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Novel actions of bisphosphonates in bone: Preservation of osteoblast and osteocyte viability

Teresita Bellido^{1,2} and Lilian I. Plotkin¹

¹Dept. Anatomy & Cell Biology, Indiana University School of Medicine, Indianapolis, IN

²Div. Endocrinology, Dept. Internal Medicine, Indiana University School of Medicine, Indianapolis, IN

Abstract

Bisphosphonates stop bone loss by inhibiting the activity of bone resorbing osteoclasts. However, the effect of bisphosphonates on bone mass cannot completely explain the reduction in fracture incidence observed in patients treated with these agents. Recent research efforts provided an explanation to this dichotomy by demonstrating that part of the beneficial effect of bisphosphonates on the skeleton is due to prevention of osteoblast and osteocyte apoptosis. Work of our group, independently confirmed by other investigators, demonstrated that bisphosphonates are able to prevent osteoblast and osteocyte apoptosis *in vitro* and *in vivo*. This pro-survival effect is strictly dependent on the expression of connexin (Cx)43, as demonstrated *in vitro* using cells lacking Cx43 or expressing dominant negative mutants of the protein as well as *in vivo* using Cx43 osteoblast/osteocyte-specific conditional knock-out mice. Remarkably, this Cx43-dependent survival effect of bisphosphonates is independent of gap junctions and results from opening of Cx43 hemichannels. Hemichannel opening leads to activation of the kinases Src and extracellular signal-regulated kinases (ERKs), followed by phosphorylation of the ERK cytoplasmic target p90^{RSK} kinase and its substrates BAD and C/EBP β , resulting in inhibition of apoptosis. The anti-apoptotic effect of bisphosphonates is separate from the effect of the drugs on osteoclasts, as analogs that lack anti-resorptive activity are still able to inhibit osteoblast and osteocyte apoptosis *in vitro*. Furthermore, a bisphosphonate analog that does not inhibit osteoclast activity prevented osteoblast and osteocyte apoptosis and the loss of bone mass and strength induced by glucocorticoids in mice. Preservation of the bone forming function of mature osteoblasts and maintenance of the osteocytic network, in combination with lack anti-catabolic actions, open new therapeutic possibilities for bisphosphonates in the treatment of osteopenic conditions in which decreased bone resorption is not desired.

Keywords

bisphosphonate; osteoblast; osteocyte; apoptosis; ERKs; connexin43

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Address correspondence to: Teresita Bellido, Ph.D., tbellido@iupui.edu; Lilian I. Plotkin, Ph.D., lplotkin@iupui.edu, Department of Anatomy and Cell Biology, Indiana University School of Medicine, 635 Barnhill Dr., MS 5035, Indianapolis, IN 46202.

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Introduction

Bone fragility leading to fractures and disability is associated with the bone disease caused by glucocorticoid excess, sex steroid deficiency, and advanced age. Therefore, preservation of bone strength is key in the management of these conditions. Whereas the majority of bone strength is determined by the amount of mineral, about 40% is due to other factors including bone size, the architecture of cortical and cancellous compartments, organization of the mineral and organic components of the bone matrix, presence of microdamage, as well as the prevalence of osteocyte apoptosis [1,2]. Anti-resorptive agents, best exemplified by bisphosphonates, maintain bone strength further than what is expected based on the moderate increase in bone mineral density (BMD) that they induce [3,4]. The discovery that bisphosphonates have direct anti-apoptotic effects on osteoblasts and osteocytes, summarized in this review, provides the basis for the additional beneficial actions of these compounds beyond inhibiting osteoclast activity and halting the loss of bone mineral.

Effects of bisphosphonates on osteoblastic cell function

Bisphosphonates inhibit resorption by acting on osteoclasts to reduce their activity or to increase the rate of apoptosis [5]. As a consequence of this inhibitory effect on osteoclast function, bone formation coupled to resorption is also decreased resulting in an overall reduction in the rate of bone remodeling [6]. Several pieces of evidence support the notion that bisphosphonates also influence directly the function of cells of the osteoblastic lineage, and that this effect may in turn contribute to the reduction in osteoclast formation and activity (Table 1). Thus, early studies showed that osteoblastic cells derived from neonatal mice treated with the bisphosphonate incadronate inhibit the differentiation of bone marrow or spleen osteoclast precursors into mature osteoclasts [7]. Similarly, osteoblastic CRP10/30 cells pre-treated with various bisphosphonates inhibited resorption by rat mature osteoclasts [8]. These inhibitory effects could be reproduced by conditioned media from treated osteoblastic cells, suggesting that bisphosphonates promote the release of factor(s) that inhibit osteoclast formation and activity *in vitro* [7-9]. Consistent with these early findings, bisphosphonates have been recently shown to decrease the expression of the receptor activator of NF- κ B ligand (RANKL) and increase the expression of the RANKL decoy receptor osteoprotegerin (OPG) in human osteoblastic cells [10,11]. Taken together these findings suggest the possibility that the anti-resorptive effect of bisphosphonates mediated by osteoblastic cells is due to interference with the pro-differentiation and survival of osteoclasts induced by RANKL signaling.

Several pieces of evidence indicate that bisphosphonates modulate the proliferation and differentiation rates of cells of the osteoblastic lineage (Table 1). Some studies reveal that culture of osteoblastic cells in the presence of bisphosphonates increases proliferation, stimulates differentiation towards the osteoblastic lineage, and enhances mineralization [12-25]. Other studies show that the drugs decrease proliferation and inhibit osteoblast differentiation and mineralization [26-34], or that they inhibit proliferation and increased differentiation [35]. This apparent contradiction is explained by the fact that growth and pro-differentiation effects are exerted at lower concentrations of bisphosphonates ranging from 10^{-9} M to 10^{-6} M, whereas the inhibitory effects are found at concentrations higher than 10^{-5} M.

In vivo administration of bisphosphonates has been also shown to promote early osteoblastogenesis in mice [15] and to increase the expression of osteoblast differentiation markers and promote calvarial wound closure in rats [14]. In addition, suppression of bone turnover by bisphosphonates increases the mineral and matrix bone tissue maturity [36]. Moreover, long term administration of bisphosphonates to dogs, non-human primates, and

humans increases wall thickness, an index of focally elevated osteoblast numbers or activity, not explained by a reduction in the remodeling space [37-40]. Consistent with an increase in osteoblast activity, humans treated for 3 years with zoledronic acid exhibit increased mineral apposition rate [41]. However, other reports failed to show a change in mineral apposition rate patients treated with alendronate or pamidronate [37,42]. Overall, this evidence suggests that bisphosphonates have a positive effect on bone formation, even in the face of reduced overall bone remodeling; thus, where osteoblasts are present, more bone is made.

Apoptosis and bone homeostasis

Studies during the last decade have demonstrated that apoptosis plays a central role in the maintenance of skeletal mass and strength and have uncovered novel molecular mechanisms involved in its regulation in bone cells [43]. It is now known that all osteoclasts die by apoptosis after completing a bone resorption cycle; that the majority of osteoblasts also die whereas the remainders become lining cells or osteocytes; and that osteocytes also can die prematurely. Furthermore, it is now recognized that systemic hormones, local growth factors, cytokines and pharmacological agents, as well as physical stimuli such as mechanical forces regulate the rate of bone cell apoptosis.

Changes in the prevalence of osteoblast apoptosis could have a significant impact in the number of osteoblasts present at sites of bone formation and their function. Thus, it is now appreciated that increased osteoblast apoptosis is at least partially responsible for the decreased bone formation rate associated with the osteopenia induced by glucocorticoid excess [44]; and, conversely, inhibition of osteoblast apoptosis might contribute to the anabolic effect of intermittent administration of PTH [45,46]. In addition, mice overexpressing the anti-apoptotic protein Bcl-2 under the control of the 2.3kb fragment of the collagen1a1 promoter (col2.3kb-Bcl-2 mice) exhibit increased BV/TV in the distal femur compared to WT mice and are protected from bone loss with age, adding evidence for a beneficial effect of preventing osteoblast apoptosis [47]. However, young mice lacking caspase-3, crucial molecule for apoptosis, exhibit decreased (instead of increased) BV/TV [48]. Therefore, further investigations are warranted to clarify the relationship between osteoblast apoptosis and bone mass *in vivo*. Studies using cultured rat calvaria cells demonstrate that apoptosis increases during bone-like tissue development *in vitro* [49], consisting with apoptosis being one of the fate of differentiating osteoblasts. Interestingly, calvaria cells isolated from col2.3kb-Bcl-2 mice exhibit increased adhesion and expression of osteoblast-specific genes, but reduced mineralization [50], suggesting an inverse relationship between osteoblast survival and their mineralization capability. Nevertheless, this appear not to be the case with bisphosphonates, as administration of the drugs induce an increase, rather than decrease bone mineralization [36].

In addition, osteocytes, mature osteoblasts embedded in the mineralized matrix and responsible for the adaptive response of the skeleton to both systemic and mechanical stimuli [51], also die by apoptosis [43]. Moreover, osteocyte apoptosis is increased in conditions of increased bone fragility, such as osteoporosis due to excess of glucocorticoids [44] and sex steroid deficiency [52,53]. Furthermore, reduced mechanical loading in mice leads to osteocyte apoptosis, suggesting that physiologic levels of strain provide survival signals to osteocytes [54]. The progressive loss of bone mass and strength that ensues with age is also associated with increased osteocyte apoptosis [55]. Conversely, osteocyte apoptosis is prevented by mechanical stimulation and by sex steroids and PTH *in vitro* and *in vivo* [45,52,53,56,57].

Earlier work by our group revealed that the beneficial effects of bisphosphonates on bone are accompanied by enhanced viability of osteoblasts and osteocytes. Thus, the

bisphosphonate alendronate prevented the increase in osteoblast and osteocyte apoptosis induced by glucocorticoids in mice [58,59]. More recently, risedronate and alendronate were also shown to inhibit osteocyte apoptosis in this case induced by fatigue cyclic loading in rats [60]. Delayed osteoblast apoptosis might result in an increased work time of osteoblasts leading to the gradual increase in trabecular thickness observed in animals or patients upon long-term treatment with bisphosphonates [37-40]. Preservation of osteocyte viability might contribute to the maintenance of the osteocyte network. Both effects could explain part of the anti-fracture efficacy of bisphosphonates.

Bisphosphonates affect osteoblasts/osteocytes and osteoclasts by different mechanisms

Our earlier work [58] as well as more recent evidence by us and other investigators [59,61-65] established that protection from apoptosis results from direct activation of survival signaling triggered by bisphosphonates on osteoblastic and osteocytic cells (Table 1). Mechanistic studies using osteocytic MLO-Y4 cells and primary murine calvaria cells demonstrated that bisphosphonates inhibit apoptosis induced by the glucocorticoid dexamethasone, the inhibitor of DNA repair etoposide that blocks topo-isomerase II activity, or TNF α , an activator of death receptors, [58] suggesting that bisphosphonates inhibits cell death, independently of the pro-apoptotic pathway activated.

As reviewed elsewhere in this issue, the mechanism by which bisphosphonates affect osteoclasts involves intracellular accumulation of high concentrations of the drugs. In the case of amino-bisphosphonates, this leads to inhibition of farnesyl pyrophosphate synthase, an enzyme crucial for osteoclast function [66]; whereas for non-amino-bisphosphonates, it results in the formation of toxic metabolites. In both cases, intracellular accumulation of the drugs inhibits osteoclast function and can lead to osteoclast apoptosis (Figure 1). In contrast to their actions on osteoclasts, the pro-survival effect of bisphosphonates on cells of the osteoblastic lineage is exerted at concentrations ranging from 10^{-11} to 10^{-6} M, which are several orders of magnitude lower than the ones required to inhibit osteoclast activity [66-69]. Even though bisphosphonates bind with high affinity to mineral, there is a continuous release of low levels of the drugs (as discussed elsewhere in this issue). This may result in a constant supply of bisphosphonates at low concentrations, which could maintain osteoblast and osteocyte viability. Remarkably, this survival effect is lost at high concentrations. Similar biphasic pattern of responsiveness has been shown for other responses of osteoblastic cells to the drugs, including the increases in proliferation, stimulation of formation of colony forming units for fibroblasts and osteoblasts, as well as stimulation of mineralization and alkaline phosphatase activity [12,15,23].

Another difference between the action of bisphosphonates on osteoblastic cells versus osteoclasts is that pro-survival of osteoblasts and osteocytes is exerted by all bisphosphonates, non-amino as well as amino-analogs, at similar effective doses ($EC_{50} \sim 10^{-10}$ M) [58,69]. Remarkably, anti-apoptosis by bisphosphonates appear to be unrelated to the anti-resorptive properties of these agents, as IG9402, NE11809, and several other bisphosphonates that do not affect osteoclast activity, also exhibit anti-apoptotic properties on osteoblastic and osteocytic cells *in vitro* [58,62,67-69]. As discussed below, recent evidence demonstrates that administration to mice of IG9402, one of these compounds that do not affect osteoclasts, prevented the increase in osteoblast and osteocyte apoptosis induced by glucocorticoid excess as effectively as alendronate without decreasing resorption (refer to manuscript to be submitted for this issue).

All this evidence indicates that the pro-survival effect of bisphosphonates on cells of the osteoblastic lineage is mediated by a different mechanism of action than the one by which

these drugs affect osteoclasts; and that the actions on osteoblasts and osteocytes can be dissociated from the ones on osteoclasts with novel analogs.

How do bisphosphonates trigger survival signaling in osteoblastic cells?

Studies using cultured cells showed that bisphosphonates induce the activation of the ERKs, recognized survival kinases (Figure 1). Thus, all bisphosphonates examined rapidly induce phosphorylation of ERKs in osteocytic cells as well as primary cultures of osteoblastic cells, independently of their structure, presence of amino residues in the molecule, or ability to inhibit osteoclast function [58]. Increased levels of phosphorylated ERKs were found also in bones of animals that had received a single injection of alendronate or IG9402 (shown elsewhere in this issue). Mechanistic studies have demonstrated that ERK activation is required for the prevention of osteoblast and osteoclast apoptosis by bisphosphonates, as pharmacological or genetic inhibition of MEK, the kinase that activates ERKs, abolished the anti-apoptotic effect of bisphosphonates [58,61].

Similar to bisphosphonates, ligand-induced activation of sex steroid receptors also attenuates apoptosis of osteoblasts and osteocytes by a mechanism that requires ERK activation [53]. However, the molecular events that lead to activation of these survival kinases by estrogens or androgens are different from the ones involved in bisphosphonate action. Thus, the sex steroids activate ERKs via an extra-nuclear function of their receptors that can be mediated by the ligand binding domain. In addition, kinase-dependent activation of transcription factors and gene transcription is required for the anti-apoptotic effect of sex steroids on osteoblastic cells [70,71]. On the other hand, anti-apoptosis induced by bisphosphonates requires neither transcription nor ERK-dependent transcription factors [70]. Instead, ERK-mediated anti-apoptosis induced by bisphosphonates requires the kinase activity of the cytoplasmic target of ERKs, p90^{RSK}, which in turn phosphorylates the pro-apoptotic protein BAD and the CCAAT/enhancer binding protein C/EBP β . Phosphorylation of BAD renders it inactive, whereas phosphorylation of C/EBP β leads to binding of pro-caspases, thus inhibiting apoptosis independently of the transcriptional activity of this transcription factor (Figure 1). Consistent with the evidence that estrogens and bisphosphonates phosphorylate diverse targets of ERKs probably resulting from activation of spatially distinct pools of these kinases, the two agents had additive effects on osteocyte survival [70].

Cx43 hemichannel opening by bisphosphonates leads to activation of the Src/ERK signaling pathway in osteoblasts and osteocytes

Binding of respective ligands to receptors for growth factors, cytokines, and hormones as well as engagement of integrins by extracellular matrix proteins, trigger molecular events that culminate in ERK activation. In the case of bisphosphonates, no receptors have been identified. Therefore, we probed into the mechanism by which these drugs activate ERKs by exploring the possibility that bisphosphonates affected the permeability of channels formed by connexin (Cx) 43, the major member of the connexin family of proteins expressed in osteoblasts and osteocytes [72-75].

Connexin hexamers form plasma membrane channels, named hemichannels. Hemichannels of adjacent cells can align to form a gap junction channel. Intercellular communication among osteocytes and with osteoblasts is maintained through gap junctions: clusters of thousands of channels that open only transiently to allow the exchange of small (<1 kDalton) water-soluble molecules [76]. Hemichannels are also present in unopposed cell membranes and their opening allows exchange of cytoplasmic contents with the extracellular fluid [76,77].

Using mouse embryonic fibroblasts, osteoblastic cell lines, and authentic osteoblasts expressing or deficient in Cx43, we demonstrated that Cx43 is required for induction of cell survival by bisphosphonates [61]. Furthermore, we found that Cx43 is present in non-junctional osteocyte membranes, and that exposure to bisphosphonates of osteoblastic and osteocytic cells results in opening of hemichannels without affecting gap junction communication. Whether bisphosphonates, due to their small molecular size, enter osteoblastic cells upon inducing opening of the hemichannels still remains unknown.

Transfection of Cx43, but not other connexins, into Cx43-naïve cells was also able to confer *de novo* responsiveness to bisphosphonates [61]. By transfecting mutants of Cx43, we found that the signal-transducing property of Cx43 requires the trans-membrane pore-forming region of the protein. In addition, survival by bisphosphonates depends on the presence of the C-terminal cytoplasmic domain of Cx43, by which the protein interacts with Src kinase, an upstream activator of ERKs. Using genetic and pharmacological approaches, we determined that Src expression, its kinase activity, and the SH2 and SH3 domains are required for ERK activation and prevention of osteoblast and osteocyte apoptosis by bisphosphonates. In more recent work, we have shown that Cx43 forms a complex with β -arrestin that retains activated ERKs in the cytoplasm, enabling exclusive activation of ERK cytoplasmic substrates, such as p90^{RSK} [78]. Cx43 is also required for the protective effect of bisphosphonates on osteoblastic cells *in vivo*, as alendronate was not able to prevent apoptosis in mice lacking Cx43 in osteoblasts and osteocytes [59]. This evidence adds Cx43 to the list of trans-membrane molecules capable of transducing survival signals in response to extracellular cues and raises the possibility that Cx43 may serve in this capacity for endogenously produced molecules or even other drugs [61,77].

Bisphosphonates have been shown to specifically bind to osteoblastic cells [79]. The requirement of Cx43 for anti-apoptosis by bisphosphonates have raised the possibility that interaction of bisphosphonates with Cx43 present in the cell membrane results in hemichannel opening, thereby initiating intracellular survival signaling. However, we found that, although Cx43 is necessary for bisphosphonate-induced survival of osteoblastic cells, this protein is dispensable for bisphosphonate cell binding [80]. Thus, bisphosphonates can bind to cells lacking Cx43 or cells in which connexin channels have been disrupted, suggesting that the drugs bind to another moiety that, in turn, interacts with Cx43. Because bisphosphonate binding to osteoblastic cells is displaced by protein phosphatase substrates, the unknown molecule which links bisphosphonates with Cx43 could be a phosphatase. Future studies need to be pursued towards the identification of bisphosphonate receptor proteins in osteoblastic cells.

A bisphosphonate that does not affect osteoclasts prevents osteoblast and osteocyte apoptosis and the loss of bone strength induced by glucocorticoids

We tested directly whether prevention of osteoblast and osteocyte apoptosis by bisphosphonates, in the absence of an anti-resorptive effect, had a beneficial effect on the skeleton. Towards this end, we examined the effect on glucocorticoid-induced bone disease of administration of the bisphosphonate analog IG9402, which we have previously shown *in vitro* that prevents osteoblast and osteocyte apoptosis but does not decrease osteoclast viability [69]. As shown in this issue in a separate manuscript (refer to manuscript submitted), unlike alendronate that decreases circulating levels of bone resorption and formation markers, administration of IG9402 did not affect bone remodeling. In full agreement with the *in vitro* data and in spite of its lack of anti-remodeling effect, IG9402 was as effective as alendronate in preventing glucocorticoid-induced osteoblast and

osteocyte apoptosis. Moreover, the decrease in bone mineral density, bone volume, osteoblast number and bone formation rate induced by glucocorticoids in vertebral cancellous bone were all preserved by IG9402. In addition, the ability of IG9402 to protect osteoblasts and osteocytes from the pro-apoptotic effect of glucocorticoids was associated with preservation of bone strength as assessed by mechanical testing. These findings demonstrate that osteoblast and osteocyte apoptosis can be selectively prevented by bisphosphonate analogs, opening new avenues for the treatment of bone fragility without significantly affecting the rate of bone remodeling. The outcome of long-term administration of these analogs warrants further investigation.

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Figure 1. Bisphosphonates prevent osteoblast and osteocyte apoptosis and inhibit osteoclast activity via different mechanisms

For simplicity, only one of the six molecules of Cx43 that form the hemichannel is shown. N-BPs, amino-bisphosphonates; non N-BPs, non amino-bisphosphonates. See text for details.

Table 1
Effect of bisphosphonates on osteoblastic cells

effect		cell model
osteoblast-mediated inhibition of osteoclast differentiation and resorption	decreased osteoclasts and resorption	murine calvaria cells [7], rat CRP 10/30 cells [8,9]
	reduced RANKL and increased OPG expression	primary human osteoblastic cells [10,11]
osteoblast proliferation	increased	primary human [19,21,22] and rat [23] osteoblastic cells, human MG-63 [22,24] and rat ROS17/2.8 cells
	decreased	murine MC3T3-E1 cells [13], human fetal osteoblastic cells [35], primary murine [27] and rat [28] calvaria cells, primary human adult [30,31,33] and fetal osteoblastic cells [32]
osteoblast differentiation and/or mineralization	increased	chick periosteal calvaria explants [12], murine and human bone marrow cells [15], rat calvaria bone [14], primary human osteoblastic cells [10,19,21,22,25], rat organ cultures [17], mouse [20] and human [34] mesenchymal stem cells, human osteoblast-like periosteal cells [26], murine MC3T3-E1 [13,16,17] and ST2 [17] cells, human Saos-2 [18] and MG-63 [22,24] cells
	decreased	human mesenchymal stem cells [34], primary human osteoblastic cells [10,30,31,33], murine [27] and rat [28] calvaria cells, human bone marrow stromal cells [29], chick periosteal calvaria explants [12]
osteoblast/osteocyte apoptosis	inhibited	Murine MLO-Y4 osteocytic [58,59,62,65], MC3T3E1 [64], Ob-6 [59] cells, embryonic fibroblasts [61], and primary calvaria cells [58,61], human MG63 [63] and primary osteoblast-like [63] cells, rat ROS17/2.8 [61] and UMR106 [64] cells
	increased	primary human adult [19,25] and fetal osteoblastic [35] cells, human bone marrow stromal cells [29], primary murine calvaria cells [27]