Anti-metastatic activity of aromatic aminomethylidenebisphosphonates in a mouse model of 4T1 cell-derived breast cancer

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Abstract. Breast cancer is one of the major causes of cancer-related mortality among women worldwide. It metastasizes to distant organs, particularly to bone tissue. Nitrogen-containing bisphosphonates are mainly used as an adjuvant therapy to inhibit skeletal-related events; however, there is increasing evidence to suggest that these compounds also exert antitumor effects. In previous studies, the authors synthesized two novel aminomethylidenebisphosphonates (BPs), namely benzene-1,4-bis[aminomethylidene(bisphos phonic)] acid (WG12399C) and naphthalene-1,5-bis[amino methylidene(bisphosphonic)] acid (WG12592A). Both BPs exhibited notable antiresorptive activity in a mouse model of osteoporosis. The present study aimed to assess the *in vivo* anticancer activity of WG12399C and WG12592A in 4T1 breast adenocarcinoma model. WG12399C exerted an anti-metastatic effect by reducing the number of spontaneous lung metastases by ~66% in comparison to the control. In the experimental metastasis model of 4T1-luc2-tdTomato cells, this compound reduced the incidence of tumor metastases in the lungs by approximately half in comparison to the control. Both WG12399C and WG12595A also significantly reduced the size and/or number of bone metastatic foci. Their pro-apoptotic and anti-proliferative activity may, at least in part, explain the observed effects. Incubation with WG12399C induced an almost 6-fold increase in caspase-3 activity in 4T1 cells. Moreover, cells treated with WG12399C or WG12595A exhibited a 2-fold reduction in invasiveness through Matrigel. Furthermore, both the BPs were able to sensitize the 4T1 cells to cytostatics. In summary, the results of the present study indicate that the examined aminomethylidene-BPs may be of particular interest in the context of combined treatment in breast cancer therapy.

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Abbreviations: BP, bisphosphonate; N-BPs, nitrogen-containing bisphosphonates; GGOH, geranylgeraniol, FOH, farnesol; ELISA, enzyme-linked immunosorbent assay

Key words: bisphosphonates, breast cancer, metastasis, anticancer treatment, mouse models

Introduction

Breast cancer is one of the major causes of cancer-related mortality among women worldwide. In 2020, over 2.3 million new cases of breast cancer were diagnosed (1). Recent advances in the early detection and treatment have led to improvements in the outcomes of breast cancer patients; however, these patients continue to have a high risk of developing disease recurrence, progression and mortality. The presence of distant metastases markedly worsens the prognosis and quality of life of women with breast cancer. Currently, the 5-year survival rate of patients with localized disease is 99%; however, in patients with distant metastases, this is reduced to 27% (2). Breast cancer has an affinity towards bone tissue. It has been estimated that in >70% of patients with advanced-stage breast cancer, disease progression will lead to bone metastases (3). Bone metastases are the major cause of excessive bone resorption, which is known to lead to severe skeletal complications, such as hypercalcemia, cancer-induced bone pain, spinal cord compression and pathological fractures (4). The multidirectional treatment for breast cancer comprises surgery, radiotherapy, chemotherapy and hormonotherapy. In addition, bisphosphonates (BPs) are used as an adjuvant therapy to inhibit skeletal-related events (5). Nitrogen-containing BPs (N-BPs) are a standard treatment for patients with breast cancer with bone metastases, as well as in patients who are at risk of developing treatment-induced osteoporosis (6). However, there is increasing evidence to suggest that BPs also exert direct and indirect antitumor effects. The direct mechanisms underlying the antitumor effects of BPs

comprises the inhibition of tumor cell proliferation, pro-apoptotic effects and synergism with cytostatics (7-9). In turn, the indirect anticancer effects include the inhibition of cell adhesion and motility, the stimulation of immune surveillance and anti-angiogenic activity (10,11). Nevertheless, the translation of preclinical findings to clinical settings is not straightforward. Several trials have demonstrated that adjuvant therapy with BPs may have some beneficial effects on breast cancer patients by improving their overall survival rate, disease-free survival, and by prolonging recurrence-free survival. On the other hand, others have demonstrated that BPs exhibit limited efficacy and even induce side-effects [reviewed in (3)]. Thus far, BPs have only been administered to patients with breast cancer with osteolytic bone metastatic disease. Recently, the American Society of Clinical Oncology has recommended that zoledronate or clodronate can be administered as an adjuvant therapy to post- and pre-menopausal patients prior to treatment who have ovarian suppression-induced menopause (12). The ESMO Clinical Practice Guidelines also recommend that BPs can be administered to patients with early-stage breast cancer with low estrogen levels, particularly those who are at a high risk of relapse and those with treatment-related bone loss (13). However, it remains unclear whether BPs may exert beneficial effects on non-skeletal metastases. Another clinical question is whether the effectiveness and adverse effects depend on the BPs used.

In previous studies, the authors synthesized two novel aminomethylidene-BPs, namely benzene-1,4-bis[aminomethyl idene(bisphosphonic)] acid (WG12399C) and naphthalene-1,5-bis[aminomethylidene(bisphosphonic)] acid (WG12592A) (14). Both BPs exhibited notable anti-proliferative and pro-apoptotic activity against osteoclast precursors and tumor cells (15,16). Moreover, the intravenous (i.v.) administration of WG12399C and WG12592A significantly prevented ovariectomy-induced bone loss through the reduction of osteoclastogenesis and the inhibition of the resorptive activity of mature osteoclasts (17). These findings suggest the potential beneficial effects of WG12399C and WG12592A in combined anticancer treatment, as bone-protecting agents and as drugs modulating the tumor-microenvironment interactions.

Due to the results of these previous studies, the authors were encouraged to perform an extended analysis of the biological activity of WG12592A and WG12592A in *in vivo* experiments. Therefore, the specific aim of the present study was to evaluate the *in vivo* anticancer activity of these BPs (as a tetrasodium salt; Fig. 1).

The present study used 4T1 breast adenocarcinoma cells inoculated into BALB/c mice as models of lung and bone metastasis. The effects of WG12399C and WG12592A on tumor growth and metastasis were examined in these animals. In addition, the anti-proliferative activity of these amino BPs in combination with cytostatic drugs was assessed in 4T1 cells. In addition, the effects of BPs on cell motility and the cellular mechanisms of action were investigated.

Materials and methods

Compounds. Tetrasodium salts of benzene-1,4-bis[aminometh ylidene(bisphosphonic)] acid (WG12399C) and naphthalene-1, 5-bis[aminomethylidene(bisphosphonic)] acid (WG12592A) were synthesized from appropriate acids as described in a previous study by the authors (17). The starting free bisphosphonic acids were obtained by dealkylating their octaethyl esters as previously described (15). Reference zoledronate was prepared using a previously described procedure (18).

In vivo experiments

Animals. A total of 155 female BALB/c mice (8-10 weeks old, weighing 16-23 g) were purchased from the Center of Experimental Medicine of the Medical University of Bialystok (Bialystok, Poland) and maintained in specific pathogen-free facility under controlled environmental conditions (temperature, 22±2˚C; humidity, 55±10%; exposure to a 12-h day/night cycle), with water and food (S8435-S023, ssniff-Spezialdiäten GmbH) *ad libitum*. All the experiments were performed in accordance with the EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The study procedures were approved by the first Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (Permission nos. 4/2015 and 80/2015).

Toxicity analyses. The subacute toxicity of WG12399C and WG12592A was determined according to the OECD Test Guideline no. 407 (https://www.oecd-ilibrary.org/environment/test-no-407-repeated-dose-28-day-oral-toxicity-study-in-rodents_9789264070684-en) with minor modifications. In total, 35 female mice were randomly divided into groups, each consisting of 5 BALB/c females (weighing 16-19 g). The mice were maintained in specific pathogen-free facility under controlled environmental conditions, with water and food *ad libitum*. The mice received WG12399C and WG12592A by i.v. administration. The total doses of the compounds were divided into four weekly injections: WG12399C: 18, 35 and 50 mg/kg body weight (b.w.); WG12592A: 1, 2 and 4 mg/kg b.w. The doses were established based on the 50% lethal dose (LD_{50}) value. The animals in the control group received the vehicle (saline). The mice were observed every day for 28 days, and weighed twice a week. The following were observed during the 28-day period: Food and water intake; the condition of skin, fur and eyes; signs of diarrhea; and changes in behavior. On day 28 of the experiment, blood samples for biochemical analyses were obtained under anesthesia with 2-3% isoflurane from the eye venous sinus. The animals were then euthanized by cervical dislocation, and the major visceral organs and tumors were excised for gross examination and histopathological analysis.

Cell transplantation

i) Orthotopic model. The 4T1 cells (cat. no. CRL 2539ATTC, ATCC) were suspended in Hanks' medium [Laboratory of Analytical Chemistry (IIET)] and inoculated orthotopically into the second mammary fat pad ($2x10^4$/mouse). A total of 45 female mice were subjected to the procedure of orthotopic tumor cell inoculation.

ii) Intracardiac model. At day '0', 4T1-luc2-tdTomato cells (CVCL_5J46, Caliper Life Sciences, USA) were suspended in 100 μl phosphate-buffered saline (PBS) and injected into the left ventricle of the heart ($2.5x10^4$/mouse) under general anesthesia with isoflurane (5% for induction and 2-3% for maintenance). The anterior chest wall was washed with iodine and 70% alcohol. Subsequently, a 30-gauge needle on a

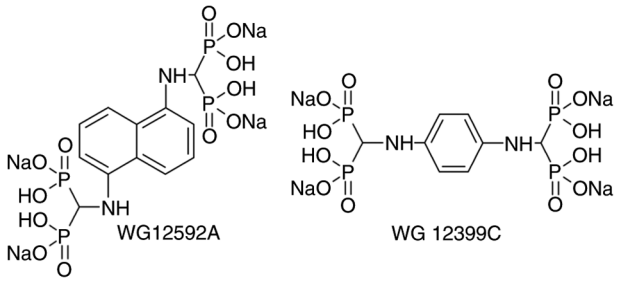


Figure 1. Chemical structures of the studied BPs.

tuberculin syringe was aimed straight and inserted through the intercostal space into the left ventricle of the heart. The proper positioning of the needle was confirmed by the spontaneous influx of blood into the syringe. A total of 75 female mice were subjected to the procedure of tumor cell inoculation. As this procedure poses a high risk of post-operative complications (19), 17 deaths were recorded. Thus, the final number of mice in each group differed at the end of the experiment.

Drug administration

i) Orthotopic model. Mice (weighing 18-22 g) were divided into five groups so that the mean tumor volume was similar in each treatment group. The mice were maintained in a specific pathogen-free facility under controlled environmental conditions, with water and food *ad libitum*. The following groups were included in the study: An untreated group and groups treated with WG12399C, WG12592A, zoledronate, or cyclophosphamide. The animals were placed in groups of 4-5 mice per cage. In the orthotopic model (n=9 per group), when the tumors reached a mean volume of ~50 mm^3, the mice began receiving i.v. injections of the vehicle or WG12399C at 50 mg/kg, WG12592A at 5 mg/kg, or zoledronate at 100 μg/kg, with the total dose divided into four and administered every 6th day (Table I) (20,21). As a reference, cyclophosphamide (25 mg/kg b.w.) was injected intraperitoneally (i.p.) three times a week. The doses of the BPs applied in the experiment were selected based on subacute toxicity experiment and previous research on the antiresorptive activity of WG12399C and WG12952A, in which they did not induce toxicity (17). The doses of cyclophosphamide and zoledronate were selected based on the literature data (20) and previous studies by the authors (21). The starting number of mice was 9 in each group. Of note, one animal in the WG12399C-treated group died during the course of experiment; however, this death was not related to treatment, tumor growth, or other experimental conditions.

ii) Intracardiac model. Mice (weighing 18-23 g) were randomly divided into five groups as follows: An untreated group and groups of mice treated with WG12399C, WG12592A, zoledronate or CY. The mice were maintained in a specific pathogen-free facility under controlled environmental conditions, with water and food *ad libitum*. The animals received i.v. injections of the vehicle or WG12399C at 50 mg/kg, WG12592A at 5 mg/kg, or zoledronate at 100 μg/kg, with the total dose divided into four (Table I). The first two doses were administered prior to tumor cell inoculation on days 6 and 1, and the following two doses were administered on days 5 and 9. Cyclophosphamide at the dose of 25 mg/kg b.w. was injected i.p. three times a week commencing on day 6.

Estimation of antitumor activity

i) Orthotopic model. All mice were observed daily and weighed three times a week throughout the experiment. When tumors became palpable, their volume was calculated three times a week according to the formula (a^2 x b)/2 (where a is the width and b is the length). Tumor growth inhibition was calculated by using the value of the control, untreated mice as 100%. The experiment was completed after 29 days of tumor cell inoculation. At the end of the experiment, the animals were anesthetized with 2-3% isoflurane and blood samples were collected. The animals were then euthanized by cervical dislocation under anesthesia with 2-3% isoflurane. Tumors and other organs (lungs, livers, ovaries and lymph nodes) were excised post-mortem, weighed and stored for further analyses. The lungs were fixed in formalin (Avantor Performance Materials Poland S.A.) and the metastatic foci were visually counted.

ii) Intracardiac model. All mice were observed daily and weighed three times a week throughout the experiment. At 11 days after tumor cell injection, the animals were anesthetized with 2-3% isoflurane and blood samples were collected. Tumors and organs (lungs, livers, kidneys, spleens, ovaries and bones) were excised post-mortem, weighed and analyzed. The post-mortem visualization of 4T1-luc2-tdTomato tumor metastases was performed using an *in vivo* MS FX PRO system (Carestream Health). Briefly, on day 11 after tumor cell inoculation, each animal received an an injection of 150 mg/kg D-luciferin potassium salt (Synchem UG & Co. KG) i.p. After 10 min, the mice were anesthetized with a 3-5% (v/v) isoflurane (Forane, Abbott Laboratories) in synthetic air (200 ml/min), euthanized by cervical dislocation, and the internal organs were excised, weighed and analyzed for the presence of neoplastic foci. X-ray images were captured with the following settings: t=2 min, f-stop=5.57 and FOV=198.6. The visualization of metastases was performed under the following conditions: t=3 min, binning 2x2, FOV=198.6, f-stop=5.57. Images were analyzed using Carestream MI SE version 5.0 software. The intensity of the luminescence was determined as the sum intensity of the region of interest and expressed in arbitrary units.

Histopathological examination. To detect micrometastases, axillary and inguinal lymph nodes and lungs were excised and fixed in 6% buffered formalin at room temperature for 1 week. To quantify the density of tumor blood vessel, samples of tumor tissue were excised and fixed in buffered formalin. Paraffin-embedded samples of tissues were cut into 4-μm-thick sections, then dewaxed with xylene and rehydrated in decreasing concentrations of ethanol. Finally, the slides were washed in distilled water. The cytoplasm was stained at room temperature with 1% eosin (Aqua Med Sp. z o.o.) for 3 sec and the nuclei were counterstained with 1% hematoxylin (Merck KGaA) for 5 min. at room temperature. The stained sections were dehydrated in an alcohol gradient and mounted with a coverslip. The number of metastases in the lungs and lymph nodes was counted at a magnification of x40 or x400. Microvessel density was quantified at a magnification of x200 in five intratumoral areas of the lesion and

Table I. Treatment strategy for mice bearing 4T1 or 4T1-luc2-tdTomato cells in orthotopic or intracardiac model, respectively.

			Commencement of treatment		Scheme of treatment	
Compound	Total dose	Route	Orthotopic model	Intracardiac model	Orthotopic model	Intracardiac model
WG12399C	50 mg/kg	i.v.	50 mm^3 tumor volume	6 Days prior tumor cells injection	Every 6 days	Day-6, -1, 5 and 9[a]
WG12592A	5 mg/kg					
Zoledronate	100 μg/kg					
CY	200 mg/kg	i.p.			Three times a week	Three times a week starting from day-6

[a]Day of tumor cell inoculation counted as day 0. CY, cyclophosphamide; i.v., intravenous.

the final score was expressed as a mean of the five analyzed areas.

Blood analysis. Blood was collected into heparinized tubes, and then analyzed using the Mythic 18 hematology analyzer (C2 Diagnostics). For biochemical analyses, blood plasma was obtained by the centrifugation of blood at 2,000 x g for 15 min at 4˚C. Alkaline phosphatase, alanine and aspartate aminotransferases, albumin, cholesterol and calcium were measured in the plasma samples using the Cobas c 111 z ISE device (Roche Diagnostics).

Measurement of plasma levels of cytokines. The expression of selected proteins was determined in tumor homogenates and plasma. Thus, blood samples were collected from the animals prior to euthanasia and immediately centrifuged at 2,000 x g for 15 min at 4˚C. The samples of plasma were stored at -80˚C for further analysis. Frozen tumor tissue was homogenized in RIPA buffer (MilliporeSigma) containing a cocktail of protease and phosphatase inhibitors (MilliporeSigma) using the Fast Prep®-24 MP Bio homogenizer (MP Biomedicals LLC). Following 20 min of incubation on ice, the samples were centrifuged (4˚C, 15 min, 12,000 x g), and the supernatants were transferred to new tubes, centrifuged again, and then kept at -80˚C for further analysis. Prior to the enzyme-linked immunosorbent assays (ELISAs), the protein concentration was quantified using the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Inc.). ELISAs were performed according to the manufacturer's protocols. The absorbance (at 450 nm) or chemiluminescence were read using the BioTek Hybrid H4 reader (BioTek Instruments, Inc.). Based on the standard curves, the concentrations of the following proteins in the test samples were calculated: Vascular endothelial cell growth factor A (VEGF-A; KMG0112, Thermo Fisher Scientific, Inc.), matrix metalloproteinase (MMP)-2 (E-EL-M0780), MMP-9 (E-EL-M0627) and transforming growth factor (TGF)-β1 (E-EL-M0051) (all from Elabscience Biotechnology Co., Ltd.).

In vitro experiments

Cells and cell culture. Mouse mammary adenocarcinoma 4T1 cells were purchased from ATCC (cat. no. CRL 2539). The cells were cultured in RPMI-1640 medium (Laboratory of Analytical Chemistry, IIET) with Opti-MEM® (Thermo Fisher Scientific, Inc.) (1:1, v/v). The medium was supplemented with 5% FBS (HyClone, Thermo Fisher Scientific, Inc.), 2 mM glutamine, 4.5 g/l glucose, 1.0 mM sodium pyruvate (all from Merck KGaA), and antibiotics (penicillin and streptomycin; Polfa Tarchomin). The 4T1-luc2-tdTomato-, luciferase- and red fluorescent protein (tdTomato)-expressing variant of the 4T1 cell line was obtained from Caliper Life Sciences, Inc. The cells were cultured in RPMI-1640 + Gluta-MAX™ medium (Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Merck KGaA) and antibiotics. Both cell lines were cultured under standard conditions (37˚C, humidified atmosphere, 5% CO_2).

Combination with cytostatics. The cells were seeded in 96-well plates (Sarstedt, Inc.) at a density of $1x10^4$ cells/well in 100 μl culture medium without FBS and antibiotics, and immediately pre-treated with WG12399C (5 μg/ml) or WG12592A (10 μg/ml), or one of the cytostatics: doxorubicin (1,000, 100, 10 and 1 ng/ml, MilliporeSigma), 5-fluorouracil (5-FU) (10, 1, 0.1 and 0.01 μg/ml, EBEWE Pharma Ges.m.b.H), or paclitaxel (10, 1, 0.1 and 0.01 μg/ml, Fluorochem Ltd.). BPs at serial concentrations of 100, 50, 25, 10, 5, 2.5, 1, and 0.1 μg/ml were added to control cells for the calibration curve. The plates were incubated under standard conditions for 24 h. Subsequently, doxorubicin, 5-FU, paclitaxel or BPs (12.5 μg/ml of WG12399C or 10 μg/ml of WG12592A) were added to obtain the following combinations: i) Pre-treatment with BPs followed by cytostatics; ii) pre-treatment with cytostatics followed by BPs; and iii) BPs and cytostatics applied simultaneously. The concentrations of the BPs applied in this assay were based on the calibration curves and did not exceed an inhibitory concentration $(IC)_{20}$. Untreated cells, as well as cells treated with BPs or cytostatics alone were used as controls. Following an additional 72 h of incubation under standard conditions, sulforhodamine B (SRB) assay was performed as previously described by Skehan *et al* (22). The absorbance of the samples was read using a Synergy H4 Hybrid Reader (BioTek Instruments, Inc.) at a wavelength of 540 nm. In total, two reference agents were used: Incadronic acid, which exhibits a chemical similarity to that of the test compounds, and zoledronic acid, with the highest antiproliferative activity among N-BPs.

To define the interactions between the BPs and cytostatics, the results of SRB assay were analyzed using the CalcuSyn program (Biosoft). Based on the method described by Chou and Talalay (23,24), the combination index (CI) was calculated. Values of CI <1, =1 and >1 indicate synergism, an additive effect and antagonism, respectively.

GGOH-FOH assay. The cells were seeded in 96-well plates at a density of $1x10^4$ cells/well in 100 μl culture medium. After 24 h, 50 μl of either 10 or 20 μM geranylgeraniol (GGOH; Enzo Life Sciences, Inc.) or farnesol (FOH; Merck KGaA) at a concentration of 5 or 10 μM was added to the cells for 1 h in 37˚C. The cells were then incubated with WG12399C or WG12592A (100, 10, 1 and 0.1 μg/ml) for an additional 48 h. Untreated cell, as well as cells treated with WG12399C, WG12592A or zoledronate alone served as controls. The anti-proliferative activity of BPs was determined using SRB assay as described above. Prolab-3 software version 4.0 Professional (INFORM-TECH) was used for the calculation of the IC_{50} values of the compounds.

Cell cycle analysis. At 24 h prior to treatment, the 4T1 and 4T1-luc2-tdTomato cells were seeded in 35-mm Petri dishes at a density of $1.5x10^4$ cells/ml in 4 ml of the culture medium. The cells were then treated with WG12399C at a concentration of 22.1 μM and WG12592A at a concentration of 16.8 μM for 24, 48 and 72 h. Untreated cells and cells treated with zoledronic acid (8.6 μM) were used as controls. The concentrations of BPs applied in this assay were based on the calibration curves from antiproliferative tests and did not exceed IC_{20}. Following incubation with compounds under standard conditions, the cells were trypsinized, washed twice with cold PBS and fixed for at least 24 h in 70% (v/v) ethanol at -20˚C. The cells were then washed again and 4'6-diamidino-2-phenylindole (DAPI) staining solution [1 μg/ml, Triton X-100 0.1% (v/v); Merck KGaA] was added to each sample. After 30 min of incubation on ice, the samples were analyzed using the BD LSRFortessa cytometer (BD Biosciences) and the ModFit LT 3.0 program (Verity Software House).

Measurement of caspase-3 activity. The 4T1 and 4T1-luc2-tdTomato cells were seeded in 24-well plates at a density of $3x10^3$ cells/ml in the culture medium. The cells were cultured overnight, and treated with WG12399C at a concentration of 23.9 μM or WG12592A at a concentration of 23.6 μM for 24, 48 and 72 h. Untreated cells and cells treated with zoledronic acid (24.1 μM) were used as controls. The concentrations of BPs applied in this assay were based on the calibration curves from antiproliferative tests and did not exceed IC_{30-40}. Following incubation under standard conditions, the cells were lysed for 30 min with ice-cold lysis buffer [50 mM HEPES, 150 mM NaCl, 10% saccharose, 5 mM EDTA, 0.1% Triton-X 100, 10 mM dithiothreitol (DTT), pH 7.5] (IIET). Subsequently, 40 μl of each sample was transferred to an opaque, 96-well plate (Corning, Inc.) containing 160 μl of the reaction buffer (10 μM Ac-DEVD-ACC substrate, 20 mM HEPES, 100 mM NaCl, 10% saccharose, 1 mM EDTA, 10 mM DTT, pH 7.5) (IIET). The fluorescence of each sample was continuously recorded for 2 h at 37˚C using the BioTek Synergy H4 Hybrid Reader (λ_{ex}=360 nm, λ_{em}=460 nm). In parallel, the SRB assay was performed in order to normalize to the protein content. The results obtained are expressed as mean relative caspase-3/7 activity in comparison to the untreated cells ± standard deviation (SD).

Annexin-V assay. The 4T1 cells were seeded in 24-well plates at a density of $3x10^3$ cells/ml in the culture medium. The cells were cultured overnight and treated with WG12399C at a concentration of 23.9 μM and WG12592A at a concentration of 23.6 μM for 24, 48 and 72 h. Untreated cells and cells treated with 24.1 μM zoledronic acid were used as controls. The concentrations of BPs applied in this assay were based on the calibration curves from antiproliferative tests and did not exceed IC_{30-40}. Following incubation under standard conditions, the cells were harvested, washed with PBS and suspended in 200 μl binding buffer (10 mM HEPES/NaOH, 140 mM NaCl, 2.5 mM $CaCl_2$, pH 7.4) containing 5 μl of APC-Annexin V (Enzo Lifesciences Ltd.). The cells were stained for 15 min in the dark at room temperature, and were then washed again in PBS, and suspended in 190 μl binding buffer. Prior to the FACS analysis, 5 μl DAPI solution (2 mg/ml) were added for 15 min at room temperature. The cells were analyzed using the BD LSRFortessa cytometer (BD Biosciences) and the Diva Software version 6.2 program. Based on the obtained two-color dot plots, cells were categorized as follows: $DAPI^+$/Annexin V^+ were apoptotic cells, $DAPI^-$/Annexin V^+ were early apoptotic cells, $DAPI^+$/Annexin $V^{+/-}$ were necrotic cells, and double-negative cells were live cells.

Migration and invasion assay. The 4T1 and 4T1-luc2-tdTomato cells were seeded in 24-well plates at a density of $5x10^3$ cells/ml in 500 μl culture medium. The cells were cultured for 24 h and then treated for an additional 48 h with WG12399C (at a concentration of 23 μM for 4T1 cells and 20.1 μM for 4T1-luc2-tdTomato cells) and WG12592A (at a concentration of 20.2 μM for 4T1 cells and 16.8 μM for 4T1-luc2-tdTomato cells). The concentrations of BPs applied in this assay were based on the calibration curves from antiproliferative tests and did not exceed IC_{20-30}. Untreated cells and cells treated with zoledronic acid were used as controls. For the migration and invasion assay, the cells in the number of $2x10^4$ cells/insert were applied on the upper side of Matrigel-coated inserts (BioCoat Matrigel Invasion Chamber, BD Biosciences) placed in the bottom chambers with the culture medium containing 20% FBS as a chemoattractant. The migration and invasion assay was performed under standard culture conditions for 24 h. The cells on the top of the membrane were then carefully removed using a cotton swab moistened with PBS, and the cells that migrated to the bottom side of the porous membrane were fixed in Diff-Quick Fix, stained with Diff-Quick I and II (for 2 min in each solution at room temperature) (Medion Diagnostics, Düdingen, Switzerland), and counted under a light microscope (Olympus Corporation). The assay was repeated at least three times.

Statistical analysis. The results were analyzed using GraphPad Prism 7.03 (GraphPad Software Inc.). The Shapiro-Wilk's normality test and Bartlett's test were used to confirm the assumptions for the analysis of variance (ANOVA). Following ANOVA, Dunnett's or Tukey's multiple comparisons test were used. For non-parametric data, the Kruskal-Wallis test with Dunn's multiple comparison test was applied. A value of $P<0.05$ was considered to indicate a statistically significant difference.

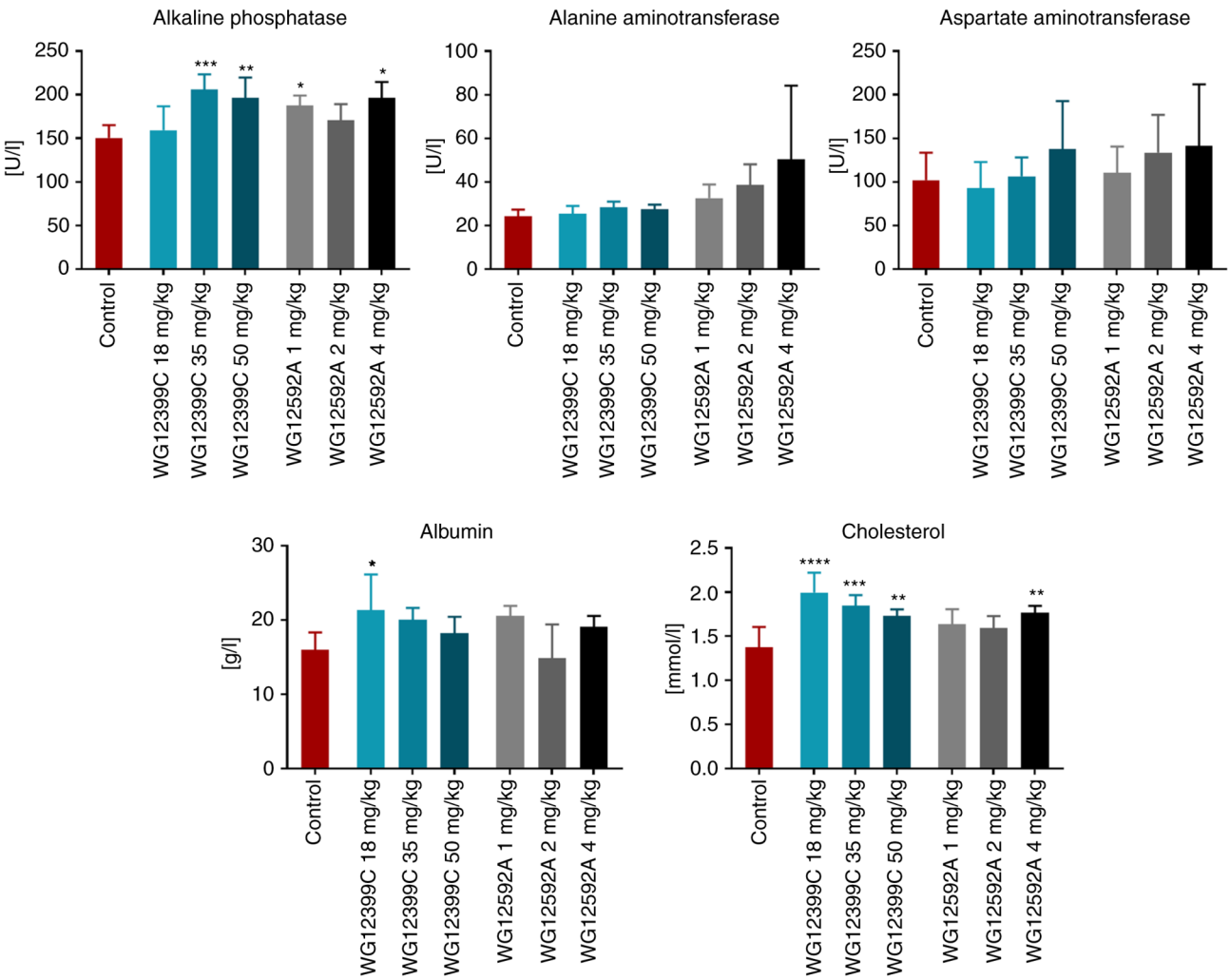


Figure 2. Changes in the biochemical parameters of blood in mice receiving WG12399C and WG12592A. Bisphosphonates were administered intravenously at the following total doses divided into four weekly injections: WG12399C at 18, 35 and 50 mg/kg; WG12592A at 1, 2 and 4 mg/kg. Data are expressed as the mean ± SD, n=5. *P<0.05, **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control, assessed using one-way ANOVA with Dunnett's multiple comparisons tests.

Results

Subacute toxicity of WG12399C and WG12592A. In previous research by the authors, the LD_{50} values of the studied BPs were determined and these were as follows: 73.8 mg/kg for WG12399C and 3.3 mg/kg for WG12592A (17). Thus, the total doses of the compounds selected for subacute toxicity tests were as follows: 18, 35 and 50 mg/kg for WG12399C, and 1, 2 and 4 mg/kg for WG12592A. These total doses were divided into four weekly i.v. injections. It was observed that neither of the compounds affected the overall well-being of the mice, as manifested by an increase in body weight during the course of the experiment (17). No treatment-related deaths were recorded. Moreover, no significant differences in the weights of internal organs excised following euthanasia were observed between the control and treated animals (Fig. S1). In addition, the studied BPs did not induce any significant changes in blood morphology that may be attributed to the toxicity of these compounds. The only significant BP-induced change in morphological parameters was an increase in the number of erythrocytes in the animals treated with the highest dose of WG12399C as compared with the control mice (Fig. S2).

The analysis of blood biochemical parameters at the end of the experiment revealed an increase in the activity of alkaline phosphatase in the groups of mice administered WG12399C and WG12592A. The concentration of albumin was also increased in the mice treated with a low dose of WG12399C (Fig. 2). The activity of alanine and aspartate aminotransferases was also found to be elevated in the groups administered higher doses of BPs (although the difference was not statistically significant). The administration of WG12399C and WG12592A (at higher doses) also led to an increase in cholesterol concentrations in the blood. Nevertheless, the weekly doses of WG12399C (e.g., 12.5 mg/kg) and WG12592A (e.g., 1 mg/kg) were well-tolerated by the animals and the BPs did not evoke any clinical signs of toxicity. Based on these observations and the data from previous research (17), the following total doses, which were divided into four and administered every 6th day, were selected for treatment of the mice: WG12399C at 50 mg/kg and WG12592A at 5 mg/kg.

Antitumor activity of WG12399C and WG12592A. The 4T1 cells were injected orthotopically into the mammary fad pads of 8- to 10-week-old syngeneic BALB/c mice. The animals were observed and the kinetics of tumor growth were recorded. As presented in Fig. 3A, the treatment applied in the experiment did not significantly affect the mean weight of the animals in any of the groups. In contrast to the reference agent, cyclophosphamide, which inhibited primary tumor growth by 46%, none of the tested BPs affected the kinetics of tumor growth in a significant manner (Fig. 3B). However, despite the lack of antitumor activity, WG12399C inhibited lung metastases. The number of macroscopically visible metastatic foci in the lungs of mice receiving WG12399C was reduced by ~66% in comparison to the control (Fig. 3C).

On day 28 of the experiment, the animals were euthanized, and the internal organs were excised, weighed and histologically analyzed. The post-mortem analysis of the internal organs revealed that the weights of the lungs, heart and ovaries were reduced in the mice receiving WG12399C as compared to the control animals (Fig. 4A). However, although these changes were evident, the differences were not statistically significant. The histopathological analysis of the lungs revealed the presence of 4T1 cell-derived metastases in 88% of the WG12399C-treated animals and in 100% of the animals in the remaining groups (Fig. 4B and C). The differences in the size and number of metastatic lesions were recorded. In the control group, as well as in the WG12592A- and zoledronate-treated mice, the metastatic foci were numerous, large subpleural and intrapleural. In the cyclophosphamide group, numerous large and small lesions were observed in the lungs, whereas in the lungs of the WG12399C-treated mice, single large or numerous, yet small foci prevailed. Moreover, WG12399C and WG12592A impaired the formation of metastases in the sentinel lymph nodes. Metastatic foci were observed only in 2 out of 8 mice, and in 5 out of 9 mice in the WG12399C- and WG12592A-treated groups, respectively, whereas in the untreated group, tumor metastases were noted in 8 out of 9 animals. Zoledronic acid did not prevent the metastasis of 4T1 tumors to the lungs or lymph nodes (Fig. 4B).

The anti-metastatic effects of the BPs were further evaluated in the experimental metastasis model of 4T1-luc2-tdTomato cells that were intracardially inoculated into a tail vein of female BALB/c mice. The 4T1-luc2-tdTomato cells are derived from a parental 4T1 cell line, labeled with fluorochrome, and they express luciferase (25). Thus, these cells may be visualized in the internal organs, and allow the monitoring of the metastasis process. The intracardiac injection of cancer cells successfully leads to their colonization of internal organs, such as the lungs, kidneys, spleen, ovaries and bones (26-29). In the present study, mice were pre-treated with BPs at 1 and 6 days prior to transplantation, and the treatment was repeated on days 4 and 9. The post-mortem visualization (day 11) of the metastatic lesions in the internal organs revealed that the administered BPs affected the metastasis of 4T1-luc-tdTomato cells in BALB/c mice at a similar rate as observed for cyclophosphamide (Figs. 5 and S3). The luminescence analysis of the lungs revealed a reduction in the size of metastatic lesions in the mice treated with either WG12399C or WG12592A (Fig. 5A). Furthermore, treatment with WG12399C reduced the incidence of tumor metastases in the lungs from 42% in the control mice to 27% in the mice

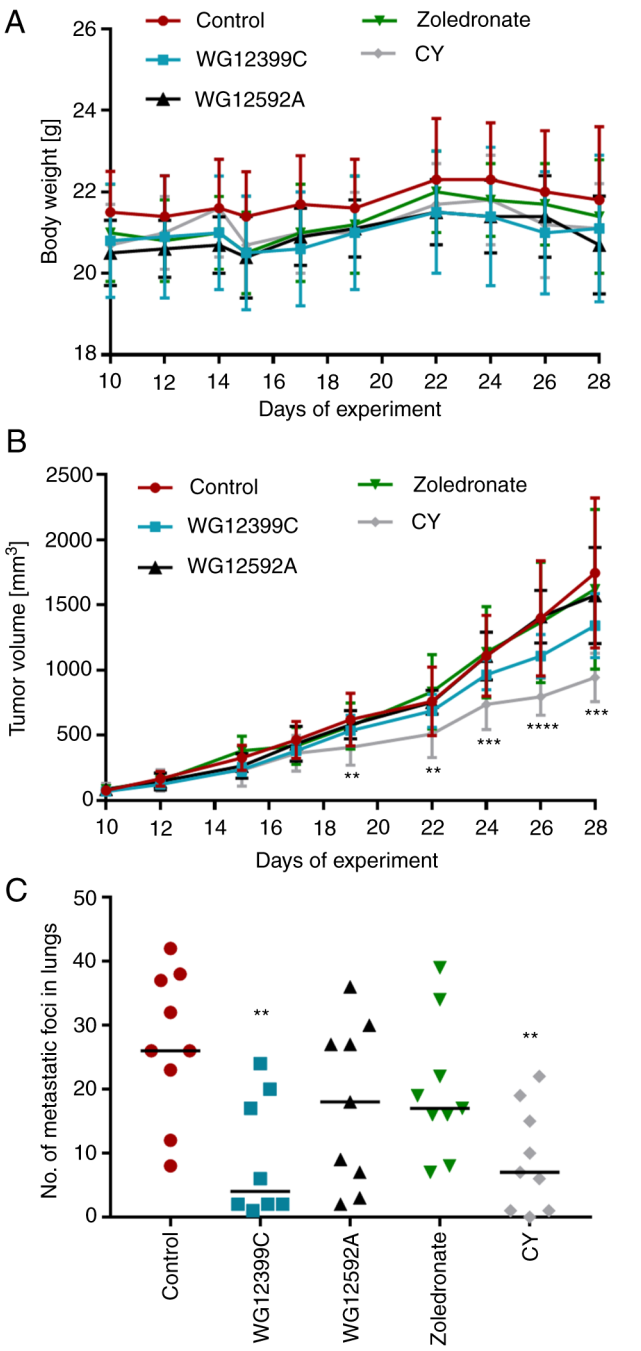
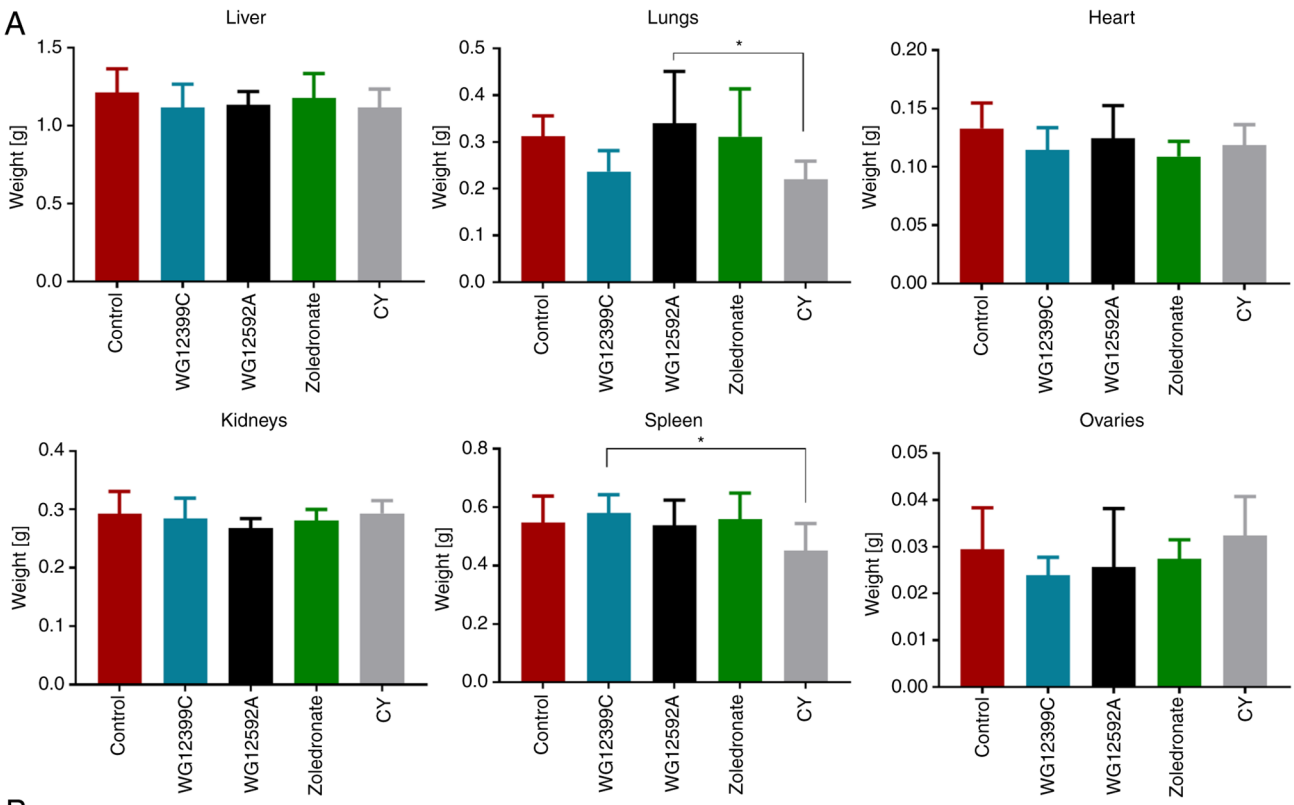


Figure 3. Effect of bisphosphonates on 4T1 tumor progression. (A) Body weight changes. (B) Kinetics of primary tumor growth. (C) Number of macroscopically visible metastatic foci in the lungs on day 28. Mice were inoculated orthotopically with 4T1 cells (day 0). From day 8, the mice began receiving treatment in the following regimens: WG12399C, total dose of 50 mg/kg, intravenous administration, every 6th day; WG12529A, total dose of 5 mg/kg, intravenous administration, every 6th day; zoledronate, total dose of 100 μg/kg, intravenous administration, every 6th day; and CY, total dose of 225 mg/kg, intraperitoneally, three times a week. n=8-9 mice per group. Data are presented as (A and C) the mean ± SD, or as (B) data for individual animals with median values. **P<0.01, ***P<0.001 and ****P<0.0001 vs. the control, assessed using one-way ANOVA with Dunnett's multiple comparisons tests. CY, cyclophosphamide.

in the WG12399C group (Fig. 5B). The effects of the BPs on other internal organs varied. In total, 2 out of the 12 control mice were diagnosed with spleen metastases, whereas no such metastases were recorded in the treated groups. However, no



B

Organ		Control	WG12399C	WG12592A	Zoledronate	CY
Lungs	Incidence of metastases	9/9 (100%)	7/8 (88%)	9/9 (100%)	9/9 (100%)	9/9 (100%)
	Histological score±SD	4±0	2.4±1.6*	4±0	4±0	3.3±1.0
Sentinel lymph nodes	Incidence of metastases	8/9 (89%)	2/8 (25%)	5/9 (56%)	9/9 (100%)	2/9 (22%)

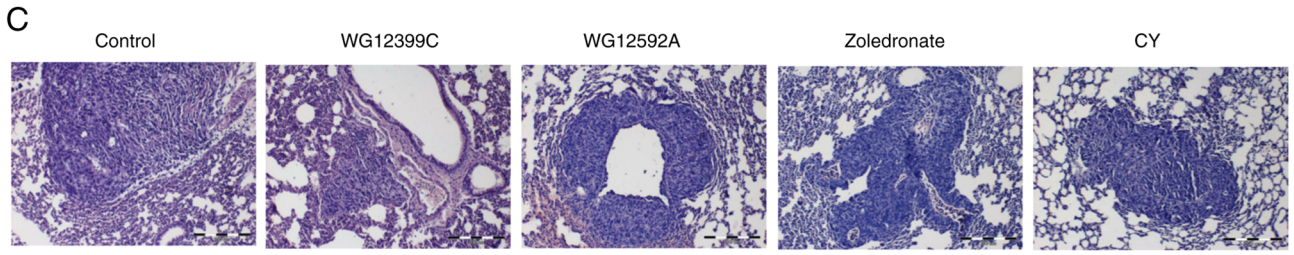
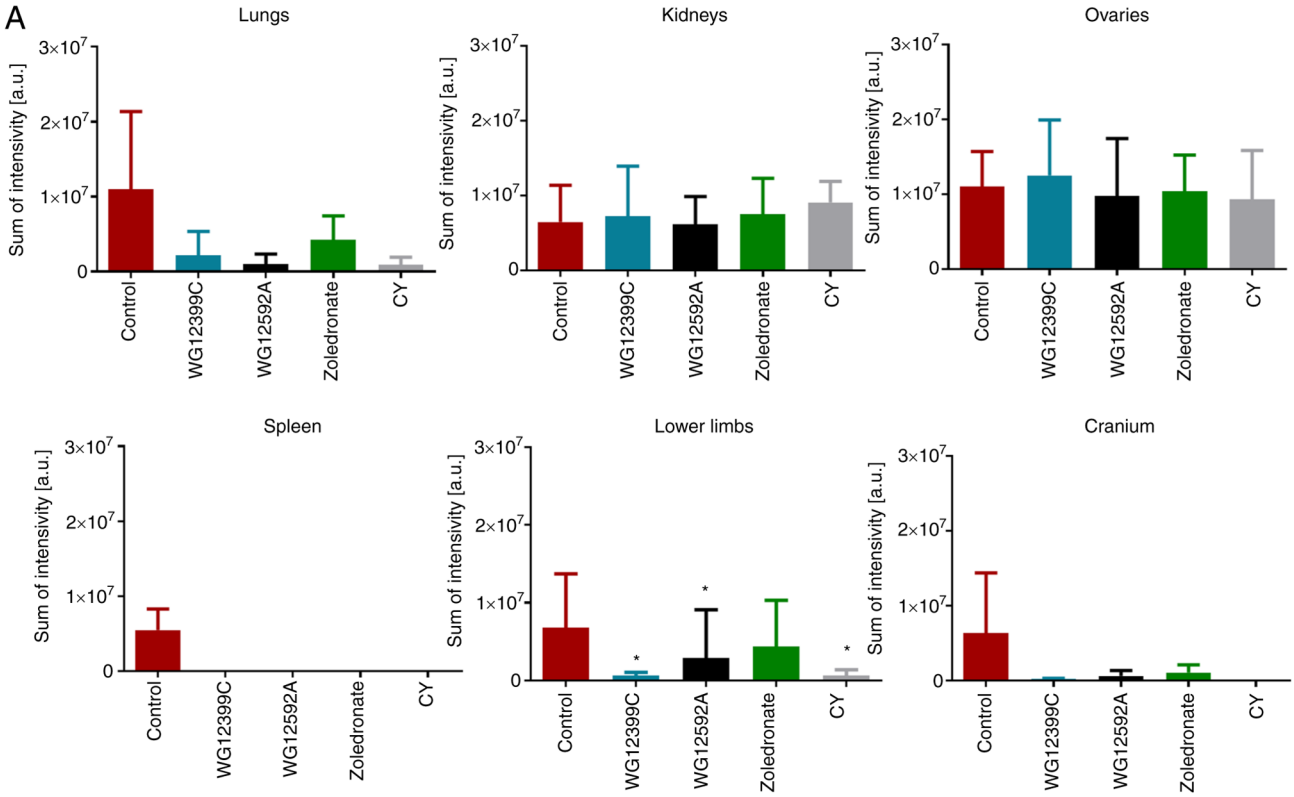


Figure 4. Effect of bisphosphonates on 4T1 metastasis. (A) Weights of the internal organs. (B) Incidence of histologically confirmed metastatic foci in the lungs and lymph nodes on day 28. (C) Representative images of metastases in the lungs; n=8-9 mice per group. Data are presented as (A and B) the mean ± SD. Histological scoring: 0, no metastatic foci; 1, single small foci; 2, single large foci; 3, numerous small foci; 4, numerous large foci. *P<0.05, assessed using one-way ANOVA with Tukey's multiple comparisons tests (weight organs, vs. control) or with the Kruskal-Wallis test with Dunn's multiple comparisons tests (histological scoring, vs. control, WG12592A and zoledronate). CY, cyclophosphamide.

marked differences were observed for metastasis in the livers, kidneys, or ovaries. In turn, treatment with BPs had a beneficial effect on tumor metastasis to the bones, although the incidence of bone metastases was not significantly affected (Fig. 5B); however, both WG12399C and WG12595A markedly reduced the size and/or the number of metastatic foci in the crania and bones of lower limbs (Fig. 5A). The reference agent, zoledronate, reduced the number of mice diagnosed with metastases in long bones; however, its effects on the size of metastases were less pronounced. These results suggest that WG12399C and WG12592A BPs significantly prevent the metastasis of 4T1 cells to secondary sites, not only including bones, but also internal organs.

Expression of proteins involved in tumor progression in plasma and tumor tissue. On day 28 following the inoculation of 4T1 cells into BALB/c mice, samples of plasma and tumor tissue were collected and the concentrations of several proteins involved



B

	Liver	Lungs	Kidneys	Spleen	Ovaries	Lower limbs	Cranium
Control	0% (0/12)	42% (5/12)	92% (11/12)	17% (2/12)	67% (8/12)	50% (6/12)	17% (2/12)
WG12399C	18% (2/11)	27% (3/11)	73% (8/11)	0% (0/11)	45% (5/11)	45% (5/11)	18% (2/11)
WG12592A	0% (0/11)	36% (4/11)	91% (10/11)	0% (0/11)	82% (9/11)	64% (7/11)	27% (3/11)
Zoledronate	8% (1/13)	31% (4/13)	85% (11/13)	0% (0/13)	62% (8/13)	31% (4/13)	23% (3/13)
CY	0% (0/11)	82% (9/11)	91% (10/11)	0% (0/11)	82% (9/11)	36% (4/11)	0% (0/11)

Figure 5. Anti-metastatic effects of BPs in the experimental metastasis model of 4T1-luc2-tdTomato cells. (A) Sums of luminescence intensity in the internal organs excised on day 11 of the experiment. (B) Incidence of metastases in the internal organs; n=11-13 mice per group. Data are presented as the (A) mean ± SD. *P<0.05 vs. control, assessed using one-way ANOVA with Dunnett's multiple comparisons tests. CY, cyclophosphamide.

in tumor growth and progression were measured. The plasma levels of VEGF and MMP-9 were significantly elevated in the untreated control mice inoculated with 4T1 cells compared to the healthy animals (Fig. 6A). In the tumor-bearing untreated control mice, the concentration of TGF-β1 was increased by 31% compared to the healthy animals; however, the difference was not statistically significant. Treatment with WG12399C and WG12592A did not affect the plasma levels of VEGF or MMPs in a significant manner. An increase in the plasma concentration of TGF-β1 was observed in the mice treated with the BPs compared to the untreated control animals, with the differences ranging from 55% (for WG12399C and WG12595A) to 151% (for zoledronate, P=0.0019). No significant changes in the levels of MMP-2 and TGF-β1 in tumor tissue were observed in the mice receiving treatment in comparison to the untreated control group (Fig. 6B). The administration of zoledronate led to a significant decrease in the tumor concentration of VEGF and MMP-9. By contrast, such effects were not observed in the animals treated with WG12399C and WG12592A. This may suggest that the antitumor activity of WG12399C and WG12592A is related to mechanisms other than the modulation of the tumor microenvironment.

Anti-proliferative activity of WG12399C and WG12592A alone and in combination with cytostatics. As previously reported, WG12399C and WG12592A exhibit antiproliferative

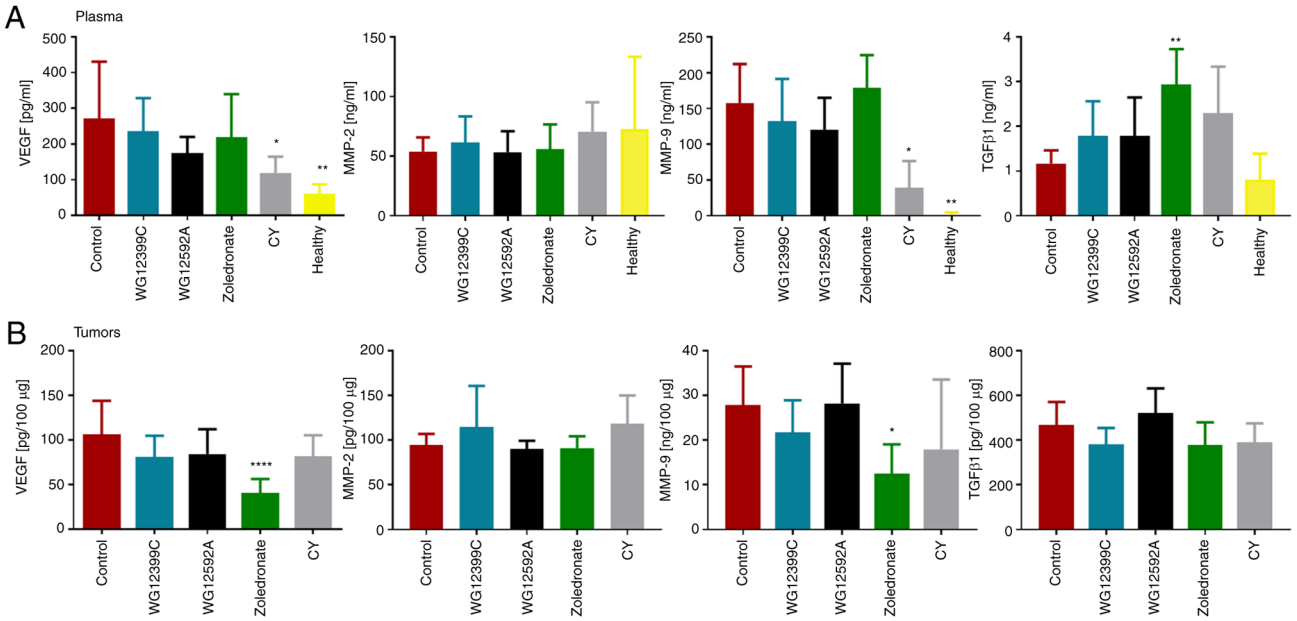


Figure 6. Expression of proteins involved in tumor progression in (A) plasma and (B) tumors of mice inoculated with 4T1 cells. Samples of plasma and tumor tissue were obtained on day 28 of the experiment. The concentrations of VEGF, MMP-2, MMP-9 and TGF-β1 proteins were assessed using ELISA; n=5-8 samples per group. Data are presented as mean ± SD. *P<0.05, **P<0.01, ****P<0.0001 vs. control, assessed using one-way ANOVA with Dunnett's multiple comparison tests (VEGF, TGF-β1, MMP-9 in tumors, and MMP-2 in plasma) or with Kruskal-Wallis test with Dunn's multiple comparisons tests (MMP-2 in tumors and MMP-9 in plasma). CY, cyclophosphamide; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinase; TGF, transforming growth factor.

activity against HL-60 promyelocytic leukemia, MCF-7 breast cancer cells and J774E macrophages, which are the osteoclast surrogates for *in vitro* studies (15). In the present study, it was observed that WG12399C and WG12592A potently inhibited the proliferation of 4T1 cells at IC_{50} values of 54.3 and 63.2 μM, respectively, in comparison to the IC_{50} value of 33.5 μM calculated for zoledronate (Fig. 7). Furthermore, the anti-proliferative effects of WG12399C and WG12592A against 4T1 cells were not abrogated by GGOH and FOH, which are intermediate metabolites in the mevalonate pathway with a proven ability to reverse the effects of amino BPs (30,31). Contrary to the positive control, zoledronate, the IC_{50} values of the WG12399C and WG12592A BPs applied alone or following pre-treatment with GGOH or FOH did not vary significantly. These results indicate the different mechanisms of action of zoledronate and tested the BPs.

Further analyses revealed that WG12399C and WG12592A augmented the anti-proliferative activity of doxorubicin, 5-FU and paclitaxel (Table II). The cells were simultaneously treated with the BPs and cytostatics or pre-treated with a BP or cytostatic 24 h prior to the addition of the second compound. The most pronounced effects were observed with the combination of BPs and paclitaxel, irrespective of the treatment schedule. The CI values for the BP + paclitaxel combinations ranged from 0.042 to 0.782. A strong synergism was also recorded for the combination of WG12592A with 5-FU or low doses of doxorubicin, regardless of the order of treatment of the cells with these compounds. The effects of WG12399C on the anti-proliferative activity of 5-FU and doxorubicin was complex, and was found to be dependent on the concentration of cytostatics and the treatment schedule. Both synergistic and antagonistic effects were observed.

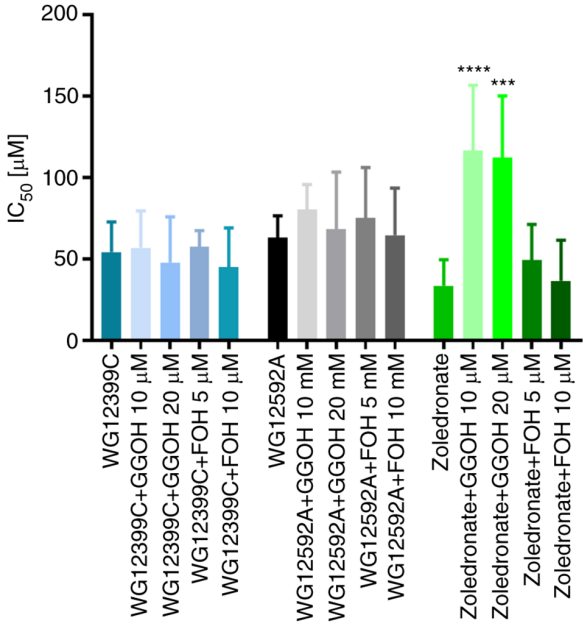


Figure 7. Effect of GGOH and FOH on the anti-proliferative activity of WG12399C, WG12592A and zoledronate. The 4T1 cells were pre-treated with either GGOH or FOH at a concentration of 10 or 20 μM for 1 h. WG12399C or WG12592A were then added at serial concentrations for a further 48 h. Cells treated with BPs alone and untreated cells served as controls. The experiment was repeated at least three times. Data are presented as the mean ± SD. ***P<0.001 and ****P<0.0001 vs. the zoledronate-treated group, assessed using one-way ANOVA with Tukey's multiple comparisons tests. GGOH, geranylgeraniol; FOH, farnesol.

Effect of BPs on cell cycle progression. To investigate the mechanisms underlying the anti-proliferative activity of WG12399C and WG12592A against 4T1 cells, the effects of these BPs on the

Table II. CI for combined treatment of WG12399C and WG12592A and cytostatics against 4T1 cells.

WG12399C[a]

	Doxorubicin (μg/ml)				5-Fluorouracil (μg/ml)				Paclitaxel (μg/ml)			
	0.001	0.01	0.1	1	0.001	0.01	0.1	1	0.001	0.01	0.1	1
WG12399C + cytostatic simultaneously	0.724	0.702	1.489	1.170	1.129	0.655	0.681	1.955	0.782	0.486	0.268	0.532
Pre-treatment with WG12399C + cytostatic	1.727	0.934	1.078	1.424	2.317	0.836	0.787	1.902	1.412	0.681	0.413	0.593
Pre-treatment with cytostatic + WG12399C	1.790	0.922	0.684	0.752	1.523	1.347	0.487	0.954	1.376	0.449	0.304	0.730

WG12592A

	Doxorubicin (μg/ml)				5-Fluorouracil (μg/ml)				Paclitaxel (μg/ml)			
	0.001	0.01	0.1	1	0.001	0.01	0.1	1	0.001	0.01	0.1	1
WG12592A + cytostatic simultaneously	0.275	0.675	1.287	1.604	0.627	0.345	0.375	2.301	0.220	0.092	0.126	1.130
Pre-treatment with WG12592A + cytostatic	0.564	0.447	0.952	1.037	0.522	0.611	0.348	1.833	0.537	0.158	0.072	0.635
Pre-treatment with cytostatic + WG12592A	0.610	0.447	1.220	1.156	0.429	0.394	0.421	1.692	0.074	0.042	0.176	1.037

[a]WG12399C was applied at a dose of 23 μM (simultaneous treatment) and 9.2 μM (pre-treatment). WG12592A was applied at a dose of 16.8 μM (pre-treatment and simultaneous treatment). CI values were calculated using the CalcuSyn program. Values <1 indicated synergism, values=1 indicated additivity, and values >1 indicated antagonism. Underlined values indicate a synergistic effect.

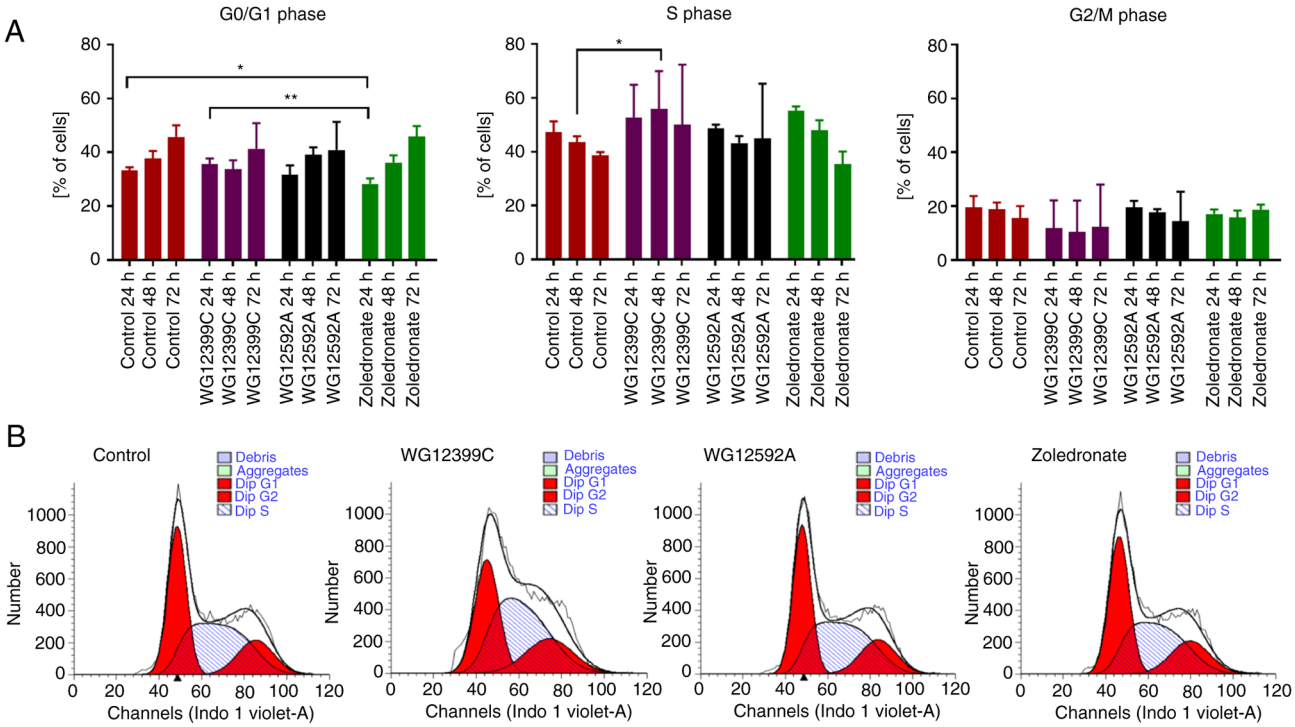


Figure 8. Effect of bisphosphonates on cell cycle progression in 4T1 cells (A) treated with 22.1 μM WG12399C, 16.8 μM WG12592A, or 8.6 μM zoledronate. Untreated 4T1 cells were used as controls. The experiment was repeated at least three times. The cellular DNA content was assessed using the BD LSRFortessa cytometer and ModFit LT 3.0 program. Data are presented as the mean ± SD. *P<0.05 and **P<0.01, assessed using one-way ANOVA with Dunnett's multiple comparisons tests. (B) Exemplary histograms obtained at 48 h of incubation.

cell cycle progression were examined. The 4T1 cells were incubated with the compounds at their IC_{20} concentrations for 24, 48 and 72 h. WG12592A did not significantly affect the progression of the cell cycle (Fig. 8). On the other hand, zoledronate led to the transitory arrest of cell cycle progression in the S phase, leading to an increase in the number of cells in this phase from 47% (control) to 55% after 24 h (although the differences were not statistically significant), as previously described (32,33). The most profound effect was observed in the 4T1 cells treated with WG12399C for 48 h, where the number of cells in the S phase increased from 44% (control) to 56% (P=0.0492).

Pro-apoptotic activity of BPs. Since the cytostatic activity may result not only from the inhibition of the cell cycle, but also from the direct pro-apoptotic effects of the compounds, the effects of BPs on apoptosis were investigated. The most substantial pro-apoptotic activity in the 4T1 cells was observed for WG12399C compared to the untreated control and the reference agent, zoledronate (Fig. 9). The pro-apoptotic activity of the WG12399C BP increased in a time-dependent manner. Following incubation for 24 h, an almost 2-fold increase in the activity of caspase-3 was noted, while following an additional 48 h, the enzyme activity increased almost 6-fold compared to the control cells (Fig. 9B). In the WG12592A-treated cells, the activity of caspase-3 increased with the time of incubation; however, the highest increase was approximately 2-fold. The enhanced activity of caspase-3 was accompanied by an increase in the percentage of early and late apoptotic BP-treated cells (Fig. 9A and C). Following 24 and 48 h of incubation with WG12399C, the percentage of Annexin V-positive cells (early + late apoptosis) increased 3-fold in comparison to the control. The observed results suggest that the pro-apoptotic effects may be one of the mechanisms of the direct antitumor activity of the WG12399C and WG12592A BPs.

Effects of BPs on the invasiveness of 4T1 cells. The motility of tumor cells is a critical biological characteristic, as it is directly associated with tumor progression and metastasis. The migratory properties of cancer cells are closely related to their invasiveness and enable the cells to leave the primary tumor and colonize distant organs. Therefore, the present study examined the effects of WG912399C and WG12592A on the migratory properties of 4T1 cells and their counterpart 4T1-luc2-tdTomato cells using Matrigel-coated wells. Notably, zoledronate stimulated the invasion of both 4T1 cell lines through Matrigel by ~90% (Fig. 10). By contrast, both WG12399C and WG12592A inhibited the migration of 4T1 cells through porous membranes; however, the effect varied depending on the cell type and compound. In the case of 4T1-luc-tdTomato cells, both WG12592A and WG12399C inhibited migration through Matrigel by >50% (P=0.0192 and P=0.0319, respectively). For the 4T1 cells, the effects were less evident; however, the effects of WG12399C and WG12592A were still beneficial compared to those of the reference agent, zoledronate. The impairment of the invasiveness of 4T1 cells may, at least in part, explain anti-metastatic effects of the WG12399C and WG12592A BPs.

Discussion

Previously, the authors developed a novel efficient method of the synthesis of N-substituted aminomethylidene-BPs (14). Two compounds, benzene-1,4-bis[aminomethylidene(bisph

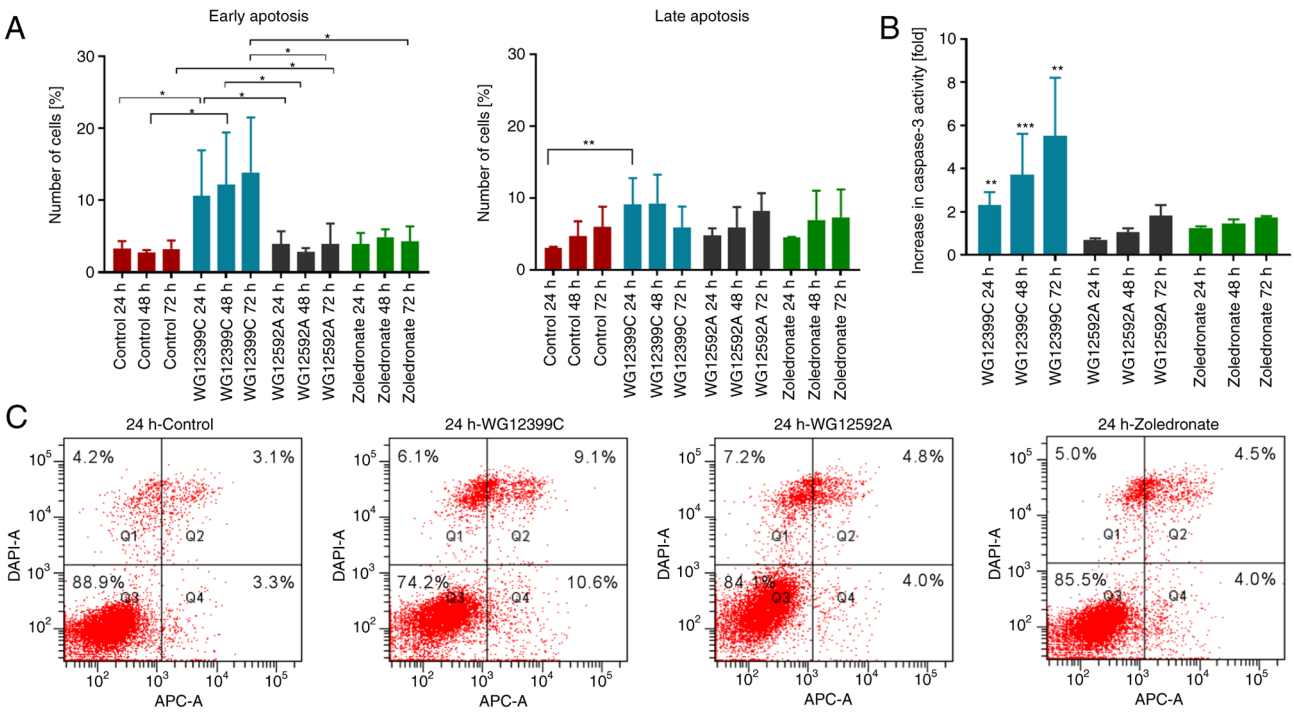


Figure 9. Pro-apoptotic activity of bisphosphonates in 4T1 cells. (A) Effect of WG12399C and on the number of apoptotic cells in the Annexin-V assay. (B) Effect of WG12399C and WG12592A on caspase-3 activity. The results were normalized to the protein content and are expressed as the mean relative caspase-3 activity compared to the untreated cells. (C) Dot plots represent data from 24 h of incubation when the most pronounced differences were recorded. In both assays, the 4T1 cells were treated with 23.9 μM WG12399C, 23.6 μM WG12592A, or 24.1 μM zoledronate for 24, 48, or 72 h. Untreated 4T1 cells were used as controls. Results are reported as mean from at least three independent experiments ± SD. *P<0.05, **P<0.01 and ***P<0.001 vs. the control, assessed using one-way ANOVA with Dunnett's multiple comparison tests (early apoptosis, late apoptosis 48 and 72 h, and caspase-3 activity) or with Kruskal-Wallis test with Dunn's multiple comparisons tests (late apoptosis 24 h).

osphonic)] acid (WG12399C) and naphthalene-1,5-bis[amin omethylidene(bisphosphonic)] acid (WG12592A), exhibited promising antiresorptive and antiproliferative properties. The present study evaluated the anticancer activity of WG12399C and WG12592A in murine models of breast adenocarcinoma. The administered weekly doses (e.g., 12.5 mg/kg for WG12399C and 1 mg/kg for WG12592A) were well-tolerated by the animals and did not induce any clinical signs of toxicity. The only exception was an increase in the activity of alkaline phosphatase and aspartate aminotransferase observed in the groups receiving higher doses of BPs, which may indicate liver damage. However, the histopathological analysis revealed only single lymphocytic infiltrates in some BP-treated mice (Fig. S1).

Much of the clinical and experimental data concerning the anti-metastatic activity of BPs refers to their influence on the metastasis to bone tissue. Local high concentrations of these compounds are achieved in the bone microenvironment and therefore, bone metastases are most likely to be positively affected by BPs (36-38). Clinical studies on the effects of BPs on non-osseous metastases have yielded conflicting results (34,35). The overall effect of BPs on tumor growth may be dependent on several conditions, including the hormone status or the drug doses and regimen (39,41,42). To analyze the effects of WG12399C and WG12592A on primary tumor growth, as well as bone and visceral metastasis, two models of metastasizing 4T1 mammary breast cancer were applied, an orthotopic and intracardiac model. Each of these models has unique advantages. Orthotopically transplanted 4T1 cells form solid primary tumors and metastasize spontaneously to the lungs and lymph nodes, while intracardially injected 4T1 cells efficiently colonize the kidneys, ovaries, brains and bones (40). In the present study, BPs were administered intravenously at the following total doses divided into four weekly injections: WG12399C at 50 mg/kg, WG12592A at 5 mg/kg and zoledronate at 100 μg/kg. A main limitation of the present study was that single doses of the BPs were applied. However, the aim was to evaluate the biological activity of WG12399C and WG12592A in comparison to zoledronate, which is the most active BP used in clinical practice. Thus, the maximum well-tolerated doses of WG12399C and WG12592A were applied in the present study. It was found that neither zoledronate nor the new aminomethylidene-BPs significantly affected the growth of 4T1-derived tumor cells in BALB/c mice. These findings are consistent with the results reported by other authors, in that zoledronate at doses similar to the one applied herein, did not inhibit the growth of murine and human transplantable breast cancers. At higher concentrations, zoledronate has been shown to stimulate the growth of MDA-MB-436 cells (42). Although no inhibitory effects on primary tumors were observed, WG12399C significantly inhibited the formation of spontaneous metastatic foci of 4T1 tumors in the lymph nodes and lungs in comparison to the untreated animals. The number of macroscopically visible foci in the lungs was significantly reduced, which was also reflected by decreased lung weights in comparison to the untreated control animals. Histopathological analyses reveled a decrease in the number of metastases-positive animals and a reduction in the metastatic

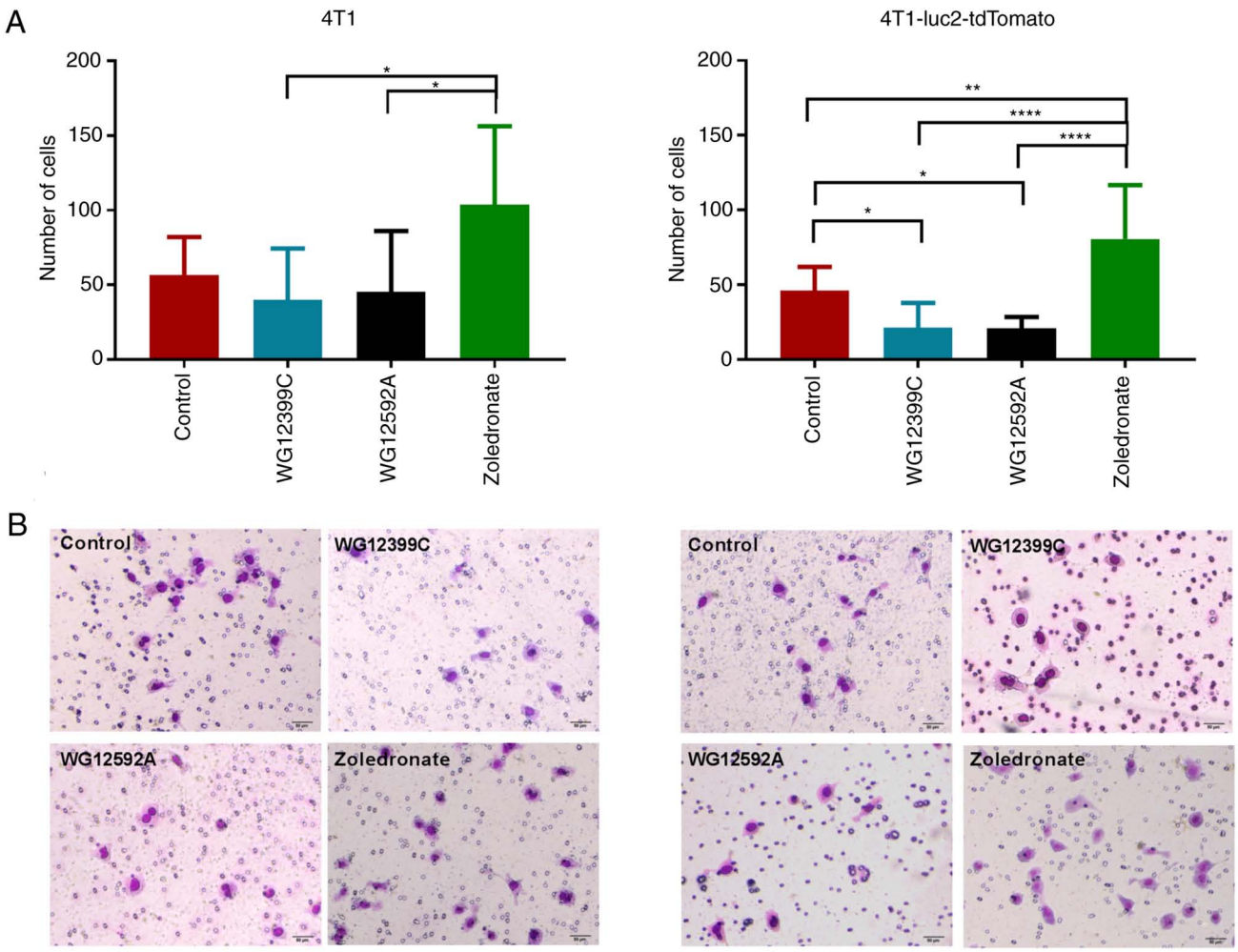


Figure 10. Effects of bisphosphonates on the migratory properties of 4T1 and 4T1-luc2-tdTomato cells (A). 4T1 cells were treated with 23 μM WG12399C, 20.2 μM WG12592A, or 17.2 μM zoledronate, and 4T1-luc2-tdTomato cells with 22.1 μM WG12399C, 16.8 μM WG12592A, or 24.1 μM zoledronate for 48 h. Untreated 4T1 cells served as controls. The cells were seeded on the upper section of the Matrigel-coated invasion chamber at a density of 2.5x104 cells/insert. The number of cells per membrane was determined and the average from at least five experiments. Data are presented as the mean ± SD. *P<0.05, **P<0.01 and ****P<0.0001 vs. the control, assessed using one-way ANOVA with Tukey's multiple comparisons tests (4T1-luc2-tdTomato cells) or with the Kruskal-Wallis test with Dunn's multiple comparisons tests (4T1 cells). (B) Representative images of cell invasion captured.

foci size. Furthermore, pre-treatment and subsequent treatment with WG12399C or WG12592A efficiently prevented the colonization of lungs, crania and bones of lower limbs by 4T1 cells. Of note, the effects observed for these BPs were comparable to those observed for cyclophosphamide and were much more profound compared to that in zoledronate-treated animals.

Several mechanisms have been postulated for the anti-cancer activity of BPs. One of these is the changes in the tumor microenvironment, including reduced vascularization. It has been demonstrated that in zoledronate-treated patients, the plasma levels of VEGF are significantly decreased and this effect is long-lasting (43,44). In the present study, elevated concentrations of plasma VEGF were observed in tumor-bearing animals in comparison to healthy animals, irrespective of the drug administered. In contrast to zoledronate, WG12399C and WG12592A did not reduce the levels of VEGF in tumor tissue. Histopathological analyses did not reveal any notable decrease in the density of tumor vasculature as a result of treatment with the BPs (Table SI).

Metastasis is a multistep process that leads to the formation of secondary tumors in organs distant to the primary site. One of its crucial steps is the invasion of secondary tissue by circulating cells. Thus, to identify the possible mechanism responsible for the anti-metastatic activity of aminomethylidene BPs, the present study evaluated their effects on the invasive potential of 4T1 cells. In the present study, 4T1-luc2-tdTomato cells treated with WG12399C or WG12595A exhibited a markedly reduced invasiveness through Matrigel by 54%. The effect observed in parental 4T1 cells was less pronounced. WG12399C inhibited the invasiveness of 4T1 cells by 29% and WG12592A inhibited this by 20% in comparison to the untreated cells. Notably, zoledronate significantly increased the metastatic potential of both cell lines by 75-83%; however, the mechanism responsible for this effect cannot be postulated based on the findings of the present study.

The exact mechanisms underlying the anti-metastatic activity of WG12399C and WG12592A remain unclear; however, the pro-apoptotic and anti-proliferative activity of these compounds may, at least in part, explain the observed effects. It was noted that WG12399C or WG12595A efficiently

inhibited the proliferation of 4T1 cells, with IC_{50} values approximately 2-fold higher than those calculated for zoledronate. It is widely accepted that N-BPs exert biological effects by inhibiting the mevalonate pathway (45). The inhibition of protein prenylation induces the apoptosis of normal and tumor cells (8,46). The present study confirmed that the addition of 10 μM GGOH to the culture medium induced a significant decrease in the anti-proliferative activity of zoledronate in 4T1 cells. By contrast, the anti-proliferative activity of WG12399C and WG12592A was not affected by the intermediate metabolites of the mevalonate pathway. This may suggest that the mechanism of action of these two amino BPs differs from that of classic N-BPs. It was hypothesized that this difference may be due to the variations in the structure of the studied BPs and zoledronate. Zoledronic acid, similar to other clinically applied N-BPs, belongs to the class of hydroxy BPs, in which the carbon atom of the bisphosphonic group is connected to the hydroxyl group, while the nitrogen atom is present in the side chain (47). WG12399C and WG12592A are aminomethylidene-BPs, in which the carbon atom of the bisphosphonic group is connected to the nitrogen atom of the amino group. Furthermore, these BPs contain two bisphosphonic groups in their structures. The presence of phenyl and naphthyl rings bound directly to the amino group decreases the basicity and increases the hydrophobicity of these compounds.

It has been widely documented that N-BPs exert pro-apoptotic effects on cancer cells by inhibiting the mevalonate pathway, as mentioned above (9,48,49). However, the exact mechanisms responsible for the pro-apoptotic activity remain to be elucidated. Herein, it was observed that despite the lack of inhibition of the mevalonate pathway, WG12399C exerted a significant pro-apoptotic effect on 4T1 cells. It increased the percentage of Annexin V-positive cells by almost 3-fold and the activity of caspase-3 by almost 6-fold in comparison to the untreated cells.

The results of several *in vitro* experiments have revealed that N-BPs can influence the anti-proliferative activity of various cytostatics, such as cisplatin, taxanes or etoposide (50-53). For example, Van Beek *et al* (54) reported that the combination of docetaxel, at a concentration minimally affecting tumor growth, with risedronate led to an almost complete inhibition of tumor growth and exerted a protective effect on bone integrity. Previous studies have demonstrated that both the simultaneous and subsequent treatment of precursors of osteoclasts with cytostatics and aminomethylidene-BPs results in a synergistic anti-proliferative effect, which is most pronounced in cells pretreated with BP (16). In the present study, it was found that both WG12399C and WG12592A, at low concentrations, enhanced the effectiveness of cytostatics commonly used as anticancer agents in breast cancer patients. The most beneficial synergistic effects were observed for 5-FU and paclitaxel, irrespective of the treatment regimen. The effects of WG12399C and WG12592A on the anti-proliferative activity of doxorubicin were found to be dependent on the concentration of the cytostatic and the treatment regimen. These results indicate the potential usefulness of the studied aminomethylidene-BPs in the adjuvant or combined treatment of cancer malignancies. However, further studies are required to evaluate the effects of these BPs on the antitumor activity of drugs in animal models.

The mechanisms regulating cell cycle progression play a role in controlling the growth and proliferation of cells. Previous studies have shown that N-BPs inhibit the progression of the cell cycle in the S phase (33,55); however, the overall effect of zoledronate on cell cycle distribution may be dependent on the cell type, proliferation rate, or the activation of particular signaling pathways. For example, Wang *et al* (56) reported that zoledronic acid significantly induced cell cycle arrest in the G_1 phase of cervical cancer cells-derived CSCs (cancer stem cells) in a concentration-dependent manner, although this compound did not affect cell cycle progression in parental cells. In the present study, it was observed that zoledronate induced the arrest of the cell cycle of 4T1 cells in the S phase, with a corresponding decrease in the number of cells in other phases; however, the changes were not statistically significant. WG12592A did not disrupt the cell cycle distribution at the concentration equaling its IC_{20-30} value. In turn, treatment with WG12399C resulted in cell accumulation in the G_0G_1 phase and a decrease in the percentage of cells in the G2M phase.

In conclusion, the findings of the present study provide some support for the adjuvant role of aminomethylidene-BPs in breast cancer therapy. WG12399C and WG12592A exhibit anti-metastatic activity in the murine breast cancer model, both in bone and in the extraosseous environment, and potentiate the anti-proliferative activity of common anticancer drugs. Further studies are required however, to elucidate the exact mechanisms underlying the anti-metastatic effects of WG12399C and WG12592A; however, it can be postulated that these compounds exert direct effects on tumor cells through their anti-proliferative and pro-apoptotic activity, as well as indirect effects by inhibiting cell motility.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ANG was involved in the conceptualization of the study, as well as in data investigation and analysis, funding acquisition, and in the writing of the original draft. WG was involved in the conceptualization of the study, as well as in data investigation, and in the writing, reviewing and editing of the manuscript. DP was involved in data investigation and analysis. MN was involved in data investigation and analysis, as well as in the writing, reviewing and editing of the manuscript. EM was involved in data investigation, and in the writing, reviewing and editing of the manuscript. JW was involved in the conceptualization and supervision of the study, and in the writing,

reviewing and editing of the manuscript. All authors have read and approved the final manuscript. ANG and WG confirm the authenticity of all the raw data.

Ethics approval and consent to participate

The *in vivo* procedures were approved by the first Local Committee for Experiments with the Use of Laboratory Animals, Wroclaw, Poland (Permission nos. 4/2015 and 80/2015).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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