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## ARTÍCULOS ORIGINALES / Originals

# $\beta$ -ARRESTIN/CONNEXIN 43 (Cx43) COMPLEX ANCHORS ERKS OUTSIDE THE NUCLEUS: A PRE-REQUISITE FOR BISPHOSPHONATE ANTI-APOPTOTIC EFFECT MEDIATED BY Cx43/ERK IN OSTEOCYTES

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### Abstract

Bisphosphonates (BPs) anti-fracture efficacy may be due in part to inhibition of osteocyte apoptosis. This effect requires opening of connexin (Cx) 43 hemichannels and phosphorylation of the extracellular signal regulated kinases (ERKs). However, unlike ERK activation by other stimuli, the Cx43/ERK pathway activated by BPs does not result in nuclear ERK accumulation. Instead, the anti-apoptotic effect of BPs depends on phosphorylation of cytoplasmic ERK targets and is abolished by forced nuclear retention of ERKs. We now report that ERKs and the scaffolding protein  $\beta$ -arrestin co-immuno-precipitate with Cx43 in MLO-Y4 osteocytic cells and that the BP alendronate increases this association. Moreover, ERK2 fused to red fluorescent protein (ERK2-RFP) co-localizes with Cx43 fused to green fluorescent protein outside the nucleus in cells untreated or treated with alendronate. Alendronate does not induce ERK nuclear accumulation in cells transfected with wild type  $\beta$ -arrestin (wtARR) or vector control, whereas it does in cells expressing a dominant negative  $\beta$ -arrestin mutant (dnARR) consisting of the  $\beta$ -arrestin-clathrin binding domain that competes with endogenous  $\beta$ -arrestin for binding to

clathrin. Alendronate activates ERKs in dnARR-transfected cells as effectively as in cells transfected with wtARR, demonstrating that dnARR only interferes with subcellular localization but not with activation of ERKs by BPs. Further, whereas alendronate inhibits apoptosis in cells expressing wtARR or vector control, it is ineffective in cells expressing dnARR. Thus, BPs induce the formation of a complex comprising Cx43,  $\beta$ -arrestin, and clathrin, which directs ERKs outside the nucleus and is indispensable for osteocyte survival induced by BPs.

**Key words:** osteocyte, bisphosphonate, beta-arrestin, apoptosis

### Resumen

**EL COMPLEJO  $\beta$ -ARRESTINA/CONEXINA 43 (Cx43) RETIENE ERKS FUERA DEL NÚCLEO: UN PASO NECESARIO PARA EL EFECTO ANTI-APOPTÓTICO DE LOS BISFOSFONATOS MEDIADO POR Cx43/ERK EN OSTEOCITOS**

La efectividad de los bisfosfonatos (BPs) en la prevención de fracturas puede deberse en parte a la inhibición de la apoptosis de osteocitos. Este efecto depende de la apertura de hemicanales de conexina (Cx) 43 y la fosforilación

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de quinasas reguladas por señales extracelulares (ERKs). Sin embargo, a diferencia de la activación de ERKs debida a otros estímulos, la vía de señalización Cx43/ERK activada por BPs no conlleva la acumulación de ERKs en el núcleo. El efecto anti-apoptótico de los BPs depende de la fosforilación de blancos citoplasmáticos de ERKs y es inhibido cuando las quinasas son retenidas en el núcleo. En este estudio hemos demostrado que ERKs y la proteína “scaffolding”  $\beta$ -arrestina co-inmunoprecipitan con Cx43 en células osteocíticas MLO-Y4 y que alendronato aumenta esta asociación. Más aún, ERK2 fusionada a la proteína roja fluorescente (ERK2-RFP) co-localiza con Cx43 fusionada con la proteína verde fluorescente fuera del núcleo en células tratadas con vehículo o alendronato. Alendronato no indujo la acumulación nuclear de ERK en células transfectadas con  $\beta$ -arrestina nativa (wtARR) o con un vector control, pero si lo hizo en células que expresan una forma dominante negativa de  $\beta$ -arrestina (dnARR), consistente en el dominio de interacción entre  $\beta$ -arrestina y clatrina, y que compete con  $\beta$ -arrestina endógena por la unión a clatrina. Alendronato activa ERKs con la misma eficiencia en células transfectadas con dnARR o wtARR, demostrando que dnARR sólo interfiere con la localización subcelular de ERKs, pero no con su activación inducida por los BPs. Más aún, mientras alendronato inhibe apoptosis en células que expresan wtARR o vector control, es inefectivo en células que expresan dnARR. En conclusión, los BPs inducen la formación de un complejo que incluye Cx43,  $\beta$ -arrestina y clatrina, el cual retiene ERKs fuera del núcleo y es indispensable para la supervivencia de los osteocitos inducida por estas drogas.

**Palabras clave:** osteocito, bifosfonatos, beta-arrestina, apoptosis

## Introduction

Bisphosphonates (BPs) are drugs used to preserve bone mass due to their inhibitory effects on osteoclast function, and therefore, act as anti-resorptive agents.<sup>1</sup> Reduced

osteoclast viability induced by BPs occurs as a consequence of the inhibition of the mevalonate pathway, or to formation of toxic intracellular metabolites. However, the efficacy of BPs on the prevention of bone fractures cannot be explained completely by their effect on bone resorption. We and others have shown that bisphosphonates prevent osteoblast and osteocyte apoptosis induced by the glucocorticoid dexamethasone, the topoisomerase inhibitor etoposide, and the cytokine tumor necrosis alpha *in vitro*, and induced by glucocorticoid excess and by lack or excessive mechanical forces *in vivo*,<sup>2,3</sup> raising the possibility that preservation of the osteocyte network might contribute to the beneficial effects of BPs on the skeleton.<sup>4</sup> The mechanism for the survival effect of these agents on osteoblastic cells differs from the one by which BPs inhibit the function of osteoclasts. Thus, the concentration required for the effect of BPs on osteoblastic cells is several orders of magnitude lower than the one required to induce osteoclast apoptosis. Further, the anti-apoptotic effect of BPs on osteoblastic cells can be dissociated from the effect of the drugs on osteoclast using BP analogs. In particular, IG9402, a BP that does not inhibit the mevalonate pathway or affect osteoclast viability *in vitro*,<sup>5,6</sup> does not affect bone remodeling but was as effective as alendronate in activating ERKs and preventing osteoblast and osteocyte apoptosis and the decrease in bone mass and strength induced by glucocorticoids.<sup>6</sup> Furthermore, IG9402 is able to prevent osteocyte apoptosis and partially preserve bone strength, without affecting bone resorption in mice unloaded by tail suspension.<sup>3</sup>

The role of connexin channels in bone cells, and in particular in osteoblasts and osteocytes, have been extensively studied, both *in vitro* and *in vivo*.<sup>7-12</sup> Cx43 is required for osteoblast and osteoclast differentiation, and for the viability of osteocytes.<sup>13-15</sup> Further, Cx43 can regulate the expression of genes

required for osteoblast differentiation directly in osteoblast altering the levels of Runx2 and osteocalcin,<sup>13,14,16</sup> or through the regulation of genes that control osteoblast differentiation in osteocytes, such as Sost/sclerostin.<sup>17,18</sup> In addition, Cx43 regulates the expression of cytokines required for osteoclast differentiation in osteoblastic cells, and Cx43 deletion results in increased expression of the pro-osteoclastogenic cytokine receptor activator of nuclear factor kappa-B ligand (RANKL) and in decreased expression of the anti-osteoclastogenic cytokine osteoprotegerin (OPG), resulting in a cytokine ratio that favors osteoclast differentiation.<sup>17-19</sup> Furthermore, Cx43 channels are required for osteoclast differentiation,<sup>20,21</sup> and deletion of Cx43 from osteoclast progenitors inhibit their differentiation *in vivo*.<sup>22</sup>

Previous evidence demonstrated that inhibition of osteocyte apoptosis by BPs is mediated via a novel paradigm of signal transduction involving connexin (Cx) 43 hemichannels and the subsequent activation of ERKs.<sup>23</sup> In addition, we found that unlike other ERK activators such as estradiol, BPs do not cause nuclear accumulation of ERKs.<sup>24</sup> And, moreover, forced nuclear retention of the kinases abolishes the survival effect of BPs. However, the mechanism of the extra-nuclear retention of ERKs remained heretofore unknown.

$\beta$ -arrestins are scaffolding proteins that bind to seven-transmembrane G protein-coupled receptors and to clathrin.<sup>25</sup> This interaction results in receptor internalization leading to recycling of the receptor to the cell surface or to its degradation, and the desensitization of the cell to the action of the receptor ligand. This mechanism modulates the actions of hormones, cytokines, and pharmacological agents, including parathyroid hormone, calcitonin, hepatocyte growth factor, and agonists of the  $\beta$ 2-adrenergic receptors. More recent evidence indicate that  $\beta$ -arrestins also act as scaffolds for intracellular

signaling kinases, interacting with Src and ERKs, resulting in extra-nuclear retention the kinases. Furthermore, we have shown that the interaction of Cx43 with  $\beta$ -arrestin is required for cAMP-mediated gene transcription and the anti-apoptotic effect of parathyroid hormone in osteoblastic cells.<sup>26</sup>

Based on the absolute requirement of Cx43 for the anti-apoptotic effect of BPs,<sup>23,27</sup> we hypothesized that ERKs activated by BPs are retained outside the nucleus by forming a complex with Cx43 and associated scaffolding proteins. In this study we examined whether  $\beta$ -arrestins associate with Cx43 and whether this complex is involved in the anti-apoptotic effect of BPs mediated by ERKs.

## Materials and Methods

### Cell culture

MLO-Y4 osteocytic cells derived from murine long bones were cultured as previously described.<sup>4,28</sup> Cells were maintained in  $\alpha$ -MEM medium containing L-glutamine and 1 % v/v penicillin/streptomycin (Gibco), 10 % heat inactivated fetal bovine serum (Hyclone).

### DNA constructs and transient transfection

The constructs encoding the nuclear green fluorescent protein (nGFP) or the nuclear red fluorescent protein (nRFP) were previously described.<sup>4,29</sup> Cx43-GFP was provided by D. Laird (University of Western Ontario, London, Ontario, Canada).<sup>30</sup> Wild-type mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) was provided by N. G. Ahn (University of Colorado, Boulder, CO).<sup>31</sup> ERK-RFP was provided by L. Luttrell (Medical University of South Carolina, Charleston, SC).<sup>32</sup> Wild type  $\beta$ -arrestin and  $\beta$ -arrestin<sup>319-418</sup> (dnARR) constructs were provided by K. DeFea (University of California, Riverside, CA).<sup>33</sup> These constructs have been shown to be normally expressed at the protein and mRNA levels. Cells were transiently transfected using Lipofectamine Plus (Invitrogen) as previously described.<sup>23</sup>



The efficiency of transfection was ~70%, as determined by quantifying cells expressing or not the fluorescent proteins under a fluorescence microscope.

### **Quantification of apoptotic cells**

Apoptosis was induced in semi-confluent cultures (less than 75% confluence). Cells were treated with vehicle or  $10^{-7}$  M alendronate for 1 h, followed by treatment with 50  $\mu$ M etoposide for 6 h, as previously reported.<sup>23</sup> The concentration of alendronate and etoposide were chosen based on previous studies showing a maximum effect for both agents in MLO-Y4 osteocytic cells. Apoptosis was assessed by enumerating MLO-Y4 cells expressing nRFP exhibiting chromatin condensation and nuclear fragmentation under a fluorescence microscope. At least 250 cells from fields selected by systematic random sampling for each experimental condition were examined. The percentage of apoptosis was calculated using the formula  $(\% DC_{e+s} - \% DC_v) / (\% DC_e - \% DC_v) \times 100$ , where DC = dead cells, e = etoposide-treated cultures, s = alendronate- or  $17\beta$ estradiol-treated cultures and v = vehicle-treated cultures, as previously published.<sup>23,29</sup>

### **Sub-cellular localization of ERK**

MLO-Y4 cells were transiently transfected with ERK2-RFP to allow the visualization of ERK and wild type MEK to anchor inactive ERK2 in the cytoplasm. Following transfection, cells were cultured in growth medium for 40 h, followed by incubation with  $\alpha$ -MEM containing  $10^{-7}$  M alendronate or  $10^{-7}$  M  $17\beta$ -estradiol for 30 min. The percentage of cells showing nuclear accumulation of ERK2-RFP was quantified by enumerating cells exhibiting increased RFP in the nucleus compared to the cytoplasm, using a fluorescence microscope. At least 250 cells from fields selected by systematic random sampling were examined for each experimental condition.

### **Western Blot analysis and immunoprecipitation**

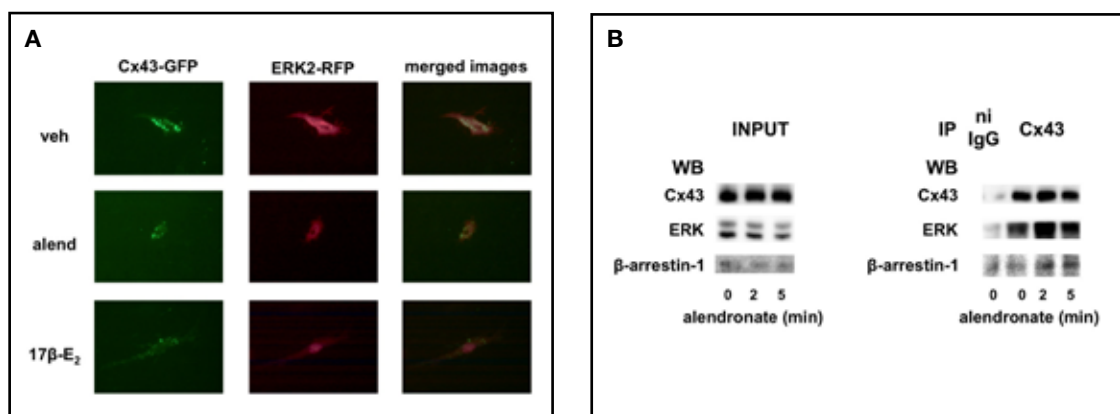
Western blots were performed using a rabbit anti-Cx43 antibody (Sigma, St. Louis, MO), rabbit anti-ERK, goat anti- $\beta$ -arrestin1, and mouse anti-GFP antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), following published protocols.<sup>23;26</sup> The intensity of the bands was quantified using the Fotodyne system (Hartland, WI). Each plate was lysed with 0.1 ml of RIPA buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, Sigma Chemical Co., St. Louis, MO) supplemented with phosphatase inhibitors (sodium fluoride, sodium orthovanadate) and protease inhibitors (leupeptin, aprotinin). Immunoprecipitation was performed using 600  $\mu$ g of extracts and 2  $\mu$ g of anti-Cx43 (Sigma), overnight at 4°C. Complexes were collected with 50  $\mu$ l of 25% slurry of protein-G agarose beads (Santa Cruz Biotechnology).

### **Statistical analysis**

Data were analyzed by one-way ANOVA, and the Student-Newman-Keuls method was used to estimate the level of significance of differences between means.

### **Results**

First, we examined whether ERKs and Cx43 exhibit the same sub-cellular localization. For this, MLO-Y4 osteocytic cells were transfected with Cx43 fused to GFP together with ERK2 fused to RFP (Figure 1A). The localization of the molecules was visualized under a fluorescence microscope. Cx43 was found in discrete areas outside the nucleus in un-stimulated cells. This distribution did not change upon treatment with alendronate or  $17\beta$ -estradiol. ERK2-RFP localized outside the nucleus in vehicle as well as alendronate-treated cells. Therefore, the merge images show that ERK2-RFP co-localizes with Cx43-GFP outside the nucleus in both untreated and alendronate-treated cells. On the other hand, ERK2-RFP localized



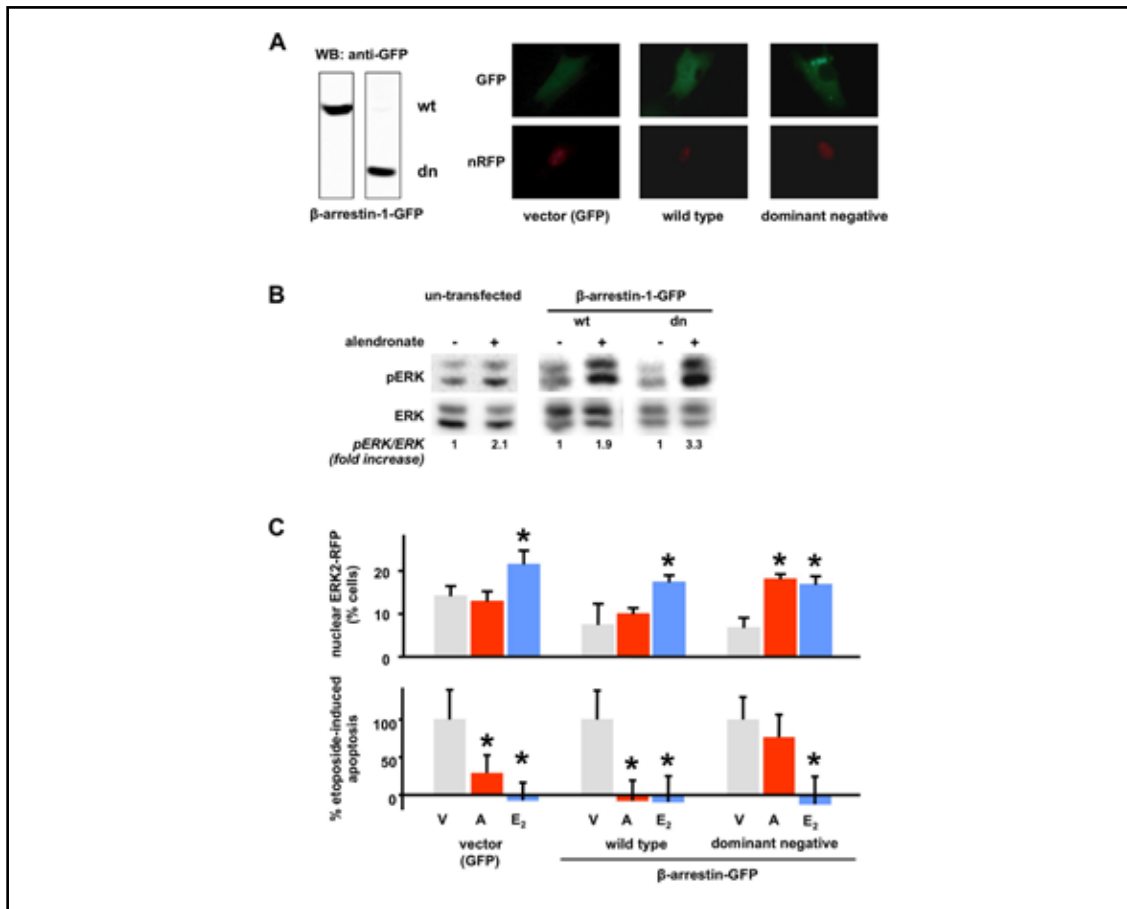
**Figure 1. Cx43 interacts with  $\beta$ -arrestin and ERKs in the cytoplasm of MLO-Y4 osteocytic cells, an interaction that is rapidly enhanced by alendronate treatment.** (A) MLO-Y4 cells were transiently transfected with the indicated constructs and treated with  $10^{-7}$  M alendronate or  $17\beta$ -estradiol for 2 minutes. Cells were fixed and observed under a fluorescence microscope. (B) Cells were treated with alendronate for the indicated times, and Cx43 was immuno-precipitated as indicated in the methods section. Western blot analysis was performed with the whole lysates (input) or with protein extracts after immunoprecipitation with non-immune (ni) IgG as control or with anti-Cx43 antibodies. Western blot (WB) analysis was performed using the indicated antibodies.

in the nucleus in  $17\beta$ -estradiol-treated cells. These results raised the possibility that ERKs interact with Cx43 in the presence of BPs. Western blot analysis showed that alendronate treatment for 2 or 5 min did not affect the total amount of ERK,  $\beta$ -arrestin-1 or Cx43 present in MLO-Y4 cell lysates (Figure 1B). In addition, we found that ERKs and  $\beta$ -arrestin-1 co-immunoprecipitated with Cx43 in the absence of BP and the association among these proteins was increased in the presence of alendronate.

We then determine the participation of  $\beta$ -arrestin in the anti-apoptotic effect of BPs. For this experiments, we used etoposide as pro-apoptotic agents since we have extensively used it and consistently increase apoptosis of MLO-Y4 osteocytic cells.<sup>4,5,23,24</sup> We transiently transfected MLO-Y4 osteocytic cells with 2 different constructs, wild type or dominant negative  $\beta$ -arrestin, both tagged with GFP (Figure 2). In addition, cells were transfected with GFP as vector control. The

wild type  $\beta$ -arrestin construct renders a protein of 75 kD, which localizes in the cytoplasm, with no detectable localization at the plasma membrane (Figure 2A). GFP-tagged dnARR consists of aminoacids 319-418 and renders a protein of 40 kD, with a punctuate distribution throughout the cell which resembles the distribution of clathrin. This mutant comprises only the clathrin binding domain of  $\beta$ -arrestin and act as a dominant negative by competing with endogenous  $\beta$ -arrestin for binding to clathrin. Both wild type and dominant negative  $\beta$ -arrestin were expressed at similar levels, as determined by Western blotting using anti-green fluorescent protein antibodies.

Overexpression of  $\beta$ -arrestin-1 constructs did not interfere with alendronate-induced ERK activation. Thus, 2-min treatment with alendronate induced similar increases in ERK phosphorylation in un-transfected cells than in cells expressing wild type or dominant negative  $\beta$ -arrestin (Figure 2B). As shown before,<sup>24</sup>



**Figure 2. Blockade of endogenous  $\beta$ -arrestin interaction with clathrin using a dominant negative  $\beta$ -arrestin abolishes ERK extra-nuclear retention and anti-apoptosis induced by BPs. (A)** MLO-Y4 osteocytic cells were transiently transfected with GFP (vector control), or GFP-tagged wild type  $\beta$ -arrestin (wt) or the dominant negative truncated molecule (dn) together with RFP targeted to the nucleus (nRFP). Forty-eight hours after transfection cells were either treated to obtain proteins lysates or fixed. Protein lysates were analyzed by Western blotting using an anti-GFP antibody. Fixed cells were observed under fluorescence microscope. **(B)** MLO-Y4 osteocytic cells were left un-transfected or transfected with wt or dn  $\beta$ -arrestin-1-GFP. Forty eight hours after transfection, cells were treated with vehicle or  $10^{-7}$  M alendronate for 2 minutes and protein lysates were prepared. The levels of phosphorylate ERKs (pERKs) were analyzed by Western blotting using specific antibodies and corrected by the level of total ERKs. **(C)** MLO-Y4 osteocytic cells were transiently transfected with the indicated constructs together with nRFP. Forty eight hours later, cells were treated with vehicle,  $10^{-7}$  M alendronate or  $17\beta$ -estradiol for 2 minutes, or treated with the reagents for 30 min, followed by a 6-h treatment with the pro-apoptotic agent etoposide ( $50 \mu\text{M}$ ). Subsequently, cells were fixed and scored under a fluorescence microscope.

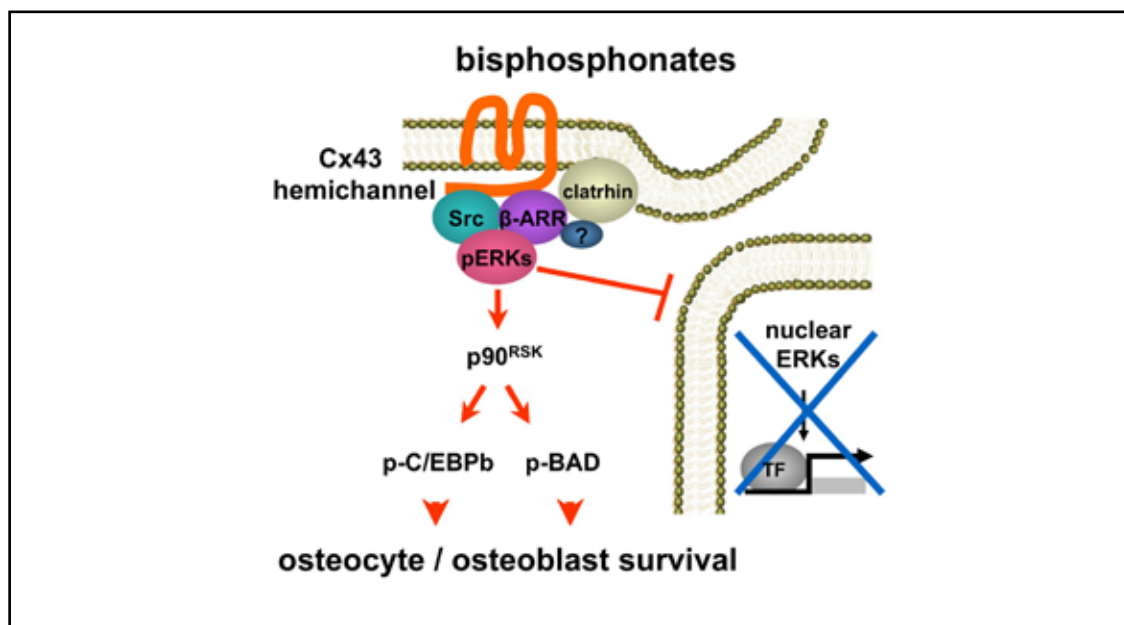
alendronate did not induce ERK nuclear accumulation in cells transfected with GFP, whereas  $17\beta$ -estradiol increase the percentage of cells exhibiting nuclear accumulation of the

kinase (Figure 2C). Similarly, alendronate did not induce nuclear accumulation of ERKs in cells expressing wild type  $\beta$ -arrestin, whereas it did increase nuclear accumulation

of ERKs in cells expressing the dominant negative construct. In contrast, neither wild type nor dominant negative  $\beta$ -arrestin altered  $17\beta$ -estradiol-induced nuclear ERK accumulation. Consistent with the requirement of extra-nuclear localization of ERKs for the survival effect of BPs, alendronate did not inhibit apoptosis induced by etoposide in cells expressing the dnARR mutant, whereas it prevented apoptosis in cells expressing GFP or wild type  $\beta$ -arrestin. The anti-apoptotic effect of  $17\beta$ -estradiol was not affected by either dominant negative or wild type  $\beta$ -arrestin. Taken together, these results indicate the specificity of the cross-talk between  $\beta$ -arrestin and the Cx43/ERK pathway.

## Discussion

The studies reported herein demonstrate that Cx43 co-localizes with ERKs outside the nucleus in untreated cells or in cells treated with alendronate; and that alendronate induces the association of Cx43 with  $\beta$ -arrestin-1 and ERKs (Figure 3). Furthermore, inhibition of the association of  $\beta$ -arrestin with clathrin prevents alendronate-induced extra-nuclear localization of ERKs and anti-apoptosis. On the other hand, wild type or dominant negative  $\beta$ -arrestin-1 did not affect ERK sub-cellular localization or anti-apoptosis induced by  $17\beta$ -estradiol, adding further evidence for the distinct mechanism of action of the two types of anti-resorptive drugs in osteocytic cells.



**Figure 3. Working model.**  $\beta$ -arrestin forms a complex with Cx43 and clathrin that also encompasses Src, which retains ERKs activated by BPs outside the nucleus. This complex favors the activation of the cytoplasmic target of ERKs p90<sup>RSK</sup>, and the phosphorylation of C/EBP $\beta$  and BAD, resulting in osteocyte (and osteoblast) survival.

BPs are widely used for the treatment of conditions associated with low bone mass.<sup>1</sup> Their beneficial effects are a consequence of the reduction in bone resorption due to

inhibition of osteoclast function. In addition, BPs prevent osteocyte and osteoblast apoptosis, an effect that can contribute to the anti-fracture efficacy of these drugs.<sup>2</sup> Indeed,





administration of a BP analog that does affect osteoclasts but prevents osteocyte and osteoblast apoptosis is able to prevent the decrease in bone mass and strength induced by glucocorticoid excess in mice.<sup>5,6,34</sup> As the classical BPs, this novel analog requires the expression of Cx43 to prevent osteocyte and osteoblast apoptosis.<sup>5</sup> Even though receptors for BPs have not been described, these agents bind to the cell surface in a specific and saturable manner.<sup>35</sup> However, the binding is independent of the expression of Cx43, indicating that another molecule binds to BPs, leading to opening of Cx43 hemichannels, and the activation the Src/MEK/ERK signaling pathway, followed by the phosphorylation of cytoplasmic targets of the kinases and cell survival. This survival signaling pathway mediated by kinases does not required new gene transcription.<sup>24</sup> This contrasts with the anti-apoptotic effect of sex steroids, which also requires Src/MEK/ERK activation, but is abolished when ERKs are retained in the cytoplasm or when new gene transcription is inhibited.<sup>24,36</sup> We now show that retention of ERKs in the cytoplasm and osteocyte survival induced by BPs requires the association among Cx43,  $\beta$ -arrestin and clathrin.

We have previously reported that the association between Cx43 and  $\beta$ -arrestin is required for the survival effect of PTH in osteoblastic cells.<sup>26</sup> However, the anti-apoptotic effect of PTH does not require ERK activation and, instead, it is mediated by new gene transcription downstream of cAMP activation.<sup>37</sup> In this context, Cx43 sequesters  $\beta$ -arrestin, preventing its binding to the PTH receptor and, therefore, the inhibition of PTH-induced, cAMP-mediate gene transcription.<sup>26</sup> Thus,  $\beta$ -arrestin contributes to osteocyte and osteoblast survival by at least two different means: by allowing the activation of the cAMP/PKA pathway in the presence of PTH, and by retaining ERK activated by BPs in the cytoplasm, without affecting the activation of the kinase. These actions of  $\beta$ -arrestins

in osteoblastic cells add to the discoveries on the novel actions of  $\beta$ -arrestins, not only as molecules involved in desensitization of G protein-coupled receptors and their internalization, but also as scaffolds for intracellular kinases and targets of novel agonists.<sup>25</sup> These agonists that can activate arrestin-selective signaling downstream of the seven-transmembrane receptors, without activating G-protein-mediated signaling are named "biased agonists". Taken together, these pieces of evidence suggest that BPs can be considered a new class of biased  $\beta$ -arrestin agonist, although the identity of the receptor activated by these agents remains to be determined.

In conclusion, our study adds to the functions of Cx43 as a scaffolding molecule that binds intracellular structural and signaling molecules. Based on the evidence presented here and our previous studies,<sup>23,24</sup> we proposed that, through its C-terminus domain, Cx43 binds to  $\beta$ -arrestin and forms a complex with clathrin, Src and MEK, leading to activation of ERKs and their retention in the cytoplasm (Figure 3). This signaling cascade leads to the activation of p90<sup>RSK</sup> and the phosphorylation of C/EBP $\beta$  and Bad. Phosphorylated C/EBP $\beta$  binds and blocks active caspase3, whereas phosphorylated Bad is inactive, resulting in anti-apoptotic signaling and osteoblast/osteocyte survival.

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**Conflicts of interest:** The authors declare no conflicts of interest.

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