Proteasome Inhibition Up-regulates p53 and Apoptosis-Inducing Factor in Chondrocytes Causing Severe Growth Retardation in Mice

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Abstract

Proteasome inhibitors (PI), a novel class of anticancer drugs, are relatively well tolerated and have recently been introduced into the clinic for the treatment of multiple myeloma. The tumor selectivity and low toxicity of PIs are surprising, given the crucial role of the ubiquitin/proteasome system in a multitude of cellular processes. Here, we show that systemic administration of PIs specifically impairs the ubiquitin/proteasome system in growth plate chondrocytes. Importantly, young mice displayed severe growth retardation during treatment as well as 45 days after the cessation of treatment with clinically relevant amounts of MG262 (0.2 μmol/kg body weight/injection) or bortezomib (1.0 mg/kg body weight/injection). Dysfunction of the ubiquitin/proteasome system was accompanied by the induction of apoptosis of stem-like and proliferative chondrocytes in the growth plate. These results were recapitulated in cultured fetal rat metatarsal bones and chondrocytic cell lines (rat, human). Apoptosis was associated with up-regulation of the proapoptotic molecules, p53 and apoptosis-inducing factor (AIF), both in vitro and in vivo. In addition to the observation that AIF is expressed in the growth plate, we also provide evidence that AIF serves as a direct target protein for ubiquitin, thus explaining its prominent up-regulation upon proteasome inhibition. Suppression of p53 or AIF expression with small interfering RNAs partly rescued chondrocytes from proteasome inhibition–induced apoptosis (35% and 41%, respectively). Our observations show that proteasome inhibition may selectively target essential cell populations in the growth plate causing significant growth failure. These findings could have important implications for the use of proteasome inhibitors in the treatment of childhood cancer. [Cancer Res 2007;67(20):10078–86]

Introduction

Longitudinal bone growth takes place in the growth plate, a thin layer of cartilage localized at each end of the long bones. The growth plate is mainly composed of chondrocytes and could be morphologically and functionally divided into three different zones: resting, proliferative, and hypertrophic. Resting zone chondrocytes, also known as stem-like chondrocytes (1, 2), are stimulated to proliferate and undergo hypertrophy, a process which involves several stages of maturation (3). Disturbed chondrocyte activity, either due to disruption in normal cell division or excessive cell death, may lead to defective longitudinal bone growth. Survivors of childhood cancer often have severe long-term complications secondary to previously administered life-saving treatment, emphasizing the importance of minimizing these undesired effects.

The proteasome is a large proteolytic complex that resides in the nucleus and cytosol of all eukaryotic cells. The selectivity of the system is safeguarded by the small protein modifier ubiquitin, which is covalently conjugated to proteins destined for proteasomal degradation. The proteasome preferentially binds and degrades ubiquitinated proteins (4). The critical involvement of the ubiquitin/proteasome system in the regulation of a number of cellular processes, as well as protein quality control, suggests that compounds or conditions that interfere with this process would be inherently toxic to eukaryotic cells. In vitro experiments confirm this notion as cells undergo apoptosis when cultured in the presence of proteasome inhibitors, making these agents attractive candidates for cancer therapy (5). Presently, there are five major classes of specific proteasome inhibitors: peptide aldehydes, peptide vinyl sulfones, peptide boronates, peptide epoxyketones, and β-lactones. Only recently, bortezomib (also known as PS341 or Velcade), a boronate proteasome inhibitor, was approved as a third-line agent in the treatment of multiple myeloma because of its profound antitumor effect (6), which was also documented in a clinical phase 1 trial in children (7). The most prevalent side effects of proteasome inhibitors, thrombocytopenia and peripheral neuropathy, are only observed in a minority of treated patients (8). However, there is no previous information available on any specific side effects of proteasome inhibitors in young, rapidly growing individuals. The molecular mechanism for proteasome inhibitor–induced cell death of cancer cells remains enigmatic and it is therefore difficult to anticipate which somatic cells may be at risk during treatment with proteasome inhibitors. To address this, the effects of two highly selective boronate proteasome inhibitors, MG262 (9) and bortezomib (7, 8), were analyzed in young mice. A transgenic reporter mouse model for the ubiquitin/proteasome system was used to monitor the in vivo proteasome inhibition. Our studies revealed functional impairment of ubiquitin-dependent proteasomal degradation in growth plate chondrocytes. Using these transgenic mice, as well as cell lines and metatarsal organ cultures as model systems, evidence for the role of caspase-dependent and -independent induction of apoptosis in chondrocytes upon proteasome inhibition has been observed. Importantly, severe growth retardation was seen in mice treated with the
Figure 1. Systemic administration of a proteasome inhibitor causes functional impairment of the ubiquitin/proteasome system in chondrocytes and bone marrow.

A. Diagram detailing the injection scheme administered to the animals. B–D, functional impairment of the ubiquitin/proteasome system in chondrocytes and bone marrow. GFP micrographs of bone sections from UbG76V-GFP/2 mice that were injected with vehicle or MG262; see Materials and Methods for details. Overview micrograph of the proximal end of the tibia and representative micrographs of (B) growth plate cartilage (tibia; resting (R), proliferative (P), and hypertrophic (H) zones, respectively), (C) articular cartilage (tibia), and (D) bone marrow of a vehicle or MG262-injected mouse. Bars, 50 μm.
proteasome inhibitors MG262 and bortezomib. These novel findings may have considerable implications for the clinical use of proteasome inhibitors in patients with cancer.

Materials and Methods

Reagents. Proteasome inhibitors, MG262 (Biomol International, SMS-gruppen) and lactacystin, fluorochrome calcein, and p53 inhibitor (pifithrin-α) were from Sigma-Aldrich, and pan-caspase inhibitor z-VAD-fmk was from R&D Systems. Bortezomib (Velcade) from Janssen-Cilag AB, was dissolved in saline. Trypsin, PBS, EDTA, fetal bovine serum, MEMs and DMEM/F12 medium were from Invitrogen.

Animal studies. All animal studies were approved by the local ethical committee. Five-week-old male B6 mice were given four injections of MG262 (0.2 μmol/kg body weight/injection) in 10 days, and then killed after 48 h or 45 days (Fig. 1A). The tibia and femur were excised and measured with a digital caliper, and growth velocity was determined as previously described (ref. 10; Fig. 1B). In a separate experiment, 5-week-old male ubiquitinG76V-green fluorescent protein (UbG76V-GFP)/2 transgenic mice (see ref. 11 for details) were given three injections of MG262 (0.2 μmol/kg body weight/injection) in 5 days, and were killed 24 h after the last injection and analyzed for accumulation of GFP reporter in different tissues. For the long-term effects, two more strains of 5-week-old male mice, C57B and NMRI, were treated with bortezomib (1.0 mg/kg body weight, two injections per week, 2 weeks treatment; ref. 12) and tibia length was measured at sacrifice, 45 days after the last injection. Serum levels of insulin-like growth factor 1 were measured using a commercial RIA kit (Mediadiagnost). Proteasome activity in blood was assessed as described previously (13).

Quantitative histology of the growth plate. Histomorphometry was done as described previously by us (14). In each tissue sample, 15 measurements of growth plate height were taken. Column density was determined as the number of chondrocyte columns per millimeter of growth plate. All measurements were done by a person blinded to the experimental details.

Organ cultures. Fetal rat metatarsal bones were cultured and monitored as described previously (15).

Cell cultures. The human chondrocytic cell line, HCS-2/8 (16) was cultured as recently described by us (17). The RCJ3.1C5.18 rat chondrogenic cell line (C5.18) was maintained in MEMs medium and supplemented with ascorbic acid and β-glycerophosphate as described previously (18, 19). After 4 to 7 days of culture, the cells acquire markers of early chondrocytic differentiation and progressively acquire markers of terminal differentiation at 7 to 10 and 10 to 14 days of culture (19, 20).

Alcian blue staining. Nodule formation, which is a chondrocyte differentiation marker, in C5.18 chondrocytes (early differentiated and differentiated/tate differentiated cells), was confirmed with 5% Alcian blue staining as described previously (ref. 21; Supporting Fig. S7).

Cell death detection ELISA and caspase-3 activity. Apoptosis was studied with the detection and quantification of cytoplasmic histone-associated DNA fragments (17) and caspase-3 activity was determined as described previously (22).

Western blot analysis. Western immunoblotting was done as described previously by us (17). The primary antibodies against p53 (1:1,000), DM2 (1:1,000), Bax (1:1,000), PARP (1:1,000), and apoptosis associated factor (AIF; 1:1,500), ubiquitin (P4D1; 1:1,000) were from Santa Cruz Biotechnology. For immunoprecipitation, cells were lysed in a radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Roche Diagnostics GmbH) and 1 mmol/L of phenylmethylsulfonyl fluoride. Supernatants were incubated with AIF antibody at 4°C for 2 h followed by the addition of protein G-Sepharose CL–4B (Amersham Bioscience). After overnight incubation, the resulting immunocomplexes were subjected to SDS-PAGE.

In situ apoptosis and cell proliferation. Apoptotic cells were detected by terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling and cell proliferation was assessed as described previously (23).

Immunohistochemistry. Antigen retrieval was done in citrate buffer (pH 6.0) containing 0.01% Tween 20 for 15 min at 94°C to 96°C. The sections were incubated with 10% normal serum for 1 h, primary antibody at 4°C overnight, secondary antibodies conjugated with immunofluorescent dyes (Alexa 488 and Alexa 546; Molecular Probes, Inc.) for 1 h at room temperature and finally counterstained with 4',6-diamidino-2-phenylindole for 15 min. Immunostaining with PC10 primary antibody (1:1,600) against proliferating cell nuclear antigens (Abcam) was done to study chondrocyte proliferation.

Small interfering RNAs. C5.18 cells (3 × 106 cells in 96-well plates or 5 × 105 cells in six-well plates) were transfected with a small interfering RNA (siRNA) double-stranded oligonucleotide designed (QiAGEN) to interfere with the expression of rat p53 sense, r(GCU ACU CAU UUU CCC UCA A) dTdT; antisense, r(UUG AGG GAA AUU GAG UAC G) dTdT; and to AIF, sense, r(GGG UAA AUG CAG AGC UUC A) dTdT; antisense, r(UGA AGC UCU GCA UUU ACC C) dGGd. Scrambled siRNA was used as negative controls.

Statistical analysis. Results are presented as mean values ± SE. Differences between the groups were tested by one-way ANOVA followed by the Newman-Keuls post-test.

Results

Systemic administration of a proteasome inhibitor causes functional impairment of the ubiquitin/proteasome system in chondrocytes. First, we evaluated the in vivo effect of proteasome inhibition using a reporter mouse model for the ubiquitin/proteasome system (11). These mice express UbG76V-GFP, which is constitutively targeted for ubiquitin-dependent proteasomal degradation by the presence of an ubiquitin fusion degradation signal. Tissues from these mice thus display low GFP fluorescence unless the cells, as a consequence of functional impairment of the ubiquitin/proteasome system, fail to degrade the UbG76V-GFP proteasome substrate.

Five-week-old male UbG76V-GFP/2 mice received i.p. injections (Fig. 1A) of 0.2 μmol MG262/kg body weight on days 1, 3, and 5 and were killed 24 h after the final injection. After screening multiple tissues, we observed the accumulation of the UbG76V-GFP proteasome substrate in the femur and tibia growth plate cartilage (Fig. 1). The strongest GFP accumulation was detected in resting zone chondrocytes in both the tibia (Fig. 1B) and femur (Supporting Fig. S1A) growth plate cartilage. A similar response was seen in articular cartilage from both the tibia (Fig. 1C) and femur (Supporting Fig. S2A). In addition, we found some increase in the reporter levels in bone marrow of MG262-treated mice (Fig. 1D).

We have previously shown that administration of a single high dose of MG262 (1 μmol/kg body weight) triggered a very strong accumulation of the GFP reporter in the liver and pancreas (11). However, we did not detect increased levels of UbG76V-GFP in the liver and pancreas of mice that had been given three low-dose injections of MG262 (0.2 μmol/kg body weight/injection), which suggests that chronic exposure affects the tissues differentially (Supporting Fig. S2). Whereas other conventional chemotherapeutics often have a general effect on proliferative tissues, we did not detect functional impairment of the ubiquitin/proteasome system in the small intestine and testis, two tissues that are known to have high indices of proliferation (Supporting Fig. S2A). Thus, MG262 displays an atypical tissue targeting primarily affecting selective populations of cells located in the growth plate, articular cartilage, and bone marrow.

Proteasome inhibition causes severe and sustained growth retardation in young mice. Our data in transgenic mice showing that MG262 causes selective accumulation of the GFP reporter in stem-like growth plate chondrocytes suggested that proteasome inhibitors might affect bone growth. To address this, mice were treated with multiple injections of MG262. We found that mice
treated with MG262 had significantly shorter femurs and tibias (Fig. 2A) and a 20% to 25% decrease in growth velocity when assessed over the 10-day period of treatment (Fig. 2C). Proteasome activity was also significantly decreased in whole blood lysates, confirming systemic proteasome inhibition (data not shown). Moreover, MG262 significantly increased apoptosis in stem-like and proliferative zone chondrocytes ($P < 0.001$; Fig. 3B) and decreased proliferating cell nuclear antigen expression throughout the growth plate ($P < 0.05$; Supporting Fig. S3). Quantitative histologic analysis of the growth plate revealed a decrease in growth plate height (Fig. 3C) and total number of chondrocyte columns in MG262-treated mice (Fig. 3D).

During treatment with MG262, food intake (3.9 ± 0.2 versus 4.2 ± 0.2 g/d in vehicle) and body weight (21.64 ± 2.1 versus 23.01 ± 2.7 g/animal in vehicle) were not significantly affected. Also, 45 days after the treatment with MG262 was terminated, food intake (3.7 ± 0.3 versus 3.7 ± 0.1 g/d in vehicle) and body weight (21.72 ± 1.2 versus 22.9 ± 1.1 g/animal in vehicle) were unaffected, indicating that the MG262-induced growth retardation was not secondary to metabolic effects or weight loss. Serum insulin-like growth factor I was not affected when assessed 2 days (232 ± 16 versus 246 ± 14 ng/mL in vehicle) or 45 days (215.4 ± 9.2 versus 213.6 ± 5.2 ng/mL in vehicle) after the last injection of MG262, further supporting the notion that the effect of proteasome inhibition on bone growth is not likely to be systemic but rather the result of direct effects on the growth plate.

Strikingly, even 45 days after the last injection of MG262, the bones of previously treated mice were still significantly shorter (Fig. 2B) and displayed reduced growth velocity (Fig. 2D) compared with vehicle-treated mice, further supporting the notion that the growth dysfunction persisted over time. To exclude that the long-term negative effect on bone growth is compound and/or mouse strain–specific, we treated both C57B and NMRI mice with bortezomib. The control group was pair-fed as bortezomib might affect food intake. Indeed, bortezomib in a clinically relevant concentration (1.0 mg/kg body weight, two injections per week, 2 weeks treatment) caused long-term growth failure (tibia length measured by digital caliper at sacrifice 45 days after last injection) in both C57B (17.38 ± 0.18 versus 18.66 ± 0.38 mm in vehicle; $P < 0.001$; $n = 10$) and NMRI (17.51 ± 0.06 versus 18.53 ± 0.09 mm in vehicle; $P < 0.001$; $n = 9$) mice. The fact that proteasome inhibition caused long-lasting negative effects on bone growth suggests irreversible damage to cells in the growth plate rather than transient cellular dysfunction.

**Inhibition of proteasome triggers apoptosis of stem-like and proliferative chondrocytes thereby causing severe growth retardation.** To further verify the direct effects of proteasome inhibition on longitudinal bone growth, we cultured fetal rat metatarsal bones and challenged them with different concentrations of the proteasome inhibitors, MG262 or lactacystin. When treated for 12 days with MG262, a dose-dependent decrease in bone growth was observed (Fig. 4A). Notably, even at the lowest concentration of MG262 (0.3 μmol/L), a significant reduction in bone growth was observed. Our *in vivo* data suggested a long-lasting negative effect of bortezomib and MG262 on bone growth. Interestingly, treatment of metatarsal bones with MG262 (1 μmol/L) for 24 h resulted in permanent growth arrest and no catch-up growth was observed when monitored up to 12 days, an effect similar to what was observed in bones continuously exposed to the proteasome inhibitor (Fig. 4A). To validate the findings observed...

![Figure 2. Proteasome inhibition causes growth retardation in young mice. Femur and tibia lengths measured (A) 48 h or (B) 45 days after a 10-day period of treatment with vehicle or MG262 ($n = 10$). Femur and tibia growth velocities (C) 48 h or (D) 45 days after the 10-day period of treatment (calcein labeling technique, see Materials and Methods; $n = 10$). ***, $P < 0.001$.](https://www.aacrjournals.org/doi/figlink/10.1158/0008-5472.CAN-06-3521)
with MG262, we also used the natural proteasome inhibitor lactacystin which also caused a dose-dependent decrease in metatarsal bone growth confirming that proteasome inhibition, indeed, directly targets bone growth (Supporting Fig. S4A). When investigating the underlying mechanisms, we found that MG262 treatment caused a dramatic increase in apoptosis in resting (81 ± 8% versus 0.5 ± 0.0% in control; P < 0.001), proliferative (54 ± 10% versus 1.7 ± 1.0% in control; P < 0.001) and hypertrophic (44 ± 1% versus 1.4 ± 0.0% in control; P < 0.001) zone chondrocytes (Supporting Fig. S5), whereas a significant reduction in bromodeoxyuridine staining was observed in resting (0.2 ± 0.0% versus 8.4 ± 1.0% in control, P < 0.001) and proliferative (0.0 ± 0.0% versus 11.9 ± 2.0% in control, P < 0.001) zone chondrocytes.

Early-differentiated chondrocytes are more sensitive to proteasome inhibition and apoptosis induction. Our detailed in vivo and in vitro studies clearly suggested that stem-like chondrocytes in the growth plate are more susceptible to undergoing apoptosis upon proteasome inhibition compared with proliferative and hypertrophic chondrocytes. To directly compare the sensitivity of early differentiated cells (comparable to stem-like chondrocytes) and late-differentiated cells (comparable to late-proliferative chondrocytes), we used the rat C5.18 chondrocytic cell line. As observed in vivo and in cultured metatarsal bones, we confirmed that MG262 induced apoptosis in C5.18 chondrocytes in a time-dependent manner (Fig. 4C and D). When compared with late-differentiated cells, early-differentiated cells were more sensitive to apoptosis when challenged with MG262 (Fig. 4C and D). The difference was most striking at the earliest time point after MG262 treatment (6 h) when there was no significant increase in apoptosis in late-differentiated cells but an ~2.5-fold increase in apoptosis in early-differentiated cells. Measurements of caspase-3 activity confirmed that proteasome inhibitors induced apoptosis, an effect which was observed earlier and was more pronounced in early-differentiated cells when compared with late-differentiated cells (Fig. 4B). MG262 also inhibited cell proliferation and stimulated chondrocyte apoptosis in human chondrocytes (Supporting Fig. S6A and B) confirming that there are no species differences related to this particular aspect of proteasome inhibitors. Taken together, these data corroborate our findings obtained in vivo and in cultured metatarsal bones, thus demonstrating that early-differentiated cells are more sensitive to proteasome inhibition.

Proteasome inhibition triggers caspase-dependent and -independent apoptosis in chondrocytes. To investigate any involvement of the caspase cascade on chondrocyte apoptosis and longitudinal bone growth, metatarsal bones were cultured with the pan-caspase inhibitor Z-VAD-fmk in combination with MG262. Notably, although Z-VAD-fmk blocked dexamethasone-induced reduction in metatarsal bone growth, this compound failed to rescue growth in bones treated with MG262 (Supporting Fig. S4B), suggesting the involvement of caspase-independent apoptosis. However, we observed a tendency towards improved growth in bones treated with the pan-caspase inhibitor, suggesting that caspase activation may contribute to the overall effects of proteasome inhibitors in this model (Supporting Fig. S4B). To further verify the activation of caspase-dependent or -independent apoptosis, we coincubated rat and human chondrocytes with Z-VAD-fmk and MG262. These experiments showed that the pan-caspase inhibitor partly prevented MG262-induced apoptosis in both early- and late-differentiated rat chondrocytes (Fig. 4C and D)
and also in human chondrocytes (Supporting Fig. S6C). Altogether, our data suggest that proteasome inhibition activates caspase-dependent and -independent apoptosis in chondrocytes.

**Genetic or pharmacologic targeting of p53 rescues chondrocytes from apoptosis induced by proteasome inhibition.** Next, we asked whether the deleterious effects of proteasome inhibition in chondrocytes could be reversed or suppressed. The tumor suppressor gene p53 plays an important role in the regulation of apoptosis and its stability is controlled by the ubiquitin/proteasome system. Therefore, we investigated whether there was any effect of proteasome inhibition on p53 in growth plate chondrocytes. Indeed, in mice treated with MG262, we found increased p53 levels throughout the entire growth plate (Fig. 5A), an effect which was verified in rat (Fig. 5B) and human (data not shown) chondrocytes. Accumulation of p53 was observed as soon as after 3 h in early-differentiated cells and after 12 h in late-differentiated cells (Fig. 5B). The p53-regulated gene products, Mdm-2 and Bax (data not shown), were also increased in parallel with p53. Suppression of p53 expression using siRNAs resulted in a 33% decrease in apoptosis in MG262-treated cells (P < 0.005 versus MG262 alone), indicating that p53 is involved in the induction of apoptosis (Fig. 5C). In further support of this conclusion, we found that the small molecule inhibitor of p53, pifithrin-α (24) also suppressed MG262-induced apoptosis (Fig. 5C).

**AIF is ubiquitinated and up-regulated through proteasome inhibition in vitro and in vivo.** We then decided to further explore the mechanism(s) underlying caspase-independent apoptosis signaling in chondrocytes. To this end, we investigated the role of AIF, a proapoptotic protein responsible for initiating caspase-independent destruction of DNA and subsequent cell death (25). We observed a strong up-regulation of AIF throughout the growth plate in MG262-treated mice (Fig. 5B). These results were further verified in rat chondrocytes in different stages of differentiation, i.e., in early-differentiated, late-differentiated, and hypertrophic cells. Indeed, AIF was found to be significantly up-regulated after 12 and 24 h (Fig. 5D; data not shown for hypertrophic chondrocytes). We hypothesized that AIF might act as a target protein for ubiquitin, thus explaining its prominent up-regulation upon proteasome inhibition. Indeed, we noted that AIF was ubiquitinated both in cell culture and in the bones of mice following the administration of MG262 (Fig. 5E). Finally, in support of a role for AIF-mediated apoptotic cell death, suppression of AIF with sequence-specific siRNAs decreased the apoptosis of rat chondrocytes by 41% (P < 0.05 versus MG262 alone; Fig. 5E).

**Discussion**

We report the finding that systemic administration of proteasome inhibitors in mice specifically target the growth plate, decrease growth velocity, and significantly impair further growth potential. Using a wide array of experimental models, we have also characterized the underlying mechanism. Here, we show that proteasome inhibition triggers apoptosis in stem-like chondrocytes in a caspase-dependent and -independent manner. Furthermore, our preclinical data showing that inhibition of p53 and AIF expression partly rescues chondrocytes from apoptosis could lead to the prevention of growth failure induced by proteasome inhibitors in patients.

Using a transgenic mouse model to monitor the in vivo status of the proteasome system, we found that the ubiquitin/proteasome system remained functional in liver and other proliferative tissues (pancreas, testis, and colon) that are often affected by conventional chemotherapy (26–29). The systemic administration of a proteasome inhibitor caused tissue-specific impairment of the ubiquitin/proteasome system. Hence, accumulation of the reporter substrate was observed only in cartilage within the long bones (growth plate...
and articular cartilage) and bone marrow of proteasome inhibitor-treated mice. Accumulation of the GFP reporter was evidenced mainly in stem-like chondrocytes of the growth plate. The stem-like chondrocytes are essential as this small population of cells give rise to proliferative chondrocytes and provides the basis for normal longitudinal bone growth. Indeed, we found that mice treated with MG262 or bortezomib (clinically relevant dose; refs. 7, 8) resulted in significant and permanent impairment of bone growth. Further studies showed that the stem-like chondrocytes in the growth plates of these mice are highly sensitive to apoptosis upon proteasome inhibition. Experimental data in cultured rat metatarsal bones confirmed the sensitivity of stem-like chondrocytes to apoptosis and showed that proteasome inhibitors act locally in the growth plate to suppress longitudinal bone growth. Using cultured human chondrocytes, we were able to reproduce the in vivo and organ culture data showing massive chondrocyte apoptosis and

Figure 5. Proteasome inhibition up-regulates p53 and AIF in vitro and in vivo and induces apoptosis. A, fluorescence immunohistochemistry of p53 and AIF in the tibia growth plate of mice treated with vehicle or MG262 (10 d), see Materials and Methods for details. Left, p53 (red) and nuclei (blue; DAPI); right, AIF (green), and nuclei (blue; DAPI). Bars, 50 μm. B, C5.18 chondrocytes in an early-differentiated stage and late-differentiated stage were treated with MG262 (1 μmol/L) for 3, 6, 12 or 24 h and the levels of p53 were assessed by Western immunoblotting and expressed as relative arbitrary units (RAU). C, apoptosis analysis (cell death detection ELISA) after suppression of p53 with a sequence-specific siRNA and or pifithrin-α (10 μmol/L) in C5.18 early-differentiated chondrocytes treated with MG262 (1 μmol/L) for 12 h (n = 4). Suppression of p53 with sequence-specific siRNA in C5.18 cells was verified by Western immunoblot. D, Western immunoblotting for AIF in C5.18 chondrocytes treated with MG262 (1 μmol/L) for 24 h and in front leg metatarsal bones from vehicle- or MG262-treated mice (in vivo). Immunoprecipitation was done with an AIF antibody and Western immunoblots were done with an antitubulin (Ub) antibody. Apoptosis analysis (OD, 405 nm) after suppression of AIF with a sequence-specific siRNA in early-differentiated C5.18 cells treated with MG262 (1 μmol/L). * , P < 0.05; **, P < 0.005; ***, P < 0.001.
decreased cell proliferation after proteasome inhibition. Therefore, we believe that our findings may be related to the clinical situation in which pediatric patients might suffer from decreased growth rate and reduced final height if treated with proteasome inhibitors.

Our finding that bone growth is negatively influenced by proteasome inhibitors is supported by a recent report of decreased growth in metatarsal bones cultured with the proteasome inhibitor-I, an observation linked to its effects on hypertrophic chondrocytes (30). In contrast, our data obtained both in vivo and in vitro clearly show that proteasome inhibition targets stem-like chondrocytes in the growth plate, and as a consequence, causes significant growth failure. It should be pointed out that proteasome inhibitor-I is not in clinical use and belongs to the peptide aldehyde class of proteasome inhibitors which are less specific and also exert nonspecific effects on the cell (9). In contrast, both bortezomib and MG262 belong to the highly specific peptide boronate class of proteasome inhibitors in which bortezomib has been approved for the treatment of multiple myeloma and a phase 1 trial in children was recently reported (6–8, 31).

Our current observation that a very short exposure to proteasome inhibition is sufficient to cause permanent growth arrest is likely explained by the induction of massive apoptosis in stem-like and proliferative chondrocytes. These cell populations are essential for bone elongation, especially during periods of rapid growth. Each stem-like chondrocyte in the growth plate is believed to be programmed to give rise to one chondrocyte column and have the capacity to undergo a limited number of cell divisions (2). Consequently, loss of stem-like chondrocytes will decrease the number of chondrocyte columns and the total number of chondrocytes. In accordance, we observed decreases in the number of columns and proliferating cell nuclear antigen protein expressions in animals treated with the proteasome inhibitor. We also found that proteasome inhibition impaired the ubiquitin/proteasome system in articular cartilage, which is of particular clinical importance because excessive apoptosis in articular cartilage has been linked to cartilage degeneration (32) and joint diseases (33).

Apoptosis is required for the sculpting of organs during embryogenesis and the maintenance of tissue homeostasis in the adult; consequently, a deregulation of apoptosis may contribute to various disease processes (34). Indeed, the occurrence of massive apoptosis in stem-like chondrocytes and concomitant decrease in cell proliferation supports our hypothesis that excessive apoptosis of these cells may cause permanent growth retardation when exposed to proteasome inhibition. We found that early-differentiated, i.e., stem-like chondrocytes, are not only more susceptible to apoptosis but are also more prone to up-regulating p53 and AIF upon proteasome inhibition. Our finding that AIF is expressed in chondrocytes, is a target protein for ubiquitin, and is up-regulated upon proteasome inhibition is of particular interest. It will be important to confirm this novel observation in other model systems. In an attempt to rescue chondrocytes from proteasome inhibition–induced apoptosis, we used a pan-caspase inhibitor, sequence-specific siRNAs against p53 and AIF, as well as a small molecule inhibitor of p53 (pifithrin-α) and were able to partially prevent apoptosis. These results, which could also serve as a basis for cytoprotection strategies during anticancer treatment with proteasome inhibitors, further validated our hypothesis that proteasome inhibition causes both caspase-dependent and -independent apoptosis in chondrocytes. Taken together, the current data obtained in a number of different model systems all point to an increased sensitivity of stem-like chondrocytes to impairment of the ubiquitin/proteasome system, and consequently, that the loss of these chondrocytes will result in a diminished growth potential.

Clinical follow-up reports reveal that survivors of childhood cancer often have long-term severe complications secondary to previously administered life-saving treatments (26). The selective targeting of chondrocytes reported herein suggests that proteasome inhibitors may target essential cell populations causing long-lasting and potentially irreversible side effects. Because of encouraging results of proteasome inhibitors in cancer treatment, it is anticipated that more selective and highly potent agents will soon be developed. The low systemic toxicity of proteasome inhibitors also makes these compounds attractive for the treatment of childhood cancer. However, our observation that growth failure is not restricted to treatment duration but might also result in long-lasting effects on bone growth warrants careful assessment of linear growth in children treated with proteasome inhibitors.

In conclusion, we report that the systemic administration of a proteasome inhibitor impairs chondrogenesis by triggering apoptosis and decreasing chondrocyte proliferation, which leads to long-term growth failure in treated mice. These preclinical observations suggest that children treated with proteasome inhibitors may be at risk for permanent side effects limiting bone growth potential. In addition, our findings may also open new possibilities to target bone sarcomas with proteasome inhibitors in adults.

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References

10. Chrysis D, Rütten EM, Savendahl L. Growth retardation
induced by dexamethasone is associated with increased apoptosis of the growth plate chondrocytes. J Endocrinol 2003;176:331–7.


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