Proteasome inhibitors in progressive renal diseases

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ABSTRACT

Proteasome (PS) is a sophisticated protein degradation machinery comprising a 20S proteolytic core particle provided with caspase-like, trypsin-like and chymotrypsin-like activities on ubiquitinylated proteins. The products of this selective, complex, controlled and strictly coordinated system play a crucial role in cell cycle progression and apoptosis; activation of transcription factors, cytokines and chemokines; degradation and generation of MHC class I-presented peptides. PS has recently emerged as a promising drug target in cancer therapy, and bortezomib has been approved for refractory multiple myeloma. PS proteolysis is crucial for the degradation of the inhibitory protein IkB of nuclear factor kB (NF-kB), and hence, an interesting field of research has been developed on possible benefits of drugs with anti-PS activity in disease conditions with hyper-expression of NF-kB. PS inhibitors are being adopted in pilot studies in antibody-mediated renal rejection and in AL amyloidosis, with increasing scientific interest in possible applications in lupus, IgA nephropathy, idiopathic nephrotic syndrome and renal fibrosis. The most often used PS inhibitor, bortezomib, has a severe peripheral neurotoxicity, and the search for effective and less toxic PS-targeted drugs is a challenging area also in nephrology.

Keywords: proteasome, antibody-mediated renal rejection, glomerular diseases, progression of renal diseases, proteasome inhibitors, renal transplantation

THE RAISING INTEREST IN PROTEASOME INHIBITORS IN NEPHROLOGY

A dysregulation of the ubiquitin–proteasome (PS) pathway has been implicated in the pathogenesis of inherited and acquired diseases, including multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer’s disease, asthma, cancer, ischemia–reperfusion injury, cachexia, autoimmune thyroid disease, type I diabetes, hepatitis B, inflammatory bowel disease and sepsis [1, 2]. Hence, PS may be a reasonable target for the treatment of several diseases [3, 4]. Also renal immune-mediated diseases are included in the promising field of potential application of PS inhibitor drugs.

Some basic science concepts of the physiological role of the ubiquitin–PS system should be considered to understand the mechanism of action of this new category of drugs. PS is a sophisticated protein degradation machinery comprising a 20S proteolytic core particle and two 19S regulatory particles [1, 2]. The proteasomal core is composed of four seven-member rings, two outer rings formed by α subunits and two inner rings, which contain β subunits. The β1, β2 and β5 subunits have caspase-like, trypsin-like and chymotrypsin-like activities for cleavage of acidic, basic and hydrophobic aminoacids, respectively. The proteasomal degradation was firstly considered a waste disposal system, but, thanks to the seminal works on the ubiquitin–PS pathway, which made A. Hershko, A. Ciechanover and I. Rose deserve the Nobel Prize in 2004 [5], it became evident that it is a very selective, complex, controlled and strictly coordinated system that plays a crucial role in processes essential to life, including cell cycle progression and apoptosis, activation of transcription factors, activation of cytokines and chemochines and degradation and the generation of MHC class I-presented peptides [6].

The gamma type interferon (IFN-γ)—and to a lesser extent also IFN-α—induces a switch to a new PS form, immunoproteasome (iPS), by replacing the catalytic subunits with β1i (or LMP2), β2i (MECL-1) and β5i (LMP7) subunits, respectively. The switch to iPS improves the catalytic proteasomal activities and leads to optimal MHC-I peptide presentation to reactive T cells [7]. This goal is reached by preparing the C-terminus of the peptide residues to the high-affinity bond with the MHC class I cleft. iPS is up-regulated in dendritic cells when they migrate to the lymph nodes to stimulate T cells.

The first benefits for targeting PS emerged in cancer therapy, but the interest for nephrologists was raised when bortezomib, a PS inhibitor, was approved for refractory multiple myeloma (MM), and proved to be beneficial also in cases with myelomatous renal involvement [8]. Most of the
therapeutic effect in MM was thought to be due to the degradation of the inhibitory protein IκB of nuclear factor κB (NF-κB), a factor that is essential for the life of stromal bone marrow cells, which in turn are crucial for the growth of MM plasma cells [9]. Hence, an interesting field of research was developed on possible benefits of drugs provided with antiproteasomal activity in disease conditions with hyper-expression of NF-κB, e.g. autoimmune diseases including lupus erythematosus or rheumatoid arthritis. NF-κB inhibition can reduce the expression of many genes encoding key inflammatory mediators, such as cytokines [tumour necrosis factor (TNF) and interleukin (IL)-1], leukocyte adhesion molecules (intracellular adhesion molecule, vascular cell adhesion molecule) and enzymes (cyclooxygenase, nitric oxide synthetase). Therefore, PS inhibitors are potentially indicated for most immune-mediated renal diseases, in which these mediators are playing a pathogenetic role [10–12]. In pre-clinical animal models, selective PS inhibitors effectively suppress inflammatory arthritis and other inflammatory conditions [13, 14] and PS inhibitors are considered potential remedies for autoimmune and inflammatory diseases.

PS inhibition can cause cellular apoptosis by affecting short-living proteins, resulting, in addition to NF-κB activity inhibition, in increased activity of p53 and Bax proteins and accumulation of cyclin-dependent kinase inhibitors p27 and p21. Their effect in MM has also suggested a possible benefit in antibody-mediated renal rejection with anti-donor-specific antibodies (DSA) [15], even though the effectiveness of PS inhibitors in non-malignant, transformed and proliferating short-living proteins, resulting, in addition to NF-κB activity inhibition, in increased activity of p53 and Bax proteins and accumulation, of cyclin-dependent kinase inhibitors p27 and p21. Their effect in MM has also suggested a possible benefit in antibody-mediated renal rejection with anti-donor-specific antibodies (DSA) [15], even though the effectiveness of PS inhibitors in non-malignant, transformed and proliferating cells is much lower.

Recent research has evidenced a role for inhibitors of iPS β5i in immune-mediated diseases, reducing cell infiltration, cytokine production and autoantibody levels in experimental arthritis and lupus. These studies provide a rationale for future applications of selective iPS inhibitors in autoimmune disorders [16].

**PS INHIBITOR TYPES AND ACTIONS**

Several natural or synthetic compounds can inhibit protein degradation by PS. Natural PS inhibitors include lactacystin and epoxketones such as epoxomicin and eponemycin [17]. The first-discovered PS inhibitors were peptide aldehydes, such as the calpain inhibitor I, which reversibly inhibits 20S PSs by modifying the catalytic hydroxyl group of threonines and the chymotryptic-like activity of PS. Peptide aldehyde inhibitors dissociate rapidly from PS, producing a short-effect PS inhibition but, since they inhibit serine and cysteine proteases including calpains and cathepsins, they are not safe for use in patients. Peptide vinyl sulfones and natural inhibitors of PS have less non-specific activity than peptide aldehydes; however, all bind irreversibly to the 20S core particle and cannot be used in humans [17].

Synthetic inhibitors of PS include peptide aldehydes such MG132, ALLN and peptide vinyl sulfones. All of these compounds bind to, and directly inhibit, active sites within the 20S core particle. Peptide boronic acids are much more potent than their peptide aldehyde analogues. Dipeptidyl boronic acid, bortezomib (PS-341), is an extremely potent, stable, reversible and selective inhibitor of chymotryptic threonine protease activity of 26S PS [18]; however, it carries high neural tissue toxicity with painful neuropathy developing in more than 30% of patients.

PR-171 (carfilzomib) is a novel epoxyketone-based irreversible PS inhibitor [19], which can be given with intensive daily schedules that inhibit PS activity by >80% in most tissues without excessive toxicity. Hence, carfilzomib may be a valid alternative to bortezomib, and comparative pre-clinical studies suggest reduced toxicity and improved pathology.

AM-114, a novel boronic chalcone derivative, exhibits potent anticancer activity through inhibition of the chymotrypsin-like activity of 20S PS. Salinosporamide A (NPI-0052) is a new generation promising PS inhibitor [20].

The antiproteasomal agents most frequently evaluated in research and clinical trials include the non-peptide inhibitors lactacystin, PS-519, peptide aldehydes (i.e. MG132) and peptide boronates, mostly dipeptidyl boronic acid bortezomib (PS-341).

It is of interest to note that calcineurine inhibitors have PS inhibitor activity. Cyclosporine acts as an uncompetitive inhibitor of the chymotrypsin-like activity of the 20S PS in vitro and tacrolimus interacts with the ubiquitine proteolitic activity [21]. Also HIV protease inhibitors have PS activity inhibition in vitro [22].

**PS INHIBITORS IN MM AND PRIMARY AL AMYLOIDOSIS**

Malignant plasma cells (terminally differentiated B-cells) are specialized for production of monoclonal immunoglobulins in MM. PS regulates synthesis of transcription factors, including NF-κB, mediators which are crucial for MM growth and angiogenesis, and inhibition of apoptosis [9, 23]. Bortezomib, a reversible PS inhibitor, was developed for this rationale, and approved by the FDA in 2003, after the initial great success in treating MM. Bortezomib proved to be effective in the treatment of relapsed MM [8] with clear superiority over dexamethasone, a previous standard of care in relapsed myeloma. However, since MM cells develop resistance to bortezomib, a second generation of PS inhibitors has been produced, including carfilzomib, an irreversible PS inhibitor, and marizomib, which is available for oral intake [24]. Renal impairment is a common complication of MM, affecting 20–40% of new cases, and it may manifest with severe acute renal injury requiring dialysis. In addition to improved overall survival, there is evidence that PS inhibition has improved the renal prognosis. Treatment with high-dose dexamethasone and bortezomib can rapidly reduce light-chain production and provide an opportunity for renal recovery [25].

Primary systemic light-chain (AL) amyloidosis is caused by clonal plasma cell dyscrasia-producing immunoglobulin light chains, which are deposited in an almost-insoluble fibrillar matrix [26]. The treatment in AL is aimed at destroying the plasma cell clone using regimens proven to be effective in MM [27, 28]. Owing to the success of bortezomib in MM, recent
trials have adopted this drug in the treatment of patients with AL amyloidosis. An additional point of rationale for its use was that the ubiquitin–PS pathway plays an essential regulatory role in the degradation of ubiquitylated cellular proteins, and hence, it was suggested that amyloidogenic plasma cells may be particularly sensible to PS inhibitors. Bortezomib with or without dexamethasone has been reported to be active in AL amyloidosis and has induced high rates of hematologic and organ responses [29]. Of interest are the recently published benefits in patients with advanced cardiac involvement [30].

**PS INHIBITORS IN LUPUS ERYTHEMATOSUS**

In systemic lupus erythematosus (SLE), autoantibodies to double-stranded DNA and IFN-α are considered the clue for disease development and progression. B cell depletion has been considered a rationale target [31]; however, only a minority of SLE patients showed a sustained response after anti-CD20 monoclonal antibody [32]. These failures may be explained because plasma cells lack the expression of CD20 and thus are not responsive to rituximab. Rituximab has a variable effect on autoantibodies levels, as anti-double-strand DNA, which in SLE form immune complexes activating the IFN pathway. Plasma cells are very sensitive to changes in protein load and endoplasmic reticulum stress. Blockade of PS can lead to apoptosis via activation of the unfolded protein response (UPR) due to endoplasmic reticulum stress. Blockade of PS also inhibits the activation of NF-κB via accumulation of the regulatory protein IkB alpha. Both mechanisms are pivotal in the development of SLE. Autoantibody production and activation of the NF-κB pathway, leading to release of inflammatory cytokines, perpetuate the proliferation of autoantigen-specific T and B cells.

The PS inhibitor peptidyl boronic acid, bortezomib, was used in a murine model of lupus nephritis [33] and proved to induce UPR in plasma cells, killing both short-living and long-living plasma cells with elimination of autoantibodies. Since bortezomib is severely neurotoxic, interest has been focused on new PS inhibitors, particularly carfilzomib, which inhibits the chymotrypsin-like active sites of PS, but also affects iPS, predominantly expressed in immune cells [19]. Indeed carfilzomib, and also selective inhibitors of iPS like the iPS-irreversible inhibitor ONX 0914, are effective in autoimmune arthritis models in mice and in murine lupus [33]. These PS and iPS inhibitors have a dual effect of inhibiting autoantibody production by plasma cells, and also the pathway activated in dendritic cells by TLR signalling, triggered by immune complexes binding and leading to release of a large amount of INF-α. In female MRL/lpr mice treated with these newly developed drugs, a significant inhibition of severity of glomerular damage and interstitial inflammation were observed, indicating that PS inhibitors and iPS inhibitors result in decreasing serum and histological features of mouse models of SLE [34].

Bortezomib is not FDA approved for treatment of SLE, but there is an open phase IV study on www.clinicaltrials.gov for testing bortezomib in proliferative lupus nephritis, however it results withdrawn. The limitation for the use of bortezomib is the severe peripheral neuropathy and cardiovascular toxicity associated with this drug, and hence, there is high interest in newly developed PS inhibitors for SLE.

**PS INHIBITORS IN IGA NEPHROPATHY**

A trial (www.clinicaltrials.gov) is recruiting patients with IgA nephropathy (IgAN) on therapy after at least 4 months with angiotensin-converting enzyme inhibitors and/or angiotensin receptor blocking agents and proteinuria >1 g/day to receive bortezomib (Velcade®) at 1.3 mg/m², on Days 1, 4, 8 and 11. A second cycle is to be given a month later for non-responders. The primary endpoint for this study is reduction in proteinuria.

Studies from our group provided the rationale for this trial which is still recruiting patients [35]. We reported in mononuclear cells from patients with IgAN a significant switch in the expression of trypsin- and chymotrypsin-like PS subunits to corresponding iPS, and this was particularly evident in patients with proteinuria >0.5 g/day. The switch from PS to iPS suggests a hyperactivation of the PS system, hence providing the rationale for the ongoing trial. Moreover, we observed in these patients an increased nuclear translocation of the p50 active subunit of NF-κB [35].

A switch from PS to iPS has been reported in some systemic autoimmune disorders and in IFN-induced antiviral response. In patients with IgAN, it may reflect a response to infectious challenges, e.g. viral infections, with an increased release of IFNs. This scenario fits in with a Th1 lymphocyte prevalence in IgAN and a Th1 prevalence in experimental animal models. An increase in iPS, besides being possibly caused by viral induction of IFNs, has more recently been thought of as being part of a response to oxidative stress, which has been detected in IgAN, in proteinuria and progressive cases [36]. It is of interest that genes involved in the iPS pathway have been found to be activated in IgAN during phases of clinical activity.

**PS INHIBITORS IN IDIOPATHIC NEPHROTIC SYNDROME**

In a pilot study [37], we recently reported that children with steroid-dependent idiopathic nephrotic syndrome (NS) underwent remission and a significant steroid-sparing effect when treated with a HIV protease inhibitor (saquinavir) provided with PS-inhibiting activity [22]. The rationale was to limit the activation of NF-κB, which was demonstrated in circulating mononuclear cells of patients with NS [38] and confirmed by transcriptome analysis of T lymphocytes [39]. Since classic PS inhibitors have severe toxic side effects, while HIV protease inhibitors have proved to be safe over prolonged periods in seropositive subjects of any age, we decided to treat with saquinavir a few, very difficult cases of NS with insufficient response to steroids. We observed a significant reduction in annual...
need of steroids, in parallel with down-regulation of NF-kB activation, while the MECL-1/beta2 mRNA iPS/PS ratio was reversed to normal values. Saquinavir blunted NF-kB activation in cultured peripheral lymphomonocytes and in human podocytes induced by LPS and TNF-α [37]. This finding suggests that saquinavir’s beneficial effects in patients with NS might not be limited to modulating the synthesis of permeability factors, produced by circulating immune cells, but also to a direct impact on podocyte protein synthesis.

The beneficial effects were obtained with a combination of saquinavir, small doses of steroids and calcineurin inhibitors, which may result in an adequate cumulative immunosuppressive effect, strengthening the final effect and limiting the adverse effects of individual drugs.

**PS INHIBITORS AND RENAL FIBROSIS**

PS inhibitors are under investigation as inhibitors of renal fibrosis, since transforming growth factor beta (TGF-β), a key factor in renal fibrosis, is regulated by the ubiquitin–PS pathway, through degradation of TGF-β-signalling molecules [40]. PS inhibition can block SnoN degradation, a negative regulator that interacts with Smad, target for TGF-β receptor kinase. In cultured rat fibroblasts and tubular epithelial cells, PS inhibitors—MG132 or lactacystin—attenuate TGF-β signalling by inhibiting Smad signal transduction [41]. However, these favourable effects in cell cultures have not yet been proved effective in experimental models of renal fibrosis [42].

**PS INHIBITORS IN RENAL TRANSPLANTATION**

Antibody-mediated rejection (AMR) is a relevant risk factor for graft loss in kidney transplanted patients. The presence of donor-specific HLA antibodies at the time of rejection is a poor prognostic factor for graft survival. The development of DSA after renal transplantation reduces the long-term graft survival [43]. Traditional therapies for AMR include i.v. immunoglobulins, plasmapheresis and rituximab, but the results are inconclusive [44]. PS inhibition attracted much interest, because of the major immuno modulatory effects of these drugs: (1) inhibition of NF-kB activity preventing nuclear translocation and transcription activity of IL-1, IL-6 and TNF, (2) inhibition of proliferation and induction of apoptosis via cell cycle arrest, (3) inhibition of MHC class I-restricted antigen presentation and (4) induction of apoptosis via endoplasmic reticulum stress. The last effect is relevant when targeting the therapy to normal plasma cells producing DSAs. Endoplasmic reticulum stress results from accumulation of misfolded or unfolded proteins in the endoplasmic reticulum [15]. This leads to a protective mechanism named the UPR. PS inhibitors induce UPR and this effect decreases protein synthesis, increases transcription of folding enzymes and enhances trafficking of ubiquitinylated proteins to PS. Persistent and progressive accumulation of misfolded proteins leads to induction of apoptosis. This mechanism is considered to play a major role in deletion of normal or transformed plasma cells by PS inhibitors.

The utility of bortezomib was demonstrated in the treatment of sporadic cases of refractory AMR [45, 46] in which this PS inhibitor reduced DSA levels by >50%, with improvement or stabilization of renal function and improvement of AMR. A large multicentre study (START) is ongoing [47] in 96 patients with AMR treated with bortezomib, and the initial report claimed a 50% reduction in DSA in 40% of the cases with improvement in renal biopsy feature in 60% of the patients. Toxicity (neural, gastro-intestinal and haematologic) was the most relevant issue.

It is of interest that the efficacy of bortezomib may be enhanced by combination regimens with other agents to enhance the final therapeutic effect, including p-glycoprotein inhibitors, histone deacetylase inhibitors, BAFF or April inhibitors [46, 48].

**CONCLUSION**

PS inhibitors are a fascinating research area and promising field for treating progressive renal disease in native kidney as well as in renal grafts. These drugs are very powerful, and their drawback is an unselective action on pathways critical to health. Tissue toxicity limits a wide clinical use of some PS inhibitors. Bortezomib, the most commonly used in published reports, has a severe toxicity, particularly peripheral neurotoxicity, in protocols used by haematologists. Reducing the cycles from six to two, as done in patients carrying a renal transplant, resulted in acceptable toxicity, considering the severe conditions in which this drug was adopted and the related risk of graft loss. Protocols are being developed to evaluate the benefits versus the side effects of using PS inhibitors in mild clinical conditions, as in an early phase of development of DSA or in glomerular diseases with persisting proteinuria without advanced chronic kidney damage. Before the publication of these results, the use of PS inhibitors should be limited to severe clinical conditions and considered a rescue therapy.

There is the need of developing less toxic compounds of the family of PS or iPS inhibitors. Several drugs are under development and in phase II trials. It is of interest that other drugs may be provided with anti-PS activity, such as the anti HIV protease inhibitors (saquinavir and ritonavir) [22], which we tried with some benefits in difficult cases of NSs [37] and which had placebo-like side effects. We used this drug in association with small doses of calcineurin inhibitors and steroids. Notably, calcineurin inhibitors act also as 20S PS inhibitors and interact with the ubiquitine proteolitic activity. Steroids have a still not fully understood potentiating effect. This cumulative effect was observed also in transplanted patients taking bortezomib for AMR [48]. It is likely that the cumulative immunosuppressive effect strengthens the final result and limits the adverse effects of individual drugs. Both development of new, less toxic and easier to manage anti-PS compounds and advancement in understanding the optimal combination of small doses of classic drugs, which could
potentiate the final effect, represent a promising area for improving treatment of progressive renal diseases.

**CONFLICT OF INTEREST STATEMENT**

In this review no original result is published, and hence, the sentence 'the results presented in this paper have not been published previously in whole or part' is not applicable.

**REFERENCES**


A perspective on anti-CCN2 therapy for chronic kidney disease

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ABSTRACT

Kidney fibrosis is the common end point of chronic kidney disease independent of aetiology. Currently, no effective therapy exists to reduce kidney fibrosis. CCN2 appears to be an interesting candidate for anti-fibrotic drug targeting, because it holds a central position in the development of kidney fibrosis and interacts with a variety of factors that are involved in the fibrotic response, including transforming growth factor (TGF) β and Bone morphogenetic proteins. Although CCN2 modifies many pathways, it does not appear to have a membrane receptor of its own. Numerous experimental and clinical studies lowering CCN2 bioavailability have shown promising results with minimal adverse side effects. This review aims to provide an overview of the current state of CCN2 research with a focus on anti-fibrotic therapy.

Keywords: CCN2, CKD, fibrosis, intervention, kidney

INTRODUCTION

The common and important feature of chronic kidney disease (CKD), independent of aetiology, is development of fibrosis. Fibrotic processes are characterized by an increase in the number of myofibroblasts and a change in extracellular matrix (ECM) composition, quality and quantity. Accumulation of ECM (e.g. collagen, fibronectin and proteoglycans), increase in non-degradable collagen cross-linking and a decrease in ECM degradation are a hallmark of fibrosis and thought to be mostly mediated by myofibroblasts.

CCN2 functions in normal physiology

CCN2 is a matricellular matrix molecule consisting of four distinct, conserved domains. Domain 1 is an insulin-like growth factor (IGF)-binding protein domain, Domain 2 a von Willebrandt factor type C repeat, Domain 3 a thrombospondin type 1 repeat and Domain 4 a cysteine knot [4]. Located between Domains 2 and 3 is a linker region susceptible to proteolytic cleavage.

Myofibroblasts are activated fibroblasts with a specialized contractile phenotype. The exact origin of the interstitial myofibroblasts remains unclear, but evidence suggests that they might originate from resident fibroblasts, pericytes, circulating fibrocytes or through endothelial- or epithelial-to-mesenchymal transition [1–3].

Currently, although many different approaches with varying results have been taken, no clinical therapy exists that targets the progression of fibrosis effectively.

A promising potential target might be the matricellular protein CCN2 (also known as CTGF, the second member of the Cyr61, CTGF, Nov family). A wide and still growing variety of pro-fibrotic properties has been attributed to CCN2, and the anti-fibrotic efficacy of CCN2 inhibition observed in many pre-clinical models is now being studied in clinical trials. This review aims to describe the physiological role of CCN2, its main pro-fibrotic properties and the current scientific evidence regarding CCN2 inhibition as an effective method for hampering the development of kidney fibrosis.