Calcified Tissue International (2019) 105:497–505
https://doi.org/10.1007/s00223-019-00590-5

ORIGINAL RESEARCH

Bone-Bound Bisphosphonates Inhibit Proliferation of Breast Cancer Cells

Young-Eun Park[1] · Usha Bava[1] · Jian-ming Lin[1] · Jillian Cornish[1] · Dorit Naot[1] · Ian R. Reid[1]

Received: 11 March 2019 / Accepted: 12 July 2019 / Published online: 19 July 2019

Abstract

Bisphosphonates are used in treating patients with breast cancer. In vitro studies have shown that bisphosphonates act directly on tumour cells, inhibiting cell proliferation and inducing apoptosis. In most such studies, drugs were added to culture media exposing cells to high bisphosphonate concentrations in solution. However, since bisphosphonates bind to bone hydroxyapatite with high affinity and remain bound for very long periods of time, these experimental systems are not an optimal model for the action of the drugs in vivo. The aim of this study was to determine whether bone-bound zoledronate has direct effects on adjacent breast cancer cells. Bone slices were pre-incubated with bisphosphonate solutions, washed, and seeded with cells of the breast cancer cell lines, MCF7 or MDA-MB-231. Proliferation was assessed by cell counts and thymidine incorporation for up to 72 h. Inhibition of the mevalonate pathway was tested by measuring the levels of unprenylated Rap1A, and apoptosis was examined by the presence of cleaved caspase-8 on western blots. The proliferation rate of breast cancer cells on zoledronate-treated bone was significantly lower compared to cells on control bone. Other bisphosphonates showed a similar inhibitory effect, with an order of potency similar to their clinical potencies. Unprenylated Rap1A accumulated in MCF7 cells on zoledronate-treated bone, suggesting zoledronate acted through the inhibition of the mevalonate pathway. Accumulation of cleaved caspase-8 in MDA-MB-231 cells on bisphosphonate-treated bone indicated increased apoptosis in the cells. In conclusion, bone-bound zoledronate inhibits breast cancer cell proliferation, an activity that may contribute to its clinical anti-tumour effects.

Keywords Bisphosphonates · Breast cancer cells · Zoledronates · Bone-bound zoledronate · MCF7 · MDA-MB-231

Introduction

Bone is a common site of metastasis in patients with breast cancer, with approximately 80% of advanced breast cancer metastasising into bone tissue [1, 2]. Dissemination to bone is a crucial step for metastasis of tumour cells [3]. It involves initial adhesion of tumour cells at the metastatic site, extravasation, colonisation and growth on this site [4]. Morbidity associated with bone metastases remains a major clinical problem even with advances in treatment options [5].

Bisphosphonates are potent inhibitors of bone resorption and are used as a standard treatment for breast cancer patients, aiming to mitigate the risk of skeletal complications in metastatic disease [6]. Bisphosphonates have been shown to inhibit multiple steps in the metastatic process, including tumour cell adhesion and proliferation, and in addition they inhibit cell invasion by modifying the bone microenvironment [7]. Bisphosphonates have a high affinity

✉ Ian R. Reid
i.reid@auckland.ac.nz

Young-Eun Park
y.park@auckland.ac.nz

Usha Bava
u.bava@auckland.ac.nz

Jian-ming Lin
j.lin@auckland.ac.nz

Jillian Cornish
j.cornish@auckland.ac.nz

Dorit Naot
d.naot@auckland.ac.nz

[1] Department of Medicine, Faculty of Medical and Health Sciences, University of Auckland, Private Bag 92019, Auckland, New Zealand

for the hydroxyapatite in the bone extracellular matrix and once administered into the body they quickly bind to bone hydroxyapatite, where they remain bound for a very long time, with a half-life of up to 10 years [8]. The most commonly used bisphosphonate in the treatment of metastatic breast cancers is the nitrogen-containing bisphosphonate zoledronate. Other clinically used nitrogen-containing bisphosphonates are alendronate, ibandronate and pamidronate, and non-nitrogen-containing bisphosphonates that have been used clinically include clodronate, etidronate and tiludronate. The two groups of bisphosphonate have different mechanisms of action; the nitrogen-containing compounds inhibit the enzyme farnesyl pyrophosphate synthase (FPPS) in the mevalonate pathway, whereas the non-nitrogen-containing bisphosphonates inhibit the mitochondrial ADP/ATP transporter and are pro-apoptotic [9–11]. Interestingly, some clinical studies have found that bisphosphonate treatment not only reduces skeletal complications but also affects cancer progression. Gnant et al. [12] found that adjuvant zoledronate treatment decreased the relative risk of cancer progression by 36%, and reduced the number of breast cancer metastases. A number of clinical studies have demonstrated that adjuvant zoledronate treatment increases cancer-free survival [12–15], decreases recurrence of breast cancer [12, 16–18] and decreases the incidence of cancer in normal older women [19].

In vitro studies have shown that bisphosphonates can act directly on tumour cells, inducing apoptosis and inhibiting cell proliferation, adhesion and migration [20–24]. Most in vitro studies investigating the effect of bisphosphonates on tumour cells were performed by adding the drugs to tumour cell culture media, directly exposing cells to bisphosphonates in solution [24–26]. Studies were carried out with high concentrations of bisphosphonates and prolonged incubation periods [27, 28]. However, since bisphosphonates remain bound to bone for a very long time, experimental systems where cells are directly treated with bisphosphonates in the culture solution are not an optimal model for the action of the drugs in vivo.

Thus, the aim of the present study was to determine whether bone-bound zoledronate has direct effects on adjacent breast cancer cells, thus investigating the possible mechanisms of the beneficial effects of this drug on breast cancer in vivo.

Methods

Preparation of Bisphosphonate-Coated Bovine Bone Slices

Zoledronate (Novartis), pamidronate (Mayne Pharma, Melbourne), clodronate (Boehringer Mannheim), ibandronate (Roche) and alendronate (Sigma-Aldrich, St. Louis) were prepared as stock solutions of 100 μM in phosphate buffered saline (PBS; pH 7.4) and filter sterilised. Cortical bovine bone slices were prepared as previously described [29]. Briefly, bovine cortical bone pieces cut to a thickness of 70 μm and to a size of 4×4 mm were sonicated, sterilised in 70% ethanol and pre-incubated in either PBS or different concentrations of bisphosphonate solutions (1, 10, 30, 60 or 100 μM) overnight at room temperature. At the end of the incubation, the bone slices were washed with PBS and transferred to 96-well plates (1 bone slice/well) with cell culture media.

Cell lines and Cell Culture Conditions

The human breast cancer MCF7 cell line was used. MCF7 cells were maintained and cultured in Minimum Essential Medium Alpha (αMEM; Gibco, Life Technologies) containing 5% fetal bovine serum (FBS; Gibco, Life Technologies), 0.01 mg/mL recombinant human insulin (Sigma-Aldrich, St. Louis, MO) and 100 U/mL penicillin and 100 μg/mL streptomycin (Gibco, Life Technologies). Cell cultures were maintained and cultured in T75 flasks (Corning) at 37 °C in a humidified atmosphere containing 5% CO_2 until subconfluent. Cells were seeded drop-wise onto bisphosphonate pre-treated bovine bone slices in 96-well plates (20,000, 40,000 or 60,000 cells/bone). Cells were allowed to adhere for 18–22 h and then bone slices with adherent cells were transferred to new wells containing fresh culture media, then visualised or used for analysis of cell proliferation or protein expression.

MDA-MB-231 cells, a human breast cancer cell line, were maintained and cultured in αMEM containing 5% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin. The cells were seeded onto bisphosphonate pre-treated bovine bone slices in 96-well plates at the densities of 5000 and 20,000 cells/slice for proliferation and apoptosis assays, respectively. The other culture conditions were the same as for MCF7 cells above.

Visualisation of Bone Slices

For visualisation of adherent cells on bone, the bone slices were fixed with 10% neutral buffered formalin (LabServ Pronalys) and stained with toluidine blue dye for 30 s at the end of each culture. Cells were visualised on an Olympus SZ61 stereo microscope.

Analysis of Cell Proliferation

Cell Numbers

At the end of the culture period, bone slices with adherent cells were incubated with 10 mg/mL collagenase

(Sigma-Aldrich) to disaggregate cell clumps. Bone slices were then incubated with trypsin/EDTA (Gibco, Invitrogen) at 37 °C until cells detached. An aliquot of the cell suspension was counted in a hemocytometer and results were expressed as numbers of cells/bone.

^{3}H-Thymidine Incorporation Assay

Six hours before the end of each culture period, ^{3}H-thymidine (0.1 µCi/well; PerkinElmer, Waltham, MA) was added into the culture media. At the end of the culture period, thymidine incorporation was terminated by the addition of 10% trichloroacetic acid. Bone slices with adherent cells were washed with ethanol:ether mix, dried, dissolved in 2 M potassium hydroxide at room temperature for 1 h, neutralized with 1 M hydrochloric acid and the entire dissolved sample was counted for radioactivity.

Western Blots

Following removal of culture media, the bone slices with the adherent cells were washed twice with PBS then protein expression was assessed using Western blot methodology. For each treatment group, cells from 11 bone slices were directly lysed with RIPA buffer (ThermoScientific, Rockford, IL) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany) and pooled together. Samples were resolved on 4–15% stain-free precast gel (Bio-Rad, Hercules, CA) and transferred to PVDF membranes (Bio-Rad, Hercules, CA). The membranes were blocked with 5% (w/v) non-fat milk powder in TBS-Tween buffer. Membranes were then incubated overnight at 4 °C with either goat polyclonal anti-Rap1A antibody (SC-1482, Santa Cruz Biotechnology, Santa Cruz, CA, 1:200) which reacts with the unprenylated form of Rap1A or rabbit monoclonal anti-caspase-8 antibody (MA5-15054, Invitrogen, Thermo Fischer Scientific, MA, 1:300) which reacts with the cleaved forms of caspase-8. Mouse anti-α-tubulin monoclonal antibody (T-5168, Sigma-Aldrich, 1:500) and mouse anti-β-actin monoclonal antibody (SC-69879, Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000) were used for internal loading controls. After 1-h incubation with the secondary antibody of anti-goat peroxidase-conjugated IgG (A5420, Sigma-Aldrich, 1:8000) or anti-mouse IgG (A9044, Sigma-Aldrich, 1:10,000; ab97046, Abcam, 1:10,000), chemiluminescence was visualised using Amersham ECL Plus Western Blotting Detection Reagents (GE Healthcare, Buckinghamshire, UK).

Statistical Analysis

The statistical tests used are indicated in the figure legends. All tests were two-tailed and a 5% significance level was maintained throughout. Data were analysed using GraphPad Prism 5.0 (GraphPad, Inc., San Diego, CA, USA). Representative graphs from two or three repeated experiments are shown.

Results

Initial adhesion of MCF7 cells to bone slices was assessed by microscopy and by counting the cells 4 h after seeding (Fig. 1). The cell numbers on control and zoledronate-treated bone were comparable. MCF7 cells were incubated on bones slices for up to 72 h in order to investigate the effect of bone-bound zoledronate on cell number. Cell numbers were similar on control and zoledronate-treated bones at 24 h but, at 48 and 72 h, significantly fewer cells were seen on the zoledronate-treated bones (Fig. 2). Analysis of ^{3}H-thymidine incorporation produced similar results, demonstrating significant inhibition of DNA synthesis at 48 and 72 h of incubation on the zoledronate-treated bones compared to controls.

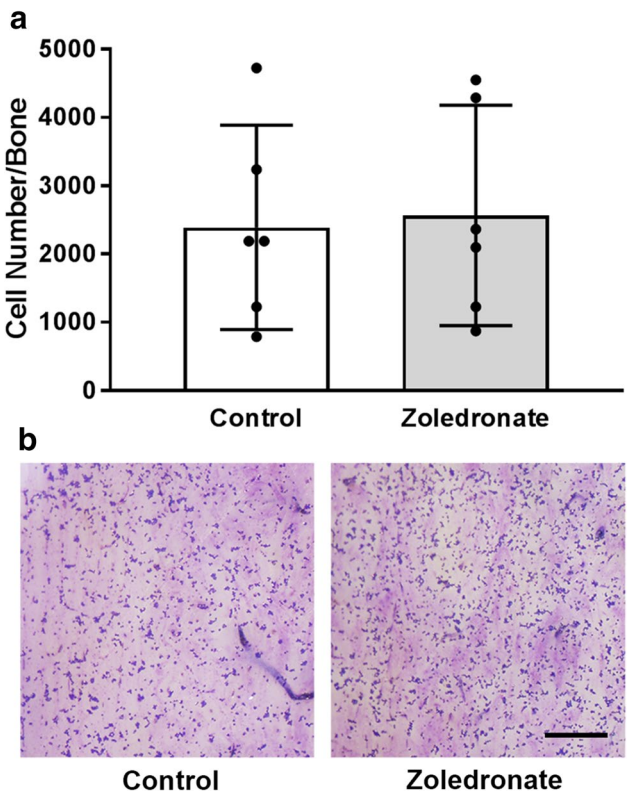

Fig. 1 Adhesion of MCF7 cells to control and zoledronate-treated bone slices. **a** Four hours after seeding MCF7 cells on bone slices, cells were harvested and counted. Zoledronate used for pre-treatment was 100 µM, bars represent mean ± 95% CI, $n = 6$. Data were analysed by Student's t test and the groups were not significantly different. **b** Representative micrographs of toluidine blue stained MCF7 cells on control (left panel) and zoledronate-treated (right panel) bone slices at 4 h. Scale bar represents 500 µm

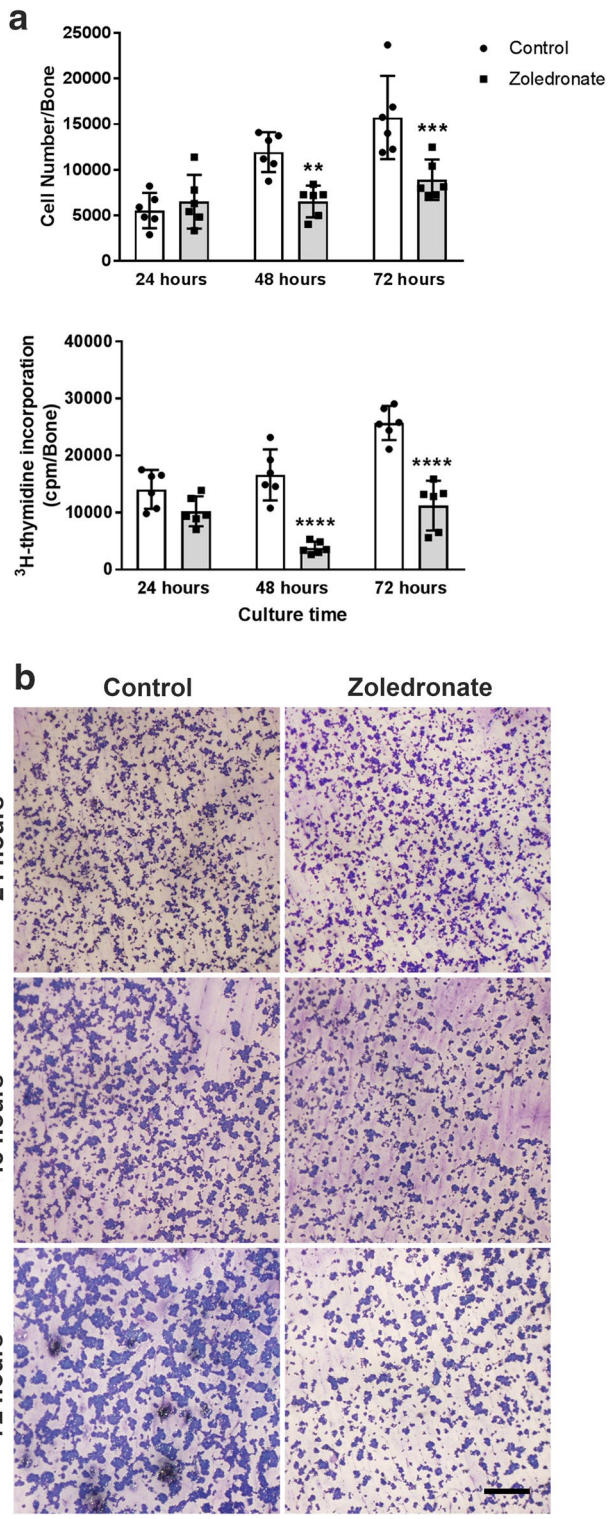

Fig. 2 Growth of MCF7 cells on control and zoledronate-treated bone slices. **a** MCF7 cells seeded on the bone slices were harvested and counted at the indicated time points (upper panel). Incorporation of ^{3}H-thymidine added 6 h prior to the end of each culture period was determined (lower panel). Concentration of zoledronate used for pre-treatment of bone slices was 100 µM, bars represent mean ± 95% CI, $n = 6$. Data were analysed by two-way ANOVA with Bonferroni's post hoc test. $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus control at the same time point. **b** Representative micrographs of MCF7 cells on control (left panels) and zoledronate-coated (right panels) bone slices at 24, 48 and 72 h. Scale bar represents 500 µm

We next determined whether the inhibition of cell proliferation was dependent on the concentration of zoledronate used in the pre-incubation of the bone slices (Fig. 3). A dose-dependent inhibitory effect was seen over a range of zoledronate pre-treatment concentrations, from 30 to 100 µM. In order to determine whether the inhibitory effect on cell proliferation could also be induced by other clinically used bisphosphonates, cell counts and ^{3}H-thymidine incorporation were determined in MCF7 cells cultured for 72 h on bone slices pre-incubated with either zoledronate, alendronate, ibandronate, pamidronate or clodronate (Fig. 4). Cell numbers were similar on control bone slices and bones pre-incubated with clodronate, whereas all the other bisphosphonates significantly reduced the number of cells. Incorporation of ^{3}H-thymidine was significantly reduced with zoledronate and alendronate, while it was not with ibandronate, pamidronate or clodronate.

To determine whether these findings were generalizable to other breast cancer cell lines, we repeated the thymidine incorporation experiment in MDA-MB-231 cells, which have a more invasive phenotype [30]. Very similar results were found, with substantial reductions in thymidine incorporation on bones pre-treated with zoledronate, alendronate and ibandronate, but no significant effects with pamidronate or clodronate (Fig. 5).

While the substantial reductions in thymidine incorporation with bisphosphonates shown here indicate that inhibition of cell proliferation is an important contributor to the reduction in cell numbers, it is also possible that this is contributed by bisphosphonate-induced apoptosis. We were unable to study apoptosis of MCF7s on the bisphosphonate-treated bone slices, as caspases, one of the most prominent players in apoptosis signalling, were difficult to detect in the cell line. However, these studies were successfully undertaken in the MDA-MB-231 cells (Fig. 6). Cleaved caspase-8 was just detectable in control cells grown on untreated bone, and these levels were significantly increased by the addition of etoposide to the media (a positive control), or by pre-treatment of bones with either alendronate or zoledronate.

Nitrogen-containing bisphosphonates have been shown to act on osteoclasts through the inhibition of the enzyme FPPS [10]. The downstream products of this enzyme are involved in the prenylation of small GTPases, including Rap1A. In order to determine whether bisphosphonates act through a similar mechanism in MCF7 cells, we compared the levels of unprenylated Rap1A in MCF7 cells cultured on plastic, bone slices, and bone slices pre-incubated with alendronate or zoledronate (Fig. 7). Unprenylated Rap1A could not be detected in MCF7

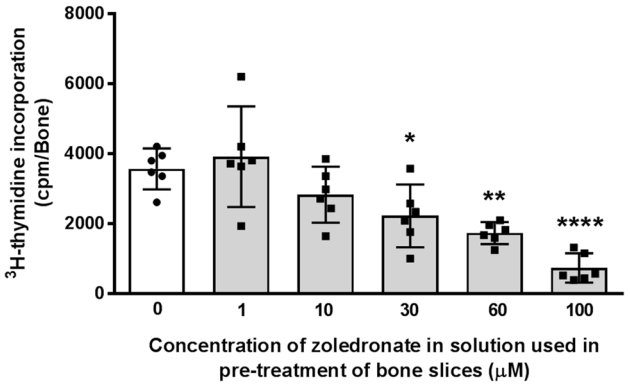

Fig. 3 Dose-response of MCF7 cell proliferation on control and zoledronate-treated bone slices. The bone slices were pre-treated with different concentrations of zoledronate overnight, and then washed with PBS before being used in the cell culture. No zoledronate was added to the culture medium. Bars represents mean ± 95% CI, $n = 6$. Data were analysed by two-way ANOVA with Dunnett's post hoc test. $^*P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$, versus control

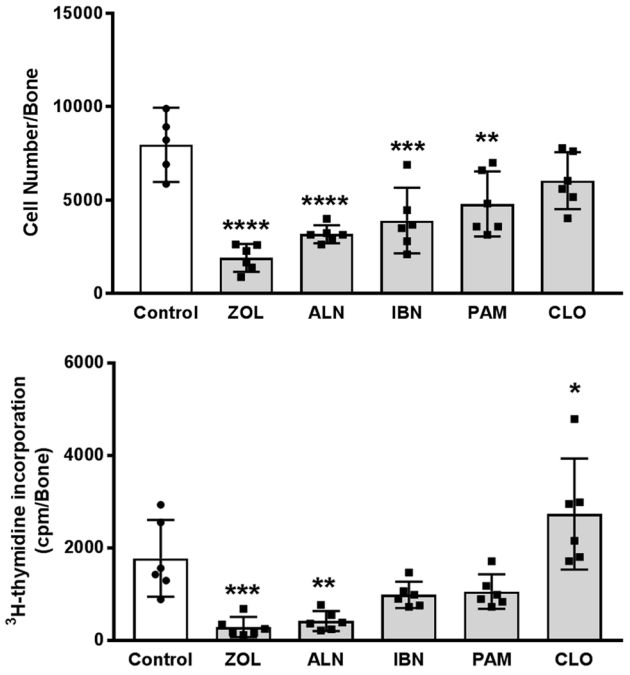

Fig. 4 Comparison of MCF7 cell proliferation on bone slices pre-incubated with different bisphosphonates. MCF7 cells seeded on bone slices were harvested and counted (upper panel). Incorporation of ^{3}H-thymidine added 6 h prior to the end of the 72-h culture period was determined (lower panel). Concentration of the bisphosphonates used in the pre-incubation was 100 μM, bars represent mean ± 95% CI, $n = 5$ or 6. Data were analysed by one-way ANOVA with Dunnett's post hoc test. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ versus control. *ZOL* zoledronate, *ALN* alendronate, *IBN* ibandronate, *PAM* pamidronate, *CLO* clodronate

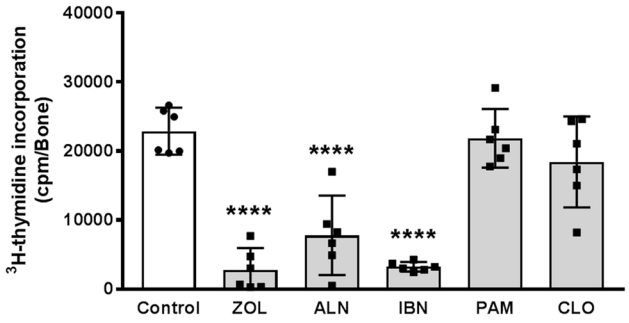

Fig. 5 Comparison of MDA-MB-231 cell proliferation on bone slices pre-incubated with different bisphosphonates. Incorporation of ^{3}H-thymidine added 6 h prior to the end of the 72-h culture period was determined. Concentration of the bisphosphonates used in the pre-incubation were 100 μM, bars represent mean ± 95% CI, $n = 6$. Data were analysed by one-way ANOVA with Dunnett's post hoc test. $^{****}P < 0.0001$ versus control. *ZOL* zoledronate, *ALN* alendronate, *IBN* ibandronate, *PAM* pamidronate, *CLO* clodronate

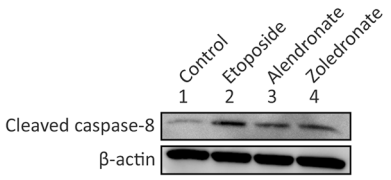

Fig. 6 Presence of cleaved caspase-8 in MDA-MB-231 cells cultured on alendronate- and zoledronate-treated bone slices. 1, control cells on PBS-treated bone; 2, cells on PBS-treated bone with 200 μM etoposide for 24 h; 3, cells on alendronate-treated bone; 4, cells on zoledronate-treated bone

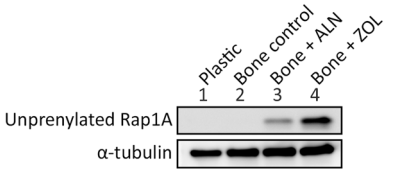

Fig. 7 Prenylation of Rap1A in MCF7 cells cultured on alendronate- and zoledronate-treated bone slices. 1, control cells on tissue culture plastic; 2, control cells on PBS-treated bone; 3, cells on alendronate-treated bone; 4, cells on zoledronate-treated bone

cells cultured on plastic or bone slices, but was present in the cells cultured on bone slices pre-incubated with the bisphosphonates. This finding suggests that similar mechanisms are involved in the effects of alendronate and zoledronate in tumour cells and in osteoclasts.

Discussion

The present studies demonstrate that bone-bound zoledronate and other bisphosphonates can inhibit the growth of adjacent breast cancer cells. These results suggest that when zoledronate is used clinically in patients with breast cancer, the cells become exposed to the drug as they adhere to bone surfaces and their growth on bone is inhibited. In this model system, similar number of cells adhered to untreated bone slices and to those pre-treated with zoledronate, but at 48 and 72 h the number of cells and their growth on the pre-treated bones were significantly lower than the controls.

We observed dose-dependent effects of zoledronate used for pre-treatment of bone slices. There was no effect of zoledronate concentration ≤ 10 µM, whereas the effects were seen at ≥ 30 µM. Similar observation was made by Tamura et al. [31] that 10 to 100 µM of zoledronate in the culture media reduced proliferation of four oral carcinoma cell lines. After intravenous injection in humans, peak serum levels of zoledronate after 4 mg reach approximately 2 µM [32], and considering the concentration of zoledronate in plasma after infusion rapidly drops due to the drug binding to bone, the concentration of bone-bound zoledronate is expected to be higher than the serum level [33]. Previously, Cornish et al. [29] have calculated the amount of zoledronate on the bone to be 150 nmol per gram of bone, for an average cancer patient administered 4 mg per month for 4 years. Also, Kuiper et al. [34] estimated the concentration of zoledronate in bone marrow to be between 20 and 100 µM in mice which had been administered a dose equivalent to that of human receiving 4 mg per month. Thus, the zoledronate concentrations used in our study appear to be within the clinically used range and the inhibitory effect on breast cancer cells is likely to be clinically relevant.

The results seen here from comparisons of clinically available bisphosphonates were consistent with the potency and affinity for hydroxyapatite of those drugs. The significant inhibitory effect of zoledronate and alendronate on MCF7 proliferation compared to less effective ibandronate and pamidronate is also seen from other studies which tested the binding affinity of bisphosphonates. The studies showed that zoledronate had the highest binding affinity to hydroxyapatite of bone, followed by alendronate and other bisphosphonates including ibandronate and clodronate [35, 36]. Clodronate, a non-nitrogen-containing bisphosphonate, did not show evidence of growth inhibition in breast cancer cells, and both cell count and thymidine incorporation results suggested clodronate on the bone surface did not have any effect on the proliferation of the cancer cells. These results are consistent with the nitrogen-containing bisphosphonates being more potent than non-nitrogen-containing bisphosphonates, which are observed in pre-clinical as well as clinical studies [37–39].

The nitrogen-containing bisphosphonates including zoledronate and alendronate inhibit osteoclasts and myeloma cells by inhibiting enzymes in the mevalonate pathway [37]. It has been established that the bisphosphonates induce apoptosis of osteoclasts by inhibiting prenylation of small GTP proteins including Rap, Ras and Rho [40]. The present results show that bisphosphonates have the same effect in breast cancer cells, and this is likely to mediate the growth inhibition shown here. This inference is supported by zoledronate having a greater effect than alendronate on both cell growth and Rap1A levels, whereas clodronate, a bisphosphonate that does not act on farnesyl pyrophosphate synthase, had no real effects on either. We have previously demonstrated similar effects of alendronate and zoledronate pre-treatment on Rap1A in Chinese hamster ovary (CHO) cells and in the Caco-2 human colorectal adenocarcinoma epithelial cell line grown on bone slices [29], so this is not specific to breast cancer cells. The mevalonate pathway is a crucial metabolic pathway for tumour growth and progression and therefore a potential therapeutic target [41]. Cancer cells are characterised by aberrant cell growth and metabolism and they upregulate the mevalonate pathway to support their proliferation [41]. The inhibitory effect of bone-bound bisphosphonate on the mevalonate pathway of adjacent breast cancer cells would be an explanation for the observations that bisphosphonates suppress the tumour burden in breast cancer patients. Zoledronate has anti-tumour effects in pre-clinical studies [26, 31, 42–44], and clinical studies found that zoledronate reduced solid tumour bone metastases [3, 45]. One study with patients with recurrent solid tumours also showed that the zoledronate-treated group had a higher proportion of bone metastases-free patients compared to the control group [46]. Also, studies show that zoledronate treatment reduces the incidence of micro-metastases in bone marrow in breast cancer [3, 47] which could explain its possible role in increasing cancer-free survival seen in these patients.

In our previous study, we have shown that bone-bound bisphosphonates directly inhibit the proliferation of primary osteoblast cells, as well as other cells including human colorectal adenocarcinoma epithelial cells and Chinese hamster ovary cells [29]. In our current study, we showed that bone-bound bisphosphonates also directly affect the proliferation of a breast cancer cell line. Our ^{3}H-thymidine results show that cell proliferation was significantly inhibited compared to the control group at the same time point. The cell count results show that, while the cell numbers on control bones increased, there was no increase in cell numbers on the zoledronate-treated bone over the culture period of 72 h. One possible explanation for these results could be that the cell

proliferation in the zoledronate group was compensated for by apoptosis of the cells exposed to the bone-bound zoledronate, as shown with the presence of cleaved caspase-8 in MDA-MB-231 cells. Zoledronate-induced apoptosis of breast cancer cells was also observed in the other studies with MCF7 and MDA-MB-231, which examined the effect of zoledronate in solution [25, 48–50]. Also, there is evidence that bisphosphonates induce apoptosis in animal models inoculated with human breast cancer cells. Hiraga et al. [51] showed ibandronate-induced apoptosis in MDA-MB-231 tumour xenograft in mammary fat pads in mice. There are several studies that have looked into the mechanism of bisphosphonates on apoptosis of human cancer cells. These studies suggest inhibition of mevalonate pathway by bisphosphonate leads to disruption of downstream signalling and failure of prenylation of small GTPases, resulting in apoptosis in human cancer cells [48, 52].

The other possible explanation for the inhibition of the cell proliferation in zoledronate-treated group compared to the control group in our study is cell cycle arrest. There is in vitro evidence that bisphosphonates, including zoledronate, induce MCF7 cell cycle arrest and hence reduce cell proliferation [53–56]. This cell cycle arrest and reduction in cell proliferation by zoledronate was also seen in other breast cancer cell line treated with zoledronate [57, 58]. Zoledronate was also found to arrest or prolong the cell cycle progression of other cancer cell lines including osteosarcoma, melanoma, myeloma, small- and non-small-cell lung cancer, mesothelioma, and cholangiocarcinoma cells, [59–67], as well as non-cancer cells including human primary oral mucosal keratinocytes [68]. However, these studies were assessing the effects of bisphosphonates in culture media, which is not an appropriate model for its clinical use where its presence in biological fluids after administration is measured in hours.

In conclusion, our study demonstrates that bone-bound zoledronate effectively reduces the growth and number of breast cancer cells, suggesting a direct anti-tumour effect of the drug. Since clinical use of zoledronate results in bone-bound drug being present for many years after dosing, and since this bone is adjacent to the marrow space where micrometastases from breast cancer are known to reside, this toxicity may contribute to zoledronate's anti-tumour effects found in clinical studies.

Acknowledgements The authors acknowledge Mr. Greg Gamble for providing statistical advice and Dr. Yi Lance Xu for providing antibodies for Western blots. This study was funded by the Health Research Council of New Zealand (Grant 15/576 – Reid Mechanisms and Management of Musculoskeletal Disease).

Author Contributions YEP and JML performed the experimental research and analysis. UB contributed to the experimental work. JC, DN and IRR designed the study and the experimental plan. YEP prepared the first draft of the manuscript, DN, JC, UB, JML and IRR provided critical feedback and contributed to the final version.

Funding This study was funded by the Health Research Council of New Zealand (Grant 15/576 – Reid Mechanisms and Management of Musculoskeletal Disease).

Compliance with Ethical Standards

Conflict of interest Dr. Reid reports grants and personal fees from Amgen, personal fees and non-financial support from Novartis, outside the submitted work. Young-Eun Park, Usha Bava, Jian-ming Lin, Jillian Cornish and Dorit Naot declare that they have no conflict of interest.

Human and Animal Rights and Informed Consent This article does not contain any studies with human participants or animals performed by any of the authors.

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