Zoledronic Acid Reverses the Epithelial–Mesenchymal Transition and Inhibits Self-Renewal of Breast Cancer Cells through Inactivation of NF-κB

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Abstract
Zoledronic acid, a third-generation bisphosphonate, has been shown to reduce cell migration, invasion, and metastasis. However, the effects of zoledronic acid on the epithelial–mesenchymal transition (EMT), a cellular process essential to the metastatic cascade, remain unclear. Therefore, the effects of zoledronic acid on EMT, using triple-negative breast cancer (TNBC) cells as a model system, were examined in more detail. Zoledronic acid treatment decreased the expression of mesenchymal markers, N-cadherin, Twist, and Snail, and subsequently upregulated expression of E-cadherin. Zoledronic acid also inhibited cell viability, induced cell-cycle arrest, and decreased the proliferative capacity of TNBC, suggesting that zoledronic acid inhibits viability through reduction of cell proliferation. As EMT has been linked to acquisition of a self-renewal phenotype, the effects of zoledronic acid on self-renewal in TNBC were also studied. Treatment with zoledronic acid decreased expression of self-renewal proteins, BMI-1 and Oct-4, and both prevented and eliminated mammosphere formation. To understand the mechanism of these results, the effect of zoledronic acid on established EMT regulator NF-κB was investigated. Zoledronic acid inhibited phosphorylation of RelA, the active subunit of NF-κB, at serine 536 and modulated RelA subcellular localization. Treatment with zoledronic acid reduced RelA binding to the Twist promoter, providing a direct link between inactivation of NF-κB signaling and loss of EMT transcription factor gene expression. Binding of Twist to the BMI-1 promoter was also decreased, correlating modulation of EMT to decreased self-renewal. On the basis of these results, it is proposed that through inactivation of NF-κB, zoledronic acid reverses EMT, which leads to a decrease in self-renewal. Mol Cancer Ther; 12(7); 1356–66. ©2013 AACR.

Introduction
Zoledronic acid is a third-generation bisphosphonate originally designed to treat osteoporosis (Fig. 1). In addition to its effects on osteoclastogenesis, clinical studies have shown that treatment with zoledronic acid increases disease-free survival (1–4) and inhibits metastatic disease (1) of postmenopausal women diagnosed with breast cancer. Preclinically, treatment with zoledronic acid inhibits viability (5–10), migration, and invasion (11, 12) of breast cancer cells. Mechanisms responsible for these effects have yet to be deduced. To date, studies have focused on the effects of zoledronic acid on cell motility. There are several steps in the metastatic cascade before cell migration and invasion, an example of which is passage through the epithelial–mesenchymal transition (EMT). Here, the effects of zoledronic acid on EMT are reported.

EMT is a cellular process whereby epithelial cells undergo cellular changes, including loss of cell–cell contact and cell polarity, gain of an elongated cell structure, and the ability to move as a single cell, to become more mesenchymal (13, 14). Mesenchymal cells will gain expression of N-cadherin and lose expression of epithelial protein E-cadherin, which correlates with upregulation of EMT transcription factor gene expression. Binding of Twist to the BMI-1 promoter was also decreased, correlating modulation of EMT to decreased self-renewal. On the basis of these results, it is proposed that through inactivation of NF-κB, zoledronic acid reverses EMT, which leads to a decrease in self-renewal.

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and 1% penicillin/streptomycin, at 37°C in 5% CO₂ and were passaged weekly. For cell treatment, each cell line was seeded and allowed to grow to 70% confluency. Cells were then treated for specified duration with either vehicle (0.9% saline) or treatment (zoledronic acid was prepared as 10⁻² mol/L stock solution at pH 7.4 and diluted). Cells and mammospheres were imaged using a Canon EOS Rebel T1i at ×10 or ×4 magnification.

**Sample collection**

Cell media were aspirated and remaining attached cells were washed twice with dPBS to remove detached cells from the culture. For Western blot analysis and PCR analyses, cells were collected by scraping directly into lysis buffer. For the rest of the experiments, attached cells were collected by trypsinization, and viability was assayed by Trypan blue exclusion. Dead cells were not removed from the sample, but cell numbers were corrected such that the same number of viable cells was used for each experiment.

**Preparation of whole-cell lysates and cell fractionation**

Whole-cell lysates were prepared as previously described (19). For cell fractionation, cells were scraped by rubber policeman in dPBS, washed twice, and centrifuged at 1,000 rpm for 5 minutes at 4°C. Pellet was resuspended in 1× hypotonic buffer (20 mmol/L Tris-HCl, pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl₂) and incubated on ice for 10 minutes. To this was added 25 μL of 10% NP40. Mixture was vortexed for 10 seconds and centrifuged for 10 minutes at 3,000 rpm at 4°C. Supernatant was collected as cytoplasmic fraction. The pellet was resuspended in 50 μL complete cell extraction buffer (cell extraction buffer with 1 mmol/L phenylmethylsulfonyl-fluoride and protease inhibitors), and mixture was vortexed every 10 minutes for 30 minutes. Mixture was centrifuged for 30 minutes at 14,000 × g at 4°C. Supernatant was collected as nuclear fraction. Protein concentrations were determined using bicinchoninic acid method.

**Western blotting**

Western blotting was conducted as previously described (19).
RNA extraction and reverse transcription
RNA was extracted and reverse transcribed as previously described (19).

PCR
mRNA and promoter expression were measured using quantitative PCR (qPCR) as previously described (19). PCRs were amplified for 50 cycles with an annealing temperature of 60°C. The following primers were used for analysis: human TWIST-1 forward: 5'-TCAGCCACTGAAAGAAAGG-3' and reverse: 5'-CCCTCAGAGGAGATGAAA-3'; human Snail forward: 5'-TTCTTCTGCGCTACTGCTGCG-3' and reverse: 5'-GGGCAGTGAGGGGAGGAAAGA-3' (20), human N-cadherin forward: 5'- AGGAGCTCTTCTCACAGAAGGA-3' and reverse: 5'-CTA-CTCAGATGCCCTCCTAAAA-3'; human E-cadherin forward: 5'-CGGGAATGCGATGAGTGGATC-3' and reverse: 5'-AGGATGTGAAGGATGGCAGTGTCAG-3'; human RelA (accession no. NM_021975) forward: 5'-TAGGCCTGAT- TATGCCTCTAG-3' and reverse: 5'-CTCAGATTTGTGAT- GTGAAGAGA-3' (21); human GAPDH forward 5'- AAGGTCCGAGTCAGCAAGGTGGT-3' and reverse: 5'- CCATGGATGACCATATTTGGAA-3' (23). Values were normalized to GAPDH and are expressed as fold change relative to control treatment.

RNAs
Cells were seeded in 12-well plates at a density of 120,000 cells/1.1 mL passage media and were allowed to briefly attach under culture conditions. RelA siRNA (20 nmol/L) was mixed with HiPerfect Transfection Reagent (6 μg/mL) for 10 minutes at room temperature. Cells were incubated for 72 hours under normal cell culture conditions. Following transfection, cell media were removed and transfected cells were used for future experiments.

Chromatin immunoprecipitation
ChIP analyses were conducted using the ChIP assay kit as per manufacturer’s instructions. Samples were incubated with either 5 μg RelA or 5 μg Twist antibody overnight at 4°C using end-over-end rotation. In addition, control samples were incubated with 5 μg normal rabbit IgG as a negative control. Products resulting from the immunoprecipitation were extracted using the QiaQuick PCR Purification Kit as per manufacturer’s instructions. Resulting DNA was used for qPCR. Primers include: human TWIST-1 promoter: forward: 5'-GGGAGGACGGAGATGTGAGCC-3' and reverse: 5'-GGGAGGAGGAGATGTGAGCC-3'; human BMI-1 promoter: forward: 5'-GGGAGGAGGAGATGTGAGCC-3' and reverse: 5'-GGGAGGAGGAGATGTGAGCC-3' (24). All primers were amplified for 50 cycles with a 60°C annealing temperature for qPCR. Samples were amplified using negative control primers upstream of the Twist-binding site on the BMI-1 promoter (24): forward: 5'-GGGAGGAGGAGATGTGAGCC-3' and reverse: 5'-GGGAGGAGGAGATGTGAGCC-3'. Values were normalized to each individual input control.

Cell-cycle analysis
Cell media were aspirated, and cells were washed twice with dPBS. Attached cells were collected by trypsinization and centrifuged for 3 minutes at 500 × g. Cells were counted and resuspended in dPBS at a cell density of 1 million cells/100 μL dPBS. Cells were incubated for 30 minutes in hypotonic cell lysis buffer (0.1% sodium citrate, 0.1% Triton X-100) in the presence of 50 μg/mL propidium iodide and 25 μg/mL ribonuclease A. Following incubation, the cells were analyzed using FlowJo software. The Watson algorithm was used to find the peak and S-phase populations from a univariate distribution curve.

Mammosphere assay
Cell media were aspirated and cells were washed twice in dPBS. Attached cells were trypsinized and collected by centrifugation at 500 × g for 5 minutes at 20°C. Cells were counted and resuspended in mammosphere media (per manufacturer’s instructions; 2.5 μg/mL amphotericin B, and 50 μg/mL gentamycin were also added) at a cell density of 10,000 cells/mL. Two milliliters of cell solution was seeded in 6-well ultra-low attachment plates. Cells were incubated for one week at 37°C in 5% CO2. Formed mammospheres were counted manually. For secondary mammospheres, mammospheres and media were collected and spun down at 350 × g for 5 minutes at 4°C. Media was aspirated and 1 mL trypan blue EDTA was added to each mammosphere pellet. The trypsin/pellet was pipetted up and down for approximately 1.5 minutes, and dPBS with 2% FBS was added. Mixture was centrifuged at 350 × g for 5 minutes at 4°C. Media was aspirated, and cells were resuspended in Mammocult media and counted. Five-thousand cells were seeded in ultra-low attachment plates and incubated for 1 week at 37°C in 5% CO2. For treated secondary mammospheres, mammospheres were formed for 72 hours before treatment.

Statistical analysis
All data are expressed as mean ± SEM. P values were calculated using unpaired t test, ANOVA, and Tukey post hoc analyses using GraphPad Prism 5. P < 0.05 was considered significant.

Results
Zoledronic acid induces changes in TNBC cellular morphology
Following treatment with zoledronic acid, TNBC cell lines, MDA-MB-231 and Hs578t, underwent morphologic changes, which occurred in a time-dependent manner. Vehicle-treated cells exhibited elongated cell structure, cellular protrusions, and lacked cell–cell contacts (Fig. 2A, i and iii). By 48 hours of treatment, protrusions were no longer visible, and increased cell–cell contact was observed (Fig. 2A, ii and iv). These results suggest that treatment with zoledronic acid drives TNBC toward a more epithelial morphology.
Zoledronic acid decreases mesenchymal and increases epithelial marker expression

Protein analyses of mesenchymal and epithelial markers were conducted on TNBC following treatment with vehicle, 1 µmol/L, or 10 µmol/L zoledronic acid for 48 hours. Only attached cells were used for analysis. Briefly, following treatment with 10 µmol/L zoledronic acid, cell media were removed and remaining attached cells were washed with dPBS to remove any additional detached cells. The remaining attached cells were used for the experiment, and exhibited decreased expression of mesenchymal proteins N-cadherin (P < 0.05 vs. vehicle in MDA-MB-231; P < 0.01 vs. vehicle in Hs578t), Snail (P < 0.01 vs. vehicle in MDA-MB-231; P < 0.001 vs. vehicle in Hs578t), and Twist (Fig. 2B and C) following both 48 and 72 hours of treatment. These changes were accompanied by a significant upregulation of E-cadherin expression following treatment for 48 hours with 10 µmol/L zoledronic acid in MDA-MB-231 cells (P < 0.05; Fig. 2D). E-cadherin expression could not be detected in either vehicle- or zoledronic acid-treated Hs578t samples, though protein lysates from MCF-7 cells (which express high levels of E-cadherin) were positive for E-cadherin protein expression (Supplementary Fig. S1A). Increased CD24 (epithelial marker) cell surface expression was observed in Hs578t following treatment for 48 hours (~40% increase in CD24; Supplementary Fig. S1B), confirming an increase in epithelial phenotype.

These results correlated with decreased mRNA expression of mesenchymal markers N-cadherin (P < 0.052 vs. vehicle in MDA-MB-231; P < 0.04 vs. vehicle in Hs578t), Snail, and Twist (P < 0.02 vs. vehicle in Hs578t; Fig. 2C and D), following 48 hours of treatment with 10 µmol/L zoledronic acid. mRNA expression of epithelial marker E-cadherin was concomitantly upregulated (P < 0.03 vs. vehicle in MDA-MB-231; P < 0.02 vs. vehicle in Hs578t; Fig. 2C and D). These results signify that zoledronic acid reduces expression of mesenchymal markers and increases expression of epithelial markers. On the basis of these findings, it is concluded that zoledronic acid reverses the EMT.

TNBC are sensitive to treatment with zoledronic acid

Previous groups report that treatment with zoledronic acid inhibits cell viability (5–10). Therefore, the effects of zoledronic acid on cell viability were assessed following

**Figure 2.** Zoledronic acid inhibits mesenchymal phenotype and induces epithelial phenotype. A, cellular morphology of Hs578t (top, i and ii) and MDA-MB-231 (bottom, iii and iv) following treatment with vehicle (i and iii) or 10 µmol/L zoledronic acid for 48 hours. Arrows indicate areas of cellular protrusion. Box indicates areas of cell-cell contact. B, mesenchymal and epithelial protein expression in MDA-MB-231 (left) or Hs578t (right) following treatment for 48 hours with vehicle, 1, or 10 µmol/L zoledronic acid. Protein levels were normalized to β-actin. C and D, mesenchymal marker mRNA expression in MDA-MB-231 (C) and Hs578t (D) following treatment for 48 hours with vehicle (V) or 10 µmol/L zoledronic acid (Z). Data are indicative of 3 independent experiments. *, P < 0.05, **, P < 0.01.
treatment with 10 μmol/L zoledronic acid in TNBC for 24, 36, 48, 72, and 144 hours. Trypan blue exclusion experiments show that cell number decreased as early as 24 hours after treatment in both cell lines, but significant changes were not observed until 72 hours of treatment in MDA-MB-231 cells (P < 0.01 vs. vehicle in 72 hours; Fig. 3A) or until after 48 hours of treatment in Hs578t cells (P < 0.01 vs. vehicle in 48 hours, P < 0.001 vs. vehicle in 72 hours; Fig. 3B). Only a small proportion of cells from either cell line remained attached following 144 hours of treatment (P < 0.001 vs. vehicle; Fig. 3A and B). Cells, which remained attached following treatment for 48 hours with 10 μmol/L zoledronic acid, were assayed for markers of apoptosis (PARP-1 and caspase-3). Resulting experiments showed that neither PARP nor caspase-3 underwent cleavage following treatment (Fig. 3C). On the basis of these results, it is concluded that TNBC are sensitive to treatment with zoledronic acid, and those cells, which remain attached following treatment, are viable and not fated for cell death.

**Zoledronic acid reverses EMT before cellular detachment**

Because such sharp decreases in cell viability were observed following treatment with zoledronic acid, it was postulated that the cells, which remained attached and became epithelial, evade loss of viability. To test this, TNBC were treated for a duration of 6 days, with detached cells collected after 72 hours (denoted 72 hours float) or 6 days (denoted 6 days float), and attached cells collected at the end of treatment (denoted attach). These samples were compared with cells treated for 6 days with vehicle for expression of mesenchymal and epithelial markers. Observed was a mesenchymal to epithelial transition in the detached cells, as evidenced by increased E-cadherin and decreased N-cadherin expression (Fig. 3D). Cells, which remain attached after 6 days (approximately 5%–10% vehicle-treated samples; Fig. 3A and B), are in transition between mesenchymal and epithelial state (Fig. 3D). Taken together, these results suggest that EMT is reversed before detachment, and those cells in which EMT

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**Figure 3.** Cells remaining attached in culture following treatment with zoledronic acid are viable and detach after reversal of EMT. A and B, percent viability of MDA-MB-231 (A) and Hs578t (B) cells following treatment with vehicle or 10 μmol/L zoledronic acid over a time course of 24, 36, 48, 72, or 144 hours. C, apoptotic protein expression in MDA-MB-231 (left) and Hs578t (right) following treatment with vehicle or 10 μmol/L zoledronic acid. Expected cleavage products: PARP, 89 kD; caspase-3, 17 kD. D, mesenchymal and epithelial protein expression in MDA-MB-231 (left) or Hs578t (right) following treatment with vehicle (Veh.) or 10 μmol/L zoledronic acid for 6 days. Seventy-two hours after the initial treatment, detached cells were collected (72 hours float) and cells were retreated. Seventy-two hours after the second treatment, both attached (attach) and detached cells (6 days float) were collected. Data are expressed as mean ± SEM and are representative of 2 independent experiments. **, P < 0.01; ***, P < 0.001. ZA, zoledronic acid.
is not reversed remain attached and may be resistant to zoledronic acid treatment.

**Zoledronic acid inhibits cell-cycle progression and decreases proliferative capacity**

Although Western blot assessment of attached cells following treatment with zoledronic acid suggested that cells were not fated for cell death, large decreases in viable cells were still observed (Fig. 3A–C). Therefore, it was hypothesized that treatment with zoledronic acid may inhibit cell proliferation. To test this hypothesis, TNBC were treated for 48 hours with 10 µmol/L zoledronic acid, and remaining attached, viable cells were analyzed for changes in cell-cycle progression. Both cell lines underwent cell-cycle arrest. MDA-MB-231 arrested in G1 phase (P < 0.001; Fig. 4A), whereas Hs578t arrested in S-phase (P < 0.001; Fig. 4B). Examination of total cell number following treatment with zoledronic acid suggested that cells treated with zoledronic acid proliferate at a slower rate when compared with vehicle-treated cells. In Trypan blue experiments, MDA-MB-231 cells treated with 10 µmol/L zoledronic acid proliferate to about 65% the amount of the vehicle-treated cells (700,000 in zoledronic acid-treated group compared with 1.1 million in vehicle-treated group; Fig. 4C), and Hs578t treated with 10 µmol/L zoledronic acid proliferate to about 30% the amount of the vehicle-treated cells (520,000 in zoledronic acid-treated group compared with 1.7 million in vehicle-treated group; Fig. 4D). These data suggest that treatment with zoledronic acid decreases proliferative capacity, which correlates with cell-cycle arrest. Taken together, these results suggest that zoledronic acid inhibits cell proliferation.

**Figure 4.** Zoledronic acid inhibits cell-cycle progression and decreases proliferative capacity. A and B, cell-cycle analysis of MDA-MB-231 (A) and Hs578t (B) following treatment for 48 hours with vehicle or 10 µmol/L zoledronic acid. C and D, total number of viable cells in MDA-MB-231 (C) and Hs578t (D) following treatment with vehicle or 10 µmol/L zoledronic acid over a time course of 24, 36, 48, 72, or 144 hours. Data are expressed as mean ± SEM and are representative of 2 independent experiments. ***: P < 0.001. ZA, zoledronic acid.
Zoledronic acid inhibits self-renewal capability

Previous findings have suggested that cells that undergo EMT acquire a self-renewal phenotype (18). However, treatment with zoledronic acid reverses EMT. Therefore, it was of interest to determine whether treatment with zoledronic acid could also inhibit self-renewal capability of TNBC. To study this in more detail, the effects of zoledronic acid on the expression of self-renewal proteins BMI-1 and Oct-4 was examined. Both markers were reduced following treatment with zoledronic acid for 48 hours (Fig. 5A). The effects of zoledronic acid on TNBC self-renewal capability were studied further using the mammosphere assay. First, the effects of zoledronic acid on TNBC prevention of mammosphere formation were investigated. TNBC were treated for 48 hours with 10 µmol/L zoledronic acid under adherent conditions. Pretreatment with zoledronic acid for 48 hours significantly reduced mammosphere formation in both cell lines (MDA-MB-231: \( P < 0.01 \) vs. vehicle; Hs578t: \( P < 0.05 \) vs. vehicle; Fig. 5B and C), signifying that treatment with zoledronic acid prevents mammosphere formation. The effects of zoledronic acid on existing mammospheres before zoledronic acid treatment were then studied. Secondary mammospheres were generated from both Hs578t and MDA-MB-231 primary mammosphere cultures. Primary mammospheres were serially passaged and were allowed to form for 72 hours. Following this 72-hour period, mammospheres were treated with vehicle or 10 µmol/L zoledronic acid and allowed to propagate for an additional 7 days in mammosphere culture. Both groups treated with zoledronic acid resulted in significantly lower number of mammospheres compared with those treated with vehicle (MDA-MB-231: \( P < 0.001 \) vs. vehicle; Hs578t: \( P < 0.05 \) vs. vehicle; Fig. 5D and Supplementary Fig. S2). Taken together, these data suggest that zoledronic acid can reduce mammosphere formation resulting from pretreated adherent cells and existing mammospheres.

Zoledronic acid has no effect on RelA expression but modulates its cellular localization

Proteins that regulate EMT were investigated as potential mechanisms explaining zoledronic acid’s modulation of EMT. The NF-κB pathway has been shown to regulate the expression of EMT transcription factors (25, 26). Therefore, it was hypothesized that zoledronic acid may modulate EMT through inactivation of NF-κB. To determine whether zoledronic acid inactivates NF-κB in TNBC, the effect on the active subunit of NF-κB signaling, RelA, was measured following treatment for 48 hours with 10 µmol/L zoledronic acid. No significant changes were observed with regard to total RelA protein expression (Fig. 6A and Supplementary Fig. S3A). However, decreased phosphorylation of RelA at serine 536 was observed (Fig. 6A and Supplementary Fig. S3A). In contrast, protein expression of p65 (RelA), p50, and p52 decreased following treatment with zoledronic acid (Fig. 6B and Supplementary Fig. S3B). Therefore, it was of interest to determine whether treatment with zoledronic acid could also inhibit self-renewal potential mechanisms explaining zoledronic acid's modulating its cellular localization.

Figure 5. Zoledronic acid inhibits self-renewal. A, self-renewal protein expression in MDA-MB-231 (left) or Hs578t (right) following treatment for 48 hours with vehicle, 1, or 10 µmol/L zoledronic acid. Protein levels were normalized to β-actin. B, Hs578t (top) and MDA-MB-231 (bottom) mammospheres resulting from cells pretreated for 48 hours with vehicle (i and iii) or 10 µmol/L zoledronic acid (ii and iv) in adherent conditions. C, quantification of mammosphere formation in B. D, quantification of existing mammospheres formed upon serial passage of vehicle-treated mammospheres allowed to form 72 hours before treatment with vehicle or 10 µmol/L zoledronic acid. Data are expressed as mean (protein) or mean ± SEM (mammospheres). Protein experiments replicated three times; mammospheres representative of 2 independent experiments. *: \( P < 0.05 \), **: \( P < 0.01 \), ***: \( P < 0.001 \).
Supplementary Fig. S3A). Next, the effect of zoledronic acid on RelA cellular localization was studied. Subcellular fractionation experiments were carried out on cells treated for 48 hours with vehicle or 10 μmol/L zoledronic acid. Observed was a decrease in nuclear RelA localization in both MDA-MB-231 and Hs578t cells (Fig. 6A and Supplementary Fig. S3A). Though decreased expression of PARP in the nuclear fraction was observed, equal amounts of lysates were loaded as evidenced by equivalent β-actin levels. Taken together, these experiments suggest that treatment with zoledronic acid inactivates NF-κB transcriptional activation.

**RelA knockdown inhibits Twist mRNA expression**

To determine the role of the NF-κB pathway plays on EMT transcription factor expression in TNBC, RNA interference (RNAi) strategies were used to decrease RelA expression in TNBC. Using this strategy, RelA mRNA expression was reduced approximately 70% in both MDA-MB-231 and Hs578t (Fig. 6B and Supplementary Fig. S3B). Following RelA knockdown, levels of Twist mRNA were measured. Twist was downregulated as compared with scramble control (P < 0.07 in MDA-MB-231, Twist: P < 0.01 in Hs578t; Fig. 6B and Supplementary Fig. S3B),...
signifying that RelA regulates Twist mRNA expression in TNBC.

**Zoledronic acid reduces interactions between RelA and the Twist promoter and Twist and the BMI-1 promoter**

To confirm transcriptional regulation of RelA on Twist expression, ChIP analyses were conducted on both Hs578t and MDA-MB-231 cell lines treated with 10 μmol/L zoledronic acid for 48 hours. In vehicle-treated cells, the Twist promoter interacted with RelA, confirming transcriptional regulation of Twist by RelA in both cell lines (Fig. 6C and Supplementary Fig. S3C). Furthermore, treatment with zoledronic acid reduced this interaction ($P < 0.05$ vs. vehicle in 231; Fig. 6C and Supplementary Fig. S3C). To determine whether the modulation of EMT played a role in decreased self-renewal, the regulation of BMI-1 by Twist, which is an established regulator of BMI-1 expression (24), was studied via ChIP. Twist interacted with the BMI-1 promoter (Fig. 6D and Supplementary Fig. S3D), and that treatment with zoledronic acid inhibited this interaction (MDA-MB-231: $P < 0.05$ vs. vehicle; Fig. 6D and Supplementary Fig. S3D). On the basis of these findings, it is concluded that zoledronic acid decreases RelA transcriptional control of Twist gene expression, leading to decreased Twist expression. Furthermore, treatment with zoledronic acid decreases Twist transcriptional control of BMI-1 gene expression, leading to decreased BMI-1 expression.

**Discussion**

Zoledronic acid has shown promise as a breast cancer therapeutic, as evidenced by its effects on increased disease-free survival (1–4), decreased metastatic disease (1), and inhibition of cell viability (5–10), invasion, and migration (11, 12) preclinically. Before this study, the mechanisms responsible for decreased migration, invasion, and metastatic disease were not well studied. The novel findings of these studies indicate that zoledronic acid can reverse EMT and decrease self-renewal capacity of TNBC. Results of these studies are the first to show loss of interaction between NF-kB active subunit RelA and the Twist promoter, providing evidence that correlates the loss of RelA activation to modulation of EMT. In addition, these studies are the first to show that treatment with zoledronic acid inhibits Twist binding to the BMI-1 promoter, providing evidence that correlates modulation of EMT to decreased self-renewal. Finally, these studies show that treatment with zoledronic acid inhibits the self-renewal capabilities of TNBC, as evidenced by decreased expression of self-renewal proteins and both prevention and elimination of mammosphere formation.

Treatment with zoledronic acid led to a decrease in mesenchymal characteristics, including loss of N-cadherin, Twist, and Snail mRNA and protein expression, as well as a loss of mesenchymal cell morphology. These results are consistent with those of other groups that have reported zoledronic acid’s effects on the expression of proteins involved in metastatic disease. For example, zoledronic acid has been shown to have differential effects on expression and activation of matrix metalloproteinases (27, 28), required for the escape of cells from a primary tumor site. Furthermore, zoledronic acid has also been reported to inactivate and downregulate both αVβ3 and αVβ5 integrins (29, 30), which are involved in cell interaction with extracellular matrices. Also observed was an increase in epithelial characteristics, including increases in cell–cell contact, E-cadherin mRNA and protein expression, and CD24 cell surface expression. These findings are novel and are reported for the first time in the current studies.

Examination of the timeline of events between reversal of EMT and detachment unveiled that cells gain epithelial characteristics before detachment. However, a small proportion of cells remain attached even after long exposure to zoledronic acid. These cells remain viable and maintain mesenchymal characteristics, suggesting that some cells are innately resistant to treatment with zoledronic acid. This may explain why some cells do not undergo cell-cycle arrest and/or why some cells maintain the ability to self-renew. Regardless, it is concluded that treatment with zoledronic acid reverses EMT before inducing cellular detachment. Furthermore, attached cells in which EMT has been reversed are not fated for cell death, suggesting that decreases in cell proliferation and not induction of apoptosis, lead to reduction in cell viability.

The conclusion that zoledronic acid inhibits cell viability through inhibition of cell proliferation and not induction of apoptosis is corroborated by the effect of zoledronic acid on cell-cycle progression, which show arrest in both G1 (MDA-MB-231) and S (Hs578) phase of the cell cycle. These effects on cell-cycle progression agree with previous results, which illustrate that treatment with zoledronic acid inhibits cell cycle in S-phase (31–33). Although arrest of cells in G1 has been observed (34–36), the mechanism explaining this finding remains unclear. Proliferative capacity of the cells was also reduced, further substantiating these findings.

In addition to its effects on EMT, treatment with zoledronic acid led to decreases in self-renewal capacity. Regulators of EMT were studied as possible leads. One such molecule is Twist, which promotes EMT by inhibiting expression of E-cadherin and increasing self-renewal through upregulation of BMI-1, a protein involved in keeping cells undifferentiated through inhibition of chromatin remodeling (24). The current study found a decrease in Twist expression at both the protein and mRNA level, which correlates with increased E-cadherin expression, decreased BMI-1 expression, and decreased interaction with the BMI-1 promoter. These results are supported by previous reports that have shown that silencing of Twist using RNAi strategies can reverse EMT and decrease metastasis (17).

The NF-κB pathway has been implicated in the regulation of EMT transcription factors, including Twist (25, 26). Although previous studies with zoledronic acid (29)
and alendronate (37), another bisphosphonate, have shown differential effects of bisphosphonates on NF-κB signaling, no work has been conducted in breast cancer cell lines. When treated with zoledronic acid, a loss in phosphorylation of RelA at serine 536 was observed. Previous reports suggest that phosphorylation at this site occurs independently of canonical NF-κB signaling (38). This noncanonical phosphorylation is in accordance with other findings that report no effect of zoledronic acid on canonical NF-κB activation (29). The interaction between RelA and the Twist promotor was also decreased following treatment with zoledronic acid, providing a direct link between zoledronic acid treatment and the inhibition of Twist expression. Although further investigation is required to determine the inhibitory effects of zoledronic acid on NF-κB activity, current findings suggest that RelA inhibition plays a role in Twist downregulation.

This study is the first to report that treatment with zoledronic acid can reverse EMT and decrease self-renewal. These findings imply that treatment with zoledronic acid may be able to inhibit metastasis by both preventing cells from leaving their primary site and abrogating their ability to repopulate at a secondary site. The findings reported by this study support the use of zoledronic acid as a treatment for breast cancer, as it may prove beneficial in the prevention of metastatic disease.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Conception and design: A.J. Schech, R.A. Gilani
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