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Zoledronic Acid Inhibits Aromatase Activity and Phosphorylation: Potential Mechanism for Additive Zoledronic Acid and Letrozole Drug Interaction

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Abstract

Zoledronic acid (ZA), a bisphosphonate originally indicated for use in osteoporosis, has been reported to exert a direct effect on breast cancer cells, although the mechanism of this effect is currently unknown. Data from the ABCSG-12 and ZO-FAST clinical trials suggest that treatment with the combination of ZA and aromatase inhibitors (AI) result in increased disease free survival in breast cancer patients over AI alone. To determine whether the mechanism of this combination involved inhibition of aromatase, AC-1 cells (MCF-7 human breast cancer cells transfected with an aromatase construct) were treated simultaneously with combinations of ZA and AI letrozole for 72 hours. This combination significantly increased inhibition of aromatase activity of AC-1 cells by compared to letrozole alone. Combination treatment of 1nM letrozole and 1μM and 10μM zoledronic acid resulted in an additive drug interaction on inhibiting cell viability, as measured by MTT assay. Treatment with ZA was found to inhibit phosphorylation of aromatase on serine 473. Zoledronic acid was also shown to be more effective in inhibiting cell viability in aromatase transfected AC-1 cells when compared to inhibition of cell viability observed in non-transfected MCF-7. Estradiol was able to partially rescue the effect of 1μM and 10μM ZA on cell viability following treatment for 72 hours, as shown by a shift to the right in the estradiol dose response curve. In conclusion, these results indicate that the combination of ZA and letrozole results in an additive inhibition of cell viability. Furthermore, ZA alone can inhibit aromatase activity through inhibition of serine phosphorylation events important for aromatase enzymatic activity and contributes to inhibition of cell viability.

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Keywords

zoledronic acid; letrozole; aromatase enzyme

1. Introduction

Estrogen receptor (ER) positive breast cancer makes up approximately 70% of all breast cancer cases in the United States. Aromatase inhibitors have been successful as first line therapy for ER positive cancers. However, treatment with aromatase inhibitors results in increased bone loss and bone pain, due to the lack of estrogen's agonist action on the bone. To treat this side effect, aromatase inhibitors have been combined with bisphosphonates, a class of compounds designed to treat osteoporosis and skeletal related malignancies. Bisphosphonates, specifically zoledronic acid (ZA), significantly improves bone-loss [1-3] and quality of life with respect to bone pain [4,5]. In combination with aromatase inhibitors, zoledronic acid has been shown to improve disease free survival in postmenopausal breast cancer patients [1,2,6].

The anti-tumor activity of zoledronic acid, the most potent of the bisphosphonates, has been illustrated in many types of cancer, including breast [7-12], prostate [13], and multiple myeloma [14]. Zoledronic acid has been shown to inhibit cell proliferation and induce apoptosis in breast cancer cell lines, as evidenced by caspase activation [8], DNA fragmentation [7], PARP cleavage [7], and release of cytochrome-c [10]. To date no molecular mechanism has been proposed to explain these cellular changes, though many groups have correlated loss of small GTPases from the membrane and cell death, as a result of zoledronic acid's primary mechanism of action [10,13].

Little work has been carried out with respect to the combination of letrozole and zoledronic acid in estrogen receptor positive breast cancer. Neville-Webbe and colleagues [15] published data which shows that the combination of letrozole at 100nM and zoledronic acid at 10 μ M results in a synergistic effect on decreasing cell viability when used in MCF-7Ca (MCF-7 cells, which are human estrogen receptor positive breast cancer cells and transfected with human aromatase construct) breast cancer cells with concurrent treatment of 24h letrozole followed by 24h zoledronic acid. Although the results of this study provided insight into the pharmacological nature of this combination, no mechanism was proposed to explain the effects on cell viability. Further examination of this combination may not only suggest a rationale for why an increase in disease free survival was observed following treatment with the combination in the ABCSG-12 and ZO-FAST clinical trials, it may also shed light on the molecular mechanism of zoledronic acid as a single agent.

The current study hypothesized that the combination of zoledronic acid and letrozole would inhibit cell viability in either an additive or synergistic manner. To study this hypothesis, the effects of ZA and letrozole, as single agents and in combination, on cell viability were studied.

2. Materials and Methods

2.1. Materials

Zoledronic acid and letrozole were kindly provided by Novartis Pharma (Basel, Switzerland). Androstenedione was purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Modified Eagle Medium (DMEM), trypsin/EDTA, penicillin/streptomycin (P/S), dPBS, and reverse transcriptase reagents (5x reverse transcriptase buffer, DTT, dNTPs, BSA, random primers, and MMLV reverse transcriptase) were purchased from Invitrogen

(NY, USA). Fetal bovine serum (FBS) was purchased from Atlanta Biologicals (Lawrenceville, GA). MTT reagent was purchased from Amresco (Cleveland, OH). Androst-4-ene-3, [1- β - 3 H]-, 17-dione (3 H Δ 4A) was purchased from PerkinElmer (Waltham, MA). Qiashredder kit and RNeasy kit were purchased from Qiagen (Valencia, CA). DyNAmo SYBR green qPCR mix was purchased from MJ Research (Boston, MA). BCA kit and Pierce Co-Immunoprecipitation kit were purchased from Thermo Scientific (Rockford, IL). CYP19 antibody was purchased from Serotec (Kidlington, Oxford, UK). p-tyrosine and β -actin antibodies were purchased from Cell Signaling (Danvers, MA). p-serine and CYP19 antibodies, and normal rabbit IgG were purchased from Santa Cruz Technologies (Santa Cruz, CA). Protease (Complete TM) and phosphatase (PhosSTOP) inhibitors were purchased from Roche (Mannheim, Germany). Polyvinylidene fluoride (PVDF) membrane was purchased from Fisher Scientific (Pittsburgh, PA). All other western blotting materials were purchased from BioRad (Hercules, CA). All other reagents were purchased from Sigma-Aldrich (St. Louis, MO).

2.2. Cell Culture & Treatment

AC-1 cells (MCF7 [American Type Culture Collection, (Manassas, VA)] stably transfected with aromatase [transfected by Luciana Macedo, aromatase construct gift from S. Chen, [16]]) were used for all experiments. Cells were routinely maintained in DMEM, 5% FBS, 1% P/S, 800 μ g/mL G418, grown at 37°C in 5% CO₂, and were passaged weekly. Cell media was changed to phenol red-free media containing charcoal-dextran treated serum 48 hours prior to seeding. For cultures used in western blot analysis, cells were seeded and allowed to grow to 70% confluency. Cells were then treated for specified duration. For cultures used for MTT assay, cells were seeded into 96-well plates at a density of 5000 cells/well and allowed to attach overnight. Cells were maintained in phenol red-free media containing charcoal-dextran treated serum, supplemented with 25nM androstenedione [17], during all assays. Cells were treated with individual treatments, with retreatment occurring 3 days following the first treatment for all 6 day experiments.

2.3. MTT Assay

At the conclusion of treatment, treatment media was aspirated and cells were washed with dPBS. Cells were then incubated in serum-free media containing 0.5mg/mL 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide, MTT) for three hours. MTT-containing media was aspirated and 100 μ L DMSO was added to each well. Plate was shaken on plate-shaker for 10 minutes, allowing formazan dye trapped within the cells to dissolve into DMSO. Plate was then read using a plate reader at an absorbance of 560nm. Data points were fitted to a sigmoidal dose-response curve with using $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{-(\text{LogIC}_{50} - X)})$. Values are expressed as percent change $\pm$ standard error of the mean, compared to vehicle treated control.

2.4. Cellular Aromatase Assay

Cellular aromatase assay was performed as described by Long and colleagues [18]. Briefly, cell media was changed to phenol red-free media containing charcoal-dextran treated serum 48 hours prior to seeding. Cells were then seeded into 6-well plates at a density of 150,000 cells/well and allowed to attach overnight. Cell media was then changed from phenol red-free media containing charcoal-dextran treated serum to 1mL of 1% phenol red-free charcoal-dextran treated serum. Cells were treated with individual treatments in the presence of 10nM Δ 4A and 0.5 μ Ci/mL androst-4-ene-3, [1- β - 3 H]-, 17-dione (3 H Δ 4A) for 2 hours in 5% CO₂. Following treatment, conditioned media was collected and added to 300 μ L trichloroacetic acid which removed proteins. The assay was terminated following addition of 2mL chloroform to the supernatant. The aqueous phase was removed and mixed with 2.5% charcoal suspension, which removed unconverted steroids. Tritiated water formed during the

aromatase reaction was measured by counting radioactivity of 500 μ L supernatant. Resulting CPM data was converted to fmol/mg using GraphPad Quick Calcs Software and is expressed as fmol per mg protein*hr, where mg protein is the total protein concentration as determined from cold control.

2.5. Preparation of Human Placental Microsomes

Microsomes were collected from human term placenta by differential centrifugation. Placenta was washed and rinsed in distilled water, then cut into pieces and weighed. 0.25M sucrose solution was added to the placental pieces at a volume 1.5 times the weight, and the tissue was homogenized. Homogenate was centrifuged at 10,000 $\times$ g for 30 minutes at 4°C. The supernatant was removed and further centrifuged using an ultracentrifuge at 109,000 $\times$ g for 1 hour at 4°C. Following centrifugation, the supernatant was removed and the remaining pellet (microsomes) was stored in 2mL of 0.1M phosphate buffer (2.78g sodium phosphate monobasic, 21.48g sodium phosphate dibasic heptahydrate, 12mg dithiothreitol).

2.6. Microsomal Aromatase Assay

Microsomal aromatase assay was performed as described by Long and colleagues [18]. Individual treatments, 10nM androstenedione (Δ 4A), and 10 μ Ci/mL 3H- Δ 4A were added to borosillate glass tubes and airdried for 10 minutes. To each tube was added 200 μ g microsomes, cofactors (20mg glucose-6-phosphate, 5mg β -NADP, and 25U glucose-6-phosphate dehydrogenase per 1mL distilled H₂O) at 100 μ L cofactors/tube, and 0.1M phosphate buffer to a final volume of 1mL. Tubes were placed in a 37°C water bath in the presence of oxygen and incubated for 30 minutes. Reaction was terminated as described in Cellular Aromatase Assay above.

2.7. RNA Extraction and Reverse Transcription

Cells were washed twice with ice cold dPBS, collected in buffer RLT, and lysed by centrifugation through QIAshredder spin columns. Total RNA was extracted and purified from lysates using the RNeasy Mini Kit. RNA concentration and purity was determined from 260nm and 280nm absorbance, diluted with molecular grade water to 0.08 μ g/ μ L, and reverse transcribed as described by Kazi and colleagues [19]. The reverse transcription reaction mixture consisted of 6 μ L diluted RNA, 4 μ L 5x strand reverse transcriptase buffer, 4 μ L deoxynucleotide triphosphate mix, 2 μ L DTT, 2 μ L of 1mg/mL BSA, 1 μ L of 0.5 μ g/ μ L random primers, and 1 μ L MMLV reverse transcriptase. The mixture was incubated for 1h at 37°C. Reverse transcription products were stored at -20°C until further use.

2.8. RNA Stability Experiments

Cells were plated and allowed to grow to 70% confluency. Cells were treated with zoledronic acid alone or in combination with 5 μ g/mL actinomycin-D over a time course. Following treatment, cells were collected and lysed as described in RNA Extraction and Reverse Transcription.

2.9. real time-Polymerase Chain Reaction

mRNA expression was measured using real time-PCR. Methods were first described by Kazi and colleagues [20]. Real time analysis was performed using a DNA Opticon System (MJ Research). Each 30 μ L reaction mixture included 3 μ L cDNA, 15 μ L DyNAmo SYBR green qPCR mix, 1.2 μ L primer mix, and 10.8 μ L molecular grade water. Each sample was assayed in duplicate. A standard curve was generated by serially diluting control cDNA. The yield of product for each unknown sample was calculated by applying its threshold cycle to the standard curve using the Opticon Monitor analysis software (version 1.01, MJ Research). The following primers were used for analysis: *human* CYP19 (accession no. NM031226):

forward: 5'-GAATATTGGAAGGATGCACAGACT-3' and reverse: 5'-GGGTAAAGATCATTTCAGCAGTGT-3'. Samples were amplified for 50 cycles, with 1 minute denaturation at 94°C, 1 minute annealing at 60°C, and 1.5 minutes polymerization at 72°C [18]. Values were normalized to corresponding GAPDH values and are expressed as the fold change relative to control treatment.

2.10. Preparation of Whole Cell Lysates

Cell media was aspirated and cells were washed twice with ice cold dPBS containing 1x protease inhibitors. Cells were scraped by rubber policeman in 400μL RIPA buffer (50mM Tris-HCl [pH 7.4], 1% Nonidet P-40, 0.25% deoxycholic acid, 1mM EDTA) containing 1x protease inhibitors and 1x phosphatase inhibitors. Samples were sonicated for 15 pulses on ice and incubated by end over end rotation at 4°C for 30 minutes. Samples were then cleared by centrifugation for 30 minutes at max speed at 4°C. Protein concentrations were determined using BCA method.

2.11. Immunoprecipitation

Immunoprecipitation was performed using the Pierce Co-Immunoprecipitation kit as per manufacturer's protocol. Antibodies (5μg CYP19: Santa Cruz #sc-14245 or 5ug normal goat IgG) were purified using the Antibody Cleanup Kit (Thermo). Immunoprecipitated samples were analyzed using western blot.

2.12. Western Blotting

Western blotting was performed as described by Thiantanawat and colleagues [17]. Primary antibodies used include: CYP19 (Serotec #MCA2077S, 1:250), p-serine (Santa Cruz #sc-81514, 1:200), p-tyrosine (Cell Signaling #9411, 1:2000), and β-actin (Cell Signaling #4970, 1:1000). Quantification was performed using Molecular Dynamics software (ImageQuant). Densitometric values were normalized to loading control.

2.13. Statistical Analysis

All data are expressed as mean±SEM. p values were calculated using unpaired t test (2 samples), ANOVA (3+ samples) and Tukey's post hoc analyses using GraphPad Prism 5. All experiments (unless noted) were performed three independent times and data calculated as one data set. p<0.05 was considered significant.

3. Results

3.1. The effect of zoledronic acid and letrozole on cell viability

AC-1 cells were treated with increasing concentrations of zoledronic acid or letrozole (1nM→100μM) for 72 hours or 6 days, and cell viability was measured using the MTT assay. Both drugs induced a dose- and time-dependent decrease of cell viability as shown in Figure 1. Letrozole was more potent at inhibiting cell viability than zoledronic acid. The inhibitory concentration of 50% viability [IC₅₀] of letrozole was 2.5μM±1.98 in 72h and 87nM±3.27 in 6 days. The IC₅₀ of zoledronic acid was 16μM±2.11 in 72 hours and 6μM±2.55 in 6 days.

3.2. Combination zoledronic acid and letrozole inhibit cell viability in an additive manner

To determine the pharmacological relationship between the two compounds, combination analyses were performed using combinations of each drug at different doses. Based on single agent dose response data, combination experiments were performed following 72 hours of treatment, which was chosen because there were not large changes in viability observed at this time. This allowed for better assessment of changes in cell viability. AC-1

cells were treated for 72 hours with a fixed concentration of letrozole (1nM) combined with 1 μ M or 10 μ M zoledronic acid. The drug doses used in the combination were chosen as they were the lowest doses which decreased cell viability. Each combination was compared to the inhibition of cell viability achieved by each drug at the specified dose (summarized in Table 1). The combination experiments demonstrated that the two compounds inhibited viability in an additive drug interaction ($p < 0.001$, Figure 2) as the expected reduction in cell viability was within the standard error of the expected inhibition effect.

3.3. Zoledronic acid potentiates the aromatase inhibition of letrozole

As the combination of ZA and letrozole was determined to function in an additive manner, it was of interest to study the mechanisms involved in the interaction. The inhibitory effects of letrozole on aromatase activity have been established. Therefore, it was of interest to determine whether zoledronic acid had any additional effects on enzymatic activity. Aromatase activity assays were performed on AC-1 cells treated with 1nM letrozole, 1 μ M or 10 μ M zoledronic acid alone or in combination for 1 hour in the presence of 0.5 μ Ci 3 H-androstenedione. The resulting conditioned media was measured for tritiated water, the byproduct of the radioactive aromatase enzymatic reaction [21]. Observed was a decrease in aromatase activity following treatment with both drugs, though only treatment with letrozole was significantly different from vehicle ($p < 0.05$ vs. vehicle treated control; Figure 3). Furthermore, aromatase activity resulting from the combinations show that the addition of zoledronic acid to letrozole treatment further decreased aromatase activity ($p < 0.001$ in presence of 1 μ M zoledronic acid, $p < 0.01$ in presence of 10 μ M zoledronic acid, both vs. vehicle treated control; Figure 3). Although the 10 μ M dose of zoledronic acid does not seem to inhibit aromatase activity as effectively as that of the 1 μ M dose, the differences in aromatase activity between the two combinations are not significantly different from each other, either alone or in combination. Both combinations are significantly different from vehicle treated control. Although the inhibition of aromatase activity following treatment with letrozole has been well established, the effect of zoledronic acid on aromatase activity has not yet been reported. Because of this, it was of interest to study the effect of zoledronic acid on the aromatase enzyme.

3.4. Zoledronic acid inhibits aromatase activity in cells but not in enzymatic preparation

As the effects of zoledronic acid exhibited a trend following 1 hour of treatment with zoledronic acid, longer treatment duration was investigated. To test this, AC-1 cells were treated for 2 hours with increasing concentrations of zoledronic acid (1nM $\rightarrow$ 100 μ M), and the effects on aromatase activity were measured using the aromatase assay. Aromatase activity was inhibited approximately 50% following treatment with 10nM zoledronic acid, with each subsequent dose of zoledronic acid inhibiting to the same level (Figure 4A). These results confirmed that there was a significant effect of zoledronic acid on the aromatase enzyme, though the inhibition following treatment with letrozole was superior ($\sim$ 100% inhibition with 1 μ M letrozole compared to 53% inhibition with 1 μ M zoledronic acid, Figure 4A). To investigate the mechanism of how this inhibition may occur, the direct effects of zoledronic acid on the aromatase enzyme were studied using an aromatase enzyme preparation. Microsomes (which are enriched in the aromatase enzyme) isolated from placental tissue were treated for 30 minutes with increasing concentrations of zoledronic acid (1nM $\rightarrow$ 100 μ M) in the presence of 0.5 μ Ci 3 H-androstenedione and required cofactors. Zoledronic acid had no effect on the aromatase activity of enzymatic preparation, though treatment with letrozole resulted in a significant reduction of activity (Figure 4B). Therefore, zoledronic acid's inhibition of aromatase activity is not due to direct inhibition of the enzyme, and therefore can be attributed to either post-transcriptional/post translational modifications or changes in gene expression.

3.5. Zoledronic acid has no effect on aromatase mRNA stability

The transfection of aromatase into the AC-1 cell line is under control of the β -actin promoter [16]. Thus, changes in gene expression are not a mechanism for reduced aromatase activity. Therefore, the effects of zoledronic acid on post-transcriptional/post translational modifications were examined in greater detail. The effect of zoledronic acid on aromatase mRNA was first investigated. As the reduction in aromatase activity occurred within 2 hours of treatment, the effects of zoledronic acid were studied within that time frame. To test this, cells were treated with 5 μ g/mL actinomycin D, which results in the inhibition of nascent RNA synthesis. Cotreatment with actinomycin-D and zoledronic acid had no effect on aromatase stability when compared to actinomycin-D and vehicle cotreated control (Figure 5). This finding rules out any effect of zoledronic acid on aromatase mRNA stability at that time point.

3.6. Zoledronic acid reduces serine phosphorylation of the aromatase enzyme

The effect of zoledronic acid on aromatase post translational modifications was next studied. Though there is little research on post-translational modifications of aromatase, some reports suggest that aromatase undergoes a phosphorylation event which is important for its enzymatic activity [22-24]. To determine whether aromatase undergoes post-translational modification in AC-1 cells, lysates were immunoprecipitated with an aromatase antibody. Immunoprecipitated samples were then probed via western blot for pan-serine and pan-tyrosine phosphorylation. Resulting experiments showed that aromatase undergoes phosphorylation only on serine residues (Figure 6). Furthermore, treatment with 10 μ M zoledronic acid was able to inhibit this phosphorylation ($p < 0.08$, Figure 6). Western blot analyses of the whole cell lysates prior to the immunoprecipitation experiments show that all samples express equal levels of CYP19, confirming that zoledronic acid does not affect total CYP19 expression (Figure 6A, WCL-aromatase).

3.7 Zoledronic acid inhibits cell viability through inactivation of aromatase

It has been established that ZA inhibits aromatase activity. Therefore, it was of interest to determine whether this loss of aromatase activity lead to decreased cell viability. Cell viability experiments comparing the IC₅₀s of nontumorigenic breast cells (MCF10A), breast cancer cells expressing low levels of aromatase (MCF-7), or the stably transfected aromatase breast cancer cell line (AC-1) show that 10 μ M zoledronic acid is most effective in inhibiting the viability of the AC-1 cell line (IC₅₀ in AC-1 cells 11.7 μ M $\pm$ 4.33 compared to 46.8 μ M $\pm$ 3.93 in MCF-7 and no achievable IC₅₀ in MCF10A, Figure 7A). Estradiol was able to rescue the effect of 1 μ M and 10 μ M ZA on cell viability following treatment for 72 hours, as shown by a shift to the right in the estradiol dose response curve ($p < 0.05$; Figure 7B) when compared to cotreatment of Δ 4A and ZA.

4. Discussion

The combination of zoledronic acid and endocrine therapy has shown promise in the improvement of disease free survival in breast cancer patients [1,25]. It remains difficult to determine the usefulness of zoledronic acid in the clinical setting without first knowing its molecular mechanisms. The objectives of this study were not only to understand better the pharmacological relationship of the combination of zoledronic acid and aromatase inhibitor letrozole on estrogen receptor positive breast cancer cells, but also to use the combination to determine the potential molecular mechanisms of zoledronic acid as a single agent. The findings of this study indicate that the combination of zoledronic acid and letrozole results in an additive drug interaction when administered simultaneously in aromatase transfected cells. In addition, this study reports novel findings that zoledronic acid inhibits both the

activity and the serine phosphorylation of the aromatase enzyme, thereby reducing the enzyme's activity.

The first conclusion of this study is that the combination of zoledronic acid and letrozole results in an additive drug interaction when used simultaneously in ER positive breast cancer cells. This differs dramatically from the study published by Neville-Webbe and colleagues in 2010 [15], who report a synergistic drug effect following concurrent letrozole and zoledronic acid treatment and no effect following simultaneous treatment with both compounds. There are differences between the two study designs which may account for these conflicting results. Neville-Webbe and colleagues treated cells for 24 hours, allowing a rest period of 48 hours before viability changes were determined. However, the treatment period in the current study was for 72 hours with no rest period. The two single agent dose response curves for both compounds from Figure 1 illustrate that longer treatment duration improves the efficacy of both compounds. This may explain why the results from this study show better efficacy of the combination. Furthermore, the finding from this study that ZA inhibits aromatase activity is consistent with an additive ratio rather than a synergistic one in combination with an aromatase inhibitor. Regardless, the results from this study conclude that the simultaneous combination of zoledronic acid and letrozole act in an additive manner to inhibit viability. The use of both drugs simultaneously parallels the administration schedule used in both recent clinical trials which report positive results in combination of zoledronic acid and endocrine therapy agents. In the ABCSG-12 clinical trial, patients who received zoledronic acid and either AI anastrozole or tamoxifen (selective estrogen receptor modulator) simultaneously experienced improved disease free survival compared to endocrine therapy alone [2,25]. In addition, the ZO-FAST trial, which looks at the simultaneous combination of letrozole and ZA, also reports improved disease free survival in BC patients [1]. Additionally, administration of zoledronic acid upfront versus delayed administration significantly improved the efficacy of the compound to inhibit bone loss and improve disease-free survival [1]. Therefore, the results from this study support the two successful clinical trials and provide strong evidence for the use of this combination in the clinical setting.

The second conclusion of this study is that zoledronic acid as a single agent can significantly inhibit aromatase activity. The results suggest that this is through inhibition of serine phosphorylation of the aromatase enzyme. These findings are supported by observations that decreased aromatase phosphorylation reduces aromatase activity [22,23]. Yue and colleagues illustrated that treatment with kinase inhibitors for both the MAPK and PI3K pathways significantly inhibited aromatase activity [22]. In addition, Miller and colleagues showed that phosphorylation of aromatase at serine 118 was important for aromatase activity, as mutation of this site resulted in decreased aromatase activity [23].

Because aromatase inhibition has proven to be beneficial in the abrogation of ER+ breast cancer growth, it was hypothesized that the effects of ZA on aromatase would also play a role in the anti-tumor mechanism of ZA. Comparison of cells, including non-tumorigenic, and low and high aromatase expressing cells confirm that ZA is more effective in aromatase expressing cells. Furthermore, rescue experiments using estradiol to circumvent the need for the aromatase enzyme demonstrate a significant rescue of viability following treatment for 72 hours. Therefore, it is concluded that ZA's effects on aromatase are involved in its modulation of cell viability.

The three main clinical trials which have investigated the efficacy of zoledronic acid as an adjuvant treatment for breast cancer have all concluded that zoledronic acid is only effective in the postmenopausal setting [2]. This suggests that the mechanism of zoledronic acid may be hormone linked. The findings of this study support that conclusion. After menopause,

estrogen production from the ovary is markedly reduced and occurs mainly in non-ovarian tissues, such as adipose tissue. Based on the mechanism proposed in this study, zoledronic acid would further lower estrogen production in postmenopausal women through inhibition of aromatase activity.

In conclusion, these studies report that the combination of zoledronic acid and letrozole results in an additive inhibition of cell viability, which corroborates both the ABCSG-12 and ZO-FAST clinical trials. In addition, this study is the first to report that zoledronic acid acts by inhibiting the activity of the aromatase enzyme. This inhibition is postulated to be due to inhibition of serine phosphorylation. Furthermore, the inhibition of aromatase activity by ZA is involved in its anti-tumor mechanism. Overall, this study supports the use of zoledronic acid for the treatment of breast cancer.

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Abbreviations

ER	Estrogen receptor
ZA	Zoledronic acid
ABCSG-12	Austrian Breast and Colorectal Cancer Study Group trial 12
ZO-FAST	Zometa-Femara Adjuvant Synergy Trial

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Highlights

- Combination zoledronic acid and letrozole results in an additive drug interaction
- Zoledronic acid inhibits aromatase activity of cells but not enzymatic preparation
- Zoledronic acid does not inhibit aromatase mRNA stability
- Zoledronic acid inhibits serine phosphorylation of aromatase enzyme

Figure 1. Zoledronic acid and letrozole inhibit cell viability

AC-1 cells were treated for 72 hours (A) or 6 days (B) with increasing concentration of zoledronic acid (dashed line) or letrozole (solid line). Data are expressed as mean $\pm$ SEM and are representative of three independent experiments.

Figure 2. Combination zoledronic acid and letrozole inhibits cell viability better than each agent alone

AC-1 cells were treated for 72 hours with 1 nM letrozole alone (white bars), 1 μ M or 10 μ M zoledronic acid alone (black bars) or in combination (grey bars). Data are expressed as mean $\pm$ SEM and are representative of three independent experiments. * p <0.05, *** p <0.001.

Figure 3. Combination zoledronic acid and letrozole inhibits aromatase activity

AC-1 cells were treated for 1 hour with 1nM letrozole (Let), 1μM, or 10μM zoledronic acid (ZA) alone or in combination. Data are expressed as mean±SEM and are representative of three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

Figure 4. Zoledronic acid inhibits aromatase activity in cells but not in enzymatic preparation
 (A) AC-1 cells were treated for 2 hours with increasing concentrations of zoledronic acid or 1 μ M letrozole (Let). (B) 200 μ g microsomal protein was incubated for 30 minutes with increasing concentrations of zoledronic acid or 1 μ M letrozole. Data are expressed as mean $\pm$ SEM and are representative of three independent experiments. * p <0.05, ** p <0.01, *** p <0.001.

Figure 5. Zoledronic acid does not affect aromatase mRNA stability

AC-1 cells were treated for 2 hours with 5ng/mL actinomycin-D (Act. D) in the presence (black bars) or absence (white bars) of 10μM zoledronic acid (ZA). mRNA levels were measured by qPCR and were normalized to GAPDH. Data are expressed as mean±SEM and are indicative of two independent experiments.

Figure 6. Zoledronic acid inhibits serine phosphorylation of the aromatase enzyme

(A) AC-1 cells were treated for 2 hours with vehicle, 1 μ M or 10 μ M zoledronic acid (ZA). Lysates were immunoprecipitated with either normal rabbit IgG or aromatase antibody. Immunoprecipitation products were probed for either pan-tyrosine or pan-serine phosphorylation. Non-immunoprecipitated samples were run as whole cell lysates (WCL) controls to show aromatase expression. (B) Densitometry analysis from (A). Data are expressed as mean $\pm$ SEM and are indicative of two independent experiments of three replicates each.

Figure 7. Zoledronic acid inhibits serine phosphorylation of the aromatase enzyme

(A) MCF7, MCF10A, and AC-1 cells were treated for 72 hours with vehicle or increasing concentrations of zoledronic acid, (B) AC-1 cells were treated with zoledronic acid in the presence of 25nM androstenedione (solid line) or 25nM estradiol (dashed line) for 72 hours. Data are expressed as mean $\pm$ SEM and are indicative of two independent experiments.

* $p < 0.05$.

Table 1

Dose-response data from Figure 2.

Treatment Type	% Viable	Expected % Viable	Actual % Viable
1nM Letrozole	81.1 ± 4.4	-	-
1μM Zoledronic Acid	72.1 ± 6.9	-	-
10μM Zoledronic Acid	59.2 ± 3.9	-	-
1nM Let + 1μM ZA		53.2%	48.7 ± 4.5
1nM Let + 10μM ZA		40.3%	37.7 ± 4.0