

## ABSTRACT

### COMPARATIVE ANALYSES OF OSTEOTROPIC HUMAN BREAST CANCER CELLS IN RESPONSE TO A BISPHOSPHONATE DRUG

Of those women who progress to an advanced stage of breast cancer, over 80% will develop bone metastases. Currently, bisphosphonates are used for the treatment of metastatic bone disease. Our preliminary results indicate that zoledronic acid directly acts on osteotropic cells by inhibiting angiogenesis and proliferation, and inducing cell death. In this project we used a proteomic approach to define the differences between (MDA-MB-231) and its osteotropic clone (MDA-231 BO) before and after treatment with zoledronic acid. We hypothesize that the osteotropic cells have properties that are more consistent with osteoclast-like cells. Our results indicate a concentration of 200  $\mu$ M ZA induced a strong cell death in 70-80% cells within 48 hr. Cells were then treated for 48 hr and the mitochondria fraction was extracted from both treated and non-treated cell lines. Extracted proteins were separated by SDS-PAGE and 2-dimensional gel electrophoresis. Finally differentially expressed proteins were identified using a MALDI-TOF Mass Spectrometer.

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May 2010



COMPARATIVE ANALYSES OF OSTEOTROPIC HUMAN  
BREAST CANCER CELLS IN RESPONSE TO A  
BISPHOSPHONATE DRUG

by  
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APPROVED

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## INTRODUCTION

### The Problem of Breast Cancer and Bone Metastasis

The division of Cancer Prevention and Control has determined that “breast cancer is the second most common cancer among US women after skin cancer” (1). One in eight women with cancer suffers from breast cancer. Men suffer as well, but at much lower risk, representing 1% of all breast cancers (2). Bone metastasis is the most common obstacle of breast cancer. Eighty percent of patients with advanced breast cancer disease develop bone metastasis (2).

In 2009, an estimated 192,370 new cases of metastatic breast cancer and 62,280 new cases of non-invasive breast cancer (*carcinoma in situ*) were diagnosed among United States women (3). Of those women diagnosed with breast cancer, 40,610 women died (1). Furthermore, it is anticipated that 350,000 people die with bone metastases due to breast and other cancers each year in United States (3).

### Breast Cancer Progression and Metastasis

The female breast mainly consists of lobules (milk-producing glands), ducts, stroma, blood vessels, and lymphatic vessels (4). DeLeonars reports that “breast cancer is the proliferation of malignant-appearing cells of the ducts and terminal lobular units of the breast” (5). The current model of tumorigenesis suggests that breast cancer malignancy is a result of activation and inactivation events of a set of genes over long periods of time (6).

Breast cancer can either be invasive or non-invasive. When they are non-invasive (*in situ*), cancer cells remain limited to ducts (ductal carcinoma *in situ*) or lobules (lobular carcinoma *in situ*); but when they are invasive, cancer cells invade

beyond the layer of cells where it started (6). Most breast cancers are invasive and spread to other parts of the body, which include bones, liver, and brain. It has been shown in different studies that cancer cells that metastasize to distant organs exhibit different behaviors from those of primary tumor (7).

Breast cancer development happens through a multi-step process, which displays itself as a sequence of pathologically defined stages. A normal breast terminal ductal lobular unit (TDLU) encloses lobules and ducts that are composed of a bi-layered epithelium of luminal and myoepithelial cells. Breast cancer initiates as the pre-malignant stage of Atypical Ductal Hyperplasia (ADH), when breast cells become abnormal in size, shape, and growth pattern, progress into the pre-invasive stage of Ductal Carcinoma *in situ* (DCIS), and terminate in the potentially lethal stage of Invasive Ductal Carcinoma (IDC), which leads to metastasis (6). This model of breast cancer progression has been used in detection methods such as mammography for early detection and treatment of breast cancer at its earlier stages (Fig. 1).

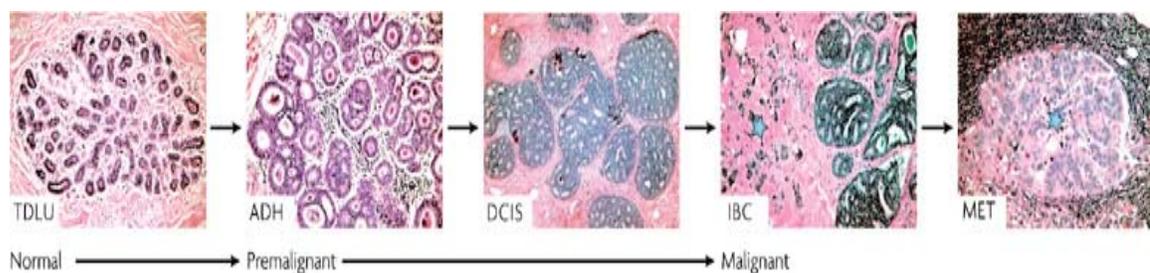


Fig. 1. Basic stages of breast cancer progression: Mutations change the properties of normal breast cells or terminal ductal lobular unit (TDLU) and progresses into the pre-malignant stage of atypical ductal hyperplasia (ADH), then progresses into the pre-invasive stage of ductal carcinoma in situ (DCIS), then culminates in the potentially lethal stage of invasive ductal carcinoma (IDC) and finally become metastatic. Modified from Vargo et al. (8).

Breast cancer frequently spreads to bone. Over 80% of women who proceed to an advanced stage of breast cancer will suffer from bone metastases (9). Paterson reports that “cancer cells enter bone via nutrient arteries and form metastases in the axial skeleton” (9). In general, cancer cells form in the vertebrae, skull, the pelvis, and the proximal ends of the long bones (10). When breast cancer metastasizes to bone, the cancer is terminal and patients suffer from nerve compression, skeletal fractures, and hypercalcemia. The average survival time for a patient with metastatic bone disease from time of diagnosis is less than 2 years (10).

Breast cancer and bone micro environment play an important role in the development of bone metastases. Breast cancer spreads to the bone by expressing chemokine receptors, bone resorbing, bone-forming factors, integrins, and cadherins. Additionally, bone microenvironment is rich in growth factors and cell types that makes bone a good target for breast cancer growth (11). Generally, it has been recognized that metastatic cancer cells show a different behavior from the primary tumor (12). Consistent with this, bone metastatic cancer cell lines show altered properties such as parathyroid hormone-related protein (PTHrP) and expression of PTH/PTH-rP receptors, which stimulate osteoclasts (13). Additionally, some bone metastatic cancer cell lines are found to express mutated (constitutively active) estrogen receptors (14). Moreover, tumor cells produce a variety of growth factors that up-regulate the receptor activator of the NF- $\kappa$ B (nuclear factor kappa-light-chain-enhancer of activated B cells) ligand (RANKL) and stimulate osteoclastogenesis (15).

### Current Drug Treatment for Bone Metastasis

Varieties of therapeutic options are available for bone metastatic disease. These treatments include chemotherapy, orthopedic surgery, hormonal therapy, radiotherapy and bisphosphonates (16). At this time bisphosphonates are used extensively for the treatment of metastatic bone disease, because they effectively reduce pain, prevent pathological fractures, and treat hypercalcemia of malignancy. Moreover, in patients with bone metastases, risk and incidence of developing a skeletal-related event can be reduced by administration of bisphosphonates like pamidronate and zoledronic acid as well as chemotherapy and hormonal therapies.

Bisphosphonates inhibit osteoclast-mediated bone resorption, which can further help patients with hypercalcemia through lowering their serum calcium concentrations. Bisphosphonates bind to active sites of bone metabolism and are released during bone resorption; they are consumed by osteoclasts, thus preventing osteoclast activity and promoting survival. There are two classes of bisphosphonates: 1) those that contain nitrogen compounds; and 2) those that do not contain nitrogen. Nitrogen-containing bisphosphonates are pamidronate, alendronate, risedronate, zoledronic acid, and ibandronate. Nitrogen-containing bisphosphonates are more effective than non-nitrogen bisphosphonates, which include etidronate and clodronate (17). Pamidronate and zoledronic acid are the only bisphosphonates approved and recommended by the American Society of Clinical Oncology (ASCO) in the U.S. for the treatment of patients with bone metastasis (18). The efficacy of clodronate, pamidronate, ibandronate, and zoledronic acid has been compared in Table 1. The non-nitrogen containing bisphosphonates like clodronate inhibit bone resorption by generating the cytotoxic analog of ATP, which interferes with mitochondrial function and induces the apoptosis of osteoclasts (19).

| <b>Table 1.</b> Efficacy comparison among four commonly used bisphosphonates (20) |                                     |                       |     |                                                                                            |                           |
|-----------------------------------------------------------------------------------|-------------------------------------|-----------------------|-----|--------------------------------------------------------------------------------------------|---------------------------|
| Bis-phosphonate Drug                                                              | Dose and Schedule                   | Trial                 | N   | Efficacy                                                                                   | P Value                   |
| Clodronate                                                                        | 1600mg/day<br>14 months             | Paterson et al. 1993  | 85  | Reduced HCM,<br>Reduced SRE 1.39% ,<br>Delayed time to first bone event up to 9.9 months   | <0.001<br><0.001<br>0.022 |
| Pamidronate                                                                       | 90 mg every<br>3-4 weeks<br>2 years | Theriault et al. 1999 | 367 | Improved bone lesion response 32%<br>Decreased pain 44%<br>Delayed SRE up to 12.7 months   | 0.002<br><0.001<br><0.001 |
| Ibandronate                                                                       | 6 mg every<br>3-4 weeks<br>2 years  | Body et al. 2003      | 154 | Reduced SMRP 20%<br>Delayed SRE up to 12 months<br>Reduced the % patients with>1 new event | 0.004<br>0.089<br>0.122   |
| Zoledronic Acid                                                                   | 4 mg every<br>3-4 weeks<br>1 year   | Kohno et al. 2004     | 377 | SRE risk reduced 44%<br>Delayed SRE up to 12 months<br>Reduced SMRP 20%<br>Reduced pain    | 0.189<br>0.046<br>0.102   |

N= number of patients; P Value= significance of the test, HCM = hypercalcaemia of malignancy; SRE = skeletal-related event; SMPR = skeletal morbidity

Newer nitrogen-containing bisphosphonates, such as pamidronate, zoledronic acid, and ibandronate have novel mechanisms of action, which increase their effectiveness compared with the non-nitrogen containing bisphosphonates (21). Nitrogen-containing bisphosphonates inhibit the HMG-CoA reductase pathway *in vitro* and inhibit protein prenylation in osteoclasts *in vivo* (21). These bisphosphonates act on bone metabolism by blocking the farnesyl diphosphate synthase (FPPS) enzyme. This enzyme catalyzes sequential condensation reactions of dimethylallyl pyrophosphate with 3-isopentenyl pyrophosphate to form farnesyl pyrophosphate in the mevalonate pathway/HMG-CoA reductase pathway. Gong et al. report that “mevalonate pathway is an important cellular metabolic pathway because it is crucial for the production of dimethylallyl pyrophosphate (DMAPP) and isopentenyl pyrophosphate (IPP)” (22). Disruption of the mevalonate pathway at the level of FPPS inhibits farnesol and geranylgeraniol development, which further prevents connection of some small proteins to cell membranes. This phenomenon is known as prenylation, or addition of hydrophobic molecules to a protein, which has an important role in controlling sub-cellular protein trafficking in osteoclasts *in vivo* (23). The mechanism of action of nitrogen-containing bisphosphonates is shown in Fig. 2.

#### A New Tool for Breast Cancer Metastasis

The human breast cancer cell line MDA-MB-231 has been used for many studies and has been the source of numerous observations into the biology of breast cancer cells. MDA-MB-231 cell lines are ideal for breast cancer research because of their tumorigenicity and metastatic capacity. MDA-MB-231 human estrogen-independent breast cancer cells can metastasize mostly to bone and occasionally to the brain.

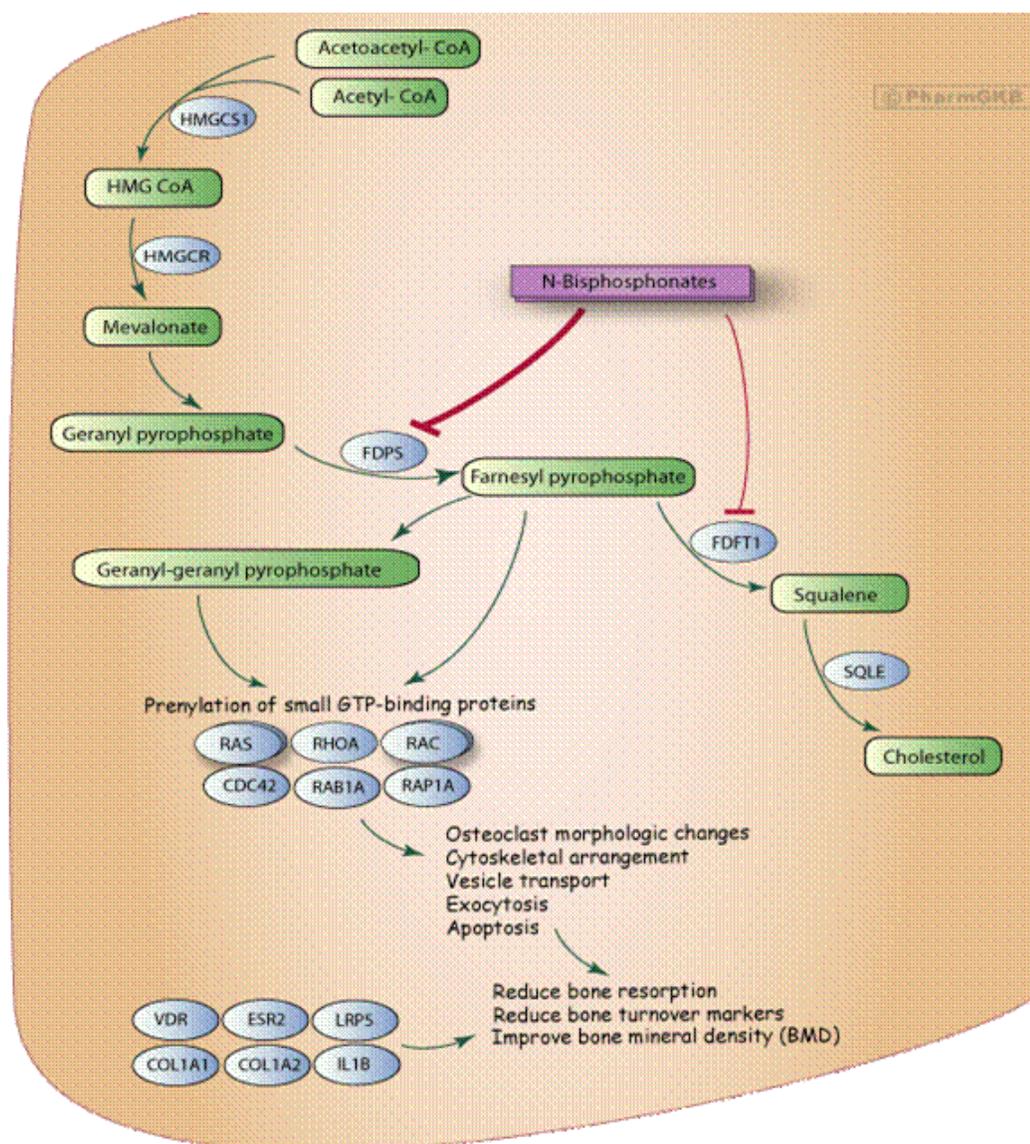


Fig. 2. Mechanism of action of nitrogen-containing bisphosphonates. Nitrogen-containing bisphosphonates block FPPS enzyme, which is an intracellular enzyme of mevalonate pathway thus prevent the prenylation of small GTPases that are essential for osteoclast function and survival. (I acknowledge the copyright to PharmGKB and state that permission has been given by PharmGKB and Stanford University) (22).

To establish an osteotropic clone, MDA-MB-231 cells in bone metastases were isolated by the explant growth technique, were grown in culture and re-inoculated into the left ventricle of the heart (24). This procedure was repeated nine times to get the osteotropic sub lines. This procedure was repeated twice more and then established MDA-231 BO cells with more selectivity for bone. Comparison of these cell lines with the parental cell lines showed they have more affinity to the bone than parental cell lines (24).

There are some distinct differences between MDA-MB-231 and MDA-231 BO cell lines. One of these differences is morphological, which is highlighted in Fig. 3. This figure shows increased branching and presence of lamellipodia in osteotropic cell lines. The other difference between bone and parental cell lines is their doubling time, which is shown in Fig. 4. This figure shows that bone cell lines grow faster than parental cell lines. Some of the known differences between primary tumor and bone metastasized cancer cells are also listed in Table 2 (24).

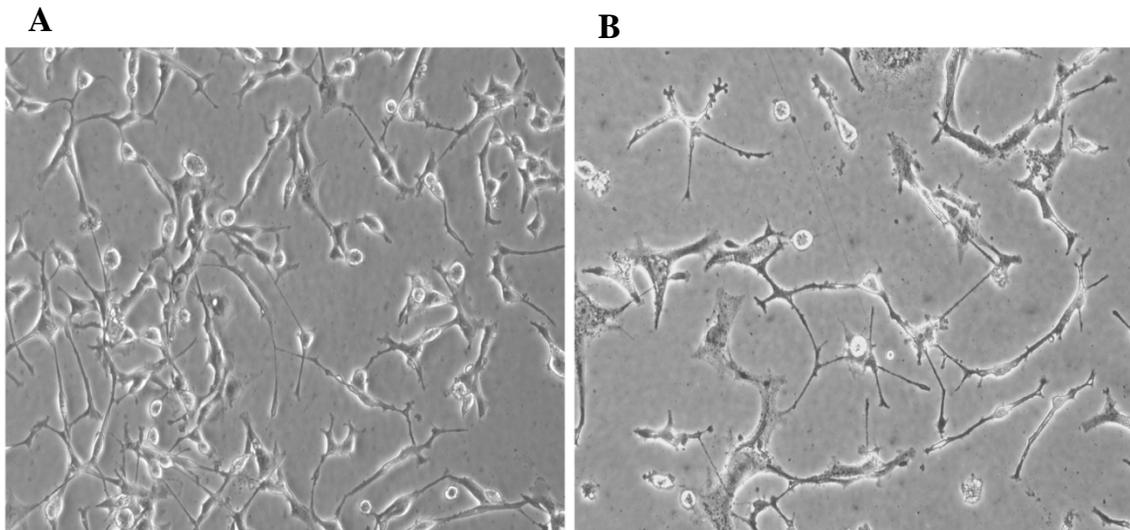


Fig. 3. Polarized images (400X) of MDA-MB-231 (A) and MDA-231 BO (B) cell lines. These images show morphological differences between MDA-MB-231 and the osteotropic MDA-MB-231 BO cell lines. Note the increased branching and presence of lamellipodia in MDA-231 BO.

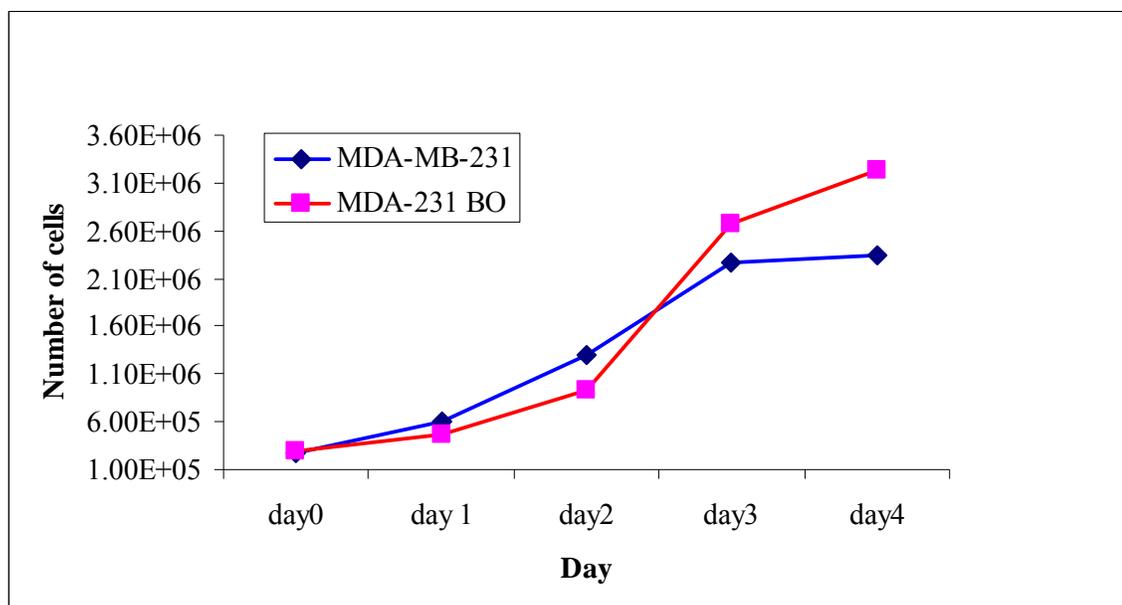


Fig. 4. Growth curve of MDA-MB-231 cell lines (parental-bone). Two Cell lines have been counted every day for 4 days. DMEM (media) used for cell culture in this experiment. No hormone was used in this experiment. This figure shows one of the differences between MDA-MB-231 cell lines is their doubling time.

**Table 2.** Comparison between some of the characteristics of osteotropic breast cancer cell lines and parental breast cancer cell lines (Modified from Yoneda et al. (24))

| Characteristics                   | Parental cell lines | Osteotropic |
|-----------------------------------|---------------------|-------------|
| Site of passage                   |                     | Bone        |
| Tumorigenicity at orthotopic site | Yes                 | Yes         |
| Site of metastasis                | Bone, Brain, Ovary  | Bone        |
| PTHrP production                  | Low                 | High        |

PTHrP= Parathyroid hormone-related protein

### Rationale and Hypothesis

Given that the majority of women with advanced stage breast cancer encounter bone metastases, we are interested in identifying molecular signatures that may define the populations that are at increased risk of developing such skeletal-related events. In this study we have used a novel cell system to help us address this critical health problem. The central question of this project is “Can we define signature molecular markers associated with an osteotropic cancer cell line?” To further assist in addressing this question, we were interested in determining the mechanism of zoledronic acid, a new nitrogen-containing bisphosphonate drug in reduction of bone metastasis.

The primary objective of this thesis was, using a proteomic approach, to define differences between a series of breast cancer cell lines which are zoledronic acid treated MDA-MB-231; zoledronic acid treated MDA-231 BO; non-treated MDA-MB-231; and non-treated MDA-231 BO. We hypothesize that zoledronic acid has a different effect on osteotropic cells versus parental cells and we expect to find proteins involved in osteoclast-specific function (25).

The rationale for this hypothesis is that zoledronic acid acts on bone metabolism by disruption of the mevalonate pathway (mitochondrial pathway) which affects proteins found in osteoclast-like cells. For this, the most effective concentration of zoledronic acid was first optimized via cell death assays. In the next step cells were treated with the optimized concentration (200  $\mu$ M) of zoledronic acid for 48 h, and the proteins either from whole cells, or mitochondria fraction, were extracted along with those from non-treated control cell line. Extracted proteins were separated either by SDS-PAGE or 2-dimensional gel electrophoresis. This was followed by performing trypsin digest on 2-D gel spots, and the Matrix-assisted laser desorption/ionization time-of flight mass

spectrometer (MALDI-TOF) was used for the identification of proteins. The ultimate goal is to find novel molecular signature biomarkers for osteotropic cells. We hope that the results of this experiment will improve our knowledge and lead to better treatment for breast cancer metastasis patients.

## MATERIALS AND METHODS

### Tissue Culture

MDA-MB-231 series (parental, bone, brain sublines) were a kind gift of Dr. Toshiyuki Yoneda (University of Texas at San Antonio). For performing a tissue culture from a pre-existing live culture, first the old media were aspirated and then cells were washed with phosphate buffered saline (PBS) to remove the serum; then trypsin-EDTA enzyme solution was used to detach the cells from the tissue culture dish. The cells were then centrifuged at 1000 rpm for 2 min, and the pellet was re-suspended with new DMEM media, Dulbecco's modified Eagle's medium (GIBCO, Grand Island, NY). The cells were pipetted into a new tissue culture plate containing an adequate volume of media and incubated at 37 °C and 5% CO<sub>2</sub>.

### Sulforhodamine B (SRB) Assay

Fifteen hundred cells in 100 µl of media were placed in each well of a 96 well flat bottom polystyrene plate. The plate was left at room temperature for 1 h and then was put in an incubator at 37 °C and 5% CO<sub>2</sub>. After 24 h, 50 µl of 200 µM zoledronic acid/DMEM media was added to each well. Each time wells were blocked at intervals of 24-48-72 h. After 72 h, 100 µl of 20% cold TCA fixative solution (trichloroacetic acid, 20% vol/vol) was added to each well and wells were incubated for 60 min at 4 °C. In the next step, the plate was washed 5 times with cold tap water by immersion. The plate was air dried overnight at room temperature. The next day, cells were stained by adding 50 µl of SRB (0.04% wt/vol) to each well and shaken for 30 min. In the next step, Sulforhodamine B (SRB) was removed by flicking and the plate was washed 4 times with 50 µl of

1% acetic acid. Washing solution was removed by flicking and 100  $\mu$ l of 10 mM tris base (pH 10.5) was added to each well to solubilize bound dye while shaking for 20 min. One hundred  $\mu$ l of tris was added to three empty wells as blank. The plate optical density was read with an endpoint assay at 564nm wavelength on a microplate spectrophotometer (SpectraMax 340, MDS, Sunnyvale, CA).

#### Zoledronic Acid Treatment

Zoledronic acid (Zometa<sup>®</sup>) was kindly supplied as the hydrated disodium salt by Novartis, Pharma AG (Basel, Switzerland). Zoledronic acid was dissolved in 0.1% dimethyl sulfoxide (DMSO) and diluted in DMEM culture medium immediately before use. The DMSO concentration in the assay did not exceed 0.1%. For control of possible adverse effects of the DMSO, 0.1% DMSO was added to the media of all control cells. MDA-MB-231 BO and MDA-MB-231 breast cancer cell lines were treated with 200  $\mu$ M of zoledronic acid (dissolved in DMEM media) for either 48 or 72 h and then were scraped for protein extraction. To have the same quantity of cells in both treated and non-treated cell plates after 48 h of treatment, the initial density of the cells in the control plates was 40% while the initial density of the cells in the treated plates was 70%.

#### Whole Protein Extraction and Quantification

For protein extraction, cells were scraped and were kept on ice then washed with PBS. After that, triple detergent (lysis buffer composed of 50 mM Tris- HCl, pH 8.0, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 5 mg/ml sodium deoxycholate, and 1 mM phenylmethylsulfonyl fluoride) and protease inhibitors were added. In the next step, cells were incubated on ice for 20 min and then centrifuged at 13-15,000 rpm for 10 min. Finally, lysate was transferred to a new tube, the standard Bradford protein assay was used for quantification, and the

protein concentration was determined using optical density (OD) reading at 595 nm. NanoVue Spectrophotometer (GE HealthCare, Wauwatosa, WI) is another method used for determination of protein content in whole cell lysates or enriched mitochondria suspensions. The instrument was blanked twice using 3  $\mu$ l of deionized water and then 3  $\mu$ l of each sample was read on NanoVue. To increase accuracy of results, each sample was read 3 times.

#### Isolation of Mitochondria Using VWR™ Pellet Mixer to Homogenize the Mixture

Each sample was grown to approximately 80% confluence in 2-150 mm tissue culture dishes to provide a harvest of approximately  $20 \times 10^6$  cells. The cells were harvested in a cold room at 4 °C by first removing the growth media and rinsing the monolayer 5 times with cold phosphate buffered saline (PBS). The cells were then dislodged from the culture dish manually using a cell scraper and placed into a new test tube. The culture dish was rinsed once with 1-2 ml of cold PBS and the suspension was added to the cell harvest. The cell suspension was then centrifuged at approximately 12,000 rpm for 2-3 min followed by removal of the supernatant. The pellet was rapidly frozen by suspending it in liquid nitrogen for approximately 1 min and then transferred to the -80°C freezer for storage. Mitochondria Reagent A (Pierce, Rockford, IL) and protease inhibitor were added to the sample and cells were incubated on ice for 2 min. In the next step, cells were homogenized on ice using the VWR™ Pellet Mixer for 3 min. In the following step, Mitochondria Reagent C (Pierce, Rockford, IL) was added to the cells and were centrifuged at 700 rpm for 10 min at 4 °C. Afterward, supernatant was transferred to a new 2 ml tube and centrifuged at 12,000 rpm for 15 min at 4 °C. For a second time, supernatant was transferred to a new tube. In the next step, Reagent C was added to the isolated mitochondria (pellet) and was centrifuged

again at 12,000 rpm for 5 min. The pellet was the mitochondria fraction. To quantify the protein concentration the pellet was re-suspended in sample rehydration buffer (8M Urea 2% CHAPS 0.5% ZOOM®Carrier Ampholytes (Invitrogen, Carlsbad, CA), 0.002% Bromophenol Blue, 20 mM dithiothreitol). The protein concentration was quantified using NanoVue™ (General Electric, Piscataway, NJ). The mitochondria isolate was then frozen at -20°C until SDS-PAGE or 2D PAGE was performed.

#### SDS-PAGE Gel Electrophoresis

To perform SDS-PAGE electrophoresis, two different size plates, spacer plate (10.1 × 7.3 cm) and short plate (10.1 × 8.3 cm) were placed into an apparatus with a spacer between them. Ten percent resolving gel (1.5 ml of 1.5 M Tris-HCl, pH 8.8, 2 ml of 30% acrylamide, 0.05 ml of 10% SDS, 2.44 ml di-ionized water, 50 µl of 10% APS and 4 µl of TEMED) was poured into the plates up to ~1.5 inches below the top. A layer of methanol was added on top of the resolving gel to help with gel polymerization. After polymerization of the resolving gel, the top of the gel was rinsed off to remove the methanol. The stacking gel was added on top of the resolving gel (0.875 ml of 1.0 M Tris-HCl, pH 6.8, 0.583 ml of 30% acrylamide, 0.035 ml of 10% SDS, 2.007 ml of water, 0.035 ml of 10% APS and 3 µl of TEMED) and a comb was inserted into the resolving gel. While waiting for polymerization of the stacking gel, samples were prepared by mixing samples with 5X sample loading buffer (60 mM of Tris-HCl, pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol and 1% bromophenol blue) to the desired protein concentration. Samples were boiled at 100 °C for 5 min and cooled down on ice for 5 min. After polymerization of the stacking gel, the electrophoresis box was

filled with running buffer, the comb was removed and samples were added to wells, and gel was run at 120 V for 90 min.

### Western Blot

For the Western blot procedure, protein samples were prepared as described in SDS-PAGE. Bis-Tris pre-cast gels (4-12%) were used for running the electrophoresis separation of the samples. The gel was run at 175 V for 50 min and then transferred to a PVDF membrane using iBlot® Dry Blotting System (Invitrogen, Carlsbad, CA). The blotted PVDF membrane was removed from the transfer apparatus and was blocked for 1 h using 1% BSA (vol/vol) in PBS. In the next step, the blot was incubated with diluted primary antibody (diluted 1:1000 in 1% BSA in PBS) and incubated on a rocker overnight at 4 °C. On the next day, the membrane was washed 3 times (20 min each) with PBST (PBS with 0.1% TWEEN 20) to remove non-bound primary antibody and then the blot was incubated with the diluted secondary antibody (1:10,000) for 1 h on a rocker at room temperature. The blot was washed 3 times (20 min) with PBST. Equal amounts of Super Signal West Pico Enhancer solution and Super Signal West Pico Stable Peroxide Solution (Thermo Scientific, Waltham, MA) were mixed and added to the blot for 5 min. The Blot was placed in a plastic membrane protector and was placed in a film cassette with the protein side facing up. An X-ray film (CL-X Posure™ Film, Thermo Scientific, Waltham, MA) was placed on top of the membrane while in a dark room and exposed for 1 min, 5 min, or 10 min using a separate piece of film for each exposure time.

### 2D Gel Electrophoresis

Two-dimensional (2D) gel electrophoresis combines two methods of separation. The first dimension of a 2D gel separates proteins according to their

charge using an immobilized pH gradient using an IPG strip (7 cm). The second dimension gel is typically an SDS gel, which separates proteins based on their mass (26).

Samples were prepared at a desired concentration using sample rehydration buffer (8M Urea, 2% CHAPS, 0.5% ZOOM®Carrier Ampholytes, 0.002% Bromophenol Blue, 20 mM dithiothreitol) to denature and solubilize the sample proteins for isoelectric focusing. Strips were inserted into the ZOOM® IPGRunner Cassette and samples were loaded on the strips; samples were sealed using sealing tape and incubated overnight at room temperature. The next day sealing tape was removed; an electrode wick was placed at each end of ZOOM® IPGRunner Cassette; 750 µl of Novex® IEF 3-10 Cathode buffer (Invitrogen, Carlsbad, CA) was added to the upper part of the electrode wick and 750 µl of Novex® IEF Anode buffer (Invitrogen, Carlsbad, CA) was added to the electrode wick which is the lower part of the cassette. Isoelectric Focusing was performed using Mini-Cell Chamber ZOOM® IPGRunner. Steps for Isoelectric Focusing were 200 V for 20 min, 450 V for 15 min, 750 V for 15 min and 2000 V for 45 min. After Isoelectric Focusing, strips were equilibrated in two steps; at the first step, 1X NuPAGE® LDS Sample Buffer (Invitrogen, Carlsbad, CA) containing 10% NuPAGE® Sample Reducing Agent was added to the strip for 30 min; at the second step 1X NuPAGE® LDS Sample Buffer containing 232 mg/10 ml of fresh iodoacetamide was added to the strips for 30 min. In the next step, the strips were inserted into the IPG well of a 1.0 mm NuPAGE® 4-12% Bis-Tris ZOOM™ Gel and covered with 300 µl of 0.5% agarose solution in appropriate buffer. The XCell SureLock™ Mini-Cell Cassette/ Buffer Core sandwich was assembled and filled with buffer. SDS-PAGE was performed at 125 V for 2 h. The gel was fixed for 1 h, stained overnight using sypro ruby stain and the next day de-stained for 30

min (20% Methanol, 7% Acetic Acid). The Alpha Imager HP Imaging system (Alpha Innotech, Santa Clara, CA) was used for fluorescent imaging and quantification.

#### In-Gel Trypsin Digestion of Protein

Spots were visualized using UV box and were excised with a spot picker (PROTEINEER sp™, BrukeDaltonics, Germany), and placed into a siliconized 1.5-ml tube. In the next step, the gel pieces were de-stained twice for 30 min at 37°C using destaining solution (50% Methanol and 10% Acetic Acid). After de-staining, the supernatant was discarded and each gel sample was incubated with 25 mM NH<sub>4</sub>HCO<sub>3</sub> for 30 min with agitation. Supernatant was discarded again and the gel piece was incubated with 100 mM NH<sub>4</sub>HCO<sub>3</sub> in 50% ACN for 30 min at 37 °C with agitation. Supernatant was discarded and 100% ACN was added to dehydrate the gel. After dehydration, 100% ACN was discarded and sample was heated at 56° C for 10 min to complete dryness. Thirty µl of 12 ng/µl trypsin (Promega , Madison, WI ) was added to cover the gel and 50 µl of 25 mM NH<sub>4</sub>HCO<sub>3</sub> was added on top of it and the gel was incubated at 37 °C for 18 h with agitation. The next day the digest solution aqueous extraction was transferred into a clean eppendorf tube. Twice, the gel pieces were incubated with 50%ACN/0.1%TFA with agitation for 30 min and then were sonicated for 5 min. Speed vacuum was used to reduce the volume of extracted digests to 10 µl. C18 Zip Tip (Millipore) Clean up was used to make the sample ready for MALDI-TOF. The cleaned sample was mixed with α-cyano-4-hydroxy-cinnamic acid (HCCA) as matrix and spotted on the metal MALDI plate to air dry.

### Mass Spectrometry (MALDI-TOF)

Matrix-assisted laser desorption/ionization mass spectrometry, or MALDI-TOF, is a new technique used in mass spectrometry, which is capable of analyzing biomolecules such as proteins and peptides (27). In proteomics, MALDI is used for the identification of proteins isolated through gel electrophoresis (2D-Page). MALDI is a very sensitive method that can detect low quantities of sample with high accuracy (0.1 -0.01). MALDI-TOF (time-of-flight mass spectrometer) is the most widely used mass spectrometer due to its large mass range. Cailleau et al. report that “the TOF measurement procedure is well-matched to the MALDI ionization process because the pulsed-laser takes individual shots rather than working in continuous operation” (27).

The mass spectrometer used in this project was an MALDI/TOF Autoflex II (Bruker Daltonics, Germany). This mass spectrometer was operated in either MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF/TOF using the FlexControl software. The instrument was calibrated with [M+H]<sup>+</sup> ions of substance P, bombesin, angiotensin I, angiotensin II, and adrenocorticotrophic hormones (clip 1-17 and clip 18-39). Each spectrum was created by adding the data from 200 consecutive laser shots. The samples were analyzed by both PMF and LIFT-TOF/TOF MS/MS from the same target. For MS/MS analysis a maximum of three precursor ions per sample was chosen. The produced spectra were interpreted using the Mascot search engine software (Matrix Science Ltd., London, UK) and database searches were performed via BioTools 2.2 interface software (Bruker, Billerica, MA) that parsed either the International Protein Index (IPI-Human), SwissProt, or non-redundant NCBI databases. A mass tolerance of 100 ppm and two missing cleavage sites for PMF were allowed and carboxymethylation of cystine, carbamidomethylation of cystine,

and oxidation of methionine residues was considered. The probability score was used as decisive factor for correct identification (28). Cailleau et al. report that “The algorithm used for determining the probability of a false positive match with a given mass spectrum is described elsewhere” (27). The experimental workflow used in this project is shown in Figure 5.

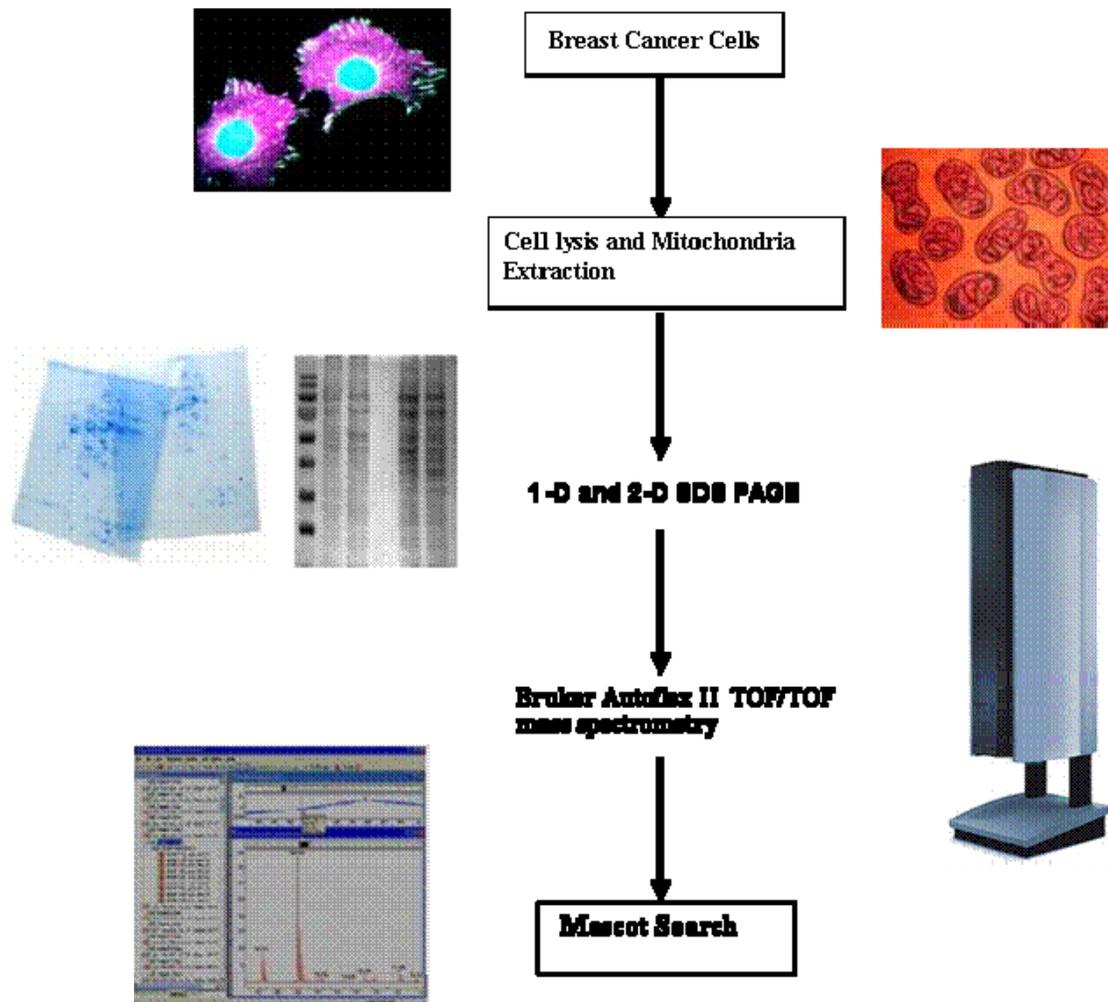


Fig. 5. Experimental workflow. This procedure starts with tissue culture cells and continues with mitochondria extraction, 1-D and 2-D SDS PAGE Gel and finally mass spectrometry (MALDI-TOF) and analysis by Mascot Search. In this experiment MDA-MB-231 and MDA-231 BO cell lines were treated with 200  $\mu\text{M}$  of zoledronic acid and the mitochondria fraction was isolated to investigate the effects of zoledronic acid on mitochondria. Extracted proteins were separated by SDS-PAGE and 2-Dimensional gel electrophoresis. This investigation was followed by performing trypsin digest on 2-D gel spots, and the matrix-assisted laser desorption/ionization time-of flight mass spectrometer (MALDI-TOF) was used for identification of proteins.

## RESULTS

### Initial Cytotoxicity of Zoledronic Acid

Initially, Sulforhodamine B assays (SRB assays) were performed for MDA-MB-231 and MDA-231 BO cell lines to optimize the most effective concentration of zoledronic acid. Results indicate that at the concentration of 200  $\mu\text{M}$ , zoledronic acid led to 70-80% death in both cell lines within 72 h, while at 100  $\mu\text{M}$  it induced 60% cell death in bone cell lines and 40% cell death in parental cell lines within 72 h (Figs. 6 & 7).

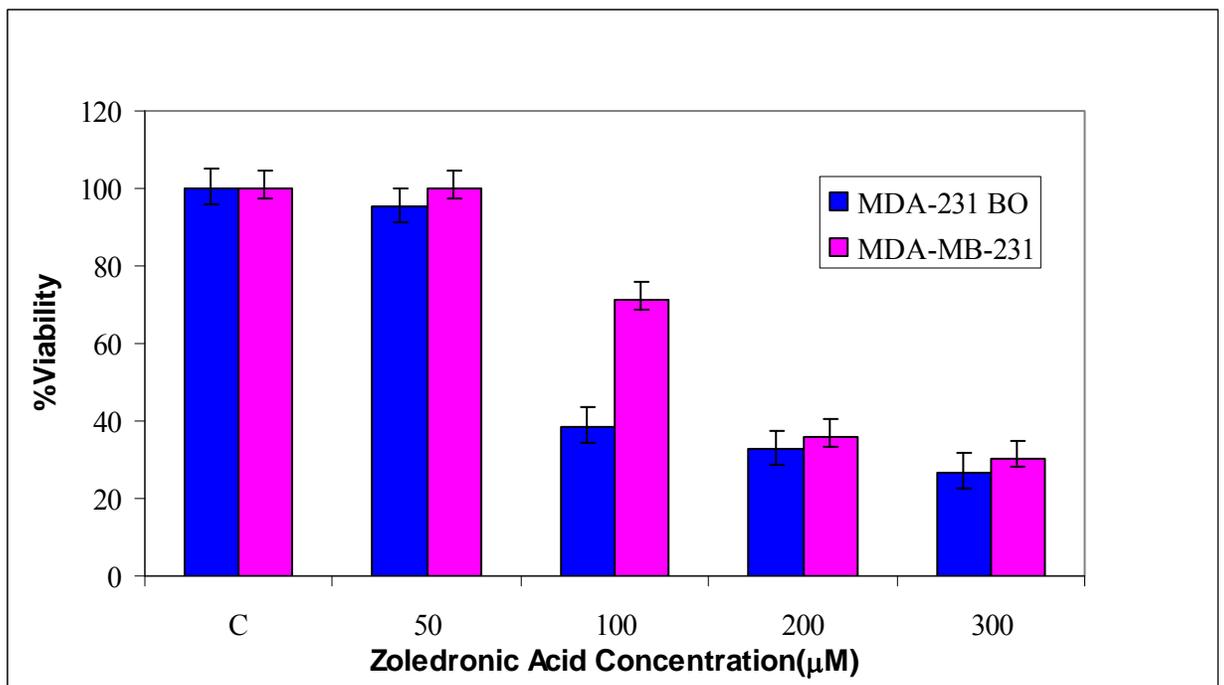


Fig. 6. Cytotoxicity assay and concentration. Using the Sulforhodamine B assay to assess cell viability, cells were exposed to different concentrations of zoledronic acid (50  $\mu\text{M}$ , 100  $\mu\text{M}$ , 200  $\mu\text{M}$  and 300  $\mu\text{M}$ ) for 72 h.

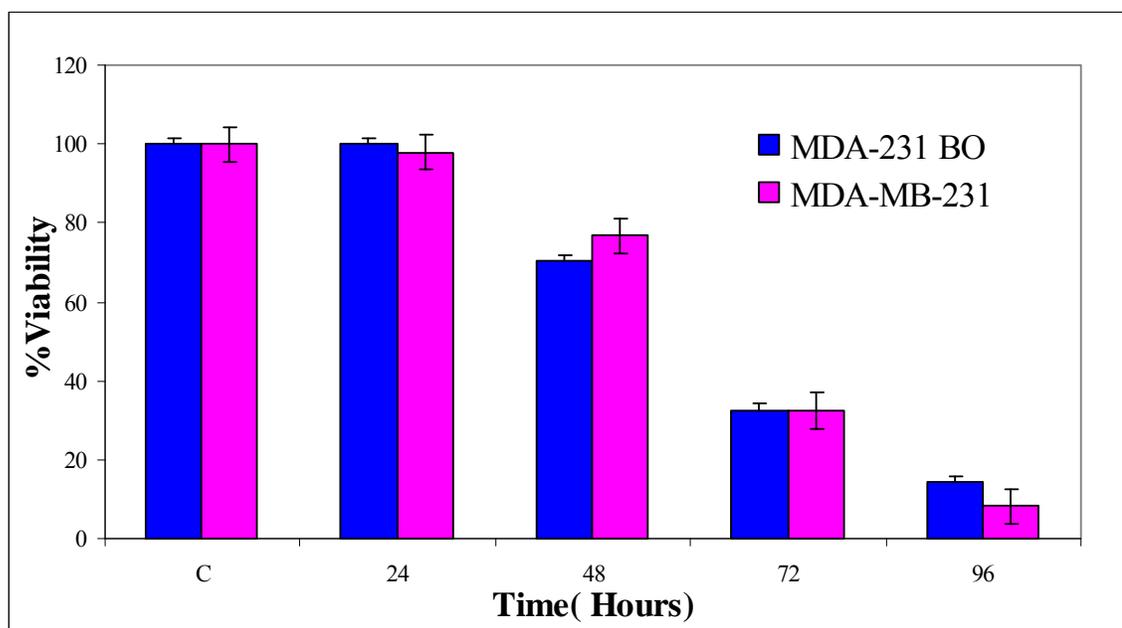


Fig. 7. Cytotoxicity assay and time. Using the Sulforhodamine B assay to assess cell viability, MDA-MB-231 and MDA-231 BO cells were exposed to 200  $\mu$ M zoledronic acid for 24, 48, 72, and 96 h.

#### Cytotoxicity of Zoledronic Acid with Calcium

In the next part of the experiment, SRB assays were performed on MDA-MB-231 and MDA-231 BO cell lines to investigate if there is a correlation between calcium concentration and zoledronic acid cytotoxicity. Results showed that calcium plays little role in zoledronic acid cytotoxicity (Fig. 8).

#### Morphology of ZA-Treated Cells Compared to Untreated Cells

MDA-231 BO cells were treated with 200  $\mu$ M of zoledronic acid for 72 h to compare morphology of control with zoledronic acid treated cells. Pictures of the cells were taken after 24, 48, and 72 h. After 48-72 h the treated cells were less confluent than control cells (Fig. 9).

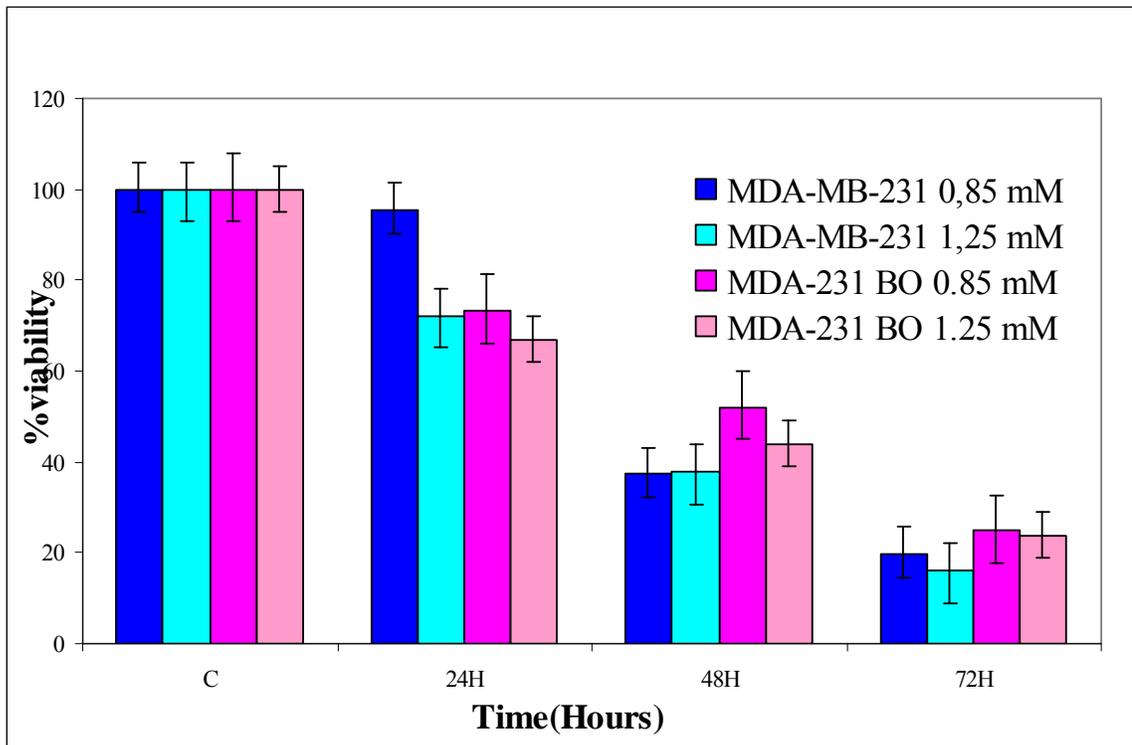


Fig. 8. Cytotoxicity assay using different concentrations of calcium. Using the sulforhodamine B assay to investigate the relation between calcium concentration and zoledronic acid cytotoxicity, cells were exposed to 200  $\mu$ M zoledronic acid with different concentrations of calcium in the form of calcium chloride for 24, 48 and 72 h.

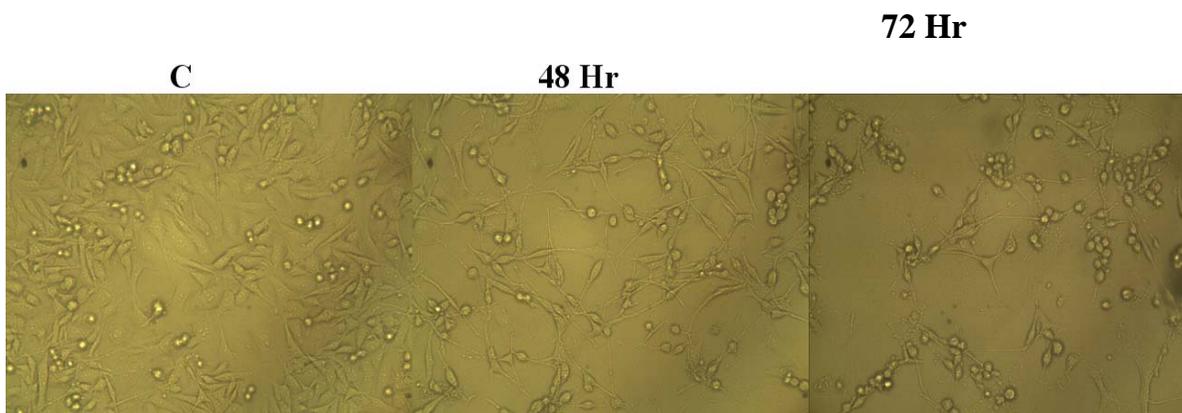


Fig. 9. Zoledronic acid treated MDA-231 BO morphology. Pictures of cells were taken every 24 h. Control cells picture was taken after 72 h. After 48 and 72 h the treated cells were less confluent than the control cells. Note: increased dead cells as indicated by rounding up. C=Control.

Control and treated MDA-MB-231 and MDA-231 BO cell lines were scraped and the whole proteins were extracted using mammalian extraction buffer (GE HealthCare, Wauwatosa, WI) and the proteins were quantified using NanoVue Spectrophotometer (GE HealthCare, Wauwatosa, WI). To compare protein expression of control versus treated cells, 200 mg of each sample (MDA-MB-231-Control, MDA-MB-231-Treated, MDA-231 BO-Control, and MDA-231 BO-Treated) was loaded on a 1-D SDS-PAGE gel (Fig. 10). The gel was stained with coomassie blue overnight and de-stained the next day. The picture of the gel was taken using Alpha Imager HP Imaging system (Alpha Innotech, Santa Clara, CA).

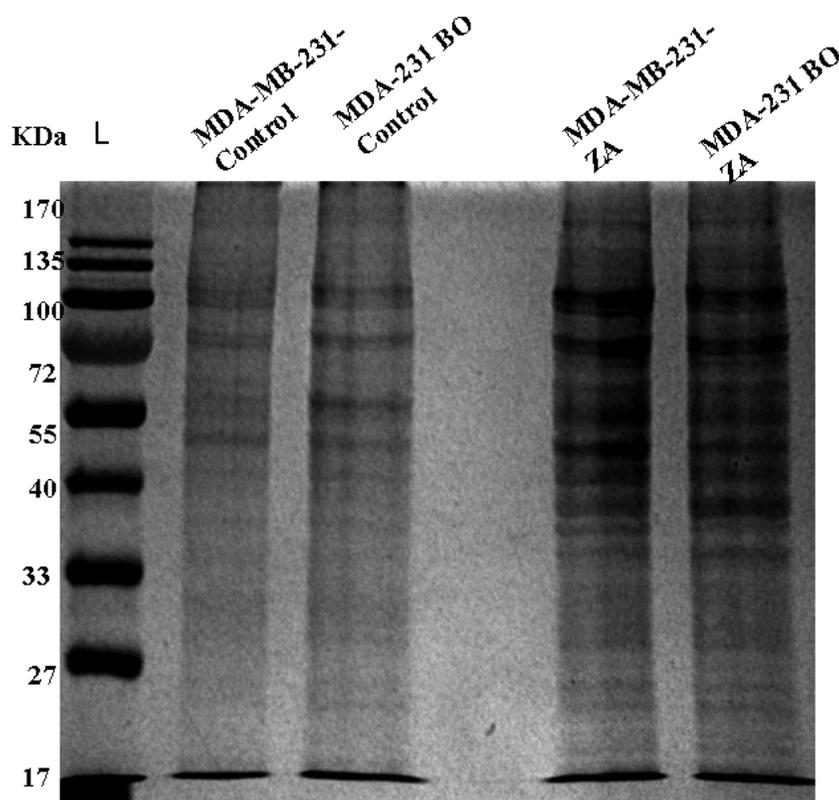


Fig. 10. 1-D SDS-PAGE of MDA-MB-231 and MDA-MB-231 Bone cells. cells were cultured in DMEM and treated with 200  $\mu$ M zoledronic acid for 48 h compared with non-treated control cells. L=Ladder or protein marker.

To further investigate proteomic differences between control and treated cell lines and to investigate the effect of zoledronic acid in mitochondrial function, mitochondria isolation kit (Pierce, Rockford, IL) was used to isolate the mitochondria from both parental and bone cell lines.

#### Confirmation of Mitochondria Isolation and Buffer Selection

Superoxide Dismutase 1 antibody (SOD1), is found in both mitochondria and cytosol. The results showed that SOD1 was present in both the mitochondria and the cytosol fractions, and also the three buffers have different abilities to solubilize the protein samples. The sample rehydration buffer showed the maximum amount of protein present (Fig. 11).

Voltage-dependent anion selective channel protein (VDAC) is found only in the mitochondria. To test our mitochondria isolation kit, mouse anti-VDAC1 was used. The results indicated that only sample rehydration buffer was effective in solubilizing the VDAC mitochondrial protein (Fig. 11). The immunoblot also confirmed that the mitochondria were enriched using the Pierce isolation kit. Based upon these results, the sample rehydration buffer was used to re-suspend the mitochondria pellets prior to 1D or 2D electrophoresis. Although mitochondria enrichment was showed for VDAC, there is no protocol for mitochondria extraction that can provide clear mitochondria fraction. This explains the presence of non-mitochondrion proteins in identified proteins. (Note: This experiment was done in collaboration with Julie Hale.)

Another SDS-PAGE was run after mitochondria isolation to compare mitochondria fractions. This SDS-PAGE gel compares MDA-MB-231-Control, MDA-MB-231-Treated with Ibandronate, MDA-MB-231-Treated with zoledronic acid, MDA-231 BO-Control, MDA-231 BO Treated with Ibandronate and MDA-

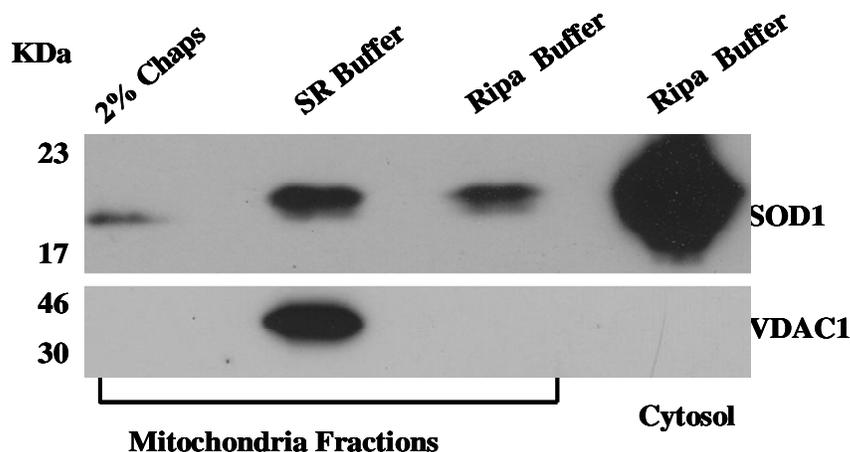


Fig. 11. Mitochondrial enrichment protocol validation. voltage-dependent anion selective channel protein (VDAC) presence in mitochondria fraction dissolved in sample rehydration buffer showed that mitochondria fraction was successfully extracted from breast cancer cells. SOD1= Superoxide Dismutase 1, SR Buffer= Sample Rehydration Buffer, 2% Chaps Buffer = 2%CHAPS in Tris buffered saline (TBS; 25 mM Tris, 0.15 M NaCl; pH 7.2).

231 BO Treated with zoledronic acid. Ten  $\mu$ g of each protein was loaded and the gel was stained with Sypro-ruby overnight and de-stained the next day. The results showed distinct proteomic differences between treated and control mitochondrial fractions and also between ibandronate and zoledronic acid treated mitochondrial fractions (Fig. 12).

In the next step, 2D gels were run to identify some of the differentially expressed proteins in ZA treated versus control cells. For this purpose, 30  $\mu$ g of each protein was loaded and the gels were stained with Sypro-ruby overnight and de-stained the next day. Differentially expressed proteins were identified from 2D gel spots excised and digested with trypsin using mass spectrometry (Figs. 13 & Table 3; Fig. 15 & Table 4; Fig. 17 & Table 5).

The spectra of succinyl-coA ligase (SUCA), one of the proteins identified in the MDA-MB-231 control versus MDA-MB-231-Treated 2-D gel, are shown below (Fig. 14).

The spectra of egl nine homolog 1 (EGLN1), one of the down-regulated proteins in the MDA-MB-231 bone-treated cells, are shown below (Fig. 16).

The spectra of miofusin 1 (MFN1), one of the up-regulated proteins in the MDA-MB-231 bone-treated cells, are shown below (Figure. 18).

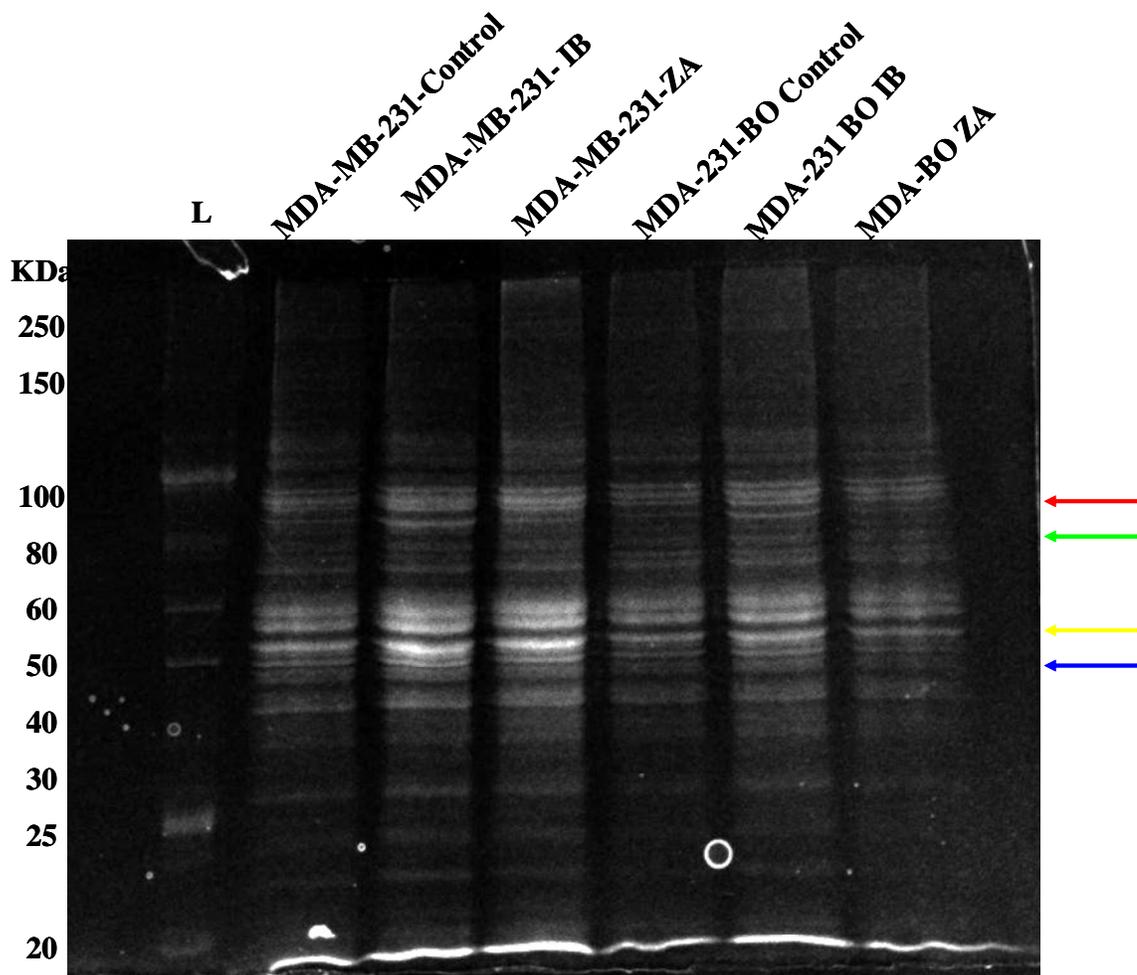


Fig. 12. 1-D SDS-PAGE of MDA-MB-231 and MDA-231 BO cells treated with 200  $\mu$ M IB or ZA for 48 h compared with control cells. There are distinct proteomic differences between treated and control mitochondrial fractions. Note: The initial density of the cells for the control plates was 40% while the initial density of the cells for the treated plates was 70%. This was done to be able to harvest the same quantity of cells from treated and control plates after 48 h. Note: arrows show the differentially expressed proteins.

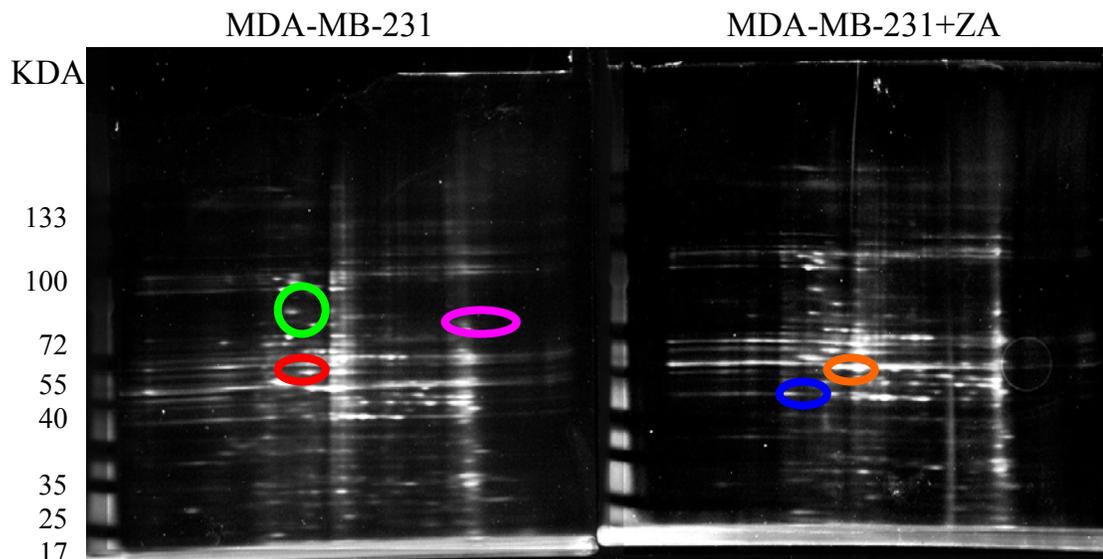


Fig. 13. 2D-PAGE comparing MDA-MB-231 cells treated with 200  $\mu$ M ZA with untreated cells: There are distinct differences between control cells and cells treated with ZA.

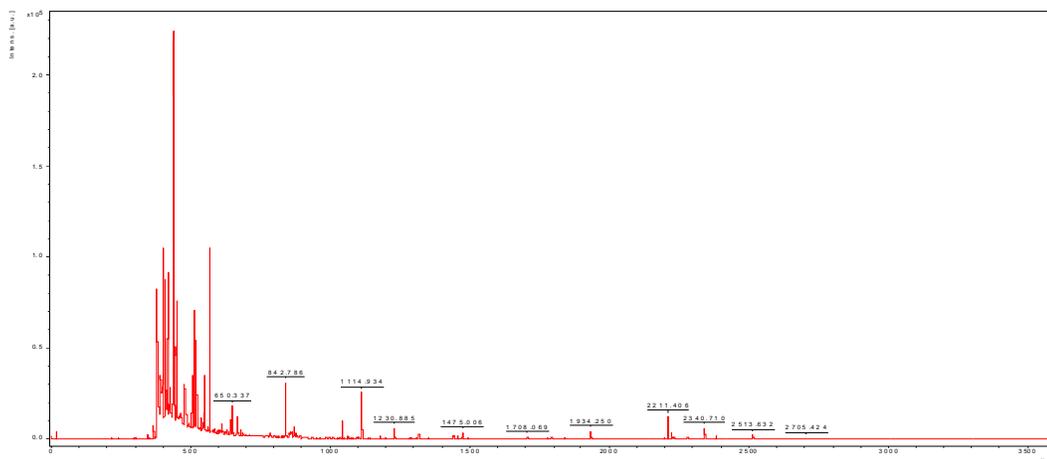


Fig. 14. MS spectra of mitochondrial component Succinyl-CoA Ligase (SUCA). SUCA is one of the proteins identified in MDA-MB-231 cell lines.

**Table 3.** Identified proteins from MDA-MB-231-control versus MDA-MB-231-treated 2-D gel. (Colors correspond to the spots on the 2-D gel), MDA-231 P=MDA-MB-231.

| Protein ID         | Common Name | Mass (Da) | ~MW on gel (kDa) | GO Functions                        | Relative Up-Regulation* | Mowse Score | Cellular Location |
|--------------------|-------------|-----------|------------------|-------------------------------------|-------------------------|-------------|-------------------|
| <b>IPI00184064</b> | DTX3        | 37964     | 45               | Notch signaling pathway             | MDA-231 P+ZA            | 32          | Cytoplasm         |
| <b>IPI00908386</b> | ADH7        | 39980     | 45               | Xenobiotic/fatty acid metabolism    | MDA-231 P+ZA            | 35          | Cytoplasm         |
| <b>IPI00872762</b> | SUCA        | 35025     | 40               | Acetylation, GTP-binding, TCA cycle | MDA-231 P+ZA            | 38          | Mitochondria      |
| <b>IPI00926163</b> | RBM25       | 32801     | 35               | mRNA binding                        | MDA-231 P+ZA            | 36          | Cytoplasm         |
| <b>IPI00748058</b> | STAC3       | 37114     | 40               | Intracellular signaling             | MDA-231 P+ZA            | 32          | Protein level     |

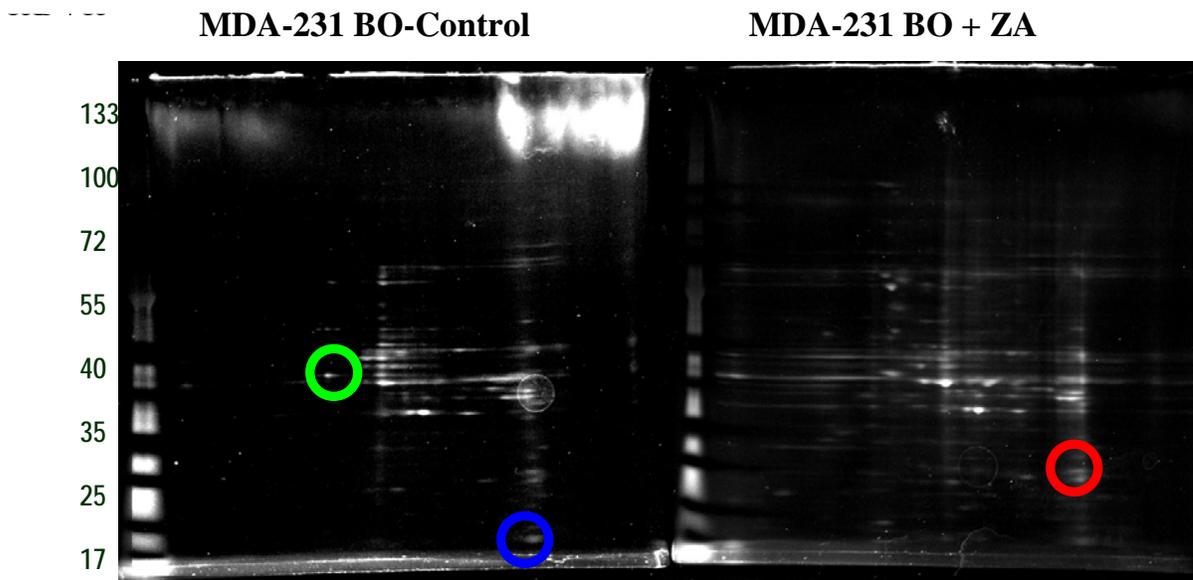


Fig. 15. 2D-PAGE comparing MDA-231 BO cells treated with 200  $\mu$ M zoledronic acid with untreated cells: There are distinct differences between control cells and cells treated with ZA.

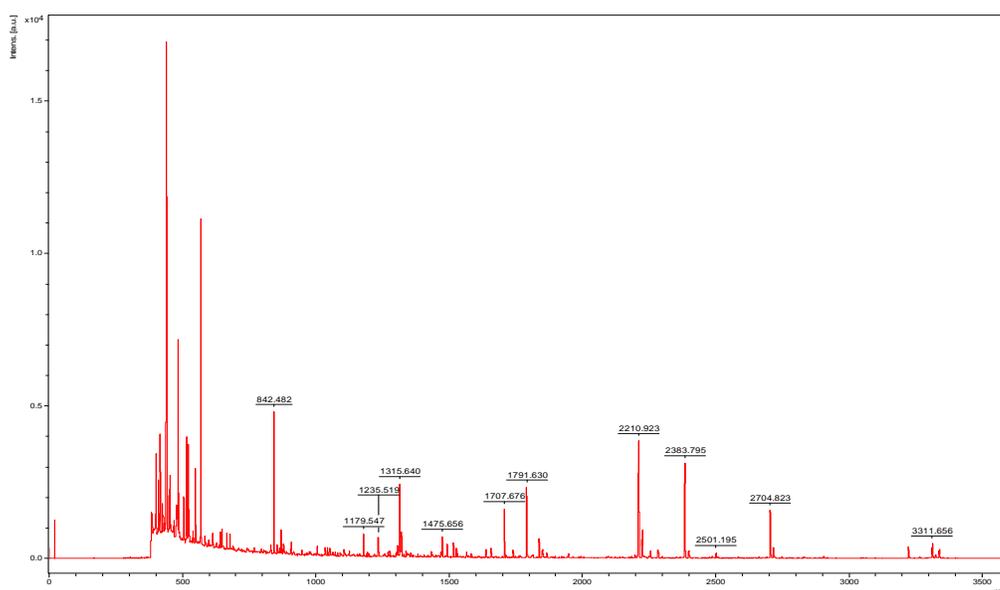


Fig 16. MS spectra of cytosolic component EGLN1. Egl nine homolog 1 is one of the identified proteins in MDA-MB-231 BO cell lines.

| <b>Table 4.</b> Identified proteins from the MDA-231 BO-control versus MDA-231 BO-treated 2-D gel. |               |           |                  |                                                          |                          |             |                   |
|----------------------------------------------------------------------------------------------------|---------------|-----------|------------------|----------------------------------------------------------|--------------------------|-------------|-------------------|
| ProteinID                                                                                          | Common Name   | Mass (Da) | ~MW on gel (kDa) | GO Functions                                             | Relative Up-Regulation * | Mowse Score | Cellular Location |
| <b>IPI00797027</b>                                                                                 | ARNTL2        | 10336     | 10               | Transcription regulation, signal transducer activity     | MDA-231 BO + ZA          | 52          | Nucleus           |
| <b>IPI00158157</b>                                                                                 | NFX1 Isoform2 | 11354     | 10               | Negative regulation of MHC Class 2 biosynthetic process  | MDA-231 BO + ZA          | 40          | Nucleus           |
| <b>IPI00004928</b>                                                                                 | EGLN1         | 45992     | 45               | Negative regulation of transcription, Oxygen homeostasis | MDA-231 BO + ZA          | 37          | Cytosol           |
| <b>IPI00552857</b>                                                                                 | RBCK1         | 29349     | 25               | Modification-dependent protein, Catabolic process        | MDA-231 BO + ZA          | 35          | Intracellular     |

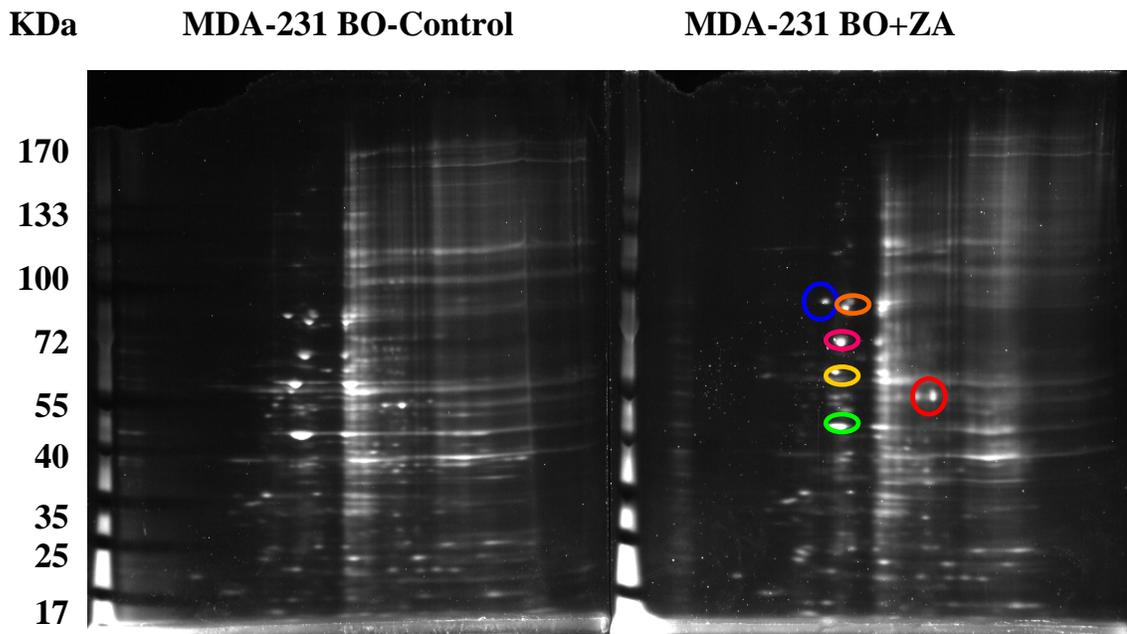


Fig. 17. 2D-PAGE comparing MDA-231 BO cells treated with 200  $\mu$ M zoledronic acid with untreated cells. There are distinct differences between control cells and cells treated with ZA.

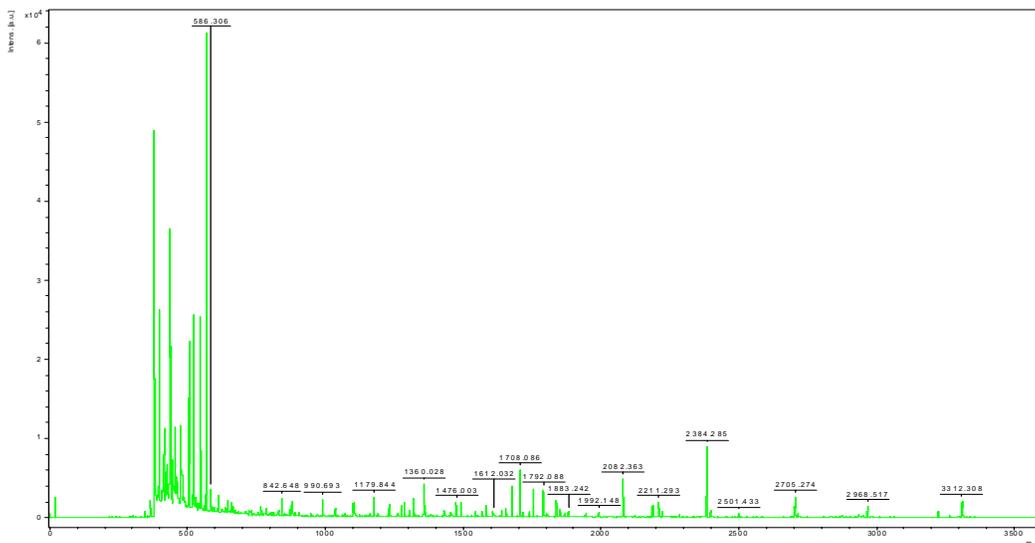


Fig. 18. MS spectra of cytosolic component MFN1. Mitofusin1 is one of the identified proteins in MDA-MB-231 BO cell line.

**Table 5.** Identified proteins from the second MDA-231 BO-control versus MDA-MB-231 BO-treated 2-D gel. ER=endoplasmic reticulum

| Protein ID         | Common Name | Mass (Da) | ~MW on gel (kDa) | GO Functions                                                                                             | Relative Up-Regulation * | Mowse Score | Cellular Location              |
|--------------------|-------------|-----------|------------------|----------------------------------------------------------------------------------------------------------|--------------------------|-------------|--------------------------------|
| <b>IPI0003362</b>  | HSPA5       | 72377     | 60               | Assembly of multimeric protein complex inside ER, anti-apoptosis, negative regulator of caspase activity | MDA-231 BO+ZA            | 68          | Nucleus, endoplasmic reticulum |
| <b>IPI00292950</b> | SERPIN D1   | 60140     | 60               | Heparsin binding, serine-type endopeptidase inhibitor activity                                           | MDA-231 BO+ZA            | 32          | Extracellular region           |
| <b>IPI00791512</b> | DDX55       | 52180     | 60               | Probable ATP-binding RNA helicase, nuclear and mitochondrial splicing                                    | MDA-231 BO+ZA            | 37          | Mitochondrion<br>Nuclear       |
| <b>IPI00027228</b> | PET112 L    | 61825     | 55               | Translation, ATP-binding, Ligase Activity                                                                | MDA-231 BO+ZA            | 39          | Mitochondrion                  |
| <b>IPI00797961</b> | RNFT2       | 48933     | 55               | Zinc ion binding, protein binding.                                                                       | MDA-231 BO+ZA            | 42          | Membrane                       |
| <b>IPI00031392</b> | CARD1 4     | 48475     | 55               | Positive regulator of cell apoptosis and NF-Kappa B activation, Regulation of apoptosis                  | MDA-231 BO+ZA            | 27          | Cytoplasm                      |
| <b>IPI00549229</b> | TACC1       | 67031     | 50               | May represent a breast cancer candidate gene, cell cycle, cell division                                  | MDA-231 BO+ZA            | 68          | Cytoplasm,<br>Nucleus          |
| <b>IPI00641910</b> | RABGA P1L   | 43733     | 50               | Regulation of Rab GTPase activity                                                                        | MDA-231 BO+ZA            | 32          | Nucleus                        |

**Table 5. Continued.**

| Protein ID         | Common Name | Mass (Da) | ~MW on gel (kDa) | GO Functions                                                      | Relative Up-Regulation * | Mowse Score | Cellular Location |
|--------------------|-------------|-----------|------------------|-------------------------------------------------------------------|--------------------------|-------------|-------------------|
| <b>IPI00917575</b> | HSPD1       | 55049     | 50               | Positive regulation of T cell activation, regulation of apoptosis | MDA-231 BO+ZA            | 53          | Mitochondrion     |
| <b>IPI00395973</b> | MFN1        | 41856     | 40               | Mediator of mitochondrial function                                | MDA-231 BO+ZA            | 30          | Mitochondrion     |
| <b>IPI00007168</b> | VASH1       | 40931     | 40               | Negative regulator of cell proliferation, anti-angiogenesis       | MDA-231 BO+ZA            | 32          | Cytoplasm         |
| <b>IPI00792971</b> | IDH3A       | 31361     | 38               | TCA Cycle, Oxidoreductase, Carbohydrate Metabolic process         | MDA-231 BO+ZA            | 30          | Mitochondrion     |
| <b>IPI00465248</b> | ENO1        | 47139     | 38               | Negative regulator of cell growth, transcription repressor        | MDA-231 BO+ZA            | 33          | Cytoplasm         |
| <b>IPI00419824</b> | IL7R        | 31361     | 38               | Negative regulation T cell mediated cytotoxicity                  | MDA-231 BO               | 30          | Mitochondrion     |
| <b>IPI00427595</b> | MFAP3 L     | 34077     | 30               | Negative of angiogenesis, negative regulation of proliferation    | MDA-231 BO+ZA            | 32          | Cytoplasm         |

## DISCUSSION

In this study we anticipated that we could find proteins involved in osteoclast-specific functions, which can further lead to better understanding of bone metastasis. To test our hypothesis we used MDA-MB-231 and MDA-231 BO cell lines as our model system and treated them with zoledronic acid to investigate the differences between bone and parental cell lines. A proteomic approach was used to identify differentially expressed proteins and to determine their role in physiological functions.

### Cytotoxicity of Zoledronic Acid

Estrogen Receptor (ER)-Negative MDA-MB-231 breast cancer cell lines (parental and bone) were chosen for this experiment based upon their sensitivity to zoledronic acid. A previous study has shown that ER-negative MDA-MB-231 cells are highly sensitive to zoledronic acid-induced apoptosis, whereas ER-positive MCF-7 cells were found to be relatively resistant (29). In this study, we demonstrate that 200  $\mu\text{M}$  zoledronic acid induced a strong cell death in 70-80% cells within 72 h (Fig. 6, 7, 9, pp. 21-23) while 100  $\mu\text{M}$  zoledronic acid induced 50% cell death within 72 h.

In previous studies, it has been shown that 100  $\mu\text{M}$  of zoledronic acid is required to induce significant anti-proliferative and apoptosis effects (30–32). Additionally, other *in vitro* studies have shown maximal inhibitory effects of bisphosphonates at doses higher than what is attainable in the serum of patients. According to another study, the concentration of bisphosphonates in plasma ranges from 1 to 3  $\mu\text{M}$  (33). The rapid decline of plasma concentrations is

primarily caused by the fast incorporation of bisphosphonates into the skeleton, where a local concentration of 100  $\mu\text{M}$  to 1 mM is achievable (34).

Bisphosphonates incorporated into bone disturb osteoclasts resulting in areas of highly concentrated bisphosphonates. High local concentrations of bisphosphonates may be a prerequisite for their ability to exert direct anti-tumor effects. Although the zoledronic acid concentration used for in this experiment is much higher than the plasma concentration, high concentrations of bisphosphonate can be attainable in skeleton.

#### Cytotoxicity of Zoledronic Acid with Calcium

Bone matrix is composed of hydroxyapatite (calcium phosphate) mineral crystals (35). High concentrations of free calcium (8-40 mM) have been detected during bone remodeling (36). Sanders et al. report that “breast cancer cells express calcium-sensing receptors, CaSR, which further increases PTHrP expression by breast cancer cells” (37). As a result, calcium may have a role in breast cancer bone metastasis.

Two different concentrations of calcium chloride were added to zoledronic acid (0.85 mM and 1.25 mM), and another sulforhodamine B assay was performed to investigate the effect of calcium on zoledronic acid cytotoxicity. Our results showed that calcium doesn't make a noticeable difference on zoledronic acid cytotoxicity (Fig. 8, p. 23). In another study, it was shown that low calcium concentration, 30  $\mu\text{mol/l}$  zoledronic acid didn't have an evident effect on zoledronic acid efficacy while higher concentrations of calcium extensively increased the inhibitory effects of zoledronic acid and promoted cell apoptosis induced by zoledronic acid (38).

Effect of Zoledronic Acid in Stress Response/  
Cell Death

Heat Shock Protein 60KDa (HSPD1)

This protein was up-regulated in bone-treated cell lines versus non-treated cell lines. Heat shock protein 60 KDa 1 is a mitochondrial protein that may have a role in the innate immune system by acting as a signaling molecule (39). Many types of heat shock proteins are found throughout the cell (39). They are highly conserved and have a fundamental role in maintaining homeostasis and viability. They are noted for their function in protein folding but are also associated with other functions, such as protein transport and assembly, triggering the protein degradation process and apoptosis (39). HSP60 has been shown to induce apoptosis via toll-like receptors (40). The presence of heat shock protein in treated bone cells may suggest that zoledronic acid induces apoptosis in cancer cell lines.

Caspase Recruitment Domain Family,  
Member14 (CARD14)

Caspase recruitment domain family, member 14 (CARD14) is another protein identified as an up-regulated protein in bone-treated cell lines. This cytosolic protein belongs to the membrane-associated guanylate kinase (MAGUK) family and CARD protein family. MAGUK proteins have an important role in multiprotein complex assembly at particular regions of the plasma membrane. CARD family members, on the other hand, are characterized by their caspase-associated recruitment domain. CARD14 shares a domain structure with CARD11 protein and both these proteins have been shown to be positive regulators of cell apoptosis and activators of NF-kappaB by interacting with BCL10 (41). It has been shown that activation of NF-kappaB can further lead to

BCL20 phosphorylation. Two different isoforms of this protein have been reported (41). This suggests that zoledronic acid induces apoptosis.

#### Heat Shock 70KDa Protein 5(HSPA5)

This protein was identified as an up-regulated protein in zoledronic acid treated bone cell lines. Heat shock 70 kDa protein 5 (HSPA5) or 78 kDa glucose-regulated protein (GRP-78) is a protein localized at cytoplasm. This protein is a highly conserved member of the 70-kDa heat shock protein family, which might facilitate multimetric protein assembly inside the endoplasmic reticulum.

Dana et al. report that “heat shock protein family plays a role in facilitating the assembly of multimeric protein complexes inside the endoplasmic reticulum(ER)” (42). Proteins stably bound to GRP78/BiP are subsequently translocated from the ER into the cytosol for proteasome-dependent degradation (43). The induction of GRP78/BiP by conditions of stress, which further result in the accumulation of misfolded or underglycosylated proteins in the ER, provides further evidence that GRP78/BiP plays an important role in protein synthesis (44). This protein also has an anti-apoptosis activity and is a negative regulator of caspase activity (42). This protein was up-regulated in treated cell lines in response to stress.

#### Alcohol Dehydrogenase 7(ADH7)

Alcohol dehydrogenase 7 was also up-regulated in parental treated cell lines. This is a cytosolic protein, which is a member of the alcohol dehydrogenase family. Members of alcohol dehydrogenase family are responsible to metabolize substrates such as ethanol, aliphatic alcohols, hydroxysteroids, retinol, and lipid peroxidation products (45). This enzyme is not efficient in ethanol oxidation, but has its maximum activity as a retinol dehydrogenase; thus, it may have an

important role in retinoic acid synthesis, which is an essential hormone for cellular differentiation (46). Also, this protein has an important role in xenobiotic metabolism. Adding zoledronic acid as an extra-cellular component to the cells has led to up-regulation of ADH7, which may further prove the role of ADH7 in xenobiotic metabolism.

#### Effect of Zoledronic Acid in Mitochondrial Function

It has been shown in different studies that zoledronic acid induces apoptosis through mitochondrial pathways (47). In one study it has been shown that zoledronic acid treatment is associated with release of mitochondrial cytochrome c into the cytosol, which further initiates the caspase 3, 7, 8, and 9 activities (48). In another study, it has been shown that Bax translocation into the mitochondria, reduction of Bcl-2 expression, and Bid activation induces apoptosis through the mitochondrial pathway (49). In this study, we identified some of the differentially expressed mitochondrial proteins that might be involved in induction of apoptosis by zoledronic acid.

#### Mitofusin1(MFN1)

Mitofusin 1 is a mitochondrial protein that was up-regulated in zoledronic acid treated bone cell lines. Mitofusin 1 is an important transmembrane GTPase with the ability to mediate mitochondrial fusion (50). Mitochondria fusion plays an important role in mitochondria morphology and has been seen in many cell types. Preventing mitochondrial fusion causes mitochondrial fragmentation by increasing mitochondrial division. It has been shown that the apoptosis induction stimulates mitochondrial divisions which further leads to formation of fragmented mitochondria; which is associated with progression of apoptosis (51).

Isocitrate Dehydrogenase 3 (NAD<sup>+</sup>)  
Alpha (IDH3A)

Isocitrate dehydrogenase 3 (NAD<sup>+</sup>) alpha is a mitochondrial protein, which was up-regulated in zoledronic acid treated bone cell lines. Isocitrate dehydrogenases catalyze the third step of citric acid cycle: “the oxidative decarboxylation of isocitrate to 2-oxoglutarate cycle and acts as an oxidoreductase” (52).

Probable Glutamyl-tRNA(Gln)  
Amidotransferase Subunit B  
(PET112L)

This mitochondrial protein was up-regulated in bone treated cell lines. Probable glutamyl-tRNA(Gln) amidotransferase subunit B develops properly charged Gln-tRNA(Gln) in the mitochondria through the transamidation of misacylated Glu-tRNA(Gln) (53). This reaction occurs through an activated gamma-phospho-Glu-tRNA(Gln) with the presence of ATP and glutamine. In addition this protein has a role in translation, ATP-binding, and Ligase activity (53). PET112L functions during translation by binding nucleic acids during polypeptide synthesis at the ribosome.

DEAD Box Polypeptide 55(DDX55)

DEAD (Asp-Glu-Ala-Asp) box polypeptide 55 DEAD box protein (DDX55) is another mitochondrial protein that was up-regulated in bone treated cell lines. DEAD box proteins, with their conserved motif Asp-Glu-Ala-Asp (DEAD), are presumed RNA helicases (54). DEAD box proteins are involved in lots of cellular functions, which include modification of RNA secondary structure, inhibition of translation process, assembly of ribosomes, and nuclear and mitochondrial splicing. It has been shown that some members of this family might play a role in cellular growth, division, and probably embryogenesis (54).

### Effect of Zoledronic Acid in Angiogenesis

The anti-angiogenic activity of zoledronic acid has been suggested in different studies. In one study it has been shown that zoledronic acid affects protein prenylation by inhibiting farnesyl pyrophosphate synthase through the mevalonate pathway, which further affects various signaling pathways including those implicated in the regulation of the vascular endothelial growth factor (VEGF) and vascular endothelial growth factor receptor 2 (VEGFR2) expressions (55). The VEGF/VEGFR2 pathway significantly contributes to multiple myeloma angiogenesis and growth (56), supporting this belief that effective anti-angiogenesis could be achieved via VEGF/VEGFR2 inhibition (Fig.19). In another study, it has been shown that “zoledronic acid inhibits proliferation of human endothelial cells promoted with fetal calf serum, basic fibroblast growth factor (bFGF), and vascular endothelial growth factor” (57). It has also been shown that Cysteine-rich, angiogenic inducer, 61 (CYR61), a growth factor-inducible, is down-regulated in zoledronic acid treated MDA-231 BO and MDA-MB-231-parental cell lines (Jason Bush, personal communication). In this study, we have identified proteins with anti-angiogenic activity in zoledronic acid treated bone and parental cell lines. These proteins are shown in the next section.

#### Vasohibin1 (VASH1)

Zoledronic acid treated bone cell lines showed up-regulation of vasohibin1 (VASH1) in comparison to its expression in the non-treated bone cell lines. VASH1 is localized at cytoplasm or endoplasmic reticulum. Vasohibin1 is a negative regulator of angiogenesis induced by vascular endothelial growth factor (VEGF)-A, which explains the presence of this protein in zoledronic acid treated bone cell lines (58). VASH1 also inhibits migration, proliferation, and network formation by endothelial cells. VASH1's inhibitory effects only work on

“endothelial cells and does not affect the migration of smooth muscle cells or fibroblasts” (58).

#### Egl Nine-Homolog 1(EGLN1/PHD2)

This protein was up-regulated in bone-treated cell lines versus non-treated cell lines. Egl nine-homolog 1 is located at cytosol. This protein has a role in catalyzing the post-translational formation of 4- hydroxyproline in hypoxia-inducible factor (HIF) alpha proteins (59). EGLN1 functions as an oxygen sensor in cells and, under normal conditions, targets “HIF through the hydroxylation for proteasomal degradation via the von Hippel-Lindau ubiquitination complex” (59). The hypoxia inducible factor (HIF) has a crucial role in the progression of several oxygen-dependent pathophysiological processes, which include tumorigenesis. Sometimes the function of the HIF is under the influence of other regulatory pathways. Misregulation of these regulatory pathways can result in improper HIF expression, which can further lead to human cancer progression by inducing genes capable of promoting angiogenesis, metastasis, cell survival, and glycolysis (60). Presence of ELGN1 in treated bone cell lines shows that zoledronic acid can be an inhibitor of angiogenesis.

#### Enolase1 (ENO1)

Enolase1 (ENO1) was identified as a protein that appeared to be up-regulated in the bone-treated cell lines compared to bone-control cell lines. Enolase 1 is located at cytoplasm or nucleus. This multifunctional enzyme plays a role in glycolysis and is a negative regulator of cell growth. Another study has shown that enolase 1 is able to bind to the myc promoter-binding protein-1(MBP-1) myc promoter-binding protein-1(MBP-1) and down-regulate “expression of a luciferase reporter gene under the control of the c-myc P2 promoter and act as a

transcriptional repressor” (61). An alternative product of the ENO1 gene is considered to be a transcriptional repressor of the c-myc oncogene, which suggests that ENO1 is a potential candidate for tumor suppression (61). The presence of ENO1 in zoledronic acid treated bone cell lines supports the idea of anti-angiogenic activity of zoledronic acid.

#### Microfibrillar-Associated Protein 3-Like (MFAP3L)

Microfibrillar-associated protein 3-like was identified as an up-regulated protein in the bone-treated cell lines. This protein is a cytosolic protein and is a negative regulator of angiogenesis and negative regulator of proliferation (62). The presence of MFAP3L in treated bone cell lines also suggests that zoledronic acid has anti-angiogenic activity.

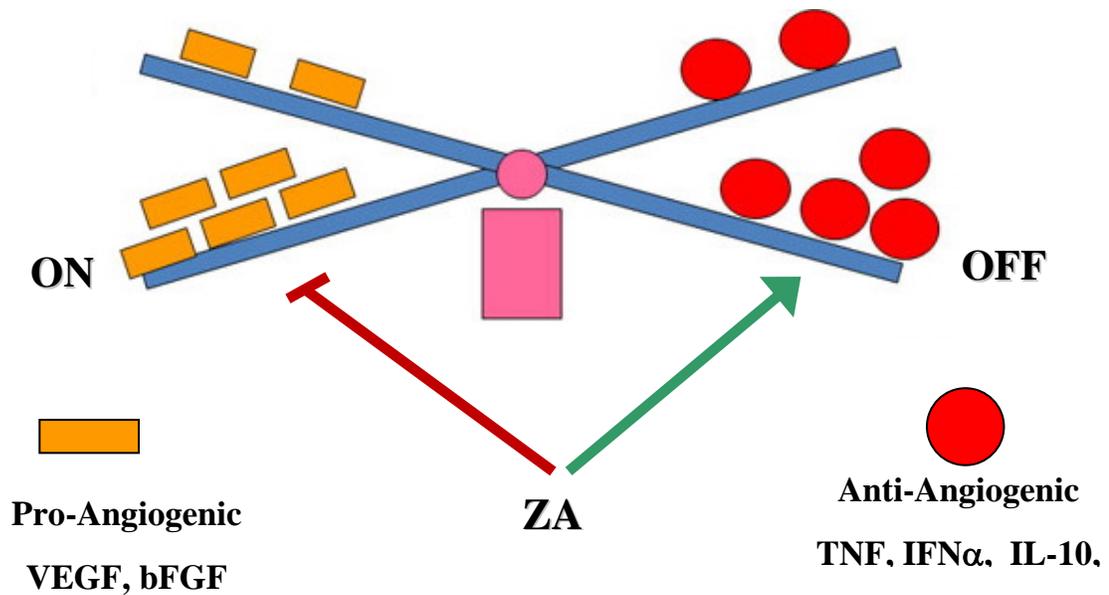


Fig. 19. The angiogenic switch. This is a representation of the factors controlling the balance between turning angiogenesis 'ON' or 'OFF'. Angiogenesis is turned off by increasing anti-angiogenic factors and requiring a concomitant decrease in pro-angiogenic factors. Zoledronic acid is believed to act by increasing anti-angiogenic factors. VEGF = Vascular endothelial growth factor; bFGF = basic fibroblast growth factor; TNF= Tumor necrosis factor-alpha, IFN $\alpha$ = interferon-alpha; IL-10= interleukin-10, ZA= zoledornic acid.

## CONCLUSION

In summary, this thesis established a workflow to identify proteins involved in the function of osteoclast-like cells. The results demonstrated that 200  $\mu$ M of zoledronic acid can induce 70-80% cell death in MDA-MB-231 and MDA-231 BO cell lines. We further showed that there is a proteomic difference between zoledronic acid treated and non-treated bone and parental cell lines, and specific mitochondrial proteins were identified using MALDI-TOF mass spectrometer. Most of the identified proteins have a role in regulating apoptosis or have anti-angiogenic activity. These findings suggest that zoledronic acid has anti-angiogenic activity and it acts presumably by inhibiting the VEGF/VEGFR2 pathway, which further prevents proliferation and metastasis of cancer cell lines. This project can be continued by further identification of mitochondrial proteins that have anti-angiogenic effects, validation of these proteins using immunoblotting, and functional studies to confirm biological significance.

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