

Anti-cancer IAP antagonists promote bone metastasis: a cautionary tale

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Abstract The bone microenvironment is complex, containing bone-forming osteoblasts, bone-resorbing osteoclasts, bone-maintaining osteocytes, hematopoietic lineage cells, as well as blood vessels, nerves, and stromal cells. Release of embedded growth factors from the bone matrix via osteoclast resorption has been shown to participate in the alteration of bone microenvironment to facilitate tumor metastasis to this organ. Many types of malignancies including solid tumors and leukemias are associated with elevated levels of inhibitor of apoptosis (IAP) proteins, and IAP antagonists represent an important emerging class of anti-cancer agents. IAPs exert anti-apoptotic roles by inhibiting caspases and upregulating pro-survival proteins, at least in part by activating classical NF- κ B signaling. In addition, IAPs act as negative regulators in the alternative NF- κ B pathway, so that IAP antagonists stimulate this pathway. The role of the classical NF- κ B pathway in IAP antagonist-induced apoptosis has been extensively studied, whereas much less attention has been paid to the role of these agents in the alternative pathway. Thus far, several IAP antagonists have been tested in preclinical and early stage

clinical trials, and have shown promise in sensitizing tumor cells to apoptosis without significant side effects. However, recent preclinical evidence suggests an increased risk of bone metastasis caused by IAP antagonists, along with potential for promoting osteoporosis. In this review, the connection between IAP antagonists, the alternative NF- κ B pathway, osteoclasts, and bone metastasis are discussed. In light of these effects of IAP antagonists on the bone microenvironment, more attention should be paid to this and other host tissues as these drugs are developed further.

Keywords Bone metastasis · IAP antagonist · Osteoclast · NF- κ B · NIK

Introduction

Bone is not an inert scaffold in the body, and its constituent cells are involved in many complex biological processes including serum calcium and phosphate regulation [1], glucose metabolism [2], male reproduction [3], hematopoietic stem cell regulation [4], immune system modulation [5], and skeletal muscle regeneration [6]. There are four major types of cell in bone, osteoblasts (OB), osteoclasts (OC), osteocytes, and chondrocytes. In normal, homeostatic conditions the activities of OBs and OCs are coupled to maintain bone mass. An imbalance between OBs, the cells building bone, and OCs, the cells resorbing bone, is a key feature of many diseases such as osteoporosis and osteopetrosis, and is important in many other conditions including tumor metastasis to bone. OBs enhance the survival and differentiation of OC precursor cells by expressing receptor activator of nuclear factor κ B ligand (RANKL) and macrophage-colony stimulating factor (M-CSF), and inhibiting osteoclastogenesis via secretion of osteoprotegerin (OPG), a decoy receptor for RANKL. The local ratio of

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RANKL:OPG is likely an important determinant of OC function in vivo, although global whole bone or serum ratios may be less meaningful. OBs may also be important local sources of inflammatory cytokines such as TNF α that can modulate OC differentiation and function. OCs modulate OB functions by mobilizing OBs to resorption sites through released cytokines such as transforming growth factor β (TGF- β), or activating OB activity by direct cell contact via ephrinB2-EphB4 engagement [7, 8]. The delicate balance between OBs and OCs is tightly regulated by many signaling pathways including NF- κ B.

Components of the NF- κ B signaling pathway are expressed in all mammalian cells, although most of this pathway's function has been studied in the immune system. In recent years, the NF- κ B pathway has been found to play major roles in regulating bone homeostasis. The NF- κ B transcription factor family consists of 5 subunits—p65 (RelA), p105/p50, c-Rel, RelB, and p100/p52—acting as dimers in two distinct pathways—the classical (or canonical) pathway and alternative (or non-canonical) pathway. In OC lineage cells, the key osteoclastogenic cytokine RANKL activates both pathways, which have distinct effects. While the classical pathway promotes the survival of OC lineage cells [9], the alternative pathway regulates OC differentiation [10] and function [11]. In contrast, activation of either NF- κ B pathway in OBs inhibits their differentiation [12, 13].

Although initially thought to regulate apoptosis via direct inhibition of caspases, cellular inhibitor of apoptosis (cIAPs) 1 and 2 are now thought to promote cell survival primarily via activation of classical NF- κ B signaling, leading to upregulation of other survival proteins including Bcl-2 and Bcl-xL. High levels of inhibitor of apoptosis (IAP) proteins are seen in many tumors, and this is often associated with chemoresistance, metastasis, and poor prognosis [14, 15], leading to the development of IAP antagonists as a new class of anti-cancer agents. Some IAP antagonist compounds, also known as second mitochondria-derived activator of caspases (SMAC) mimetics after the endogenous inhibitor, have reached phase I or II clinical trials according to ClinicalTrials.gov, and a large number of companies are pursuing drugs in this class. Inhibition of IAPs causes some signals such as TNF α or DNA damage to become potentially apoptotic, particularly in tumor cells with elevated IAP expression. However, cIAPs are broadly expressed, and the effects of inhibitors on the host can lead to side effects or change the host microenvironment, altering the potential for tumor survival and growth.

The bone microenvironment is a fertile soil for tumor seeds to grow, especially when OC activity is elevated. When some tumor cells such as breast cancer cells circulate into bone, they have a tendency to activate OC through the secretion of cytokines. Activated OC then resorb bone and

release embedded growth factors into the microenvironment. These factors support the growth of tumor cells, resulting in increased secretion of osteoclastogenic cytokines. Thus, tumor cells and OCs form a self-supporting cycle, the so-called “vicious cycle”, leading to enhanced tumor growth in bone [16]. We have recently demonstrated that IAP antagonists impact the vicious cycle, causing osteoporosis and increased bone metastasis in mice [17], and promote the differentiation of both murine and human OC precursors in vitro. In this review, we will discuss the roles of NF- κ B in bone, how IAP antagonists enhance bone metastasis by activating NF- κ B, and possible co-treatments to avoid host effects in bone.

NF- κ B in bone

Complexity of NF- κ B signaling pathways

NF- κ Bs are transcription factors that modulate numerous cellular processes in response to a number of stimuli. Like many other signaling pathways, the NF- κ B pathways are able to exert distinct actions in different cell types or in response to different stimuli. The 5 subunits—p65 (RelA), p105/p50, c-Rel, RelB, and p100/p52—share the Rel homology domain, which is a characteristic feature of NF- κ Bs and contains the DNA binding and nuclear localization sequences. Only p65, RelB, and c-Rel have transactivation domains. p105 and p100 are precursors that bear I κ B domains, and their partial degradation to p50 and p52, respectively, generates active partners for p65, RelB, and c-Rel which only bind DNA as dimers. I κ B α and the I κ B domain in p100 are major factors restraining NF- κ Bs to the cytosol in basal conditions. While many signals and a wide array of inflammatory cytokines activate classical NF- κ B, only a few including RANKL are also able to activate the alternative pathway. p65, p105/p50, and c-Rel generally act within the classical pathway, whereas RelB and p100/p52 participate in the alternative pathway. Distinct kinetics of the two pathways contributes to the complexity; the classical pathway can be activated within minutes of a signal and then quickly returns to baseline, but the alternative pathway requires hours to be switched on and lasts for many hours or even days. Even with similar protein domain structures, p65 and RelB, key effectors in classical and alternative pathways, respectively, can drive distinct sets of target genes upon translocation to nucleus [18]. Whether this is due to specific interactions through their less well-conserved transactivation domains or their different kinetics is not yet clear. Cross-talk between the pathways adds more complexity to the NF- κ B network. Excessive accumulation of p100, which primarily binds RelB, can also be inhibitory to the classical pathway due to binding of its I κ B

domain to p65 [19]. Likewise, activation of the IKK complex, the upstream kinase complex regulating classical pathway activation, causes upregulation of RelB and p100 expression, and can lead to nuclear accumulation of RelB [20]. The stabilization of NF- κ B-inducing kinase (NIK), the dominant kinase activating the alternative NF- κ B pathway, can cause activation of classical NF- κ B signaling as well via the formation of a NIK-IKK complex [21].

Role of NF- κ B in bone

Unlike OCs which originate from hematopoietic progenitor cells, the other bone cells such as OBs, osteocytes and chondrocytes are of mesenchymal origin. NF- κ B has been shown to modulate the differentiation and function of a majority of bone cells, with OCs being most extensively studied. In OCs, RelB is an important upstream regulator of master osteoclastogenic factor nuclear factor of activated T cells c1 (NFATc1). NFATc1 expression is blunted and OC differentiation is blocked in RelB deficient osteoclast precursor cells in vitro [10]. p65, a key player in classical pathway, on the other hand, suppresses a RANKL-induced apoptotic JNK pathway to sustain precursors during a critical window for differentiation [9]. In OBs, however, the activation of NF- κ B inhibits both differentiation and activity of OB. Nuclear p65 can disrupt the SMAD complex, a key transcription complex driving OB differentiation, at the promoters of SMAD target genes, and blockage of classical NF- κ B has been shown to increase basal bone mass [22, 23]. Similarly, mice with disruption of the alternative pathway have increased OB numbers and bone formation rates [12, 13]. In osteocytes, NF- κ B has been postulated to participate in β 1 integrin-modulated responses to mechanical strain [24]. At the growth plate, insulin-like growth factor 1 (IGF-1) mediated chondrogenesis is partially regulated by p65 [25]. In articular cartilage, NF- κ B has been shown to play a critical role in the pro-inflammatory stress-related responses of chondrocytes in osteoarthritis, and in the control of chondrocyte differentiation [26].

Alternative NF- κ B pathway in osteoclast

NF- κ B signaling in osteoclasts

The activation of NF- κ B signaling pathways is achieved when NF- κ B members translocate to nucleus. This can be a result of many stimuli, including cytokines, growth factors, radiation, infection, mechanical shear, and certain chemical compounds. In contrast to the classical pathway found in all cells, the alternative pathway is confined to certain cell types, including OC lineage cells. Unlike the classical

pathway that can be turned on by many stimuli, only a few cytokines can activate the alternative pathway, including RANKL, the key cytokine driving osteoclastogenesis. Its receptor RANK, a member of TNF receptor superfamily, is expressed on the plasma membrane of OC lineage cells including macrophages and monocytes. Like many other types of TNF receptors, after trimerization induced by ligand binding, RANK uses adaptor proteins bound to its intracellular domains to transduce signals. In OC lineage cells, RANKL is able to activate both NF- κ B pathways with assistance from different adaptors.

Tumor necrosis factor receptor-associated factor 6 (TRAF 6) is one of the key RANK binding adaptors that initiates classical pathway signaling. A protein complex composed of TGF β -activated kinase 1 (TAK1) and TAK1-binding proteins 2/3 (TAB 2/3) relays to a kinase complex containing I κ B kinase $\alpha/\beta/\gamma$ (IKK $\alpha/\beta/\gamma$). In basal conditions, I κ B α binds p65/p50 dimers and retains them in the cytoplasm (Fig. 1a) RANKL activates a phosphorylation cascade downstream of TRAF6, causing I κ B α to be ubiquitinated on K48 and targeted for proteosomal degradation, releasing primarily p65/p50 dimers into nucleus (Fig. 1b).

NIK is the dominant kinase of the alternative pathway, and its activation is regulated by protein stabilization rather than phosphorylation. In unstimulated OC lineage cells, NIK binds to a protein complex consisting of TRAF3, TRAF2, and cIAP1/2 through its TRAF3-binding domain. This interaction brings cIAP1/2 into the proximity of NIK, leading to K48-type ubiquitination of NIK by cIAP1/2, and its proteosomal degradation (Fig. 1a). Thus, NIK levels are very low and alternative NF- κ B signaling is actively repressed in unstimulated conditions. Upon RANKL stimulation, a conformational change of RANK likely recruits TRAF3. This new interaction with RANK causes ubiquitination of TRAF3 by cIAP1/2, and the ensuing TRAF3 degradation releases NIK from the complex, allowing accumulation of newly translated NIK [27, 28]. This explains why alternative pathway activation takes hours and is blocked by protein synthesis disrupting agents. In addition to cIAP1/2 that have been identified as negative regulators of alternative NF- κ B, recent studies have shown that NLRP12 and OTUD7B are capable of facilitating the degradation of NIK as well, although the molecular mechanisms remain unclear [29, 30] and their activity in OCs is unknown.

Among the many homo- and hetero-dimers of NF- κ Bs, p65/p50 and RelB/p52 are the major types in OC lineage cells. Classical pathway p65/p50 dimers increase Gadd45 β expression to restrain apoptotic JNK activation as a way to counterbalance pro-apoptotic effects of RANKL [9]. Alternative pathway RelB/p52 dimers promote transcription of NFATc1, and osteoclast differentiation ensues. This demonstrates distinct roles for the two NF- κ B pathways during RANKL-driven osteoclastogenesis [9, 10].

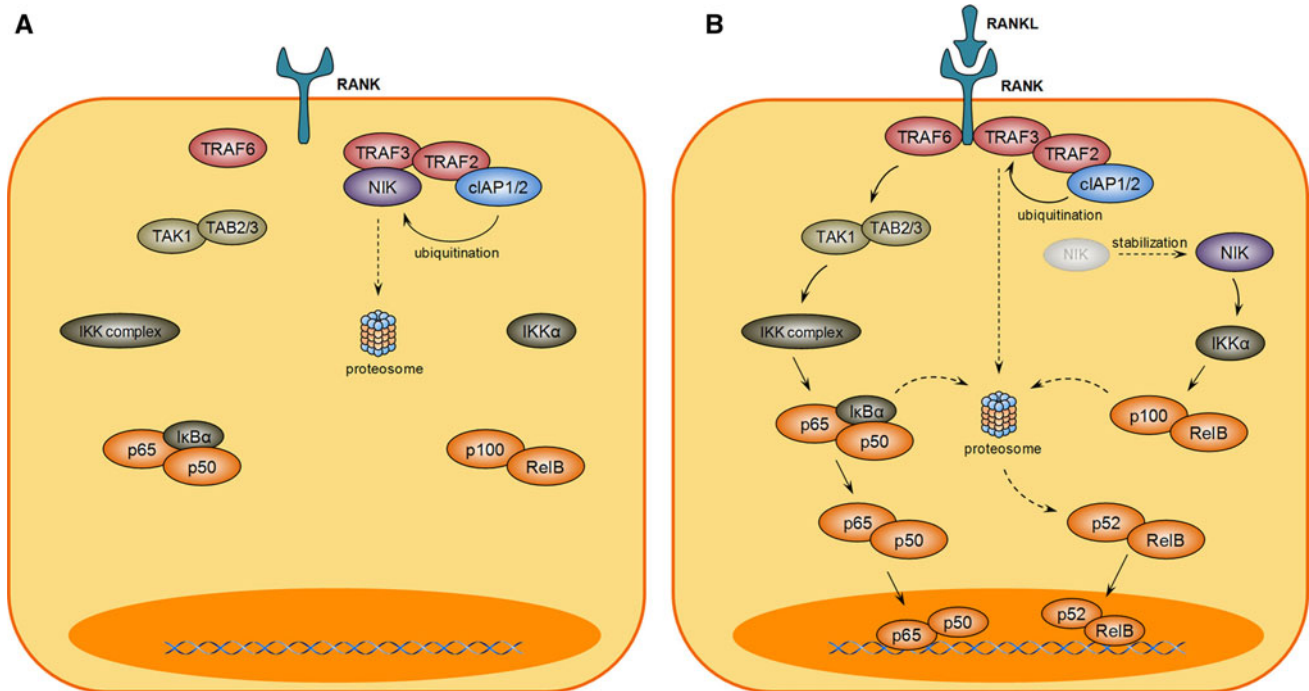


Fig. 1 NF-κB signaling pathways in osteoclast lineage cells. **a** In basal conditions, IκBα sequesters p65 and p50 in the cytosol, keeping the classical NF-κB pathway off. TRAFs 2 and 3 bring NIK and cIAP1/2 together, causing ubiquitination and degradation of NIK, preventing activation of the alternative NF-κB pathway. **b** Both pathways are activated by RANKL. In the classical NF-κB pathway, adaptor proteins TRAF2/5/6 support the activation of TAK1 and

IKKβ, leading to proteosomal degradation of IκBα. Released p65/p50 dimers then move into the nucleus, where p65 regulates the transcription of target genes. In the alternative NF-κB pathway, TRAF3 is degraded by cIAP1/2 ubiquitination after it binds RANK. The stabilized NIK leads to partial degradation of p100. Without sequestration by the IκB domain in p100, p52/RelB dimers translocate to the nucleus, marking the activation of the alternative pathway

Activation of the alternative NF-κB pathway increases OC function

Although the alternative pathway is required for OC differentiation *in vitro*—RelB or NIK deficient macrophages fail to form OCs when cultured with M-CSF and RANKL—basal OC numbers in these mice are normal [10]. However, upon challenge, pathological bone loss, including tumor-induced osteolysis, is blunted in RelB or NIK deficient mice [10, 13, 19]. The pivotal role of the alternative pathway is strengthened by further studies showing that over-activation of alternative pathway from a constitutively active form of NIK results in enhanced osteoclastogenesis, elevated OC resorptive activity, osteoporosis, increased pathological osteolysis, and a larger tumor burden in bone [11, 17]. Moreover, the accumulation of p100 by TNF stimulation has been shown to inhibit RANKL-induced osteoclast formation, and TNF-Tg/p100^{-/-} mice exhibit more joint erosion than do TNF-Tg littermates, indicating an inhibitory role for p100 in inflammatory osteolysis [31]. These findings suggest that the alteration of alternative NF-κB may be a common feature in many conditions of pathological bone loss, such as osteoporosis, osteolysis, and bone metastasis.

Development of IAP antagonists

IAP family and its features

Before IAP proteins were identified as regulators of NF-κB, they were found to be upregulated in many types of cancer, and associated with chemoresistance and poor prognosis [32]. IAP proteins were first found when a portion of baculovirus was able to rescue apoptosis caused by mutated p35 [33], and soon after many other proteins with a similar structure were identified, forming the IAP family [34]. Among eight IAP proteins in humans, cIAP1, cIAP2, X chromosome-linked IAP (XIAP), survivin, and melanoma IAP (ML-IAP) are the most thoroughly studied. The baculovirus IAP repeat (BIR) domain is the defining structure of IAP proteins, and is responsible for protein interactions (Fig. 2). For instance, cIAPs bind to TRAF2 via the BIR domain to negatively regulate alternative NF-κB. cIAPs, however, positively regulate classical NF-κB via facilitating the activation of the IKK complex by the TAK1 complex. XIAP activates the classical pathway by interacting with TAB1 through its BIR domain.

Some IAP family members harbor additional domains enabling their multifunctionality. The RING finger domain

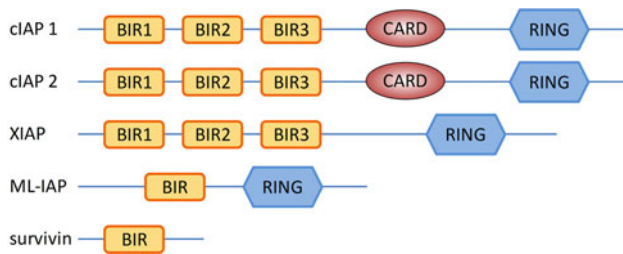


Fig. 2 Major IAP proteins in the IAP family. Schematic presentation of cIAP1, cIAP2, XIAP, ML-IAP, and survivin are shown. BIR (baculoviral IAP repeat) domain is the defining characteristic of the IAP family. CARD (caspase recruitment domain) domain is involved in protein–protein interactions. RING (really interesting new gene) domain possesses E3 ubiquitin ligase activity

in cIAPs has ubiquitin E3 ligase activity, causing proteosomal degradation of NIK when cIAPs bind the TRAF2/TRAF3/NIK complex [35, 36]. IAP proteins exert their pro-survival features through both caspase-dependent and -independent mechanisms. XIAP is the only IAP protein that is capable of direct binding and inhibition of caspases, blocking both intrinsic and extrinsic apoptosis pathways [37]. cIAPs, on the other hand, instead of direct inhibition of caspase activity, cause degradation of certain caspases via ubiquitination [38]. In the classical NF- κ B pathway, taking TNF receptor 1 (TNFR1) as an example, many studies have shown cIAP1/2 are required for signaling down to the IKK complex to ultimately activate the pathway. Binding of TNF α to TNFR1 recruits a protein complex including TNF receptor-associated death domain (TRADD) adaptor protein, TRAF2 (or TRAF 5), cIAP1/2, and receptor-interacting protein kinase 1 (RIPK1). Activated cIAP1/2 K63-ubiquitinates TRADD and RIPK1, which, instead of marking the protein for proteosomal degradation, provides a scaffold that activates the TAK1–TAB2/3 and IKK $\alpha/\beta/\gamma$ complexes. Then IKK β initiates a phosphorylation cascade leading to the degradation of I κ B α , and allows p65/p50 nuclear translocation [39]. The inability of TNF α alone to kill cells in most circumstances is due to cIAP1/2-mediated anti-apoptotic effects, including increased expression of anti-apoptotic factors such as Bcl-2 and Bcl-xL by activation of classical NF- κ B [40], and blocking the formation of RIPK1-dependent, caspase 8-activating complex [41], both depending on E3 ligase activity of cIAP1/2. cIAP1 and cIAP2 are highly redundant proteins as evidenced by the fact that knockouts of either of them present no noticeable phenotypes. Loss of both cIAP1 and 2 strongly impairs TNF α -mediated activation of classical NF- κ B pathway [42].

Due to their profound anti-apoptotic effects, it is not surprising that the expression levels of several IAP family members have been shown to be elevated in many types of cancer, including leukemia, lymphoma, and solid tumors [14]. However, tumors such as multiple myeloma with

decreased levels of IAPs and activation of alternative NF- κ B have been reported [43–45]. Like many other balanced regulators in the body, IAPs have their natural antagonists as well. Second mitochondria-derived activator of caspase (Smac), a pro-apoptotic mitochondrial protein, inhibits IAPs by direct interaction when it is released to the cytosol in response to diverse apoptotic stimuli. However, the correlation between altered levels of Smac and tumorigenesis is not as well-defined as that in IAPs [46].

NF- κ B-mediated apoptotic effects of IAP antagonists

Given the close relationship between cancer and IAPs, many researchers in both academic and industrial institutions have been working to target IAPs for anti-cancer therapy. This new class of agents named IAP antagonists or Smac mimetics [47] includes small compounds, short peptides, and antisense nucleotides. They disrupt IAP interaction with caspases, or decrease intracellular levels of IAPs with distinct approaches, such as auto-ubiquitination-induced degradation, damaging the translation of IAP proteins, or inhibiting the transcription of IAPs. Many IAP antagonists, especially small molecules, have been shown to exhibit strong apoptotic effects on tumor cells, but not healthy cells, both in vitro and in vivo [15]. In both pre-clinical studies and clinical trials, most IAP antagonists are being tested as cell death sensitizing agents in combination with other well-established anti-cancer agents, such as doxorubicin, gemcitabine, or imatinib [48–50]. Currently, more than ten IAP antagonists have entered clinical trials (Table 1).

IAPs upregulate pro-survival proteins via classical NF- κ B signaling (Fig. 3a), facilitate tumorigenesis by escalating inflammation, and promote metastasis by modulating cell–cell interactions [51]. When cIAP1/2 are eliminated by IAP antagonists, a brief activation of the classical pathway occurs, presumably due to transient scaffolding from auto-ubiquitination, resulting in a short surge of TNF α expression (Fig. 3b). While newly secreted TNF α engages the cells in an autocrine manner, the absence of cIAPs leads to caspase 8-induced apoptosis. This apoptosis can be prevented by re-expression of cIAPs or adding TNF α blocking antibody [36]. Therefore, IAP antagonist-induced cell death is mainly NF- κ B-mediated and TNF α -induced, although TNF-independent apoptosis has been reported [52, 53]. To no surprise, there are many IAP antagonist-resistant cells, presumably due to inability to produce TNF α , or to the fast re-synthesis of IAPs [54, 55].

The alternative NF- κ B pathway, on the other hand, does not appear to be involved in IAP antagonist-induced apoptosis. In contrast to the positive role of cIAPs in the classical NF- κ B pathway, these proteins negatively regulate alternative NF- κ B and their elimination causes its

Table 1 IAP antagonists currently under development

Target	Compound	Application	Organization	Development	
cIAP1/2	BV6 ^a [36]	Cancer cell lines	Genentech	Preclinical	
	LBW242 ^a [49]	Solid and blood cancer cells	Novartis	Preclinical	
	GT-T ^a [68]	Cancer cell lines	TetraLogic Pharmaceuticals	Preclinical	
XIAP	Bestatin ^a [69] (Ubenimex)	Adult acute nonlymphatic leukemia	Nippon Kayaku	Approved in Japan	
	AEG35156 ^b [70]	Advanced pancreatic cancer, refractory chronic lymphocytic leukemia	Aegera Therapeutics	Phase 1, 2	
	1396-11	Cancer cell lines	The Burnham Institute	Preclinical	
	1396-12 ^a [71]				
	TWX006 TWX024 ^a [72]	Cancer cell lines	Scripps Research Institute	Preclinical	
	Embelin ^a [73]	Cancer cell lines	University of Michigan	Preclinical	
	JP1201 ^a [50]	Cancer cell lines	Joyant Pharmaceuticals	Preclinical	
	Birinapant (TL32711) ^a	Advanced solid tumors, ovarian cancer, acute myelogenous leukemia	TetraLogic Pharmaceuticals	Phase 1,2	
cIAP1/2 & XIAP	AT-406 ^a [74]	Advanced solid tumors, lymphomas	Ascenta Therapeutics	Phase 1	
	GDC-0152 [75]	Solid cancer	Genentech	Phase 1	
	GDC-0917 ^a				
	HGS1029 ^a (AEG40826)	Advanced solid cancer, relapsed lymphoid malignancies	GlaxoSmithKline	Phase 1	
	LCL161 ^a [76]	Breast cancer, advanced solid cancer	Novartis	Phase 1,2	
	SM-122	Cancer cell lines	University of Michigan	Preclinical	
	SM-164 ^a [77]				
	AEG40730 ^a [42]	Cancer cell lines	Aegera Therapeutics	Preclinical	
Survivin	LY2181308 ^b [78]	Lung cancer, liver cancer, prostate cancer, and leukemia	Eli Lilly	Phase 1,2	
	YM155 ^c [79]	Solid tumors, lymphoma, melanoma, advanced non-small cell lung carcinoma	Astellas Pharma	Phase 1,2	
	Terameprocol (EM-1421) ^c [80]	Solid tumors, glioma, leukemia, cervical intraepithelial neoplasia	Erimos Pharmaceuticals	Phase 1,2	
	CWP232291 ^c [81]	Acute myeloid leukemia	JW Pharmaceutical	Phase 1	
	EZN-3042 ^c [82]	Relapsed acute lymphoblastic leukemia	Enzon Pharmaceuticals	Phase 1	
	ML-IAP	BV6 ^a [83]	Melanoma cell lines	Genentech	Preclinical

Among numerous lead compounds with IAP antagonistic activities, only the ones with explored biological relevance are listed. IAP-based compounds/peptides that are used in vaccine therapy are not listed. These were search results obtained from ClinicalTrials.gov

^a The compounds that bind directly to IAPs proteins causing their degradation

^b The antisense oligonucleotides that bind IAPs mRNA, disrupting the protein synthesis

^c Transcription inhibitors that down-regulate the expression of IAPs

sustained activation. In basal conditions, cIAPs constantly ubiquitinate NIK within the cIAP/TRAF/NIK protein complex to keep a very low level of NIK (Fig. 3a). In the absence of cIAPs or with antagonist treatment, NIK is stabilized and is able to activate sustained alternative NF- κ B signaling (Fig. 3b). Some tumors, such as multiple myeloma, breast cancer, and pancreatic cancer, are associated with inappropriate activation of alternative NF- κ B, [44, 56, 57]. Nevertheless, no direct tumor-enhancing

effects of IAP antagonists have been reported. However, recent work has shown that the IAP antagonist effects on the host can promote tumor growth.

Effects of IAP antagonists in bone and bone metastasis

With the bulk of research focused on the pro-apoptotic effects of IAP antagonists, their effects on the alternative

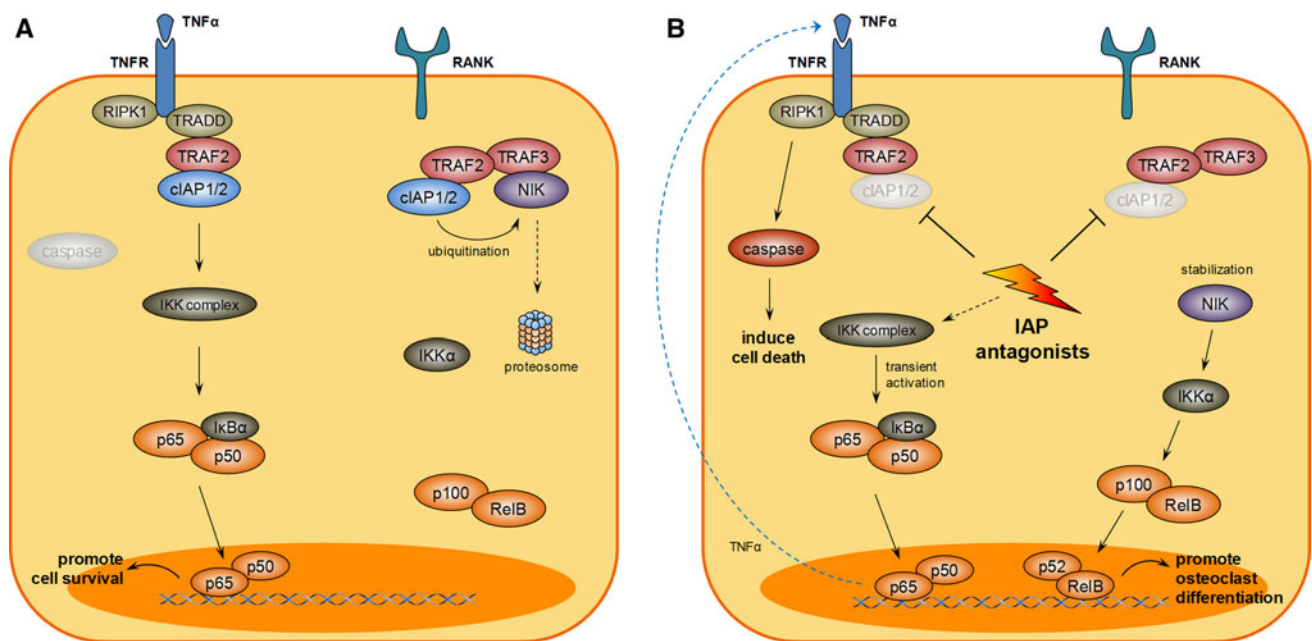


Fig. 3 IAP antagonists and NF- κ B signaling in osteoclast lineage cells. **a** NF- κ B signaling in the absence of IAP antagonists. cIAP1/2 are required to activate classical NF- κ B pathway (*left side*) downstream of TNF α , promoting cell survival. In the alternative NF- κ B pathway (*right side*), NIK is eliminated by cIAP1/2-induced proteasomal degradation, keeping this pathway off in the absence of stimulating signals. **b** NF- κ B signaling in the presence of IAP antagonists. The classical NF- κ B pathway (*left side*) is critical for IAP antagonist-induced apoptosis. In the presence of IAP antagonists,

cIAP1/2 are degraded and the IKK complex is transiently activated, resulting in a pulse of TNF α synthesis. In the absence of cIAP1/2, the new round of TNF α -initiated signaling leads to caspase activation and promotes cell death. The alternative NF- κ B pathway (*right side*) is responsible for IAP antagonists-enhanced osteoclast differentiation. IAP antagonist-induced cIAP1/2 degradation stabilizes NIK, resulting in a sustained activation of the alternative pathway, and a promotion of osteoclastogenesis

pathway have largely been ignored. In our recent studies with mouse models, both BV6, a bivalent IAP antagonist made by Genentech, and 52S, a self-synthesized monovalent IAP antagonist, caused significant decreases in trabecular bone mass within 2–4 weeks, with enhanced activity of both osteoclasts and osteoblasts [17]. The rapid kinetics are not surprising since bone is a very active organ. Other studies, using mouse models, have shown that 3 doses of RANKL decreases bone mass in 50 hours, and a 2-week treatment of anti-RANKL antibody increases bone mass [58, 59]. In *in vitro* studies, both BV6 and 52S enhanced osteoclast differentiation in not only mouse bone marrow macrophages but also human peripheral blood monocytes, suggesting a universal effect of IAP antagonists in both mouse and human. Thus, in older cancer patients, administration of IAP antagonists may accelerate the age-related deterioration in bone mineral density. This may be particularly problematic in peri- or postmenopausal women with breast cancer.

Our data indicate IAP antagonists enhance bone turnover in a fairly short period of time. Increased numbers of osteoclasts from IAP antagonist treatment can release growth factors into local microenvironment from bone matrix, creating a favorable condition in the bone for tumor growth (Fig. 4). In fact, BV6-resistant mouse breast cancer

4T1 cells grew faster when implanted into the tibias of BV6-treated mice, which were associated with extremely high levels of osteolysis locally [17]. These same tumor cells also formed more and larger bone metastases when cells were allowed to disseminate following intracardiac injection in BV6 treated mice. 4T1 tumor growth in subcutaneous and visceral locations was unaffected by the drug [17]. In experiments with the IAP-antagonist sensitive breast cancer line MDA-231, the anti-tumor efficacy of BV6 was blunted in the bone, as growth of subcutaneous tumors was blocked while bone tumors continued to grow, albeit at a slower rate than vehicle controls [17]. Together, these experiments demonstrate microenvironment-specific effects of IAP antagonists in bone.

Although the preceding experiments are purely pre-clinical, they suggest that drug-resistant tumor cells in patients might find a fertile environment in the bone, leading to formation of new bone metastases, of particular concern in breast and lung cancers which have a high propensity for bone metastasis overall. The bone marrow is thought to be an important niche for quiescent tumor cells which can potentially seed late-appearing metastases. Whether bone turnover is an important factor in the establishment of populations of quiescent tumor reservoirs in the marrow it not yet clear. However, it is certainly

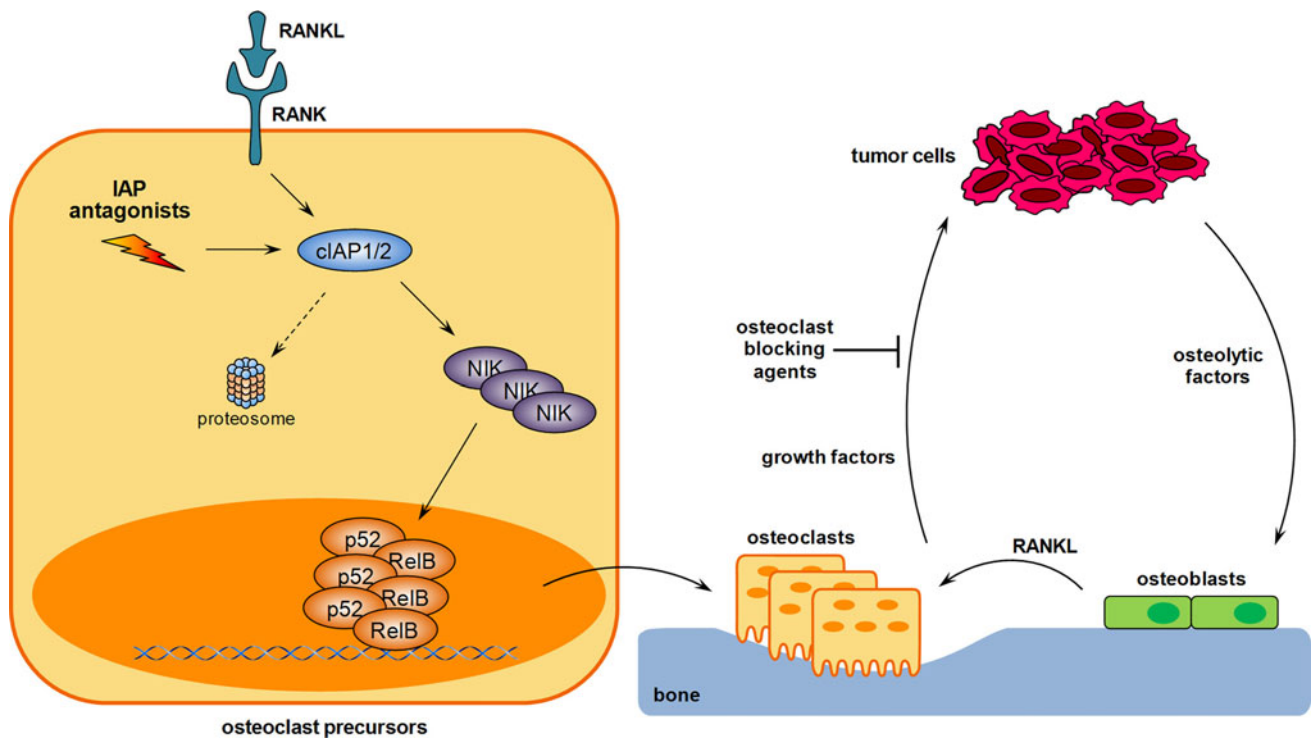


Fig. 4 IAP antagonists promote bone metastasis via activation of alternative NF- κ B pathway. The presence of IAP antagonists enhances the degradation of cIAP1/2, stabilizing more NIK, thereby increasing more osteoclastogenic signaling through p52/RelB. Elevated numbers of osteoclasts provide a favorable microenvironment

possible that IAP antagonists may impact the establishment of these long-lived cells in the marrow, and their potential for eventual formation of clinically significant metastases. Clearly considerable work remains to be done to determine whether there is any real risk for increased metastasis, either early or late, in cancer patients.

In our mouse models with IAP antagonists, increased metastases were only found in bone but not in soft tissues or visceral organs, suggesting that a bone-residing cell was the most likely culprit. In fact, the OC-blocking bisphosphonate zoledronic acid successfully reversed IAP antagonist-promoted bone metastasis, offering an approach to preserve the promise for IAP antagonists for cancer patients. Bisphosphonates are currently used in the clinic in many patients, such as those with breast cancer, to reduce the risk of bone metastasis and bone loss from chemotherapy [60]. Therefore, they may be good candidates to be used along with IAP antagonists to prevent such bone metastasis and bone loss from occurring with other cancer types, such as lung cancer, which frequently metastasize to bone (Fig. 4).

The enhanced OC activity responsible for increased bone turnover in response to IAP antagonists is due to activation of alternative NF- κ B. NIK-deficient mice were completely protected from the osteoporotic effects of BV6 treatment

for bone metastasis through release of tumor growth factors from bone matrix and a positive feedback loop from RANKL-secreting osteoblasts. Osteoclast inhibiting agents such as bisphosphonates can interrupt this vicious cycle, reducing the osteolytic and pro-tumor effects of IAP antagonists

[17]. Although activation of classical NF- κ B also occurred with BV6 treatment, this was similar in WT and NIK-deficient cultures, despite the lack of an osteoclastogenic response in the latter, and knockdown of p53 had no effect on BV6-induced OC formation. These results indicate that the specific mechanisms for IAP antagonist effects may be very tissue and cell type specific. Additionally, at least in bone, host effects result from the intended molecular mechanism of action for the compounds, and thus it may be difficult to avoid at the level of drug design. However, host effects may be mitigated by different dosing regimens or by combination therapies, such as OC inhibitors.

In light of the complex roles of IAP family proteins, beyond the regulation of apoptosis, IAP antagonists may cause a variety of unexpected effects. IAP antagonists have been shown to modulate spontaneous as well as TNF-induced inflammatory responses [61]. T cell responses and efficacy of antitumor vaccines are both augmented in response to LBW242 treatment, an IAP antagonist produced by Novartis [62]. Some types of cancers demonstrate decreased levels of IAP proteins, thus IAP antagonism may not achieve the desired outcome [43]. Agents with specific affinity to certain IAP proteins may cause different effects. For instance, BV6 ablates cIAP1/2 much more efficiently than XIAP, whereas 52S eliminates cIAP1/2 and XIAP to a

similar degree. Although the OC-related effects of these drugs appear similar, this may not be the case with other target organs. Additional functions of IAP proteins that may complicate the effects of IAP antagonists include, but are not limited to, modulation of cell migration [63], regulation of muscle integrity [64, 65], survival of pancreatic β cells [66], and development of ovarian follicles [67]. Further preclinical studies and careful evaluation of patients in clinical trials will be required to sort out the significance of these potential outcomes.

Summary

Elevated levels of IAP proteins are associated with tumorigenesis, and IAP antagonists have proven to be highly effective in inducing apoptosis of tumor cells. The classical NF- κ B pathway mediates the pro-apoptotic effects of these drugs, while the effects of activation of the alternative NF- κ B pathway by IAP antagonists has been recognized, but less well studied, especially in the context of host microenvironment. In preclinical mouse models, IAP antagonist treatment can promote bone metastasis due to enhanced OC activity, a result of over-activation of the pro-osteoclastogenic alternative NF- κ B pathway in OC lineage cells. Bisphosphonates effectively blocked IAP antagonist-promoted bone metastasis, suggesting that a combination of bisphosphonates and IAP antagonists is a possible approach to avoid the potentially significant side effect of enhanced bone metastasis. For new and ongoing clinical trials with IAP antagonists, measures should be taken to assess bone effects in human subjects including tracking bone quality using bone remodeling biomarkers and DEXA, and long term follow-up of the subjects to document possible fractures or bone metastases. Given the growing list of roles for IAP proteins, only time will tell if other host effects emerge that will impact the use, for better or worse, in cancer treatment.

Conflict of interest All authors have no conflicts of interest.

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