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

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**ABSTRACT**

Inhibitor of apoptosis (IAP) proteins play a central role in many types of cancer, and IAP antagonists are in development as anticancer agents. IAP antagonists cause apoptosis in many cells, but they also activate alternative NF-κB signaling through NF-κB-inducing kinase (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, as compared with other sites during IAP antagonist treatment. These drugs also caused osteoporosis and increased osteoclastogenesis, mediated by NIK, and enhanced tumor-associated osteolysis. Cotreatment with zoledronic acid, a potent osteoclast inhibitor, reduced IAP antagonist–enhanced tumor growth in bone and osteolysis. Thus, IAP antagonist–based cancer treatment may be compromised by osteoporosis and enhanced skeletal metastasis, which may be prevented by antiresorptive agents.

**SIGNIFICANCE:** Although IAP antagonists are a class of anticancer 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 antagonist–based therapy may require detailed examination of this potential for enhanced bone metastasis and osteoporosis, as well as possible combination with antiresorptive agents. Cancer Discov; 3(2); 212–23. ©2012 AACR.
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 NF-κB-inducing kinase (NIK), which is activated by protein stabilization rather than phosphorylation (10). In unstimulated cells, NIK is targeted for degradation by binding TNF receptor–associated factor 3 (TRAF3), which recruits the E3 ligases cellular inhibitor of apoptosis (CIAP) 1 and 2 that ubiquitinate NIK and target it to the proteasome (11, 12). Engagement of a selected number of TNF receptor (TNFR) family members such as RANK and cluster of differentiation 40 (CD40) redirects the TRAF3–CIAP
they synergize with other chemotherapeutic drugs, such as doxorubicin and imatinib, expressed in osteoclasts causes osteoporosis in vivo and stimulates osteoclastogenesis and bone resorption in vitro (14). Conversely, we found that NIK-deficient animals have increased bone mass (8) and are resistant to many forms of pathologic osteolysis (15).

In addition to negative regulation of the alternative NF-κB pathway, cIAP1 and 2 transmit signals through inhibitor of IκB kinase-β (IKK-β; classical NF-κB) to support transcription of prosurvival factors downstream of TNFFR1 (16, 17). Alterations in cIAPs have been associated with the development of tumors and resistance to chemotherapy, and IAP antagonists are in development as anticancer agents (18–20). IAP antagonists seem to exert their anticancer effects mainly by enhancing TNF-α-induced apoptosis in many cancer cell lines in vitro, and they synergize with other chemotherapeutic drugs, such as doxorubicin and imatinib, 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 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 anticancer 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 in vitro, and induced osteoporosis in vivo, via activation of NIK. Metastasis was enhanced specifically in bone, but not other sites. Antiresorptive therapy attenuated these drug effects, showing that the osteoclast is the primary effecter of IAP antagonist–enhanced bone metastasis.

RESULTS

BV6 Enhances Tumor Growth in Bone

Because IAP antagonists have been shown to cause cell death in both cancer cells and myeloid/osteoclast lineage cells (24–26), we first gave the bivalent IAP antagonist BV6 to nude mice inoculated with the 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 whereas subcutaneous tumor growth was effectively suppressed by BV6, bone metastases were only partially reduced (Fig. 1C and D). These results suggest that, in addition to its antitumor effects, BV6 may indirectly support tumor growth in bone by modulating the host microenvironment. To specifically examine the effects of BV6 in the bone microenvironment during tumor growth, distinct from its antitumor effects, we used 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). Immunocompetent BALB/c mice were administered 4 weekly doses of BV6, and 4T1-Fluc cells were inoculated directly into left tibia after the third dose of BV6. Right tibia were injected with PBS. Tumor-inoculated mice were imaged 3 times to monitor tumor growth (Fig. 1F). BLI signals from the left tibia of BV6-treated mice were 17.3- and 5.5-fold higher than signals from vehicle-treated ones on day 7 and day 10, respectively, after tumor inoculation (Fig. 1G and H), suggesting that BV6 treatment increases tumor growth in bone. Histomorphometric analysis at day 10 confirmed increased tumor in the bone marrow cavity of BV6-treated mice (Fig. 1I and J) and showed increased necrosis in these rapidly growing tumors, which may have blunted the BLI signals at this later (day 10) timepoint. Before decalcification for histologic analysis, isolated tibias were subjected to microcomputed tomography (μCT) to examine osteolysis. In vehicle-treated mice, tumor-bearing tibias had a 24% decrease in trabecular bone volume (BV/TV) compared with the PBS-injected tibias in the same animals (Fig. 1K and L). Strikingly, BV6-treated mice showed a 97% drop in BV/TV in tumor-bearing tibias, as compared with 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 the effects of BV6 on tumor growth were likely bone specific. Interestingly, in PBS-injected mice, BV6 caused an 18% decrease in bone mass, as compared with 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 BV/TV by 9% after 2 weeks and 35% after 4 weeks, compared with 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–S1D), showing 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 and H). Histomorphometric analysis also showed increased osteoclast-covered surface/bone surface (Oc.S/Bs; Fig. 2F and G). To quantify the extent of increased osteoblast activity, calcein and alizarin red were sequentially given to the
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Figure 1. BV6 increases tumor burden in bone. A, cultured MDA-MB-231-Fluc cells were incubated with BV6 (5 μmol/L) for 2 days, and then viability was measured by MTT assay. B, scheme of tumor inoculation. Female nude mice (homozygous CrtNCr-Foxn1nu) were intraperitoneally injected with BV6 (10 mg/kg) or vehicle (Veh), with 3 weekly doses before and 5 biweekly doses after tumor inoculation. Six-week-old mice were intravenicularly and subcutaneously injected with 1 x 10^6 and 1 x 10^5 MDA-MB-231-Fluc, respectively, and then imaged on days 11, 14, and 18. C and D, tumor burden of BV6- and vehicle-treated mice in the soft tissue and skeleton (legs and jaw) are plotted. Vehicle, n = 7; BV6, n = 8. E, cultured 4T1-Fluc cells were incubated with BV6 (5 μmol/L) for 2 days, and then viability was measured by MTT assay. F, scheme of tumor inoculation. Male BALB/c mice were treated with BV6 or vehicle once per week for 4 weeks, and 1 x 10^7 4T1-Fluc cells were inoculated directly into left tibias when mice were 6 weeks old. Right tibias were subcutaneously injected with 1 x 10^5 and 1 x 10^4 MDA-MB-231-Fluc, respectively, and then imaged on days 11, 14, and 18. Tumor burden of BV6- and vehicle-treated mice in the soft tissue and skeleton (legs and jaw) are plotted. Vehicle, n = 7; BV6, n = 5. G, representative images of G on day 10 are shown. H, 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 the bone marrow cavity. I, tumor; B, bone marrow. J, representative images of I are shown. K, tumor and PBS-injected tibias were scanned by μCT, and BV/TV was calculated. L, representative photos of K are shown. M, 6-week-old BALB/c mice were subcutaneously injected with 1 x 10^7 4T1-Fluc, and mice were imaged on day 10. Tumor burden is plotted. n = 5/group. Scale bar, 500 μm. Data represent the mean ± SD; n.s., not significant; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

mice 3 and 8 days following 2 weekly doses of BV6. Analysis of undecalcified, unstained sections showed 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 the monovalent IAP antagonist 52S (30) on bone. After only 2 weeks (6 mg/kg/day), this drug significantly decreased trabecular bone mass (Fig. 2L and 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

To study the direct effects of BV6 on osteoclasts, we treated differentiating bone marrow macrophages (BMM) with BV6 for 2 hours (the drug half-life in vivo) every other day, in combination with suboptimal levels of RANKL, conditions in which few osteoclasts are generated. Pulsed BV6 increased osteoclastogenesis at all doses tested except the highest doses (20–40 μmol/L; 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 osteoclasts) showed levels of proliferation similar to those in BMMs treated with the osteoclastogenic combination (Supplementary Fig. S2B). The mRNA levels of genes encoding 4 osteoclast differentiation markers, NFATc1, DC-Stamp, β3 integrin, and calcitonin receptor, were all elevated in BV6 pulse-treated cultures, compared with RANKL alone (Fig. 3B). Constant presence of BV6 progressively increased osteoclast formation up to 2 μmol/L. At 5 μmol/L, the drug is inhibitory (Supplementary Fig. S3A). To establish that the osteoclastogenic effect is universal among IAP antagonists, we tested 2 additional antagonists, 52S and ML183 (31). Both drugs significantly enhanced osteoclast differentiation from mouse BMMs (Fig. 3C and Supplementary Fig. S3B).

We also tested the effects of the drugs on human osteoclast progenitors, using peripheral blood monocytes purified...
Drug-induced bone loss enhanced osteoclastogenesis is the primary mechanism for (Supplementary Fig. S3E), supporting the conclusion that the drug might affect bone turnover in humans as well as mice. BV6 was unable to increase resorption when added to mature osteoclasts generated on bone, derived from either mouse or human precursors (Supplementary Fig. S3C and S3D). 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

Because 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 and E) and nuclear translocation of RelB and p52 (Fig. 4C and 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). By pulldown

**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-dose treatment; n = 7/group in 4-dose 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. Vehicle, n = 5; BV6, n = 6. F, histomorphometric analysis of tartrate-resistant acid phosphatase (TRAP)-stained sections to quantify osteoclast surface. n = 7/group. G, representative photos of F are shown. Red staining indicates TRAP osteoclasts. H, quantification of osteocalcin from serum drawn at the end of 4 BV6 doses. Vehicle, n = 5; BV6, n = 6. I–K, mice were given 2 weekly doses of BV6, and histomorphometric analysis of calcein/alizarin double staining sections was conducted to quantify bone formation. n = 6/group. Representative photos are shown. In K, green indicates calcein, and red indicates alizarin red. L–P, 6-week-old C57BL/6 mice were treated with daily doses of 52S (6 mg/kg) or vehicle for 14 days. Proximal tibia was analyzed by μCT and histomorphometry (Oc.S/BS, MAR, and BFR, as shown earlier). Representative photos of proximal tibias are shown. Veh, n = 6; 52S, n = 7. Scale bars, 100 μm. Data represent the mean ± SD; *, P < 0.05; **, P < 0.01.
Figure 3. IAP antagonists enhance osteoclastogenesis 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 vehicle were given in 2-hour pulses every other day, and RANKL was always present. TRAP-positive multinucleated cells were counted in each well and plotted. mOC, mouse osteoclasts. B, BMMs were cultured as in A, and RNA was collected on days 0, 2, and 4 for real-time reverse transcriptase PCR (RT-PCR) analysis of osteoclast differentiation markers. Data are plotted as fold change relative to day 0. C, mouse BMMs were treated with constant 52S (0.3 μmol/L) during osteoclast differentiation. mOC per well were counted and plotted. D and E, human peripheral mononuclear cells were cultured in osteoclastogenic media and pulse treated with BV6 (D) or 52S (E), as described earlier. Mature osteoclasts (hOC) were TRAP stained and counted. Scale bars, 1 mm. Data represent the mean ± SD; *, P < 0.05; **, P < 0.01; ***, P < 0.001.

assays in BV6-treated BMMs (Fig. 4D) showed early activation of classical NF-kB (p65/p50) and later activation of alternative NF-kB (RelB/p52) signaling. To confirm activation of classical NF-kB, we examined induction of transcription of TNF-α and IkBα, 2 well-established downstream targets (Supplementary Fig. S5A). However, p65-driven NF-kB was not required for the osteoclastogenic effect of BV6, as the drug was able to induce osteoclasts equally in p65-sufficient and -deficient cells (Supplementary Fig. S5B and S5C).

To show that NIK, the upstream kinase in the alternative NF-kB pathway, is the primary effector responsible for IAP antagonist–enhanced osteoclastogenesis, we tested the effects of BV6 in both BMMs and mice deficient in NIK (34). We have previously shown that NIK-knockout mice have osteoclasts
in vivo, but their BMMs do not differentiate in vitro when cultured in macrophage colony-stimulating factor (M-CSF) and RANKL (7), although they can form osteoclasts when exposed to TNF (15). Although wild-type (WT) controls showed enhanced osteoclast differentiation in response to RANKL and BV6, NIK-knockout BMMs were unable to differentiate in these conditions (Fig. 4G), despite equal activation of classical NF-κB (Supplementary Fig. SSD). More importantly, following treatment with 4 weekly doses of BV6, NIK-knockout mice were protected from bone loss, whereas 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 NIKxTRAF3 (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 would 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 and B). A microcomputed tomography (μCT) analysis of tibias showed that NT3.catK mice have tumor-induced osteolysis (Fig. 5C and 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...
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in a disseminated tumor model, and if cotreatment with osteoclast inhibitors can moderate the effects of BV6 on tumor growth in bone. To address the former, we conducted 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 used as an osteoclast-targeting agent. BALB/c mice were injected with BV6 and/or zoledronic acid (40 μg/kg) for 4 weeks, and 4T1-Fluc cells were inoculated into the left ventricle after the third dose (Fig. 6A). Combined BLI signals from both legs and jaws on day 13 after tumor inoculation showed that BV6 doubled tumor burden, compared with the vehicle group (Fig. 6B and C). BV6 also increased the number of distinct bone metastases per animal (Fig. 6D). Addition of zoledronic acid along with BV6 returned tumor burden to the level of that in the vehicle group, whereas zoledronic acid further decreased the tumor burden of vehicle controls (Fig. 6B and C). However, it did not change the average number of bone metastases in either control- or BV6-treated mice (Fig. 6D). The nonskeletal (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 zoledronic acid, which dramatically increased bone mass in vehicle-treated mice, with BV6, which enhanced tumor-associated osteolysis (Fig. 6E and 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 with vehicle. Zoledronic acid reversed this bone loss, with BV6+ZA-treated animals showing a significant gain in bone mass, as compared with animals given 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 metastatic effects in bone.

DISCUSSION

Although anticancer agents are typically designed to directly kill tumor cells, the effect of such drugs on the host microenvironment may have an impact on their ultimate efficacy. In the case of IAP antagonists, we found that 2 distinct drugs (BV6 and 52S) stimulated bone turnover, resulting in osteoporosis. All 3 IAP antagonists (including ML183) that were tested showed increased osteoclastogenesis in vitro. Thus, the proosteoclastogenic effect seems 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 were 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 were also observed with drug-sensitive MDA-MB-231 tumors, which showed less inhibition of growth in bone compared with soft tissue. Thus, the bone microenvironment seems to be capable of modifying the antitumor 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 in vitro, synergizing with suboptimal doses of RANKL, the primary osteoclast differentiating cytokine. Osteoclasts were also increased in vivo. Importantly, the bisphosphonate zoledronic acid prevented the tumor-enhancing effects of BV6 in bone. In further support of the osteoclast as the primary target of IAP antagonists, we found that although BV6 stimulates bone formation in vitro, it inhibits osteoclasts in vivo. Expression of constitutively active NIK (NIKTRAF3) by osteoclast lineage cells also stimulates the osteoclast compartment (14) and is sufficient to increase tumor growth in bone. Therefore, our data 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, although the proosteoclastogenic effects of BV6 were completely ablated in the absence of NIK, they were independent of p65 levels. As additional support for the importance of the alternative NF-κB pathway for IAP antagonists' effects in bone, NIK-deficient mice were completely protected from BV6-induced bone loss in vivo. We conclude that activation of alternative NF-κB signaling is the predominant mechanism whereby IAP antagonists exert their 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 antiresorptive drug, zoledronic acid, to prevent BV6-enhanced bone metastasis.

Our studies show that IAP antagonists alter the bone microenvironment in ways that affect tumor growth. In patients with breast cancer, 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-MB-231 cell line. In sum, these results suggest that, regardless of the antitumor efficacy of IAP antagonists, their direct effects on the bone microenvironment should be considered in the design of therapeutic regimens for all cancer types.
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METHODS

Reagents and Mice

BV6 was provided by Genentech, Inc. IAP antagonists S2S (compound 1; ref. 30) and ML183 (31) were provided by Robert H. Mach, Division of Radiological Sciences, Washington University (St. Louis, MO). M-CSF, in the form of CMG 14-12 cell supernatant (40), and glutathione-S-transferase RANKL (GST-RANKL) were made as described previously (41). NIK, RelB, p100/p52, and p105/p50 antibodies were from Cell Signaling Technology, Inc., and cIAP1/2 antibody was from R&D Systems, Inc. Transgenic mice bearing cDNA for NIK lacking the TRAF3-binding domain (NIKΔTRAF3), inserted into the ROSA26 locus and homozygous for the transgene (13), were mated to heterozygous Cathepsin K-Cre mice (42). Controls were Cre-negative littermates of NT3.catK mice.

Cells

The 4T1 and B16F10 cell lines were originally obtained from the American Type Culture Collection and modified to express Fluc by the Weibauer laboratory, as described (27, 35). MDA-MB-231 cells were obtained from Mbalaviele and colleagues (43) and labeled with the Weibauer laboratory, as described (27, 35). MDA-MB-231 cells were obtained from Mbalaviele and colleagues (43) and labeled with the Weibauer laboratory, as described (27, 35).

Tumor Inoculation and Bioluminescent Imaging Analysis

BV6 and zoledronic acid were injected intraperitoneally. A total of 1 × 104 tumor cells in PBS were injected into the proximal end of 1 × 103 tumor cells mixed with Matrigel (Sigma) were subcutaneously injected into the flank, or 1 × 103 tumor cells in PBS were injected into the left ventricle. Mice were imaged with a charge-coupled device (CCD) camera-based BLI system (IVIS 100, Caliper Life Sciences), as previously described (44). In accordance with BLI coupled device (CCD) camera-based BLI system (IVIS 100, Caliper Life Sciences), as previously described (44). In accordance with BLI coupled device (CCD) camera-based BLI system (IVIS 100, Caliper Life Sciences), as previously described (44). In accordance with BLI coupled device (CCD) camera-based BLI system (IVIS 100, Caliper Life Sciences), as previously described (44). In accordance with BLI coupled device (CCD) camera-based BLI system (IVIS 100, Caliper Life Sciences), as previously described (44).

Histomorphometric Analysis

Calcine (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 methyl methacrylate for sectioning. Histomorphometric analysis was conducted 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 µCT-40 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 1,000 were used for all analyses. ROIs were selected 20 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

Before bleeding, mice were starved of food, but not water, overnight. Blood was collected from the 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 was measured by sandwich ELISA (Biomedical Technologies, Inc.).

Osteoclast Culture and BV6 Treatment

Mouse BMs (7 × 103/well in 96-well plates) were plated in the indicated doses of GST-RANKL with 1:25 dilution of CMG 14-12 cell supernatant (M-CSF source), with media changes every 2 days. Ten milliliters of human whole blood was drawn from a healthy volunteer. Leukocytes were purified with Ficoll (Sigma) and then selected.
with anti-CD14 magnetic beads (Miltenyi Biotec). Human CD14+ cells were seeded into 96-well plates at the density of \(8 \times 10^4\) cells/well for osteoclastogenesis with 20 ng/ml human M-CSF and 100 ng/ml GST-RANKL. This protocol was approved by the Human Studies Committee at Washington University School of Medicine, permit 201107087. BV6 was added into the osteoclastogenic media, incubated for 2 hours, then washed 3 times before osteoclastogenic media was replaced, and this procedure was 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).

**xB Pulldown Assay**

Nuclear extract (30 μg) was incubated with streptavidin-coated agarose beads preincubated with biotinylated xB oligonucleotide for 30 minutes at 4°C on a rotator in 1× binding buffer [30 mmol/L NaCl, 10 mmol/L Tris, pH 7.4, 1 mmol/L EDTA, 5% glycerol, 1 mg/ml bovine serum albumin (BSA), and 1 mmol/L dithiothreitol (DTT)] with 1 μg polydeoxynucleosinic-deoxyctydilic acid (poly-dIdC). Beads were then washed in 1× binding buffer 3 times before SDS-PAGE and immunoblotted for RelB, p52, p65, and p50 (8).

**Real-Time RT-PCR**

Total RNA was extracted from differentiating osteoclast lineage cells using the NucleoSpin RNA II Kit (Machery-Nagel), and cDNA was generated with Sprint RT Complete PCR tubes (Clontech). Reverse transcriptase PCR (RT-PCR) was conducted on an ABI 7300 Real-Time PCR System (Applied Biosystems) using SYBR Advantage Premix (Clontech) and the following sets of primers: NFATc1a, 5′-GTGATGACCCCAGCATGCACCAGT-3′ and 5′-GACTCTGGAGCACAATTGTCCTT-3′; NFATc3, 5′-GGTAACTCTGTCTT-AAGACG-3′ and 5′-TGAGTCCTCTCAGACATTGGTGCTC-3′; and NFκB1, 5′-GACGGACACATTGGGGGTAG-3′ and 5′-GTGGCCTCAGATGAGACTTTGTC-3′. PCR reactions were carried out for 40 cycles, with denaturation at 95°C for 5 seconds, annealing/extension at 60°C for 31 seconds. Melt curve analysis was conducted after each run. The relative abundance of each target was calculated as 1,000\(^{\frac{C_t\text{ target gene}}{C_t\text{ Gapdh}} + g}\), in which \(g\) represents the threshold cycle for each transcript, and Gapdh is the reference.

**Statistical Analysis**

Values reported graphically are expressed as mean ± SD, with numbers of sample size indicated in legends. A \(P\) value was obtained through the use of unpaired one-tailed Student t test. \(P\) values are indicated in each figure, and values less than 0.05 were considered significant.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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**Acknowledgments**

The authors thank Domagoj Vucic at Genentech, Inc., for providing BV6, Manolis Pasparakis for the p65\(^{fl/fl}\) mice, Crystal Idleburg for expert histology, and Steven Teitelbaum for critical review of the article.

**Grant Support**

This study was primarily supported by the NIH, grant AR052705 (to D.V. Novack), with additional support from ARS2921 and AR53628 (to R. Faccio), CA100730 (to K.N. Weilbaecher), and the Barnes-Jewish Foundation (to D.V. Novack). Histologic and \(\mu\)CT analysis was supported in part by the Washington University Center for Musculoskeletal Research and the NIH/National Institute of Arthritis and Musculoskeletal and Skin Diseases (NIAMS), grant AR057235. The Molecular Imaging Center was supported by NIH grant P50 CA94056.

Received June 15, 2012; revised December 13, 2012; accepted December 13, 2012; published OnlineFirst December 26, 2012.

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