Title: Antagonism of Inhibitor of Apoptosis Proteins Increases Bone Metastasis via Unexpected Osteoclast Activation

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Abbreviations list: IAP, inhibitor of apoptosis; cIAP, cellular IAP; NIK, NF-κB inducing kinase; RANKL, Receptor Activator of NF-κB Ligand; BMM, bone marrow macrophage; CTX, C-terminal collagen telopeptide.

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ABSTRACT

IAP (inhibitor of apoptosis) proteins play a central role in many types of cancer, and IAP antagonists are in development as anti-cancer agents. IAP antagonists cause apoptosis in many cells, but they also activate alternative NF-κB signaling through NIK, which regulates osteoclasts. In bone metastasis, a positive feedback loop between tumors and osteoclasts promotes tumor growth and osteolysis. We therefore tested the effect of IAP antagonists on the bone microenvironment for metastasis. In both drug-sensitive and drug-resistant tumors, growth in bone was favored compared to other sites during IAP antagonist treatment. These drugs also caused osteoporosis and increased osteoclastogenesis, mediated by NIK, and enhanced tumor-associated osteolysis. Co-treatment with zoledronic acid, a potent osteoclast inhibitor, reduced IAP antagonist-enhanced tumor growth in bone and osteolysis. Thus, IAP-based cancer treatment may be compromised by osteoporosis and enhanced skeletal metastasis which may be prevented by anti-resorptive agents.

SIGNIFICANCE

While IAP antagonists are a class of anti-cancer agents with proven efficacy in multiple cancers, we show that these agents can paradoxically increase tumor growth and metastasis in the bone by stabilizing NIK and activating the alternative NF-κB pathway in osteoclasts. Future clinical trials of IAP antagonists-based therapy may require detailed examination of this potential for enhanced bone metastasis and osteoporosis, as well as possible combination with anti-resorptive agents.
INTRODUCTION

Bone metastasis is a major complication for patients with malignant tumors, especially breast, prostate and lung cancer (1, 2). Interactions among tumor cells, osteoclasts, and osteoblasts play critical roles in providing a favorable environment for tumor growth. Tumor-produced cytokines directly stimulate osteoclasts, and also promote Receptor Activator of NF-κB Ligand (RANKL) production by osteoblasts, enhancing osteoclastogenesis. Activated osteoclasts release growth factors stored in bone, further expanding tumors, and generating a potent positive feedback loop that supports bone metastasis. Bisphosphonates and RANKL-targeted compounds, potent osteoclast blocking agents, decrease the incidence and severity of bone metastasis (3-6).

Osteoclast differentiation and function are stimulated by activation of the alternative NF-κB pathway (7-9). The primary regulatory kinase for this pathway is NIK, which is activated by protein stabilization rather than phosphorylation (10). In unstimulated cells, NIK is targeted for degradation by binding TRAF3, which recruits the E3 ligases cIAP1 and 2 that ubiquitinate NIK and target it to the proteasome (11, 12). Engagement of a select number of TNFR family members such as RANK and CD40 redirects the TRAF-cIAP destruction complex, releasing NIK and allowing its levels to rise. NIK can be constitutively activated by deletion of its TRAF3 binding domain (NIKΔTRAF3) (13). We have previously shown that expression of the NIKΔTRAF3 mutant in osteoclasts causes osteoporosis in vivo, and stimulates osteoclastogenesis and bone resorption in vitro (14). Conversely, we find that NIK-deficient animals have increased bone mass (8), and are resistant to many forms of pathological osteolysis (15).

In addition to negative regulation of the alternative NF-κB pathway, cIAP 1 and 2 transmit signals through IKKβ (classical NF-κB) to support transcription of pro-survival factors
downstream of TNFR1 (16, 17). Alterations in cIAPs have been associated with the development of tumors and resistance to chemotherapy, and IAP antagonists are in development as anti-cancer agents (18-20). IAP antagonists seem to exert their anti-cancer effects mainly by enhancing TNFα-induced apoptosis in many cancer cell lines \textit{in vitro}, and they synergize with other chemotherapeutic drugs, such as doxorubicin and imatinib, \textit{in vivo} (21, 22). Macrophage lineage cells have also been shown to undergo TNF-induced apoptosis in response to IAP antagonists, and osteoclasts are also susceptible to TNF-mediated death when NF-κB is inhibited (23). Thus, cIAPs may play a significant role in maintaining osteoclast survival downstream of TNF. However, given the opposing roles for cIAPs in classical and alternative NF-κB signaling, and the distinct actions of these pathways in osteoclast survival and differentiation/function, it is difficult to predict the effect of IAP antagonists on osteoclasts.

Because IAP antagonists are candidate anti-cancer agents, and osteoclasts play an important role in skeletal metastasis, we evaluated the effect of IAP antagonism on the bone microenvironment and its effect on metastasis. We found that IAP antagonists stimulated osteoclastogenesis, \textit{in vitro}, and induced osteoporosis, \textit{in vivo}, via activation of NIK. Metastasis was enhanced specifically in bone, but not other sites. Anti-resorptive therapy attenuated these drug effects, demonstrating that the osteoclast is the primary effector of IAP antagonist-enhanced bone metastasis.
RESULTS

**BV6 enhances tumor growth in bone**

Since IAP antagonists have been shown to cause cell death in both cancer cells and myeloid/osteoclast lineage cells (24-26), we first gave bivalent IAP antagonist BV6 to nude mice inoculated with human breast cancer cell line MDA-MB-231 to evaluate its antitumor effects in soft tissue and bone. MDA-MB-231 cells have been stably transduced with firefly luciferase (Fluc), enabling real-time monitoring of tumor burden by quantification of bioluminescence signals (27). Similar to previous reports (25, 28), we found BV6 caused significant apoptosis in MDA-MB-231-Fluc cells (Fig. 1A). Female nude mice were pretreated with 3 weekly doses of BV6 (10 mg/kg), and then were subcutaneously and intraventricularly inoculated with MDA-MB-231-Fluc cells, followed by 5 biweekly doses of BV6 (Fig. 1B). Quantification of tumor burden by bioluminescence imaging (BLI) indicated that while subcutaneous tumor growth was effectively suppressed by BV6, bone metastases were only partially reduced (Fig. 1C,D). These results suggest that, in addition to its anti-tumor effects, BV6 may indirectly support tumor growth in bone by modulating the host microenvironment. In order to specifically examine the effects of BV6 on the bone microenvironment during tumor growth, distinct from its anti-tumor effects, we employed the osteolytic tumor cell line 4T1-Fluc, a murine breast cancer line derived from a BALB/c mouse (29), which retains its viability in the presence of BV6 (Fig. 1E). Immuno-competent BALB/c mice were administered 4 weekly doses of BV6, and 4T1-Fluc cells were inoculated directly into left tibias after the 3rd dose of BV6. Right tibias were injected with PBS. Tumor-inoculated mice were imaged 3 times to monitor tibial tumor growth (Fig. 1F). BLI signals from the left tibia of BV6-treated mice were 17.3-fold and 5.5-fold higher than vehicle-treated ones on day 7 and 10 after tumor inoculation (Fig. 1G,H), suggesting BV6 treatment...
increases tumor growth in bone. Histomorphometric analysis at day 10 confirmed increased tumor in the bone marrow cavity in BV6-treated mice (Fig. 1I,J), and demonstrated increased necrosis in these rapidly growing tumors, which may have blunted the BLI signals at this later (day 10) timepoint. Prior to decalcification for histological analysis, isolated tibias were subjected to μCT to examine osteolysis. In vehicle-treated mice, tumor-bearing tibias had a 24% decrease in trabecular bone volume (BV/TV) compared to the PBS-injected tibias in the same animals (Fig. 1K,L). Strikingly, BV6-treated mice showed a 97% drop in BV/TV in tumor-bearing tibias compared to PBS controls, suggesting synergism between BV6 and tumor-mediated signals for osteolysis. To determine whether the effects of BV6 on the growth of 4T1 tumors were specific to the bone microenvironment, we injected tumor cells subcutaneously with the same regimen of drug treatments used previously. Tumors were imaged on day 10 after inoculation, and we found no differences in subcutaneous tumor growth, as measured by BLI, between drug and vehicle treated animals (Fig. 1M), indicating that BV6 effects on tumor growth were likely bone-specific. Interestingly, in PBS-injected tibias, BV6 caused an 18% decrease in bone mass compared to vehicle controls, suggesting a possible tumor-independent effect of the drug (Fig. 1K).

**IAP antagonists cause high bone turnover osteoporosis**

To explore the effects of IAP antagonism directly on bone, we administered 6 week old BALB/c mice 2 or 4 weekly doses of BV6 (10 mg/kg) or vehicle, and analyzed femurs by μCT. BV6-treated mice showed lower trabecular bone mass (BV/TV), by 9% after 2 weeks and 35% after 4 weeks, compared to vehicle-treated controls, accompanied by a decrease in both trabecular bone mineral density and cortical bone area (Fig. 2A-D). We also gave BV6 to C57Bl/6 mice and found a similar 23% decrease in BV/TV after 4 weeks (Supplementary Fig. S1A-D),
demonstrating that the bone loss was not strain specific. ELISA-based assays for both C-terminal collagen telopeptide (CTX), a marker of osteoclast activity, and osteocalcin, a marker of osteoblast activity, were increased 7 days after the last BV6 injection, indicating that BV6 treatment stimulated both cell types (Fig. 2E,H). Histomorphometric analysis also demonstrated increased osteoclast-covered surface/bone surface (Oc.S/BS) (Fig. 2F,G). To quantify the extent of increased osteoblast activity, calcein and alizarin red were sequentially given to the mice 3 and 8 days following 2 weekly doses of BV6. Analysis of undecalcified, unstained sections demonstrated that both mineral apposition (MAR) and bone formation rates (BFR) were increased (Fig. 2I-K), but to levels insufficient to counteract the enhanced bone resorption by osteoclasts. To establish that the osteoporotic effect is universal among IAP antagonists, we tested the effect of monovalent IAP antagonist 52S (30) on bone. After only 2 weeks (6 mg/kg/day), this drug significantly decreased trabecular bone mass (Fig. 2L,M) and bone mineral density (not shown), and increased Oc.S/BS, MAR, and BFR (Fig. 2N-P). Thus, IAP antagonists induce high turnover osteoporosis characterized by enhanced osteoclast and osteoblast activities, in mice.

**IAP antagonists enhance osteoclast differentiation in vitro**

In order to study the direct effects of BV6 on osteoclasts, we treated differentiating bone marrow macrophages (BMMs) with BV6 for 2 hours (the drug half-life in vivo) every other day, in combination with suboptimal levels of RANKL, in which few OC are generated. Pulsed BV6 increased osteoclastogenesis at all doses tested except the highest doses (20-40 μM) (Fig. 3A), at which most cells were killed (Supplementary Fig. S2A). BV6 alone, however, was not able to induce osteoclasts in the absence of RANKL. The synergistic effect of BV6 with RANKL on differentiation was not due to increased precursor proliferation, as BMMs treated with BV6 or
RANKL alone (which failed to induce OCs) showed similar levels of proliferation to those treated with the osteoclastogenic combination (Supplementary Fig. S2B). The mRNA levels of 4 osteoclast differentiation markers, NFATc1, DC-STAMP, β3 integrin, and calcitonin receptor, were all elevated in BV6 pulse-treated cultures, compared to RANKL alone (Fig. 3B). Constant presence of BV6 progressively increased osteoclast formation up to 2 μM. At 5 μM the drug is inhibitory (Supplementary Fig. S3A). To establish the osteoclastogenic effect is universal among IAP antagonists, we tested two additional antagonists, 52S and ML183 (31). Both drugs significantly enhanced osteoclast differentiation from mouse BMMs (Fig. 3C, Supplementary Fig. S3B).

We also tested the effects of the drugs on human osteoclast progenitors, using peripheral blood monocytes purified with anti-CD14 magnetic beads (32, 33) incubated with suboptimal levels of RANKL. Human osteoclastogenesis was enhanced by both BV6 and 52S (Fig. 3D,E), suggesting that the drug might affect bone turnover in humans as well as mice. BV6 was unable to increase resorption when added acutely to mature osteoclasts generated on bone, derived from either mouse or human precursors (Supplementary Fig. S3C,D). Thus, the bone loss observed in vivo is likely due to an increase in osteoclastogenesis rather than a direct stimulation of bone resorption. Enhanced osteoblast activity was also observed in vivo, but BV6 was modestly inhibitory for osteoblast differentiation and function in vitro (Supplementary Fig. S3E), supporting the conclusion that enhanced osteoclastogenesis is the primary mechanism for drug-induced bone loss in vivo.

Enhanced osteoclastogenic effects of BV6 are NIK-dependent

Since cIAPs ubiquitinate and destabilize NIK, IAP antagonists can activate the alternative NF-κB pathway by preventing NIK degradation. Thus, we examined the activation status of the
alternative NF-κB pathway in BV6-treated cells. BV6 caused rapid and prolonged degradation of cIAP1/2 and stabilized NIK, leading to increased processing of p100 to p52 (Fig. 4A,E) and nuclear translocation of RelB and p52 (Fig. 4C,F), in both mouse BMMs and human monocytes. Similarly, 52S treatment decreased cIAP levels and increased p52 production (Fig. 4B). Furthermore, suboptimal doses of RANKL and BV6 were able to synergize in the generation of p52 (Supplementary Fig. S4). κB pulldown assay in BV6-treated BMMs (Fig. 4D) demonstrated early activation of classical NF-κB (p65/p50) and later activation of alternative NF-κB (RelB/p52). To confirm activation of classical NF-κB, we examined induction of transcription of TNFα and IκBα, two well-established downstream targets (Supplementary Fig. S5A). However, this p65-driven NF-κB was not required for the osteoclastogenic effect of BV6, as the drug was able to induce OCs equally in p65 sufficient and deficient cells (Supplementary Fig. S5B,C).

To demonstrate that NIK, the upstream kinase in the alternative NF-κB pathway, is the primary effector responsible for IAP antagonist-enhanced osteoclastogenesis, we tested BV6 effects in both BMMs and mice deficient in NIK (34). We have previously shown that NIK KO mice have osteoclasts in vivo, but their BMMs do not differentiate in vitro when cultured in M-CSF and RANKL (7), although they can form osteoclasts when exposed to TNF (15). While WT controls showed enhanced osteoclast differentiation in response to RANKL and BV6, NIK KO BMMs were unable to differentiate in these conditions (Fig. 4G), despite equal activation of classical NF-κB (Supplementary Fig. S5D). More importantly, following treatment with 4 weekly doses of BV6, NIK KO mice were protected from bone loss, while their WT littermates became osteoporotic (Fig. 4H). These findings indicate that BV6-enhanced osteoclastogenesis occurs via alternative, but not classical, NF-κB.

Osteoclast expression of constitutively active NIK promotes tumor growth in bone
To further support our hypothesis that NIK activation in osteoclasts enhances the microenvironment for bone metastasis, we turned to a genetic model of conditional NIK activation in which expression of NIKΔTRAF3 (13), a constitutively active form of NIK flanked by flox sites, is predominantly restricted to osteoclasts by a cathepsin K promoter driving cre recombinase (NT3.catK). We have shown that NT3.catK mice exhibit increased osteoclastogenesis and decreased bone mass (14), similar to BV6 effects. To investigate if constitutively active NIK will increase tumor burden in a fashion similar to BV6, we directly inoculated B16-Fluc, a mouse melanoma cell line with constitutive expression of luciferase (35), into the tibias of 6 week old NT3.catK mice, then monitored the tumor growth by BLI. Tumor burden was increased by 2.2-fold on day 12 in NT3.catK mice (Fig. 5A,B). μCT analysis of tibias showed that NT3.catK mice have tumor-induced osteolysis (Fig. 5C,D).

**BV6 preferentially promotes bone metastasis, which is effectively blocked by bisphosphonates**

In our final set of experiments, we sought to determine if BV6 has differential effects on bone versus visceral metastasis in a disseminated tumor model, and if co-treatment with osteoclast inhibitors can moderate the effects of BV6 on tumor growth in bone. To address the former, we performed intracardiac injection of 4T1-Fluc cells (27), which leads to both bone and visceral metastasis in a large percentage of recipients over a 2 week period. The bisphosphonate zoledronic acid (ZA) inhibits skeletal metastasis in both humans and mice (4, 36, 37), and thus was employed as an osteoclast targeting agent. BALB/c mice were injected with BV6 and/or ZA (40 μg/kg) for 4 weeks, and 4T1-Fluc cells were inoculated into the left ventricle after the 3rd dose (Fig. 6A). Combined BLI signals from both legs and jaws on day 13 after tumor inoculation showed that BV6 doubled tumor burden compared to the vehicle group (Fig. 6B,C).
BV6 also increased the number of distinct bone metastases per animal (Fig. 6D). Addition of ZA along with BV6 returned tumor burden to the level of vehicle group, while ZA further decreased the tumor burden of vehicle controls (Fig. 6B,C). However, it did not change the average number of bone metastases in either control or BV6-treated mice (Fig. 6D). The non-skeletal (visceral) tumor burden was not different in any group (not shown), confirming the bone-specific effects of BV6. Femurs were then analyzed by μCT to examine the interactions of ZA, which dramatically increased bone mass in vehicle treated mice, with BV6, which enhanced tumor-associated osteolysis (Fig. 6E,F). Similar to the effects of BV6 in the intratibial injection model, animals treated with BV6 alone had a 78% decrease in BV/TV compared to vehicle. ZA reversed this bone loss, with BV6+ZA animals showing a significant gain in bone mass compared to vehicle or BV6 alone. Thus, BV6 preferentially enhanced the growth of tumor in bone but not in viscera, and inhibition of osteoclasts with bisphosphonates opposed these pro-bone metastatic effects.
DISCUSSION

Although anti-cancer agents are typically designed to directly kill tumor cells, the effect of such drugs on the host microenvironment may impact their ultimate efficacy. In the case of IAP antagonists, we found that two distinct drugs (BV6 and 52S) stimulated bone turnover, resulting in osteoporosis. All 3 IAP antagonists (including ML183) tested increased osteoclastogenesis \textit{in vitro}. Thus, the pro-osteoclastogenic effect appears to be universal among IAP antagonists. In keeping with other examples of an activated bone microenvironment, BV6 treatment increased both the rate of tumor growth and the degree of related osteolysis when 4T1 breast cancer cells were injected into the tibia. Similarly, bone metastases following intracardiac injection was also augmented by the drug. In contrast, growth of subcutaneous tumors and formation of abdominal visceral metastases were not affected by the same regimen, indicating that the tumor-enhancing effects of BV6 are specific to the bone microenvironment. The differential effects of IAP antagonists on bone versus other sites was also observed with drug-sensitive MDA-MB-231 tumors, which showed less inhibition of growth in bone compared to soft tissue. Thus, the bone microenvironment appears to be capable of modifying the anti-tumor potency of IAP antagonists.

The osteoclast, a multinucleated myeloid lineage cell that is unique in its ability to remove both the organic and inorganic phases of bone, is central to the interaction between cancer and the skeleton (38). By degrading bone, the cell releases matrix-residing growth factors that encourage local tumor expansion (1). IAP antagonists directly stimulated osteoclastogenesis \textit{in vitro}, synergizing with suboptimal doses of RANKL, the primary osteoclast differentiating cytokine. Osteoclasts were also increased \textit{in vivo}. Importantly, the bisphosphonate ZA prevented the tumor-enhancing effects of BV6 in bone. Further supporting the osteoclast as the
primary target of IAP antagonists, we found that although BV6 stimulates bone formation in vivo, it inhibits osteoblasts in vitro. Expression of constitutively active NIK (NIKΔT3) by osteoclast lineage cells also stimulates the osteoblast compartment (14) and is sufficient to increase tumor growth in bone. Therefore, our data therefore suggest that the osteoclast is the dominant cell mediating the effects of IAP antagonists on skeletal metastasis.

Both cIAPs and IAP antagonists have been shown to modulate classical and alternative NF-κB (20). Therefore, a priori, it was difficult to predict whether the effects of IAP antagonism on bone would be mediated by one or both pathways. Indeed we found that both NF-κB pathways were potently stimulated by IAP antagonists BV6 and 52S in osteoclast precursors, in vitro. However, while the pro-osteoclastogenic effects of BV6 were completely ablated in the absence of NIK, they were independent of p65 levels. Further supporting the importance of alternative NF-κB for IAP antagonist effects in bone, NIK-deficient mice were completely protected from BV6-induced bone loss, in vivo. We conclude that activation of alternative NF-κB is the predominant mechanism of IAP antagonist effects on bone.

The model emerging from this study is illustrated in Fig. 7. IAP antagonists increase osteoclast activity by stabilizing NIK, causing pro-osteoclastogenic RelB/p52-mediated transcription. Osteoclasts, in turn, release growth factors embedded in bone matrix, thereby expanding cancer cells. The abundant tumor secretes more osteolytic factors that target osteoclasts directly, or indirectly through osteoblasts, ultimately promoting metastatic growth. The therapeutic significance of this model and the central role of the osteoclast were underscored by the capacity of an anti-resorptive drug, ZA, to prevent BV6-enhanced bone metastasis.

Our studies demonstrate that IAP antagonists alter the bone microenvironment in ways that affect tumor growth. In breast cancer patients, isolated, apparently dormant, tumor cells can
be found in the bone marrow during and after chemotherapeutic treatment, representing a seed for future symptomatic metastases (39). Thus, the effects of IAP antagonists on the host, providing a more hospitable environment for tumor growth in bone, may be clinically significant even in patients with IAP antagonist-sensitive tumors, as we observed in the MDA-231 cell line. In sum, these results suggest that, regardless of the anti-tumor efficacy of IAP antagonists, their direct effects on the bone microenvironment should be considered in the design of therapeutic regimens for all cancer types.
MATERIALS AND METHODS

Reagents and mice.

BV6 was provided by Genentech, Inc. IAP antagonists 52S (compound 1) (30) and ML183 (31) were provided by Robert H. Mach, Division of Radiological Sciences, Washington University in St Louis. Macrophage-colony stimulating factor (M-CSF), in the form of CMG 14-12 supernatant (40), and glutathione-S-transferase RANKL (GST-RANKL) were made as described previously (41). NIK, RelB, p100/p52, p105/p50 antibodies were from Cell Signaling Technology, Inc., cIAP1/2 antibody was from R&D Systems, Inc. Transgenic mice bearing cDNA for NIK lacking the TRAF3 binding domain (NIKΔT3) inserted into the ROSA26 locus and homozygous for the transgene (13) were mated to heterozygous cathepsin K Cre mice (42). Controls were Cre- littermates of NT3.catK mice. C57BL/6 and BALB/c mice were from Harlan Laboratories, Inc. Nude mice (homozygous CrTac:NCr-Foxn1nu) were from Taconic Farms, Inc. All experimental protocols were approved by the Institutional Animal Studies Committee at Washington University School of Medicine, permit numbers 20080131 and 20110067.

Cells

4T1 and B16F10 cell lines were originally obtained from American Type Culture Collection and modified to express Fluc by the Weilbaecher lab as described (27, 35). MDA-231 cells were obtained from T. Yoneda (43), and labeled with Fluc in a similar manner. Low passage stocks were utilized for all cell lines and regularly tested for Mycoplasma and maintenance of growth characteristics.

Tumor inoculation and bioluminescent imaging analysis.

BV6 and zoledronic acid were injected intraperitoneally. 1x10^4 tumor cells in PBS were injected into proximal end of tibia, 1x10^5 tumor cells mixed with Matrigel (Sigma) were subcutaneously
injected into flank, or 1x10^5 tumor cells in PBS were injected into left ventricle. Mice were imaged with a charge-coupled device (CCD) camera-based bioluminescence imaging system (IVIS 100, Caliper Life Sciences, Hopkinton, MA) as previously described (44). For bioluminescence imaging of living animals, mice were injected intraperitoneally with 150 mg/g D-luciferin (Biosynth, Naperville, IL) in PBS, anesthetized with 2.5% isofluorane, and then imaged (exposure time 1-60 seconds, binning 8, field of view 15, f/stop 1, open filter, anterior side). Signal was displayed as radiance (photons/sec/cm^2/sr). Regions of interest (ROI) were defined around tumors in hind legs and mandibles using Living Image and IgorPro Software (Version 2.50) to determine photon flux (photons/sec). Ex vivo images of dissected organs were obtained immediately after live animal imaging. For bone metastatic incidence analysis, any discrete tumor site on hind legs and mandibles was counted.

**Histomorphometric analysis.**

Calcein (10 mg/kg, Sigma) and alizarin red (30 mg/kg, Sigma) were intraperitoneally injected into mice 3 and 8 days after 2 doses of BV6, respectively. Dissected femurs and tibias were fixed with formalin overnight, then embedded in methy methacrylate for sectioning. Histomorphometric analysis was performed by a blinded observer using BioQuant OSTEO 2010 software (BioQuant Image Analysis Corporation) and standard parameters (45).

**µCT analysis.**

Femurs or tibias were dissected out from sacrificed mice and fixed with formalin overnight. These bones were scanned in µCT40 or vivaCT (Scanco Medical) at 55 kVp, 145 µA, and 16 µm resolution. Gauss sigma of 1.2, Gauss support of 2, lower threshold of 237, and upper threshold of 1000 were used for all the analysis. Regions of interest (ROIs) were selected 50 slices above
the growth plate of the distal femur, or 30 slices below the growth plate of the proximal tibia, to evaluate the trabecular compartment.

**CTX and Osteocalcin Assays.**

Prior to bleeding, mice were starved of food, but not water, overnight. Blood was collected from mandibular venous plexus and serum was separated with serum separator tubes (BD). CTX was measured using RatLaps EIA for serum (Immunodiagnostic Systems Ltd.), and osteocalcin by sandwich ELISA (Biomedical Technologies, Inc.).

**Osteoclast culture and BV6 treatment.**

Mouse BMMs (7 x 10^3/well in 96-well plates) were plated in the indicated doses of GST-RANKL with 1:25 dilution of CMG 14-12 supernatant (M-CSF source), with media changes every 2 days. 10 ml human whole blood was drawn from a healthy volunteer. Leukocytes were purified with Ficoll (Sigma) then selected with anti-CD14 magnetic beads (Miltenyi Biotec). Human CD14+ cells were seeded into 96-well plates at the density of 8 x 10^4/well for osteoclastogenesis with 20 ng/ml hM-CSF and 100 ng/ml GST-RANKL. This protocol was approved by the Human Studies Committee at Washington University School of Medicine, permit number 201107087. BV6 was added into the osteoclastogenic media, incubated for 2 hours, then washed 3 times before osteoclastogenic media was replaced, repeated every other day. Cultures were fixed in 3.7% formaldehyde and 0.1% Triton X-100 for 5 minutes, and stained for tartrate resistant acid-phosphatase (TRAP) according to the manufacturer’s instructions (Sigma).

**κB pulldown assay.**

Nuclear extract (30 μg) was incubated with streptavidin-coated agarose beads preincubated with biotinylated κB3 oligo-nucleotide for 30 minutes at 4°C on a rotator in 1x binding buffer (30
mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA, 5% glycerol, 1 mg/ml BSA, and 1 mM DTT) with 1 μg poly dIdC. Beads were then washed in 1x binding buffer 3 times prior to SDS-PAGE and immunoblotted for RelB, p52, p65, and p50 (8).

**Real time RT-PCR.**

Total RNA was extracted from differentiating osteoclast lineage cells with NucleoSpin RNA II kit (Machery-Nagel), and cDNA was generated with Sprint RT Complete PCR tubes (Clontech). RT-PCR was performed on an ABI7300 Real-Time PCR system (Applied Biosystems) using SYBR Advantage premix (Clontech) and the following sets of primers: *NFATc1a*, 5’-GGTAACTCTCTTTCTAACCCTAAGGCTC-3’ and 5’-GTGATGCCCTCCAGCATGCACCAGTCA-3’; *β3-integrin*, 5’-

TGTTGCTCAGATGAGACTTTGTC-3’ and 5’-GACTCTGGAGCCAATTGTCCTT-3’; *DC Stamp*, 5’-ACAAACAGTTCCAAGCTTG-3’ and 5’-TCCTTGGGT-TCTTGCTTC-3’; *Calcitonin Receptor*, 5’-CAAGAACCTTAGCTGCCAGA-3’ and 5’-AAGACGCGGACAATGG-3’; *Gapdh*, 5’-CTTCACCACCATGGGAG-3’ and 5’-GACGGACACATTGGG-3’. The amplification reaction was performed for 40 cycles with denaturation at 95°C for 5 seconds, and annealing/extension at 60°C for 31 seconds. Melt curve analysis was performed after each run. The relative abundance of each target was calculated as $1000 \times 2^{-(Ct \text{ target gene} - Ct \text{ Gapdh})}$, where $Ct$ represents the threshold cycle for each transcript, and Gapdh is the reference.

**Statistical Analysis.**

Values reported graphically are expressed as mean ± s.d., with numbers of samples indicated in figure legends. A p-value was obtained through the use of unpaired 1-tailed Student’s t-test. P values are indicated in each figure, and values less than 0.05 were considered significant.
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AUTHOR CONTRIBUTIONS

C.Y. conducted the majority of the experiments and helped write the manuscript. J.L.D., R.Z., P.V., X.S. and L.C. performed some experiments and interpreted related data. S.V. conducted chemical synthesis of compounds under the direction of R.H.M. K.N.W. and R.F. and D.P-W. contributed to experimental design and data interpretation, and edited the manuscript. D.V.N. conceived of and supervised the project and wrote the manuscript.
REFERENCES


FIGURE LEGENDS

Figure 1

BV6 increases tumor burden in bone. (A) Cultured MDA-MB-231-Fluc cells were incubated with BV6 (5 μM) for 2 days, then viability was measured by MTT assay. (B) Scheme of tumor inoculation. Female nude mice (homozygous CrTac:NCr-Foxn1nu) were intraperitoneally injected with BV6 (10 mg/kg) or vehicle (Veh), 3 weekly doses before and 5 biweekly doses after tumor inoculation. 6 week old mice were intraventricularly and subcutaneously injected with 1 x 105 and 1 x 104 MDA-MB-231-Fluc respectively, then imaged on day 11, 14, and 18. (C,D) Tumor burden of BV6 and vehicle treated mice in the skin and skeleton (legs and jaw) are plotted. Veh, n = 7; BV6, n = 8. (E) Cultured 4T1-Fluc cells were incubated with BV6 (5 μM) for 2 days, then viability was measured by MTT assay. (F) Scheme of tumor inoculation. Male BALB/c mice were treated with BV6 or vehicle once/week for 4 weeks, and 1x10⁴ 4T1-Fluc cells were inoculated directly into left tibias when mice were 6 weeks old. Right tibias were injected with PBS. Bioluminescence from tibias were measured on days 4, 7, and 10 after tumor inoculation. (G) Tumor burden of BV6 and vehicle treated mice in the tibias is plotted. Veh, n = 7; BV6, n = 5. (H) Representative images of (g) on day 10 are shown. (I) Mice from (g) were sacrificed on day 10 and the tibias were subjected to histomorphometric analysis. Tumor occupancy was measured by the ratio of tumor area over total area of bone marrow cavity. T, tumor; B, bone marrow. (J) Representative images of (i) are shown. (K) Tumor and PBS injected tibias were scanned by μCT and trabecular bone volume (BV/TV) was calculated. (L) Representative photos of (k) are shown. (M) 6 week old BALB/c mice were subcutaneously injected with 1 x 10⁵ 4T1-Fluc, and mice were imaged on day 10. Tumor burden is plotted. n =
5/group. Scale bar, 500 µm. Data represent the mean ± s.d. n.s., not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. 
Figure 2

IAP antagonists cause high turnover osteoporosis in vivo. (A) 6 week old BALB/c mice were treated with either 2 or 4 weekly doses of BV6 (10 mg/kg) or vehicle (Veh), and distal femurs were analyzed by μCT 7 days after the last dose. n= 6/group in 2 doses treatment, n=7/group in 4 doses treatment. (B-D) Bone mineral density and cortical bone area were quantified after 4 doses of BV6. Representative photos of distal femurs are shown. (E) Quantification of CTX from serum drawn at the end of 4 BV6 doses. Veh, n = 5; BV6, n = 6. (F) Histomorphometric analysis of TRAP-stained sections to quantify osteoclast surface. n = 7/group. (G) Representative photos of (f) are shown. (H) Quantification of osteocalcin from serum drawn at the end of 4 BV6 doses. Veh, n = 5; BV6, n = 6. (I-K) Mice were given 2 weekly doses of BV6 and histomorphometric analysis of calcein/alizarin double-staining sections were performed to quantify bone formation. n = 6/group. Representative photos are shown. (L-P) 6 week old C57BL/6 mice were treated with daily doses of 52S (6 mg/kg) or vehicle (Veh) for 14 days. Proximal tibia were analyzed by μCT and histomorphometry (Oc.S/BS, MAR, and BFR as shown above). Representative photos of proximal tibias are shown. Veh, n=6; 52S, n=7. Scale bars, 100 μm. Data represent the mean ± s.d. *P < 0.05, **P < 0.01.
Figure 3

IAP antagonists enhance osteoclastogenesis \textit{in vitro}. (A) BMMs were cultured with indicated doses of BV6, 20 ng/ml GST-RANKL plus vehicle (Veh), or BV6 plus RANKL for 6 days, then visualized by TRAP staining. BV6 and Veh were given in 2 hours pulses every other day, and RANKL was always present. TRAP positive multinucleated cells were counted in each well and plotted. (B) BMMs were cultured as in (a), and RNA was collected on day 0, 2, and 4 for real time RT-PCR analysis of osteoclast differentiation markers. Data is plotted as fold change relative to day 0. (C) Mouse bone marrow macrophages were treated with constant 52S (0.3 μM) during osteoclast differentiation. Mouse osteoclasts (mOC) per well were counted and plotted. (D,E) Human peripheral mononuclear cells were cultured in osteoclastogenic media and pulse treated with BV6 (D) or 52S (E) as described above. Mature osteoclasts (hOC) were TRAP-stained and counted. Scale bars, 1 mm. Data represent the mean ± s.d. \(*P < 0.05, **P < 0.01, \)**\(**P < 0.001\).
Figure 4

BV6-enhanced osteoclastogenesis is NIK-dependent. (A) Mouse BMMs were pulse treated with BV6 (5 μM) for 2 hours, then incubated in media (αMEM, 10% FBS) for indicated times. cIAP1/2 degradation, NIK stabilization, and p100/p52 processing were assessed by immunoblot of total cell lysates. (B) Mouse BMMs were treated with GST-RANKL (20 ng/ml) or 52S (0.3 μM) for indicated times. cIAP1/2 degradation and p100/p52 processing were assessed by immunoblot of total cell lysates. (C) Mouse BMMs were treated with BV6 (5 μM) for 30 minutes, 2 hours, and 16 hours (14 hours after 2-hour pulse). Cytosolic and nuclear extracts were collected to detect RelB, p52, p65, and p50. (D) Mouse BMMs were treated as described in (C). Nuclear extracts were collected for κB pulldown assay to detect DNA-bound RelB, p52, p65, and p50. (E,F) Human monocytes were treated with BV6 (5 μM) for 16 hours (14 hours after 2-hour pulse). Total cell lysates were collected for detection of cIAP1/2, NIK, p100/p52 processing (E). Cytosolic and nuclear extracts were purified to detect RelB nuclear translocation (F). (G) NIK WT and KO BMMs were pulse treated with BV6 (5 μM) every other day in osteoclastogenic media with 20 ng/ml GST-RANKL for 6 days. Mature osteoclasts were visualized with TRAP staining. (H) 6 week old NIK WT and KO mice were injected with BV6 (10 mg/kg) once a week for 4 weeks. Femoral trabeculae were measured by μCT. WT Veh, n = 7; WT BV6, n = 6; KO Veh, n = 8; KO BV6, n = 6. Scale bar, 1 mm. Data represent the mean ± s.d. n.s., not significant, *P<0.05.
Figure 5

Transgenic mice with constitutively active NIK demonstrate increased tumor growth in bone. (A) 6 week old NT3.catK and control (Ctl) littermates were injected with 1x10^4 B16-Fluc cells into left tibias, and right tibias were injected with PBS. Mice were imaged on days 5, 9, and 12 after tumor inoculation. Ctl, n = 12; NT3.catK, n = 8. (B) Representative images of (a) on day 12 are shown. (C) Tibias from (a) were scanned with μCT to measure trabecular bone volume (BV/TV). (D) Representative μCT photos of (c) are shown. Data represent the mean ± s.d. *P < 0.05, **P < 0.01.
Figure 6

BV6 increases bone metastases. (A) Scheme of tumor inoculation. BALB/c mice were treated with BV6 (10 mg/kg) and zoledronic acid (ZA) (40 µg/kg) weekly for 4 weeks, and 1x10^5 4T1-Fluc cells were injected into left ventricles when mice were 6 weeks old. Veh, n = 15; Veh+ZA, n = 12; BV6, n = 9; BV6+ZA, n = 9. (B) Bioluminescence signals were measured on days 7, 10, and 13 after tumor inoculation. Signals from both hind legs and mandibles were summed. (C) Representative images of (b) on day 13 are shown. (D) Incidence of metastases was calculated from tumor growth sites on hind legs and mandible of each mouse. (E) Femurs were scanned with µCT to measure BV/TV. (F) Representative photos of (e) are shown. Data represent the mean ± s.d. n.s., not significant. *P < 0.05, **P < 0.01, ***P < 0.001.
Figure 7

Proposed model for IAP antagonists-enhanced bone metastasis. RANKL counteracts the NIK-degrading activity of cIAP1/2, promoting RelB/p52 mediated transcription (left). IAP antagonists degrade cIAP1/2, stabilizing NIK, thereby enhancing osteoclastogenic signaling through RelB/p52 (center). Increased numbers of osteoclasts provide a favorable microenvironment for bone metastasis through release of tumor growth factors, an effect opposed by ZA (right).
Fig. 3

A

B

C

D

E

Veh  52S

Veh  BV6

Veh  52S
Antagonism of Inhibitor of Apoptosis Proteins Increases Bone Metastasis via Unexpected Osteoclast Activation

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