]. Green tea polyphenolic catechins such as (−)-epigallocatechin-3-gallate {(−)-EGCG} and its analogs have been widely studied for their possible benefits in cancer prevention. EGCG was reported to potently inhibit the chymotrypsin-like activity of the proteasome *in vitro* and in cultured tumor cells. It was suggested that free phenolic hydroxyl groups on the benzoate ring were essential for forming H-bond with the β5 subunit of the proteasome [60].

c) Other proteasome inhibiting agents

Disulfiram, a drug for the treatment of alcohol dependence, was shown to inhibit the proteasome (Fig. (5)). Because of proteasome inhibition, it inhibited the TNF-α-induced NF-κB translocation and caused the accumulation of the tumor suppressor protein p27[kip1]. Disulfiram was shown to have selective cytotoxicity against the chronic lymphocytic leukemia cells but not to normal peripheral blood mononuclear cells [61].

Acridine derivatives were known as a class of anti-cancer agents primarily targeting DNA and topoisomerase II. The structure of tetra-acridine is shown in Fig. (5). Recently, a study showed that tetra-acridine derivatives possessed proteasome inhibitory activity in addition to their effects on DNA [62]. These compounds displayed equal potency of cytotoxicity with/without down-regulation of topoisomerase II activity. On the other hand, other acridine derivatives that lack proteasome inhibition activity, such as massacring, showed weaker cytotoxicity toward cells with down-regulation of topoisomerase II. Proteasome inhibition by these compounds appeared to be partly responsible for their anti-tumor activity.

Proteasome activators

In contrast to the development of proteasome inhibitors, drug-like small molecules that can activate or enhance proteasome activity are rare and are not well studied [63]. On the other hand, there are three known types of cellular proteasome activators, PA28, PA200 and PA700 [6–8, 64, 65]. Over-expression of PA28 was shown to enhance the survival of Huntington's disease (HD) neuronal model cells [66]. Dysfunction of the ubiquitin-proteasome system was indicated in patients with Huntington's disease. The beneficial effects of PA28 over-expression on the HD cell model suggested that small molecule proteasome activators might be developed as useful therapeutic agents.

Several types of small molecules, including denaturing reagents (SDS), lipids and peptide-based activators were shown to activate the proteasome at relatively high concentrations. For

example, SDS at 0.05%, polylysine at 1 mg/ml, and some other peptide-based activators were shown to activate 20S proteasome at 100 μM [67]. Fatty acids such as oleic, linoleic, and linolenic acids were also shown to activate the proteasome [68, 69]. Fatty acids modulated proteasome activity in a similar pattern to that of SDS by partially denaturing the proteasome. On the other hand, synthetic peptidyl alcohols, esters, p-nitroanilides, and nitriles were reported to activate the proteasome in a concentration range of 50–150 mM through binding to the same site as PA28 [70]. Cellular lipid components such as ceramides, lysophosphatidylinositol, and cardiolipin were found to activate the proteasome [71–73]. In addition, the arginine-rich histone H3, a protein found in chromatin, was reported to selectively enhance protein degradation by the proteasome [74].

In contrast to the above discussed proteasome activators with either relatively low potency or high molecular mass, oleuropein and betulinic acid (BA) are two small molecules that can activate the proteasome at low micromolar concentrations (Fig. (6)). Oleuropein is a natural product isolated from *Olea europaea* [75]. Oleuropein was shown to enhance all three proteasome activities and delay replicative senescence of human embryonic fibroblasts. Unlike PA28, SDS, or oleuropein that activates all three proteasome proteolytic activities, BA preferentially activated the chymotrypsin-like activity and with no or minimal effect on trypsin-like and caspase-like activities [76]. In addition to activation of the proteasome, BA and BA derivatives have been extensively studied for their potential as anti-HIV agents [77–82]. BA, a lupene-type pentacyclic triterpene derived from many plant species such as birch tree [83], was also extensively studied as a potential anti-tumor agent due to its selective cytotoxicity towards some cancer cell lines [84–86].

Triterpene derivatives as either proteasome inhibitors or activators

Although BA is a proteasome activator, it can be structurally modified to become an inhibitor. In contrast to BA, many BA derivatives inhibited the proteasome. For example, BA derivatives, such as DSB (3′,3′-dimethylsuccinyl betulinic acid) and compound **4,** inhibited the proteasome (Table 1). In a cell-based assay, DSB effectively inhibited the chymotrypsin-like proteasome activity by 50% (EC$_{50}$) at 5.5 μM [76]. DSB is a potent anti-HIV-1 agent targeting viral maturation [77]. However, the proteasome inhibition activity of this compound does not appear to be responsible for its anti-HIV-1 activity.

Triterpenes other than BA can be used as scaffolds to synthesize potent proteasome inhibitors. For example, Glycyrrhetinic acid (GLA) is a β-amyrin type pentacyclic triterpene that shares structural similarities to BA and is better for the synthesis of proteasome inhibitors (Table 1). The major differences between GLA and BA are on the size of E-ring, and the extra α, β conjugated ketone on its C-ring. GLA and the 3-diglucuronide derivative glycyrrhizin can be extracted from *Glycyrrhiza glabra* (Leguminosae). Biological activities including anti-inflammatory, anti-ulcer, anti-tumor and anti-viral activity have been reported for GLA related compounds [87]. In contrast to BA, GLA did not activate proteasome, it inhibited the chymotrypsin-like activity of the proteasome (Table 1).

Various C-3 modified derivatives of GLA have been synthesized and tested for proteasome inhibition [88]. With the same C-3 side chain modifications, GLA derivatives were in general more potent than BA derivatives as seen in **1** versus DSB, and in **2** versus **4** (Table 1). Among the GLA derivatives, GLA monoisophthalate (**3**) exhibited a 2-log improvement in potency over GLA. Glycyrrhizic acid, the glycoside of GLA, is a major component from licorice species and has been used as food sweetener and clinical treatment of HBV with low toxicity [89]. Thus, GLA derivatives might have the potential to be developed as clinically useful proteasome inhibitors.

Summary

Due to the importance of proteasome in multiple cellular processes, activation or inhibition of the proteasome is expected to affect many vital cellular functions. Potential applications and biomedical implications of proteasome regulators could be interesting and significant in many areas including cell cycle control, apoptosis, stress and heat shock responses, regulation of gene expression, modulation of antigen presentation for appropriate immune responses, inhibition of virus replication by proteasome inhibitors [90–92], inflammatory diseases [93, 94], neurodegenerative diseases, and aging. Many factors such as cell type, proliferation status, and specificity could affect the ultimate effects of proteasome regulators. Although proteasome regulators may affect diverse biological responses, potent and specific activities can be developed for biological and clinical applications as seen with bortezomib.

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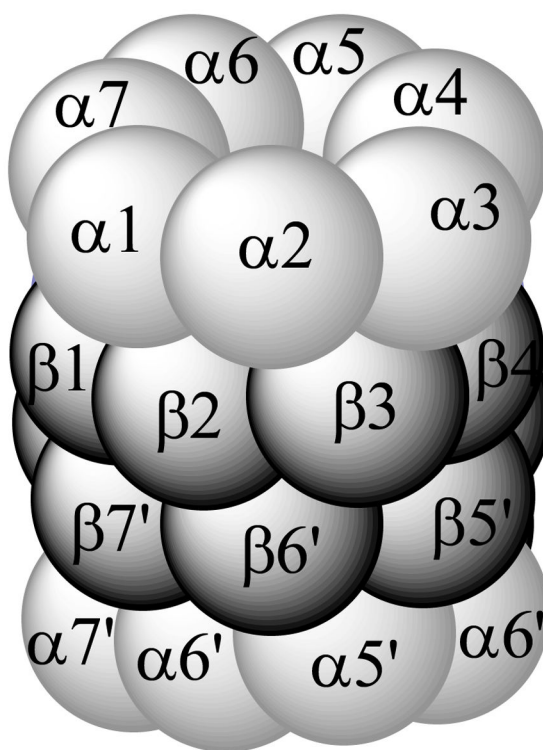
Figure 1.
Model of the 20S proteasome. Active sites are located at the N-termini of β1, β2 and β5.
The substrate preferences of these sites are: β1 – caspase-like substrates; β2 – trypsin-like
substrates; β5 – chymotrypsin-like substrates.
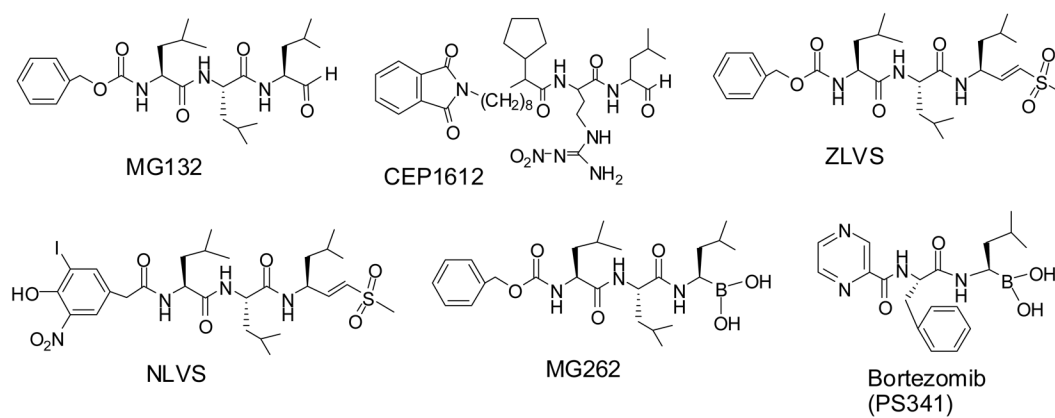
Figure 2.
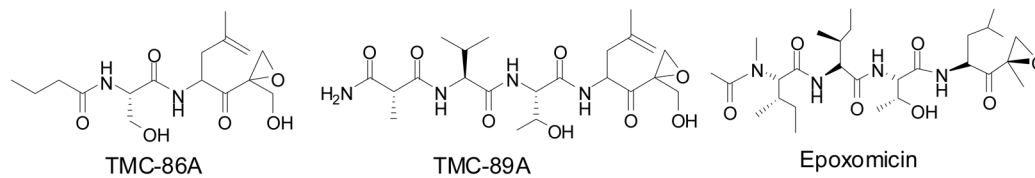Synthetic peptide derivatives

Peptide aldehyde

Tyropeptin A

Peptide expoxide

TMC-86A

TMC-89A

Epoxomicin

Carfilzomib

Lactones

Lactacystin

Omuralide

Belactosin A

Salinosporamide A
(NPI-0052)

Cyclic peptides

TMC-95A

Argyrin A
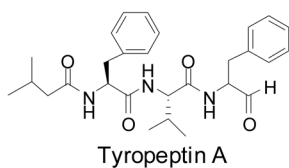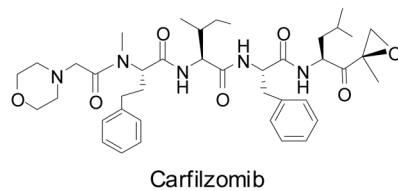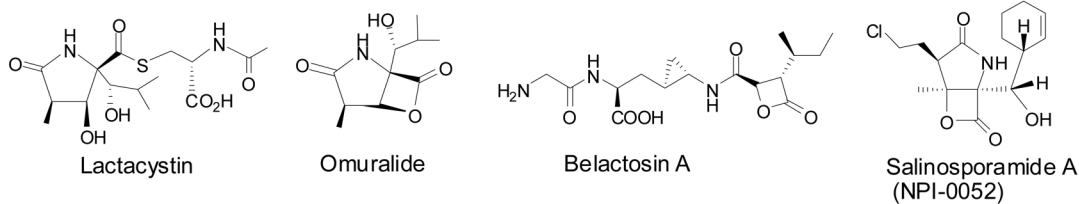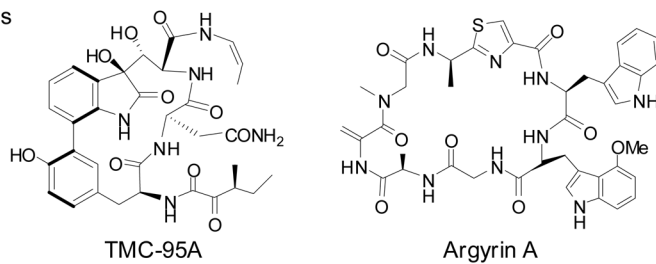
Figure 3.
Natural product derived proteasome inhibitors

Celastrol   Withaferin A   Gliotoxin   (-)-EGCG
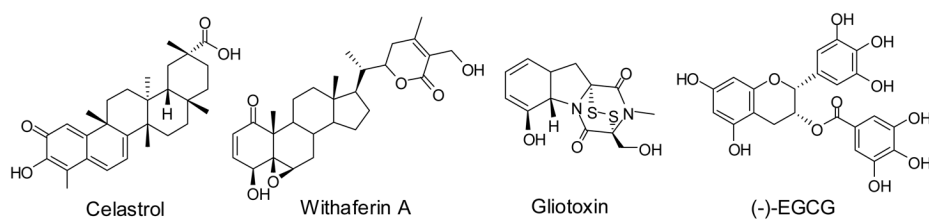
Figure 4.
Non-peptide proteasome inhibitors

Disulfiram

Tetra-acridine
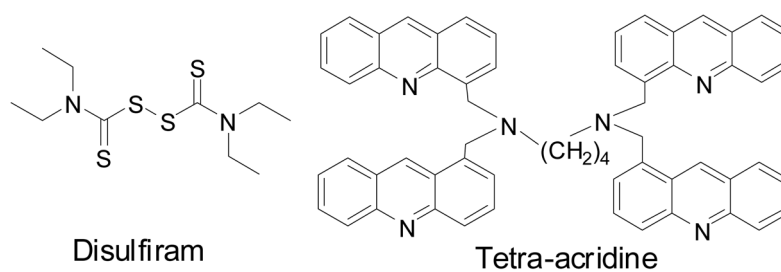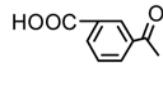
Figure 5.
Other proteasome inhibitors
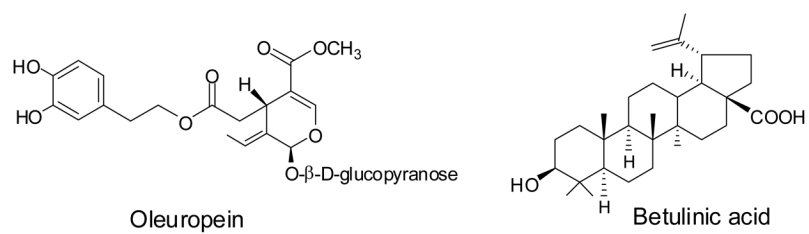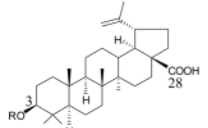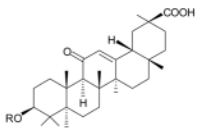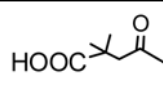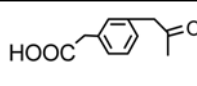
Figure 6.
Proteasome activators

Table 1

Regulation of the chymotrypsin-like proteasome activity by triterpene derivatives.

Betulinic acid derivative (BA)   18-β-glycyrrhetinic acid derivative (GLA)

R	BA derivatives	EC$_{50}$ (μM)	GLA derivatives	EC$_{50}$ (μM)
H	BA	Activator	GLA	22.3
	DSB	5.5	1	0.87
	4	2.9	2	1.05
			3	0.22