Protective Role of Humanin on Bortezomib-Induced Bone Growth Impairment in Anticancer Treatment

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Background

Bortezomib is a proteasome inhibitor currently studied in clinical trials of childhood cancers. So far, no side effects on bone growth have been reported in treated children. However, bortezomib was recently found to induce apoptosis in growth plate chondrocytes and impair linear bone growth in treated mice. We hypothesize that [Gly\textsubscript{14}]-humanin (HNG), a 24-amino acid synthetic antiapoptotic peptide, can prevent bortezomib-induced bone growth impairment.

Methods

Mice with human neuroblastoma or medulloblastoma tumor xenografts (9–13 animals/group) received one 2-week cycle (2 injections/week) of bortezomib (0.8 mg/kg or 1.0 mg/kg), or HNG (1 μg/mouse), or the combination of HNG/bortezomib, or vehicle. Cultures of human growth plate cartilage, chondrogenic- and cancer cell lines, and immunohistochemistry for detection of proapoptotic proteins were also used. Statistical significance was evaluated by two-sided Mann–Whitney U test or by parametric or nonparametric analysis of variance.

Results

Bortezomib efficiently blocked the proteasome and induced pronounced impairment of linear bone growth from day 0 to day 13 (0.09 mm/day, 95% confidence interval [CI] = 0.07 to 0.11 mm/day; vs 0.19 mm/day, 95% CI = 0.15 to 0.23 mm/day in vehicle; \( P < .001 \)), an effect significantly prevented by the addition of HNG (0.15 mm growth/day, 95% CI = 0.14 to 0.16 mm/day; \( P < .001 \) vs bortezomib only; \( P = 0.03 \) vs vehicle). Bortezomib was highly toxic when added to cultures of human growth plate cartilage, with markedly increased apoptosis compared with control (\( P < .001 \)). However, when combining with HNG, bortezomib-induced apoptosis was entirely prevented, as was Bax and PARP activation. Bortezomib delayed tumor growth, and HNG did not interfere with the anticancer effect when studied in human tumor xenografts or cell lines.

Conclusions

HNG prevents bortezomib-induced bone growth impairment without interfering with bortezomib’s desired anticancer effects.


Adult survivors of childhood malignancies often display secondary long-term complications after previous life-saving treatments (1,2); short stature is a well-known recorded long-term sequela (3). Increasing survival rates and progressively more intensive treatment regimens make it even more important to evaluate long-term effects on normal bystander tissues (4). Linear bone growth occurs at the growth plate by endochondral ossification, a process in which bone formation begins from a cartilage template that is later replaced by bone (5,6). An exact balance between different systemic and local factors affecting chondrocyte proliferation, differentiation/hyper trophy, matrix synthesis, and cell death within the growth plate must exist to secure normal linear bone growth. Chemotherapy can interfere with any of these processes, thereby affecting chondrogenesis and bone growth negatively (7–10). Despite the prevailing clinical evidence, there have been few investigations into the direct effects of anticancer agents and potential ways to rescue bone growth during treatment for childhood cancers.

Medulloblastoma (MBL) and neuroblastoma (NBL) are embryonal tumors of the central and peripheral nervous systems, respectively. These are the most common and deadly tumors of childhood, with many patients still developing therapy resistance with fatal outcome despite intensive multimodal therapy (11). In addition, survivors frequently suffer from disease and treatment-related long-term side effects, underscoring the need for new therapeutic options specifically targeting cancer cells. The proteasome is a potential target for such a novel cancer therapy (12).

The ubiquitin/proteasome system is the major proteolytic site in mammalian cells, and its coordinated functions are essential to many cellular processes (13,14). The first proteasome inhibitor tested in humans was bortezomib (Velcade), which was shown to be a highly successful treatment for adult hematologic malignancies (15–17). Bortezomib is able to suppress cancer cell growth in childhood malignancies such as leukemias and lymphomas (18), NBL (19,20), MBL (21,22), rhabdomyosarcoma (11), and...
Ewing’s sarcoma (23). Recent phase I studies in pediatric patients demonstrated that bortezomib is well tolerated with promising therapeutic activity (24–26) (n = 50 patients), and phase II clinical trials are ongoing. So far, any undesired secondary side-effects are yet unknown. Nonclinically used proteasome inhibitors negatively affect chondrogenesis and impair linear bone growth in mice (27–29). We recently reported that bortezomib is cytotoxic to the human growth plate and induces permanent growth impairment in mice (30), suggesting that bone growth could potentially be impaired in children treated with bortezomib.

Humanin was identified as a neuroprotective peptide in the brain of patients with Alzheimer’s disease (31). It has been shown to be a wide-spectrum survival molecule in different cell types with antiapoptotic (32–34), metabolic (35), and anti-inflammatory (36) properties. Recent studies using animal models have demonstrated its effectiveness in diabetes (34,37), stroke (38), and atherosclerosis (39). Mechanistic studies have confirmed that humanin interacts with Bax, a proapoptotic protein, and thereby contributes to the observed antiapoptotic activity (40–42).

This study was designed to investigate the potential for humanin to rescue bone growth from bortezomib-induced impairment without interfering with the desired anticancer effect. A wide range of methodologies were applied, including human tumor xenografts, in vitro cultures of human growth plate cartilage and rat metatarsal bones, and use of chondrogenic and cancer cell lines.

Methods

Ethics Statement

The protocol for human growth plate tissue collection was approved by the local medical ethics committee. Informed consent was obtained from all patients and their legal guardians according to the Helsinki Declaration and documented in the medical records. All animal studies were carried out in strict accordance with the institution guidelines.

Drugs

Bortezomib (Velcade, PS-341; Millennium Pharmaceuticals, Cambridge, MA), and the synthetic humanin analog, [Gly14]-humanin (HNG) (Sigma-Aldrich, Schnelldorf, Germany), were dissolved in sterile saline (0.9%), aliquoted, and stored at −80°C.

Cell Line Cultures

The nontransformed rat chondrogenic cell-line, RCJ3.1C5.18 (43), was cultured as described previously (30,44). Human NBL (SH-SY5Y, SK-N-BE(2), SK-N-AS, SK-N-SH, SK-N-DZ, and IMR32), and MBL supratentorial primitive neuroectodermal tumor (D-283, D-324 [also called DAOY], D-425, D-458, UW-228-3, and PFSK-1) cell lines were grown as detailed elsewhere (45,46). Lung cancer (A549), prostate (PC-3), colon (HCT116), breast cancer (MCF-7), and acute lymphatic leukemia (CCRF-CEM) were cultured as described previously (47,48). More information and culture conditions can be found in the Supplementary Methods (available online).

Organ Cultures

The three middle metatarsal bones were dissected from the hind paws of 20-day-old rat embryos (E20), randomly divided into groups, cultured with bortezomib (25 nM) and/or HNG (100 nM) for 12 days, and measured as described earlier (49). The measurements were based on two individual experiments, with each experiment containing six bones per group. Culture conditions and bone growth measurements are further described in the Supplementary Methods (available online).

Human Growth Plate Cultures

Growth plate tissues collected from the proximal tibiae and distal femur in 5 pubertal children (n = 2 boys and 3 girls) undergoing epiphysal surgery were treated for 24 hours with HNG (100 nM) and/or bortezomib (1000 nM) as described previously (30) and in the Supplementary Methods (available online).
cells served as secondary controls (n = 5). Additional methods are given in detail in the Supplementary Materials (available online).

Statistical Analysis
Results are presented as means with 95% confidence intervals (CIs). Differences between two groups were evaluated by the Mann–Whitney U test, whereas differences between several groups were tested by one-way analysis of variance followed by the Tukey test or the Kruskal–Wallis analysis of variance on ranks followed by Dunn’s multiple comparison test. TVI growth curves were constructed by the Kaplan–Meier method, and statistical differences between treatment groups were performed using the log rank test. All statistical tests were two-sided, and the minimal level of statistical significance was P less than .05.

Results
Metatarsal Bone Growth In Vitro
Pronounced metatarsal growth impairment was observed after 12 days of exposure to 25 nM bortezomib (P < .001 vs control) (Figure 1). When combining HNG with bortezomib, partial rescue of bone growth was observed (P < .001 vs bortezomib only; P = .002 vs control).

Pharmacodynamics
One hour after the first intravenous injection with either bortezomib (0.8 mg/kg) or HNG/bortezomib, proteasome inhibition was found to be 57.0% and 56.8%, respectively (P < .001 vs vehicle for both treatments). Intraperitoneal treatment with bortezomib (1.0 mg/kg) resulted in less proteasome inhibition (37.2%). Proteasome activity recovered to baseline levels when assessed 48 hours after the last injection of bortezomib (both after intravenous and intraperitoneal injections) and was not affected at any time in mice injected with vehicle or HNG alone (data not shown).

Linear Bone Growth In Vivo
Mice engrafted with NBL (SK-N-BE(2)) cells were randomized into groups when tumors reached 0.15 mL, which was designated as day 0, and bortezomib injections were given on days 1, 4, 8, and 11 (Figure 2A). In bortezomib-treated mice, mean femur growth (from day 0 to day 13) was 0.09 mm/day (95% CI = 0.07 to 0.11 mm/day), which was statistically less than in the vehicle group, in which mean femur growth was 0.19 mm/day (95% CI = 0.15 to 0.23 mm/day; P < .001) (Figure 2B). When added in combination with bortezomib, HNG partially rescued femur growth (0.15 mm growth/day; 95% CI = 0.14 to 0.16 mm/day; P < .001 vs bortezomib only; P = .03 vs vehicle) (Figure 2B). Mice subjected to HNG only grew similar to vehicle-treated animals. Tibia growth plate height was decreased after bortezomib treatment (88.9 µm, 95% CI = 80.9 to 96.9 µm; vs 103.4 µm, 95% CI = 98.1 to 108.7 µm in vehicle; P < .001) (Figure 2, C, E, and G), an effect prevented when bortezomib was combined with HNG (98.9 µm, 95% CI = 94.9 to 103.0 µm) (Figure 2, F and G). The combined height of the resting and proliferative zones was also decreased after bortezomib treatment (35.8 µm; 95% CI = 27.5 to 44.1 µm) compared with vehicle (53.6 µm; 95% CI = 46.3 to 60.9 µm; P < .001), whereas the combination with HNG prevented this effect of bortezomib (48.4 µm; 95% CI = 42.9 to 53.9 µm) (Figure 2H).

Bortezomib induced apoptosis of resting/stemlike chondrocytes (3.0%; 95% CI = 2.1% to 3.9%) when compared with vehicle (0.5%; 95% CI = 0.2% to 0.8%; P = .001) (Figure 2I). This effect was completely prevented when combining bortezomib with HNG (0.6%; 95% CI = 0.4% to 0.8%) (Figure 2I). HNG prevention of bortezomib-induced bone growth impairment was confirmed in two additional in vivo studies (NBL and MBL tumor growth curves expressed as the mean bone length increase (mm) compared with day 0, and error bars represent 95% confidence intervals (n = 12 bones per group). Statistical test was two-sided and evaluated by Mann–Whitney U test.

Figure 1. Effects of [Gly\(^{\text{N}}\)]-humanin (HNG) on linear bone growth in cultured metatarsals exposed to bortezomib (Btz). Fetal (E20) rat metatarsal bones were dissected and cultured ex vivo with or without HNG (100 nM) and/or bortezomib (25 nM) for 12 days. Bortezomib caused sustained bone growth impairment (**P < .001 vs control on day 12). Combining HNG with bortezomib could partially rescue bone growth from bortezomib-induced impairment (***P < .001 vs bortezomib 25 nM; **P = .002 vs control on day 12). The experiment was repeated twice with results expressed as the mean bone length increase (mm) compared with day 0, and error bars represent 95% confidence intervals (n = 12 bones per group). Statistical test was two-sided and evaluated by Mann–Whitney U test.
In xenografts (in which bortezomib was administered intraperitoneally (Supplementary Figure 1, available online). Mice without tumors showed similar bone growth as those with established tumors, verifying that the tumors themselves did not affect growth (data not shown). Serum insulin-like growth factor 1 levels did not differ between vehicle and any of the treatment groups (data not shown).

Human Growth Plate Chondrocyte Apoptosis In Vitro
Bortezomib (1000 nM) increased chondrocyte apoptosis (22.4%; 95% CI = 16.7% to 28.1%) in cultured human growth plates when compared with control (4.8%; 95% CI = 3.2% to 6.4%; P < .001) (Figure 3A). This effect was mainly observed in resting/stemlike and early proliferative chondrocytes based on blinded observations during the evaluation procedure. When added in combination, HNG (100 nM) efficiently rescued from bortezomib-induced apoptosis (7.4%; 95% CI = 5.4% to 9.4%), whereas HNG alone was similar to control (3.4% apoptosis; 95% CI = 2.2% to 4.6%) (Figure 3A). Bax and PARP were activated in bortezomib-treated human growth plate cartilage (Figure 3, D and H) but not when bortezomib was combined with HNG (Figure 3, E and I), in control (Figure 3, B and F), or in HNG alone (Figure 3, C and G).

Human Cancer Cell Lines
Bortezomib-treatment decreased NBL and MBL cell survival in a dose-dependent (Figures 4A and 5A) and time-dependent (data not shown) manner in all tested cell lines. HNG alone did not affect cell survival except at the highest concentration (100 nM) in SH-SY5Y NBL (53.3%; 95% CI = 52.9% to 53.7%) and D-283 MBL (50.1%;
human cancer cell lines (acute lymphatic leukemia, lung, prostate, colon, and breast) (Supplementary Figure 2, available online).

**Human Tumor Xenografts**

NBL tumor growth (TVI > 4) was markedly delayed by bortezomib (1.0 mg/kg, intraperitoneally; *P* = .002 vs vehicle) (Figure 6A). Combination with HNG did not interfere with the antitumor effect of bortezomib when compared with vehicle in NBL and MBL xenografts (Figures 6A and 7A). HNG alone delayed NBL tumor growth (day 13; *P* = .004 vs vehicle) (Figure 6A). Mice engrafted with a one-sided hindflank NBL-tumor showed a more pronounced delay of tumor growth (TVI > 2) when treated with bortezomib (0.8 mg/kg, intravenously), and the combination with HNG did not interfere with the antitumor effect (Figure 6B). Interestingly, HNG alone suppressed NBL tumor doubling time (day 13; *P* = .001 vs vehicle) (Figure 6B). HNG in combination with bortezomib showed delayed NBL TVI (day 13; *P* = .005 vs vehicle) (Figure 6C), and HNG alone also reduced TVI in both NBL experiments (day 13; *P* = .008 and *P* = .02, respectively vs vehicle) (Figure 6, C and D).

Histologic examination of bortezomib-treated NBL tumors revealed significantly reduced number of microvessels (3.0; 95% CI = 2.3 to 3.7) when compared with vehicle (13.7; 95% CI = 4.9 to 22.5; *P* = .002) (Figure 6F). Apoptosis was also increased after bortezomib treatment (3.7%; 95% CI = 2.6 to 4.8%) compared with vehicle (2.3%; 95% CI = 1.8% to 2.8%; *P* = .02) (Figure 6G). When added in combination, HNG did not interfere with bortezomib effects on angiogenesis and tumor cell apoptosis (Figure 6, F and G). Besides delaying tumor growth, HNG alone decreased angiogenesis (2.9%; 95% CI = 1.1% to 4.7%; *P* = .003), and increased apoptosis (5.0%; 95% CI = 2.8% to 7.2%; *P* = .002) compared with vehicle (Figure 6, F and G). Tumor cell proliferation did not differ between the groups (Figure 6E). Bortezomib was found to upregulate Bax in chondrocytes but not in NBL cancer cells (Supplementary Figure 3, available online).

**Discussion**

Bortezomib has negative effects on bone development. We made the novel observation that HNG can rescue bone growth during such treatment. Importantly, HNG did not interfere with the desired anticancer effect of bortezomib as tested and verified in mouse tumor xenograft models as well as several human tumor cell lines. The cytoprotective effect of HNG was found to be most prominent in resting/stemlike growth plate chondrocytes being protected from bortezomib-induced apoptosis, an effect mediated through interference with the proapoptotic protein, Bax. We also confirmed that HNG can protect cultured human growth plate cartilage from the cytotoxic effects of bortezomib, emphasizing the potential clinical relevance of these findings.

The serious negative effects of proteasome inhibitor treatment on bone development documented in this study are in line with previous reports (27–30). A direct action of bortezomib in growth plate cartilage was confirmed in organ cultures of rat metatarsal bones as well as in human cartilage. We also discovered that HNG efficiently prevents bortezomib-induced bone growth impairment. Our data were consistent in all applied model systems, supporting a cytoprotective effect of HNG in resting/stemlike chondrocytes.
Mechanistic studies revealed that HNG prevents bortezomib-induced activation of proapoptotic Bax and PARP, proteins earlier shown by us to play important roles in bortezomib-triggered apoptosis (30). Our finding that HNG inhibits Bax, thereby protecting chondrocytes from apoptosis, is supported by recent findings in other cell types (40,41). Our finding that bortezomib increases Bax accumulation in chondrocytes but not in human NBL cells suggests that the specific chondrocyte-rescuing effect of HNG might be linked to a Bax-dependent effect.

Our data verify an anticancer effect of bortezomib in preclinical NBL and MBL models, in line with previous reports (19,22,51). We observed a delay of tumor growth in response to bortezomib but no apparent evidence of tumor regression, also consistent with previous reports (20,52). Importantly, our data demonstrate that HNG does not diminish the anticancer effect of bortezomib. Interestingly, HNG by itself showed an antitumor effect as documented in two different NBL tumor xenograft experiments where the effect was linked to decreased angiogenesis and increased tumor cell apoptosis. We speculate that...
this might be related to an anti-inflammatory effect by humanin (eg, binding to proinflammatory cytokines as previously shown) (32). The differential effect of HNG in protecting normal cells but not tumor cells but rather having direct antitumor properties may be analogous to effects of dietary interventions, as recently described (53, 54).

The highest tolerated dose of bortezomib (1 mg/kg), earlier shown to have the greatest antitumor effect in different mouse models (19, 21, 51, 52), corresponds to 3.0 mg/m² in a mouse by using the formula by Reagan-Shaw and colleagues (55), which is more than twice the dose used in humans (1.3 mg/m²). However, the basal metabolic rate per gram of body weight is seven times higher in mice compared with humans (56). The common bortezomib dose used in mice (1 mg/kg) is associated with some toxicity (30, 52, 57) and also death, as indicated in our study. Because the nutritional situation may affect growth, all our mice were given the same amount of food.

Measurement of proteasome inhibition is the clinical marker of therapeutic effect and should be within the 50% to 80% range (58). In our study, a similar level of proteasome inhibition was demonstrated, although a slightly higher range of inhibition was observed in some cell lines. In conclusion, HNG, which is a naturally occurring peptide, was shown to have a protective effect on normal cells in vitro, resulting in a differential cell survival effect when compared with tumor cells treated with both HNG and bortezomib. The protective effect of HNG in normal cells was associated with a selective increase in the expression of DAB2IP and the downregulation of cyclin D1, two genes associated with cell cycle regulation and tumor suppression.

**Figure 5.** Effect of [Gly⁸]-humanin (HNG) on bortezomib (Btz)-treated medulloblastoma (MBL) cell lines. Six MBL cells were treated with increasing concentrations of Btz (1–20 nM) (A) or HNG (1–100 nM) (B) for 48 hours, and cell survival was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazoleum (MTT) assay. C–H Survival of D-283 (C), D-324 (D), D-425 (E), D-458 (F), PFSK-1 (G), and UW-228-3 (H) MBL cells after combined treatment for 48 hours with HNG (100 nM), and/or Btz (1–20 nM). The horizontal dashed line indicates the concentration of the drug that caused 50% inhibition of growth for each cell line. Six parallel wells were measured for each cell line and concentration, and the experiment was repeated twice. Data are expressed as the mean percentage of cell survival compared with the mean of untreated cells for each individual experiment. Error bars indicate 95% confidence intervals. ***P < .001 vs control (Ctrl). Statistical analyses were two-sided and evaluated by one-way analysis of variance followed by the Tukey test.
Figure 6. Effects of [Gly\textsuperscript{14}]-humanin (HNG) on neuroblastoma (NBL) xenograft growth in nude mice treated with bortezomib (Btz). NMRI nu/nu mice were subcutaneously engrafted with 20\times10\textsuperscript{6} NBL (SK-N-BE(2)) cells and treated with Btz 1.0 mg/kg (intraperitoneally (A, C) or 0.8 mg/kg (intravenously) (B, D), HNG (1 \(\mu\)g/mouse, intraperitoneally), the combination of HNG/Btz, or vehicle at the appearance of palpable tumors of 0.10 mL (A, C) or 0.15 mL (B, D). A) Treatment with intraperitoneally injected Btz (**\(P = .002\)), the combination of HNG/Btz (**\(P < .001\)), or HNG (**\(P = .004\)) delayed tumor growth compared with vehicle, thereby increasing the time until tumors reached tumor volume index (TVI) greater than 4 (n = 8–10). The table indicates number of tumors at risk in each treatment group at various time points. B) Tumor growth, TVI greater than 2, was further delayed after intravenously injected Btz (**\(P < .001\)), HNG/Btz (**\(P < .001\)), or HNG (**\(P = .001\)), compared with vehicle (n = 9–11). The table indicates number of tumors at risk in each treatment group at various time points. C) The combined treatment of HNG/Btz decreased TVI vs vehicle (**\(P = .003\)). Treatment with HNG alone suppressed TVI in both NBL experiments compared with vehicle (**\(P = .008\)) (C) and (**\(P .02\)) (D). Quantification of proliferation by Ki-67 immunostaining (E), angiogenesis by BS-1 (F), and apoptosis by applying the TUNEL method (G) in one-sided hind flank NBL tumors (n = 5 tumors per group); *\(P = .02\), **\(P = .002\), ***\(P < .001\) vs vehicle. Data represent means with 95% confidence intervals. Statistical tests were two-sided and evaluated by logrank test (A, B) or by Kruskal–Wallis analysis of variance on ranks followed by Dunn's multiple comparison test (C–G).
Figure 7. Effect of [Gly^2]-humanin (HNG) on medulloblastoma (MBL) xenograft growth. NMRI nu/nu mice subcutaneously engrafted with 15 × 10^6 (D-283) MBL cells and treated with bortezomib (Btz, 1.0 mg/kg, intraperitoneally), HNG (1 μg/mouse, intraperitoneally), the combination, or vehicle at the appearance of palpable tumors of 0.1 cm³ (vehicle (**, p < 0.0001), evaluated by two-sided log rank test. The table indicates number of tumors at risk in each treatment group at various time points. A) TVI means with 95% confidence intervals (n = 9–10).

A limitation of the in vivo studies is that no long-term follow-up and/or dose–response studies were made. Nevertheless, the relevance of our data is supported by our previous long-term follow-up study where normal mice were followed for 6 months after a similar treatment regimen with bortezomib and permanent growth impairment was documented (30). Besides the local actions of bortezomib in growth plate cartilage reported herein, it is possible that this treatment also has systemic effects influencing bone growth despite serum insulinlike growth factor 1 levels not being affected. The clinical significance of the growth rescuing effect of HNG reported herein is limited by the fact that the combination of bortezomib in culture has been reported from the few ongoing clinical trials in children. However, the fact that we found bortezomib to induce massive apoptosis in cultured human growth plate cartilage and that HNG prevented this strengthens the clinical relevance of our findings. Further studies are needed to confirm whether HNG has the capacity to prevent bone growth impairment caused by other drugs used to treat childhood cancers.

In summary, this preclinical study demonstrates that the mitochondrial protein HNG efficiently prevents bortezomib-induced bone growth impairment and chondrocyte apoptosis without interfering with the desired anticancer effect of bortezomib. Based on our experimental data, we propose careful monitoring of longitudinal bone growth in children being treated with proteasome inhibitors.

References


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