Recent insights how combined inhibition of immuno/proteasome subunits enables therapeutic efficacy

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Abstract

The proteasome is a multicatalytic protease in the cytosol and nucleus of all eukaryotic cells that controls numerous cellular processes through regulated protein degradation. Proteasome inhibitors have significantly improved the survival of multiple myeloma patients. However, clinically approved proteasome inhibitors have failed to show efficacy against solid tumors, neither alone nor in combination with other therapies. Targeting the immunoproteasome with selective inhibitors has been therapeutically effective in preclinical models for several autoimmune diseases and colon cancer. Moreover, immunoproteasome inhibitors prevented the chronic rejection of allogeneic organ transplants. In recent years, it has become apparent that inhibition of one single active center of the proteasome is insufficient to achieve therapeutic benefits. In this review we summarize the latest insights how targeting multiple catalytically active proteasome subunits can interfere with disease progression in autoimmunity, growth of solid tumors, and allograft rejection.

Introduction

The proteasome is the main protease in the cytosol and nucleus of all eukaryotic cells and exerts numerous essential regulatory functions in nearly all cell biological pathways. The 26S proteasome degrades poly-ubiquitylated protein substrates and consists of a 19S regulator bearing ubiquitin receptors and an ATPase ring in charge of protein unfolding as well as a 20S proteolytic core complex. Structurally, the 20S proteasome core particle consists of four stacked rings, each consisting of seven subunits. The inner two rings of the constitutive proteasome are composed of β -subunits and bear the catalytically active subunits proteasome subunit beta (PSMB)6 (β 1c), PSMB7 (β 2c), and PSMB5 (β 5c) [1]. These three proteolytically active subunits are responsible for at least three peptidase activities: caspase-like (exerted by β 1c), trypsin-like (exerted by β 2c), and chymotrypsin-like (exerted by β 5c) activities. Immunoproteasomes are formed by replacing the standard

proteasome subunits β 1c, β 2c, and β 5c with the immunoproteasome subunits low molecular mass polypeptide (LMP) 2 (β 1i; PSMB9), multicatalytic endopeptidase complex-like (MECL)-1 (β 2i; PSMB10), and LMP7 (β 5i; PSMB8) in cells of hematopoietic origin and in most other tissues after the stimulation with the pro-inflammatory cytokine interferon (IFN)- γ . These exchanges alter the cleavage-specificity of the 20S proteasome. In immunoproteasomes the caspase-like activity, exerted by β 1c in standard proteasomes, is strongly reduced, whereas the chymotrypsin-like activity is enhanced. Whereas B cells, T cells, and monocytes mainly express immunoproteasomes [2, 3], immunoproteasome expression in tumors varies depending on cancer type and differentiation status. Furthermore, mixed structures consisting of standard- and immunoproteasome subunits have been described [4].

Due to the role of proteasomes in the regulation of numerous cellular processes, inhibition of the proteasome is an attractive strategy to anticancer therapy. Several proteasome inhibitors of different structural classes have been described in the last three decades [5–8]. Based on whether or not they form a covalent bond with the active site threonine, these inhibitors can be divided into two large groups (covalent inhibitors, non-covalent inhibitors), which can be further subdivided into structural classes, like aldehydes, boronates, epoxyketones, α -ketoaldehyde, β -lactones, vinyl-sulfones, syrbactins, and oxa-thiazolones. The reversibly binding peptide boronate proteasome inhibitor bortezomib (Velcade®) (Fig. 1a), which targets

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A Bortezomib **B Carfilzomib** **C Ixazomib** **D ONX 0914** **E Marizomib** **F Glidobactin A** **G Luminmycin A** **H Glidobactin C** **I PRN1126** **J LU-001i** **K ML604440** **L KZR-504** **M Compound 10 Ref. [96]** **N Compound 8 Ref. [96]** **O KZR-616** **P LU-005i** **Q DPLG3**

Fig. 1 Chemical structures of proteasome inhibitors described in this review. Chemical structures of (a) bortezomib, (b) carfilzomib, (c) ixazomib, (d) ONX 0914, (e) marizomib, (f) glidobactin A, (g) luminmycin A, (h) glidobactin C, (i) PRN1126, (j) LU-001i, (k) ML604440, (l) KZR-504, (m) compound 10 Ref. [96], (n) compound 8 Ref. [96], (o) KZR-616, (p) LU-005i, and (q) DPLG3 are shown.

both the constitutive proteasome and immunoproteasome, is the first clinically approved proteasome inhibitor for relapsed and/or refractory myeloma and mantle cell lymphoma [9]. To overcome intrinsic or acquired resistance and off-target toxicity of bortezomib, the irreversibly binding peptide epoxyketone-based proteasome inhibitor carfilzomib (Kyprolis®) (Fig. 1b) has been developed [10]. Later, the boronate-based, orally available compound ixazomib (Ninlaro®) (Fig. 1c) was approved for treatment of relapsed/refractory multiple myeloma. Its faster off-rate for proteasome binding than bortezomib allows to associate and dissociate with multiple proteasomes, thus, leading to a broader tissue distribution [11]. In addition to cancer, proteasome inhibitors were also evaluated in autoimmune diseases [12, 13] and transplantation medicine [14–16] in humans. Although proteasome inhibitor treatment might have had some positive effects in these trials the rather severe side effects of proteasome inhibition, such as anemia, thrombocytopenia, and neutropenia, limits its therapeutic applicability for autoimmune diseases and interference with transplant rejection.

Immunoproteasomes are constitutively expressed in hematopoietic cells, induced in cells stimulated with IFN- γ , and upregulated in certain cancers. Therefore, immunoproteasome inhibitors can be more selective and have fewer toxic side effects than standard proteasome inhibitors. Immunoproteasome inhibitors have been very effective in dampening the activation of lymphocytes [2, 17, 18], in altering macrophage polarization [19], in reducing secretion of pro-inflammatory cytokines [20–25], and in dampening T helper (Th)₁₇ differentiation [20, 24–28]. Furthermore, the immunoproteasome has been shown to be a promising drug target in several preclinical disease models for autoimmunity [20–22, 24, 28–33], for brain inflammations [34, 35], colitis-associated cancers [26, 36], angiogenesis [37], viral myocarditis [38], acute kidney injury [39], ischemic stroke [25], preterm birth [40], abdominal aortic aneurysm [41], atherosclerosis [42], cardiac remodeling [43], Alzheimer's disease [44, 45], and organ transplantation [17, 46, 47]. Hence, immunoproteasome inhibitors might emerge as a new treatment option for several diseases (summarized in Table 1) with reduced side effects compared to inhibitors targeting the standard proteasome.

Indeed, the peptide-ketoepoxide immunoproteasome inhibitor ONX 0914 (formerly named PR-957) (Fig. 1d) has been associated with reduced neurotoxicity compared to bortezomib [48].

Proteasome co-inhibition in solid tumors

Many proteasome target proteins, such as tumor suppressor protein p53, cyclins, cyclin dependent kinase inhibitor p27, pro-apoptotic protein Bax, and the NF- κ B inhibitor I κ B- α ,

are involved in carcinogenesis and cancer survival. Proteasome inhibition in multiple myeloma causes a toxic build-up of proteins leading to apoptosis [49]. Initially, experiments performed in yeast identified the β 5 subunit of the proteasome as the rate-limiting subunit in proteasomal protein degradation [50, 51]. Consequently, proteasome inhibitors were designed to target the β 5 subunit. However, targeting only β 5 has little effects on protein degradation [52]. More than 50% inhibition of protein degradation is achieved only when both β 5 and either β 1 or β 2 sites are inhibited [53, 54]. Hence, the β 1 and β 2 sites should be considered as co-targets of anticancer drugs. Indeed, co-inhibition of either trypsin-like and/or caspase-like sites together with β 5 inhibition is needed to achieve maximal cytotoxicity in multiple myeloma cell lines [52, 55–58]. Bortezomib targets the β 5 and β 1 activity, but not the β 2c activity of the proteasome [9, 59]. Carfilzomib displays greater specificity than bortezomib for β 5 activity relative to β 1 activity [60]. However, greater occupancy of multiple 20S subunits was associated with an increased likelihood of achieving a clinical response at higher carfilzomib doses in multiple myeloma patients, indicating that targeting multiple proteasome sites is beneficial [61]. Due to its success in multiple myeloma therapy, bortezomib was used in many clinical studies to investigate its potential as an antitumor agent in different solid tumors, like f.i. lung cancer, prostate cancer, and breast cancer (summarized in [62]). However, bortezomib as monotherapy or in combination with other therapies has not enabled a clinical benefit in patients with solid tumors. Although bortezomib is effective in the treatment of hematological malignancies, the reason for the unresponsiveness in solid cancers remains elusive. In vitro, cell lines derived from solid tumor cells are as sensitive to proteasome inhibitors as multiple myeloma cells [9]. Pharmacodynamics data obtained in clinical trials show that bortezomib equally inhibits proteasomes in solid tumors and blood, suggesting poor tumoral penetration not to be the reason for the lack of activity in solid tumors in vivo [63].

A possible explanation for the lack of response to proteasome inhibitors in solid tumors is insufficient potency. Indeed, Kisselev and co-workers showed that inhibiting a second site of the proteasome, particularly the β 2-site, sensitized triple-negative breast cancer cell lines to bortezomib and carfilzomib [64]. Like other solid tumors, bortezomib was not effective in patients suffering from breast cancer [63, 65]. However, in vitro, many different triple-negative breast cancer cell lines were more sensitive than multiple myeloma cells to treatment with bortezomib under identical conditions resembling clinically achievable proteasome inhibition [64]. Interestingly, the viability correlated not with inhibition of the β 5 site, but with the onset of inhibition of β 1. Greater cytotoxicity was usually observed only at concentrations at which the β 1 and β 2 sites were at

Table 1 Table of diseases investigated in preclinical models with immunoproteasome-selective inhibitors.

Disease	Preclinical model	Immunoproteasome inhibitor	Treatment	Effect	Citation
Rheumatoid arthritis	Collagen antibody-induced arthritis	ONX 0914	Therapeutic	Amelioration of disease symptoms	[20]
	Collagen-induced arthritis	ONX 0914	Therapeutic	Amelioration of disease symptoms	[20]
Type I diabetes	LCMV RIP-GP model	ONX 0914	Preventive	Reduction in blood glucose level	[20]
Colitis	DSS-induced colitis	ONX 0914	Preventive	Amelioration of disease symptoms	[21]
	DSS-induced colitis	DPLG3	Preventive	Amelioration of disease symptoms	[33]
Systemic lupus erythematoses	Lupus prone MRL/lpr mice	ONX 0914	Preventive	Prevents disease progression	[29]
	Lupus prone NZB/W mice	ONX 0914	Preventive	Prevents disease progression	[29]
Hashimoto's thyroiditis	Sodium iodide injection into NOD-H2 ^{b4} mice	ONX 0914	Preventive	Reduced thyroiditis scores	[30]
	Sodium iodide injection into NOD-H2 ^{b4} mice	ONX 0914	Therapeutic	Reduced thyroiditis scores	[30]
Graves' hyperthyroidism	Immunization of susceptible BALB/c mice with Ad-TSHR289	ONX 0914	Preventive	No effect	[30]
Multiple sclerosis	MOG ₃₅₋₅₅ induced EAE	ONX 0914	Preventive	Strongly delays disease induction	[22]
		ONX 0914	Therapeutic	Ameliorates disease progression	[22]
	Transfer of MOG ₃₅₋₅₅ reactive T cells	ONX 0914	Preventive	Reduction of disease symptoms	[22]
	PLP ₁₃₉₋₁₅₁ -induced EAE	ONX 0914	Therapeutic	Prevents disease progression and relapse	[22]
	Transfer of PLP ₁₃₉₋₁₅₁ reactive T cells	ONX 0914	Therapeutic	Prevents disease progression and relapse	[22]
Neuritis	Immunization with the neuritogenic synthetic P0 peptide 180-199 in rats	ONX 0914	Preventive	Reduced clinical score	[31]
Myasthenia gravis	Immunization of rats with the R97-116 peptide	ONX 0914	Preventive	Reduced clinical score	[32]
Autoimmune myocarditis	Cardiac troponin I-directed autoimmune myocarditis	ONX 0914	Preventive	Diminished troponin I autoimmune myocarditis	[118]
	Cardiac troponin I-directed autoimmune myocarditis	ONX 0914	Therapeutic	Diminished troponin I autoimmune myocarditis	[118]
Kidney transplantation	Chronic rejection of kidneys transplanted from Fischer to allogeneic Lewis rats	ONX 0914	Starting after transplantation	Survival of grafted kidney	[46, 47]
Heart transplantation	BALB/c hearts transplanted into fully allogeneic C57BL/6 recipients	DPLG3	Starting after transplantation	Prolonged graft survival	[17]
Preterm birth	LPS-induced preterm birth in mice	ONX 0914	Co-application of LPS and ONX 0914	Reduced inflammation	[40]
Alzheimer's disease (AD)	LPS-induced mouse model of neuroinflammation	YU102 (selective for LMP2 and β 1c)	5 days post LPS	Improved cognitive function	[44]
		PR-924 (LMP7 selective)	Preventive	Could not be assessed due to strong toxicity in this model	[44]
		YU102 (selective for LMP2 and β 1c)	Starting at the age of 9 months	Ameliorates AD-related cognitive impairment	[44]

Table 1 (continued)

Disease	Preclinical model	Immunoproteasome inhibitor	Treatment	Effect	Citation
	Transgenic mouse model Tg2576, which expresses human amyloid precursor protein (APP) with the Swedish double mutation (KM670/671NL)	DB-310 (LMP2 selective inhibitor)	Starting at the age of 9 months	Ameliorates cognitive deficits	[45]
Ischemic stroke	Middle cerebral artery occlusion (MCAO) in mice	ONX 0914	After MCAO	Neuroprotective effects	[25]
Cardiac remodeling	DOCA-salt cardiac remodeling mouse model	ONX 0914	1-day pre-surgery	Ameliorates DOCA-salt-induced cardiac remodeling and dysfunction	[43]
Abdominal aortic aneurysm (AAA)	Infusion of angiotensin II into ApoE knockout mice	ONX 0914	Starting at angio-tensin II infusion	Reduces AAA incidence and severity	[41]
Acute kidney injury	Kidney pedicle clamp	ONX 0914	Before kidney pedicle clamp	Anti-inflammation and oxidative stress potency	[39]
Atherosclerosis	Atherogenic diet in apolipoprotein E (ApoE) knockout mice	ONX 0914	Preventive	Suppression of diet-induced atherosclerosis	[42]
Viral meningitis	Intracranial LCMV infection	ONX 0914	Preventive	Reduced clinical score	[35]
Viral myocarditis	Coxsackievirus B3-induced myocarditis in A/J mice	ONX 0914	Preventive	Increased survival	[38]
	Coxsackievirus B3-induced myocarditis in NMRI mice	ONX 0914	Preventive	Elevated viral cytotoxicity	[119]
	Coxsackievirus B3-induced myocarditis in NMRI mice	ONX 0914	Therapeutic	No anti-inflammatory effect in the acute or chronic stages	[119]
Colitis-associated cancer	Chemically induced (AOM/DSS) colon carcinogenesis	ONX 0914	Preventive	Reduced clinical symptoms and tumor numbers	[36]
		ONX 0914	Therapeutic	Reduced tumor numbers	[36]
		ONX 0914	Preventive	Reduced tumor numbers	[26]
		ONX 0914	Preventive	Reduced clinical symptoms and tumor numbers	[36]
	Transgenic mouse model (ApcMin/+) of colon carcinogenesis.	ONX 0914	Therapeutic	Reduced clinical symptoms and tumor numbers	[36]
<i>Candida albicans</i> infection	Intravenous injection of <i>Candida albicans</i>	ONX 0914	Preventive	Decreased survival and increased <i>candida albicans</i> titers	[120]
Adenovirus infection	Adenovirus type 1 infection of neonatal C57BL/6 mice	ONX 0914	Preventive	No effect on viral load and virus-induced inflammation in the heart	[121]

least partially inhibited. Using site-specific proteasome inhibitors, the authors could show that selective inhibition of the β 2-site sensitized triple-negative breast cancer cells to β 5 inhibitors better than site-specific inhibition of the β 1 site. Confirming these results, genetic inactivation of the PSMB7/ β 2 gene sensitized cells to β 5 inhibition more than silencing β 1. Co-inhibition of β 5 and β 2 increased the accumulation of ubiquitylated proteins compared to β 5 inhibition alone. Interestingly, after an initial accumulation of ubiquitylated proteins in carfilzomib treated cells after 6 h, these ubiquitin conjugates disappeared at later time points. This phenomenon went along with a recovery of β 5 activity, indicating the emergence of newly assembled proteasomes. Under homeostatic conditions, the inactive transcription factor nuclear respiratory factor 1 (Nrf1) is continuously retro-translocated from the ER lumen to the cytoplasm, where it is degraded by the proteasome [66, 67]. Upon partial proteasome inhibition, Nrf1 is proteolytically processed by the aspartyl protease DNA-damage inducible 1 homolog 2 (DDI2) to a soluble, active C-terminal fragment in the cytoplasm that has transcription factor activity and translocates to the nucleus. There, it enhances the concerted transcription of genes of the ubiquitin proteasome system [68, 69]. Among others, mature Nrf1 activates transcription of all genes encoding subunits of the 26S proteasome. This results in the assembly of more 26S proteasomes, which allows cells to escape the cytotoxic effects of rapidly catabolized proteasome inhibitors. Bortezomib most efficiently induces Nrf1 processing and 26S subunit expression at low concentrations that only partially block the chymotrypsin-like site and cause only a minor (<40%) decrease in proteolysis. By contrast, higher bortezomib concentrations that completely block the β 5 site and also cause a large (>50%) inhibition of the caspase-like site and protein breakdown, inhibit Nrf1 processing leading to insoluble Nrf1 aggregation. This prevents Nrf1 nuclear entry and, thus, 26S induction [70, 71]. In the study by Weyburne et al. on breast cancer cells, carfilzomib alone or in combination with an inhibitor of the β 1 site led to Nrf1 activation by DDI2 cleavage and, consequently, to an upregulation of 26S genes and survival of breast cancer cells [64]. In contrast, co-inhibition of the β 5 site and the β 2-site suppressed production of soluble, active Nrf1 and prevented induction of proteasome genes. In line with these observations, partial knockdown of Nrf1 increased cell death when the β 5 site alone or in combination with the β 1 was targeted, but not with β 5/ β 2 co-inhibition. Apart from breast cancer cells, inhibition of β 2 when combined with carfilzomib and bortezomib also sensitized cell lines originating from other solid tumor types derived from lung, renal, and ovarian cancer. Taken together, co-inhibition of β 5 and β 2 causes aggregation of Nrf1, which blocks upregulation of proteasome genes and prevents recovery of

Fig. 2 Co-inhibition in solid tumors. Co-inhibition of 20S proteasome subunits β 5 and β 2 causes aggregation of Nrf1, which blocks upregulation of proteasome genes and prevents recovery of proteasome activity, and, thus, leads to cell death in solid tumors [64].

proteasome activity, and, thus, leads to cell death in solid tumors (Fig. 2).

The FDA-approved proteasome inhibitors bortezomib, ixazomib, and carfilzomib do not inhibit the β 2-site at therapeutically relevant concentrations [72]. In contrast, marizomib (Fig. 1e), a natural irreversible proteasome inhibitor purified from marine *actinomycete* bacteria, potently inhibits all three 20S proteasome subunits and is currently in development for treatment of multiple myeloma and malignant glioma [73, 74]. Marizomib localizes to the CNS and significantly inhibits proteasome activity in the brain [75]. Targeting all subunits of the proteasome by marizomib would fulfill the requirements as delineated by Weyburne et al. to efficiently treat solid tumors [64]. The compound was studied in clinical trials in patients with metastatic solid tumors and advanced hematologic malignancies. Although marizomib can penetrate the blood–brain barrier it has a good safety profile and significant peripheral neuropathy, unlike in bortezomib regimens, was not noted. Marizomib is showing promising signs of clinical benefit and compared with the current standard-of-care agents such as thalidomide, lenalidomide, bortezomib, carfilzomib, and ixazomib, marizomib has a non-cross-reactive toxicity profile. Renal and CNS toxicity were described only at higher marizomib dose levels and infusion over shorter time periods [76–79]. The efficacy of marizomib in solid tumors was investigated in different cell lines and preclinical animal models [80–83]. Thereby, marizomib displayed superior activity to bortezomib in several solid tumor xenograft models and more potently impacted several hallmarks of cancer, including angiogenesis and invasion.

Kisselev and colleagues have demonstrated that for optimal proteasome inhibition in solid tumors and multiple myeloma, β 5c/ β 5i and β 2c/ β 2i sites have to be targeted, respectively [64, 84]. Hence, there is a strong need to develop proteasome inhibitors simultaneously targeting β 5 and β 2. Recently, a syringolin-based chemical probe was used to screen for potent inhibitors of the 20S proteasome in crude metabolite extracts from a *burkholderiales* bacteria strain [85]. In this screen, the previously known proteasome inhibitors glidobactin A (Fig. 1f) and luminmycin A (Fig.

1g) [86–88] as well as the novel proteasome inhibitor glidobactin C (Fig. 1h) were identified [85]. Glidobactin C displayed an unprecedented $\beta 2/\beta 5$ co-inhibition profile with single-digit nanomolar potency. Glidobactin A and lumimycin A were poorly active in live cell experiments. However, glidobactin C, which differs from glidobactin A only by encompassing two additional methylene groups in its acyl-chain, considerably inhibited substrate cleavage in live cells, suggesting that its slightly increased chain length sufficiently enhanced membrane penetration. The attractive $\beta 2/\beta 5$ subunit co-inhibition profile in combination with its cell permeability renders glidobactin C a promising anticancer drug candidate. Indeed, glidobactin C induced accumulation of ubiquitin conjugates in a fibrosarcoma cell line, inhibits cytokine secretion and blocks proliferation of different human breast cancer cell lines with comparably low immunotoxicity. Taken together, glidobactin C [85] exactly fulfills the requirement for optimal proteasome inhibition in solid tumors as determined in the study by Weyburne et al. [64].

Furthermore, the recently described non-covalent amide derivate proteasome inhibitors simultaneously target multiple proteasome subunits, and, therefore bear the potential to be effective in solid tumors [89]. Amide derivatives targeting the $\beta 5i$ and $\beta 2i$ have been shown to induce cell cycle arrest in multiple myeloma MM.1R cells [90].

Immunoproteasome co-inhibition in autoimmunity

ONX 0914 (formerly called PR-957) was the first immunoproteasome inhibitor for which an ameliorating effect in preclinical models of autoimmune diseases could be demonstrated [20]. In two different mouse models of rheumatoid arthritis, collagen antibody-induced arthritis and collagen-induced arthritis, immunoproteasome inhibition strongly reduced disease symptoms. These effects were attributed to reduced inflammatory cytokine secretion and impeded Th17 differentiation. In a model for type I diabetes, ONX 0914 reduced blood glucose levels, probably due to alterations in MHC-I presentation [20]. In a human leukemia cell line, which expresses both forms of the proteasome, ONX 0914 was shown to be 20- to 40-fold more selective for LMP7 over the next most sensitive sites, $\beta 5$ or LMP2. Furthermore, in human peripheral blood mononuclear cells (PBMCs), in which standard proteasome subunits are barely expressed, ONX 0914 at concentrations <100 nM inhibited LMP7 activity by >80% with minimal inhibition of LMP2 or MECL-1. However, higher concentrations resulted in a substantial inhibition of LMP2 and MECL-1. In order to demonstrate specificity of ONX 0914, LMP7-deficient cells were used in this study [20]. In contrast to wild-type cells, no reduction in cytokine secretion

and MHC-I surface expression could be observed under ONX 0914 inhibition in cells lacking LMP7. Therefore, the authors concluded that the observed effects in this study were LMP7-specific. In vivo, mice were treated with 2, 6, or 10 mg/kg body weight. Inhibition at 2 mg/kg was sufficient to block disease progression. However, the subunit specificity in vivo at the used concentrations was not reported in this study. In future studies, ONX 0914 was used in vitro at 200–300 nM and in vivo at 10–20 mg/kg [21, 22, 29, 91].

During a medicinal chemistry effort focused on the discovery of inhibitors with exclusive selectivity for LMP7, PRN1126 (Fig. 1i) was developed at the pharmaceutical company Principia Biopharma [92]. PRN1126 is a reversible covalent inhibitor of LMP7, covalently anchoring to its peptide cleavage site via reversible binding to Cys48 on LMP7. In vitro assays showed that PRN1126 was 13-fold and 30-fold more selective for mouse and human LMP7 over $\beta 5c$, respectively. No selectivity for other standard or immunoproteasome subunits could be observed. Hence, PRN1126 can be considered LMP7-specific at the applied concentrations. However, in contrast to the previously used LMP7 inhibitor ONX 0914, PRN1126 could not reduce MHC-I surface expression on splenocytes, had no effect on IL-6 secretion by lipopolysaccharide (LPS)-stimulated mouse splenocytes and human PBMCs. Moreover, it did not affect Th17 differentiation and could neither ameliorate dextran sodium sulfate (DSS)-induced colitis nor experimental autoimmune encephalomyelitis (EAE) [92]. To elaborate the difference between ONX 0914 and PRN1126, the specificity profile of the two inhibitors was compared. Studies with fluorogenic proteasome activity substrates and electrophoretic mobility shifts of immunoproteasome subunits revealed that ONX 0914 significantly inhibited both LMP2 and LMP7 at concentrations above 100 nM when cells were incubated with this irreversible epoxyketone inhibitor for longer time periods, whereas PRN1126 remained highly selective for LMP7 and did not inhibit LMP2. It had already been shown that LMP2 inhibition alone had no effect on cytokine secretion and autoimmunity [24, 93]. Therefore, it was hypothesized that inhibition of both LMP7 and LMP2 might be required to induce broad anti-inflammatory effects in vitro and in vivo. A combination of PRN1126 and the well-characterized LMP2-specific inhibitors LU-001i (Fig. 1j) [24, 94] or ML604440 (Fig. 1k) [95] were used to investigate this hypothesis. Indeed, it was found that a co-inhibition of both LMP2 and LMP7 is required to reduce MHC-I surface expression on splenocytes, to decrease IL-6 secretion, to impair Th17 differentiation, and to ameliorate experimental colitis and EAE [92] (Fig. 3).

A similar study by Johnson et al. reached the same conclusion [96]. In this study, a library of immunoproteasome subunit-specific inhibitors was employed to probe the role of individual subunits in regulation of cytokine secretion and inflammation. Compared to ONX 0914, selective

Fig. 3 Co-inhibition in autoimmunity. Co-inhibition of 20S immunoproteasome subunits LMP2 and LMP7 (upper panel) reduces MHC-I surface expression, impairs Th17 differentiation, decreases cytokine secretion, and ameliorates different autoimmune diseases [92, 96]. Co-inhibition of 20S immunoproteasome subunits MECL-1 and LMP7 (lower panel) reduces cytokine secretion [96].

single-site LMP2 (KZR-504; (Fig. 1l)), MECL-1 (compound 10; [96] (Fig. 1m)), and LMP7 (compound 8; [96] (Fig. 1n)) inhibitors insufficiently reduced cytokine expression in LPS or T cell receptor stimulated human leukocytes. Since the design of a cross-species selective MECL-1 inhibitor proved to be difficult, an analysis of MECL-1 in mouse disease models was precluded. Instead, LMP7 and LMP2 selective inhibitors were used to explore the therapeutic effect in active collagen antibody-induced arthritis, a mouse model of inflammatory arthritis. Single-agent treatment of diseased mice had a negligible impact on disease progression, indicating that individual subunit inhibition is insufficient to suppress inflammation *in vivo*. However, applying a combination of LMP7 with either MECL-1 or LMP2 inhibitors *in vitro* achieved an optimal cytokine inhibition profile. *In vivo*, dual LMP7 and LMP2 inhibition was therapeutically effective in collagen antibody-induced arthritis (Fig. 3). Interestingly, ONX 0914 achieved activity in a mouse arthritis model via complete LMP7 inhibition and ~40% LMP2 inhibition, suggesting that only partial inhibition of a secondary immunoproteasome subunit is required for a multi-cytokine inhibitory effect. Such a dual proteasome inhibition profile is a hallmark of the immunoproteasome inhibitor KZR-616 (Fig. 1o) [96], developed by Kezar Life Sciences, which proved to have a favorable safety profile in healthy humans and is now in a phase 2 clinical trial for treatment of the severe autoimmune disease lupus nephritis [97–99].

Why has the requirement for dual proteasome inhibition for regulating inflammation not been discovered previously? The original publication describing ONX 0914 showed an LMP2 inhibition by ONX 0914 of ~35% at 200 nM and 75% at 500 nM. This was considered not to be crucial for the observed immunological effects since no

influence of ONX 0914 on MHC-I surface expression and cytokine secretion could be observed with LMP7-deficient splenocytes. However, LMP7-deficient mice barely incorporate LMP2 and MECL-1 into immunoproteasomes [100]. Therefore, LMP7-deficient cells can only be used to detect non-immunoproteasome related off-target effects. Furthermore, the crystal structure of ONX 0914-soaked immunoproteasome supported the view of LMP7 selectivity [1]. Although the hydrophobicity of the substrate-specificity pockets of LMP2 supports ONX 0914 binding, atomic distances indicate that Phe31 creates a steric barrier to the P1 phenyl moiety of ONX 0914. Hence, ONX 0914 was assumed to bind only against significant repulsive forces mirrored in an unfavorable orientation of Phe31 toward the carbonyl oxygen of Asp32 in LMP2.

These new insights [92, 96] will allow the design of new generations of immunoproteasome inhibitors simultaneously targeting LMP2 and LMP7 and possessing optimal therapeutic efficacy for the treatment of autoimmunity. The inhibitor LU-005i (Fig. 1p) fulfills the requirements developed in the study by Basler et al. and Johnson et al. [92, 96]. In a structure-based design approach to obtain proteasome inhibitors with considerably improved selectivity for $\beta 5i$ over $\beta 5c$ and $\beta 2i$ over $\beta 2c$ LU-005i was found [94]. LU-005i is an effective inhibitor of human and mouse MECL-1, LMP2, and LMP7 with at least tenfold selectivity over $\beta 2c$, $\beta 1c$, and $\beta 5c$ and, therefore, can be considered an immunoproteasome-selective inhibitor targeting all active sites of the immunoproteasome [24]. Treatment of splenocytes with LU-005i reduced MHC-I surface expression only in wild-type cells but not in LMP7-deficient cell, which barely express immunoproteasomes [100], demonstrating that LU-005i is immunoproteasome-selective. LU-005i treatment of endotoxin- or T cell receptor stimulated mouse splenocytes strongly reduced IL-6 or IFN- γ secretion as well as IL-6 and IL-23 production from LPS-stimulated human PBMCs [24]. Differentiation of naive T cells to Th17 cells under Th17-polarizing conditions was impaired in LU-005i treated cells. LU-005i was also active *in vivo* in DSS-induced colitis. Treatment of mice with LU-005i in this colitis model resulted in reduced body weight loss, disease activity index, colon length shortening, cytokine expression in the colon, and histological scores compared to vehicle-treated mice. This study confirms that multiple immunoproteasome subunits have to be targeted and that the immunoproteasome is an attractive drug target in inflammatory diseases.

Proteasome inhibition in transplantation. Do we need co-inhibition?

Organ transplantation has overcome major technical limitations to become the success it is today. Allogeneic organs

are acutely rejected in the absence of pharmacological immunosuppression. Current immunosuppressive therapies target T cells nonspecifically and have to be taken lifelong, leaving patients more susceptible to infections and cancers, in addition to having toxic off-target effects. Chronic antibody-mediated rejection is a leading cause of kidney allograft failure years after transplantation. Currently, suitable pharmacological agents for the suppression of chronic antibody-mediated rejection are lacking. Bortezomib inhibits the chronic active antibody-mediated rejection in experimental renal transplantation in the rat [101]. In humans, several smaller studies have suggested efficacy of the proteasome inhibitor bortezomib in preventing antibody-mediated kidney transplant rejection in humans (summarized in [102]). However, a randomized placebo-controlled trial of bortezomib in late antibody-mediated kidney transplant rejection failed to show significant differences between bortezomib- ($n = 21$) and placebo- ($n = 23$) treated groups [16]. The immunoproteasome inhibitor ONX 0914 impeded the chronic rejection of kidneys transplanted from Fischer to allogeneic Lewis rats [46] (summarized in [102]). Chronic nephropathy was ameliorated and renal allograft function preserved, enabling long-term survival of recipient rats. Alloantibodies attacking the transplanted organ are a major driver for rejection of transplanted kidneys. Proteasome inhibitors have been rather successful in treating antibody-driven diseases in preclinical models [29, 103–105] and in small clinical trials [12, 13, 106]. However, immunoproteasome inhibitors are preferred over broad-spectrum proteasome inhibitors to treat antibody-mediated diseases since immunoproteasomes are mainly expressed in hematopoietic cells and, therefore, have fewer toxic side effects. Plasma cells are the main producers of alloantibodies. The success of proteasome inhibitors in suppressing antibody-mediated diseases is probably due to the fact that plasma cells need a high proteasome capacity to degrade misfolded antibody chains. Hence, proteasome inhibition leads to the induction of the unfolded protein response and ultimately to the induction of apoptosis. Indeed, in a rat kidney transplantation model it was shown that immunoproteasome inhibition with ONX 0914 induced plasma cell apoptosis and preserved kidney allografts by activating the unfolded protein response and suppressing plasma cell survival factors [47]. Plasma cells isolated from diseased rats showed high expression of LMP2 and LMP7, although standard proteasome subunits were readily detectable. ONX 0914 treatment induced accumulation of protein-ubiquitin-conjugates in plasma cells, indicating that the proteasome capacity is exceeded. As evidenced by upregulation of central downstream molecules of inositol-requiring enzyme 1 α and protein kinase RNA-like ER kinase signaling like X-box binding protein-1, activating transcription factor 4, and C/EBP homologous protein, the

unfolded protein response was activated in these cells. This ultimately resulted in enhanced caspase-3 cleavage and apoptosis of plasma cells. Is an inhibition of more than one immunoproteasome subunit required to be effective in transplantation? It has been shown that for cytokine secretion and T helper cell differentiation a co-inhibition of LMP7 and LMP2 or MECL-1 is required [92, 96]. Since such processes also play a role in transplant rejection one can assume that co-inhibition is also required to suppress these processes in order to preserve allografts. Is co-inhibition also required to kill antibody producing plasma cells in transplantation? Investigation on immunoproteasome inhibition in rat kidney transplantation has been performed with ONX 0914 [46, 47], which inhibits both LMP7 and to a certain degree LMP2. In the studies by Li et al., LMP7 was inhibited more than 80% in bone marrow and spleen, whereas $\beta 5c$ activity was barely impaired [46]. Although the degree of LMP2 inhibition was not addressed in these studies, one can assume that LMP2 in rats is similarly affected as in mice [92]. Due to the limitations in available cell numbers of plasma cells the determination of proteasome inhibition in these cells remains challenging. Multiple myeloma cells are degenerated neoplastic plasma cells. Therefore, proteasome inhibition-induced cytotoxicity in plasma cells and multiple myeloma cells should be rather similar. Besse et al. demonstrate that proteasome inhibitors targeting $\beta 5$ subunit are cytotoxic only when $\beta 1$ or $\beta 2$ is co-inhibited [52]. However, the selective $\beta 2/\beta 5$ inhibition of both standard and immunoproteasome is the most cytotoxic. Therefore, one can assume that immunoproteasome inhibitors have to co-inhibit $\beta 5i$ and another immunoproteasome subunit to prevent antibody-mediated rejection in organ transplantation. However, experiments combining site-specific immunoproteasome inhibitors in kidney transplantation experiments remain to be performed.

Immunoproteasome inhibition in transplantation has also been performed in a preclinical heart transplant mouse model [17]. In this study, the novel LMP7-selective inhibitor DPLG3 (Fig. 1q) was used. DPLG3 (IC₅₀ for LMP7 of 4.4 nM) is a non-covalent reversibly acting N,C-capped dipeptide inhibitor of LMP7 that has a 1000-fold selectivity over the standard subunit $\beta 5c$ and shows <50% inhibition of $\beta 1i$, $\beta 2c$, $\beta 2i$, and $\beta 2c$ at 33.3 μ M. No cytotoxic activity of DPLG3 was observed in human PBMCs or mouse bone marrow-derived macrophages. In vitro, DPLG3 inhibited secretion of IFN- α and IP-10 by CpG-activated PBMCs, blocked proliferation of T cells, and reduced activation of human plasmacytoid dendritic cells. In vivo, Sula Karecci et al. used a stringent preclinical heart transplant mouse model in which BALB/c hearts are transplanted into fully allogeneic C57BL/6 recipients. C57BL/6 recipients were treated with DPLG3 (25 mg/kg, i.p., per day) for 14-days beginning on the day of transplantation. Although the survival of the allograft

was significantly prolonged compared with vehicle-treated recipients (median survival time, 13 vs. 7 days) the transplanted heart was finally rejected. Co-application of DPLG3 with an anti-CTLA4 antibody in different treatment regimens strongly increased the graft survival time. Why is a co-inhibition of different immunoproteasome subunits not required in this transplantation model? In contrast to the kidney transplantation model [46, 47], in which rejection is mediated by alloantibodies within weeks, rejection of transplanted hearts occurs within days and is mainly mediated by T cells. In vitro, DPLG3 was used in the low μM range, a concentration which should, according to the reported IC50 values, barely affect LMP2 or MECL-1. In vivo, the degree of immunoproteasome subunit inhibition in hearts or relevant cell types was not reported. Hence, it remains unclear whether co-inhibition of other immunoproteasome subunits is required in this heart transplantation model. Experiments using LMP2 or MECL-1 site-specific inhibitors in addition to DPLG3 would clarify whether inhibition of an additional proteasome site can further improve the outcome of heart transplantation.

Cooperation of immunoproteasome subunits for optimal MHC class I cell surface expression

Co-inhibition of LMP7 and LMP2 was also required to reduce MHC-I surface expression on spleen lymphocytes [92]. LMP2-deficient mice [107] display, in contrast to LMP7-deficient mice [108], no difference in MHC-I surface expression compared to wild-type mice [109]. This indicates, that LMP2 alone is not rate limiting in producing MHC-I ligands. However, as mentioned above, LMP7-deficient mice have reduced LMP2 incorporation into mature proteasomes [100]. Experiments using site-specific inhibitors for LMP2 and LMP7 indicate that both subunits together are rate limiting in MHC-I ligand production in steady-state [92]. Mouse MHC-I molecules only accommodate peptides with hydrophobic C-terminal residues. Since both LMP2 and LMP7 contribute to the chymotrypsin-like activity (and thus to the production of ligands with hydrophobic C-terminus) it seems quite obvious that both subunits together contribute to MHC-I ligand production. Nevertheless, it would be of interest to determine the contribution of MECL-1 to MHC-I antigen presentation with site-specific inhibitors.

Concluding remarks

The reason to target more than one proteasome subunit in multiple myeloma seems rather clear. Proteasome inhibition in multiple myeloma causes a toxic build-up of proteins

leading to apoptosis. The more proteasome subunits are targeted the less proteins can be degraded by the proteasome, leading to an accumulation of proteins destined to be degraded. The cell counteracts by the induction of the unfolded protein response and by the expression of newly synthesized 26S proteasome components. If the cell does not succeed, apoptosis is induced in proteasome inhibited cells. In diseases which are mediated by antibody producing plasma cells, like chronic rejection of transplanted organs or systemic lupus erythematosus, a similar mechanism induced by proteasome inhibition might underlie. In solid tumors, the threshold to induce apoptosis by proteasome inhibition is probably higher compared to myeloma cells, since the excessive production of antibodies does not occur in solid tumors. Hence, more sites of the proteasome have to be blocked for prolonged periods to reach a similar degree of protein accumulation as observed in myeloma cells. This can be accomplished by combining current clinically approved proteasome inhibitors with additional $\beta 2$ -site-specific inhibitors. In addition, targeting multiple proteasome sites might impede the development of proteasome inhibitor resistant cells, which emerge due to mutations in genes of the targeted proteasome subunits thus preventing proteasome inhibitor binding. However, joint targeting of proteasome sites for longer time periods might increase toxic side effects in proteasome inhibitor treated patients. Therefore, it is necessary to find treatment regimens in which proteasome inhibition in solid tumors is sufficient to induce protein accumulation-induced cell death while retaining low toxicity in non-neoplastic cells. Targeting multiple sites of the immunoproteasome with immunoproteasome-specific inhibitors might be an alternative treatment strategy for solid tumors expressing high levels of immunoproteasomes. Immunoproteasome inhibitors can reach high inhibition rates in tumors without affecting nonneoplastic cells. However, hematopoietic cells, like CD14⁺ monocytes have been shown to be particularly sensitive to immunoproteasome inhibition [3]. Apart from targeting multiple catalytic proteasome subunits additional factors, such as bioavailability (discussed in [110]) contribute to clinical efficacy. Furthermore, immunoproteasome content of target cells varies among tumor type, differentiation state, and patient. Hence, in contrast to autoimmunity, in which target cells (cells derived from the hematopoietic system) mainly express immunoproteasomes, targeting both constitutive and immunoproteasomes might be beneficial for tumors to induce optimal proteasome inhibition capacity in these cells. In order to measure proteasome activity in vitro and in vivo different methods have been applied. These methods encompass, among others, the use of a cocktail of activity-based probes that enables simultaneous gel-based detection of all six catalytic human proteasome subunits [72], assays based on the cleavage of

proteasome-specific peptides linked to a fluorophore [111], the use of cell permeable fluorescent proteasome substrates [24], a proteasome immunoprecipitation-based assay [112], and the ELISA based ProCISE (proteasome constitutive immunoproteasome assay subunit ELISA) assay [113, 114].

The reason to target multiple active proteasome sites in autoimmunity, apart from antibody-mediated diseases, is not that obvious. Immunoproteasome inhibition impairs cytokine secretion, T cell activation [2], and T helper cell differentiation [115]. How does immunoproteasome inhibition affect these processes? Naive T and B cells express nearly exclusively immuno- or mixed proteasomes but no standard proteasomes [2]. Activation of B and T cells treated with ONX 0914 (inhibits LMP7 and LMP2) led to a transient accumulation of ubiquitin protein aggregates which were resolved 20 h post treatment [2]. T cell receptor-triggered activation of T cells strongly increases the translation rate in T cells [116]. Hence, proteasomes have to be fully active to maintain protein homeostasis in activated T cells. Indeed, ONX 0914 treatment induced mild proteostasis stress in T cells, which was dependent on protein neo-synthesis. However, in contrast to MG-132, which inhibits all subunits of the immunoproteasome [117], ONX 0914 did not induce apoptosis in activated T cells [2]. Hence, in order to block T cell activation without inducing cell death, only a partial inhibition of the immunoproteasome is required. This is crucial in the treatment for autoimmunity, since in contrast to tumor cells, induction of cell death in immune cells is not intended. Apparently, the reduced proteasome capacity in ONX 0914 treated T cells is resolved via Nrf1-mediated upregulation of standard proteasomes [2]. Interestingly, ONX 0914 treatment of LMP2-deficient T cells only had a minor effect on T cell activation, indicating that inhibition of both LMP7 and LMP2 is required to reduce T cell activation. To investigate whether co-inhibition in T cell activation is indeed required, experiments combining site-specific immunoproteasome inhibitors have to be performed. T cell activation is the first step in Th17 differentiation. Hence, a similar mechanism as described for T cell activation might also contribute to T cell differentiation. Taken together, reducing immunoproteasome capacity in activated T cells leads to a transient proteostasis stress. Whether such a mechanism also exist in vivo, and thereby suppress autoimmunity, remains to be investigated. Furthermore, how immunoproteasome inhibition reduces cytokine secretion in endotoxin stimulated cells, and whether proteostasis stress processes play a role in cytokine secretion remains to be investigated.

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Compliance with ethical standards

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