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Inhibitors of the Immunoproteasome: Current Status and Future Directions

Zachary Miller, Lin Ao, Kyung Bo Kim, and Woojin Lee

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, 789 South Limestone, Lexington, Kentucky 40536-0596

Abstract

The ubiquitin-proteasome system (UPS) plays a vital role in maintaining protein homeostasis and regulating numerous cellular processes. The proteasome, a multi-protease complex, is the key component of the UPS and has been validated as a therapeutic target by the FDA's approval of bortezomib and carfilzomib. These proteasome inhibitor drugs have substantially improved outcomes in patients with hematological malignancies and are currently being investigated for other types of cancer as well as several other diseases. These approved proteasome inhibitors target the catalytic activity of both the constitutive proteasome and the immunoproteasome indiscriminately, and their inhibitory effects on the constitutive proteasome in normal cells are believed to contribute to unwanted side effects. In addition, selective immunoproteasome inhibition has been proposed to have unique effects on other diseases, including those involving aberrant immune function. Initially recognized for its role in the adaptive immune response, the immunoproteasome is often upregulated in disease states such as inflammatory diseases and cancer, suggesting functions beyond antigen presentation. In an effort to explore the immunoproteasome as a potential therapeutic target in these diseases, the development of immunoproteasome-specific inhibitors has become the focus of recent studies. Owing to considerable efforts by both academic and industry groups, immunoproteasome-selective inhibitors have now been identified and tested against several disease models. These inhibitors also provide a valuable set of chemical tools for investigating the biological function of the immunoproteasome. In this review, we will focus on the recent efforts towards the development of immunoproteasome-selective inhibitors.

Keywords

Immunoproteasome; Proteasome inhibitors; Molecular probes

1. Introduction

The ubiquitin-proteasome system (UPS) is a key pathway responsible for maintaining protein homeostasis and regulating a wide variety of cellular processes such as cell cycle progression, differentiation, inflammatory response, antigen presentation and apoptosis [1–3]. The central player of the UPS is the proteasome, a multi-protease complex which consists of a 20S core particle with regulatory particles attached to one or both ends of the core. The mature 20S proteasome core is made up of four stacked rings, two inner α -rings

Correspondence to: Woojin Lee.

CONFLICT OF INTEREST

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containing the catalytic subunits and two outer α -rings, to form a barrel-shaped structure [4, 5]. As a key member of the N-terminal nucleophilic (Ntn) hydrolase family, the proteasome has a unique catalytic mechanism that utilizes the N-terminal Thr residues of the catalytic subunits as both the proton acceptor ($-\text{NH}_2$) and the nucleophile ($-\text{OH}$) to carry out the hydrolysis of peptide bonds [2, 6]. In vertebrates, there are two major types of proteasomes: the constitutive proteasome that is normally expressed in all cell types, and the immunoproteasome that can be induced upon exposure to inflammatory cytokines such as tumor necrosis factor alpha (TNF- α) or interferon gamma (IFN- γ).

Since the initial discovery of the proteasome nearly three decades ago, our understanding of the biological and pathophysiological roles of the proteasome has come a long way. The use of synthetic and natural proteasome inhibitors has not only helped to unveil the diverse and complex nature of the proteasome, but has also provided groundwork for the development of therapeutic agents targeting the UPS. Such efforts have culminated in the successful development of bortezomib and carfilzomib as multiple myeloma therapies, and a number of next-generation proteasome inhibitors are currently in pre-clinical and clinical development (Figure 1). Despite their promising therapeutic efficacies, many of these proteasome inhibitors are frequently associated with narrow therapeutic windows or unwanted side effects, possibly due to their inhibitory effect on the constitutive proteasome in normal cells. On the other hand, it has been increasingly recognized that the immunoproteasome is highly expressed in a number of diseases including cancer and autoimmune diseases and it may be involved as a pathogenic mechanism [7–9]. These findings further justify the development of immunoproteasome-selective inhibitors as a strategy to selectively target diseased cells with minimal impact on the viability of normal cells. This approach is supported by the recent reports that selective inhibition of the immunoproteasome can offer therapeutic benefits without causing major side effects [10, 11]. The recent advances in the development of proteasome inhibitors and their chemical and pharmacological aspects have been more extensively reviewed elsewhere [8, 12, 13]. In this review, we will provide a brief overview of recent literature reporting the crucial role of the immunoproteasome in several diseases and the advances made in developing immunoproteasome-selective inhibitors and molecular probes.

2. The Immunoproteasome

Since its initial discovery in virtually all eukaryotic cell types, the constitutive proteasome has been extensively studied for its biological roles as well as its potential to serve as a therapeutic target. The constitutive proteasome contains three distinct catalytic subunits;

1 (β , *PSMB6*), 2 (β , *PSMB7*), and 5 (β , *PSMB5*), which are known to cleave peptide bonds after acidic, basic, and hydrophobic residues, respectively. Consequently, these subunits are commonly referred to as mediating the caspase-like (C-L), trypsin-like (T-L), and chymotrypsin-like (CT-L) activities of the constitutive proteasome [14]. A decade after the proteasome was first described, an alternative form of the proteasome was identified in cells treated with IFN- γ [15]. This new subtype, named the immunoproteasome, is formed by the replacement of the constitutive catalytic subunits, 1, 2 and 5 with alternate catalytic subunits, 1i (LMP2, low molecular weight protein 2, *PSMB9*), 2i (MECL1, multicatalytic endopeptidase complex-like 1, *PSMB10*), and 5i (LMP7, low molecular weight protein 7, *PSMB8*), respectively. The immunoproteasome was named based on its inducibility by IFN- γ as well as the location of the 1i and 5i genes in close proximity to other genes of the major histocompatibility complex II (MHC-II), including the *TAP1* and *TAP2* genes which encode the transporters associated with antigen presentation [4].

The immunoproteasome, which is expressed abundantly in cells of hematopoietic origin [16], is believed to be more efficient at eliciting immune responses compared to the

constitutive proteasome by generating peptides with improved binding to the MHC-I molecules for antigen presentation [17]. In particular, the incorporation of immunoproteasome catalytic subunits has been linked with enhanced CT-L and T-L activities and a decreased C-L activity as compared to the constitutive proteasome [4, 18]. The reported crystal structures of the 20S proteasome provide valuable structural insights underlying these altered cleavage preferences. For instance, the β 1i subunit carries important amino acid substitutions that alter its substrate binding pocket to be more hydrophobic and constricted relative to the β 1 counterpart [5, 19]. These substitutions appear to explain the decreased C-L activity of the β 1i subunit compared to β 1. Recently, additional key structural differences in the binding pocket were also reported in the case of the β 5i subunit with respect to the β 5 subunit [19]. Results from the crystallographic analyses showed that configuration changes in key residues may be responsible for enhancing the CT-L activity of β 5i as compared to its constitutive homologue, further contributing to an increased production of peptides suitable for MHC-I presentation [19]. These dissimilarities in the cleavage preferences and structures of the immunoproteasome and constitutive proteasome are certainly intriguing and prompt further investigations of unique functional roles of the immunoproteasome distinct from those of the constitutive proteasome.

3. The Immunoproteasome in Human Diseases

Although initially recognized for its role in the adaptive immune response, the immunoproteasome has also been implicated in other biological and pathological processes. It is now recognized that immunoproteasome upregulation in diseased cells may not be a mere consequence of elevated cytokine levels, but may have functional implications during disease development and progression. For example, it was reported that the immunoproteasome plays an important role in the degradation of damaged proteins formed as a result of oxidative stress or cytokine-stimulated protein synthesis during an inflammatory response [20]. As additional roles of the immunoproteasome outside of antigen presentation are uncovered, the list of disease states where the immunoproteasome has been implicated as a potential pathogenic mechanism is ever growing. The development of agents targeting the immunoproteasome can provide not only valuable tools to further elucidate the biological and pathological roles of the immunoproteasome, but also potential therapeutic agents [9, 16, 21]. Summarized below is a list of various human diseases where the pathological roles of the immunoproteasome have been implicated and the therapeutic potential of immunoproteasome-targeting approaches has been explored.

Inflammatory and Autoimmune Diseases

Given the well-known role of inflammatory cytokines in the formation of the immunoproteasome, the functions of the immunoproteasome have been most extensively investigated in the context of inflammatory and autoimmune diseases. In recent years, elevated immunoproteasome levels have been reported in a number of inflammatory and autoimmune diseases, such as ulcerative colitis [22], Crohn's disease [23], inflammatory bowel disease (IBD) [24, 25] and hepatitis [26]. For instance, samples from patients with IBD showed higher levels of β 1i and β 2i protein in the inflamed colon tissues compared to normal tissues [24, 25]. Furthermore, the β 1i subunit was found to be upregulated in a mouse model of dextran-sulfate sodium (DSS)-induced colitis. Transgenic mice lacking the β 1i subunit did not develop DSS-induced colitis and selective inhibition of the immunoproteasome mitigated damages to colon mucosa [22, 24, 25]. Together, these findings further indicate that β 1i plays an important role in the pathogenesis of IBD. Similarly, a report using mouse models of rheumatoid arthritis and transgenic mice lacking the β 5i subunit indicated that the immunoproteasome plays a key role in the pathogenic immune response [10]. The authors further demonstrated that the β 5i-selective inhibitor, ONX 0914 (PR-957) attenuates various inflammatory immune responses and reduces the

severity of disease symptoms [10]. These findings clearly support the involvement of the immunoproteasome as a pathogenic mechanism in these inflammatory and autoimmune diseases and provide a promising case that selective inhibition of the immunoproteasome can offer a therapeutic benefit.

Cancer

Cancer cells are generally associated with increased proteasome activity compared to non-malignant cells, likely due to factors including rapid cell proliferation, increased oxidative stress, and elevated cytokine levels [27]. With regard to immunoproteasome expression levels in cancer, apparent discrepancies however exist in the literature across differing cancer types. For example, the immunoproteasome is reported to be downregulated in esophageal, renal, skin, and head and neck cancers [28–32]. Most of these studies did not further investigate the functional consequences of immunoproteasome downregulation. Therefore, it remains unclear whether these changes in immunoproteasome expression levels have any functional significance in terms of cancer cell survival and proliferation. On the other hand, upregulation of the immunoproteasome has been observed in multiple myeloma, prostate cancer, and lung cancer [11, 33, 34]. Recent investigations using *in vitro* and *in vivo* models of multiple myeloma and prostate cancer showed that selective inhibition of the immunoproteasome leads to anticancer activity with an improved toxicity profile over broadly-acting proteasome inhibitors [11, 35]. These findings strongly suggest that the immunoproteasome plays an important functional role in cancer cell survival and thus serves as a viable target for chemotherapeutics.

Diseases of the Central Nervous System (CNS) and Aging

Diseases of the CNS are frequently associated with the accumulation of protein aggregates, often due to an imbalance between protein synthesis and proteolysis. Several studies have shown that immunoproteasome levels are elevated in these disease settings. Specifically, upregulation of the β 1 and β 5i subunits has been observed in animal models of neurodegenerative diseases, such as Alzheimer's disease, Huntington's disease, and amyotrophic lateral sclerosis [36–40]. In addition, Zheng *et al.* recently reported that immunoproteasome upregulation is linked to changes in the proteasomal activity observed in a mouse model of experimental autoimmune encephalomyelitis (EAE) as well as in clinical samples of multiple sclerosis patients [41, 42]. Interestingly, it was also noted that the levels of the immunoproteasome catalytic subunits increased in acute EAE and that the levels of the constitutive proteasome decreased during chronic EAE, implying that the immunoproteasome may be potentially involved in handling damaged proteins in inflammatory demyelinating disorders [43]. The immunoproteasome in human brain tissues is also known to exhibit age-related upregulation, which may be associated with chronic inflammation of the aged brain [44]. It is interesting to note that the immunoproteasome has been recently shown to play a crucial role in the clearance of oxidatively damaged proteins and defective ribosomal products (DRiPs) [20]. These findings support a potential role for the immunoproteasome in aging and neurodegenerative diseases as well as a general role in the handling of oxidized, misfolded, and aggregated proteins. However, the specific functions carried out by the immunoproteasome are not fully understood in aging and neurodegenerative diseases and the possible therapeutic implications require further exploration.

4. Immunoproteasome and Clinically Relevant Proteasome Inhibitors

Over the past decades, extensive efforts have been made towards the identification and development of proteasome inhibitors that can be used both as molecular probes to investigate proteasome biology and as potential therapeutic agents to treat disease

conditions. Such efforts have resulted in a number of small molecule proteasome inhibitors, including the FDA-approved bortezomib and carfilzomib. Most of these proteasome inhibitors are peptide-based small molecules which contain an electrophilic warhead at the C-terminus. These mechanism-based inhibitors can be attacked by the nucleophilic hydroxyl side chain of the Thr1 residue of the catalytic subunits. The majority of proteasome inhibitors currently used in clinical settings or as research tools are broadly-acting inhibitors that target both the constitutive proteasome and the immunoproteasome (Figure 1). As the roles of the immunoproteasome in many cellular processes are uncovered, researchers have attempted to isolate the contribution of immunoproteasome catalytic subunits to the pharmacological activity and toxic effects of these broadly-acting inhibitors. As an initial effort, recent investigations have characterized the inhibition profiles of these broadly-acting proteasome inhibitors against the individual catalytic subunits of the constitutive proteasome and immunoproteasome. Here, we briefly summarize the inhibition profiles of clinically relevant proteasome inhibitors including bortezomib and carfilzomib against the constitutive proteasome as well as the immunoproteasome (the inhibitory potencies of these clinically relevant inhibitors for individual catalytic subunits from the literature are summarized in Table 1).

Bortezomib

Since its FDA approval in 2003, Bortezomib (Velcade®, Millenium: The Takeda Oncology Company) has drastically improved therapeutic outcomes in patients with multiple myeloma, validating the proteasome as a therapeutic target [45]. Bortezomib is a dipeptidyl boronic acid that inhibits the catalytic activities of both the constitutive proteasome and the immunoproteasome by forming a slowly reversible tetrahedral adduct with the catalytic Thr1 residue at the -OH [12]. Based on kinetic analyses, bortezomib has been shown to potently inhibit proteasomal CT-L and C-L activities, with minimal impact on the T-L activity [46]. Recently, Blackburn *et al.* provided a more detailed profile of the selectivity of bortezomib towards the proteasome catalytic subunits [47]. Using subunit-specific fluorogenic substrates, bortezomib was shown to have similar potencies in inhibiting the β_1 , β_2 , and β_5 subunits, indicated by IC₅₀ values of 5.5, 8.2, and 3.3 nM, respectively. The remaining catalytic subunits, β_1 , β_2 , and β_5 can also be inhibited by bortezomib, but with much higher IC₅₀ values of 140, 940, and 1500 nM, respectively [47]. Currently, the contribution of individual catalytic subunits to the pharmacological activity and toxicity of bortezomib is not fully understood, but it may well depend on the cellular context including the relative abundance of the constitutive proteasome and the immunoproteasome. Peripheral neuropathy, a commonly occurring dose-limiting side effect with bortezomib therapy, is recently reported to be unrelated to the inhibition of the constitutive proteasome, but rather due to the inhibition of a non-proteasomal target, a serine protease expressed in neurons [48]. Nonetheless, the development and use of molecular probes targeting individual catalytic subunits may help dissect the molecular mechanism underlying the pharmacological activity and toxicity of bortezomib.

Carfilzomib

Carfilzomib (Kyprolis®, Onyx Pharmaceuticals Inc.) is a tetrapeptide epoxyketone-based proteasome inhibitor belonging to the large α , β -epoxyketone peptide family of proteasome inhibitors and it was approved as the second proteasome inhibitor drug by the FDA in 2012. Like other epoxyketone-based inhibitors, carfilzomib inactivates the proteasome irreversibly by forming a morpholino ring between its C-terminal epoxyketone pharmacophore and the catalytic Thr1 residue of the proteasome [49]. Carfilzomib was initially shown to inhibit the CT-L activity of the proteasome [46]. Later inhibition profiling studies revealed that carfilzomib inhibits both β_5 and β_1 with a similar potency (IC₅₀ values of less than 10 nM), while minimally affecting the other catalytic subunits [50]. Interestingly, carfilzomib

induced cell killing with a similar potency following the selective knockdown of the $\beta 5$ subunit in the lymphoma cell line HS-sultan [50]. These findings suggest that the inhibition of $\beta 5$ may be sufficient to exert the anticancer effect of proteasome inhibitors and provide a rationale for the possible development of $\beta 5$ -selective inhibitors as anticancer agents.

Other proteasome inhibitors under clinical development

Following the success of bortezomib and carfilzomib, there are a number of proteasome inhibitors currently under clinical evaluation. These compounds include delanzomib, oprozomib, ixazomib, and marizomib, all of which show encouraging efficacy against hematopoietic cancers as single agents or in combination with other therapies. Despite the wide variety of these next generation inhibitors progressing towards clinical use, the specific targets and mechanisms through which they exert their anticancer effects remain to be understood. In particular, the activities of many of these compounds toward the immunoproteasome require further investigations.

Delanzomib (CEP-19770), like bortezomib, binds to the proteasome irreversibly via a boronic acid pharmacophore and is shown to potently inhibit all of CT-L, C-L and T-L activities [51]. In preclinical models of multiple myeloma, delanzomib is shown to be effective in suppressing tumor growth and has demonstrated similar potencies in inhibiting proteasomal activities when compared to bortezomib. When tested against several types of normal human cells including bone marrow progenitor cells, delanzomib was found to in fact be less toxic than bortezomib when used at equipotent concentrations. Despite this, delanzomib treatment showed equal or greater activity in several mice xenograft models compared to bortezomib [51, 52]. Other significant advantages of delanzomib include potent activity following oral dosing, efficacy in cells with acquired bortezomib resistance, and, possibly, the ability to inhibit bone destruction in multiple myeloma patients via effects on osteoclasts [51, 52]. Based upon these results delanzomib is currently being studied in two phase 1/2 trials in patients with multiple myeloma.

Oprozomib (ONX 0912) is an orally bioavailable epoxyketone proteasome inhibitor and it is currently under clinical investigations for the treatment of hematologic malignancies and some solid cancers. During the development process, carfilzomib served as a starting point for having poor oral bioavailability due to its chemical/metabolic instability and low membrane permeability via P-glycoprotein-mediated efflux [53]. A large number of analogs containing a variety of side chain substitutions were synthesized and evaluated for their suitability for oral administration. This process led researchers at Onyx Pharmaceuticals to remove the P4 residue of carfilzomib, to modify the N-terminal cap, and to substitute the small hydrophilic O-methylserine residue at both the P2 and P3 positions. Lastly, the substitution of phenylalanine for leucine at the P1 position provided oprozomib with potent activity at both $\beta 5$ and $\beta 5i$. The end result was the first reported orally-available tripeptide epoxyketone proteasome inhibitor [53]. The activity profile of oprozomib towards the proteasome is similar to its parent compound carfilzomib, mainly targeting the CT-L-like activity of the $\beta 5$ and $\beta 5i$ subunits and oral dosing of oprozomib produced significant antitumor activity in two mouse xenograft cancer models [53]. *In vitro* results examining the efficacy of oprozomib in Waldenström macroglobulinemia (WM) cells showed potent inhibition of the CT-L activity of both the immunoproteasome and the constitutive proteasome [54]. Interestingly, it was also noted that WM cells expressed markedly elevated levels of immunoproteasome subunits compared to normal CD 19⁺ B cells, suggesting that inhibition of $\beta 5i$ may induce apoptosis more specifically in this type of malignant B-cells [54].

Ixazomib (MLN9708), a proteasome inhibitor currently being examined in phase 3 clinical trials, is an esterified prodrug of the boronic acid MLN2238. It mainly targets the $\beta 5$ subunit

with an IC₅₀ value of 3.4 nM, but has been shown to inhibit the T-L and C-L activities of the constitutive proteasome as well at higher concentrations (Table 2) [55]. The active metabolite, MLN2238 is shown to bind to the proteasome reversibly with a faster dissociation rate than bortezomib. Ixazomib has shown encouraging results in refractory multiple myeloma patients both as a single agent as well as in combination with lenalidomide and dexamethasone. The improved bioavailability of ixazomib also allows it to be dosed orally, an advantage over bortezomib and carfilzomib, which are restricted to intravenous or subcutaneous routes of administration [55].

Marizomib (NPI-0052, Salinosporamide A) is a non-peptidyl proteasome inhibitor with a β -lactone- β -lactam bicyclic ring structure. While marizomib shares a β -lactone pharmacophore with the previously reported natural product proteasome inhibitor omuralide, marizomib is shown to have an approximately 35-fold improved potency over omuralide [56]. The high potency of marizomib has been attributed to its unique substituents in the bicyclic ring, most notably the presence of the C2 chloroethyl group [57]. This substitution was shown to allow a unique two-step reaction mechanism with the proteasome which produces irreversible inhibition [58]. Marizomib was reported to inhibit the CT-L and T-L activities of the constitutive proteasome with a greater potency than bortezomib (Table 2) [57]. Marizomib is also reported to inhibit the immunoproteasome, although its potency towards specific subunits is not well characterized. Importantly, when tested in tumor cells from multiple myeloma patients, marizomib remained active even in samples with resistance or decreased sensitivity to bortezomib [59]. Based on these promising *in vitro* results, marizomib is currently under evaluation in clinical trials for the treatment of multiple myeloma, lymphomas, leukemias, as well as some solid cancers [60].

5. Immunoproteasome-selective Inhibitors

As the roles of the immunoproteasome expand beyond immune response and become implicated in various disease processes, considerable efforts have been spent on the development of immunoproteasome-selective inhibitors in the recent years. This led to the development of several inhibitors selectively targeting the immunoproteasome (Figure 2). Most of these inhibitors contain the chemical structure of a peptide backbone and selectively target individual subunits of the immunoproteasome (Table 2 and Figure 2). A brief summary of the activity and biological relevance of reported β 1i- and β 5i-selective inhibitors can be found in the sections below.

β 1i-Selective Compounds

UK-101—UK-101, a peptide epoxyketone, is the first reported inhibitor to selectively target β 1i [34]. In identifying UK-101 as the lead compound, the previous work on the structure activity relationship (SAR) of the related natural product proteasome inhibitors epoxomicin and eponemycin provided crucial structural insights. Previously, it had been shown that compared with epoxomicin, eponemycin preferentially inactivates the catalytic subunits of the immunoproteasome over those of the constitutive proteasome with β 1i being a primary target [61]. Thus, the N-terminal long hydrocarbon tail and C-terminal hydroxyl group of eponemycin were kept as a structural scaffold for designing a small molecule library of peptide epoxyketones. Upon screening this library, the lead compound UK-101 containing a *tert*-butyldimethylsilyl (TBDMS) ether group at the C-terminus, an alanine residue at the P2 position, and the hydrophobic heptanoic tail at the N-terminus was identified as the most promising β 1i-selective inhibitor of the series [34]. *In vitro* assays confirmed that UK-101 covalently modifies β 1i and inhibits the CT-L activity of the immunoproteasome. Furthermore, it is shown that UK-101 does not inhibit angiogenic sprouting of human endothelial cells, which is known to rely on the activity of the constitutive proteasome [34].

Taken together, these findings support that UK-101 selectively inhibits the immunoproteasome without affecting the activity of the constitutive proteasome.

Computational modeling studies further revealed the structural basis for the observed selectivity of UK-101 toward the β 5 subunit [62]. Briefly, the results from the computational modeling studies suggested that the linear hydrocarbon group of UK-101 at the P3 position fits well into a long hydrophobic pocket formed by the interface of the β 5 subunit with the adjacent β 2 subunit. The corresponding pocket between the β 5 subunit and its adjacent β 6 subunit was predicted to be considerably less hydrophobic and to interact less favorably with UK-101. In addition, the TBDMS group of UK-101 was shown to be positioned to interact favorably with the entrance to the hydrophobic pocket of the β 5 active site. To the contrary, UK-101 was predicted to have a specific steric clash with Phe169 in the β 5 subunit, which disrupts hydrogen bonding between the epoxide oxygen and a key residue of the oxyanion hole and makes the binding of UK-101 to the β 5 subunit energetically unfavorable [62].

The availability of UK-101 as a β 5-selective inhibitor provided a new strategy for exploring the potential of the immunoproteasome as whole or the β 5 subunit alone as a therapeutic target. Initially, UK-101 was shown to induce apoptotic cell death in prostate cancer cell lines expressing β 5 [34]. More recent results confirmed that UK-101 dosing can suppress *in vivo* tumor growth using a mouse xenograft model of human prostate cancer [11]. In this particular study, additional experiments were carried out to further probe the mechanisms underlying the anticancer effect of UK-101 and to validate the β 5 subunit as the major pharmacological target of UK-101. Interestingly, genetic knockdown of β 5 using siRNA was found to significantly decrease the proliferation of prostate cancer cells, similar to the UK-101 treatment [11]. Analyses of additional clinical samples and established cancer cell lines of multiple cancer types also indicated that the β 5 subunit is frequently upregulated in cancer cells, but not in non-malignant cells [11]. Together, these results suggest that the immunoproteasome may have an important role in the development and progression of cancers derived from solid organs and that targeting the immunoproteasome may serve as a viable therapeutic strategy for cancer treatment.

In addition to being a potential therapeutic agent, UK-101 also provided a tool to selectively modulate the β 5 activity and explore the biological function of the β 5 subunit. The involvement of β 5 in the activation of the canonical NF- κ B pathway has been controversial in the literature over the past decade [63–65]. Traditional gene deletion approaches do not allow for interrogation of the involvement of β 5 in this process, since the deletion of β 5 would impact the proteasome assembly process and thereby disable the function of the entire multi-subunit protein complex. Using UK-101 and other small molecule inhibitors as chemical tools, Jang *et al.* have shown that β 5 catalytic activity is not required for the activation of the canonical NF- κ B pathway [66]. More recently, UK-101 has proven to be a useful tool in investigating the roles of the immunoproteasome in the degradation of CYP2B1 and the maintenance of pluripotency in human embryonic stem cells [67, 68].

IPSI-001—IPSI-001 (immunoproteasome-specific inhibitor 001, Z-LnL-CHO), another β 5-selective inhibitor, was identified among a panel of carbobenzyloxy-capped (Z-capped) dipeptide aldehydes [35]. IPSI-001 was found to have a greater than 100-fold selectivity for the immunoproteasome over the constitutive proteasome as measured by K_i values for the inhibition of both the CT-L and branched chain amino acid preferring (BrAAP) activities [35]. At concentrations of up to 50 μ M, IPSI-001 inhibited β 5 activity with little to no effect on other catalytic subunits. IPSI-001 was shown to induce apoptosis in multiple myeloma cell line models and in samples from patients with hematological malignancies [35]. Interestingly, IPSI-001 effectively induced apoptosis in multiple myeloma cells resistant to

bortezomib and was less toxic towards nonmalignant cells than bortezomib [35]. This study provided additional evidence that the immunoproteasome, specifically the β 5i subunit, is a promising target for the treatment of hematologic malignancies, especially multiple myeloma.

YU-102—YU-102 is a tetrapeptide epoxyketone and it was first reported as an inhibitor of the C-L activity of the proteasome based on its ability to selectively block the proteasomal hydrolysis of the fluorogenic substrate Z-LLE-AMC [69]. However, we recently found that YU-102 preferentially targets β 1i and to a lesser extent, β 1. These findings were obtained using molecular shift assays as well as *in vitro* enzyme kinetics with purified proteasome preparations and specific fluorogenic substrates (unpublished results from this laboratory). Despite potent β 1i inhibition, YU-102 was found to have minimal activity against the β 5 and β 5i subunits and low cytotoxicity when tested against certain cancer cell lines (unpublished results). The unique characteristics and inhibition profiles of YU-102 may provide an additional tool in investigating the biology of the immunoproteasome and in pursuing drug discovery efforts. Interestingly, YU-102 is structurally distinct from other β 1i-selective inhibitors, such as UK-101 and IPSI-001, as it contains a γ -turn amino acid proline in its backbone. Thus, understanding the structural basis for the interaction between YU-102 and β 1i may shed light on the future designs of novel β 1i-selective inhibitors with greater potency, specificity, and therapeutic utility.

β 5i-Selective Compounds

ONX 0914 (PR-957)—ONX 0914 (PR-957), a tripeptide epoxyketone, is the first β 5i-selective compound to be reported in the literature. In the initial study, the selectivity of ONX 0914 was assessed by quantifying the occupied proteasome active sites in human leukemic cells treated with ONX 0914 via a subunit-specific ELISA and the results indicated that ONX 0914 is 20- to 40-fold more selective for the β 5i subunit than the next most sensitive subunits, β 5 or β 1i [10]. ONX 0914 was found to inhibit the remaining catalytic subunits, but at even higher concentrations (Table 2) [10]. The selectivity of ONX 0914 for β 5i was further verified using knockout mice lacking β 5i. Specifically, the inhibitory effect of ONX 0914 on the inhibition of CT-L substrate hydrolysis and on the presentation of MHC class I molecules was observed in wild-type animals, but not in β 5i knockout mice [10]. Using *in vivo* dosing studies, intravenously administered ONX 0914 was shown to achieve selective inhibition of β 5i in the proteasomes isolated from whole blood and kidney [10].

Using ONX 0914 as a β 5i-selective inhibitor, several investigations have reported the promising potential of the immunoproteasome-targeting approaches, in particular for inflammatory and autoimmune diseases. Targeted inhibition of β 5i *in vivo* using ONX 0914 was found to offer significant therapeutic benefits in mouse models of rheumatoid arthritis (RA) [10]. ONX 0914 significantly inhibited the production of pro-inflammatory cytokines such as IL-23 and TNF in activated monocytes and blocked disease progression and alleviated the severity of disease symptoms. By multiple measures, ONX 0914 proved to be even more effective than the clinically important RA treatment etanercept as well as the β 5-selective inhibitor PR-825 and carfilzomib [10]. Specifically, PR-825 showed no effect on disease severity whereas carfilzomib had modest efficacy but only at the maximum tolerated dose (MTD). In contrast, ONX 0914 was effective at the 2 mg/kg dose level, far below the MTD of 30 mg/kg. In addition to RA, similar anti-inflammatory activity of ONX 0914 was observed in a mouse model of colitis [22]. ONX 0914 was found to decrease the secretion of pro-inflammatory cytokines and effectively blocked the loss of body weight and improved other measures of disease severity in dextran sulfate sodium-induced colitis [22]. More recently, ONX 0914 was also investigated as a potential therapy for systemic lupus

erythematosus (SLE), a complex autoimmune disease characterized by dysregulation in multiple arms of the immune system [70]. Previously, bortezomib was shown to be effective in depleting autoantibody-producing plasma cells and ameliorate SLE-like diseases in mice [71]. When tested using lupus-prone mice, ONX 0914, with a potentially less systemic toxicity, was as highly effective as bortezomib or carfilzomib in preventing disease progression and reducing symptoms of nephritis [71]. Together these findings strongly indicate that β 5i inhibition is a viable strategy for the treatment of a potentially diverse set of important inflammatory and autoimmune diseases, each with their own unique pathophysiology.

Structural insights into the observed selectivity of ONX 0914 toward the β 5i subunit can be found in a recent report on the crystal structure of the murine immunoproteasome in complex with ONX 0914 [19]. The findings from the crystal structure of the ONX 0914:immunoproteasome complex suggested that the N-terminal morpholinyl acetyl cap of ONX 0914 makes no apparent contacts with the β 5i subunit and likely serves to improve the solubility and possibly the pharmacokinetic properties of ONX 0914 [19]. Other findings from these crystallographic studies may provide crucial insights into the design of immunoproteasome inhibitors with improved subunit selectivity and potency. (A more detailed summary on structural findings and lessons for drug development is provided in a separate section below.)

PR-924 (IPSI)—PR-924 is another epoxyketone peptide-based, β 5i-targeting inhibitor with structural similarities to ONX 0914. When tested using the ELISA-based ProCISE assay, PR-924 was found to potently and selectively inhibit β 5i with an IC_{50} value of 22 nM [50] (Table 2). The selectivity of PR-924 for β 5i was greater than 100-fold over β 5 and little or no inhibition was observed for other catalytic subunits (β 1, β 1i, β 2, or β 2i) even at high μ M concentrations (Table 2). Although recent studies suggest that PR-924 can be more potent and selective than the related inhibitor ONX 0914 [10, 19, 50], some discrepancies appear to exist in terms of the pharmacological activity of PR-924 in the literature. The initial study reported that one-hour pulse treatment with PR-924, a condition causing β 5i inhibition by greater than 90%, does not induce apoptotic cell death in any of the three hematologic cancer cell lines tested [10]. In line with this observation, PR-924 did not induce Noxa upregulation or the accumulation of polyubiquitinated proteins, well-known markers of proteasome inhibition [10]. In contrast, when combined with selective β 5 inhibition by PR-825 (CPSI) or genetic knockdown of β 5, cell death and other classical effects of proteasome inhibition were evident [10]. Surprisingly, a more recent study reported that PR-924 can effectively induce apoptosis in both clinical samples from multiple myeloma patients and in numerous multiple myeloma cell lines [72]. In two multiple myeloma cell lines, PR-924 was shown to induce apoptosis via the intrinsic pathway, accompanied by increased cleavage of caspases-3, -8 and -9 and several signs of mitochondrial loss of permeability [72]. No significant toxicities were observed in normal peripheral blood mononuclear cells following exposure to as much as 20 μ M PR-924 [72]. More importantly, strong antitumor activity of PR-924 was shown in two mouse xenograft models of multiple myeloma without observable weight loss or signs of neurological damage. It is unclear at this point how to reconcile these apparent discrepancies in *in vivo* and *in vitro* responses of PR-924 [50, 72]. Although PR-924 has not yet been investigated in clinical trials, these studies highlight the need for further investigation of the role of β 5i subunit in cancer.

6. The Structure of the Immunoproteasome and Lessons for Drug Development

Since Science published the first crystal structure of the proteasome by Lowe et al. from an archeal species [73], numerous additional crystal structures have been reported including those of mammalian constitutive proteasomes [74]. Despite this, the design of immunoproteasome inhibitors has been hampered until recent years by the lack of structural information about the immunoproteasome. Recently, Huber et al. [19] reported the structures of murine constitutive proteasome and immunoproteasome in complex with the 5i-selective inhibitor ONX 0914. These crystallographic studies revealed key structural differences between the constitutive catalytic subunits and their inducible counterparts, providing important molecular insights into how specificity/selectivity can be achieved for both substrates and inhibitors of the immunoproteasome [19].

While the structures of the 2 and 2i subunits were essentially identical in terms of their substrate binding pockets, the 1i subunit showed a structure distinct from its partner, 1. Specifically, the 1i subunit carries several important substitutions which alter the character of its S1 and S3 substrate binding pockets relative to the 1 subunit. For example, Arg45 located in the S1 pocket of 1 is replaced with a leucine, explaining the loss of preference for negatively-charged 'C-L' substrates in the 1i subunit of the immunoproteasome. Additional substitutions serve to make the 1i S1 pocket more hydrophobic and less spacious and to alter the character of the S3 pocket. Despite these changes, it appears that the S3 pockets of 1 and 1i are similar as both readily bind inhibitors containing the -turn amino acid proline (unpublished results) [19]. In the case of 5i, its S1 pocket is similar in sequence to that of the constitutive 5 subunit but has a larger volume for ligand binding due to a change in the configuration of the critical Met45 residue. The conformation of this residue in 5 has been reported to be involved in 'tuning' the size of S1 pocket as crystal structures have depicted various poses for Met45 depending on the inhibitor used in complex with the proteasome [19]. In contrast, 5i is shown to have a distinct Met45 conformation due an apparent hydrophobic interaction with the side chain of nearby Gln53 which is altered from 5's Ser53. Additionally the S2 pocket of 5i is decreased in size relative to 5 due to the substitution of the constitutive subunit's Gly48 residue by a cysteine or serine residue in 5i depending on the species. The S3 pocket of the immunoproteasome subunit is also slightly restricted by an alanine to serine substitution at position 27. Other changes include the A46S and V127T substitutions which are hypothesized to improve the catalytic activity of 5i by stabilizing the tetrahedral intermediate and these changes are highly conserved in the 5i subunits across multiple species [19].

The 5i specificity of ONX 0914 was readily justified by the X-ray structure of the ONX 0914:immunoproteasome complex. As expected based upon prior specificity data, the three subunits which lack CT-L character, 1, 2, and 2i, were found to lack structural requirements for strong inhibitor binding. Specifically, it was revealed that ONX 0914's P1 phenylalanine residue is not well stabilized by the large and hydrophilic S1 binding pockets of 2 and 2i. Similarly the constitutive proteasome's 1 subunit was found to lack the required P1 hydrophobicity and inhibitor binding was further hampered by a steric clash with the Arg45.

In contrast to the above subunits, 1i and 5i can be inhibited by ONX 0914 albeit with inhibitor IC₅₀ values approximately 15-fold larger than that for 5i [10]. In the case of 1i, the P1 site does carry the hydrophobic residues needed to stabilize ONX 0914 but the presence of Phe31 may sterically oppose binding [19]. Analysis of binding to the 5 subunit further confirmed the importance of Met45. According to the study, the side chain of Met45 must undergo rotation upon the binding of ONX 0914 to 5 but not to 5i. This rotation

eventually leads to the displacement of the two β -sheets, S4 and S5, as well as the H1 α -helix which together form the majority of the S1 pocket, making the ONX 0914 binding to S5 less favorable. In addition, the X-ray structure demonstrated that the formation of the initial hemiketal adduct between the inhibitor and the Thr10 nucleophile may depend chiefly on the P1-S1 interaction. This suggests that favorability of P1 interactions may play an especially large role in determining specificity thus increasing the penalty associated with Met45 rotation [19]. Although x-ray crystallography cannot fully reflect the dynamic process of inhibitor binding, this first crystal structure of the immunoproteasome with an inhibitor will certainly provide insight into the design of immunoproteasome-specific inhibitors in the future.

7. Immunoproteasome-selective probes

Over the years, small molecule inhibitors have been successfully modified to yield valuable molecular probes for studying various cellular processes. Examples include affinity reagent- or fluorescent group-labeled small molecules that target specific proteins. Recently, the availability of several immunoproteasome-selective inhibitors has made it feasible to design immunoproteasome-selective affinity reagents, imaging probes, and fluorogenic substrates. The following is a brief summary of the recent advances in the development of these immunoproteasome-selective probes.

Fluorogenic Peptide Substrates

One of the commonly used methods to assess the activity of proteasome inhibitors is to measure their ability to block the hydrolysis of fluorogenic substrates by the proteasome. These fluorogenic substrates are typically tri- or tetrapeptides linked to a fluorogenic group at the C-terminus via a peptide bond, which can be hydrolyzed by the proteasome. Commonly used fluorogenic groups include 7-amino-4-methylcoumarin (AMC), 7-amino-4-trifluoromethylcoumarin (AFC), 2-naphthylamine (NA), and 4-methoxy-2-naphthylamine (MNA). These peptide substrates bind to the catalytic subunit active sites and undergo peptide bond hydrolysis in a manner analogous to that of natural peptide substrates. This method is simple and lends itself to high throughput screening methodologies. Initially, a number of peptide substrates were developed to measure each of the CT-L, T-L and C-L activities of the proteasome [75]. For example, the fluorogenic substrate Suc-LLVY-AMC is frequently used to measure the CT-L activity of the proteasome which is contributed by multiple subunits, namely β_5 , β_5i , and to a lesser extent, β_1i . With increasing interest in the function of the immunoproteasome, there is now more than ever a clear need to selectively measure the activity of the immunoproteasome catalytic subunits. In this regard, Blackburn *et al.* reported that peptide substrates, Ac-PAL-AMC and Ac-ANW-AMC can be used to measure the activity of β_1i and β_5i , respectively [47]. In this study, the authors validated the selectivity of Ac-PAL-AMC and Ac-ANW-AMC as probes for β_1i and β_5i by demonstrating no substrate cleavage in cellular extracts prepared from cells lacking the immunoproteasome [47]. The availability of these immunoproteasome-selective substrates will provide a powerful tool for the screening of potential immunoproteasome-selective inhibitors on a large scale.

Fluorescent Imaging Probes

Inhibitor-derived imaging probes have long been useful tools for investigating protein function. For the proteasome, broadly-acting proteasome inhibitors were initially used to design activity-based fluorescent probes that target catalytically active proteasome subunits (Figure 3A) [76, 77]. These broadly-acting proteasome imaging probes have been successfully used for proteasome functional studies and inhibitor screening [76, 78, 79]. However, these probes were not adequate in distinguishing the activities of the constitutive

proteasome and the immunoproteasome. In this regard, our laboratory has recently developed immunoproteasome-selective fluorescent imaging probes for β 1i by attaching a fluorophore to the β 1i-selective inhibitor UK-101 [80]. Similarly, our laboratory generated a fluorescent probe targeting β 5i by the strategic modification of the β 5i-selective inhibitor LKS01 [81] (Figure 3B). LKS01 itself was synthesized as a derivative of the β 5i-selective inhibitor PR-924 by producing a suitable anchor site for further modification. In these efforts, the anchor sites for fluorescent residues within UK-101 and LKS01 were selected by *in silico* molecular modeling studies in order to avoid any interference with the binding of the probe to its target in the immunoproteasome. The specificity of these fluorescent probes was confirmed by molecular shift, in-gel fluorescence, and competition-based assays [81]. These probes will be especially useful in investigating the functional dynamics of the catalytically active immunoproteasome in living cells. Furthermore, immunoproteasome-selective near infrared probes developed by our laboratories can be potentially used for noninvasive detection of the immunoproteasome in small animal settings [80, 81].

In addition to these direct treatment approaches utilizing fluorescently labeled inhibitors, indirect, bioorthogonal chemical approaches have also been developed to label the desired subunits with fluorescent groups. In this approach, proteasome catalytic subunits are first tagged using proteasome inhibitors containing biologically inert chemical handles. In a second step, fluorescent groups are specifically linked to the chemical handles. This can be achieved through the classic coppercatalyzed azide-alkyne Huisgen cycloaddition (or 'click' reaction) [82], the Staudinger–Bertozzi ligation [83], or the irreversible norbornene-tetrazine Diels-Alder reaction [84]. Compared with the direct attachment of fluorophores, these novel strategies are far less likely to perturb inhibitor permeability or target binding [85]. In one relevant publication, three functionalized proteasome inhibitors were generated: a norbornene-containing β 5 inhibitor, an azide-containing β 1 inhibitor, and a non-selective, alkyne-functionalized analog of epoxomicin. Living cells were first treated with the two selective inhibitors followed by the non-selective inhibitor in order to uniquely label all three constitutive proteasome catalytic subunits. After cell lysis, ligation reactions were performed to specifically label two of the bound inhibitors with different BODIPY fluorophores and the third with biotin. This allowed the detection of all three constitutive proteasome subunits [85]. When combined with multicolor fluorescence detection, simultaneous detection of all proteasome subunits is possible, thus providing a powerful technique for monitoring proteasome activity in living systems.

It is clear that inhibitor-based probes have now reached a level where they can provide significant information about the proteasome and immunoproteasome. Although our understanding of the function of the immunoproteasome at the level of binding and catalysis has advanced, new probes may greatly enhance our ability to investigate the biology of the immunoproteasome and screen immunoproteasome-selective inhibitors.

7. Future Directions

Considerable progress has been made in the development of immunoproteasome inhibitors, probes, and substrates in recent years. With these available tools, the field has just begun to explore the intricate biology of the immunoproteasome and its therapeutic potential is yet to be fully realized. As the field of immunoproteasome biology advances, there remains a clear need to develop immunoproteasome inhibitors with higher potency and selectivity. In addition, there will be many hurdles to overcome as the therapeutic use of immunoproteasome inhibitors becomes a reality. Despite the exciting results from the dosing of immunoproteasome inhibitors in mice, no data has been published demonstrating the use of immunoproteasome inhibitors in humans. In fact, it remains unclear if the current generation of immunoproteasome inhibitors will be suitable for use in humans. In addition,

the possible beneficial and adverse effects of modulating the immune system with immunoproteasome inhibitors require careful study. The stability, distribution, and elimination of immunoproteasome inhibitors, in particular, need further investigation. Carfilzomib has been shown to have a half-life of less than 30 minutes, most likely due to rapid backbone proteolysis and epoxide group hydrolysis [86, 87]. Although proteasome inhibition with carfilzomib may persist as long as one day, this duration of inhibition would likely be unsuitable for many patients with chronic inflammatory disease [87]. It is for these reasons that the development of novel immunoproteasome inhibitors with improved properties is necessary to achieve true clinical utility.

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Figure 1.
Broadly acting proteasome inhibitors. A. FDA-approved proteasome inhibitor drugs bortezomib and carfilzomib and other compounds under clinical development. B. Proteasome inhibitors that are widely used as research tools.

Figure 2.
Chemical structures of immunoproteasome-selective inhibitors targeting 1i (A) and 5i (B).

Figure 3. Fluorescently labeled activity-based small molecule probes of the proteasomes. A. Probes targeting both the constitutive proteasome and immunoproteasome. B. Probes selectively targeting the immunoproteasome.

Inhibitory potencies of clinically relevant and other broadly-acting proteasome inhibitors toward the proteasomal subunits.

Table 1

Classification	Inhibitor	IC ₅₀ (nM) for individual subunits					
		1	1i	2	2i	5	5i
FDA-approved Inhibitors	Bortezomib ^{a,b}	74–140	5.5	1,500–4,200	940	7–8.2	3.3–4
	Carfilzomib ^{a,i}	2,400	2,420	3,600	620	6	33
In clinical development	Marizomib ^c	430	n.r.	28	n.r.	3.5	n.r.
	Delanzomib ^d	3–10	1–3	~30	10–30	3–20	3–20
	Oprozomib ^e	n.r.	n.r.	n.r.	n.r.	36	82
	Ixazomib ^f	31	n.r.	3,500	n.r.	3.4	n.r.
Research tools	MG-132 ^f	2,240	n.r.	9,215	n.r.	24	n.r.
	Epoxomicin ^g	153	n.r.	78	78	18	18
	Omuralide ^g	2,600	n.r.	760	n.r.	20	n.r.
	ZLVS ^h	>10,000	n.r.	>10,000	n.r.	~2,000	n.r.

n.r. denoting that IC₅₀ values are not reported.

^{a–h} denoting the experimental methods used in determining IC₅₀ values.

^aIC₅₀ values obtained from hydrolysis of fluorogenic substrates Suc-LLVY-AMC, Z-LLE-AMC, and Boc-LRR-AMC in purified 20S preparations of the constitutive proteasome (from erythrocytes) and immunoproteasome (from peripheral blood mononuclear cells);

^bIC₅₀ values obtained from hydrolysis of fluorogenic substrates Z-LLE-AMC, Ac-KQL-AMC, Ac-WLA-AMC, Ac-PAL-AMC, Ac-ANW-AMC in purified preparations of the constitutive proteasome and immunoproteasome;

^cIC₅₀ values obtained from hydrolysis of fluorogenic substrates Suc-LLVY-AMC and BZ-VGR-AMC in purified 20S proteasomes from erythrocytes;

^dIC₅₀ value for 1 was obtained from hydrolysis of fluorogenic substrate Z-LLE-AMC, whereas values for 1i, 2, and 2i were approximated using a fluorescent probe labeling competition assay. IC₅₀ value ranges for 5 and 5i were approximated from the results of both methods. All assays were performed in lysates from RPMI-8226, H929, and JIN3 multiple myeloma cells;

^eIC₅₀ values obtained using ProCISE assay in purified human 20S proteasomes;

^fIC₅₀ values from hydrolysis of Suc-LLVY-AMC, Z-VGR-AMC, and LLE-AMC in blood cell lysates (Ixazomib) or purified 20S proteasomes (MG-132), IC₅₀ values were obtained from *in vitro* treatment of MLN2238, the hydrolyzed, biologically active form of Ixazomib (MLN9708) ;

g IC₅₀ values obtained from hydrolysis of luminescent peptide substrates Suc-LL VY-aminoluciferin, Z-LRR-aminoluciferin, and Z-nLPnLD-aminoluciferin in U266 myeloma cell lysates (epoxomycin) or purified 20S proteasome (omuralide);

h IC₅₀ values obtained from hydrolysis of fluorogenic substrates Suc-LL VY-AMC, Ac-nLPnLD-AMC, Ac-RLR-AMC, and Ac-RQR-AMC in purified 26S proteasomes from rabbit muscle;

i IC₅₀ values obtained from hydrolysis of fluorogenic substrates Suc-LL VY-AMC, Ac-nLPnLD-AMC, Ac-RLR-AMC, and Ac-RQR-AMC in purified immunoproteasomes from rabbit spleen.

Table 2

Inhibitory potencies of immunoproteasome-selective inhibitors toward the proteasomal subunits.

Classification	Inhibitor	K _i or IC ₅₀ for individual subunits (nM)						Ref.
		1	1i	2	2i	5	5i	
1i-selective Inhibitors	UK-101 ^a	>10,000	100–500	>10,000	>10,000	>500	>10,000	[11, 34]
	IPSI-001 ^b	239,000*	1450*	n.r.	n.r.	105000*	1030*	[35]
	YUI-102 ^c	209	46	>5,000	>5,000	>1,000	>5,000	
5i-selective Inhibitors	ONX 0914 ^d	~9000	~500	~3000	~1000	~500	~10	[10]
	PR-924 ^e	>30,000	8200	>30,000	>30,000	2900	22	[50]

n.r. denoting that IC50 / Ki values are not reported.

^a – ^e denoting the experimental methods used in determining IC50 values.

^a IC50 values approximated from mobility shift electrophoretic assays and biotinylated-inhibitor competition assays;

^b Ki values for the hydrolysis of Z-GGF-pAB and an unknown BrAAP substrate in purified constitutive and immunoproteasomes;

^c IC50 values for inhibition of hydrolysis of fluorogenic substrates Ac-nLpLD-AMC, Ac-GPFL-AMC, Boc-LRR-AMC, and Suc-LLVY-AMC using purified constitutive and immunoproteasomes (unpublished data);

^d IC50 values from ProCISE assay using MOLT-4 cells;

^e IC50 values from ProCISE assay using purified human constitutive proteasome and immunoproteasome.