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In vitro and *in vivo* studies using non-traditional bisphosphonates

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Abstract

Non-traditional bisphosphonates, that is, bisphosphonates that do not inhibit osteoclast viability or function, were initially reported in the 1990s by Socrates Papapoulos' group. Originally designed to study the role of the R1 residue of aminobisphosphonates on bisphosphonate affinity for hydroxyapatite, these modified bisphosphonates retained similar affinity for mineralized bone as their parent compounds, but they lacked the potential to inhibit the mevalonate pathway or bone resorption. We found that, similar to classical bisphosphonates, these non-traditional compounds prevented osteoblast and osteocyte apoptosis *in vitro* through a pathway that requires the expression of the gap junction protein connexin 43, and the activation of the Src/MEK/ERK signaling pathway. Furthermore, one of those compounds named IG9402 (also known as amino-olpadronate or lidadronate), was able to inhibit osteoblast and osteocyte apoptosis, without affecting osteoclast number or bone resorption *in vivo* in a model of glucocortidoid-induced osteoporosis. IG9402 administration also ameliorated the decrease in bone mass and in bone mechanical properties induced by glucocorticoids. Similarly, IG9402 prevented apoptosis of osteoblastic cells in a model of immobilization due to hindlimb unloading. However, in this case, the bisphosphonate was not able to preserve the bone mass, and only partially prevented the decrease in bone mechanical properties induced by immobilization. The effect of IG9402 administration was also tested in a mouse model of masticatory hypofunction through the induction of masseter muscle atrophy by unilateral injection of botulinum toxin type A (BoNTA).

IG9402 partially inhibited the loss of trabecular bone microstructure in the mandibular condyle, but not the decrease in masseter muscle mass induced by BoNTA administration. In summary, these non-traditional bisphosphonates that lack anti-resorptive activity but are able to preserve osteoblast and osteocyte viability could constitute useful tools to study the consequences of preventing apoptosis of osteoblastic cells in animal models. Furthermore, they could be used to treat conditions associated with reduced bone mass and increased bone fragility in which a reduction of bone remodeling is not desirable.

Introduction

Bisphosphonates have been used as anti-resorptive agents for over 40 years, not only to treat osteoporosis, but also for other conditions including osteogenesis imperfecta, primary bone tumors and bone metastases [1]. However, early observations made by physicians led to the idea that the small increase in bone mass induced by bisphosphonates could not completely explain the potent anti-fracture efficacy of these agents. Based on these observations, we tested the hypothesis that part of the bisphosphonate anti-fracture efficacy is due to prevention of osteoblast and osteocyte apoptosis [2]. Maintaining osteoblast viability would lead to an increase in bone-forming activity and preservation of osteocyte viability should increase the mechanosensory function of these cells, resulting in improved bone strength. To test this hypothesis, we and others used modified bisphosphonates, first described in the 1990s [3, 4], which still bind to the bone mineral but lack the ability to decrease osteoclast number and function. Therefore, these compounds, named non-traditional bisphosphonates, should lack anti-resorptive effects. As detailed in this manuscript, unlike the traditional amino-bisphosphonates, the non-traditional compounds do not inhibit enzymes of the mevalonate pathway but still maintain the ability to prevent osteoblast and osteocyte apoptosis [5, 6]. Further, one of the compounds tested, IG9402 (also known as amino-olpadronate or lidadronate), was able to prevent, at least partially, the deleterious effects of glucocorticoids and immobilization in bone [2], as well as the consequences of masticatory muscle atrophy in the mandible. In this review, we will describe the published evidence for the efficacy of these novel bisphosphonates *in vitro* and *in vivo*.

Early studies using modified classical bisphosphonates

Bisphosphonates consist of a basic P-C-P (phosphate-carbon-phosphate) structure and two side chains bound to the carbon molecule, named R1 and R2. These two side chains can be modified, providing different properties to the bisphosphonates. Originally, it was thought that the R1 chain was responsible for the binding to calcium in the hydroxyapatite molecules, whereas the R2 conferred activity to the bisphosphonate [1]. R1 could be -Cl, as in chlodronate, or -OH, as in eitdronate and amino-bisphosphonates such as alendronate and olpadronate. Early work by Socrates Papapoulos' group aimed to test the effect of substituting the –OH group in the R1 chain by an $-NH_2$, with the hypothesis that this change should not alter either the binding of the compounds to the mineral or the biological activity [3]. They found that, indeed, replacing the –OH by an $-NH_2$ group in etidronate or pamidronate did not alter the affinity for hydroxyapatite, whereas amino-olpadronate (named IG9402 in this report) exhibit a slight reduction in affinity to bone mineral when compared

to olpadronate. On the other hand, IG9402 did not inhibit bone resorption *in vivo*, as measured by ^{45}Ca released from fetal metacarpals [3] nor did alter the growth of the cellular slime mould Dictyostelium discoideum, a model used to test bisphosphonate potency [4]. Further, later *in vitro* studies showed that, unlike olpadronate, IG9402 does not induce the activation of γδT cells, which highly correlate with the potency of the bisphosphonate to inhibit enzymes of the mevalonate pathway [6].

IG9402, a model non-traditional bisphosphonate that has similar activity and potency as alendronate on osteoblastic cells

Prompted by evidence suggesting that the anti-fracture efficacy of bisphosphonates could not be completely explained by the mild increase in bone mass resulting from bisphosphonate administration, we hypothesized that, in addition to their recognized anti-resorptive properties through inhibition of osteoclasts, bisphosphonates exhibit beneficial effects on osteoblasts and osteocytes. In particular, we tested *in vitro* whether the drugs are able to prevent osteoblastic cell apoptosis. We found that both non-amino- (etidronate) and amino- (alendronate, olpadronate, pamidronate) bisphosphonates were able to prevent apoptosis induced by the topoisomerase II inhibitor etoposide, the glucocorticoid dexamethasone, and the activator of death receptors TNFα [7]. This survival effect of bisphosphonates was independent of inhibition of the mevalonate pathway, as it was also exerted by IG9402, and was achieved at concentrations 3-orders of magnitude lower than the ones required to induce osteoclast apoptosis *in vitro*. Bisphosphonates induced the activation of the extracellular signaling regulated kinases ERKs, and inhibition of the kinase prevented the anti-apoptotic effect of these drugs.

The dissociation between the anti-osteoclastic effect and the inhibition of osteoblast and osteoclast apoptosis was later confirmed using another modified bisphosphonate, NE11809, which has limited effect on the enzymes of the mevalonate pathway [8], as well as by 5 additional compounds that do not induce osteoclast apoptosis even at concentrations as high as 10^{-4} M [5].

A novel mechanism of action of bisphosphonates independent of the inhibition of the mevalonate pathway.

The survival effect of bisphosphonates on osteoblastic cells is associated with rapid and transient activation of the survival kinases ERKs in cultured cells (Figure 1) [7]. ERK activation was also seen in bone preparations of mice treated with a single injection of alendronate or IG9402 [9]. In addition, pharmacological and genetic inhibition of ERK activation prevented the anti-apoptotic effect of the bisphosphonates [7, 10]. Further mechanistic studies showed that bisphosphonates activate a signaling pathway involving the kinases Src and MEK upstream of ERK activation. ERKs, in turn, activate the cytoplasmic kinase p90^{RSK}, which phosphorylates the pro-apoptotic protein BAD, rendering it inactive; and C/EBPβ, creating a caspase-binding box that inhibits caspase activation [10, 11]. All these signaling events occur in the cytoplasm and are independent of new gene transcription [11]. Moreover, forced expression of ERKs in the nuclei prevents bisphosphonate survival

effect on osteocytic cells. This signaling pathway contrasts with the one activated by estrogens, which also requires transient ERK activation, but it is dependent on new gene transcription and requires ERK-mediated gene transcription.

ERK activation results from binding of ligands, including growth factors, hormones, and cytokines to their respective receptors. However, no receptor has been identified for bisphosphonates. Connexin (Cx) channels and, in particular, Cx43 are potential candidates to mediate bisphosphonate effects, since they are membrane structures that form channels allowing the passage of water-soluble molecules smaller than 1 kD, and that favor negatively charged compounds (such as bisphosphonates) [12]. Six Cx43 molecules form hemichannels in unopposed cell membranes, able to mediate the exchange of molecules between the cytoplasm and the extracellular milieu [13]. In addition, hemichannels in neighboring cells align to form gap junction channels that mediate cell-to-cell communication. To test the role of connexins on bisphosphonate action, we utilized pharmacologic inhibitors, genetic tools including connexin encoding and dominant-negative constructs, as well as primary cells and cell lines expressing or not connexins [10]. We found that Cx43, but not other connexins, conferred responsiveness to bisphosphonates to connexin-deficient cells. Inhibition of connexin channels *in vitro* or deletion of Cx43 *in vitro* or *in vivo*, abrogated the anti-apoptotic effect of bisphosphonates [10, 14]. Further, Cx43 hemichannels, and no gap junctions, mediate the effect of these drugs in osteoblastic and osteocytic cells [10]. However, although the drugs bind to saturable, specific and high-affinity binding sites in the cells, Cx43 is not required for bisphosphonate binding [15, 16]. Interestingly, bisphosphonates inhibit phosphatases in the cell membrane, and their binding to the cells is displaced by phosphatase substrates. This evidence suggests bisphosphonates bind to phosphatase in the cell membrane, triggering intracellular signaling leading to preservation of cell viability.

Additional mechanistic studies showed that the survival effect of bisphosphonates requires both the transmembrane domain and the cytoplasmic tail of Cx43, and that formation of a complex comprising Cx43, βarrestin, and Src/MEK/ERK is required to retain ERKs in the cytoplasm and to prevent osteoblast and osteocyte apoptosis (Figure 1) [10, 17].

Non-classical effects of bisphosphonates - *in vivo* approaches

In vivo studies confirmed the lack of IG9402 anti-resorptive activity. Indeed, whereas daily alendronate injections reduced both bone formation and bone resorption circulating markers, as well as osteoblast and osteoclast numbers and bone formation rate in vertebral bone, IG9402, administered at an equimolar dose did not alter any of these parameters [9]. However, as found *in vitro*, the IG9402 was as effective as alendronate in preventing osteoblast and osteocyte apoptosis in a murine model of glucocorticoid-induced bone disease. Further, both alendronate and IG9402 prevented glucocorticoid-induced loss of vertebral bone mass and strength.

In another *in vivo* study in which bone disease was induced by hindlimb unloading, IG9402 did not reverse the increase in bone resorption, whereas alendronate decreased circulating levels of the resorption marker CTX and osteoclast surface *in vivo* and osteoclastogenesis *ex*

vivo in both ambulant control and tail suspended mice [18]. On the other hand, both bisphosphonates were equally able to prevent osteoblast and osteocyte apoptosis, and partially prevented the reduction in bone strength induced by immobilization.

Taken together, these studies suggest that preservation of osteoblast and osteocyte apoptosis contributes to the overall effect of bisphosphonates in bone.

A new application for non-classical bisphosphonates: preservation of the mandibular bone

The botulinum toxin type A (BoNTA), an agent that induces skeletal muscle paralysis by selectively blocking the release of acetylcholine in the neuromuscular junction, is widely used in dentistry for aesthetic and therapeutic purposes [19]. In particular, BoNTA is intramuscularly injected in the masticatory muscles (i.e. the masseter and the temporalis muscles) as an *off-label* use to manage undesirable consequences of sleep bruxism (teeth grinding) such as the loss of dental hard tissue and myofascial pain [19, 20]. However, the masticatory muscles hypofunction induced by BoNTA modifies the bone remodeling process of the mandible in adult individuals [21–23]. In adult mice, the unilateral injection of BoNTA in the masseter muscle causes its atrophy and results in both bone loss and morphological alterations of the mandibular condyle from the experimental side, two weeks after intervention [24, 25]. For these studies, we performed 2D histological assessment at the mandibular condyle and found that the paralyzed side shows a significant reduction of the bone area per tissue area (B.Ar/T.Ar) and the trabecular thickness (Tb.Th) [24]. In addition, 3D evaluation of the mandibular condyle in this animal model was performed [25]. We found that, in addition to shape changes, BoNTA administration resulted in a significant reduction of the bone volume fraction (BV/TV) and Tb.Th, as determined from high-resolution X-Ray microtomography (microCT) data (DIONDO d3 microCT system, DIONDO GmbH, Hattingen, Germany; scan parameters: voxel size, 5.11 μm, isotropic; voltage 80 kV; current 60 mA;0.5 mm aluminium filter) [25]. Figure 2A shows the volume of interest (VOI), i.e. the mandibular condyle (See Supplementary video 1 for a 360° view) obtained from the 3D rendering of a complete mouse mandible sample. The 3D characterization of the Tb.Th in the mouse mandibular condyles was performed using Paraview v.5.4.1 [26] and can be observed in the Supplementary video 2 (mandibular condyle from intra-individual control side, i.e. saline injected side) and Supplementary video 3 (mandibular condyle from BoNTA injected side), that shows both VOI (experimental and intra-individual control) from the same representative animal. Using this preclinical model, the IG9402 was preliminarily tested as a systemic co-intervention during the masseter muscle atrophy induced by BoNTA, in order to determine its effects on the mandibular condyle subchondral bone.

To achieve this aim, 9-week-old male BALB/c mice received one BoNTA injection (0.2 U/10 μl; *Onabotulinumtoxin A*; BOTOX®, Allergan Chile) in the right masseter muscle and the same volume of BoNTA vehicle (saline solution) in the left masseter muscle (Day 0). All animals were randomly located in three different groups, according to the intervention/co-intervention approach, as follows: BoNTA (n=6); BoNTA + vehicle (n=6), BoNTA +

IG9402 (n=5). A group of mice with the same age and sex (n=5) was used as control of the systemic administration of IG9402, without BoNTA intervention. IG9402 (0.6 mg/kg/day) or its vehicle (saline solution) were administered daily as subcutaneous injection, starting three days before BoNTA intervention. The mandibular samples were collected 14 days after single intramuscular injection of BoNTA and were analyzed using microCT [27], as previously described [25]. BV/TV and Tb.Th were quantified using BoneJ [28]. All procedures were approved by the local ethical committee at the Universidad de Chile (certificate N° 17011-OD-UCH).

Both BV/TV and Tb.Th were significantly reduced in the mandibular condyles from BoNTA-injected sides in the BoNTA, BoNTA + vehicle and BoNTA + IG9402 groups (Figure 2B). In the latter group, however, the co-intervention with IG9402 partially prevented the loss of BV/TV by 36 % (p-value < 0.05), after analyzing the differences (deltas) between sides. Nevertheless, using the same analysis, the reduction in Tb.Th was not rescued in the mandibular condyles from BoNTA-injected sides when compared with vehicle, in both BoNTA + vehicle and BoNTA + IG9402 groups (Table 1). Interestingly, a significant increase of BV/TV was detected between both sides of IG9402 control group (without BoNTA intervention) and the control side (saline-injected) of the BoNTA group (Figure 2B).

To examine the effect of the treatments on cell viability, paraffin sections were obtained and immunostained for active caspase3, following established protocols [29]. Qualitative analysis of the histological sections stained for active caspase3 indicate an increased proportion of active caspase3 negative (alive) osteocytes in the BoNTA + IG9402 group compared to BoNTA + vehicle group, 14 days after administration (Figure 3). Current studies are under way to quantify the effect of the treatments on cell survival (caspase3 activation).

In addition, a qualitative improvement of the Tb.Th distribution in the central region of the mandibular condyle (BoNTA-injected side) in the BoNTA + IG9402 group was seen when the three-dimensional rendering of Tb.Th in the BoNTA groups was compared (Figure 4). Taken together, these results suggest that IG9402 prevents bone loss in the mouse mandibular condyle during the masseter muscle atrophy induced by BoNTA.

Another potential application of the non-classical bisphosphonate is the prevention of bisphosphonate-related osteonecrosis of the jaw (BRONJ). Indeed, in dentistry, BRONJ is a complication of the long-term use of traditional bisphosphonates such as zolendronate, with a high prevalence after any bone or teeth trauma at mandibular level (e.g. oral surgery) [30]. The cellular and molecular mechanisms of the BRONJ are not still fully understood. In rodents, zoledronate impairs bone healing in the mandible after tooth extraction, by reducing the vascularization and by altering the bone remodeling process [31–33]. One of the potential explanations of these deleterious effects is the inhibition of the formation and the maturation of osteoclasts, promoted by the traditional bisphosphonates (e.g. zoledronate) [34]. The osteoclasts are required for the proper vascularization of the newly formed bone during bone healing/remodeling [35]. Therefore, the fact that the bisphosphonate analog IG9402 does not affect osteoclast viability but prevents osteoblast/osteocyte apoptosis [9,

18] represents a promising alternative to the treatment of patients under BRONJ risk due to specific oral conditions that require surgical interventions or alter the mandibular bone remodeling. However, additional studies are required to test the possibility of using compounds such as IG9402 in order to reduce the risk of osteonecrosis of the jaw after invasive dental procedures.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Highlights

- Bisphosphonates (BPs) are potent inhibitors of osteoclastic bone resorption.

- BPs also prevent osteoblast/osteocyte apoptosis *in vitro* and *in vivo*.

- BP analogs do not inhibit enzymes of the mevalonate pathway or osteoclast activity.

- These non-traditional BPs inhibit osteoblastic cell apoptosis *in vitro* and *in vivo*.

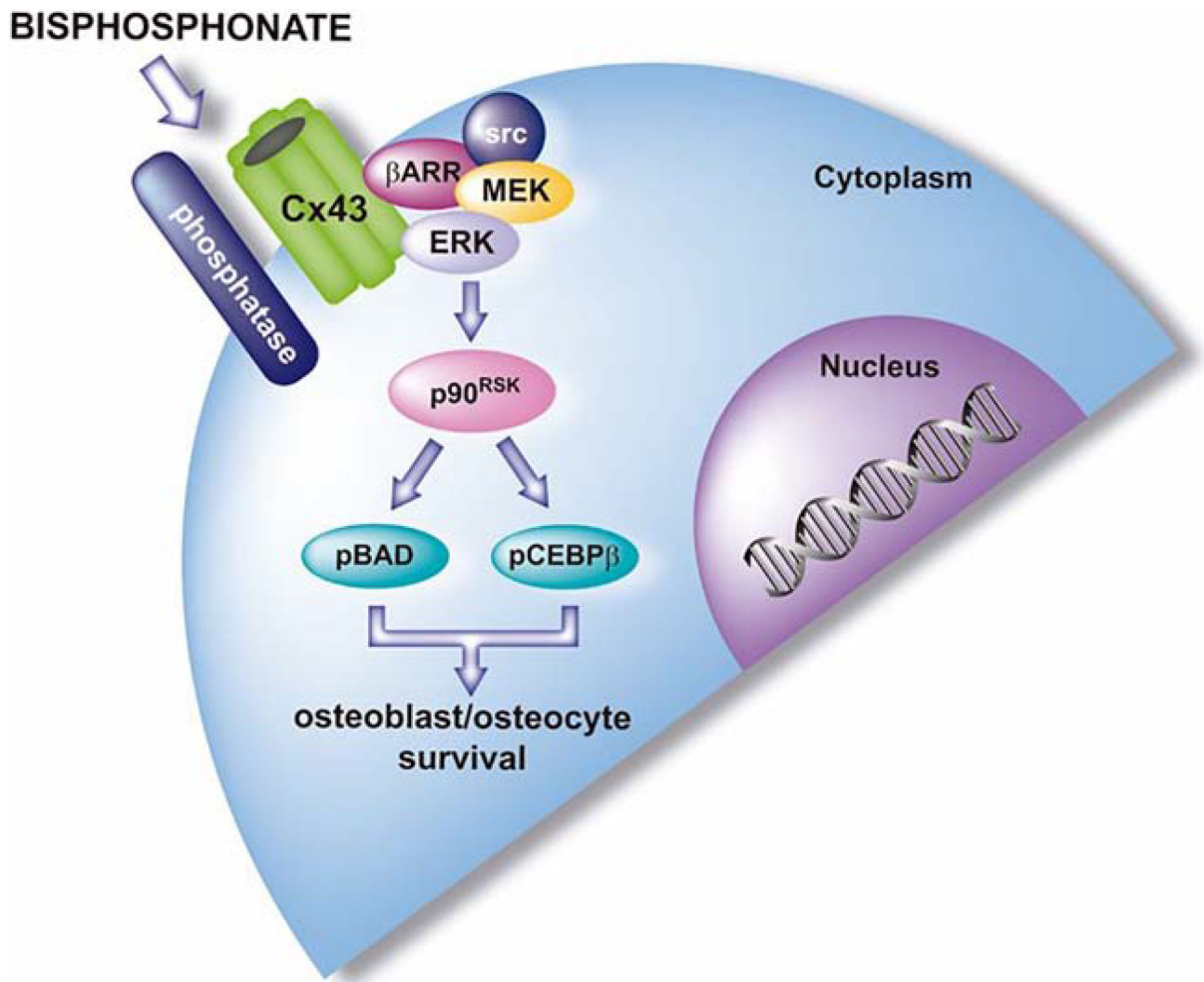- BP analogs are useful to set the role of osteoblastic cell death in animal models.

Figure 1. Model of action of bisphosphonates on osteoblasts and osteocytes.
Bisphosphonates, likely through binding to a membrane-bound phosphatase, induce the opening of Cx43 hemichannels, leading to the activation of the Src/MEK/ERKs signaling pathway in the cytoplasm of osteoblasts and osteocytes. Activated ERKs phosphorylate p90^{RSK}, which in turn phosphorylates and inactivates the pro-apoptotic protein BAD. p90^{RSK} also phosphorylates C/EBPβ leading to the formation of a caspase-binding domain that prevents caspase activation and apoptosis.
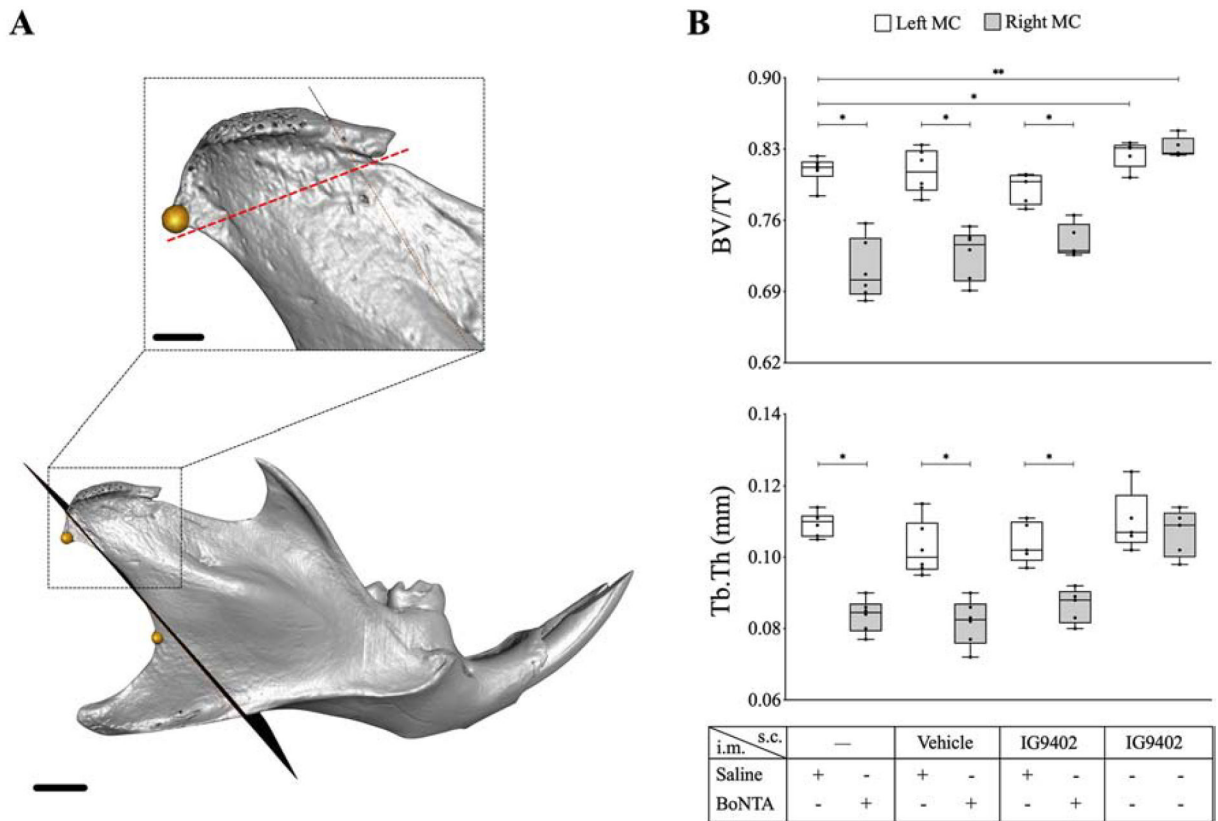
Figure 2. Systemic co-intervention with IG9402 during the BoNTA-induced masseter muscle atrophy partially prevents the bone loss in the mandibular condyle, 14 days after intervention. (A) A representative image of the volume of interest (VOI; mandibular condyle; *Scale bar: 500 μm*) obtained after 3D rendering of the mouse mandible (*Scale bar: 1 mm.*) is shown. (B) The masticatory hypofunction caused by the masseter muscle atrophy after BoNTA intervention results in a significant reduction of the 3D bone parameters (BV/TV and Tb.Th) in all BoNTA groups (BoNTA, BoNTA + vehicle and BoNTA + IG9402), when compared between sides (BoNTA versus Saline, intragroup comparison). The IG9402 significantly increased de BV/TV of the mandibular condyles from both sides in the control group (without BoNTA intervention) compared with the control side of BoNTA group (n = 5–6; Min to Max; *: *p*-value < 0.05; Wilcoxon matched-pairs signed rank test between sides of the same group; Mann Whitney test between sides from different groups). *Abbreviations: MC, mandibular condyle; BoNTA, botulinum toxin type A; BV/TV, bone volume fraction; Tb.Th, trabecular thickness; i.m. intramuscular; s.c. subcutaneous.*
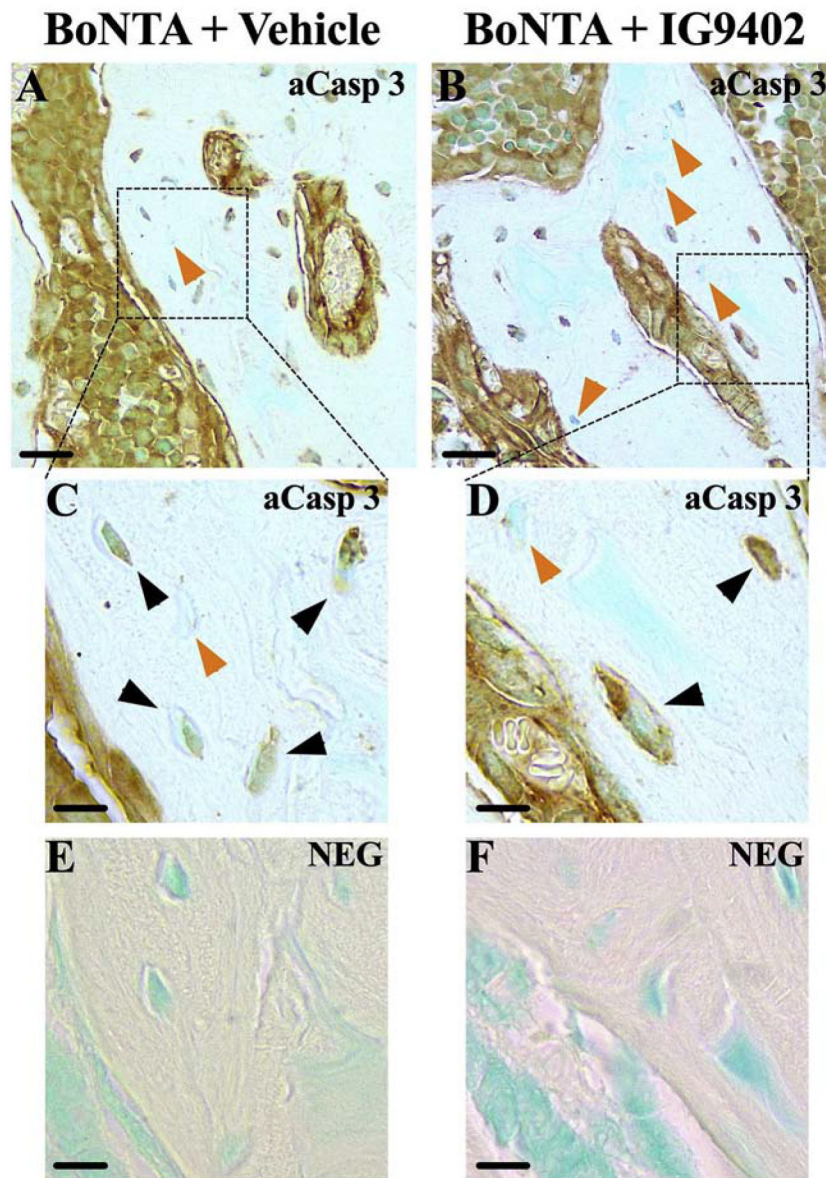
Figure 3. The bisphosphonate IG9402 reduces osteocyte apoptosis in mandibular condyle evoked by masseter muscle paralysis.

Representative digital 40X images (LEICA ICC50W, Leica Mycrosystems) of subchondral bone (middle portion of the mandibular condyle, coronal plane, plane of section indicated in Figure 2A) showing active Caspase3 (aCasp3) negative osteocytes (alive; orange arrow heads) in experimental (BoNTA) sides from both groups, BoNTA + vehicle (**A**) and BoNTA + IG9402 (**B**). *Scale bar: 25 μm.* (**C** and **D**) Detailed 100X digital images of representative zones (dotted line square) from **A** and **B**, respectively, showing both alive (orange arrow heads) and apoptotic (aCasp3 positive; black arrow heads) osteocytes. (**E** and **F**) Negative control (non-reactive IgG) of **C** and **D**, respectively. *Scale bar: 5 μm. Abbreviations: BoNTA, botulinum toxin type A.*
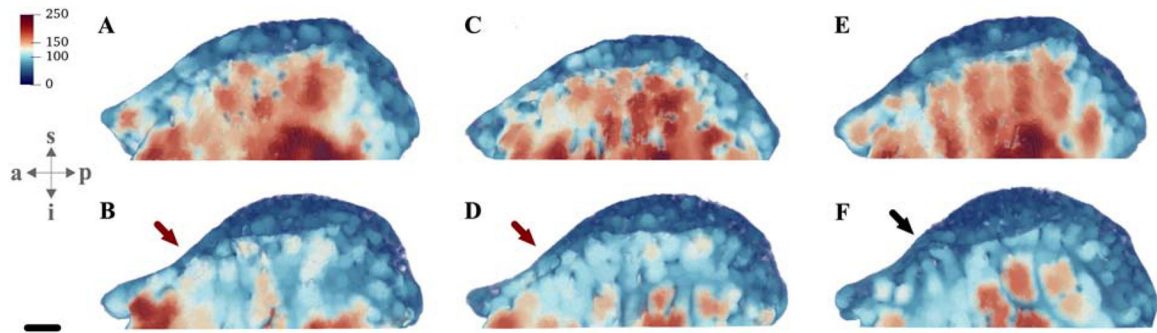
Figure 4. Qualitative regional Tb.Th changes of the mandibular condyles after BoNTA intervention are partially controlled with IG9402 co-intervention.

Medial views of 3D rendering of Tb.Th from the mandibular condyles in all BoNTA groups: Saline-injected (BoNTA, A; BoNTA + vehicle, C; BoNTA + IG9402, E) and BoNTA-injected (BoNTA, B; BoNTA + vehicle, D; BoNTA + IG9402, F) sides. The color bar included in this figure was obtained using the 3D rendering in Paraview v.5.4.1. and the encoded grayscale from the original microCT data. In this analysis, 0 corresponds to dark voxels and 255 to white voxel [36]. The files were changed to 8-bit images and the grayscale was adjusted from 0 to 250, and color was implemented (instead gray) in order to improve the contrast between regions. A qualitative increase in the Tb.Th and its regional distribution in the central portion is observed in the BoNTA + IG9402 mandibular condyles (E and F) when compared with the other BoNTA groups. In addition, the concave shape in the anterior-superior portion detected after 2 weeks in the BoNTA and BoNTA + vehicle groups (red arrows; the concavity suggests local bone loss) is partially reversed in the mandibular condyles from the BoNTA-injected side in the BoNTA + IG9402 group (black arrow). *Color bar in grayscale. Scale bar: 200 µm. Abbreviations: s, superior; i, inferior; a, anterior; p, posterior.*

Table 1.

IG9402 co-intervention during the BoNTA-induced masseter atrophy prevents the reduction of BV/TV but not Tb.Th when comparing deltas between sides (p-value < 0.05; Mann-Whitney test).

Bone Parameters ＼Groups	BoNTA + Vehicle (Mean ± SEM)	BoNTA + IG9402 (Mean ± SEM)	p-value
BV/TV	0.080 ± 0.013	0.051 ± 0.005	0.032
Tb.Th	0.020 ± 0.003	0.017 ± 0.003	0.372

Abbreviations: BoNTA, botulinum toxin type A; BV/TV, bone volume fraction; Tb.Th, trabecular thickness; SEM: Standard error of the mean.