Zoledronic acid blocks the interaction between mesenchymal stem cells and breast cancer cells: implications for adjuvant therapy of breast cancer

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**Background:** Zoledronic acid (ZA) reduces locoregional and distant metastases in estrogen receptor (ER)-positive breast cancer patients. Since ZA rapidly concentrates in the bone following i.v. administration, we hypothesized that this phenomenon involves the mechanism of action of ZA in bone tissue.

**Materials and methods:** Migration assays were carried out in fibronectin-coated Boyden chambers. Activation of signaling proteins was analyzed with a phosphoprotein array. Chemokines and growth factors were measured by immunoassays and real-time PCR.

**Results:** ZA significantly reduced in bone marrow-derived mesenchymal stem cells (MSCs) the activation of AKT and mitogen-activated protein kinase and their ability to migrate. Conditioned medium (CM) from ZA-treated MSCs

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showed a reduced capacity to promote the migration of ER-positive MCF-7 breast cancer cells as compared with CM from untreated MSCs. The levels of the chemokine (C-C motif) ligand 5 (CCL5, also known as RANTES - Regulated upon Activation, Normal T-cell Expressed, and Secreted) and interleukin (IL)-6 were significantly reduced in MSC-CM following treatment with ZA. Anti-RANTES and anti-IL-6 antibodies almost completely abolished the migration of MCF-7 cells induced by MSC-CM. Recombinant RANTES and IL-6 significantly induced MCF-7 cell migration and their combination showed a cooperative effect. Similar results were observed in different breast cancer cell lines.

**Conclusion:** ZA might exert its antitumor activity by inhibiting MSC migration and blocking MSCs' secretion of factors involved in breast cancer progression.

**Key words:** breast cancer, mesenchymal stem cells, metastases, tumor microenvironment, zoledronic acid

**introduction**

The nitrogen-containing bisphosphonate zoledronic acid (ZA) represents the mainstay of treatment of bone disease associated to a range of solid tumors, including breast cancer [1]. ZA blocks osteoclast-mediated bone resorption by inhibiting the key enzyme of the mevalonate pathway, farnesyldiphosphatase synthase. This prevents the synthesis of farnesyl diphosphate and its downstream metabolite geranylgeranyldiphosphate that are required for the post-translational modification of small GTPases, including Ras, Rho and Rab, which regulate osteoclast function [2].

Preclinical and clinical findings suggest that ZA might also have an antitumor effect [3]. In particular, the phase III Austrian Breast and Colorectal Cancer Study Group trial 12 (ABCSG-12) has recently demonstrated that the addition of ZA to endocrine therapy increases the disease-free survival of estrogen receptor (ER)-positive breast cancer patients by reducing both locoregional and distant metastases, suggesting that this compound might act directly on micrometastasis of breast cancer cells [4]. Conflicting results have been reported in other trials in which ZA was added to the adjuvant therapy of breast cancer, with still positive results only from the largest Zometa-Femara Adjuvant Synergy Trial (ZO-FAST)[5]. More recently, analysis of the results of the Adjuvant Zoledronic acid to redUce RECurrence (AZURE) trial has also suggested that ZA might increase the activity of adjuvant endocrine therapy only in postmenopausal patients [6].

While the clinical activity of ZA in the adjuvant therapy of breast cancer needs still to be explored, the mechanism through which ZA might exert its antitumor activity in this setting has not been clarified yet. In fact, following i.v. administration, bisphosphonates spread both in calcified and noncalcified tissues, but they decline quickly in noncalcified tissues, proportionately to the decrease of plasma concentration that shows an half-life of ~15 min [7, 8]. In agreement with these findings, a single injection of ZA had no effects on tumor growth outside the bone in experimental models [9], whereas the sequential treatment with doxorubicin followed by ZA [10] or repeated injections of ZA (every 4 days) [11] elicited antitumor effects in soft tissues. Since ZA was administered once every 6 months in the ABCSG trial, we hypothesize that the antitumor effects of ZA in the adjuvant setting are related to its mechanism of action within the bone.

Mesenchymal stem cells (MSCs) reside predominantly in the bone marrow, where they give rise to different specialized connective tissue cells such as 'reticular cells', osteoblasts, chondrocytes, adipocytes and smooth muscle cells [12, 13]. MSCs are recruited by developing breast tumors where they can enhance the metastatic potential of weakly tumorigenic breast cancer cells through the secretion of the chemokine CCL5/RANTES [14]. More recently, interleukin (IL)-17B has been also shown to mediate the interaction between MSCs and breast cancer cells [15]. In addition, MSCs may sustain the growth and survival of cancer cells within the bone microenvironment where they can contribute to form 'niches' for dormant micrometastases that may later seed distant metastases [16].

In order to assess the mechanism of the antitumor activity of ZA, we analyzed the effects of this drug on the interaction between MSCs and breast cancer cells.

**materials and methods**

**materials**

ZA was from Novartis Pharma AG (Basel, Switzerland), anti-human CCL5/RANTES and IL-6 mAbs from R&D Systems (Minneapolis, MN) and recombinant IL-6 and RANTES from Peprotech (London, UK).

**cell culture**

Bone marrow-derived human MSCs were purchased from Lonza (Verviers, Belgium). These cells were positive for CD29, CD44, CD105 and CD166 and negative for markers of the hematopoietic lineage, such as CD14, CD34 and CD45. Cells were maintained in MSCGM bullet kit (Lonza).

Breast cancer cell lines were from the American Type Culture Collection.

**cell growth inhibition assays**

MSCs were seeded in a 48-well plate (11 000 cells/well) and treated with ZA. At the indicated time points, cells were trypsinized and counted with an automated cell counter.

**immunoassays for quantification of signaling protein phosphorylation**

Levels of the phosphorylated and total mitogen-activated protein kinase (MAPK), AKT, c-Jun, c-SRC and signal transducer and activator of transcription 3 (STAT3) signaling proteins were analyzed using the Bio-Plex phosphoprotein array (Bio-Rad, Milan, Italy). Target proteins were quantified by using the Bio-Plex 200 System and data were analyzed with the Bio-Plex Manager Software 5.0. Results were recorded as mean fluorescence intensities (MFIs). Relative MFI was calculated by normalizing values of phosphoproteins for the levels of total proteins in each sample.

**western blot analysis**

Antibodies anti-phospho-p42/p44MAPK (Thr202/Tyr204), anti-p42/p44MAPK, anti-phospho-AKT (Ser 473), anti-AKT and anti-phospho-STAT3 (Tyr705, clone 3E2) were from Cell Signaling Technology (Beverly, MA). The anti-α-tubulin antibody (clone DM1A) was from Sigma (Milan, Italy).

**preparation of conditioned media and immunoassays**

MSCs were cultured in serum-containing medium in the absence or in the presence of ZA for 48 h. Then, cells were washed twice with
phosphate-buffered saline and cultured for 16 h in serum-free medium. The conditioned media (CMs) were collected, sterile filtered and stored in aliquots at −80°C. The levels of RANTES, IL-6 and angiogenic factors were assessed with the xMAP Bio-Plex Cytokine array system (Bio-Rad).

real-time PCR
Total RNA was extracted from untreated or ZA-treated MSCs using TRI Reagent solution (Ambion/Applied Biosystems, Milan, Italy). Real-time PCR was carried out using the SYBR Green PCR Master Mix (Applied Biosystems) and the RT² PCR Primer Sets for human RANTES, IL-6 and glyceraldehyde-3-phosphate dehydrogenase (SAbiosciences, Frederick, MD).

migration assays
Cells (MSCs: 50,000 cells/insert; breast cancer cells: 110,000 cells/insert) were seeded in serum-free medium in the upper wells and allowed to migrate for 20 h through a fibronectin-coated Boyden chamber using the QCM-FN Haptotaxis Cell Migration Assay-Fibronectin, Colorimetric (Chemicon/Millipore, Milan, Italy). Serum-containing media (for MSCs); or CMs from untreated or ZA-treated MSCs without or with different concentrations of anti-RANTES antibody (0.5, 1, 2, 4 μg/ml) or anti-IL-6 antibody (100, 200, 400 ng/ml); or serum-free medium added with recombinant human RANTES (50 ng/ml) or IL-6 (50 ng/ml) alone or in combination were used as chemoattractants in the lower Boyden chambers.

statistical analysis
Significance was determined using two-tailed Student’s t-test. P values <0.05 were considered statistically significant.

results
effects of ZA on MSCs
We analyzed the effects of ZA on MSC proliferation by exposing cells to increasing concentrations of ZA for 24–72 h (Figure 1A). An ~25% growth inhibition following treatment with 20 μM ZA for 48–72 h was observed. Conversely, ZA produced a significant dose-dependent reduction in the ability of MSCs to migrate.
through a fibronectin-coated membrane in response to serum (Figure 1B). In particular, an ~45% inhibition was observed following treatment with 20 μM ZA.

The effects of ZA on the activation of various signaling proteins in MSCs were assessed by using a Luminex-based phosphoprotein array and western blot analysis. Treatment with 20 μM ZA produced a significant reduction in the levels of phosphorylation of MAPK and AKT (Figure 2A and C), whereas it did not affect the levels of activation of STAT3, c-SRC and c-Jun (data not shown). The results obtained with the phosphoprotein array were confirmed by using western blot analysis (Figure 2B and D).

**ZA affects the ability of MSCs to sustain breast cancer migration**

We next investigated whether ZA affects the ability of breast cancer cells to migrate in response to factors secreted by MSCs. For this purpose, we carried out migration assays in which ER-positive MCF-7 breast cancer cells were allowed to migrate in response to CM from untreated or ZA-treated MSCs (Figure 3A). CM from ZA-treated MSCs showed a reduced ability to promote the migration of MCF-7 cells as compared with CM from untreated cells, and this phenomenon was dose dependent (Figure 3A).

**Figure 3.** Effects of ZA on the ability of MSCs to support breast cancer migration. (A) Migration of MCF-7 cells in response to CM from MSCs treated with 10, 15 or 20 μM ZA (*P < 0.05). (B) Analysis of factors secreted by MSCs after treatment with 20 μM ZA as assessed by xMAP Bio-Plex Cytokine array system. Nd indicates that the levels of RANTES protein in this sample were below the threshold of sensitivity. ZA, zoledronic acid; MSCs, mesenchymal stem cells; CM, conditioned medium.

**Figure 4.** Effects of 15 or 20 μM ZA on (A and B) RANTES/CCL5 and (C and D) IL-6-secreted protein and mRNA (*P < 0.05). Nd indicates that the levels of RANTES protein in this sample were below the threshold of sensitivity. ZA, zoledronic acid; IL, interleukin; mRNA, messenger RNA.
MSCs secrete factors that can promote tumor cell migration as well as angiogenic growth factors that are involved in tumor progression [14, 17]. Levels of secretion of cytokines and angiogenic factors in CM from untreated and ZA-treated MSCs were measured using the xMAP Bio-Plex Cytokine array system. To account for the difference in the cell number following treatment with ZA, the levels of secreted factors in CM from MSCs were normalized to viable cell number. A significant reduction in the levels of secretion of RANTES and IL-6 and a marginal decrease of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) were observed in CM from MSCs treated with 20 μM ZA, whereas the drug did not affect the levels of secretion of IL-8 (Figure 3B). A dose-dependent effect of ZA on the secretion of RANTES and IL-6 in MSCs was found. As shown in Figure 4A, treatment with 15 μM ZA for 48 h produced a significant reduction in the levels of secreted RANTES in MSCs, and the secretion of this growth factor was almost completely suppressed at 20 μM ZA. In agreement with these results, we observed a significant decrease in the levels of RANTES messenger RNA (mRNA) following treatment with ZA (Figure 4B). A significant reduction of secreted IL-6 protein and IL-6 mRNA was also found following treatment of MSCs with 20 μM ZA for 48 h (Figure 4C and D).

role of RANTES and IL-6 in breast cancer cell migration

To evaluate whether IL-6 and RANTES secreted from MSCs are able to sustain the migration of MCF-7 cells, we carried out migration assays in which MCF-7 cells were induced to migrate in response to MSC-CM in the presence of different concentrations of anti-IL-6 and anti-RANTES antibodies. Both antibodies significantly inhibited the migration of MCF-7 cells induced by MSC-CM in a dose-dependent manner (Figure 5A and B). Treatment with the combination of anti-IL-6 and anti-RANTES antibodies almost completely abolished MSC-CM-induced migration of MCF-7 cells (Figure 5C; supplemental Figure S1, available at Annals of Oncology online).

Next, we assessed whether RANTES and IL-6 could induce migration of breast cancer cells belonging to different subtypes of breast carcinoma. The ER-positive MCF-7, BT-474 and T47D breast cancer cell lines; the basal-like MDA-MB-231 and the ER-negative, ErbB-2 overexpressing SK-BR-3 cell lines were allowed to migrate in response to recombinant human RANTES and IL-6, alone or in combination. Both recombinant proteins produced a significant increase of the migration in all breast cancer cell lines, with the combination showing a greater effect (Figure 6).

The effects of RANTES and IL-6 on the activation of signaling pathways were investigated in breast cancer cells (Figure 7; supplemental Figure S2, available at Annals of Oncology online). RANTES was able to increase the levels of phosphorylation of MAPK (p-MAPK), AKT (p-AKT) and STAT3 (p-STAT3) with a first peak after 15–30 min of treatment. A second peak of activation at 4–6 h for AKT and/or STAT3 was observed in some cell lines. Treatment with IL-6 resulted in significant activation of STAT3 in all cell lines after ~30 min and at lesser extent of AKT in selected cell lines.

These findings are in agreement with previous reports showing that RANTES and IL-6 are able to activate AKT and STAT3 signaling in breast cancer cells [14, 17]. Interestingly, combined treatment with RANTES and IL-6 produced in MCF-7 cells a more significant and persistent activation of both AKT and STAT3 (Figure 7).

discussion

Increasing evidence suggests that MSCs play an important role in the pathogenesis of breast cancer. MSCs are recruited to the stroma of developing breast tumors where they might contribute to the progression of breast cancer [14, 15]. The interaction...
between breast cancer cells and MSCs can also occur within the bone marrow microenvironment [16]. MSCs play a fundamental role in the pathogenesis of bone metastasis and they have been shown to facilitate the entry of breast cancer cells in the bone marrow [18]. Micrometastatic foci within the bone marrow might give rise to distant metastases, and MSCs might facilitate this phenomenon.

Our data suggest that ZA can significantly affect the interaction between MSCs and breast cancer cells. We have demonstrated for the first time that ZA is able to significantly reduce the migration of MSCs. Therefore, ZA might affect the recruitment of MSCs by primary tumors thus blocking an important mechanism involved in tumor progression. In this regard, the concentrations of ZA that we used in our experimental model (10–20 μM) are much higher of the plasma levels that are observed following i.v. administration of the drug (1–2 μM) [7]. Bisphosphonates concentrate in the bone from which they are released during bone resorption. Therefore, it is likely that ZA can reach quite high concentrations within the bone microenvironment in cancer patients. Actually, bone resorption is enhanced in patients receiving adjuvant therapies that produce estrogen deprivation [19]. Previous reports have shown antiproliferative and pro-apoptotic effects of ZA in bone marrow stromal cells or purified MSCs [20–22]. However, induction of apoptosis occurred in most studies for concentrations of ZA ranging between 50 and 100 μM, whereas significant effects on MSC proliferation with concentrations of 20 μM were reported only in one study [22]. The different origin of MSCs and the seeding density of cells may account for the slighter effects that we observed on cell proliferation as compared with this latter study.

This paper also demonstrated for the first time that treatment with ZA significantly affects the ability of MSCs to sustain cancer cell migration. Several different studies have shown that MSCs can increase the ability to migrate of breast cancer cells and that this phenomenon is mediated by different chemokines such as RANTES/CCL5, IL-17B, and CCL2 [14, 15, 23].
direct contact between MSCs and breast cancer cells induces in this latter cell type the expression of epithelial-to-mesenchymal-specific markers thus resulting in increasing metastatic potential [24]. In this regard, we identified two factors that are significantly affected by ZA in MSCs, RANTES and IL-6. Karnoub et al. [14] demonstrated that MSCs cause weakly metastatic breast cancer cells to acquire enhanced metastatic abilities through the secretion of RANTES. IL-6 has been shown to be a potent growth factor for breast cancer cells [17], to promote breast cancer cell invasion [25, 26] and to mediate self-seeding of circulating tumor cells to developing breast tumors [27]. We confirmed that RANTES and IL-6 promote migration in a panel of breast cancer cell lines from different histological subtypes. More importantly, we showed for the first time that RANTES and IL-6 cooperate in inducing the migration of breast cancer cells. The effects of ZA on the secretion of these proteins was quite specific since no significant reduction of IL-8, bFGF and VEGF secretion was observed following treatment with ZA. Therefore, RANTES and IL-6 might represent important biomarkers of activity of ZA on MSC-induced tumor cell migration and might allow to select patients for therapeutic approaches aimed to block this specific mechanism.

Interestingly, it has been shown that the levels of secretion of RANTES increased following coculture of MSCs and breast cancer cells, suggesting that a cross talk between these two cell types might occur within the tumor microenvironment [14]. In this regard, we have recently demonstrated that activation of epidermal growth factor receptor (EGFR) signaling in MSCs produces a significant increase in the levels of secretion of both IL-6 and VEGF [28]. Since the majority of breast tumors express high levels of EGFR ligands [29], it is possible that the EGFR receptor/ligand system might play an important role in the interaction between MSCs and breast cancer cells. We have also previously shown that EGFR signaling affects in MSCs the secretion of the receptor activator of NF-κB ligand and macrophage colony-stimulating factor, which are involved in osteoclast activation and, therefore, in the pathogenesis of bone metastases [30].

In conclusion, this study showed that ZA has biological activity on MSCs and significantly affects the ability of MSCs to secrete two growth factors RANTES and IL-6, which promote breast cancer cell migration. We hypothesize that two different mechanisms might be involved in the activity of ZA in the adjuvant treatment of breast cancer. Since ZA inhibits the migration of MSCs, the recruitment of MSCs by breast cancer cells could be significantly impaired, and this might result in reduced progression of micrometastatic foci in soft tissues. Alternatively, ZA could act on breast cancer micrometastasis within the bone marrow. Although ZA in this latter model might also directly inhibit breast cancer cell growth, the significant effects that this drug has on the secretion by MSCs of factors that promote breast cancer cell migration might significantly hamper the ability of tumor cells to form distant metastasis.

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**disclosure**

The authors declare no conflict of interest.

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