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Erik Henke, Bettina Brendel, Herbert Stolz, Elke Butt-Dörje, Alma Zernecke-Madsen, Andreas Beilhack & Prof Dr. Friedrich Schardt

Universitätsklinikum Würzburg

ABSTRACT

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In this study we explored the glycolysis blocking properties of the bisphosphonate zoledronic acid in leukemia and breast cancer cells. Although, zoledronic acid had little effect at normoxic conditions, it significantly inhibited lactate production at reduced oxygen levels. Under these hypoxic conditions, that resemble the oxygenation levels in many tumors, zoledronic acid was also of significantly higher toxicity to the tumor cells. Moreover, we show that it strongly increased sensitivity to chemotherapy.

These results support the Warburg hypothesis and encourage further testing in vivo to explore a potentially beneficial effect of zoledronic acid on the response to chemotherapy.

Keywords: bisphosphonates, hypoxia, tumor cells, anaerobic glycolysis, bone metastasis.

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Keywords: bisphosphonates, hypoxia, tumor cells, anaerobic glycolysis, bone metastasis.

Author α: Institute of Anatomy and Cell Biology, Universität Würzburg.

σ ρ: Central Laboratory, Universitätsklinikum Würzburg.

¥ §: Institute of Experimental Biomedicine, Universitätsklinikum Würzburg.

χ: Department of Internal Medicine II, Center for Experimental Molecular Medicine, Universitätsklinikum Würzburg nikum Würzburg.

ν: Betriebsärztliche Untersuchungsstelle, Universitätsklinikum Würzburg.

I. INTRODUCTION

Bisphosphonates are stable synthetic analogues of pyrophosphate that are resistant to metabolic hydrolysis and applied as inhibitors of osteoclasts against bone resorption. In this function they are applied as palliative treatment in patients with manifested bone metastases in the context of breast cancer (reviewed in [1, 2]). However, a potential effect in preventing bone metastases or as adjuvant treatment to prevent therapy induced bone loss has been discussed [3-5].

Osteoclasts disassemble and digest the composite of hydrated protein and mineral in bones at a molecular level by secreting acid phosphates and collagenases [6]. They are found in pits, so called resorption bays or Howship's lacunae, and in niches of the bone marrow, locations that are characterized by a reduced oxygen partial pressure (pO_2) [7]. These particular micro-environments resemble niches for hematopoietic stem cells.

Because of their hypoxic environment, the metabolism of osteoclasts and malignant tumor cells depend on an increased rate of glycolysis, which is sustained by a very high glucose import rate as the glucose molecule has the highest percentage of oxygen comparable to fat and proteins [8-11]. Therefore, substances that inhibit glycolysis are prone to interfere with the metabolism of these cells and appear particularly attractive for supporting cancer treatment. This holds especially true for the targeting of cancer stem cells (CSCs) or tumor initiating cells (TICs) in certain cancer types. Especially CSCs/TICs breast, colon and hepatocellular cancer rely

heavily on glycolysis [12-16], whereas in glioblastoma, lung and pancreatic cancer CSCs/TICs revert more to oxidative phosphorylation for ATP-synthesis [17-19]. The CTCs seem to depend on their respective metabolic program to maintain their stem-like properties. Beside their ability to initiate and repopulate tumors, CSCs are characterized by chemoresistance. Therefore, we asked whether targeting glycolysis in types of cancers, in which CSCs and TICs depend on this metabolic process would make them vulnerable, increase treatment sensitivity and decrease metastasis.

As bisphosphonates can interfere with glycolysis, we investigated whether their application could interfere with glycolysis in cancer under hypoxic conditions. Here we report that zoledronic acid, a potent bisphosphonate, reduced lactate production in leukemia and breast cancer cell lines under hypoxic conditions. Importantly, zoledronic acid potentiated cytotoxic effects of chemotherapeutic agents against breast cancer under hypoxic conditions.

II. RESULTS

To test our hypothesis that bisphosphonates can interfere with glycolysis, a metabolic pathway activated under hypoxic conditions and necessary for stem cell maintenance, we first examined the effect of zoledronic acid on cell lines of acute and chronic myeloid leukemia (AML, CML). Leukemia cells generally express high levels of *bona fide* stem cell markers [20]. Inhibition of glycolysis activity should affect levels of lactate produced by the cells. Indeed, lactate concentration in the supernatant of cells treated with zoledronic acid at various concentration (1μM, 10μM and 100μM) were significantly lower than in untreated cells (**Figure 1**). However, we observed this effect only under cell culture conditions of reduced oxygen levels at or below 2% O_2 (**Figure 1E-H**). Under normoxia (20% O_2) overall lactate production was lower and not affected by treatment with zoledronic acid **(Figure 1A-D)**. Within the four tested lines, THP-1 cells produced by far the least amount of lactate and were the only line showing inconclusive response to zoledronate-treatment. THP-1 cells are a well-differentiated line that can be readily differentiated [21]. We next tested the response of breast cancer lines to zoledronate-treatment, given the reliance of breast cancer CTCs on glycolysis. Again, a solid effect of zoledronic acid on lactate production was observed in three of the four tested lines at reduced oxygen levels (**Figure 2A**). At normoxia, zoledronate-treatment again had no significant effect on lactate production (**Figure 2B**). The breast cancer lines required higher zoledronate concentrations than the leukemia cells to reduce lactate levels.

To determine effects of zoledronic acid on viability and proliferation, the three sensitive breast cancer cell lines were treated for 72h with a range of concentrations of the drug. All three lines were significantly more sensitive under hypoxic conditions (**Figure 3**).

CTCs are characterized by chemoresistance and contribute significantly to the tumors ability to sustain and rebound after cytotoxic treatment. Sensitivity of breast cancer lines to the microtubule-stabilizing drug paclitaxel was tested in the presence of 1μM zoledronate at normoxic and hypoxic conditions. Cultivation at low oxygen levels significantly reduced the cells to the cytotoxic drug (**Figure 4A-B**). Co-treatment with zoledronic acid re-sensitized the cells as the EC_{50}-levels were reduced to values similarly to those found for cells cultivated at normoxia.

III. DISCUSSION

Zoledronic acid can block the phosphorylation from glyceraldehyde-3-phosphate to 1,3-bispho sphoglycerate. This reaction proceeds through a thioester intermediate, which allows the oxidation of glyceraldehyde to be complied to 3-phos phoglycerate. Cysteine reacts normally with the aldehyde group of the substrate, forming a hemithioacetal and takes place with the transfer of a hydride ion to NAD. This reaction is facilitated by the transfer of a proton to the imidazole ring of histidine. Zoledronic acid can attach to the thioester instead of an orthophosphate and thereby stops the continuation of the anaerobic glycolysis. The following part of the glycolysis would gain a ΔG

value of – 43.9. However, the anaerobic glycolysis can proceed only if the ΔG values of all reactions are negative. In contrast, the first part of anaerobic glycolysis has a positive ΔG of 5,7 (-30.9 + 36.6). Additionally, NAD from the reduction of pyruvate to lactate is not available for this process of the glycolysis in cytosol of the cell.

In this study, we examined the glycolysis blocking properties of zoledronic acid in cancer. Accordingly, we observed reduced lactate production in different cancer cell lines upon zoledronate treatment. Notably, this effect happened only under hypoxic conditions, when anaerobic glycolysis gains relevance to sustain the high metabolic demands of cancer cells. As a consequence, the breakdown of pyruvate to lactate was interrupted. We observed this effect in less differentiated cancer cells, whereas more differentiated cancer cell lines such as THP-1 or MCF-7 were less affected. These results emphasize that less differentiated cancer cells, including CTCs and TICs in certain cancer types depend more on glycolysis, which makes them more vulnerable to disruption of glycolysis. Indeed, when we combined zoledronate with paclitaxel as a chemotherapeutic agent to increase the cellular stress level for CTC-like breast cancer cells, they became markedly more vulnerable upon adding the bisphosphonate. These results are encouraging to be tested in vivo, as metastases to hypoxic bone niches and ensuing chemoresistance pose a therapeutic hurdle to effectively treat breast cancer patients.

IV. MATERIAL AND METHODS

Chemicals were acquired from standard commercial suppliers (Sigma Aldrich, Merck). Zoledronic acid was acquired as a 0.8 mg/mL solution from Denk Pharma (Munich, Germany) and diluted from this stock accordingly. Cell culture media and supplements were purchased from Thermo Fisher (Germany).

4.1 Cell lines

All cell lines were acquired from ATCC. Breast cancer cell lines (MDA-MB231, MDA-MB435s, MDA-MB-468, MCF-7 and SkBr-3) were maintained in DMEM supplemented with 10% FBS and penicillin/streptomycin. The leukemia cell lines (MO7, HL-60, THP-1 and K-562) were maintained in RPMI 1640 media supplemented with 10% FBS and penicillin/streptomycin.

4.2 Lactate measurement

Adherent growing cells were seeded at $2x10^4$ cells in 24-well MWD dishes in 500 µL in lactate and pyruvate free media. To attach, cells were maintained at normoxia for 24 h. Then one set of cells were transferred to a hypoxia incubator set at 2% O_2, while a second set was maintained at normoxia. Cells were allowed to adjust to the conditions for 24h, before they were supplied with fresh media containing the appropriate amount of zoledronic acid. Media was collected after 24h and frozen at – 80°C.

Cells growing in suspension were treated analogously. For media exchange and to harvest growth media at the end of the experiment cells were separated by centrifugation (300 x g for 5 min).

Lactate concentration and LDH activity was determined vs. pyruvate levels using standard conditions given by the manufacturer on a Cobas 8000 modular analyzer (Roche, Mannheim, Germany):

4.3 Assessment of cell viability

In cell toxicity studies each concentration was tested in a 6-fold replicate. Cells were incubated with the therapeutics for 72 h before media was removed and cells stored at -80 °C until further quantification using the CyQuant assay kit (ThermoFisher, Germany) according to the manufacturer's instructions.

4.4 Cytotoxicity of zoledronic acid

Adherent growing cells were seeded at $1x10^3$ cells in 96-well MWD dishes in 200 µL in standard media. To attach, cells were maintained at normoxia for 24 h. Then one set of cells were transferred to a hypoxia incubator set at 2% O_2, while a second set was maintained at normoxia. Cells were allowed to adjust to the conditions for 24h, before they were supplied with fresh media supplemented with zoledronic acid. Zoledronic

acid was applied at 9 different concentrations at a range from 4.6 nM to 30 µM. Cells were re-incubated at the respective O2-levels (2% or 20%) and cell viability was assessed 72 h later.

For each tested cell line (MDA-MB-231 and MDA-MB468) four 96-well MWDs were prepared, seeding cells at $1x10^3$ cells in 200 µL using standard media. In two of those plates media was supplemented with 1µM zoledronic acid. Two plates, one with and one without added zoledronic acid, were transferred to a hypoxia incubator set at 2% O_2, the two other plates were maintained at normoxia. Cells were allowed to adjust to the conditions for 24h, before they were supplied with fresh media supplemented with paclitaxel. Paclitaxel was applied at 9 different concentrations at a range from 0.46 nM to 3 µM. Cells were re-incubated at the respective O2-levels (2% or 20%) and cell viability was assessed 72 h later.

4.5 Statistical Analysis

All statistical analysis was done using the Prism5 Software (GraphPad, LaJolla, CA). Differences between two groups were analyzed using an unpaired, two-tailed Student's T-test. In parallel the samples were tested for significant variation of variance, and if necessary, a Welch correction was included in the statistical analysis.

All authors reviewed this article.

The authors declare no competing interest.

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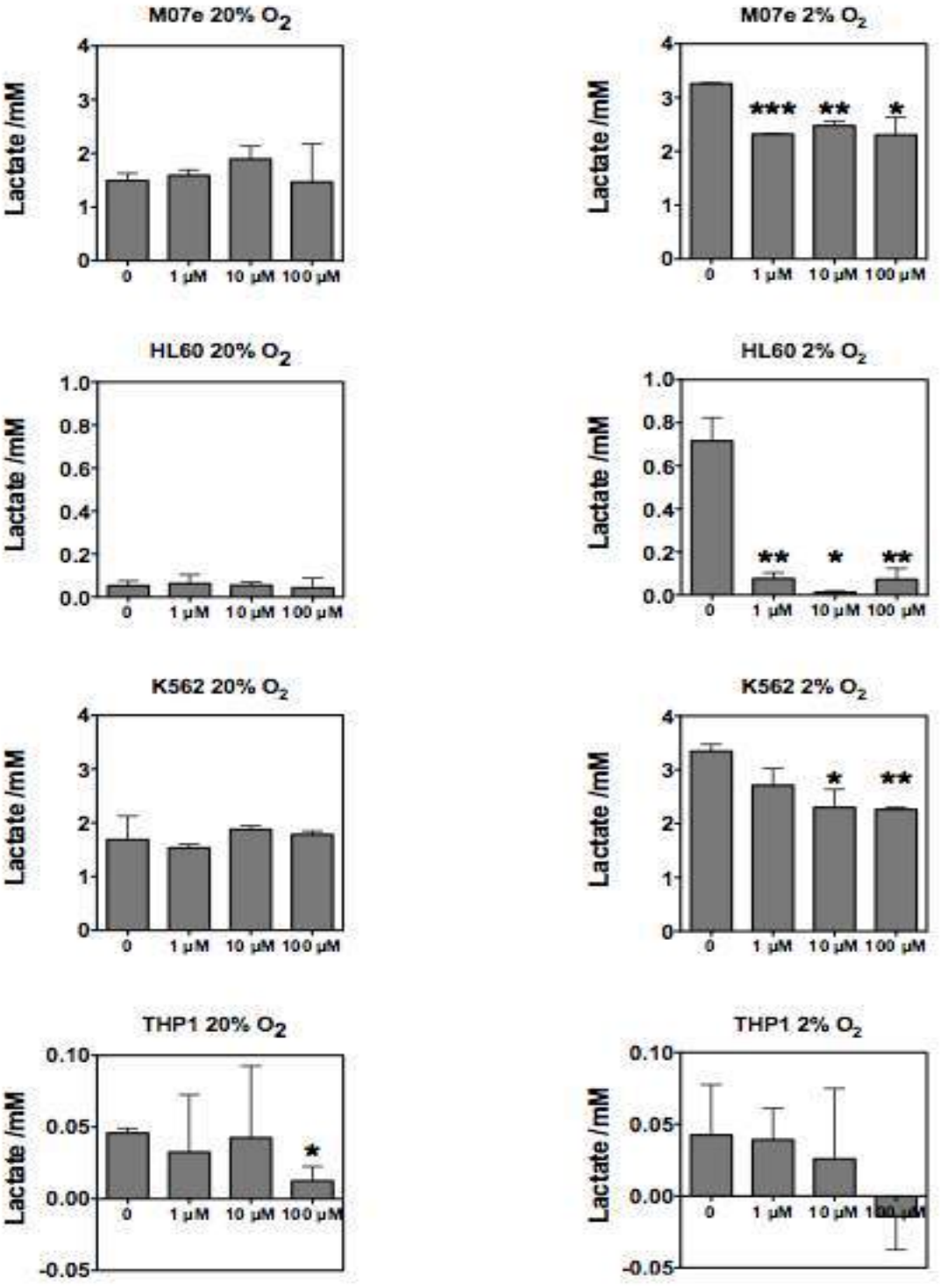

Figure 1: Zoledronic acid treatment increases lactate production in myeloid leukemia cell lines under hypoxic conditions

(**A-D**) Lactate concentration in the supernatant of four myeloid leukemia cell lines cultivated under normoxia (20 % O_2) and treated with increasing concentrations of zoledronic acid.
(**E-F**) Lactate concentration in the supernatant of the same four myeloid leukemia cell lines cultivated under hypoxia (2 % O_2) and treated with increasing concentrations of zoledronic acid.
Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

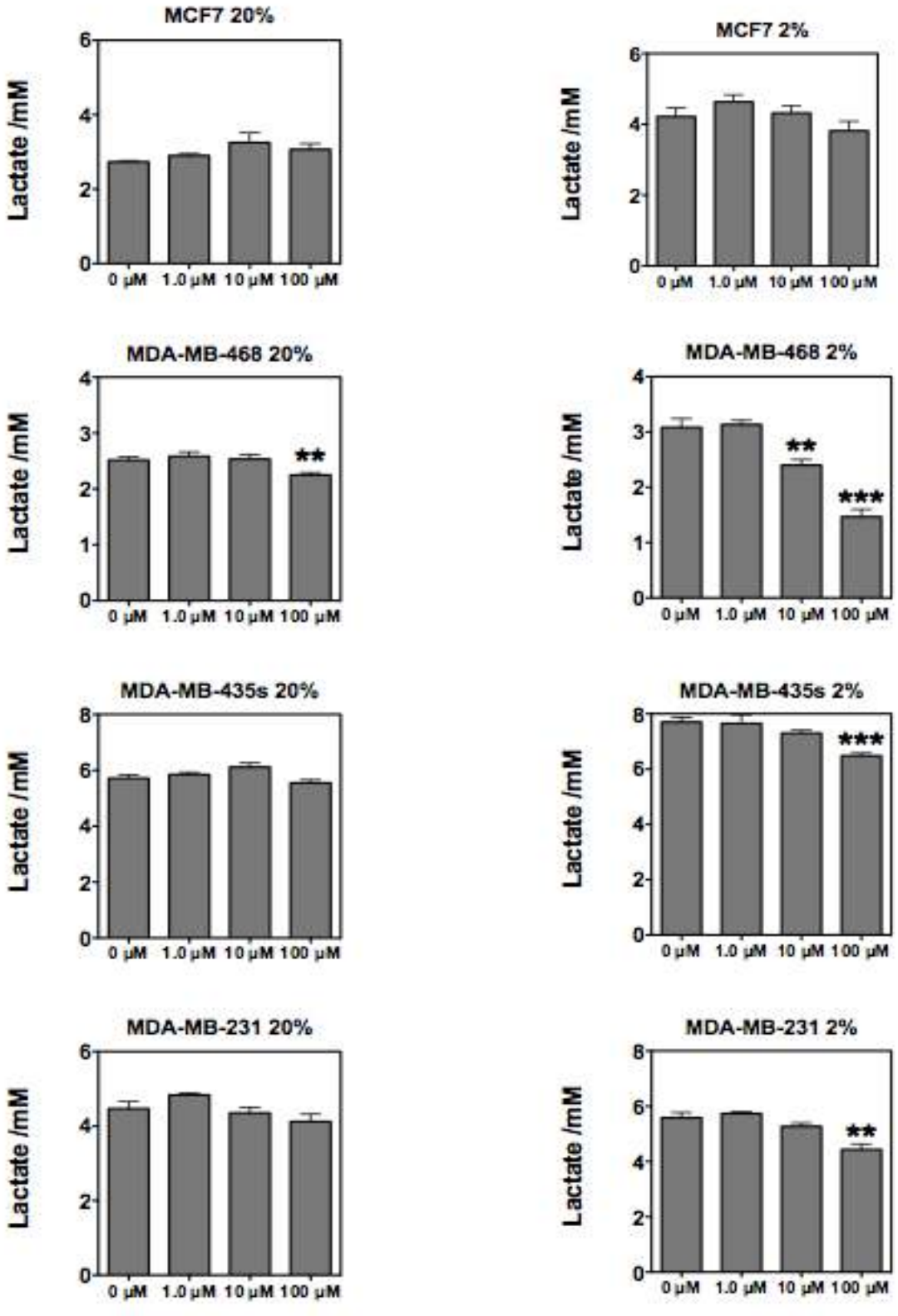

Figure 2: Effect of zoledronic acid on lactate production in breast cancer cell lines.

(**A**) Lactate concentration in the supernatant of four breast cancer cell lines cultivated under hypoxia (2 % O_2) and treated with increasing concentrations of zoledronic acid.
(**B**) Lactate concentration in the supernatant of the same four breast cancer cell lines cultivated under normoxia (20 % O_2) and treated with increasing concentrations of zoledronic acid.
Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

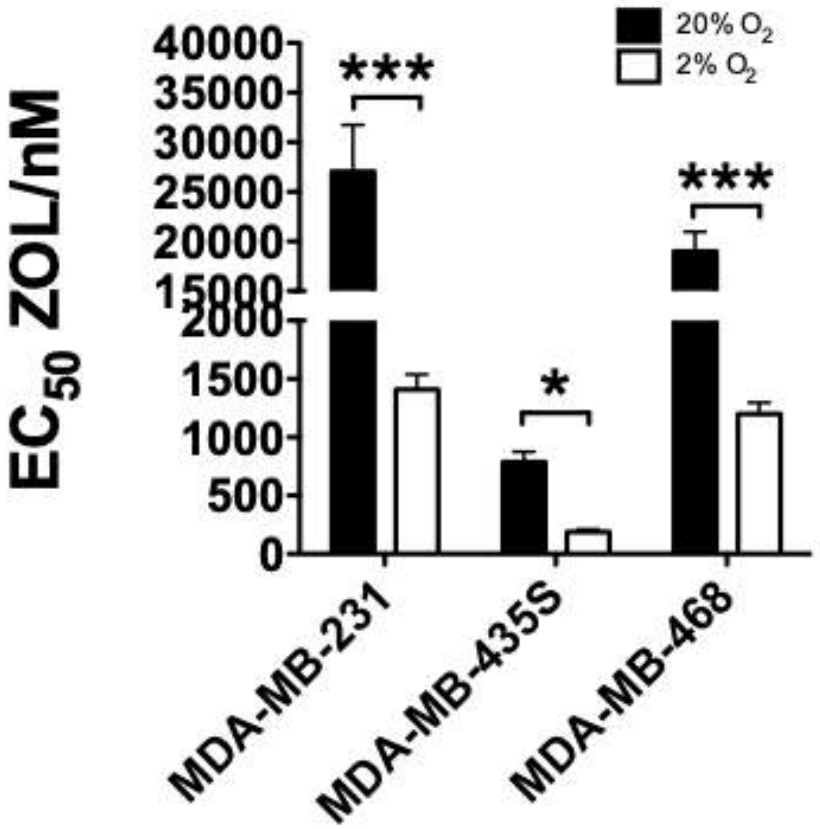

Figure 3: Cell toxicity of zoledronic acid on breast cancer cell lines

EC50-values of zoledronic acid in three breast cancer cell lines under normoxia (20 % O_2) and hypoxia (2 % O_2) Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.

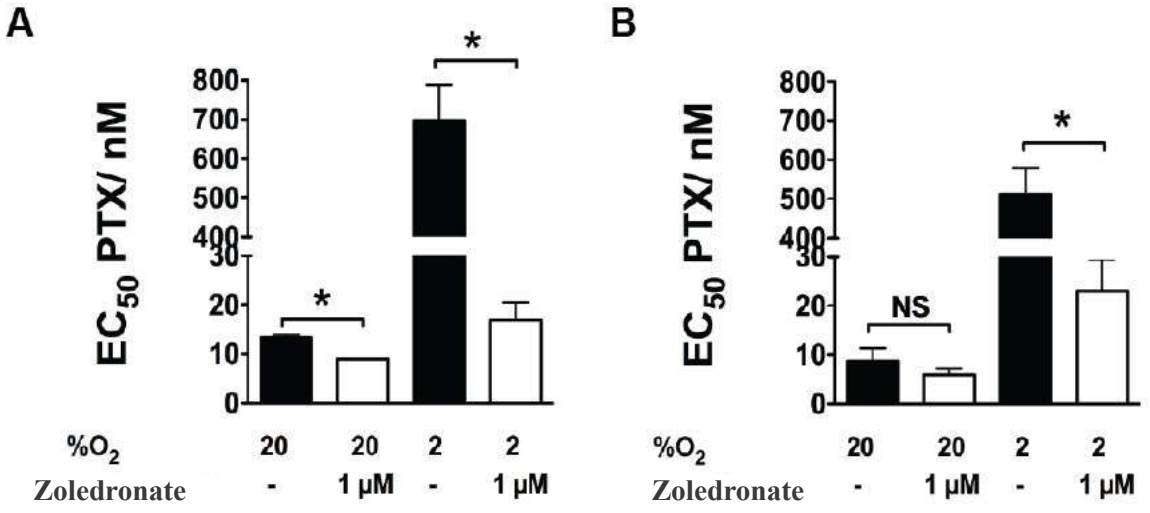

Figure 4: Cell toxicity of paclitaxel (PTX) on breast cancer cell lines pre-treated with zoledronic acid

(**A**) EC50-values of PTX in MDA-MB-231 breast cancer cells in dependence of pre-treatment with 1 µM zoledronic acid and of oxygenation status (20 % O_2 vs. 2 % O_2).
(**B**) EC50-values of PTX in MDA-MB-468 breast cancer cells in dependence of pre-treatment with 1 µM zoledronic acid and of oxygenation status (20 % O_2 vs. 2 % O_2).
Error bars: +/- SEM, *: P < 0.05, **: P < 0.01, ***: P < 0.001.