Chapter 3

Human stromal cells are required for an anti-breast cancer effect of zoledronic acid

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Abstract

Previous studies suggested that bisphosphonate zoledronic acid (ZOL) exerts an anti-tumor effect by interacting with the microenvironment. In this study, we elucidated the mechanism behind the anti-breast cancer effect of ZOL.

We showed that ZOL did not influence in vitro human breast cancer cell survival, but did affect human stromal cell survival. In co-culture, the addition of stromal cells to breast cancer cells induced tumor cell death by ZOL, which was abolished by transforming growth factor (TGF)-β. In the in vivo chicken chorioallantoic membrane model, ZOL reduced the breast cancer cells fraction per tumor only in the presence of human stromal cells. ZOL decreased TGF-β excretion by stromal cells and co-cultures. Moreover, supernatant of ZOL-treated stromal cells reduced phospho-Smad2 protein levels in breast cancer cells. Thus, ZOL exerts an anti-breast cancer effect via stromal cells, accompanied by decreased stromal TGF-β excretion and reduced TGF-β signaling in cancer cells.

Significance: Our study is the first to show, both in vitro and in vivo, that ZOL can exert an anti-cancer effect only in the presence of human stromal cells.
**Introduction**

Breast cancer accounts for the highest cancer incidence and cancer mortality among women worldwide (1). Despite great advances in breast cancer treatment, including increasingly targeted systemic treatment, development of metastatic disease still cannot be prevented in all patients. To tackle this problem, the focus has shifted to the breast cancer microenvironment (2), in which immune cells, fibroblasts, adipose cells and endothelial cells are involved in tumor growth and metastasis. Preclinical studies have also shown that the microenvironment can modulate treatment effects (3).

The bisphosphonates are thought to mediate an anti-cancer effect in the microenvironment. A recent meta-analysis (4) concluded that this class of compounds has a survival benefit in postmenopausal women being treated for breast cancer. Although the anti-cancer effect of bisphosphonates is apparently not limited to their anti-resorptive role in bone lesions (5), the exact mechanism that effectuates this anti-cancer response remains unclear. Recent research by Junankar et al. has elucidated parts of this mystery (6). In an *in vivo* study, they showed that the bisphosphonates risedronate and pamidronate are internalized by tumor-associated macrophages, but not by mouse tumor cells 4T1; the authors concluded that these compounds target macrophages, but not tumor cells. These results support the hypothesis that bisphosphonates have an indirect anti-tumor effect.

Our research complements the work of Junankar et al. by providing functional evidence of the role of stromal cells in zoledronic acid (ZOL) mediated breast cancer cell survival. By using *in vitro* and *in vivo* models consisting of human stromal cells as well as human breast cancer cells, we studied the role of stromal cells in breast cancer bisphosphonate sensitivity.

**Results**

*Stromal cells are required for the anti-breast cancer effect of ZOL in vitro*

High concentrations of ZOL were required to decrease cell survival of the human breast cancer cell lines. The SCP2 line was the least sensitive to ZOL, with a 50% inhibitory concentration (IC$_{50}$) of 486 µM after 96 hours incubation. The human breast tumor cell lines SUM-149, H2N, MCF-7 and MDA-MB-231 had an IC$_{50}$ of 194, 155, 145 and 86 µM respectively. However, ZOL affected stromal cell survival at concentrations far below the concentrations directly affecting breast cancer cell lines: IC$_{50}$ for human stromal cell line Hs27a was reached at a concentration of only 8 µM (Suppl. Fig. 1).

To investigate the indirect effects of ZOL, we used a fluorescence-based *in vitro* co-culture model. Representative nuclear structures of a viable and a dead SCP2 cell are depicted in Fig. 1A. At 24 hours (Fig. 1B), 50 µM of ZOL increased breast cancer cell death in the
co-culture group (SCP2 and Hs27a) compared to the mono-culture (SCP2) cancer cell group (18.9 ± 1 % vs 6.8 ± 3.5 %, \( P < 0.01 \)). This effect was ZOL dose-dependent in the co-culture group, increasing breast cancer cell death to 21.6 ± 0.6 % for 100 µM \( P < 0.01 \) and 27.6 ± 7.8 % \( P < 0.001 \) for 500 µM. In mono-culture, increasing the dose of ZOL did not increase breast cancer cell death (9.6 ± 1.6 % for 100 µM and 10.3 ± 1.7 % for 500 µM of ZOL). At 48 hours, the stromal-dependent breast cancer cell death induced by ZOL was even more pronounced than at 24 hours (Fig. 1B). At a ZOL dose of only 10 µM, breast cancer cell death in the co-culture group (23.5 ± 2.8 %) was higher compared to the mono-culture group (5.1 ± 3.1 %, \( P < 0.001 \)). And the effect became more pronounced
as the dose of ZOL increased, with breast cancer cell death of 6.5 ± 2 % for 50 µM, 11.8 ± 2.3 % for 100 µM and 18.4 ± 3.3 % for 500 µM in the mono-culture group versus 37.0 ± 0.4 % for 50 µM, 38.0 ± 3.4 % for 100 µM and 44.0 ± 4.6 % for 500 µM in the co-culture group (P < 0.001 for all doses). In mono-cultures of SCP2, ZOL increased breast cancer cell death after 48 hours compared to control from 4.3 ± 1.4 % to 11.8 ± 2.3 % (P < 0.05) for 100 µM and 18.4 ± 3.3 % (P < 0.001) for 500 µM ZOL (Fig. 1B).

**Stromal cells are required for anti-breast cancer cell effect by ZOL in vivo**

On day 14 of the in vivo CAM assay, vehicle-treated tumors containing SCP2 plus Hs27a cells were heavier (42.7 ± 14.7 mg vs 21.6 ± 10.3 mg, P < 0.001) and larger (55.5 ± 21.7 mm³ vs 31.8 ± 15.5 mm³, P < 0.05) compared to tumors containing only SCP2 cells (Fig. 2A and 2B). Tumors containing only SCP2 cells had a higher weight after treatment with ZOL compared to vehicle (33.9 ± 17.1 vs 21.6 ± 10.3 mg (P < 0.05). The tumor size was not affected by ZOL compared to vehicle treatment for tumors consisting of only SCP2 cells. However, tumors containing SCP2 plus Hs27a cells were sensitive to ZOL. On day 14, these tumors weighed less and were smaller when treated with ZOL compared to vehicle-treated SCP2 plus Hs27a tumors (tumor weight: 23.0 ± 8.6 mg vs 42.7 ± 17.7 mg P < 0.01, size: 32.4 ± 19.8 mm³ vs 55.5 ± 21.7 mm³ P < 0.05) (Fig. 2A and 2B).

ZOL treatment showed similar results for tumors consisting of only MCF-7 cells and tumors containing MCF-7 plus Hs27a cells. On day 14, tumors comprising MCF-7 plus Hs27a cells were heavier (36.9 ± 8.8 mg vs 21.4 ± 6.2 mg P < 0.01) and larger (54.4 ± 15.5 mm³ vs 18.6 ± 5.6 mm³ P < 0.001) (Fig. 2C and 2D) compared to tumors consisting of only MCF-7 cells. Moreover, ZOL had no effect on tumors containing only MCF-7 cells. However, in tumors containing MCF-7 plus Hs27a cells, ZOL treatment resulted in reduced tumor weight and size compared to vehicle-treated tumors (weight: 23.5 ± 6.4 mg vs 36.9 ± 8.8 mg P < 0.01, size: 28.2 ± 10.7 vs 54.4 ± 15.5 mm³ P < 0.01) (Fig. 2C and 2D). ZOL did not affect size and weight of tumors containing only Hs27a cells (Suppl. Fig. 2).

The breast cancer cell fraction of tumors containing only SCP2 or MCF-7 cells was not affected by ZOL. However, SCP2 and MCF-7 tumors that also contained stromal cells showed a higher corrected weight. ZOL reduced the corrected weight of the co-cultured tumors (P < 0.01 SCP2, P < 0.05 MCF-7). Moreover, the size of ZOL-treated SCP2 and MCF-7 co-culture tumors was reduced to the size of their respective mono-culture tumors (Fig. 2E and 2F). Hematoxylin & eosin (H&E) staining showed no difference in tumor viability between any tumor group (Suppl. Fig. 3).
Fig. 2
Scatter plots illustrating weight (mg), size (mm$^3$) and quantified breast cancer cell fraction (breast cancer cells/mg tumor) of in ovo tumors harvested on day 14 after a single gift of zoledronic acid (200 µM) or vehicle (phosphate buffered saline(PBS)) on day 10. Tumors consisted of SCP2 alone or SCP2 with Hs27a stromal cells (A, B, E) and MCF-7 alone or MCF-7 with Hs27a stromal cells (C, D, F). Tumor weight, size and breast cancer cell fraction are depicted for every individual tumor.
Reduced TGF-β signaling in stromal cells is required for anti-breast cancer effect of ZOL
Transforming growth factor (TGF)-β1 excretion levels in supernatant of stromal cells and co-cultures were reduced by 48 hours treatment with ZOL (Suppl. Table 1). TGF-β1 levels in supernatant of stromal cells decreased after treatment with 50, 100 and 500 µM ZOL (Fig. 3A). In the supernatant of SCP2 co-cultured with Hs27a, TGF-β1 levels also decreased after treatment with ZOL 50 µM, 100 µM and 500 µM (Fig. 3B). In co-cultures of H2N with Hs27a and MCF-7 with Hs27a, TGF-β1 levels were reduced after treatment of 100 µM and 500 µM ZOL (Fig. 3C and 3D). However, 48 hours of ZOL treatment did not reduce TGF-β1 levels in the supernatant of SCP2 and H2N breast cancer cells in the absence of stromal cells. In the supernatant of MCF-7 breast cancer cells without stromal cells, TGF-β1 levels were undetectable (< 0 ± 60 pg/mL) (data not shown). One day of

![Graphs showing TGF-β1 excretion (pg/mL) after 48 hours of exposure to 0–500 µM zoledronic acid of Hs27a stromal cells in mono-culture (A) or co-cultured with SCP2 (B), H2N (C) or MCF-7 (D).]
Fig. 4 Viability (%) of SCP2 mono-culture or co-culture with Hs27a stromal cells after 48 hours of treatment with 0–500 µM zoledronic acid with or without TGF-β1. B, Western blotting of pSmad2 was performed on lysates of SCP2 cells. β-actin served as a loading control. SCP2 cells were incubated for 1 hour with medium, TGF-β, or supernatant of Hs27a cells treated for 48 hours with 0–500 µM zoledronic acid. The relative density of the pSmad2 bands compared to the loading control is shown.
ZOL treatment did not alter TGF-β1 levels in the supernatant of monocultures of stromal Hs27a cells and SCP2, H2N and MCF-7 cells. This was also the case for the TGF-β1 levels in the supernatant of co-cultures of stromal and breast cancer cells treated for 24 hours with ZOL (Suppl. Fig. 4).

ZOL treatment resulted in dose-dependent increased death of SCP2 cells in co-culture with Hs27a cells in vitro (Fig. 4A). The addition of TGF-β1 to the in vitro co-culture model of SCP2 and Hs27a cells treated with ZOL almost completely abolished the ZOL-induced SCP2 cell death. Two days after the addition of TGF-β1, SCP2 cell death decreased from 24.3 ± 2.9 % to 9.5 ± 2.3 % (P < 0.001) for 10 µM ZOL, 35.6 ± 2.9 % to 13.3 ± 2.5 % (P < 0.001) for 50 µM ZOL, 37.7 ± 2.9 % to 15.3 ± 3.8 % (P < 0.001) for 100 µM ZOL and 47.8 ± 8.6 % to 32.9 ± 1.9% (P < 0.001) for 500 µM ZOL. The addition of TGF-β1 to monocultures of SCP2 cells treated with ZOL did not have this effect.

TGF-β signaling activity in SCP2 breast cancer cells, measured by pSmad2 levels, was increased after incubation with Hs27a supernatant (Fig. 4B). Also addition of TGF-β to the SCP2 cells resulted in Smad2 phosphorylation. In contrast, supernatant of Hs27a cells treated with all dosages of ZOL decreased pSmad2 levels. This indicates that ZOL can indirectly reduce TGF-β signaling in breast cancer cells, via an effect on stromal cells.

Discussion

Our study provides functional proof that the bisphosphonate ZOL can exert an anti-breast cancer effect only in the presence of stromal cells. These results complement research by Junankar et al. showing that the bisphosphonates risedronate and pamidronate target macrophages in the breast cancer microenvironment of mouse 4T1 xenografts (6). In their mouse model, intravital imaging revealed bisphosphonate uptake in calcium-rich tumor regions, while no uptake was seen in non-cancerous mouse mammary glands. Together with the data by Junankar et al., our study supports the concept of a microenvironment-mediated anti-tumor effect of bisphosphonates, and further clarifies the mechanism behind this effect.

These results suggest that bisphosphonate treatment not only modulates the bone environment, but also affects non-bone disease. This is in line with clinical data. A recent meta-analysis showed that breast cancer recurrence decreased in early breast cancer patients treated with a bisphosphonate (4). The clear interaction of ZOL with stromal cells in our study supports the broader effect of bisphosphonates outside of the bone environment.

We have shown that TGF-β secreted by stromal cells is an important mediator for induction of the anti-cancer effect of ZOL via stromal cells. Previous research suggested a strong role for the microenvironment with respect to bisphosphonate sensitivity
and TGF-β signaling (7). In mice, biphosphonate pamidronate treatment applied to established in-bone metastases of the cell line SCP28 evidently reduced active TGF-β signaling in these metastases. In that study, however, treatment of SCP28 tumor cells with pamidronate in vitro – without the presence of stromal cells – affected neither TGF-β signaling nor tumor cell death. Our study explains this apparent paradox by demonstrating the mediating role of the stromal cells. TGF-β transcriptional activity can also be inhibited by ER activation (8), thereby linking our research with pre-clinical and clinical studies showing that bisphosphonates only exert an anti-tumor effect under low estradiol concentrations (9, 10). If, under high estradiol levels, TGF-β signaling is already repressed, adding another TGF-β suppressing agent like ZOL would have no additional anti-cancer effect.

To our knowledge, ours is the first study to evaluate the effect of ZOL on the breast cancer-stroma interaction by assessing the separate components. This analysis was made possible by using the CAM model. Due to its low immunogenicity, xenografts can be grown on the CAM model up to day 18 of embryonic development (11). Moreover, in the CAM model, growth of host stroma into the human xenograft is limited. In contrast, tumors of mice inoculated with human cell lines or patient-derived xenografts consist of around 40% mouse DNA (12, 13). We observed good experimental reproducibility of the CAM assay between separate experiments.

In conclusion, the anti-breast cancer effect of ZOL is dependent on the presence of stromal cells. This is accompanied by decreased stromal cell TGF-β excretion and reduced TGF-β signaling in cancer cells.

**Methods**

**Cell lines and reagents**

Different human cell lines were used. ER positive breast cancer cell line MCF-7 and immortalized stromal cell line Hs27a (American Type Culture Collection (ATCC)) were cultured in Roswell Park Memorial Institute (RPMI) medium (Invitrogen), supplemented with 10% fetal calf serum (FCS). Hs27a cells were cultured up to a maximum of 30 passages during which these cells remained phenotypically stable and viable. The inflammatory triple negative breast cancer cell line SUM-149 (Asterand) was cultured in HAM’s Nutrient Mixture-F12 (HAM) supplemented with 5% FCS, 5 µg/mL insulin and 1 µg/mL hydrocortisone. Triple negative breast cancer cell line MDA-MB-231 (ATCC), the daughter cell line MDA-MB-231-H2N (H2N) (transfected to stably overexpress HER2) (14), luciferase transfected MDA-MB-231-SCP2 (SCP2) (provided by Dr Y Kang, Princeton University, NJ) (15) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) (Invitrogen) with 10% FCS and 1% glutamine. Cell lines were cultured at 37 °C in
a humidified atmosphere containing 5% CO₂ and were routinely tested for *Mycoplasma*. Short tandem repeat profiling (BaseClear) was used to authenticate the cell lines.

**Breast cancer and stromal cell proliferation in vitro in response to ZOL**

Hs27a, MCF-7, MDA-MB-231, H2N, SCP2 and SUM-149 cells were plated in 96-well plates with a density of 4,000 – 12,500 cells/well. ZOL (SelleckChem) was added in different concentrations (1-1,000 µM) and cells were incubated for 96 hours. Thereafter, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT) was added and formazan production as read out of cell number and metabolic activity was measured as described previously (16).

**Breast cancer cell death in an in vitro co-culture model in response to ZOL**

This fluorescence-based *in vitro* co-culture model was described previously (17). To distinguish the cancer cells from the stromal cells, SCP2 cells were pre-labeled with fluorescent marker DiI (Molecular Probes, Invitrogen). Hs27a cells were grown as monolayer on glass slides inserted in 24-well plates. Subsequently, 15,000 SCP2 cells were added per well, with or without a pre-cultured stromal cell monolayer and allowed to attach to the glass insert or the stromal layer for 24 hours. Attached cells were treated with ZOL (10 - 500 µM) for 24 or 48 hours. To study whether the effects of ZOL could be counteracted by TGF-β1, a condition with active TGF-β1 addition was included. The cells were cultured for 48 hours following the addition of 1 ng/mL TGF-β1 (PeproTech) concurrently with ZOL.

After incubation, the plates were centrifuged at 300 g for 7 minutes. Glass slides were collected, fixed with methanol:acetone (1:1) and stained with 1:1000 4',6-diamidino-2-phenylindole (DAPI). The results were analyzed by fluorescent microscopy. Tumor cell viability was assessed with nuclear DAPI staining based on observation of the nuclear structure (intact versus fragmented nuclei). For each condition, 6 - 12 fields of view were counted and the average percentage of dead cells was calculated.

**Breast cancer cell death in an in vivo co-culture model in response to ZOL**

To study the effect of ZOL *in vivo*, the CAM model was used as described previously (18). Two breast cancer models, SCP2 and MCF-7, were studied in this way. Eggs (het Anker BV) were incubated at 38°C and after 3 days the CAM was lowered by puncturing the top of the eggs. On day 6 of embryonic development, a window was made in the egg shell to access the CAM. The CAM was damaged using a cotton tissue (Celltork) and each egg was inoculated with a total of 5·10⁶ cells in 50 µL culture media and Matrigel (BD Biosciences) (1:1). On day 10 of embryonic development, a 4 mm latex ring (Dentsply
International) was placed on the CAM and 15 µL of ZOL (200 µM) or vehicle was pipetted in the ring. Each experiment comprised 3 subgroups; group 1) breast cancer cells only, SCP2 or MCF-7 cells treated with ZOL (SCP2 n = 15, MCF-7 n = 6) or vehicle (SCP2 n = 17, MCF-7 n = 8), group 2) Hs27a cells treated with ZOL (n = 8) or vehicle (n = 7) and group 3) a mixture of breast cancer cells, SCP2 or MCF-7, with Hs27a cells (1:1) treated with ZOL (SCP2 n = 17, MCF-7 n = 9) or vehicle (SCP2 n = 12, MCF-7 n = 6). On day 14 of embryonic development, tumors were harvested, weighed and measured with a caliper. Tumor tissue was formalin-fixed and paraffin embedded for immunohistochemical analyses. H&E staining was performed to analyze tissue viability and morphology. Slides (5 µm) were stained with an antibody against cytokeratin (CK) 8, 18 and 19 (Abcam 1:100). Substitution of the 1st antibody by bovine serum albumin was used as negative control. To calculate the breast cancer cell fraction in the tumors, the ratio of CK positive cells on the total number of cells was determined per tumor. Then this number was multiplied by the total tumor weight.

**Stromal and breast cancer TGF-β1 excretion in mono- and co-culture models in vitro in response to ZOL**

TGF-β1 levels were determined in supernatant of Hs27a cells in mono-culture or in co-culture with MCF-7, SCP2 or H2N cells after 24 and 48 hours incubation with ZOL. Cell culture supernatant was removed, centrifuged for 15 minutes at 240 g to remove any residual cells or cell remnants, and subsequently stored frozen in aliquots. TGF-β1 levels were measured with enzyme-linked immunosorbent assays (ELISA) according to manufacturer’s instructions (Quantikine, R&D Systems). The absorbance of each well was measured by a microplate reader (Bio-Rad).

**Breast cancer TGF-β signaling in vitro in response to ZOL**

As read out for TGF-β signaling activity protein expression of phosphorylated Smad2 was measured by Western blot. Hs27a cells were grown as monolayer and treated with ZOL (10 - 500 µM) for 48 hours. Subsequently, 500,000 SCP2 cells were plated in 6 wells plates and incubated for 24 hours. The supernatant of Hs27a cells was harvested and added to the confluent SCP2 cells. After 1 hour incubation, the supernatant was removed. Total cell lysates were size fractionated and transferred to a membrane as described previously (19). Membranes were exposed to primary antibodies (anti-pSmad2; Cell Signaling Technology, anti-β-actin; MP Biomedicals). Binding of antibodies was determined using horseradish
peroxidase (HRP)-conjugated secondary antibodies (DAKO) and visualized with Lumi-light™ (Roche Diagnostics). Band density was evaluated by ImageJ software.

**Statistical analysis**
Data are presented as mean ± SD. Statistical analysis was performed using the one-way or two-way ANOVA test with Tukey’s or Bonferroni’s post hoc test (GraphPad Prism, version 5). Differences were considered significant when \( P < 0.05 \).
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Supplemental Fig. 1

Relative survival (%) curves after 96 hours of exposure to 1–1000 µM zoledronic acid of the stromal cell line Hs27a and breast cancer cell lines MCF-7, SUM-149, SCP2, H2N and MDA-MB-231. The 50% inhibitory concentration (IC$_{50}$) is depicted for every cell line (dashed red line).
Supplemental Fig. 2
Scatter plots illustrating weight (mg) and size (mm$^3$) of in ovo tumors harvested on day 14 after a single gift of zoledronic acid (200 µM) or vehicle (PBS) on day 10. Tumors consisted of Hs27a stromal cells. Tumor weight and size are depicted for every individual tumor.
Supplemental Fig. 3

Representative images of H&E (A) and cytokeratin staining (B) of SCP2, SCP2 and Hs27a, MCF-7, MCF-7 and Hs27a in ovo tumors on day 14 at x20 magnification. Images depicted represent vehicle treated tumors.
Supplemental Fig. 4
TGF-β1 excretion (pg/mL) after 24 hours of exposure to 0–500 µM zoledronic acid of Hs27a stromal cells in mono-culture (A) or co-cultured with SCP2 (B), H2N (C) or MCF-7 (D).
### Supplemental table 1: TGFβ levels in supernatant of stromal cells and co-cultured breast cancer cells treated with ZOL for 48 hours

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**Abbreviations:** Zoledronic acid (ZOL), transforming growth factor (TGFβ)
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References


