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Anti-tumor and anti-osteolysis effects of the metronomic use of zoledronic acid in primary and metastatic breast cancer mouse models

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ABSTRACT

This study aims to determine the effect of metronomic (0.0125 mg/kg twice a week for 4 weeks) zoledronic acid (ZOL) on cancer propagation and osteolysis against both metastatic and primary breast cancer in mice model. From our results, metronomic ZOL resulted in a significant reduction of tumor burden and did not promote lung or liver metastasis. The metronomic ZOL appeared to be more effective than the conventional regimen (0.1 mg/kg once in 4 weeks) in reducing breast cancer tumor burden, and regulating its movement to lung and liver. This dosing schedule of ZOL showed great potential against metastatic breast cancer.

1. Introduction

Cancer is a major health problem worldwide, and the incidence and mortality rate showed an upward trend. Breast cancer is one of the leading causes of cancer death, which ranks the top of most frequent cancers in both sexes in 2008 [1]. The disease is characterized by a high incidence of bone metastasis causing significant morbidity including pain, fracture and spinal cord compression [2], with over 70% of patients dying of breast cancer have bone metastasis and develop severe bone destruction [3]. Breast cancer induced-bone metastasis frequently produces osteolytic bone lesions by activating local osteoclasts. During bone metastasis, parathyroid hormone-related peptide (PTHrP) is secreted by tumor cells and potentially stimulates osteoclasts. Activated osteoclasts degrade bone matrix and release growth factors including transforming growth factor-β (TGF-β), which in turn increase PTHrP secretion, and eventually promote a vicious cycle of bone destruction and tumor expansion [4]. Although many significant advances on the frontline breast cancer research and chemotherapy have been developed, the efficacies of current therapies are limited by a range of adverse side effects, toxicity and drug resistance. Therefore, novel therapeutic strategies and more effective drugs for advanced disease are still urgently needed.

Zoledronic acid (ZOL), the potent third-generation nitrogen-containing bisphosphonate, is effective in prevention and treatment of bone destruction caused by metastatic spread of primary cancer to the skeleton [5]. ZOL inhibits osteoclastic bone resorption by preventing prenylation of GTPases and ultimately induce cell death in osteoclasts [6]. In addition to the anti-resorptive efficacy of ZOL, there is an increasing number of reports describing the potential direct and indirect anti-tumor effects of ZOL in both *in vitro* and *in vivo* models. ZOL could dose-dependently inhibit proliferation of leukemia, breast cancer, prostate cancer and osteosarcoma cells *in vitro* and induce apoptosis in these tumor cells [7,8]. ZOL

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could also reduce tumor cell adhesion, invasion and angiogenesis activities [9]. Besides, treatment with ZOL alone or in combination with doxorubicin in animal models of breast cancer metastasis has demonstrated bone protection and inhibition of tumor growth [10]. However, previous studies showed that conventional administration of ZOL resulted in no effect on lung metastases but promoted in a xenograft mouse model that closely mimics the clinical outcome of patients with osteosarcoma [5,11]. In addition, some reports showed that long-term use of ZOL could lead to the osteonecrosis of jaw, an increased risk of oesophageal cancer, and femoral insufficiency fracture [12–14]. However, these side effects of ZOL are manageable and avoidable with standard treatment [15].

One of the strategies to potentiate the anti-tumor effects of ZOL could be administration of the drug at a metronomic way which means lower doses given more frequently on a prolonged schedule [16]. Recent clinical studies showed that the metronomic use of low dose ZOL appeared to be more effective than the conventional regimen in breast cancer patients in the long-lasting reduction of biomarkers, such as VEGF and NTx [17,18]. Metronomic administration of zoledronic acid and taxotere combination in castration resistant prostate cancer patients showed promising anti-tumor activity [19]. In addition, a recent study demonstrated that weekly administration of ZOL had greater anti-tumor effects as compared with conventional single administration in nude mice xenografted with breast cancer cells, even if the total administered dose is the same [20]. Nevertheless, very few reports on the effect of metronomic ZOL on metastatic and primary breast cancer were found. Therefore, we aimed to investigate the anti-tumor and anti-osteolysis activities of metronomic ZOL against both metastatic and primary breast cancer. In this study, the human breast cancer MDA-MB-231-TXSA-TGL cells were used. MDA-MB-231-TXSA-TGL cells, tagged with a luciferase reporter construct, were enabled for sensitive, non-invasive bioluminescence imaging tracking of the cell growth and its metastatic spread to organs after injected with the substrate of luciferase [21]. Here, we compared the anti-tumor and anti-osteolysis activities between the metronomic (0.0125 mg/kg twice a week for 4 weeks) and conventional (0.1 mg/kg once in 4 weeks) ZOL in mouse models of breast cancer development in terms of bone marrow and orthotopic mammary tissue.

2. Materials and methods

2.1. Cells and reagents

The MDA-MB231 derivative cell line, namely MDA-MB-231-TXSA-TGL [21], was cultured in DMEM medium containing 10% (v/v) fetal bovine serum and 1% (v/v) penicillin–streptomycin (Life Technologies, USA) at 37 °C in 5% CO_2 humidified incubator.

Zoledronic acid (ZOL) was purchased from Novartis Pharma Stein, Switzerland, with a Reg. No. of 031390. D-luciferin was purchased from Biosynth, Switzerland.

2.2. Intratibial breast cancer-induced osteolysis model

Four-week-old female nude mice were provided by Laboratory Animal Services Center, The Chinese University of Hong Kong (CUHK), and were housed under pathogen-free conditions, approved by Animal Experimentation Ethics Committee of CUHK. MDA-MB-231-TXSA-TGL cells (1×10^6) resuspended in 10 μl PBS, were injected into the marrow space of the proximal tibia with a 27-gauge needle coupled to a Hamilton syringe. After cancer cell implantation, mice were divided randomly into three groups ($n = 10$): control group ($1 \times$ PBS, i.p. injected twice a week for 4 weeks), ZOL-C group (0.1 mg/kg ZOL, i.p. injected once only, as conventional single dose), ZOL-M group (0.0125 mg/kg ZOL, i.p. injected twice a week for 4 weeks, as metronomic dose). During ZOL treatment, mice were imaged weekly using the *In Vivo* Imaging System (IVIS) 200 bioluminescence system (Xenogen, USA). After 4 weeks treatment, mice were sacrificed, lungs and livers were removed for bioluminescence imaging and quantification of tumor burden. Both the tibias of each animal were removed for X-ray, micro-computed tomography and histological analysis.

2.3. In vivo bioluminescence imaging

Tumor growth in live animals was assessed by the IVIS 200 system (Xenogen, USA). Mice were imaged after i.p. injected with D-luciferin solution at 150 mg/kg body weight and gas anaesthetized. Bioluminescence images were taken 30 min after the D-luciferin injection, acquired for 1–30 s and the photon emission was quantitated using the software, Living image 3.2 (Xenogen, USA), and graphed according to the average radiance (photons/s/cm^2/sr).

2.4. X-ray and micro-computed tomography (μ-CT) analysis

Tibias removed from mice were scanned with X-ray (MX-20, Faxitron X-ray, WI, USA) and a high resolution microtomographic system, μ-CT 40 (Scanco Medical, Switzerland). The tibia specimens were measured at room temperature and placed inside the X-ray chamber of the X-ray machine. The voltage and exposure time of the X-ray were 32 kV and 10 s, respectively. Then the samples were exposed to the μ-CT. Each three-dimensional image data were consisted of approximately 500 micro-CT slide image (8 μm/slide) starting from the growth plate of tibial interface and moving down the tibia. The bone density was expressed as percentage of BV/TV, which was generated and compared with each groups using the formula: (Bone volume/Tissue volume) × 100% [20].

2.5. Histology

Lungs and livers were fixed in 10% buffered formalin for 7 days at room temperature. As for the tibia, after fixed in 10% buffered formalin, the tibia was decalcified in decalcification buffer (14% EDTA w/v in distilled water, pH 6.8–7.2) for 21 days. Then samples were paraffin embedded, sectioned longitudinally at 5 μm, and stained with H&E. Stained sections were examined and photographed using an Olympus IX71 microscope (Japan) and were analyzed using SPOT advanced (version 3.5.6) software. Tumor burden, defined as the tumor area, was calculated from the section of the lung or liver and expressed as an average tumor area per group in absolute units (mm^2).

2.6. Mouse mammary tumor model

Female nude mice (6–8 weeks of age) were provided by Laboratory Animal Services Center, CUHK. MDA-MB-231-TXSA-TGL cells (5×10^6) resuspended in 0.2 ml PBS, were subcutaneously (s.c.) inoculated at the mammary fat pad of each nude mouse. After the tumor size reached 80 mm^3, the tumor-bearing nude mice were randomly assigned into three groups ($n = 16$): control group ($1 \times$ PBS, i.p. injected twice a week for 4 weeks), ZOL-C group (0.1 mg/kg ZOL, i.p. injected once only, as conventional single dose), ZOL-M group (0.0125 mg/kg ZOL, i.p. injected twice a week for 4 weeks, as metronomic dose). Tumor size and body weight of each mouse were measured twice a week during the 4-week treatment period. At day 28, mice were injected (i.p.) with D-luciferin solution at 150 mg/kg body weight, and then sacrificed, and the lungs and livers were removed for bioluminescence imaging and quantification of tumor burden. Tibias of mice from different groups were removed for micro-CT analysis.

2.7. Statistical analysis

All data were expressed as mean ± SEM. Statistical analysis was performed using one way ANOVA (unless otherwise specification), with p-value (p) < 0.05 as considered statistically significant.

3. Results

3.1. Effect of ZOL on tumor burden, metastasis, and cancer-induced bone destruction in metastatic breast cancer

To evaluate the efficacy of ZOL against tumor growth within bone, cancer-induced bone destruction and tumor metastasis, an intratibial breast cancer-induced osteolysis model was employed. In this model, the MDA-MB-231-TXSA-TGL cells were injected into the tibial marrow cavity of nude mice directly. After ZOL treatment, no significant body weight loss was found in ZOL-treated groups (data not shown). As shown in Fig. 1A, an increase of photon emission (expressed as average radiance) from day 7 onwards associated with an increase in tumor burden of bone. Unlike ZOL-C, treatment with metronomic ZOL (ZOL-M) resulted in the slowing down of tumor growth and a significant difference was shown at day 28 (Fig. 1B). Metronomic use of ZOL resulted in a reduction of tumor burden in the bone, while the conventional ZOL had no effect.

To assess the effect of ZOL on breast cancer-induced osteolysis, the tibias of mice were subjected to X-ray and μ-CT analysis. Both X-ray and μ-CT images showed that the tumor-bearing tibia of control group was severely destroyed, while the non-tumor-bearing tibia was intact (Fig. 2A and B), suggesting the breast cancer-induced bone lesion. After treatment with ZOL, the bone structure was protected completely with a significant increase in the percentage of BV/TV. ZOL-M treatment showed greater increase in the percentage of BV/TV in both non-tumor and tumor-bearing tibias, and a significant difference was shown in the percentage of BV/TV in non-tumor-bearing tibias, as compared to conventional ZOL (ZOL-C) (Fig. 2C and D). Histological analysis of the tibias showed that the bone structure of control group could not be observed,-while the bone structure of tibias from ZOL-treated groups could be clearly observed (Fig. 2E). These results suggested that ZOL treatment protected the bone significantly from breast cancer-induced osteolysis. Besides, the tumor size in ZOL-M was the smallest among the three groups (Fig. 2E).

At the end of the experiment, lungs and livers of mice were excised to assess the tumor burden of metastasis, as evaluated by bioluminescence imaging and histological analysis. As shown in Fig. 3A, the photon emission of lungs in conventional ZOL was very strong, and the fractional number reflected the frequency of lung metastasis in this group was 9/10, showing the highest incidence of lung metastasis among three groups (Fig. 3A). Fig. 3C represented the mean luciferase activity in lungs, and a significant increase was observed in ZOL-C versus control, while ZOL-M decreased the frequency of lung metastasis and showed less luciferase activity from TXSA-TGL cells. Consistent results were found in histological analysis (Fig. 3B and D). Hence, the metronomic ZOL was effective in slowing down metastasis to lungs. In addition, the tumor burden of liver was also assessed. No luciferase activity was detected in both control and ZOL-M groups, while two animals in ZOL-C were luciferase positive (Fig. 3E and G). The result was confirmed by the histological analysis that there was no noticeable tumor cells found in livers of both control and ZOL-M groups (Fig. 3F and H).

3.2. Effect of ZOL on tumor growth, metastasis, and cancer-induced bone destruction in orthotopic breast cancer xenografts

To investigate the activity of ZOL against tumor growth, metastasis and cancer-induced osteolysis in orthotopic breast cancer, a mammary fat pad model was employed, in which cells were injected into the mammary fat pad of female nude mice. During ZOL treatment, no significant body weight loss was found in ZOL-treated groups (data not shown). Tumors at mammary tissue were found to grow steadily in both control and ZOL-C groups, while the growth slowed down in ZOL-M group starting from day 8 after treatment, with a significant difference against ZOL-C at day 28 (Fig. 4A).

Tibias of mice from different groups were removed for μ-CT analysis. After ZOL treatment, percentage of BV/TV was increased significantly in mice tibia, with a net increasing of 9.7% and 9.9% in ZOL-C and ZOL-M, respectively (Fig. 4B and C). However, no significant difference was found between the two groups.

Lungs and livers of animals were removed for bioluminescence imaging and histological analysis. Bioluminescent images from Fig. 5A showed that the signal emission was strong in ZOL-C with an incidence of 12 mice in 16 developed lung metastasis. While metronomic ZOL-treated group presented less photon emission with a lower incidence of lung metastasis, as compared with ZOL-C (Fig. 5C). In order to further confirm the results of lung metastasis, histological analysis was included. As shown in Fig. 5B, MDA-MB-231-TXSA-TGL tumors were present in lungs of different groups, with a bigger area in ZOL-C group. Besides, ZOL-M showed remarkable inhibition of lung metastasis in mice as compared to ZOL-C (Fig. 5D). In addition, the liver metastasis was also assessed. ZOL-M significantly decreased frequency of liver metastasis in 6/16 mice, as compared to 9/16 mice in ZOL-C group (Fig. 5E). With the mean bioluminescence measurement in livers, significant increase was observed in ZOL-C versus control and ZOL-M groups (Fig. 5G). Furthermore, histological analysis showed that the tumor burden in liver was the highest in ZOL-C group, with a significant difference to ZOL-M (Fig. 5F and H).

4. Discussion

A metronomic dose means repeated low doses, which is based on more frequent and low-dose drug administration compared with conventional therapy [22]. Early in 2000, Klement and Browder published two pioneering articles showing that mice bearing subcutaneous tumors would respond to continuous repeated low doses of chemotherapy [23,24]. During the last decade, clinical studies have shown that the metronomic treatment represent an interesting alternative for either primary systemic therapy or maintenance therapy, e.g. positive results were reported with various metronomic chemotherapy regimens for patients with metastatic breast cancer, recurrent ovarian cancer, advanced multiple myeloma, recurrent malignant glioma, metastatic or locally advanced neuroendocrine carcinoma [25–27]. The effectiveness of metronomic regimen in patients with differ-

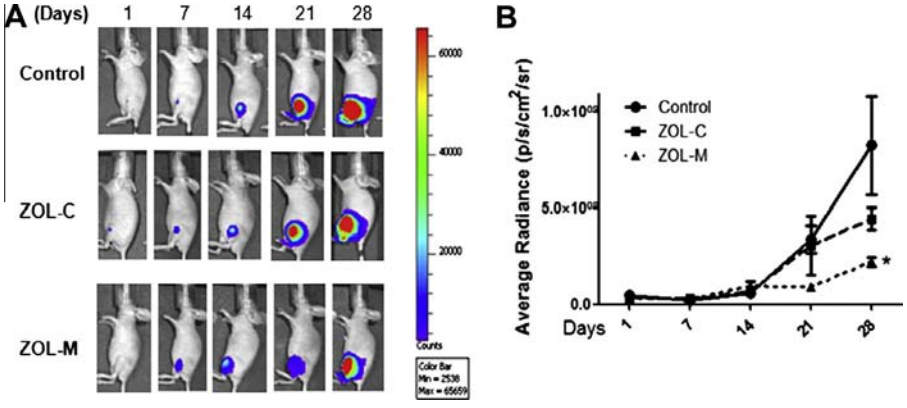

Fig. 1. Tumor burden change during conventional and metronomic ZOL treatment in intratibial breast cancer model. (A) Representative images of tumor burden obtained from each group at different time points as assessed by IVIS system. (B) Graph showed the bioluminescence measurements according to the Average Radiance. ZOL-M slowed down the tumor burden in bone, and a significant difference was presented at day 28. Data were expressed as mean ± SEM, $n = 10$. $^{*}p < 0.05$, as compared with control.

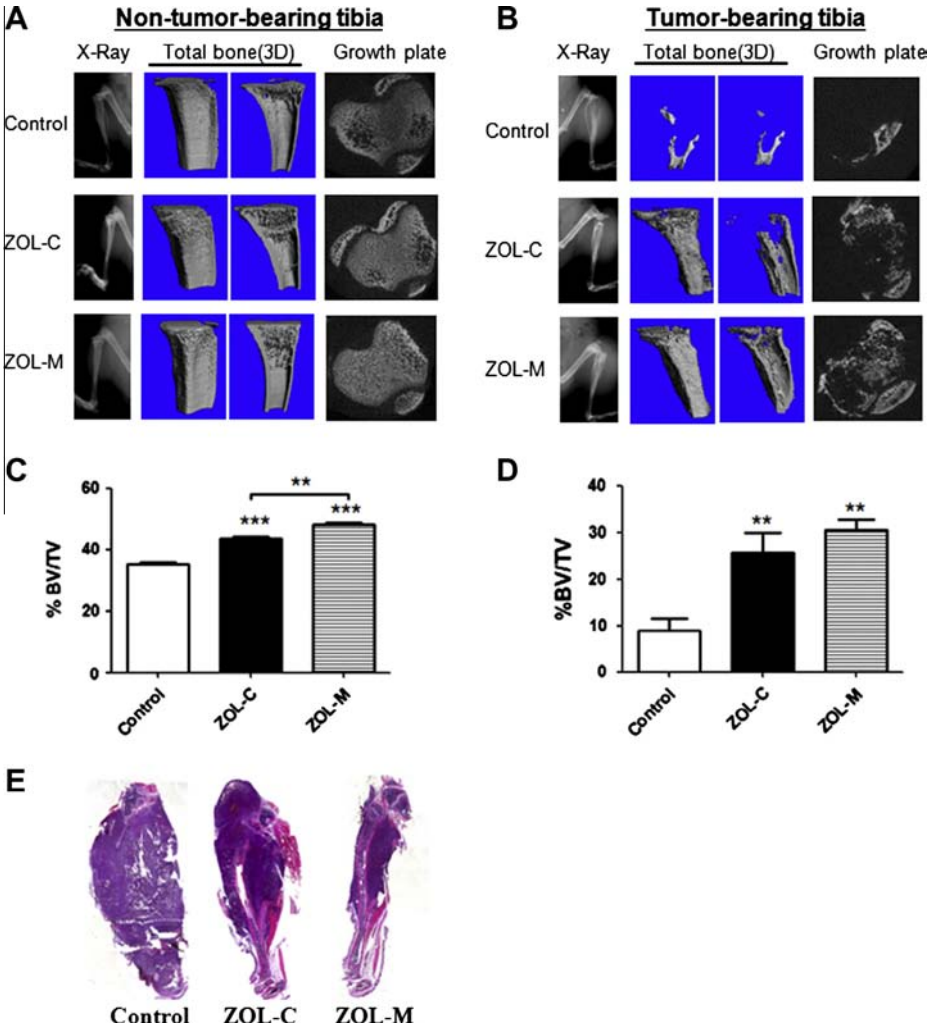

Fig. 2. Qualitative and quantitative assessment of bone structure in both tibias after conventional or metronomic administration of ZOL. (A and B) Representative X-ray, μ-CT 3D images, section-cut and growth plate of (A) non-tumor-bearing and (B) tumor-bearing tibias obtained from different groups. (C and D) Graphs showed the bone volume density (% BV/TV) of (C) non-tumor-bearing and (D) tumor-bearing tibias. Both ZOL-C and ZOL-M increased the % BV/TV significantly in both tibias, and ZOL-M showed better result in bone protection in non-tumor-bearing tibias. (E) Hematoxylin and eosin (H&E) stain of formalin-fixed sections from tumor-bearing tibias obtained from different groups. Representative images from different groups showed bone, soft tissue and tumor structures. Data were expressed as mean + SEM, $n = 10$. $^{**}p < 0.01$ and $^{***}p < 0.001$, as compared with control.

ent cancer types, may account to the prolonged duration of clinical benefit obtained with metronomic way, and the chronic administration of chemotherapeutic agents at relatively low, minimally toxic doses [28]. Accumulating evidence suggests that the efficacy of repeated low doses treatment could prevent tumor angiogenesis [29], and new mechanisms of action were also reported, such as restoration of anticancer immune response and induction of tumor dormancy [30]. However, in some cases, metronomic chemotherapy failed to improve patient survival [31].

ZOL was effective in treatment of various bone diseases associated with enhanced bone resorption, but had long-term use risks and the conventional regimen of ZOL has been reported to increase lung metastasis to certain extent in an osteosarcoma mouse model [11]. In this study, a new way of administration of metronomic (repeated low dose) ZOL was investigated on anti-tumor and anti-osteolysis effects against metastatic and primary breast cancer of MDA-MB-231-TXSA-TGL cells.

In the present study, the anti-tumor and anti-osteolysis effects of conventional (ZOL-C) and metronomic (ZOL-M) ZOL were investigated in an intratibial breast cancer-induced osteolysis model, which closely mimics breast cancer cells metastasized to bone and developed severe bone destruction [21]. No significant body weight loss was found in ZOL-treated groups. Metronomic ZOL treatment resulted in significant inhibition of tumor growth in bone (Fig. 1A and B), which was in consistent with previous report that daily or weekly therapy with clinical doses of ZOL inhibited skeletal tumor growth in a mouse model of bone metastasis implanted with human B02/GFP.2 breast cancer cells [20]. Besides, the metronomic ZOL protected bone structure significantly, with greater increase in the percentage of BV/TV in both tumor-bearing and non-tumor-bearing tibiae (Fig. 2). A slight significant difference of percentage of BV/TV was shown in non-tumor-bearing tibia between the conventional and metronomic ZOL. In addition to the bone protection effect, the metronomic use of ZOL did not increase lung and liver metastasis as the conventional ZOL did (Fig. 3). Our results showed that conventional administration of ZOL had no effect in inhibiting tumor growth, but to some extent promoted metastasis to lung and liver, which were in line with our previous study [11] and Dass and Choong's findings [32]. The results indicated that the metronomic use of ZOL showed greater effects in slowing down of tumor growth and metastasis to lung and liver than the conventional ZOL. These observations may account to the prolonged duration of clinical benefit obtained with metro-

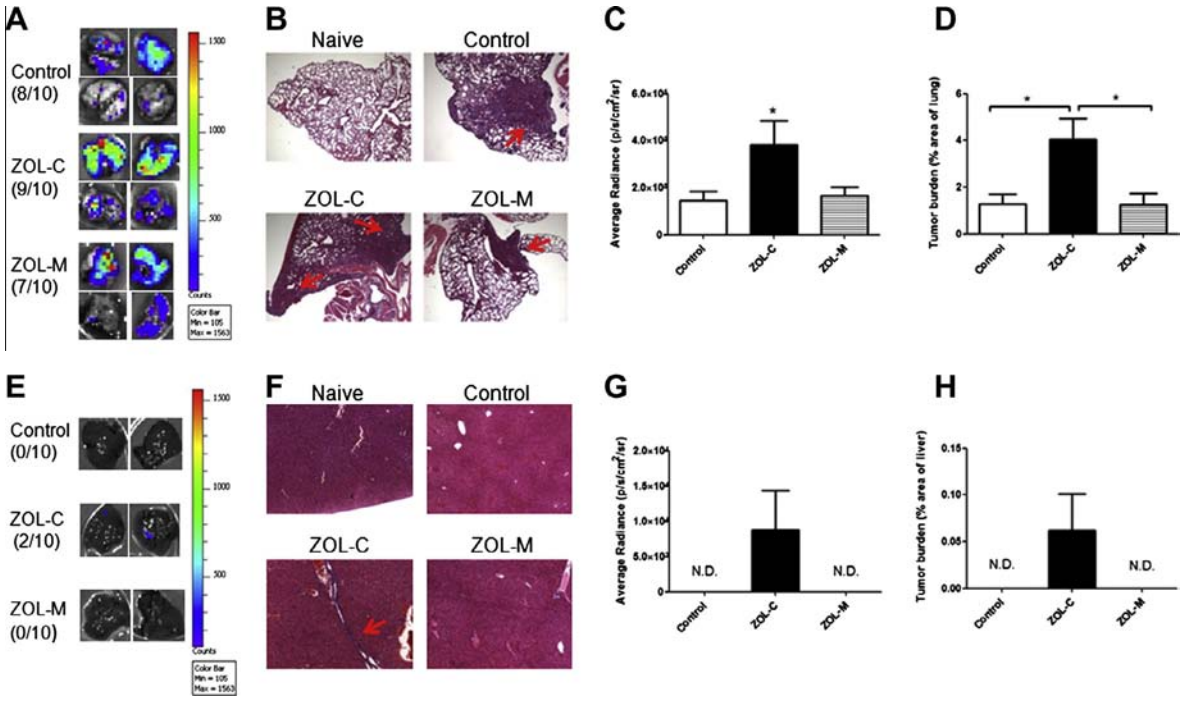

Fig. 3. Effects of conventional and metronomic ZOL treatment on lung metastasis in metastatic breast cancer. (A) Representative images of lungs obtained from different groups at end point of IVIS scan, and the fractional number in each group revealed the frequency of metastasis. (B) Photographs were representative of H&E-stained sections of mouse lungs at the end of experiment with arrows showing the tumor cells. (C) Graph represented the bioluminescence measurements in lungs from different groups. A significant increase was shown in ZOL-C, as compared to control. Data were expressed as Average Radiance. (D) Graph showed the tumor burden in lungs as assessed by histological analysis, and expressed as an average percentage per group. (E-F) Representative images of livers obtained from different groups of (E) bioluminescence imaging and (F) H&E staining. (G) Graph represented the bioluminescence measurements in livers. No luciferase positive cells were found in control and ZOL-M groups. (H) Graph represented the tumor burden in livers as assessed by histological analysis. Unlike ZOL-C, non-detectable tumor cell was found in control and ZOL-M groups. Data were expressed as mean + SEM, $n = 10$; $^{*}p < 0.05$; N.D. means non-detectable.

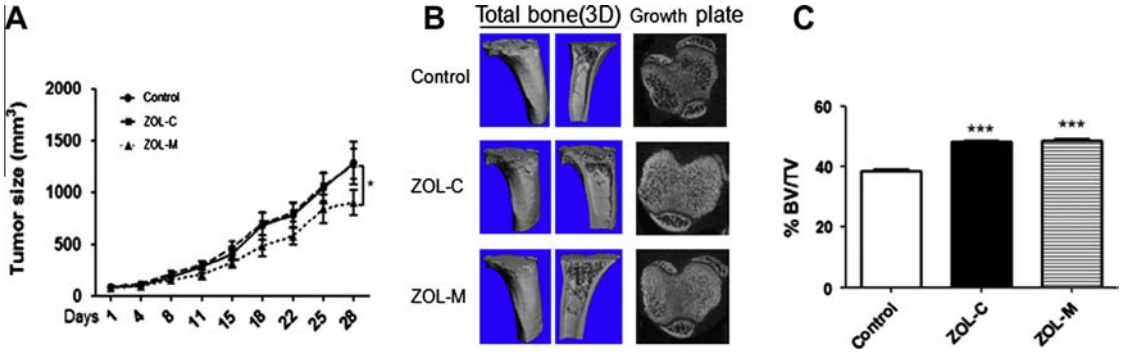

Fig. 4. Body weight and tumor size change during conventional and metronomic ZOL treatment in orthotopic breast cancer xenograft. (A) The anti-tumor effects of ZOL-C and ZOL-M against orthotopic mammary tumors. ZOL-M slowed down the tumor growth after treatment, and a significant difference was observed at day 28 as compared to ZOL-C. $^{*}p < 0.05$, analyzed by Students' *t*-test. (B) Qualitative 3D μ-CT images of representative animals from each group. (C) Quantitative assessment of the bone volume density in tibias from different groups. ZOL-treated groups increased the % BV/TV significantly. Data were expressed as mean + SEM, $n = 16$; $^{***}p < 0.001$, as compared with control.

nomic way. Accumulating evidence suggested that the metronomic treatment could prevent tumor angiogenesis [29], underlying mechanism including selective inhibition of proliferation of activated endothelial cells, selective inhibition of endothelial cell migration, increase of the angiogenesis inhibitor and sustained decrease of bone marrow-derived endothelial progenitor cells [16]. The anti-tumor and anti-metastasis effects of metronomic ZOL against metastatic breast cancer may result from inhibition of tumor angiogenesis due to metronomic dosing. Angiogenesis is a key involvement in tumor growth and metastasis in breast cancer, which is likely to change with tumor growth, regression and tumor site [33]. On the other hand, pharmacokinetic studies of ZOL indicated that approximately half of the dose reached the skeleton with an early half-life of ten days [34]. That means the metronomic regimen of ZOL prolonged the duration of clinical benefit in host at relatively low doses, and may result in better effect in anti-tumor and anti-metastasis.

In order to substantiate the observations in metastatic breast cancer, the anti-tumor and anti-osteolysis effects of ZOL against primary breast cancer was investigated in an orthotopic breast cancer xenografted model. The consistent results were also observed. Metronomic ZOL was demonstrated to be effective in bone protection from breast cancer-induced osteolysis (Fig. 4). Moreover, metronomic ZOL showed significant inhibition on tumor growth in orthotopic mammary tissue, while the lung and liver metastasis did not increase. In contrast, ZOL-C did not inhibit tu-

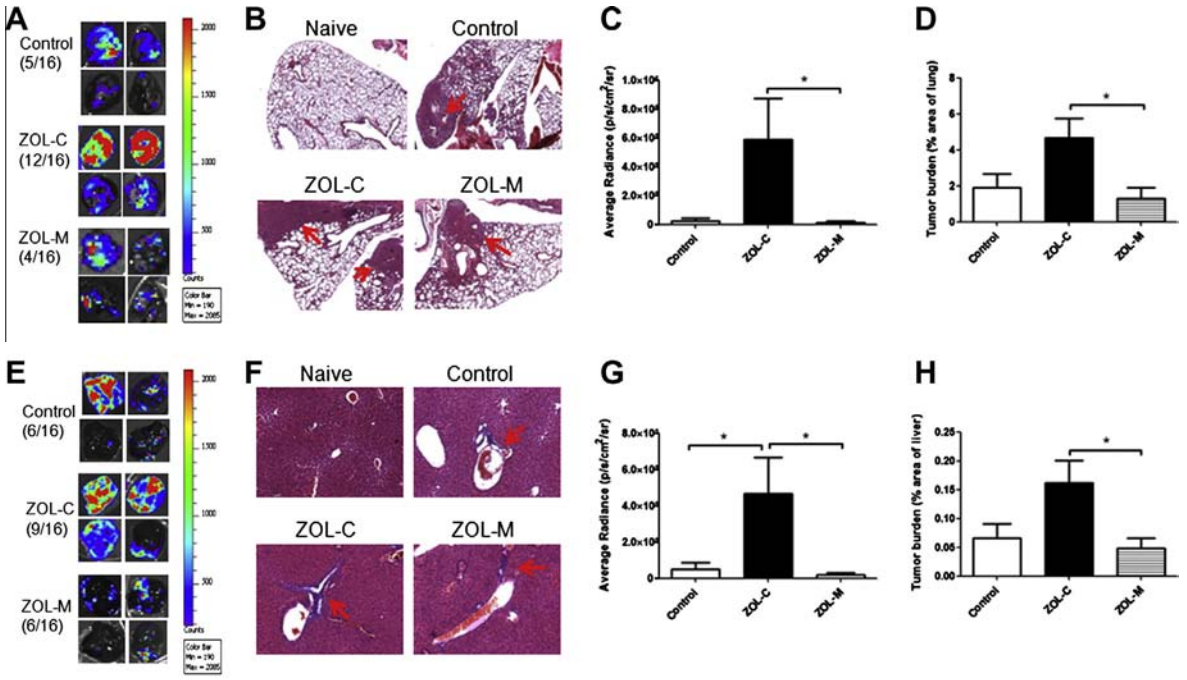

Fig. 5. Effects of conventional and metronomic ZOL treatment on lung metastasis in orthotopic breast cancer xenograft. (A) Representative images of lungs obtained from different groups at end point of IVIS scan, and the fractional number in each group revealed the frequency of metastasis. (B) Photographs were representative of H&E-stained sections of mouse lungs with arrows showing the tumor cells. (C) Graph represented the bioluminescence measurements in lungs from different groups. A significant increase was shown in ZOL-C, as compared to ZOL-M. Data were expressed as Average Radiance. (D) Graph showed the tumor burden in lungs as assessed by histological analysis, and expressed as an average percentage per group. (E–F) Representative images of livers obtained from different groups of (E) bioluminescence imaging and (F) H&E staining. (G) Graph represented the bioluminescence measurements in livers. A significant increase of bioluminescence measurement was observed in ZOL-C, as compared to control and ZOL-M. (H) Graph represented the tumor burden in livers as assessed by histological analysis. Contrast to ZOL-C, ZOL-M showed less liver metastasis. Data were expressed as mean + SEM, $n = 10$; $^{*}p < 0.05$; N.D. means non-detectable.

mor growth but increased the incidence of lung and liver metastasis (Fig. 5). Taken together, the metronomic use of low dose ZOL appeared to be more effective than the conventional regimen in reducing the tumor burden and decreasing metastasis to lung and liver in both of the primary and metastatic breast cancer.

In this study, two mouse models of breast cancer were employed. The intratibial breast cancer-induced osteolysis model mimics metastatic breast cancer that the breast cancer cells already metastasized to bone. In this model, the breast cancer MDA-MB-231-TXSA-TGL cells were injected into the tibial marrow cavity of nude mice directly [21]. While in mammary tumor model, MDA-MB-231-TXSA-TGL cells were injected orthotopically into the mammary fat pad of female nude mice and developed primary breast cancer. Both of the mouse models could induce bone destruction. In intratibial model, the injected breast cancer cells in bone marrow could destroy the balance of the bone formation and resorption, and promote a vicious cycle of bone destruction and tumor expansion [4]. In mammary tumor model, once the primary breast cancer tumor metastasize to bone, osteolysis would be induced. Therefore, the two animal models are completely different and thus the cell response in bone is different after treated with ZOL. This may possibly explain that the metronomic ZOL treatment resulted in a significant increase in % BV/TV in non-tumor-bearing tibias in intratibial model as compared to conventional ZOL (Fig. 2), while no significant difference was observed between the two groups in mammary tumor model (Fig. 4).

Apart from the anti-osteolysis effect, the effects of conventional and metronomic ZOL on lung and liver metastasis were also compared in this study. Our results showed that the conventional clinical relevant dose of ZOL showed no effect but to some extent promoted lung metastasis, which was in complete agreement with Labrinidis et al.'s finding on osteosarcoma [5], Dass and Choong's findings [32], and Wolfe et al.'s findings on osteosarcoma OSCA40 [35]. On the other hand, the metronomic low dose of ZOL inhibited lung metastasis significantly as compared to the conventional treatment. This result was in line with other similar reports on other kinds of tumor. Koto et al. found that high dose of ZOL (80 μg/kg three times/week) inhibited the growth of osteosarcoma at the primary site, while the low dose of ZOL (80 μg/kg once a week) significantly prevented lung metastasis [36]. Besides, in Li et al.'s report, it was demonstrated that low-dose zoledronic acid reduces spinal cord metastasis in pulmonary adenocarcinoma [37]. Interestingly, our study first demonstrated that metronomic ZOL inhibited liver metastasis significantly. However, current clinical data seldom showed that treatment of ZOL resulted in induction of lung or liver metastasis in cancer patients. A possible reason may be due to the differences in human versus nude mice which are immunodeficient. In this regards, it was demonstrated that ZOL could directly stimulate gamma–delta T cells in human peripheral blood mononuclear cells which suggest that immune system may play a role [38,39].

In conclusion, our data showed that metronomic regimen of ZOL, (i.e. total administered dose remains the same as the clinically relevant dose), could protect the bone from breast cancer-induced osteolysis, and presented great potential in inhibiting tumor growth and metastasis to lung and liver in both of primary and metastatic breast cancer. This metronomic dosing schedule revealed promising results and should be further assessed in clinical trials.

Conflict of Interest

None.

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References

[1] J. Ferlay, H.R. Shin, F. Bray, D. Forman, C. Mathers, D.M. Parkin, Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008, Int. J. Cancer 127 (2010) 2893–2917.
[2] R.E. Coleman, Metastatic bone disease: clinical features, pathophysiology and treatment strategies, Cancer Treat. Rev. 27 (2001) 165–176.
[3] L.J. Suva, R.J. Griffin, I. Makhoul, Mechanisms of bone metastases of breast cancer, Endocr. Relat. Cancer 16 (2009) 703–713.
[4] P.S. Steeg, D. Theodorescu, Metastasis: a therapeutic target for cancer, Nat. Clin. Pract. Oncol. 5 (2008) 206–219.
[5] A. Labrinidis, S. Hay, V. Liapis, D.M. Findlay, A. Evdokiou, Zoledronic acid protects against osteosarcoma-induced bone destruction but lacks efficacy against pulmonary metastases in a syngeneic rat model, Int. J. Cancer 127 (2010) 345–354.
[6] F.L. Zhang, P.J. Casey, Protein prenylation: molecular mechanisms and functional consequences, Annu. Rev. Biochem. 65 (1996) 241–269.
[7] S.G. Senaratne, K.W. Colston, Direct effects of bisphosphonates on breast cancer cells, Breast Cancer Res. 4 (2002) 18–23.
[8] A. Evdokiou, A. Labrinidis, S. Bouralexis, S. Hay, D.M. Findlay, Induction of cell death of human osteogenic sarcoma cells by zoledronic acid resembles anoikis, Bone 33 (2003) 216–228.
[9] J. Wood, K. Bonjean, S. Ruetz, A. Bellahcene, L. Devy, J.M. Foidart, V. Castronovo, J.R. Green, Novel antiangiogenic effects of the bisphosphonate compound zoledronic acid, J. Pharmacol. Exp. Ther. 302 (2002) 1055–1061.
[10] P.D. Ottewell, J.K. Woodward, D.V. Lefley, C.A. Evans, R.E. Coleman, I. Holen, Anticancer mechanisms of doxorubicin and zoledronic acid in breast cancer tumor growth in bone, Mol. Cancer Ther. 8 (2009) 2821–2832.
[11] A. Labrinidis, S. Hay, V. Liapis, V. Ponomarev, D.M. Findlay, A. Evdokiou, Zoledronic acid inhibits both the osteolytic and osteoblastic components of osteosarcoma lesions in a mouse model, Clin. Cancer Res. 15 (2009) 3451–3461.
[12] P. Ryan, I. Saleh, L.F. Stassen, Osteonecrosis of the jaw: a rare and devastating side effect of bisphosphonates, Postgrad. Med. J. 85 (2009) 674–677.
[13] J. Green, G. Czanner, G. Reeves, J. Watson, L. Wise, V. Beral, Oral bisphosphonates and risk of cancer of oesophagus, stomach, and colorectum: case-control analysis within a UK primary care cohort, BMJ 85 (2010) 674–677.
[14] D.E. Sellmeyer, Atypical fractures as a potential complication of long-term bisphosphonate therapy, JAMA 304 (13) (2010) 1480–1484.
[15] A. Lipton, The safety of zoledronic acid, Expert Opin. Drug Saf. 6 (3) (2007) 305–313.
[16] E. Pasquier, M. Kavallaris, N. Andre, Metronomic chemotherapy: new rationale for new directions, Nat. Rev. Clin. Oncol. 7 (2011) 455–465.
[17] X. Zhao, X. Xu, Q. Zhang, Z. Jia, S. Sun, J. Zhang, B. Wang, Z. Wang, X. Hu, Prognostic and predictive value of clinical and biochemical factors in breast cancer patients with bone metastases receiving "metronomic" zoledronic acid, BMC Cancer 11 (2011) 403.
[18] X. Zhao, X. Xu, L. Guo, J. Ragaz, H. Guo, J. Wu, Z. Shao, J. Zhu, X. Guo, J. Chen, B. Zhu, Z. Wang, X. Hu, Biomarker alterations with metronomic use of low-dose zoledronic acid for breast cancer patients with bone metastases and potential clinical significance, Breast Cancer Res. Treat. 124 (2010) 733–743.
[19] G. Facchini, M. Caraglia, A. Morabito, M. Marra, M.C. Piccirillo, A.M. Bochicchio, S. Striano, L. Marra, G. Nasti, E. Ferrari, D. Leopardo, G. Vitale, D. Gentilini, A. Tortoriello, A. Catalano, A. Budillon, F. Perrone, R.V. Iaffaioli, Metronomic administration of zoledronic acid and taxotere combination in castration resistant prostate cancer patients: phase I ZANTE trial, Cancer Biol. Ther. 10 (2010) 543–548.
[20] F. Daubine, C. Le Gall, J. Gasser, J. Green, P. Clezardin, Antitumor effects of clinical dosing regimens of bisphosphonates in experimental breast cancer bone metastasis, J. Natl Cancer Inst. 994 (2007) 322–330.
[21] I. Zinonos, A. Labrinidis, M. Lee, V. Liapis, S. Hay, V. Ponomarev, P. Diamond, A.C. Zannettino, D.M. Findlay, A. Evdokiou, Apomab, a fully human agonistic antibody to DR5, exhibits potent antitumor activity against primary and metastatic breast cancer, Mol. Cancer Ther. 8 (2009) 2969–2980.
[22] O.G. Scharovsky, L.E. Mainetti, V.R. Rozados, Metronomic chemotherapy: changing the paradigm that more is better, Curr. Oncol. 16 (2009) 7–15.
[23] G. Klement, S. Baruchel, J. Rak, S. Man, K. Clark, D.J. Hicklin, P. Bohlen, R.S. Kerbel, Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity, J. Clin. Invest. 105 (2000) R15–R24.
[24] T. Browder, C.E. Butterfield, B.M. Kraling, B. Shi, B. Marshall, M.S. O'Reilly, J. Folkman, Antiangiogenic scheduling of chemotherapy improves efficacy against experimental drug-resistant cancer, Cancer Res. 60 (2000) 1878–1886.
[25] L. Orlando, A. Cardillo, R. Ghisini, A. Rocca, A. Balduzz, R. Torrisi, G. Peruzzotti, A. Goldhirsch, E. Pietri, M. Colleoni, Trastuzumab in combination with metronomic cyclophosphamide and methotrexate in patients with HER-2 positive metastatic breast cancer, BMC Cancer 6 (2006) 225.
[26] O. De Weerdt, N.W. van de Donk, G. Veth, A.C. Bloem, A. Hagenbeek, H.M. Lokhorst, Continuous low-dose cyclophosphamide-prednisone is effective and well tolerated in patients with advanced multiple myeloma, Neth. J. Med. 59 (2001) 50–56.
[27] M.P. Brizzi, A. Berruti, A. Ferrero, E. Milanesi, M. Volante, F. Castiglione, N. Birocco, S. Bombaci, D. Perroni, B. Ferretti, O. Alabiso, L. Ciuffreda, O. Bertetto, M. Papotti, L. Dogliotti, Continuous 5-fluorouracil infusion plus long acting octreotide in advanced well-differentiated neuroendocrine carcinomas. A phase II trial of the Piemonte oncology network, BMC Cancer 9 (2009) 388.
[28] M.W. Kieran, C.D. Turner, J.B. Rubin, S.N. Chi, M.A. Zimmerman, C. Chordas, G. Klement, A. Laforme, A. Gordon, A. Thomas, D. Neuberg, T. Browder, J. Folkman, A feasibility trial of antiangiogenic (metronomic) chemotherapy in pediatric patients with recurrent or progressive cancer, J. Pediatr. Hematol. Oncol. 27 (2005) 573–581.
[29] K.D. Miller, C.J. Sweeney, G.W. Sledge Jr., Redefining the target: chemotherapeutics as antiangiogenics, J. Clin. Oncol. 19 (2001) 1195–1206.
[30] S. Brem, H. Brem, J. Folkman, D. Finkelstein, A. Patz, Prolonged tumor dormancy by prevention of neovascularization in the vitreous, Cancer Res. 36 (1976) 2807–2812.
[31] M.K. Krzyzanowska, I.F. Tannock, G. Lockwood, J. Knox, M. Moore, G.A. Bjarnason, A phase II trial of continuous low-dose oral cyclophosphamide and celecoxib in patients with renal cell carcinoma, Cancer Chemother. Pharmacol. 60 (1) (2007) 135–141.
[32] C.R. Dass, P.F. Choong, Zoledronic acid inhibits osteosarcoma growth in an orthotopic model, Mol. Cancer Ther. 6 (2007) 3263–3270.
[33] P. Carmeliet, R.K. Jain, Angiogenesis in cancer and other diseases, Nature 407 (2000) 249–257.
[34] D.B. Kimmel, Mechanism of action, pharmacokinetic and pharmacodynamic profile, and clinical applications of nitrogen-containing bisphosphonates, J. Dent. Res. 86 (2007) 1022–1033.
[35] T.D. Wolfe, S.P. Pillai, B.E. Hildreth, L.G. Lanigan, C.K. Martin, J.L. Werbeck, T.J. Rosol, Effect of zoledronic acid and amputation on bone invasion and lung metastasis of canine osteosarcoma in nude mice, Clin. Exp. Metastasis 28 (4) (2011) 377–389.
[36] K. Koto, N. Horie, S. Kimura, H. Murata, T. Sakabe, T. Matsui, M. Watanabe, S. Adachi, T. Maekawa, S. Fushiki, T. Kubo, Clinically relevant dose of zoledronic acid inhibits spontaneous lung metastasis in a murine osteosarcoma model, Cancer Lett. 274 (2) (2009) 271–278.
[37] Y.Y. Li, J.W. Chang, L.L. Hsieh, S.M. Chen, K.Y. Yeh, Low-dose zoledronic acid reduces spinal cord metastasis in pulmonary adenocarcinoma with neuroendocrine differentiation, Anticancer Drugs 23 (9) (2012) 970–978.
[38] Z.X. Li, L.L. Sun, R.L. Cheng, Z.W. Sun, Z.M. Ye, Amplification of γδ T cells in PBMCs of healthy donors and osteosarcoma patients stimulated by zoledronate, Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi 28 (8) (2012) 822–824 (Chinese).
[39] K. Sato, S. Kimura, H. Segawa, A. Yokota, S. Matsumoto, J. Kuroda, M. Nogawa, T. Yuasa, Y. Kiyono, H. Wada, T. Maekawa, Cytotoxic effects of gammadelta T cells expanded *ex vivo* by a third generation bisphosphonate for cancer immunotherapy, Int. J. Cancer 116 (2005) 94–99.