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Proteasome Targeted Therapies in Rheumatoid Arthritis

Aisha Siddiqah Ahmed
*Karolinska Institutet
Sweden*

1. Introduction

Rheumatoid arthritis (RA) is a chronic, systemic, autoimmune disease that primarily affects the joints. Approximately 0.5% of the adult population worldwide suffer from RA. The functional disability that results from progressive joint destruction is associated with substantial cost, significant morbidity and premature mortality [Carmona et al, 2010]. Pain and inflammation are initial symptoms followed by various degree of bone and cartilage destruction. During the last few decades' tremendous improvements have been made in search of therapies against RA. Disease modifying anti-rheumatic drugs (DMARDs) and biological therapies such as antagonists against TNF- α or IL-1 have provided efficient treatments and changed the shape of this disorder. However, the side effects, availability, and their focused approach to reduce inflammation have limited their scope. Thus there is a need of therapies targeting inflammation as well as reducing inflammatory pain and joint destruction in RA.

Cytokines are key players in pathogenesis of RA [Brennan & McInnes, 2008]. Synovial fluid from RA joints contains large quantities of cytokines secreted by macrophages, dendritic cells, neutrophils and synovial fibroblasts [Raza et al, 2005]. Cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1) and interleukin-17 (IL-17) stimulate the production of destructive proteases. Synthesis of these pro-inflammatory mediators is regulated by the transcription factor NF- κ B, controlled by the ubiquitin proteasome system (UPS) [Baldwin, 1996]. UPS is a multicatalytic system of protein degradation and present in all cell types including neurons and glia cells and regulates numerous cellular functions by selectively degrading cellular proteins.

2. Ubiquitin proteasome system

The degradation and processing of cellular proteins is critical for cell survival, growth, and cell division. Proteolysis via the proteasome pathway plays an important role in a variety of basic cellular processes. These processes are regulation of cell cycle and division, modulation of the immune and inflammatory responses, intracellular signaling, and development and differentiation [Goldberg, 2003].

Cellular proteins are mainly degraded in two ways: lysosomal degradation and ubiquitin-mediated degradation. Proteolysis in lysosomes is a non-specific process. In higher

eukaryotes, membrane-associated and extracellular proteins captured during endocytosis (e.g. viral, bacterial) are destroyed in lysosomes. Degradation of the vast majority (80-90%) of intracellular proteins is proteasome mediated [Ciechanover, 2005]. The ubiquitin proteasome system (UPS) controls the degradation of proteins in the cytosol, nucleus as well as in the luminal endoplasmic reticulum in eukaryotic cells (Goldberg, 2003).

Ubiquitin-mediated degradation of a protein involves two discrete and successive steps: first, the conjugation of multiple moieties of ubiquitin (Ub) to the protein substrate; multiple copies of ubiquitin covalently bind to available lysine residues on target proteins in a three-step process. Second, recognition of polyubiquitinated proteins by the 19S proteasome complex; Ub chain is cleaved by deubiquitinated enzymes (DUB), the substrate protein is unfolded and enters the 20S core for degradation. Then the substrate protein is cleaved into smaller peptide chains (5-20 amino acids), which are further degraded into constituent amino acids and are recycled by the cell [Goldberg, 2003]. The polyubiquitin chain is also broken down by the hydrolase enzymes and free Ub molecules are recycled by the cell [Kisselev et al., 1999] (Fig. 1).

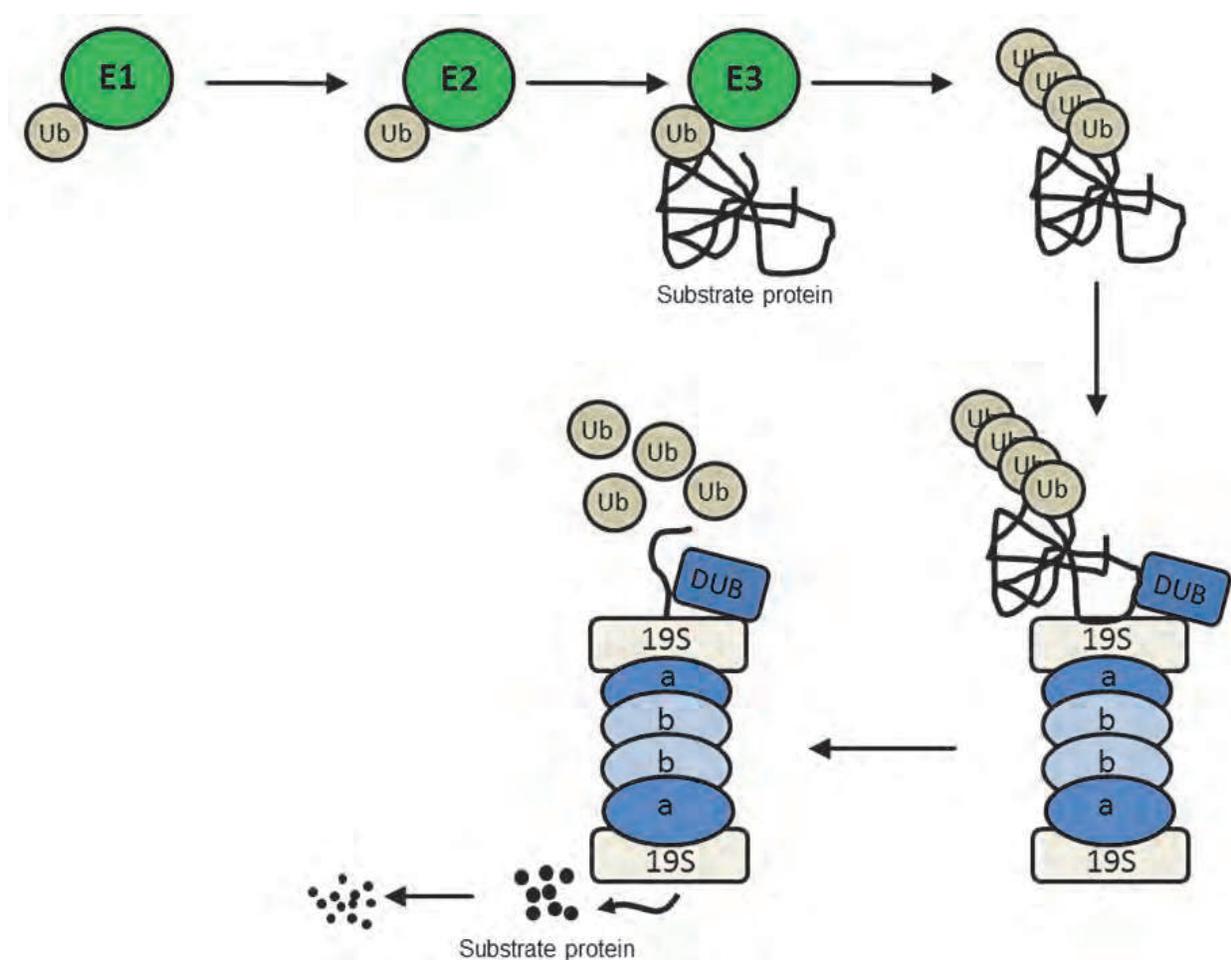


Fig. 1. The ubiquitin-dependent degradation of protein

This process has been named the “ubiquitin-dependent degradation of protein” and was first discovered by A. Ciechanover, A. Hershko, and I. Rose who were later awarded the Nobel Prize in 2004 [Sorokin et al., 2009].

2.1 Enzymatic cascade

Ubiquitin is a 76 amino acid protein conserved across eukaryotic cells. The covalent attachment of ubiquitin to a substrate protein is a highly regulated process and can be controlled at multiple points. Ubiquitin is first activated by an activating enzyme, E1. This step requires ATP to generate a high-energy thioester intermediate, E1-S~ubiquitin. The thioester attachment induces a conformational change in the E1 that promotes association with an ubiquitin carrier protein, E2. Next, activated ubiquitin is transferred to the E2 via formation of an additional high-energy thiol intermediate, E2-S~ubiquitin, leading to dissociation from the E1 [Huang et al, 2007]. In the third step, a substrate-specific ubiquitin E3 ligase interacts with the target protein-E2 ubiquitin complex to transfer ubiquitin to the target protein (Fig 2). Additional ubiquitin proteins are attached to the initial ubiquitin via a lysine linkage forming polyubiquitin chains that may be linear or branched [Kim et al, 2007; Pickart et al, 2004]. The protein must be polyubiquitinated for Ub-dependent protein degradation by the proteasome.

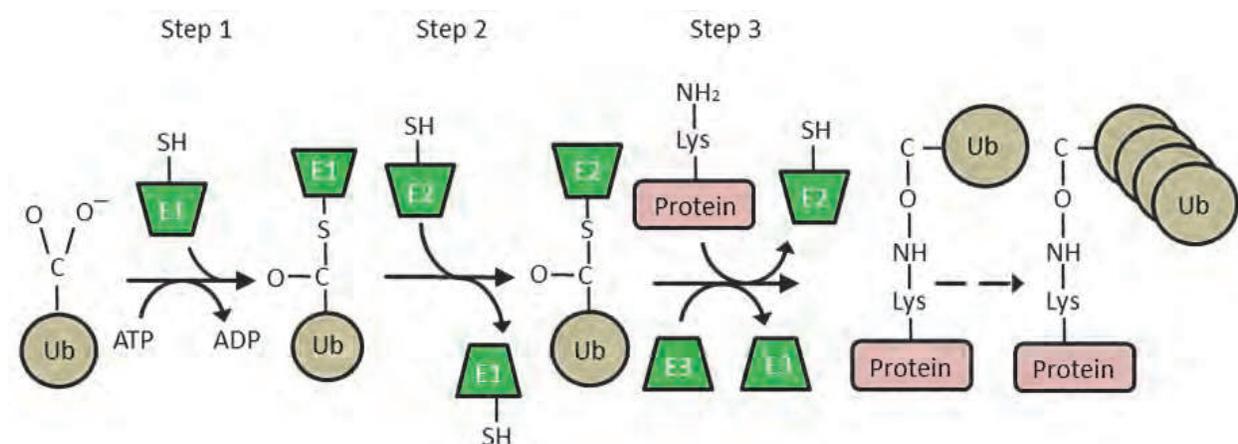


Fig. 2. Polyubiquitination of substrate protein. Ubiquitin (Ub) is activated by enzyme E1 and translocated to enzyme E2. In the last stage, E3 ligase conjugates Ub to the substrate protein.

In mammals, there are only two E1 ligases [Jin et al, 2007], but dozens of E2 ligases, and hundreds of E3 ligases. Ubiquitination specificity is determined principally by this large variety of E3 ligases, which generate the vast number of E2/E3 combinations that each target specific groups of protein substrates.

2.2 Proteasome structure

The proteasome is a cylindrical shaped structure with a molecular weight of 1,500 to 2,000 kD, located both in the cytoplasm and in nucleus in eukaryotes. It consists of two 19S regulatory complex and a core 20S catalytic complex (Fig. 3). It is also denoted the 26S proteasome [Orlowski, 1990; Ciechanover, 1998].

2.2.1 The 19S regulatory complex

Ubiquitin-tagged proteins are recognized by the 19S regulatory complex, where the ubiquitin tags are removed. ATPases with chaperone-like activity at the base of the 19S regulatory complex then unfold the protein substrates and feed them into the inner catalytic compartments of the 20S proteasome cylinder [Ciechanover, 2005]. The opening into the 20S catalytic chamber is small (approximately 1.3 nm), and significant unfolding of the substrate

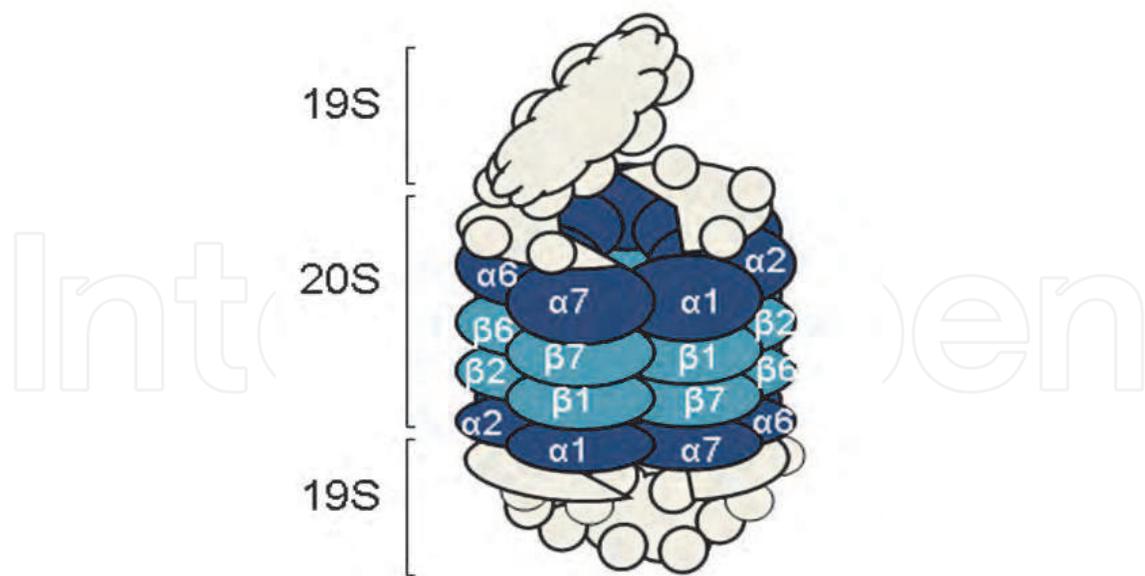


Fig. 3. The 26S proteasome, composed of two regulatory 19S and one catalytic 20S subunits.

is required for successful entering into the 20S subunit [Pickart, 2000]. A molecular gate (N-terminal tail of the α 3-subunit) also guards the opening, but it is constitutively open when the 19S regulatory units are bound to the 20S proteasome [Groll, et al., 2000]. There are also multiple different 11S regulatory complexes that can replace the 19S regulator [Hill et al, 2002]. These alternate regulators do not have ATPase function and do not bind polyubiquitin chains. Proteasomes with 11S substitutions for 19S regulators have higher levels of proteolytic activity [Cascio et al, 2002; Fruh et al, 1994].

2.2.2 The 20S proteasome subunit

The 20S proteasome subunit consists of two outer and two inner rings that are stacked to form a cylindrical structure with three compartments [Lowe et al., 1995]. Each outer ring has seven alpha-subunits (α 1 to α 7), whereas each inner ring contains seven beta-subunits (β 1 to β 7) (Fig 4).

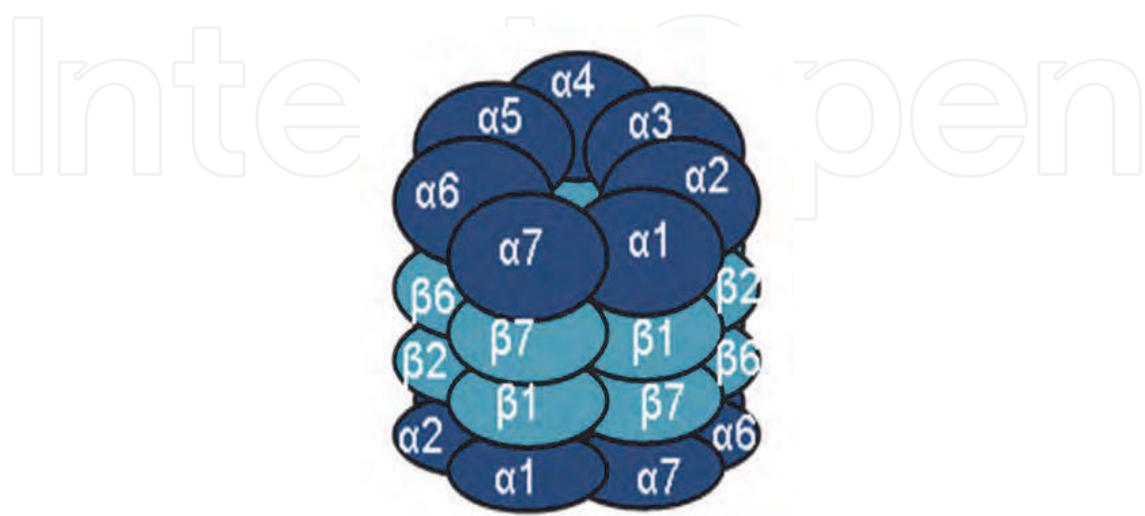


Fig. 4. The 20S proteasome.

The 20S proteasome complex has chymotryptic, tryptic, and peptidylglutamyl-like activities [Ciechanover, 2005; Orłowski, 1990]. It is conformationally flexible with active catalytic sites located on the inner surface of the cylinder where protein substrates bind. Proteins unfolded and without Ub tag, enter the inner chamber, where they are hydrolyzed by six active proteolytic sites on the β subunits (two sites each on the β 1-, β 2-, and β 5-subunits) into small polypeptides ranging from three to 22 amino acids in length. Proteins cannot enter the inner cylinder through the outer walls of the 20S proteasome because the gaps between the rings are tight [Lowe et al., 1995; Stein et al., 1996]. In eukaryotic cells, 26S proteasomes are localized both in the cytoplasm and in the nucleus. This distribution is tissue-specific [Lowe et al., 1995].

3. UPS in immune and inflammatory response

A role for UPS in the pathogenesis of human diseases was first suggested some two decades ago. With the broad spectrum of protein substrates and the complex enzymatic machinery involved in targeting them and practically all intracellular processes being controlled by the UPS, it is not surprising that the proteasome pathway is involved in the pathogenesis of malignant, autoimmune, and neurodegenerative diseases.

The UPS plays significant role in immune and inflammatory processes. It has been shown that UPS takes part in the antigen processing in antigen presenting cells, regulates the transmission of signals from T-cell antigen receptors and the co-stimulatory CD28 molecule and is involved in activation of transcription factor- κ B (NF- κ B). NF- κ B is the key regulator of the activity of genes of many inflammatory cytokines, chemokines and cell adhesion molecules [Sorokin, 2009]. The function of UPS in the activation of NF- κ B is the most important and will be discussed here in details.

NF- κ B is a family of dimeric transcription factors. The NF- κ B family consists of five members: p50, p52, p65/RelA, c-rel, and RelB [Neumann & M. Neumann, 2007]. p50 and p52 are formed as a result of processing from precursors p105 and p100, respectively. The processing of p105 can be performed both by the Ub-dependent pathway by the 26S proteasome [Coux & Goldberg, 1998] and by the ATP-/Ub-independent pathway by the 20S proteasome [Moorthy, 2006]. NF- κ B activation promotes the expression of variety of target genes involved in the immune response, reparation reactions, and apoptosis. These include the pro-inflammatory cytokines IL-1 β and TNF- α , extracellular matrix metalloproteinase (MMPs), prostaglandins and nitric oxide. IL-1 β and TNF- α , in particular, have been shown to play pivotal roles in the pathogenesis of RA both in preclinical [Han et al., 1998] and clinical studies using biological agents such as etanercept and infliximab [Carteron, 2000; Cunnane, 2001].

The UPS activate NF- κ B in two stages. At first, the proteasome performs ubiquitin dependent processing of phosphorylated precursors p105 and p100 with the formation of active subunits of transcription factors p50 (NF- κ B1) and p52 (NF- κ B2). NF- κ B is composed of p50 and p65 subunits, and in non-stimulated cells it is retained in the cytoplasm in a latent form associated with inhibitory protein I κ B. Following exposure of the cell to a variety of extracellular stimuli such as cytokines, viral and bacterial products and stress, I κ B is phosphorylated, poly-ubiquitinated (which is recognized by the 19S regulatory subunit of Proteasome) and is finally rapidly degraded by the 26S proteasome. The released active heterodimer is translocated into the nucleus where it activates the transcription of corresponding genes [Van Waes et al., 2007] (Fig 5).

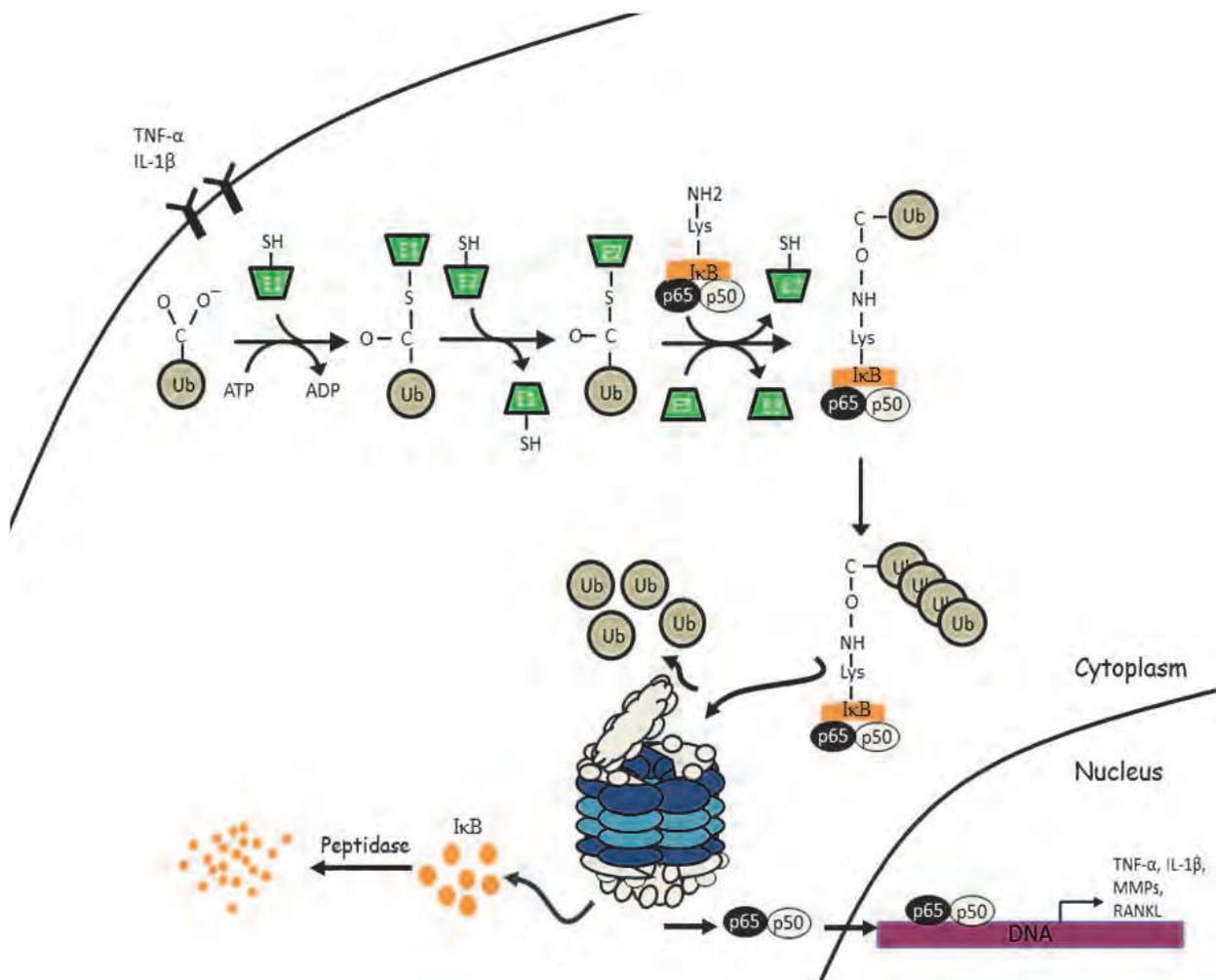


Fig. 5. Activation of Nuclear factor-κB by the proteasome system

NF-κB promotes transcription of genes which encode cytokines (TNF-α, IL-6, IL-1), stress response factors (Cyclooxygenase-2, NO), cell cycle regulators, and anti-apoptotic proteins (IAP-1, Bcl-2 family) [Delhalle et al., 2004]. The pathological activation of NF-κB is a cause of many inflammatory diseases including RA and has been an important target for therapeutic drug research in recent years [Elliott et al., 2003].

3.1 Activation of NF-κB in RA

NF-κB is one of the best-characterized transcription factors and regulates the expression of many genes, most of which encode proteins that play crucial roles in the processes of immunity and inflammation. The activation of NF-κB has been associated with the up-regulation of pro-inflammatory genes involved in several inflammatory conditions [Baldwin, 1996], and has been implicated in pathogenesis of RA [Firestein, 2004]. NF-κB activation has been studied in animal models of arthritis [Han et al., 1998; Palombella et al., 1998] and in the synovium of RA patients [Handel et al., 1995; Firestein, 2004]. NF-κB is essential for TNF-induced synovial cell activation and proliferation as several studies indicated that treatment of synovial cells with an antioxidant agent inhibited TNF-α induced NF-κB activation and transcription [Fujisawa et al., 1996]. Moreover, nuclear extracts from IL-1β stimulated human synovial fibroblasts contained p65 DNA-binding

NF- κ B complexes and both the NF- κ B classical oligonucleotide decoy and antisense oligonucleotide specific to p65, and they produced a concentration dependent decrease in IL-1-stimulated PGE2 production [Handel, 1995]. Additionally, NF- κ B activator, IL-18 can indirectly stimulate osteoclast formation through up-regulation of RANKL production from T cells in RA synovitis [Dai et al., 2004]. Blocking of IKK β *in vitro* with a dominant negative adenoviral construct was shown to inhibit the induction of IL-6, IL-8, and intercellular adhesion molecule-1 (ICAM-1) after stimulation with IL-1 or TNF- α [Aupperle et al., 2001].

The significance of NF- κ B in inflammatory joint disease has been validated by numbers of arthritis models such as carrageenan-induced paw edema, collagen-induced arthritis and adjuvant-induced arthritis [Min et al, 2009, Campo et al, 2011, Ahmed et al., 2010]. In animal models of arthritis the activation of NF- κ B appears to precede the onset of disease, and the blockade of NF- κ B decreases arthritis severity [Tsao et al., 1997; Ahmed et al., 2010]. Intra-articular gene transfer of IKK β -wild type into the joints of normal rats resulted in significant paw swelling and accompanied synovial inflammation. Increased IKK activity was detectable in the IKK β -wt-injected ankle joints which was coincident with enhanced NF- κ B-DNA-binding activity. Intra-articular gene transfer of IKK β -dominant negative significantly ameliorated the severity of adjuvant arthritis, accompanied by a significant decrease in NF- κ B DNA expression in the joints of adenoviral IKK β -dominant negative-treated animals [Tak et al., 2001].

3.1.1 NF- κ B in RA joint destruction

Progressive destruction of bone and articular cartilage plays a pivotal role in the pathogenesis of RA. During joint inflammation, the inflamed synovium forms a pannus tissue, which grows into the bone and causes destruction, initially as marginal erosions at the site of synovial proliferation where bone is unprotected by hyaline cartilage. Subsequent bone destruction leads to subluxation and deformity. Cytokines such as TNF- α , IL-1 β and IL-17 stimulate the activation of bone destroying osteoclasts, and the production of destructive proteases - matrix metalloproteinases (MMPs). MMPs have been suggested to be involved in the pathogenesis of RA and OA through their ability to degrade proteoglycans [Flannery et al., 1992; Humbry et al., 1995].

NF- κ B is essential for osteoclast formation and survival through the receptor activator of the nuclear factor kappa-B ligand (RANKL) pathway [Soysa et al., 2009]. Abnormal activation of NF- κ B signalling in osteoclasts has been observed in osteolytic conditions, including arthritis, Paget's disease of bone, and periodontitis [Xu et al., 2009]. Inhibition or deletion of RANKL prevents bone destruction [Zwerina et al., 2004]. Further, it is demonstrated that inhibition of I κ B-kinase complex can suppress RANKL stimulated NF- κ B activation and osteoclastogenesis both *in vitro* and *in vivo*. Additionally, this peptide significantly reduced the severity of collagen-induced arthritis in mice by reducing levels of TNF- α and IL-1 β , and thereby abrogating joint swelling and reducing destruction of bone and cartilage [Jim et al., 2004]. Elevated levels of MMP-1 (collagenase-1) in the synovial fluid and serum of RA patients [Green et al., 2003; Yamanaka et al., 2000] and MMP-3 (stromelysin) in the synovial fluid from RA patients has been determined [Hembry et al., 1995]. Interestingly, it has been reported that NF- κ B regulates synthesis of MMPs including MMP-1 and MMP-3 [Thurberg et al., 1998].

4. UPS in neuronal signalling

In the nervous system, UPS is present in neurons, glia and synapses and regulates numerous functions including neuronal signalling, synapse assembly, maintenance, and function [Mengual et al., 1996]. Recent work utilizing the powerful genetic tools in *C. elegans* and *Drosophila* as well as synaptic assays in mammalian neuronal culture systems has unravelled the critical role of UPS in neuronal signaling. Active E3 ligases are identified at the synapse which participates in synaptic plasticity [Myat et al., 2002]. Moreover, localization of many E3 ligases in nucleus and synapse suggests the interplay between UPS regulation of transcriptional programs that function in synaptic modulation and local synaptic regulation of protein degradation.

UPS regulates synaptic functions by controlling levels of pre-synaptic proteins [Speese et al., 2003]. At the post-synaptic levels, the UPS regulates the surface expression and internalization of NMDA- and AMPA-glutamate receptors [Moriyoshi et al., 2004]. It has been implicated that mechanical allodynia and hyperalgesia can be prevented with NMDA-receptor antagonists [Laughlin et al., 1997]. During pathological pain UPS regulates neuronal signalling by controlling levels of synaptic proteins [Ossipov et al., 2007]. Much future work is needed to identify exact role of UPS in acute and chronic pain conditions.

5. Proteasome inhibition

Proteasome inhibitors are considered as a potential remedy for cancer, inflammation-related disorders and neurodegenerative diseases. Proteasome inhibitors can cause cellular apoptosis in proliferating cancer cells by affecting various short-lived proteins, resulting in inhibition of NF- κ B activity, increased activity of p53 and Bax proteins, and accumulation of cyclin- dependent kinase inhibitors p27 and p21 [Moriyoshi et al., 2004; Van Waes et al., 2007]. Preclinical studies show that malignant, transformed, and proliferating cells are more susceptible to proteasome inhibition than cells in a resting state [Adams, 2002; Sherr, 1996].

Bortezomib is the first inhibitor of the ubiquitin-proteasome pathway to enter clinical studies [Adams et al., 1999; Richardson et al., 2003]. On the basis of a large, multicenter phase II clinical trial in which approximately one third of patients with advanced multiple myeloma (MM) had a significant response to therapy with bortezomib, on May 13th 2003, the US Food and Drug Administration granted approval for use of this drug in the treatment of patients with MM [Richardson et al., 2003]. The promising preclinical and clinical activity exhibited by bortezomib in MM and non-Hodgkin lymphomas (NHL) has confirmed the proteasome as a relevant and important target in the treatment of cancer. Several proteasome inhibitors are being tested and are in the pre-clinical and clinical phase of testing.

In the case of RA, up-regulation of the most important pro-inflammatory mediators such as TNF- α , IL-1 β , IL-6, iNOS and endothelial cell adhesion molecules (e.g. vascular cell adhesion molecule 1 (VCAM-1)) are regulated by NF- κ B [Van Waes et al., 2007; Han et al 1998]. Therefore RA qualifies as a potential target for proteasome inhibitors. *In vitro* and *in vivo* studies have presented encouraging results by the use of different proteasome inhibitors to reduce the NF- κ B activation.

5.1 Proteasome inhibitors

Proteasome inhibitors include a variety of natural and chemically synthesized molecules which exclusively inhibit proteasome activity. The structure and function of some important classes of proteasome inhibitors are described here.

5.1.1 Peptide aldehydes

Peptide aldehydes were the first proteasome inhibitors to be developed [Palombella et al., 1994; Rock et al., 1994]. These include MG132 (Z-Leu-Leu-Leucinal-) (Fig 5), MG115 (Z-Leu-Leu-norvalinal-) and calpain inhibitor I (*N*-acetyl-Leu-Leu-norleucinal). These compounds are potent, reversible and cell permeable. MG132 is a reversible inhibitor of the chymotrypsin like activity of the proteasome.

5.1.2 Boronic acid peptides

Boronate inhibitors are much more potent than their structurally analogous peptide aldehydes [Adams et al., 1998]. These includes MG262 (Z-Leu-Leu-Leu-boronate; analogous to MG132) and PS-341 (pyrazylcarbonyl-Phe-Leu-boronate; analogous to the aldehyde PS-402). MG262 is a cell permeable and reversible inhibitors of the chymotrypsin like activity of the proteasome. PS-341 is clinically the most advanced proteasome inhibitor and inhibits the chymotrypsin like active site of the proteasome β -subunit. Its boronic acid group binds the active site threonine in the proteasome with high affinity and specificity (Fig 5).

5.1.3 Lactacystin

Lactacystin is a naturally occurring compound produced by *Streptomyces lactacystinaeus*. It selectively targets the $\beta 5$ subunit of the proteasome [Fenteany et al., 1995] by covalent acylation of the amino-terminal threonine residues and is considered as an irreversible inhibitor of the proteasome. The active component of lactacystin is the highly reactive *clasto*-lactacystin β -lactone and PS-519 (Fig 5).

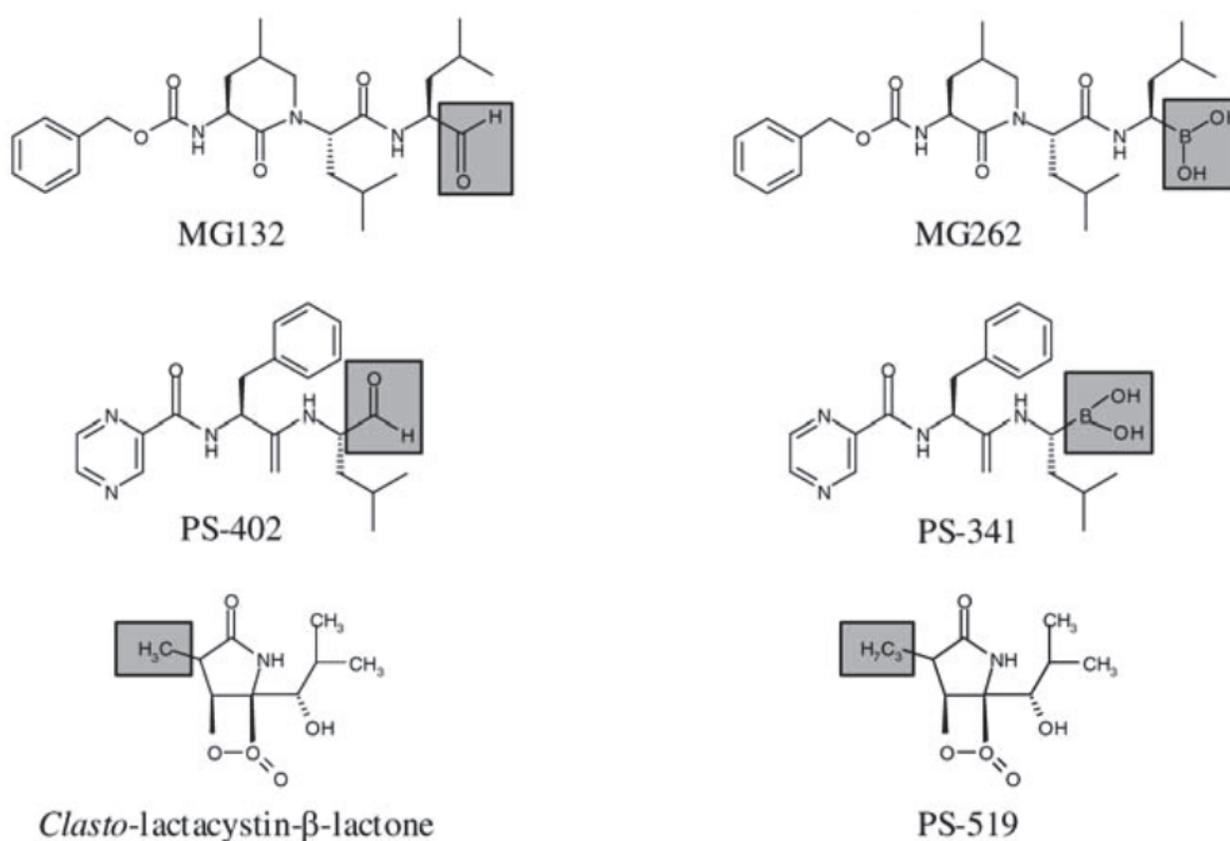


Fig. 6. Structures of selected proteasome inhibitors [Elliott et al., 2003].

5.1.4 Epoxyketones

These are naturally product proteasome inhibitors isolated from actinomycete fermentation broths by screening for antitumor activity in mice. Examples of this class include Epoxomicin and eponemycin. These compounds inhibit the chymotrypsin like site only or the chymotrypsinlike and caspase-like, respectively. In contrast to previously mentioned inhibitors, epoxomicin initially forms a covalent bond between the proteasome's amino-terminal threonine hydroxyl at its C-terminal ketone carbonyl. This primary adduct formation is followed by formation of a stable six-membered ring adduct by a second attack by the terminal free amino group [Groll et al., 2000].

5.2 Proteasome inhibition in animal model of arthritis

Proteasome inhibitors exhibit anti-inflammatory and anti-proliferative effects. Their use in diseases characterized by these processes is thought to be promising but the effects of proteasome inhibitors on the pathogenesis of inflammatory joint disorder such as RA remain quite limited. To date the effects of proteasome inhibition have been studied only in animal models of arthritis; streptococcal cell wall induced polyarthritis in rats, collagen induced arthritis and adjuvant induced arthritis (Table 1). These animal models have several clinical and pathological similarities with human rheumatoid arthritis regarding inflammation, pain, swelling, synovial hyperplasia and destruction of cartilage and bone [Kannan et al., 2005].

Animal models	Proteasome inhibitor	References
streptococcal cell wall induced polyarthritis in rat	PS-341	Palombella et al., 1998
collagen induced arthritis in mice	PS-341	Lee et al., 2009
Adjuvant induced arthritis in rat	MG132	Ahmed et al, 2010
Adjuvant induced arthritis in rat	PS-341	Yannaki et al., 2010

Table 1. List of proteasome inhibitors used in animal models of RA.

5.2.1 Proteasome inhibition and joint inflammation

Proteasome inhibitors PS-341 and MG132 have been tested in different animal models of arthritis (Table 1) with pronounced anti-inflammatory effects. Here effects of proteasome inhibitor MG132 in adjuvant induced arthritis (AIA) rat model will be discussed in details. MG132 was administered subcutaneously daily at the onset of arthritis. Two weeks of administration significantly reduced signs of inflammation including swelling, redness and warmth in ankle joints compared to vehicle treated arthritis animals. Similar effects were observed in studies where proteasome inhibitor PS-341 was administered in different arthritis models. PS-341 significantly attenuated the arthritis severity and the clinical progression of the T cell dependent chronic phase of the disease. The chronic phase of arthritis was also associated with increased serum levels of NF- κ B dependent pro-inflammatory factors such as IL-1, IL-6, and nitric oxide metabolites [Palombella et al., 1998]. The expression of TNF- α , IL-1 β , IL-6, MMP-3, COX-2 and iNOS were decreased in PS-341-treated animals compared to untreated [Lee et al., 2009].

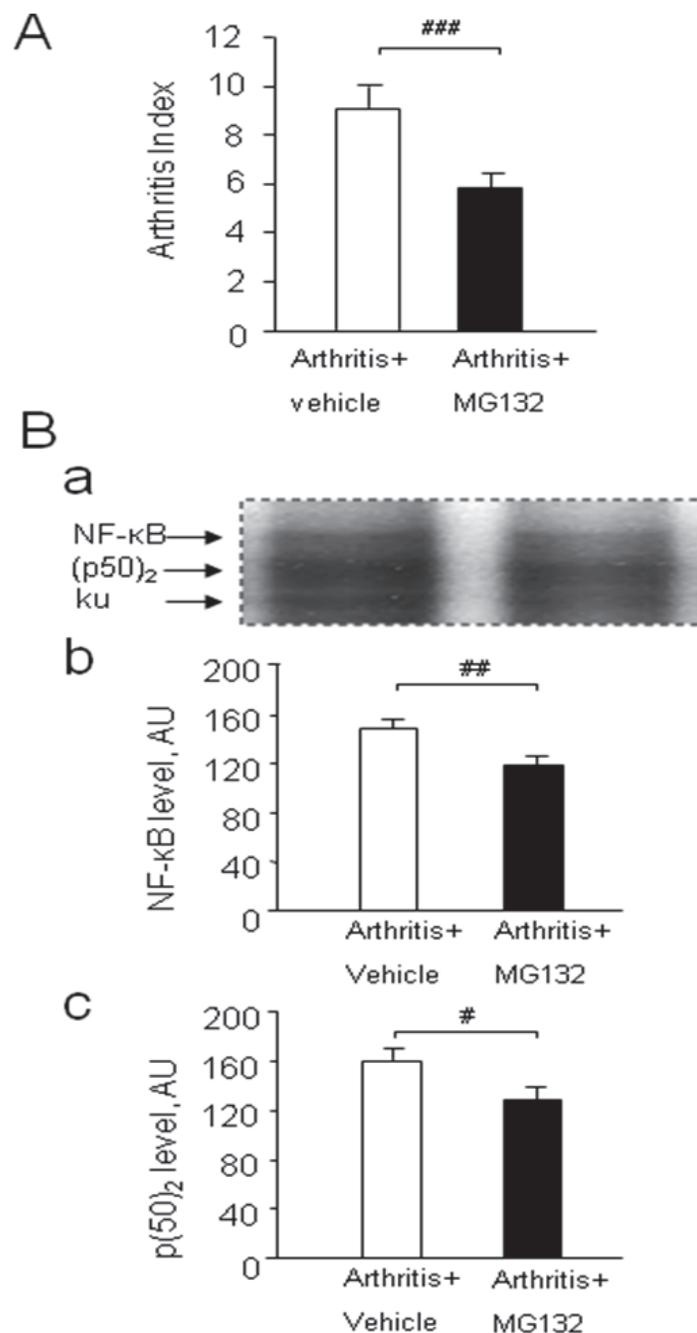


Fig. 7. Effects of proteasome inhibitor MG132 on (A) arthritis index; severity of arthritis was scored using a macroscopic scoring system according to changes in erythema and oedema in each paw (B) NF-κB and p50 activation in arthritic ankle joint of AIA rat. (a) autoradiograph of electrophoretic mobility shift assay. The upper two bands represent NF-κB and p50 homodimer complexes (indicated by arrows), (b) and (c) semi-quantification of the NF-κB and (p50)₂ levels.

MG132 treatment significantly down-regulated the expression of NF-κB1 (p50) in inflamed ankle joints as well as the DNA binding activity of both NF-κB and p50 homodimer in arthritic ankle joints (Fig 7 A and B). These results indicate that MG132 hinders the nuclear localization of NF-κB by retaining them in the cytosol in an inactive form bound to the inhibitory protein IκB, and also blocks UPS-mediated processing of the p105 precursor to

mature p50 [Magnani et al., 2000], which is a subunit of mature NF- κ B. Significantly lower levels of NF- κ B dependent proinflammatory factors such as IL-1, IL-6, and nitric oxide metabolites were found in PS-341-treated animals than in control rats. Thus supporting the concept that the profound anti-inflammatory effects of PS-341 result, in part, from inhibition of NF- κ B activity [Palombella et al., 1998]. Proteasome inhibitor MG132 and PS-341 treated animals gained significantly more body weight than the vehicle treated controls indicating that proteasome inhibitors given at therapeutically relevant doses were well tolerated.

5.2.2 Proteasome inhibition and joint destruction

Progressive destruction of bone and cartilage plays a pivotal role in the pathogenesis of RA. Effect of proteasome inhibitor MG132 on joint destruction was studied in AIA model [Ahmed et al., 2010]. The radiographic and histological analysis revealed that augmented cartilage and bone resorption, which is a characteristic feature of arthritis, was mitigated by the MG132 (Fig 8).

Bone resorption is a collective result of osteoclast stimulation and suppression of osteoblast precursors within the bone marrow. Previous studies have shown that NF- κ B controls osteoclast activation through RANKL signalling [Soysa & Alles, 2009], while inhibition or deletion of RANKL prevents bone destruction [Zwerina et al., 2004; Pettit et al., 2001]. The protective effect of MG132 may be a consequence of with interfering osteoclast activation through the RANKL signalling pathway that is under control of NF- κ B, or by enhancing the osteoblast activity. This assumption is supported by *in vitro* and *in vivo* studies indicating that the proteasome inhibitor bortezomib directly suppressed human osteoclast formation and promoted maturation of osteoblasts [Zangari et al., 2006; Mukharjee et al., 2008] and reduced joint destruction and preserved bone density in CIA mice [Lee et al., 2009].

5.2.3 Proteasome inhibition and inflammatory pain

Chronic pain is a major feature of RA and is maintained in part by long-lasting neuroplastic changes in the central and peripheral nervous system. Recent, pre-clinical studies demonstrated that the UPS is one of the systems involved in the maintenance of chronic pain by regulating proteins at pre- and post-synaptic levels [Speese, 2003; Mengual et al., 1996]. Effects of proteasome inhibitor MG132 on inflammatory pain was studied in the AIA animal model. Inflammation in joints significantly reduced the pain bearing capacity in arthritic animals as measured by the paw withdrawal threshold (PWT). Administration of MG132 significant increased PWT in arthritic animals compared to vehicle treated group (Fig 9).

Central and peripheral neuronal mechanisms are thought to play a critical role in inflammatory joint disorders, particularly with regard to inflammation and pain [Benrath, et al., 1995; Levine et al., 1985]. Sensory neuropeptides, substance P (SP) and calcitonin gene-related peptide (CGRP), are shown to participate not only in pain modulation but also in inflammatory processes. An up-regulation in the SP and CGRP expression in ankle joints and their corresponding dorsal root ganglia was demonstrated in adjuvant arthritis [Ahmed et al., 1995]. The development and progress of joint inflammation in adjuvant arthritis was significantly attenuated by using the neurotoxin capsaicin, which specifically down regulates sensory innervation [Ahmed et al., 1995]. The beneficial effects of capsaicin on joint inflammation were correlated with reduced levels of SP and CGRP in the ankle joints and corresponding DRG. Methotrexate treatment has been shown to reduce the severity of joint inflammation and destruction, partly due to its inhibitory effect on sensory

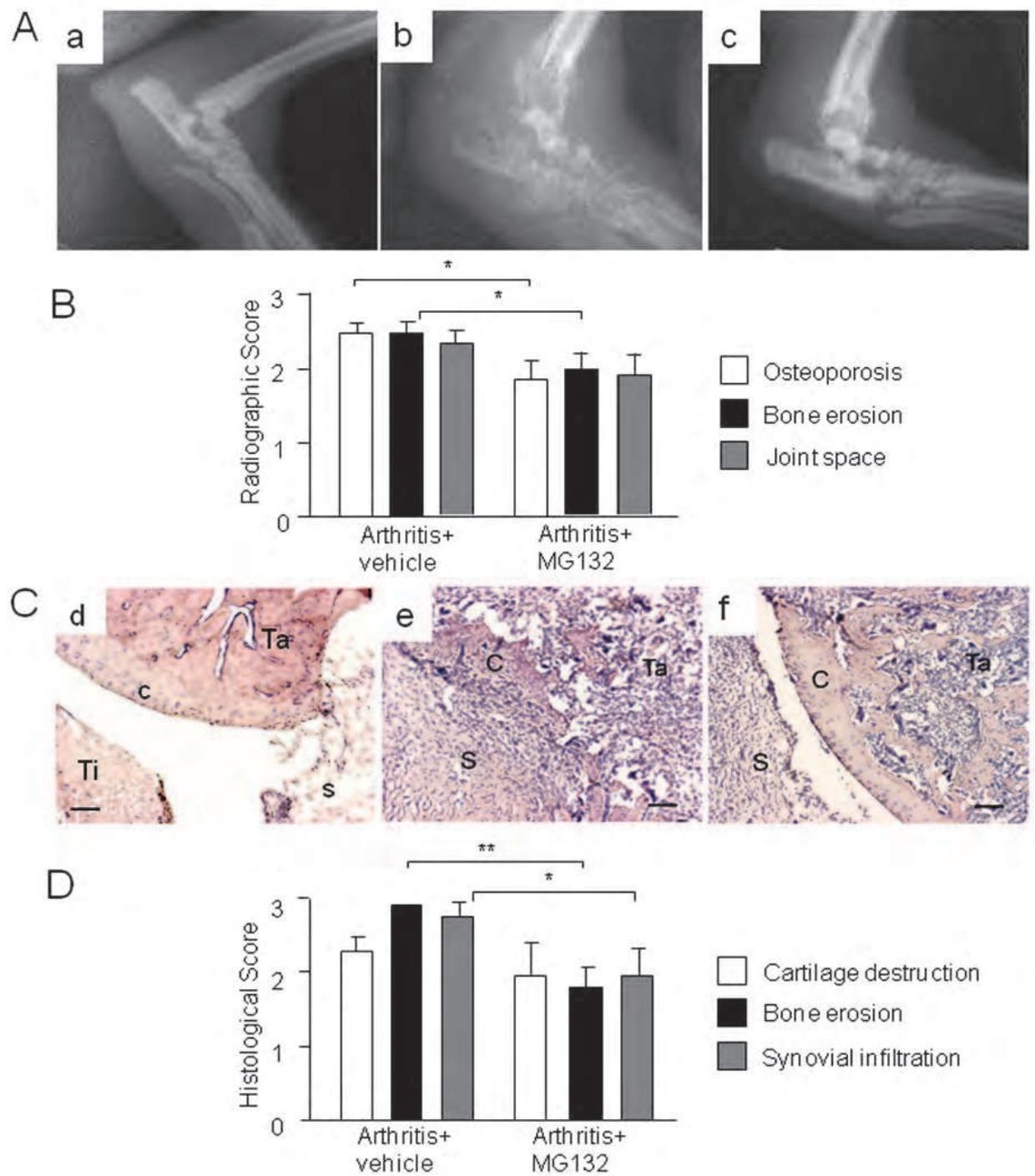


Fig. 8. Radiologic and histologic analysis of bone and cartilage destruction. A, Representative lateral view radiographs of ankle joint of (a) normal, and (b) vehicle- or (c) MG132-treated arthritic animals. B, Changes in the radiographic parameters of osteoporosis, bone erosion and joint space in ankle joints of arthritic animals treated with vehicle or MG132. C, Photomicrographs of haematoxylin and eosin stained ankle joints from (d) control rat; (e) vehicle-treated arthritic rat; and (f) MG132-treated arthritic rat. D, Changes in histologic parameters of cartilage and bone resorption and synovial infiltration in arthritic rats treated with vehicle or MG132. (c; articular cartilage, s; synovial membrane, Ti; tibia and Ta; talus). Modified results from Ahmed et al, 2010.

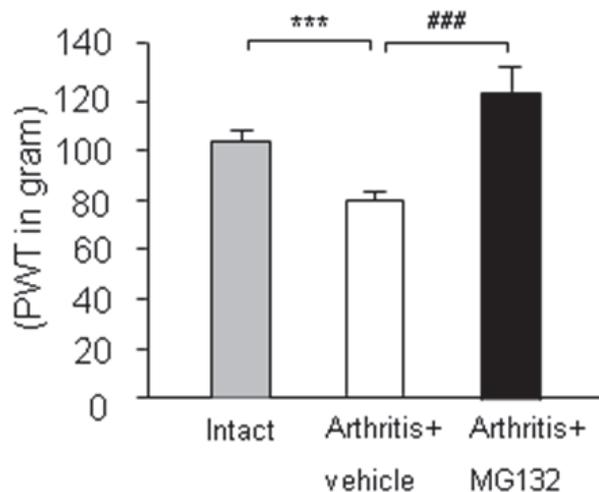


Fig. 9. Hind paw withdrawal threshold (PWT) in control and arthritic groups treated with MG132 and vehicle.

neuropeptides [Ahmed et al., 1995]. In the AIA model, strong up-regulation of SP and CGRP in the periosteum and synovium structures, which are pain sensitive and prone to inflammation, was observed. This increased SP and CGRP expression coincided with decreased pain thresholds. Administration of MG132 resulted in the normalization of pain responses as well as significantly down-regulating the expression of SP and CGRP in arthritic ankle joints (Fig 10). Results also indicate that UPS regulates inflammation induced pain behaviour and that UPS-mediated protein degradation is involved in the peripheral sensitization.

Previously it has been shown that proteasome inhibitors MG132 and epoxomicin can prevent the development of behavioural signs of neuropathic pain and abolish abnormal pain induced by sustained morphine exposure [Ossipov et al., 2009; Moss et al., 2008]. These compounds inhibited the release of DYN and CGRP and normalized molecular changes in the spinal cord contributing to central sensitization [Ossipov et al., 2009; Moss et al., 2008]. Although the cause and neurobiological mechanisms underlying neuropathic and inflammatory pain are different, the common mechanism for the effects of proteasome inhibitors in these pathological conditions is the similar central neuronal mechanism and the activation of neurotransmission mediated by the sensory neuropeptides including SP, CGRP and dynorphins.

The dorsal root ganglia (DRG) and the spinal cord actively participate in the peripheral and central sensitization. DRG neurons have very long t-shaped axons with one end forming a sensory terminal at the skin or joints and other end synapsing in the dorsal horn of the spinal cord. In the spinal cord these neurons project to the outermost region of the spinal dorsal horn (lamina I and outer lamina II) and terminate largely on spinal neurons that project to higher-order pain centers such as the cortex and the hypothalamus in the brain.

In AIA rats, a significant increase in the SP and CGRP expression has been reported in the DRG [Ahmed et al, 1995a]. In the spinal cord an enhanced release of SP and CGRP has been recorded in the lumbar dorsal horn during inflammation [Garry & Hargreaves, 1992]. Up-regulated SP expression in the DRG correlated with arthritis severity and nociceptive behavior of arthritic rats [Ahmed et al, 1995a]. This agrees with other observations that the altered expression of SP and CGRP is critical for the modulation of pain and inflammation (Ambalavanar et al., 2006; Hutchins et al., 2000). Moreover, it has been reported that

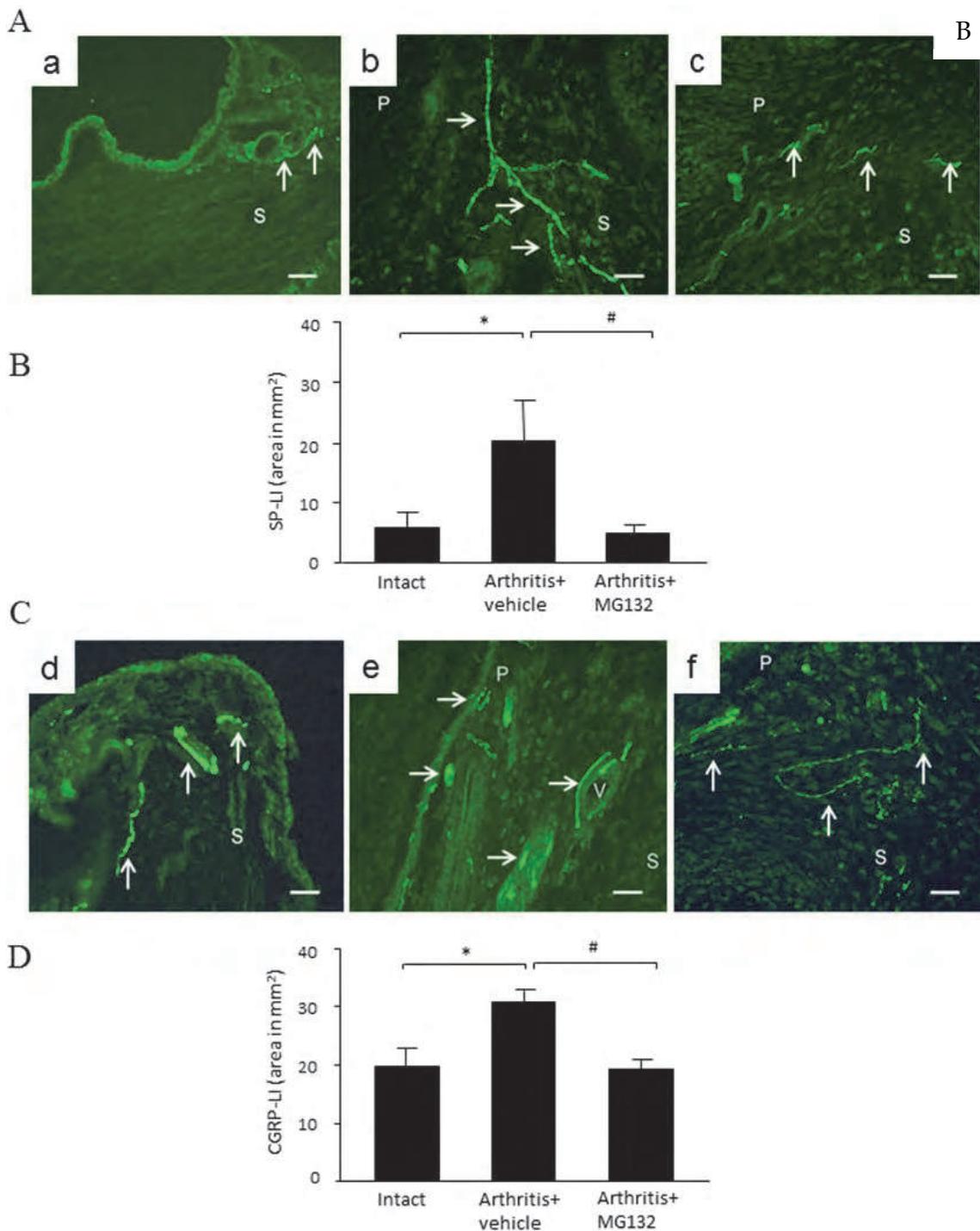


Fig. 10. Immunofluorescence micrographs and semi-quantitative analysis of SP and CGRP in rat ankles. A, Nerve fibres positive to SP in the vehicle-treated control rats (a), and in the vehicle- (b) or MG132- (c) treated arthritis rats. B, Semi-quantitative analysis of SP immunoreactive nerve fibres (immunofluorescent area) in ankle joints of the control and arthritic rats treated with vehicle or MG132. C, Nerve fibres positive to CGRP in the vehicle-treated control rats (d), and in the vehicle- (e) or MG132- (f) treated arthritis rats. D, Semi-quantitative analysis of CGRP immunoreactive nerve fibres (immunofluorescent area) in ankle joints of the control and arthritic rats treated with vehicle or MG132. (s; synovial membrane, p; periosteum and v; blood vessel). Modified results from Ahmed et al, 2010.

peripheral inflammation induces a dramatic up-regulation of PDYN biosynthesis in nociceptive neurons of the spinal dorsal horn (Przewlocki, 1987; Marvizon et al., 2009). As a future perspective it will be interesting to observe the effects of proteasome inhibition in the DRG and SC in inflammation. In the monosodium-induced model of osteoarthritis, which is a well-recognized model of osteoarthritis, MG132 treatment has normalized the up-regulated expression of SP and CGRP in the inflamed knee joints and their corresponding DRG, with reduced pain behavior [Ahmed et al, unpublished data].

6. Toxicity

The clinical application of proteasome inhibitors might be limited due to potential side effects of available compounds following chronic administration. Toxic effects might result from the accumulation of ubiquitinated proteins after inhibition of the 26S proteasome. The proteasome inhibitor bortezomib (PS-341) induced mild-to-moderate neurotoxic effects in rats [Cavaletti et al., 2007] and peripheral sensory neuropathy in cancer patients when given this compound chronically [Cata et al., 2006]. The features of bortezomib neuropathy are characteristic for a small fiber neuropathy and are characterized by a more sensory than motor neuropathy. Several observations, however, argue against these possibilities. First, in rats, the neurotoxic effects were observed when bortezomib was administered at maximum tolerated, sub-lethal doses in rats [Cavaletti et al., 2007]. Bortezomib might have induced neurotoxic effects because of the presence of a component in its activity that is blocked by the polyhydroxyl compound Tiron, this component is not involved in MG132 activity [Fernandez et al., 2006]. No effects of MG132 toxicity were apparent on motor performance during rotarod, posture, gait, exploratory and locomotor activity, or on cell death in the spinal cord, when MG132 was administered at higher doses [Ossipov et al., 2006]. Moreover, MG132 treated animals gained significantly more body weight than the vehicle treated arthritic controls [Ahmed et al., 2010]. These results indicate that proteasome inhibitor MG132 given at therapeutically relevant doses was well tolerated. This is a significant finding as the proteasome plays a central role in many intracellular functions and its inhibition might theoretically be expected to induce numerous side effects.

7. Future perspectives

Taking into account that the UPS controls important functions in eukaryotic cell, proteasome inhibitors could have been considered as toxins without any therapeutic value. Unexpectedly, proteasome inhibitors are well-tolerated drugs and do not produce adverse effects in normal cells even at high doses. Though, clinical trials indicate that use of bortezomib induces peripheral sensory neuropathy in patients, which might limit its therapeutic use. However, the reversible proteasome inhibitor such as MG132 apparently did not produce any toxic effect and was well tolerated. The use of reversible proteasome inhibitors can therefore be considered as a better alternative. It will be a future challenge to develop drugs specifically targeting the UPS, or more specifically UPS E3 ligases that select proteins for the UPS-mediated degradation, in order to treat inflammatory joint disorders. Non-toxic proteasome inhibitors alone and/or in combination with conventional RA therapies might be more effective to treat patients with this painful and debilitating arthritic disease.

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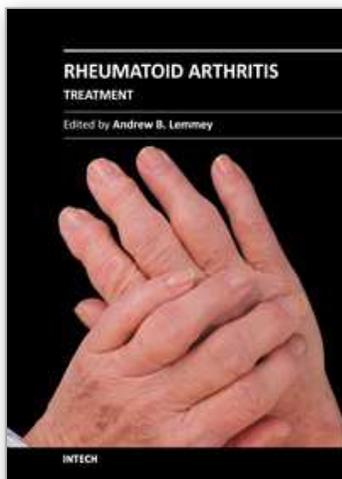
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The purpose of this book is to provide up-to-date, interesting, and thought-provoking perspectives on various aspects of research into current and potential treatments for rheumatoid arthritis (RA). This book features 17 chapters, with contributions from numerous countries (e.g. UK, USA, Canada, Japan, Sweden, Turkey, Bosnia and Herzegovina, Slovakia), including chapters from internationally recognized leaders in rheumatology research. It is anticipated that Rheumatoid Arthritis - Treatment will provide both a useful reference and source of potential areas of investigation for research scientists working in the field of RA and other inflammatory arthropathies.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
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Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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