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Subunit specific inhibitors of proteasomes and their potential for immunomodulation

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Abstract

Specialized variants of the constitutive 20S proteasome in the immune system like the immunoproteasomes and the thymoproteasome contain active site-bearing subunits which differ in their cleavage priorities and substrate binding pockets. The immunoproteasome plays a crucial role in antigen processing and for the differentiation of pro-inflammatory T helper cells which are involved in the pathogenesis of autoimmunity. Selective inhibitors of the immunoproteasome and constitutive proteasome have recently been generated which interfere with the development and progression of autoimmune diseases. Here we describe these inhibitors and their therapeutic potential as predicted from preclinical models.

Introduction

The ubiquitin-proteasome pathway is the major quality-control pathway for newly synthesized proteins in every eukaryotic cell. The 26S proteasome is a large (2.5 MDa), multi-subunit, ATP-dependent proteolytic complex that processively degrades proteins into peptides. It consists of a hollow cylindrical 20S proteolytic core and one or two 19S regulatory particles (RP). The 19S RP recognizes ubiquitylated substrates and prepares them for proteolysis, which occurs inside the 20S core. The 20S cores are hollow cylindrical structures comprising two pairs of 14 different polypeptides arranged in four stacked rings. Three subunits on each inner ring carry catalytic residues for the proteolytic sites (Figure 1). The catalytic residues of the chymotrypsin-like sites are located on β5 subunits and cleave peptide bonds predominantly after hydrophobic residues. The caspase-like sites are located on the β1 subunits which cleave peptide bonds after acidic residues. The third pair of sites is located on the β2 subunits. They cleave after basic residues and are referred to as trypsin-like sites. The lymphoid tissues contain immunoproteasomes, in which β5, β2, β1 subunits

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Conflict of interest

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are replaced with β5i (LMP7), β2i (MECL1), β1i (LMP2) subunits, respectively (Figure 1). Moreover, epithelial cells in the cortex of the thymus express thymoproteasomes consisting of the active site subunits β5t, β2i and β1i. Here we describe the recent development of inhibitors for these tissue specific proteasome variants and how they can be exploited for immunomodulation in antigen processing and the therapy of autoimmune diseases.

The majority of proteasome inhibitors inhibit multiple active sites

The inhibitors of proteolytic sites of the 20S core have served as indispensible tools to identify proteasome substrates and establish proteasome involvement in a variety of biochemical, immunological, and physiological processes for two decades (see [1••] for review). One of the earliest findings with these inhibitors was that transformed cells rapidly undergo apoptosis upon treatment with proteasome inhibitors, while much higher concentrations are needed to induce death of their non-transformed counterparts. These discoveries stimulated development of proteasome inhibitors as anti-neoplastic agents and, in 2003, bortezomib was approved by the FDA for the treatment of multiple myeloma. It took few additional years to realize that exquisite sensitivity of this particular cancer to proteasome inhibitors is caused by an exceptionally high load on the proteasome in myeloma cells, which secrete large amounts of immunoglobulins [2]. In 2012, another proteasome inhibitor, carfilzomib [3], has been approved for the treatment of multiple myeloma.

Because results of site-directed mutagenesis in yeast revealed that the chymotrypsin-like sites are most important in protein degradation, and because of the ability of hydrophobic peptides to enter cells, the various synthetic proteasome inhibitors were optimized according to their capacity to block the β5 sites, which cleave preferentially after hydrophobic residues [1••]. The primary target of all natural product inhibitors is also the chymotrypsin-like site. All compounds targeting chymotrypsin-like sites (*e.g.*, MG-132, bortezomib, epoxomicin, clasto-lactacystin-β-lactone) effectively inhibit protein degradation inside the cells. However, none of these inhibitors can be considered chymotrypsin-like sites selective, and inhibition of protein degradation occurs at concentrations that co-inhibit either caspase-like or trypsin-like sites [4], raising the possibility that contribution of these two sites to protein degradation is larger than previously appreciated. This observation, together with the fact that bortezomib inhibits the caspase-like activity of the proteasome [4–6], stimulated the interest in the development of specific inhibitors of the individual active sites, to be used as tools to dissect contributions of individual sites to protein degradation, and to define their individual roles as targets for anti-neoplastic and anti-inflammatory agents.

Subunit-specific proteasome inhibitors

The great majority of site-specific proteasome inhibitors consist of an electrophilic trap that interacts with the catalytic threonine at the N-terminus of mature β-type subunits and a peptide moiety. A peptide epoxyketone YU-101 (Figure 2A) was the first highly potent and specific inhibitor of the chymotrypsin-like sites to be developed [7]. Carfilzomib is a derivative of this inhibitor [3]. It is the most specific inhibitor of the chymotrypsin-like sites among FDA-approved agents or agents undergoing clinical trials. NC-005 is even more

specific than YU-101 and carfilzomib [8] while IPSI-001 inhibits both β1i and β5i [9]. Further efforts to improve selectivity have challenged the common dogma that active-site specificity is determined by the peptide portion of the inhibitor but not by the active-site electrophile. It was found that replacing epoxyketone in NC-005 (Figure 2A) with a vinyl sulfone moiety increases specificity for the chymotrypsin-like sites [10•]. Finally, utilization of fluorinated amino acids had led to another compound with improved specificity for the chymotrypsin-like activity, LU-005 (Figure 2A) [11].

Systematic modification of the peptide epoxyketone scaffold led to the development of the β5-specific inhibitor PR-825 (Figure 2F), and two β5i inhibitors, PR-924 and PR-957 (later renamed ONX-0914, Figure 2E) [12•,13••]. Replacement of phenyl side chains in the P1 position in PR-924 and PR-957 with a cyclohexyl generated the highly selective β5i inhibitors LU-015i and LU-005i (Figure 2E) [14]. Oxathiazolones (Figure 2E) were first discovered as inhibitors of mycobacterial proteasomes but a recent study revealed that they show considerable selectivity toward β5i over β5 sites [15•]. The residues involved in the stabilization of the oxathiazolone covalent adducts with the mycobacterial proteasomes and the β5i site of human immunoproteasomes are similar. Oxathiazolones have a short half-life in aqueous solutions [15•], which may limit their *in vivo* activity. Lastly, a number of non-peptide inhibitors of β5i were identified recently by virtual screening [16].

YU-102 was the first inhibitor of caspase-like sites to be developed [17], followed by the more specific NC-001 and compound 1 [18] (Figure 2C). All three epoxyketones inhibit both the β1 and β1i sites. Replacement of the isopropyl side chain in the P1 position with a cyclohexyl moiety, and fluorine substitutions in the 3-postion of the P3-Pro residue converted NC-001 into the β1i-specific inhibitor LU-001i (Figure 2D). LU-001i is more β1i-specific than another peptide epoxyketone UK-101 (Figure 2D), which also inhibits β5i sites [14,19]. Dipeptide boronate ML604440 is a more potent and specific inhibitor of β1i sites [20]. All inhibitors are cell-permeable.

A bigger challenge has been the development of cell-permeable inhibitors of the trypsin-like sites. The first specific inhibitors of these sites were cell impermeable [21,22]. Another structural class, peptide vinyl esters [23], initially reported as cell-permeable inhibitors of the trypsin-like sites, did not have any inhibitory activity when re-synthesized by another group [10•]. Finally two cell-permeable peptide epoxyketones, NC-002 and NC-022 (Figure 2B), were discovered a few years ago [24]. They were followed by the more potent vinyl sulfone LU-102 (Figure 2B), which also has a better cell permeability [25••]. Just as with inhibitors of the chymotrypsin-like sites, these studies revealed that replacing the epoxyketone electrophile with vinyl sulfone dramatically increases the specificity of compounds for its primary target.

Most of these specific inhibitors were initially used to define the role of different active sites as drug targets in cancer. For example, it was found that increasing the specificity of compounds for the chymotrypsin-like sites dramatically decreases the cytotoxicity for HeLa cells [10•]. Inhibitors of trypsin-like and caspase-like sites, while not cytotoxic to cells when used as a single agent, sensitized multiple myeloma cells to inhibitors of the chymotrypsin-like sites, *e.g.* NC-005 and LU-005 [8]. Furthermore, inhibitors of the trypsin-like sites

selectively sensitized myeloma cells to bortezomib and carfilzomib [24]. In other words, cytotoxicity was observed only when two types of active sites were inhibited. The lack of cytotoxic effect of site-specific inhibitors offers opportunities for using them for immunomodulation.

Selective inhibition of the immunoproteasome allows *in vivo* modulation of antigen processing and T cell generation

When cells are stimulated with interferon (IFN)-γ and tumor necrosis factor (TNF)-α, they produce vast amounts of the inducible β-type subunits β1i, β2i, and β5i. In such a pro-inflammatory environment virtually all newly synthesized 20S proteasomes incorporate these inducible subunits rather than their constitutive homologues β1, β2, and β5 to form immunoproteasomes. In mice infected with viruses, bacteria or fungi it has been shown that in the liver and other organs (but not the brain [26]) constitutive proteasomes are largely replaced by immunoproteasomes [27,28]. The proteasome is centrally involved in the generation of peptide ligands of major histocompatibility complex (MHC) class I molecules which are recognized by the antigen receptors of cytotoxic T lymphocytes (CTL) (Figure 3). Under stimulation with IFN-γ or TNF-α the cell surface expression of class I molecules is up-regulated approximately tenfold. The immunoproteasome is required to produce this surplus of class I peptide ligands needed because deletion of β5i alone or all three immunosubunits (β1i, β2i, β5i) together reduces class I surface expression in spleen, lymph nodes and thymus by approximately 50% [29]. Moreover, the treatment of cells or mice with the β5i selective inhibitor ONX-0914 (formerly called PR-957) leads to a similar reduction in MHC class I surface expression indicating that β5i is pivotal for the elevated generation of class I ligands [13••,30]. ONX-0914 exerts this effect in wild type but not in $\beta5i^{-/-}$ mice indicating an on target effect of this first selective inhibitor of the immunoproteasome.

The most likely reason why immunoproteasomes produce more or better suited class I ligands is that compared to constitutive proteasomes they alter the cleavage preferences such that class I ligands with higher binding affinities for the peptide binding groove of class I molecules are generated. The caspase-like activity of β1, for example, produces weaker class I ligands than the chymotrypsin-like activity of β1i because their C-terminal anchor residues must possess hydrophobic or basic but not acidic side chains to enable tight binding into the peptide cleft of class I molecules [31]. Since β5 and β5i both exert a chymotrypsin-like activity, the requirement for this exchange was less obvious. However, the recently reported high resolution structures of mouse constitutive- and immunoproteasomes revealed that the S1 substrate pocket of β5i accommodates better bulky aliphatic and aromatic hydrophobic side chains [32••] which are most frequently found in peptides eluted from class I molecules. In spite of this valuable structural insight, the consequence of replacing β2 with β2i has remained elusive as they both harbor an equivalent trypsin-like activity [33,34] and no obvious differences between the peptide binding pockets of β2 and β2i have been noted. Accordingly, the generation of selective inhibitors for β2 and β2i will be a formidable task.

The effect of immunoproteasome deficiency or inhibition on the bulk supply of MHC class I ligands on immune defence is limited. Mice lacking the inducible subunits are immunocompetent except for an enhanced susceptibility to experimental infections with

Toxoplasma gondii [35] and *Listeria monocytogenes* [36]. However, on the level of the unique epitope the presence of immunoproteasomes can be crucial either because the activity of β1i, β2i, or β5i is required for generating the epitope [37] or because epitope destruction through the constitutive subunits β1, β2, or β5 needs to be prevented by replacement with the respective immuno-subunits [20]. The important role of immunoproteasome subunits in defining the repertoire of peptides presented on MHC class I molecules has been underlined by the mass spectrometric analysis of peptides eluted from two class I molecules of splenocytes of wild type and β1i/β2i/β5i triply gene deleted mice [29]. The advent of subunit specific inhibitors has therefore enabled the pharmacological modulation of peptide antigen generation and presentation as well as the development and expansion of cognate T cells *in vivo* [13••,30]. Whether this potential of subunit selective proteasome inhibitors can be exploited for pharmacological intervention with infectious or autoimmune diseases remains to be determined.

Immunoproteasome subunits as novel targets for the suppression of autoimmunity

Accumulating evidence indicates that the immunoproteasome plays a crucial role for the pathogenesis of several autoimmune diseases and that this role is unrelated to the MHC class I antigen processing pathway. It was found that both, $CD8^+$ CTLs and $CD4^+$ T helper cells, need the immunoproteasome to survive in a pro-inflammatory environment [38]. Consequently, the inhibition of β5i, β2i, or β1i may be a means to ameliorate pro-inflammatory autoimmune diseases which rely on these cells. In fact, the treatment of mice with the β5i selective inhibitor ONX-0914 prevented the development or progression of experimental arthritis [13••], diabetes [13••], inflammatory bowel disease [39], systemic lupus erythematosus [40], and experimental autoimmune encephalomyelitis (EAE) [41••] in preclinical mouse models. Unexpectedly, β5i-deficient mice did not display the same protection profile as ONX-0914 treated wild type mice in each of these models. Both, β5i inhibition and deficiency protected mice from dextrane sulfate sodium (DSS) induced colitis indicating that β5i plays an essential role in disease development which cannot be sustained by β5 activity [39,42]. In the multiple sclerosis model EAE, in contrast, protection from disease progression and relapse was observed in ONX-0914 treated wild type but not in $\beta5i^{-/-}$ mice [41••]. However, the treatment of $\beta5i^{-/-}$ but not wild type mice with the β5-selective inhibitor PR-825 suppressed EAE. Since β5 completely occupies the β5/β5i position in the proteasome of $\beta5i^{-/-}$ mice, this result strongly suggests that inhibition with PR-825 suppressed the chymotrypsin-like activity in $\beta5i^{-/-}$ mice to a degree which blocked the pathogenic cells. This interpretation is consistent with high immunoproteasome expression in bone marrow-derived cells which cause the disease. The lesson learnt from these experiments is that β5i selective inhibitors may not only be of therapeutic potential for diseases which rely on a non-redundant function of β5i but for all pro-inflammatory diseases which rely on leukocytes expressing high levels of immunoproteasomes.

At first sight one may argue that more general inhibitors of the proteasomal chymotrypsin-like activity could be applied. However, immunoproteasomes are largely confined to sites of inflammation and leukocytes and therefore β5i selective therapy will block them and not the

majority of tissues which express mainly constitutive proteasomes. Consistently, it has been shown that ONX-0914 is effective tenfold below its maximally tolerated dose while inhibitors of β5i and β5 like bortezomib or carfilzomib have to be applied *at* the maximally tolerated dose. The resulting adverse effects are acceptable for the treatment of neoplastic diseases like multiple myeloma or mantle cell lymphoma but are probably too severe for the long term treatment of chronic autoimmune diseases.

Conclusion

The selective inhibition of proteasome subunits is an interesting approach especially if specific subunits of the immunoproteasome but perhaps also of the thymoproteasome [37,43] are targeted. The high resolution structures of the 20S constitutive- and immunoproteasome have suggested that immunoproteasomes should be druggable and the first inhibitor of β5i has proven the principle in preclinical models of autoimmunity. Immunoproteasomes are expressed in lymphocytes, antigen presenting cells and innate immune cells and it will be important to elucidate which cells are primarily targeted in which type of disease. A similarly big challenge will be to find out how the immunoproteasome is mechanistically involved on a molecular level in the pathogenesis especially in those disease models which rely on a non-redundant function of the immunoproteasome. Hopefully site specific inhibitors of the immunoproteasome will help to identify selective substrates or cleavage activities of the immunoproteasome. Based on the reported preclinical results research teams in academia and pharmaceutical industry have intensified the search for selective proteasome inhibitors which will hopefully soon be subjected to clinical testing.

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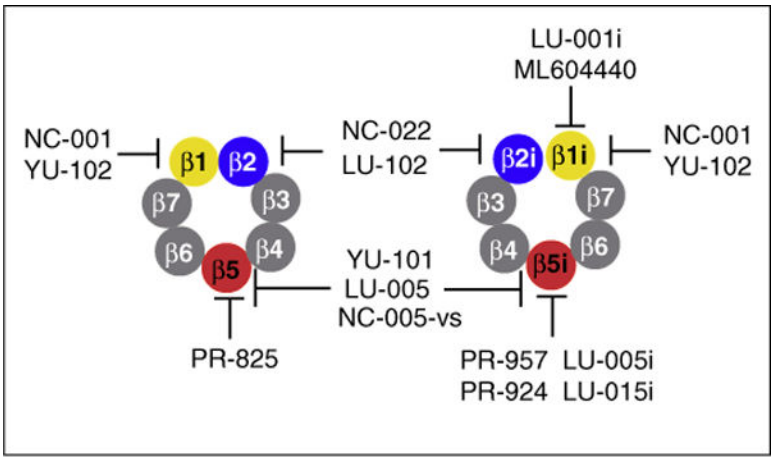

Figure 1.

Subunits of the constitutive proteasome (left) and the immunoproteasome (right) with assignment of their respective inhibitors as mentioned in the text.

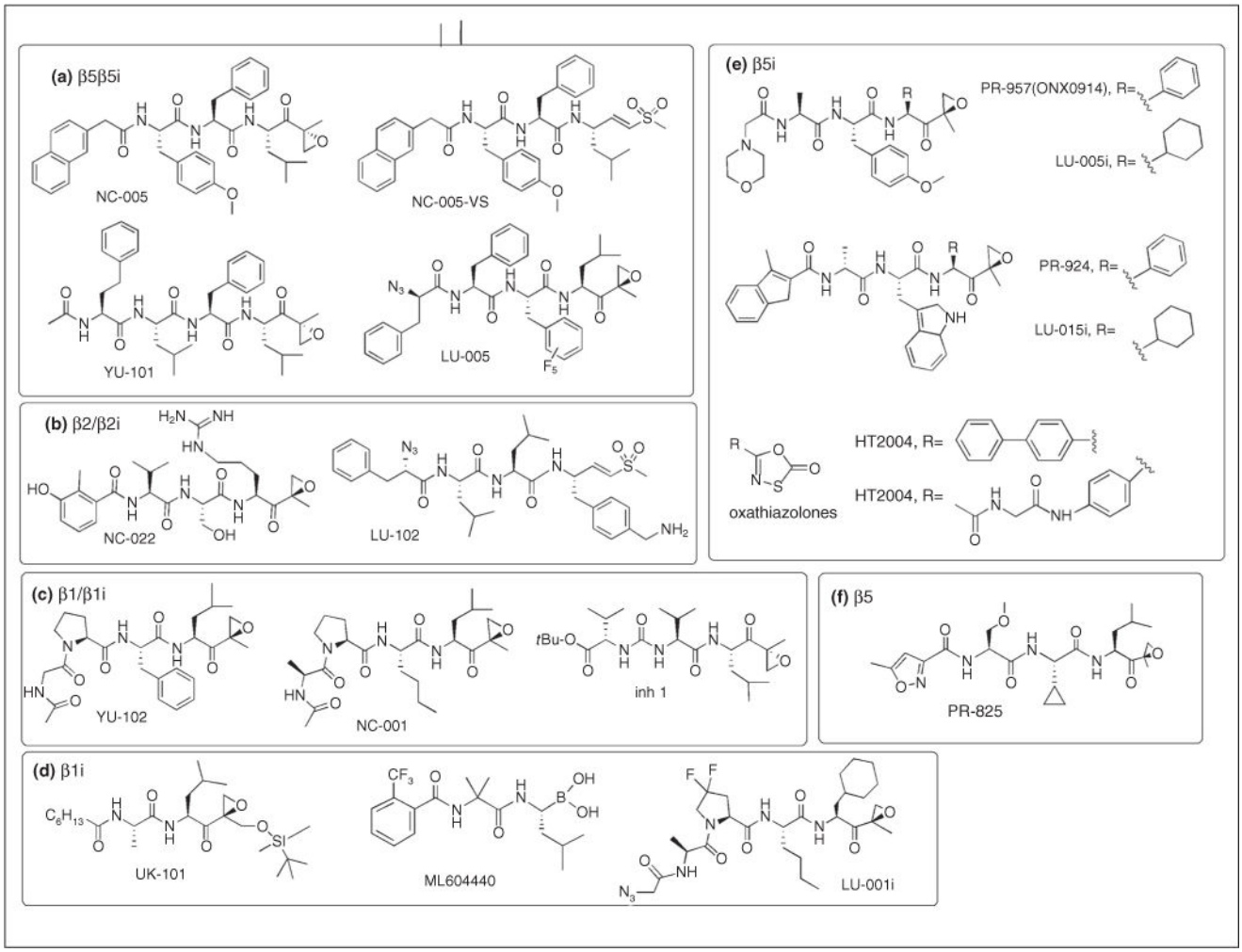

Figure 2.

Structures of proteasome inhibitors mentioned in this review grouped according to their proteasome subunit specificities.

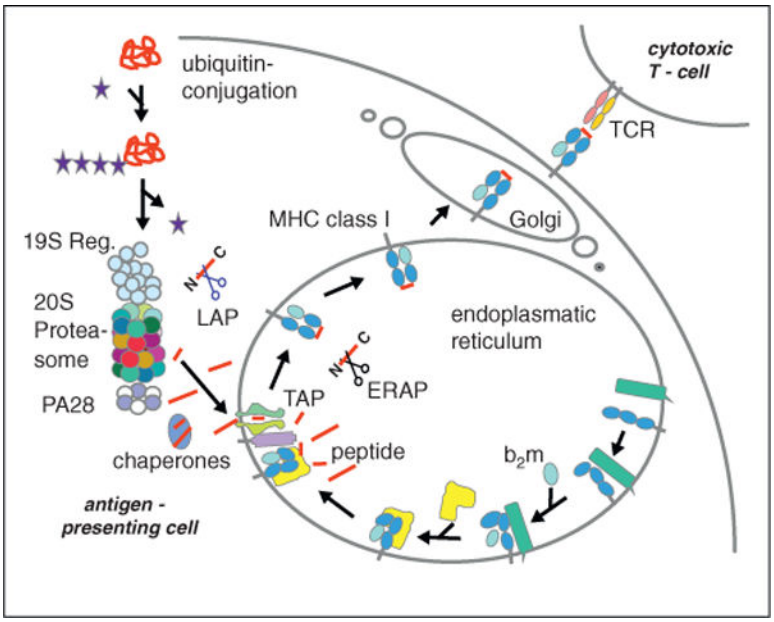

Figure 3.

Antigen processing along the MHC class I pathway. Proteins synthesized in the cell are polyubiquitylated and degraded by the proteasome. Peptides produced are either of the ideal length for class I binding (8–9 amino acids) or are N-terminally extended precursors which can be cleaved by amino peptidases in the cytoplasm. The transporter associated with antigen processing (TAP) transports the peptides into the endoplasmic reticulum (ER) where they can be further trimmed by ER amino peptidase (ERAP)1/2. Peptides binding with high affinity to the MHC class I heavy chain/β2-microglobulin (b2m) complex induce a final folding and release from the ER lumenal chaperone calreticulin (in yellow) and the transmembrane chaperon calnexin (in green) to allow exit from the ER to the plasma membrane.