Bortezomib attenuates murine collagen-induced arthritis

S-W Lee, J-H Kim, Y-B Park, S-K Lee

ABSTRACT
Objectives: Nuclear factor kappa B (NF-κB) is a major regulator of pivotal proinflammatory cytokines in the pathogenesis of rheumatoid arthritis (RA). Bortezomib inhibits NF-κB activation by blocking the degradation of the NF-κB inhibitor, IκB. In this study, the efficacy of bortezomib on murine collagen-induced arthritis (CIA) was investigated.

Methods: Thirty-five male DBA/1 mice were divided into five groups. All mice except controls were injected with type II collagen. Mice in the bortezomib-treated groups were injected intraperitoneally with 0.01, 0.1 and 1 mg/kg bortezomib twice a week for 2 weeks. Controls and mice in the untreated group were also injected intraperitoneally with phosphate-buffered saline in the same manner. Arthritis score and paw thickness were measured and histopathological assessment of joint sections was performed. The expression of proinflammatory cytokines and enzymes was evaluated by immunohistochemical staining. Joint destruction was confirmed using three-dimensional micro-computed tomography (CT). Blood cells were counted and liver and kidney functions were monitored.

Results: Bortezomib significantly attenuated the severity of arthritis and histopathological findings in CIA mice. The expression of tumour necrosis factor alpha, IL-1β, IL-6, matrix metalloproteinase 3, cyclooxygenase 2 and inducible nitric oxide synthase decreased in bortezomib-treated mice compared with untreated mice. In addition, micro-CT confirmed that bortezomib reduced joint destruction. No adverse effects in blood cells, liver or kidneys were observed with bortezomib treatment.

Conclusions: These data suggest that bortezomib may play an anti-inflammatory role in the pathophysiology of RA and serve as a new therapeutic modality for RA.

A proteasome functions to induce ubiquitin-mediated proteolysis of intracellular apoptosis-regulatory proteins. Bortezomib was the first proteasome inhibitor to enter clinical trials in cancer patients. Bortezomib induces apoptosis, attenuates drug resistance in multiple myeloma cells, and alters the expression of cytokines, cell adhesion proteins and angiogenesis. Furthermore, bortezomib inhibits nuclear factor kappa B (NF-κB) activation by blocking the degradation of the NF-κB inhibitor (IκB) and causes upregulation of proapoptotic regulators as well as downregulation of anti-apoptotic proteins.

Rheumatoid arthritis (RA) is a chronic inflammatory disorder affecting multiple synovial joints. Inflammation is mainly driven by the overproduction of pivotal proinflammatory cytokines that are important in the pathophysiology of RA: tumour necrosis factor alpha (TNFα), IL-1β and IL-6. These cytokines mediate long-term cartilage degradation and bone erosion, resulting in pain and joint dysfunction. NF-κB is a major regulator of the pivotal proinflammatory cytokines TNFα and IL-1β in the pathophysiology of RA. The inactivation of NF-κB is thus important to reduce inflammation in RA, suggesting that bortezomib might act as a negative regulator in inflamed joints.

To address these issues, we investigated the effect of bortezomib on the macro and microscopic severity of arthritis and the expression of TNFα, IL-1β, IL-6, matrix metalloproteinase 3 (MMP-3), cyclooxygenase 2 (COX-2) and inducible nitric oxide synthase (iNOS) in inflamed joints of mice with collagen-induced arthritis (CIA). We also assessed the extent of joint destruction using three-dimensional micro-computed tomography (CT).

MATERIALS AND METHODS
Induction of CIA
All animals were treated in accordance with the guidelines and regulations for the use and care of animals of Yonsei University, Seoul, Korea. Thirty-five male DBA/1 mice at 8 weeks of age (SLC, Shizoka, Japan) were evenly divided into five groups (group 1, controls; group 2, untreated; group 3, 0.01 mg/kg bortezomib-treated; group 4, 0.1 mg/kg bortezomib-treated; group 5, 1 mg/kg bortezomib-treated). All mice except controls were given an intradermal injection of 100 μg bovine type II collagen emulsified in complete Freund’s adjuvant (Arthrogen-CIA, Redmond, Washington, USA) (1 : 1, w/v) to the base of the tail. Two weeks later, the mice were given a booster intradermal injection of 100 μg bovine type II collagen in incomplete Freund’s adjuvant (DIFCO, Detroit, Michigan, USA) (1 : 1, v/v). The control mice were treated with Freund’s adjuvant without bovine type II collagen.

Treatment protocol for CIA
Treatment with bortezomib (Velcade; Millennium Pharmaceuticals, Cambridge, Massachusetts, USA) began 4 weeks after primary immunisation (following the full development of arthritis). Doses of 0.01, 0.1 and 1 mg/kg bortezomib were injected intraperitoneally once a week for the first 2 weeks and observed for 3 more weeks. Controls and untreated mice were injected intraperitoneally with the same volume of phosphate-buffered saline twice a week during the bortezomib treatment period.

Assessment of arthritis severity
Mice were observed twice a week for 65 days after primary immunisation. Arthritis severity was...
evaluated by visual inspection. All four legs of the mice were evaluated and scored from 0 to 4 according to the following scale: 0, no signs of arthritis; 1, swelling and/or redness of the paw or one digit; 2, two joints involved; 3, more than two joints involved; and 4, severe arthritis of the entire paw and all digits.\(^3\)

Paw thickness was measured with a Vernier caliper. Arthritis scoring and paw thickness measurement were performed by two independent observers.

### Histopathological and immunohistochemical examination

Mice were anaesthetised and killed on day 65, and paws and knee joints were removed for histopathological examination after routine fixation, decalcification and paraffin embedding of tissue. Tissue sections were prepared and stained with haematoxylin and eosin. Sections were sequentially incubated with specific antibodies directed against murine TNF\(\alpha\) (Hycult Biotec, Uden, The Netherlands), IL-1\(\beta\), IL-6, MMP-3, iNOS, or COX-2 (Santa Cruz Biotec, Santa Cruz, California, USA) followed by the appropriate secondary antibodies (ISU Abxis, Seoul, Korea). All tissue samples were counterstained with haematoxylin.

After immunohistochemical staining, expression of the different markers in the synovial tissue of paw and knee joints was scored semiquantitatively on a four-point scale independently and blindly by two individual pathologists, and the average of their scores was calculated. A score of 0 represented minimal expression, 1 represented mild expression and 2 represented moderated expression, whereas 3 represented abundant expression of a marker. Minor differences between observers were resolved by mutual agreement.

### Micro-CT imaging

We conducted an additional experiment using 35 CIA mice for micro-CT imaging (controls, untreated CIA mice and CIA mice treated with 0.01, 0.1 and 1 mg/kg bortezomib) according to the same method mentioned above. Mice were observed twice a week for 65 days after primary collagen injection, killed, and their legs were excised and fixed in 4% formalin for 2 days. The paws (from the tip of the toes to the end of the distal phalanx) obtained from experimental mice were scanned and reconstructed into a three-dimensional structure with micro-CT (SkyScan 1076; SkyScan, Antwerp, Belgium) with a voxel size of 18 \(\mu\)m. The x-ray tube voltage was 60 kV and the current was 170 \(\mu\)A, with a 0.5 mm thickness of aluminum filter. Exposure time was 1180 ms. The x-ray projections were obtained at 0.5\(^\circ\) intervals with a scanning angular rotation of 360\(^\circ\). The reconstructed dataset was segmented by an automated thresholding algorithm.\(^7\) The projection images were reconstructed into three-dimensional images using NRECON software (version 1.5.1) and CT-Analyzer (version 1.7), both from SkyScan. The parameters measured and calculated were as follows: (1) bone surface area (BS) was calculated by the marching cubes method to triangulate the surface of the bone;\(^10\) (2) bone volume (BV) was calculated using polyhedrons corresponding to the enclosed volume of the triangulated surface;\(^11\) (3) total tissue volume (TV), the volume of the whole examined sample and the normalised index was measured and bone volume fraction (BV/TV) enabled comparisons of samples of different sizes; (4) specific bone surface to volume ratio was given by bone surface density (BS/BV); and (5) mean thickness of the trabeculae, the trabecular thickness (Tb.Th), was obtained by filling maximal spheres in the structure with the distance transformation,\(^12\) and then the average thickness of all bone voxels was calculated to give the Tb.Th.

### Toxicity of bortezomib in CIA mice

We also used 21 CIA mice for investigation of the toxicity of bortezomib (controls and CIA mice treated with 0.1 and 1 mg/kg bortezomib) according to the same method mentioned above. We obtained blood from controls, 0.1 and 1 mg/kg bortezomib-treated mice after killing and examined the white blood cell count, haemoglobin, platelet count and levels of aspartate aminotransferase, alanine aminotransferase, blood urea nitrogen and creatinine for liver and kidney toxicity.

### Statistical analysis

All statistical analyses were conducted using SPSS package for Windows, version 11.5. The representative values were the means of those obtained from each mouse in each group, and all values in the experimental groups were compared with controls and untreated CIA mice. All results and measurements are expressed as the mean (SD). Statistical comparisons of arthritis score, paw thickness, semiquantitative histopathological and immunohistochemical examination and laboratory findings between the two groups were evaluated by the Mann–Whitney U test, and parameters in micro-CT imaging were compared using the t-test. Correlations between parameters measured by micro-CT were calculated using Spearman’s correlation coefficient. When we compared the values between the untreated and each treated group, we gave an asterisk (*) to the mean value of each treated group that had statistical significance (p<0.05).

### RESULTS

#### Bortezomib decreases severity of arthritis in CIA mice

Macroscopic evidence of arthritis such as erythema or swelling was markedly observed in untreated mice. Doses of 0.1 and 1 mg/kg bortezomib significantly attenuated arthritis severity in CIA mice. In a dose-dependent manner, both mean arthritis score and paw thickness in bortezomib-treated mice were significantly lower than in untreated mice (fig 1A, B). The dose of 0.01 mg/kg bortezomib also decreased the severity of arthritis significantly, but the improvement of arthritis was not remarkable and it was shown in a relatively late treatment phase in comparison with doses of 0.1 and 1 mg/kg bortezomib.

#### Bortezomib improves histopathological findings in inflamed joints of CIA mice

Histopathological evaluation of paws and knee joint sections of untreated mice revealed inflammatory cell infiltration, synovial hyperplasia and partial bone destruction (pannus). In contrast, in CIA mice treated with 0.1 and 1 mg/kg bortezomib, the extent of inflammatory cell infiltration and bone destruction was significantly reduced (fig 2A). However, somewhat inflammatory features were still observed in 0.01 mg/kg bortezomib-treated mice. The semiquantitative analysis of histopathological features including infiltration and bone destruction scores is shown in fig 2B. A dose-dependent tendency was slightly observed but there were no significant differences between mice treated with 0.1 and 1 mg/kg bortezomib.

#### Bortezomib reduces expression of TNF\(\alpha\), IL-1\(\beta\), IL-6, MMP-3, iNOS and COX-2 in inflamed joints of CIA mice

Immunohistochemical analysis of paws and knee joint tissue obtained from untreated mice exhibited markedly positive staining for TNF\(\alpha\), IL-1\(\beta\), IL-6 and MMP-3, which were localised primarily in inflamed cells around the joints. In contrast, few significant positive staining reactions for TNF\(\alpha\), IL-1\(\beta\), IL-6 or IL-6.
MMP-3 were observed in 0.1 and 1 mg/kg bortezomib-treated CIA mice, a finding that was similar in controls. Similarly, staining for iNOS and COX-2 were significantly positive in untreated and 0.01 mg/kg bortezomib-treated mice but were unlikely in 0.1 and 1 mg/kg bortezomib-treated mice (fig 2A). Semiquantitative analysis by two independent pathologists showed that the degree of stain positivity in untreated and 0.01 mg/kg bortezomib-treated mice was significantly higher than in 0.1 and 1 mg/kg bortezomib-treated mice. However, there were no significant differences between the two well-treated groups (fig 2B). In this study, we used anti-murine cytokines, enzymes and rabbit or goat antibodies as primary antibodies. In order to evaluate isotype-irrelevant primary antibodies, we treated normal rabbit or goat sera containing various non-specific antibodies and then added secondary antibodies. We could not find positive immunohistochemical staining in the joint sections.

Micro-CT scan proved efficiency of bortezomib in CIA mice

Figure 3A shows the three-dimensionally reconstructed bones of paws. Metatarsophalangeal joints were coloured red to intensify differences in bone destruction. Severe bone erosions were observed in untreated CIA mice. Although the extent of joint destruction between 0.1 and 1 mg/kg bortezomib-treated CIA mice showed subtle differences, when compared with untreated

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**Figure 1**  Severity of arthritis in mice with collagen-induced arthritis. Doses of 0.1 and 1 mg/kg bortezomib significantly decreased the mean arthritis score and paw thickness in a dose-dependent manner compared with untreated mice (A, B). The dose of 0.01 mg/kg bortezomib also decreased the severity of arthritis significantly, but the improvement of arthritis was not remarkable and it was shown in a relatively late treatment phase in comparison with doses of 0.1 and 1 mg/kg bortezomib. The bars represent the standard deviation. When we compared the values between the untreated and each treated group, we gave an asterisk (*) to the mean value of each treated group that had statistical significance (p<0.05).
mice, they showed markedly less joint destruction. CIA mice treated with 0.01 mg/kg bortezomib exhibited no improvement in comparison with untreated mice.

We analysed the four parameters of BV, BV/TV, BS/BV and Tb.Th and found that they were well correlated with one another significantly (fig 3B). BV, BV/TV and Tb.Th in 0.1 and 1 mg/kg bortezomib-treated CIA mice were significantly higher than in untreated or 0.01 mg/kg bortezomib-treated mice (fig 3C, table 1). In addition, BS/BV in 0.1 and 1 mg/kg bortezomib-treated CIA mice was significantly lower than in untreated mice (fig 3C, table 1), suggesting a higher volume and quality of preserved trabecular bone despite joint inflammation. However, CIA mice treated with 0.01 mg/kg bortezomib did not show any significant differences in parameters compared with untreated CIA mice. There were also no differences in parameters between 0.1 and 1 mg/kg bortezomib-treated mice (fig 3C).

### Toxicity of bortezomib in CIA mice

There were no differences in white blood cell count, haemoglobin and platelet count among controls and 0.1 and 1 mg/kg bortezomib-treated CIA mice. Liver and kidney toxicities were not observed in mice treated with 0.1 and 1 mg/kg bortezomib (table 2).

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**Figure 2.** Histopathological findings and immunohistochemical staining for tumour necrosis factor alpha (TNF-α), IL-1β, IL-6, matrix metalloproteinase 3 (MMP-3), inducible nitric oxide synthase (iNOS) and cyclooxygenase 2 (COX-2) in mice with collagen-induced arthritis (CIA). Histopathological evaluation revealed severe inflammation in the joint sections of untreated CIA mice. In contrast, the extent of arthritis and bone destruction was significantly reduced in the joints of mice treated with 0.1 and 1 mg/kg bortezomib (original magnification ×100, haematoxylin and eosin).

Immunohistochemical analysis of paws and knee joint tissue obtained from untreated CIA mice showed markedly positive staining for TNF-α, IL-1β, IL-6 and MMP-3, localised primarily in the inflamed cells around the joints. In contrast, few significant positive staining reactions for TNF-α, IL-1β, IL-6 or MMP-3 were found in 0.1 and 1 mg/kg bortezomib-treated CIA mice as with controls. Staining for iNOS and COX-2 showed a similar pattern of positivity in experimental mice (original magnification ×100). However, 0.01 mg/kg bortezomib did not improve arthritis (A). Semiquantitative analysis by two independent pathologists was performed and showed that 0.1 and 1 mg/kg bortezomib significantly reduced inflammation in the joints (B). The bars represent the standard deviation. When we compared the values between the untreated and each treated group, we gave an asterisk (*) to the mean value of each treated group that had statistical significance (p<0.05).
DISCUSSION
In this study, we found that bortezomib attenuated the severity of arthritis in CIA mice. The mean arthritis score as well as mean paw thickness in 0.1 and 1 mg/kg bortezomib-treated CIA mice were significantly lower than in untreated mice. More importantly, mice treated with 1 mg/kg bortezomib showed rapid remedial values compared with mice treated with 0.01 or 0.1 mg/kg bortezomib at 4–7 days in the early stage of the treatment period.

In addition, 0.1 and 1 mg/kg bortezomib significantly improved histological findings by decreasing the expression of proinflammatory cytokines and inflammation-associated enzymes in joint tissues of CIA mice compared with untreated mice. The mechanism by which bortezomib regulates the

**Figure 3** Micro-computerised tomography scan proved efficiency of bortezomib in mice with collagen-induced arthritis (CIA). Severe bone erosions were observed in untreated CIA mice but 0.1 and 1 mg/kg bortezomib-treated CIA mice showed markedly less joint destruction. CIA mice treated with 0.01 mg/kg bortezomib exhibited no improvement in comparison with untreated mice (A). Bone volume (BV), bone volume fraction (BV/TV), bone surface density (BS/BV) and trabecular thickness (Tb.Th) correlated well with one another significantly (B). BV, BV/TV and Tb.Th in 0.1 and 1 mg/kg bortezomib-treated CIA mice were significantly higher than in untreated or 0.01 mg/kg bortezomib-treated mice. In addition, BS/BV in 0.1 and 1 mg/kg bortezomib-treated CIA mice was significantly lower than in untreated mice, suggesting a higher volume and quality of preserved trabecular bone despite joint inflammation. However, CIA mice treated with 0.01 mg/kg bortezomib did not show any significant differences in parameters compared with untreated CIA mice (C). When we compared the values between the untreated and each treated group, we gave an asterisk (*) to the mean value of each treated group that had statistical significance (p<0.05).

**Table 1** Comparison among the parameters of micro CT scan

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Untreated</th>
<th>0.01 mg/kg Bortezomib</th>
<th>0.1 mg/kg Bortezomib</th>
<th>1 mg/kg Bortezomib</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>BV (mm³)</td>
<td>11.2 (0.8)</td>
<td>8.9 (1.1)</td>
<td>9.5 (0.4)</td>
<td>9.9 (0.6)</td>
<td>10.7 (0.9)</td>
<td>0.007</td>
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<tr>
<td>BV/TV (%)</td>
<td>3.7 (0.3)</td>
<td>3.0 (0.3)</td>
<td>3.1 (0.1)</td>
<td>3.3 (0.2)</td>
<td>3.6 (0.3)</td>
<td>0.138</td>
</tr>
<tr>
<td>BS/BV (mm⁻¹)</td>
<td>18.2 (1.3)</td>
<td>22.0 (2.4)</td>
<td>20.9 (0.7)</td>
<td>19.7 (1.4)</td>
<td>18.2 (1.6)</td>
<td>0.013</td>
</tr>
<tr>
<td>Tb.Th (mm)</td>
<td>0.066 (0.003)</td>
<td>0.058 (0.003)</td>
<td>0.059 (0.003)</td>
<td>0.062 (0.004)</td>
<td>0.065 (0.002)</td>
<td>0.005</td>
</tr>
</tbody>
</table>

All results and measurements are expressed as the mean (SD).

BS, bone surface area; BV, bone volume; CT, computerised tomography; Tb.Th, trabecular thickness; TV, tissue volume.
activity of NF-κB is well known; bortezomib inhibits activation by blocking the ubiquitin-mediated degradation of IκB by the proteasome, resulting in an increase in the number of NF-κB and IκB complexes that cannot enter the nucleus. Products of certain genes that are regulated by NF-κB also cause activation of NF-κB; both TNFα and IL-1β activate and are activated by NF-κB. This positive regulatory loop may amplify and perpetuate local inflammatory responses, and interruption of this cycle is the key to reducing inflammation in RA. 

Furthermore, NF-κB is a transcription factor that promotes the expression of various proinflammatory cytokines or enzymes, such as IL-6, iNOS and COX-2. It could thus be reasonably concluded that bortezomib decreases the expression of TNFα, IL-1β, IL-6, MMP-3, iNOS and COX-2 by reducing NF-κB DNA-binding activity, and that bortezomib could negatively regulate inflammation in inflamed joints of CIA mice.

These results were also confirmed by three-dimensional micro-CT. The common alterations of the bone in the affected joints in RA are bone erosion and periarticular osteopenia. We thus used four parameters of three-dimensional micro-CT to investigate these alterations quantitatively. Despite the range of the measurement of bone already being set as, there might be subtle differences in the size of the samples, BV representing total bone volume has a limitation. We thus measured not only BV, but also TV, which enables comparisons of bone samples of different sizes. These parameters reflected the loss of bone in the affected joints. BS/BV was also used to reflect the loss of bone surface due to erosion. Tb.Th was calculated to represent the extent of periarticular osteopenia induced by joint inflammation. In order to clarify the validity of the parameters measured or calculated by micro-CT, we evaluated the correlation coefficients among four parameters, BV, TV, BS/BV and Tb.Th, which were obtained from all CIA mice including control, untreated and bortezomib-treated groups, and found that they correlated well with one another with significance. Doses of 0.1 and 1 mg/kg bortezomib significantly preserved both bone density and Tb.Th. However, 0.01 mg/kg bortezomib did not improve bone destruction. From these data, we could determine a critical concentration of bortezomib, 0.1 mg/kg, to treat arthritis in CIA mice.

The adverse effects of bortezomib have recently been reported in various refractory cancers, such as multiple myeloma. Thrombocytopenia, anaemia, neutropenia and peripheral neuropathy have also been shown to be frequent and serious adverse effects of bortezomib. The mean platelet count usually exhibits a great decrease on day 11 from the first injection (the fourth injection of bortezomib in a cycle), followed by recovery to the initial count. As we did not collect serial blood samples, we could not describe the alterations in platelet count during the treatment period. However, there were no significant differences in the mean platelet count between controls and bortezomib-treated mice. Furthermore, no anaemia, leucocytopenia, or deterioration in liver and kidney function were observed in bortezomib-treated mice.

This study has a potential limitation in that bortezomib was administered only for a period of time, to those with chronic diseases. To clarify these issues, other experiments will be needed to identify the cycle-dependent efficacy and serious adverse effects of bortezomib in CIA mice.

In conclusion, bortezomib significantly attenuated the severity of arthritis and improved histological findings in CIA mice. Bortezomib decreased the expression of TNFα, IL-1β, IL-6, MMP-3, iNOS and COX-2 in inflamed joints. In addition, micro-CT confirmed that bortezomib reduced joint destruction and preserved bone density. These data suggest that bortezomib may play an anti-inflammatory role in the pathophysiology of RA and could serve as a new and additive therapeutic modality for RA.

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