Zoledronic acid induces apoptosis and changes the TRAIL/OPG ratio in breast cancer cells

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Abstract
Breast cancer has a propensity to metastasize to bone, thus causing pathological fractures. Bisphosphonates are established drugs in the treatment of bone metastasis that inhibit osteoclast activity and interrupt the vicious cycle of osteoclast–tumor cell interactions. We evaluated the direct effects of zoledronic acid on estrogen receptor (ER)-negative MDA-MB-231 and ER-positive MCF-7 breast cancer cells. While zoledronic acid (100 μM) inhibited MDA-MB-231 cell proliferation after 72 h, and induced apoptosis via activation of caspase-3 and -7, it had only minor effects on MCF-7 cells. In addition, zoledronic acid induced apoptosis by up-regulating TNF-related apoptosis-inducing ligand (TRAIL) in MDA-MB-231 cells (p < 0.01), but had no effect on the expression of its decoy receptor osteoprotegerin (OPG). In MCF-7 cells, both cytokines were suppressed by zoledronic acid. In conclusion, zoledronic acid enhanced the TRAIL-to-OPG ratio in TRAIL-sensitive MDA-MB-231 cells, indicating that the TRAIL/OPG cytokine system is a bisphosphonate-responsive target in breast cancer.

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1. Introduction

Bone metastases represent a major medical problem in the management of women with breast cancer, confer a poor prognosis, and cause a substantial decrease in life quality [1]. To inhibit the exaggerated bone loss associated with metastatic breast cancer which essentially results from a dysbalance of bone turnover, bisphosphonates are widely used potent anti-resorptive drugs that reduce the bone loss and the fracture risk of women suffering from bone metastasis [2,3]. In addition to inducing apoptosis in osteoclasts [4], bisphosphonates have been shown to reduce tumor cell invasion of breast and prostate cancer cells [5,6] and to confer anti-angiogenesis activity [7]. Furthermore, bisphosphonates appear to have direct pro-apoptotic effects on cancer cells [8], but the sensitivity towards apoptosis seems to vary between different breast cancer cell lines [9,10]. A recent clinical study demonstrated that adjuvant therapy with the potent amino-bisphosphonate zoledronic acid improved disease-free survival of women with early breast cancer without bone metastases [11].

An endogenous molecule that has been shown to prevent bone metastasis in vivo is osteoprotegerin (OPG) [12]. OPG represents a soluble decoy receptor that neutralizes
both the essential osteoclast cytokine, receptor activator of NF-κB ligand (RANKL), and the pro-apoptotic cytokine TNF-related apoptosis-inducing ligand (TRAIL), both members of the TNF ligand superfamily. The importance of the RANKL–OPG-interaction in the homeostasis of bone metabolism has been reviewed in detail [13]. However, there is emerging evidence that endogenous OPG can also act as a survival factor for breast cancer cells by protecting them against TRAIL-induced apoptosis [14]. We have previously shown that bisphosphonates modulate OPG expression in osteoblasts [15], but to date, there are no studies investigating direct effects of bisphosphonates on OPG and TRAIL expression by breast cancer cells.

Overall, the positive effects of bisphosphonates and their clinical potential in metastatic breast cancer are widely confirmed in vitro as well as in vivo [16,17]. In the present study we tested the hypothesis that the potent bisphosphonate zoledronic acid affects proliferation and apoptosis of breast cancer cells in vitro. In addition we examined its influence on TRAIL and OPG expression to further clarify the role of these two cytokines in breast cancer survival.

2. Materials and methods

2.1. Materials

Anti-poly-ADP-ribose polymerase (PARP) antibody, anti-cleaved caspase-3, and anti-caspase-7 antibody were from Cell Signaling Technology (Frankfurt Main, Germany). Anti-β-actin antibody was from Santa Cruz (Heidelberg, Germany). The ECL detection reagents were purchased from Amersham Bioscience (Little Chalfont, UK). American methyl [3H]-thymidine was from GE Healthcare UK Limited (Buckinghamshire, UK). Annexin V-FITC apoptosis detection kit was from Calbiochem (Schwalbach, Germany). The OPG kit was purchased from Immundiagnostik (Bensheim, Germany). All other reagents were obtained from Sigma–Aldrich (Munich, Germany).

2.2. Cell culture

MDA-MB-231 cells were maintained in McCoy’s medium and MCF-7 cells were maintained in RPMI 1640 medium from Bio West (Renningen, Germany). Both media were supplemented with 10% fetal bovine serum from PAN Biotech (Aidenbach, Germany) and 1% penicillin/streptomycin from Biochrom AG (Berlin, Germany). Cells were passaged after reaching 70% confluency by trypsin, and were kept at 37 °C (5% CO₂, 90% humidity).

2.3. Time-lapse microscopy

For time-lapse experiments, cells were seeded at 5 × 10⁴ cells/well and cultured in 6-well plates. Phase-contrast photographs were taken at 0, 24, 48, and 72 h by live cell imaging system with an Axiovert Inverted Microscope (Carl Zeiss, Göttingen, Germany).

2.4. Thymidine assay

DNA synthesis of MDA-MB-231 and MCF-7 cells was determined by [3H]thymidine incorporation. Both cell lines were seeded in 6-well plates. Cells were kept for 72 h in the presence of zoledronic acid. [3H]thymidine (0.5 Ci/ml) was added for the last 4 h. Acid-soluble [3H]thymidine was removed by washing the cells for 30 min in ice-cold 5% (v/v) trichloroacetic acid at 4 °C. Subsequently, the cells were treated with ethanol, air-dried, and solubilized in 500 µl/well of 0.1% NaOH and 1% sodium dodecyl sulphate (SDS) at room temperature. The incorporated radioactivity was determined by a spectrophotometric liquid scintillation counter.

2.5. Assessment of apoptosis

Apoptosis of breast cancer cells was studied through the detection and quantification of cytoplasmic histone-associated DNA fragments using the Cell Death Detection ELISAPLUS photometric enzyme immunoassay (Roche Diagnostics, IN). Apoptotic cell death was also analyzed by annexin V-FITC staining. Briefly, cells were cultured in 6-well plates and treated with 100 µM of zoledronic acid for up to 72 h. After treatment, cells were washed with cold PBS, and 1 × 10⁵ cells were resuspended in 195 µl of cold binding buffer plus 5 µl annexin V-FITC, followed by 10 min incubation at room temperature in the dark. Cells were washed and resuspended with 190 µl binding buffer. Ten µl of propidium iodide at a concentration of 20 µg/ml were added immediately before analysis. Apoptosis was assessed by dual color flow cytometry on a FACScan cytofluorometer from Becton Dickinson (Mountain View, CA) using Cell Quest software package from Becton Dickinson.

2.6. Western blot analysis

Breast cancer cells were lysed in ice-cold lysis buffer and sonicated. Insoluble cell debris was removed by centrifugation at 13,000 rpm for 5 min. Protein content was quantified by the Bio-Rad protein assay and 15 µg protein were separated by SDS–PAGE and transferred onto nitrocellulose membranes. After blocking in 5% non-fat dry milk for 1 h, membranes were incubated overnight at 4 °C with antibodies to PARP and cleaved-caspase-3 and -7 (Cell Signaling Technology). Signals were detected using horseradish peroxidase-conjugated anti-rabbit IgG antibody and chemoluminescent reaction with ECL detection reagent.

2.7. OPG protein assay

OPG protein secretion was measured using a commercial OPG assay from Immundiagnostik. Briefly, the supernatant of cells was harvested and the protocol provided by the manufacturer was used for the sandwich ELISA. The concentration was quantified by determining the absorbance with an ELISA reader at 450 nm against 690 nm as a reference.
2.8. RNA isolation

Total RNA from MDA-MB-231 and MCF-7 cells was isolated using the RNeasy kit from Qiagen (Hilden, Germany) according to the manufacturer’s instructions. RNA was quantified spectrophotometrically at 260 nm.

2.9. Quantitative real-time RT-PCR

RNA was isolated from cells using RNeasy kit from Qiagen. Omniscript RT-kit from Qiagen was used for the reverse transcription. The qRT-PCR of OPG was performed as previously described [18]. Additional primers were chosen using PrimerExpress 3.0 from Applied Biosystems (Carlsbad, CA): TRAIL sense 5’TCCACAGTGCCTGCA GT3’, TRAIL anti-sense 5’TTCGCCAACTAAAGGGCC CCC3’. Values were expressed as the mean ± SD of triplicate measurements.

2.10. Statistical analysis

Each experiment was reproduced at least three times. Values are expressed as the mean ± SD of triplicate measurements unless otherwise stated. Student’s paired t-test was used to analyze differences between the sample of interest and its control. Time courses and dose responses were compared by multiple measurements ANOVA and corrected by Student-Newman–Keul’s test for differences between groups. A p value of less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of zoledronic acid in human breast cancer cell lines

To analyze the effects of zoledronic acid on breast cancer cells, MCF-7 and MDA-MB-231 cells were exposed to increasing concentrations of zoledronic acid for 72 h. Assessment of both cell lines following exposure revealed that zoledronic acid exerted dose-dependent effects on cell proliferation and apoptosis in MDA-MB-231 cells. One hundred micromolar of zoledronic acid were more potent in inhibiting proliferation in MDA-MB-231 cells as early as 24 h after zoledronic acid exposure. After 48 h of exposure, a reduction of cell numbers was clearly visible, with an almost complete loss of cells after 72 h. In direct comparison, MCF-7 cells were less affected by the presence of zoledronic acid, indicating a certain degree of resistance (Fig. 2A). We next investigated the cellular pathways underlying the observed findings. Cell proliferation was assessed by measuring thymidine incorporation. While only mild anti-proliferative changes could be observed in MCF-7 cells after 72 h of zoledronic acid exposure, MDA-MB-231 cells showed significant inhibition of cell growth after 48 h. After 72 h of exposure, proliferation of MDA-MB-231 cells was suppressed by more than 95% (Fig. 2B, p < 0.0001).

3.2. Effects of zoledronic acid on cell proliferation in human breast cancer cell lines

We then analyzed the rate of apoptosis as an explanation for the morphological changes detected. Both cell lines were treated with 100 µM of zoledronic acid for various time intervals, and apoptosis was assessed using an immunoassay. As shown in Fig. 3A, an enhanced rate of apoptosis was observed for estrogen receptor (ER)-negative MDA-MB-231 cells, with a maximum 14-fold induction compared to untreated controls after 48 h of zoledronic acid exposure (p < 0.05). At the same time, the rate of apoptosis remained unaffected in ER-positive MCF-7 cells (Fig. 3A). To quantify the percentage of apoptotic cells, we analyzed zoledronic acid-treated MDA-MB-231 and MCF-7 cells using annexin V/PI double labeling technique (Fig. 3B). By this method, the annexin V-stained fraction increased from 3.1% to 26.3% (by 8.5-fold) in MDA-MB-231 cells after 72 h of zoledronic acid exposure, whereas the annexin V-fraction of MCF-7 cells only increased from 4.2% to 7.8%.

To assess the role of caspases in the initiation of apoptosis, caspase-3, caspase-7, and PARP cleavage were analyzed by Western blotting. As shown in Fig. 3C, zoledronic acid caused a time-dependent cleavage of caspase-3, caspase-7, and PARP in MDA-MB-231 cells. The earliest cleavage could be detected for caspase-3 after 24 h, and caspase-3 cleavage continuously increased thereafter. In addition, caspase-7 and PARP were also cleaved after 24, 48, and 72 h, respectively. After 72 h of zoledronic acid exposure PARP was completely cleaved. In MCF-7 cells no cleavage was detected after 24 h. Only after 48 and 72 h of zoledronic acid exposure, all examined caspases were partially cleaved (Fig. 3C).

Fig. 1. Assessment of proliferation and apoptosis in MCF-7 and MDA-MB-231 cells following zoledronic acid exposure with 0–100 µM for 72 h. (A) Proliferation was assessed by [3H]thymidine incorporation. Data represent the mean ± SD of three independent experiments, *p < 0.05, **p < 0.001. (B) Western blot analysis of PARP cleavage in MDA-MB-231 and MCF-7 cells following exposure to zoledronic acid as indicated. Equal protein loading was verified by β-actin staining. Representative blots are shown. PARP, poly-ADP-ribose polymerase.
3.4. Effects of zoledronic acid on OPG expression in human breast cancer cell lines

OPG is known to be abundantly expressed and secreted by a large number of breast cancers in vitro and in vivo. OPG has the ability to bind and block TRAIL-induced apoptosis. Therefore, we investigated OPG mRNA levels and protein secretion of MDA-MB-231 and MCF-7 cells in the presence of zoledronic acid. Both cell lines were stimulated with 100 \( \mu \)M of zoledronic acid for up to 72 h after which RNA was isolated and supernatant harvested. While the isolation of sufficient amounts of RNA was impossible in MDA-MB-231 cells after 72 h due to extensive cell loss, OPG mRNA levels remained unaffected after 24 and 48 h of zoledronic acid exposure (Fig. 4A). In accordance with those findings, the amount of secreted OPG protein remained unchanged after 48 h of zoledronic acid exposure (Fig. 4A). However, in MCF-7 cells, the presence of zoledronic acid induced a sustained suppression of OPG with a maximum suppression of OPG mRNA levels to 41 ± 8.7% of control (100%) after zoledronic acid exposure over 72 h (\( p < 0.001 \)). OPG protein secretion decreased by 66% from 4.7 ± 0.6 pmol/l to 1.6 ± 0.2 pmol/l after 48 h of zoledronic acid exposure (Fig. 3B, \( p < 0.01 \)).

3.5. Effects of zoledronic acid on TRAIL mRNA levels in human breast cancer cell lines

Up to 30% of primary breast cancers have been shown to express TRAIL [19], but little is known about how TRAIL expression is regulated in breast cancer cells. We therefore compared TRAIL mRNA levels of quiescent MDA-MB-231 and MCF-7 cells using qRT-PCR and assessed its modulation by zoledronic acid. Both cell lines were exposed to 100 \( \mu \)M of zoledronic acid for 24, 48, and 72 h. Interestingly, MDA-MB-231 cells had basal TRAIL mRNA levels that were about 50-fold higher compared to MCF-7 cells. In addition, zoledronic acid had opposite effects on TRAIL mRNA levels in both cell lines. While zoledronic acid suppressed TRAIL mRNA levels in MCF-7 by 50% (\( p < 0.01 \)), it enhanced TRAIL mRNA levels by 2.5-fold in MDA-MB-231 cells (Fig. 5A, \( p < 0.05 \)). This regulation significantly affected the calculated TRAIL/OPG ratio (Fig. 5B). The baseline TRAIL/OPG ratio of MDA-MB-231 cells was 7.4 ± 1.7 and was increased by zoledronic acid exposure at a dose of 100 \( \mu \)M over 48 h to 24.1 ± 7.7 (\( p < 0.05 \)). By contrast, the TRAIL/OPG ratio in untreated MCF-7 cells was 0.52 ± 0.14 and not significantly altered (0.65 ± 0.10), because zoledronic acid lowered both TRAIL and OPG to the same extent.

Fig. 2. Morphological and proliferative effects of zoledronic acid on breast cancer cell lines MCF-7 and MDA-MB-231. (A) Phase-contrast photographs were taken after 0, 24, 48, and 72 h of zoledronic acid exposure (100 \( \mu \)M). (B) MCF-7 and MDA-MB-231 cells were treated with 100 \( \mu \)M of zoledronic acid. Proliferation was assessed by \(^{3}H\) thymidine incorporation. Data represent the mean ± SD of three independent experiments. * \( p < 0.01 \), ** \( p < 0.0001 \).
4. Discussion

Bisphosphonates are commonly used to treat malignant and benign skeletal diseases characterized by excessive bone resorption. In particular, zoledronic acid has profound beneficial effects in patients with skeletal metastases [20]. Bisphosphonates act through the induction of osteoclast apoptosis, probably by inhibiting the isoprenylation of proteins required for osteoclast survival [21,22]. Moreover, direct anti-tumor effects of bisphosphonates have been demonstrated in several cancer types, including multiple myeloma [23], pancreatic cancer [24], prostate cancer [25], and breast cancer [26].

In this study, we demonstrate that the potent nitrogen-containing bisphosphonate zoledronic acid inhibits cell proliferation and induces apoptosis in breast cancer cells. Zoledronic acid inhibited cell proliferation by over 90% after 72 h of treatment in the ER-negative MDA-MB-231 breast cancer cell line, which is capable of creating osteolytic bone metastases. In addition to inhibiting cell proliferation, zoledronic acid induced apoptosis in MDA-MB-231 cells. Moreover, the local concentration of bisphosphonates in the bone microenvironment, resulting in areas of highly concentrated bisphosphonates. High local concentrations may be a prerequisite for their ability to exert direct anti-tumor effects in vivo.

Zoledronic acid appears to have different effects depending on tumor type and origin. For instance, in lung cancer cells, zoledronic acid has been shown to decrease cell proliferation without inducing apoptosis [29], whereas conflicting reports on breast cancer sensitivity to zoledronic acid exist. A study comparing the response of three breast cancer cell lines, including MCF-7 and MDA-MB-231 cells, to various bisphosphonates found that MCF-7 cells were

![Graph](image_url)
Fig. 4. Regulation of OPG mRNA levels and protein secretion in MDA-MB-231 and MCF-7 cells by zoledronic acid. Cells were treated with zoledronic acid (100 μM) for 0, 24, 48, and 72 h. OPG mRNA levels were quantified using qRT-PCR. OPG protein secretion was measured by an immunoassay using supernatants of MDA-MB-231 (A) and MCF-7 (B) cells. OPG mRNA levels were normalized to cyclophilin. Expression is given relative to control and represents the mean ± SD from three individual experiments. *p < 0.01, **p < 0.001, # no sufficient RNA could be isolated after 72 h of zoledronic acid exposure.

Fig. 5. (A) Effects of zoledronic acid on TRAIL mRNA levels in MDA-MB-231 and MCF-7 cells. Cells were treated with zoledronic acid (100 μM) for 0, 24, 48, and 72 h. Data are given relative to control as the mean ± SD from 3 individual experiments and normalized to cyclophilin. (B) TRAIL/OPG ratios before and after zoledronic acid exposure for 48 h. Data are shown as ratios after normalization to cyclophilin and represent the mean ± SD from three individual experiments. *p < 0.05, **p < 0.01, # no sufficient RNA could be isolated after 72 h of zoledronic acid exposure.
more sensitive to zoledronic acid than MDA-MB-231 cells [8]. By contrast, another study found that both cell lines were similarly sensitive to bisphosphonates [26], which is in contrast to a previous report where the bisphosphonate clodronate reduced cell survival of MDA-MB-231, but not MCF-7 cells [30]. In our hands, ER-negative MDA-MB-231 cells were highly sensitive to zoledronic acid-induced apoptosis, whereas ER-positive MCF-7 cells were found to be relatively resistant. It remains unclear why these differences exist. Some authors have proposed that bisphosphonate-induced apoptosis is caspase-dependent [26,31]. A possible explanation for the relative insensitivity of MCF-7 cells may be their lack of caspase-3 [32]. Studies with primary breast cancer samples may help to define the role a functioning ER plays in the susceptibility to zoledronic acid. This may be especially interesting in light of the limited treatment options for ER-negative breast cancer which are reminiscent of the MDA-MB-231 cells [33]. Women who suffer from bone metastases due to breast cancer benefit from bisphosphonate therapy. Of note, the addition of zoledronic acid to a neoadjuvant chemotherapy regimen improved the tumor response rate (Winter et al., personal communication), and emerging evidence suggests a benefit for preventive bisphosphonate treatment of patients with early stages of breast cancer without bone metastases [34]. Increased survival may, at least in part, be related to the direct anti-tumor effects through enhanced apoptosis as observed in this study. This concept is supported by a recent study demonstrating that zoledronic acid reduced dissemination of breast cancer cells into the bone marrow (Solomayer et al., personal communication). Thus, administration of zoledronic acid and subsequent accumulation in the skeleton may condition the bone, yielding an unfavourable microenvironment for seeding and survival of breast cancer cells.

OPG expression has been shown to positively correlate with ER expression [19], but the clinical relevance of this observation still needs to be established. Enhanced OPG expression may increase ligation of RANKL and thereby reduce the capability of tumor cells to metastasize to the skeleton [35], but could also result in a better protection from TRAIL-induced apoptosis [14]. It is conceivable that the TRAIL/OPG ratio determines tumor survival of MDA-MB-231 cells that are both sensitive to TRAIL and express high endogenous levels of TRAIL [33]. Exposure of MDA-MB-231 cells to zoledronic acid increased TRAIL, but not OPG expression. These changes translated into an enhanced TRAIL/OPG ratio. Of note, only the bisphosphonate- and TRAIL-sensitive MDA-MB-231 cells were affected in this way, while the TRAIL/OPG ratio remained unaffected in the resistant MCF-7 cells.

In conclusion, zoledronic acid displays pronounced anti-proliferative and pro-apoptotic effects in ER-negative MDA-MB-231, whereas ER-positive MCF-7 cells are relatively resistant. Furthermore, zoledronic acid increased TRAIL production and enhanced the TRAIL/OPG ratio in TRAIL-sensitive MDA-MB-231 cells, thus affecting cell integrity and survival. Our findings indicate that the TRAIL/OPG cytokine system is a bisphosphonate-responsive target in breast cancer.

Conflicts of interest
None declared.

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