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Biological effects of aromatic bis[aminomethylidenebis(phosphonic)] acids in osteoclast precursors in vitro

Running title: Biological activity of new aminobisphosphonates

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Abstract

Nitrogen-containing bisphosphonates (BPs) inhibit bone resorption by preventing osteoclast activity. Most clinically BPs are hydroxybisphosphonates with the exception of incadronate, which belongs to the class of aminomethylidenebisphosphonic acids. The aim of this study was to evaluate the anti-proliferative activity of two previously reported aminobisphosphonates (WG8185B2 and WG9001B) in combination with doxorubicin and cisplatin toward J774E cells (a model of osteoclast precursors in vitro). WG8185B2 and WG9001B BPs enhanced the cytotoxic activity of doxorubicin and cisplatin, especially when applied 24 hrs prior to cytostatics. The anti-proliferative effect of studied BPs was related to the changes in cell cycle progression. WG8185B2 leads to significant accumulation of J774E cells in S phase, whereas WG9001B causes transient arrest in G_2/M phase, followed by an increase in the percentage of cells in S phase. Moreover, WG8185B2 and WG9001B BPs showed enhanced pro-apoptotic activity in osteoclast precursors, which was manifested by an increase in caspase-3 activity and percentage of apoptotic cells. In addition, both compounds influenced the motility of J774E cells. The exact molecular mechanism of action of examined BPs remains to be determined; however, results show an interesting biological activity of these compounds, which may be of interest in the context of anti-resorptive therapy.

Introduction

Excessive bone resorption is observed in many diseases, such as Paget's disease, tumor-induced osteolysis, and postmenopausal osteoporosis (Feng & McDonald, 2011). Approximately 30% of postmenopausal women suffer from the osteoporosis, thus making it a serious health issue worldwide. Almost half of these women will experience at least one bone fracture in their lifetime. In addition, despite of the advances in early detection and treatment of breast cancer, all patients are at risk for disease recurrence, progression, and death. Across all tumor types, patients with breast cancer have the highest incidence of skeletal complications. In total, 80% of patients with metastatic breast cancer develop bone lesions (Coleman, 2009).

Bisphosphonates (BPs) are powerful inhibitors of osteoclast activity, and therefore are successfully used in the management of postmenopausal osteoporosis and cancer-related bone diseases, as an adjuvant therapy in patients with osteolytic bone metastases and patients treated with agents leading to bone loss (for a review see (Drake, Clarke, & Khosla, 2008; Gnant & Clézardin, 2012)). The molecular mechanism responsible for the cytotoxic activity of N-containing BPs toward osteoclasts is the inhibition of farnesyl diphosphate synthase (FPPS), which results in the suppression of mevalonate pathway responsible for the synthesis of cholesterol and prenyl groups. Post-transcriptional prenylation of small GTPases plays an important role in protein-protein interactions and anchoring to cell membranes (Shinde & Maddika, 2018). The inhibition of mevalonate pathway leads to the accumulation of unprenylated proteins, impairment of a downstream signaling, and in turn to the inhibition of resorptive activity of osteoclasts and often to apoptosis (K. Iguchi, Tatsuda, Usui, & Hirano, 2010; Miwa et al., 2012; Tsubaki et al., 2013).

The biological activity of BPs is closely related to their chemical structure, which enables BPs to chelate calcium ions efficiently as well as to target bone environment specifically (Ebetino et al., 2011). The character of two substituents R^1 and R^2, covalently bound to the carbon atom of the bisphosphonic group (P-C-P), play a crucial role in BP activity. In most commercial BPs, the R^1 substituent is a hydroxyl group that determines the affinity of BPs to bone mineral surface, while the anti-resorptive activity is dependent basically upon the chemical structure of R^2 chain. Third-generation BPs containing the *N*-heterocyclic ring (risedronate, minodronate, and zoledronate) are the most active compounds among all available commercial BPs, e.g. zoledronic acid is approximately 10,000 times more active than etidronate (Sharma et al., 2013).

One of the oldest and still commonly used procedure for the aminomethylidenebisphosphonic acids preparation is based on the three-component reaction of the primary or secondary amine with trialkyl orthoformate and dialkylphosphonate, and further hydrolysis of crude tetraalkylesters (Takeuchi et al. 1993). There are some other methods like reaction of trialkylphosphites with amides in the presence of phosphrous oxychloride (Olive, Le Moigne, Mercier, Rockenbauer, & Tordo, 1998), oxalyl chloride (O'Boyle et al., 2011) or triflic anhydride (Wang, Chang, Sun, & Huang, 2015), reaction of acetals of formamides with dialkylphosphonates (Groß & Costisella, 1968; Prishchenko, Livantsov, Novikova, Livantsova, & Petrosyan, 2009), phosphonylation of oximes with trialkylphosphites or dialkylphosphonates via Beckmann Rearrangement (Yokomatsu, Yoshida, Nakabayashi, & Shibuya, 1994). In contrast to 1-hydroxybisphosphonates, aminomethylidenebisphosphonates are significantly less investigated and described in the literature.

Although, the so-called 'orthoformate method' is the most often used procedure for the preparation of the aminobisphosphonic acid it has several disadvantages like elevated

temperatures (130-150^{o}C), which excludes the use of substrates with labile groups, low or average yield of the final bisphosphonic acid and problems with the separation of pure esters. Due to these disadvantages we were looking for another, mild and more efficient method for the synthesis of this group of compounds.

In 2012, we developed a very convenient and mild procedure to synthesize N-substituted aminomethylidenebisphosphonates in a reaction of trialkyl phosphites and isocyanides in the presence of a stoichiometric amount of hydrogen chloride (Goldeman, Kluczyński, & Soroka, 2012). Next, we used our method for the preparation of a series of aminomethylidenebisphosphonic acids derived from selected β-arylethylamines (Goldeman & Nasulewicz-Goldeman, 2015) and a series of diamines (Goldeman & Nasulewicz-Goldeman, 2014). The preliminary biological evaluation of these new BPs revealed that two of them, i.e. benzene-1,4-bis[aminomethylidene(bisphosphonic)] (WG8185B2) and naphthalene-1,5-bis[aminomethylidene(bisphosphonic)] acid (WG9001B) (Fig. 1.) showed high anti-proliferative activity toward J774E mouse macrophages which are a model of osteoclast precursors in vitro (Goldeman & Nasulewicz-Goldeman, 2014).

The aim of this in vitro study was to evaluate the anti-proliferative activity of above two aminobisphosphonates in combination with cytostatic drugs toward the well-defined macrophage cell line J774E cells. The influence on cell motility and cellular mechanisms of action were also studied.

Materials and methods

General information

All reagents used for the synthesis were of commercial quality and purchased from Sigma-Aldrich, while solvents were of commercial quality and purchased from a local Supplier

(POCh). Incadronate and zoledronate were synthesized according to literature procedures (Singh, Manne, Ray, & Pal, 2008; Takeuchi et al., 1993). Concentrations of WG8185B2 and WG9001B used for each assay were each time experimentally evaluated and depended on test conditions, like incubation time, area of wells and density of cells. In each case the relationship between the concentration of compound and the inhibitory effect was determined. To avoid the toxicity effect, doses lower than IC_{40} were used, unless otherwise stated.

Cell culture

J774E mouse macrophages were obtained from American Type Culture Collection (Rockville, Maryland, USA) and maintained in the Cell Culture Collection of the Institute of Immunology and Experimental Therapy (IIET), Polish Academy of Sciences, Wroclaw, Poland. Cells were maintained in RPMI-1640 GLUTAMAX (Gibco, Scotland, UK) medium containing 100 U/mL penicillin and 100 µg/mL streptomycin (both from Polfa Tarchomin S.A., Warsaw, Poland) and supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich, Darmstadt, Germany).

Combination with cytostatics

Cells were plated in 96-well plates (Sarstedt, Inc. Newton, NC, USA or Corning B.V. New York, USA) at a density of 1×10^4 cells per well in 100 µL of culture medium without FBS and antibiotics. Immediately after seeding, cells were pretreated either with doxorubicin (at serial concentrations of 1000 – 100 – 10 – 1 ng/mL) or cisplatin (at serial concentrations of 10 – 1 – 0.1 – 0.01 µg/mL) or BP at a concentration of 25 µg/mL (54 µM) for WG8185B2 and 2 µg/mL (3.76 µM) for WG9001B. The concentrations of BPs were based on previously calculated IC_{50} values and on the kinetics in the wide range of concentrations. Cells treated with BPs at serial concentrations of 100 – 50 – 25 – 10 – 5 – 2.5 – 1 – 0.1 µg/mL served as

controls for a calibration curve. After 24 hrs of incubation under standard conditions, doxorubicin (Sigma-Aldrich), cisplatin (Accord Healthcare Limited, North Harrow, UK), or BP was applied to obtain the following combinations: I. cytostatic following pretreatment with BP; II. BP following pretreatment with cytostatic; III. BP and cytostatic used together. Cells treated with BP or cytostatic alone as well as non-treated cells served as controls. An SRB assay was performed after 72 hrs, as described by Skehan et al. (Skehan et al., 1990). The optical densities of the samples were measured by a Synergy H4 Hybrid Reader (BioTek Instruments, Winooski, VT, USA) at λ = 540 nm.

The results from the SRB assay were calculated with the CalcuSyn program (Biosoft, Cambridge, United Kingdom) to determine the presence of synergy between BPs and cytostatics. Based on the method of Chou and Talalay (Chou & Talalay, 1981, 1984), the CalcuSyn program was used to determine whether the drug combination produced greater effects together than expected from a simple summation of their individual effects. The combination index (CI) values obtained from the data reflected the nature of the interaction between BPs and cisplatin or doxorubicin, i.e. < 1, synergistic activity; = 1, additive; > 1, antagonism.

GGOH-FOH assay

Cells were plated in 96-well plates at a density of 1×10^4 cells per well in 100 µL of culture medium with FBS and antibiotics. At 24 hrs after seeding, cells were pretreated for 1 hr with 50 µL of either geranylgeraniol (GGOH, Sigma-Aldrich) or farnesol (FOH, Sigma-Aldrich) at the concentration 10 or 20 µM. Next, cells were treated with WG8185B2 or WG9001B BP (at serial concentrations of 100 – 10 – 1 – 0.1 µg/mL) for further 48 hrs. Cells treated with studied or reference BPs alone as well as non-treated cells served as controls. SRB assay was

performed as described above, and the IC_{50} values were calculated based on the inhibition of proliferation rates using Prolab software (INFORM-TECH, Zabrze, Poland).

Migration and invasion assay

J774E macrophages were seeded at a density of 5×10^4 cells/mL in the culture medium in 24-well plates, cultured overnight, and treated for 48 hrs with BPs at a concentration of 27.6 μM for WG8185B2 and 3.4 μM for WG9001B. Untreated cells and cells treated with either zoledronic or incadronic acid served as controls. After incubation cells were non-enzymatically detached from the wells, centrifuged and counted. For migration assay, 2.5 x 10^4 of viable cells were suspended in Dulbecco's Modified Eagle's medium without FBS and applied to the upper section of the inserts (Transwell Permeable Supports 6.5 μm Insert, Corning Incorporated, Kennebunk, USA) coated with type I collagen at a concentration of 10 μg/mL. For invasion assay, 2.5 x 10^4 of viable cells were suspended in Dulbecco's Modified Eagle's medium without FBS and applied at the top of Matrigel-coated inserts (BioCoat Matrigel Invasion Chamber, BD Biosciences). Culture medium supplemented with 20% FBS, applied to the lower section, served as a chemoattractant. Migration and invasion assays were carried out in standard culture conditions for 6 and 24 hrs, respectively. Thereafter, the cells on the bottom side of the membrane were fixed, stained with a Diff-Quick Set (Medion Diagnostics, Düdingen, Switzerland), and counted by light microscopy. Experiment was repeated at least three times. Statistical analysis was performed using STATISTICA version 10.

Annexin-V assay

J774E macrophages were seeded at a density of 5×10^4 cells/mL in the culture medium in 24-well plates, cultured overnight, and treated with BPs at a concentration of 27.6 μM for WG8185B2 and 3.4 μM for WG9001B for 24, 48, and 72 hrs. Untreated cells and cells

treated with either incadronic or zoledronic acid served as controls (76.7 and 41.4 μM, respectively). After exposure time, cells were collected and counted. Cells (5×10^5) were washed with phosphate-buffered saline (PBS) and resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). In total, 195 μL of cell suspension was stained with 5 μL of APC-annexin V (Enzo Lifesciences Ltd., Farmingdale, NY, USA) for 15 min in dark at room temperature. Then, cells were washed in PBS, centrifuged, and suspended in binding buffer. In total, 5 μL of DAPI solution (5 mg/mL) was added prior to FACS analysis. Data analysis was performed by flow cytometry using the Diva Software program for data acquisition. Data were displayed as two-color dot plot with APC-annexin V (x-axis) vs DAPI (y-axis). Double-negative cells were live cells, $DAPI^+$/annexin $V^{+/-}$ necrotic cells, DAPI weak/annexin V+ apoptotic cells, and DAPI -/annexin V+ early apoptotic cells.

Caspase-3 activity

J774E macrophages were seeded at a density of 5×10^4 cells/mL in the culture medium in 24-well plates, cultured overnight, and treated with BPs at a concentration of 27.6 μM for WG8185B2 and 3.4 μM for WG9001B for 24, 48, and 72 hrs. Untreated cells and cells treated with either incadronic or zoledronic acid served as controls (76.7 and 41.4 μM, respectively). After exposure time, cells were lysed for 30 minutes with 50 μL of lysis buffer (50 mM HEPES, 10% saccharose, 150 mM NaCl, 5 mM EDTA, 10 mM DTT, 0.1% Triton-X 100, pH 7.5) (IIET). Then, 40 μL of lysate was transferred to a white, 96-well plate (Corning, NY, USA) and mixed with 160 μL of the reaction buffer (10 μM Ac-DEVD-ACC substrate, 20 mM HEPES, 10% saccharose, 100 mM NaCl, 1 mM EDTA, 10 mM DTT, pH 7.5) (IIET). Sample fluorescence was continuously recorded at 37°C for 2 hrs using a Biotek Synergy H4 Hybrid Reader (λ_{ex} = 360 nm, λ_{em} = 460 nm). Results were normalized to the protein content

using the SRB method and expressed as mean relative caspase-3/7 activity compared to the untreated control sample ± standard deviation (SD).

Cell cycle

J774E macrophages were seeded at a density of 5×10^4 cells/mL in the culture medium in 24-well plates, cultured overnight, and treated with BPs at a concentration of 14.7 μM for WG8185B2 and 2.5 μM for WG9001B for 24, 48, and 72 hrs. Untreated cells and cells treated with either incadronic or zoledronic acid served as controls (52.3 and 27.6 μM, respectively). After exposure time, cells were collected and counted. The cells (1×10^6) were washed twice in cold PBS and fixed for 24 hrs in 70% (v/v) ethanol at −20°C. Then, the cells were washed twice in PBS and stained for 30 min with DAPI stain solution (DAPI 1 μg/mL, Triton x100 0.1%, Sigma-Aldrich) on ice. The cellular DNA content was determined using a BD LSRFortessa instrument (Becton Dickinson, San Jose, CA, USA) and a ModFit LT 3.0 program (Verity Software House, Topsham, ME, USA).

Statistical evaluation

Statistical analysis was performed using GraphPad Prism 7.01 (GraphPad Software Inc., San Diego, CA, USA). Analysis of variance was determined by Shapiro–Wilk's normality and Bartlett's tests. Tests used for each data analysis are indicated in figure legends. $p<0.05$ was considered significant.

Results

Synthesis

Benzene-1,4-bis[aminomethylidene(bisphosphonic)] acid (WG8185B2) and naphthalene-1,5-bis[aminomethylidene(bisphosphonic)] acid (WG9001B) were prepared in the reaction of the appropriate diisocyanide (1,4-diisocyanobenzene and 1,5-diisocyanonaphthalene, respectively) with triethylphosphite in the presence of hydrochloride and further dealkylation with bromotrimethylsilane (Fig. 2) according to the authors' protocol developed in previous studies (Goldeman et al., 2012; Goldeman & Nasulewicz-Goldeman, 2014).

Anti-proliferative activity of WG8185B2 and WG9001B bisphosphonates in combination with cytostatics

As previously reported, WG8185B2 and WG9001B BPs show anti-proliferative activity against J774E macrophages (Goldeman & Nasulewicz-Goldeman, 2014). In these studies, we showed that WG8185B2 and WG9001B BPs enhance the cytotoxic activity of doxorubicin and cisplatin. Beneficial effects were observed when cells were pretreated with BPs for 24 hrs prior to cytostatics. For both BPs applied in the study, a strong synergism was recorded, with CI values ranging from 0.151 to 0.711. Simultaneous treatment of J774E macrophages with WG8185B2 or WG9001B BP and cytostatics resulted in synergistic effects; however, antagonism was observed with low concentrations of cytostatics. Similarly, pretreatment with high concentrations of cytostatics followed by the application of WG8185B2 or WG9001B BPs led to synergism, whereas pretreatment with low concentrations of cytostatics resulted in additive or even antagonistic effects (Table 1, Table 2).

GGOH-FOH assay

To investigate whether the anti-proliferative effect of WG9001B and WG8185B2 BPs is related to the inhibition of protein prenylation, we used GGOH and FOH, intermediate metabolites in the mevalonate pathway, which are proven to reverse the effects of aminobisphosphonates (Benford, Frith, Auriola, Mönkkönen, & Rogers, 1999; Denoyelle, Hong, Vannier, Soria, & Soria, 2003; Goffinet et al., 2006). J774E cells were pretreated with either GGOH or FOH at the concentration of 10 or 20 μM for 1 hr and then incubated with WG9001B or WG8185B2 bisphosphonates for 48 hrs. Cells incubated with zoledronate and incadronate served as positive controls. Neither GGOH nor FOH significantly influenced the anti-proliferative activity of WG9001B and WG8185B2 BPs (Fig. 3). IC_{50} values for WG9001B and WG8185B2 compounds alone or in combination with GGOH or FOH did not significantly vary. By contrast, GGOH significantly diminished anti-proliferative effects of zoledronate and, to a lower degree, of incadronate.

Effect of bisphosphonates on the motility of macrophages

Motility is a very important aspect of osteoclast differentiation and function. Upon stimulation, osteoclast precursors migrate and attach onto the bone surface, which enables these cells to differentiate into active resorbing osteoclasts. Thus, we assessed the effect of WG9001B and WG8185B2 BPs on migratory properties of J774E cells using the transwell system, both with collagen- and Matrigel-coated wells. In both cases, BPs inhibited the migration of J774E cells through porous membranes (Fig. 4). However, the effect was much more pronounced in the case of Matrigel-coated wells. Over 90% inhibition of migration of J774E cells through Matrigel was observed for WG9001B compound and 80% for WG8185B2 BP, which was similar for the activity of incadronate (90%) and much higher as

compared to zoledronate (38% of inhibition). In the case of migration through collagen-coated wells, the inhibitory effect was much less evident; however, the activity of WG9001B compound was comparable to that of incadronate and zoledronate.

Pro-apoptotic activity of WG9001B and WG8185B2

All the applied compounds (WG9001B, WG8185B2, incadronate, and zoledronate) caused a substantial increase in caspase-3 activity in J774E cells as compared to untreated control (Fig.5). Both WG9001B and WG8185B2 BPs showed enhanced caspase-3 activity as compared to reference incadronate and zoledronate. Treatment with WG8185B2 BP led to a significant increase in the caspase-3 activity in a time-dependent manner. Incubation for 24 hrs increased the caspase-3 activity by over twofold, and the enzyme activity increased over sevenfold after additional 24 hrs in comparison to untreated cells. In the case of WG9001B BP, the activity of caspase-3 enzyme remained stable upon the time of incubation, with the mean increase of approximately sixfold. These changes in the caspase-3 activity were reflected in the percentage of apoptotic cells assessed in annexin-V assay (Fig. 6). Incubation with BPs led to a substantial increase in the percentage of J774E cells in early and late apoptosis. Interestingly, the pro-apoptotic effect of WG9001B compound was observed much earlier as compared to the reference zoledronate and incadronate, and WG8185B2 BP. After 24 hrs of incubation, almost 60% of cells were annexin-V positive and the number of apoptotic cells increased to over 80% at the time of treatment. Both incadronate and zoledronate induced apoptosis in J774E macrophages, with the most significant effect observed after 72 hrs of incubation (58% and 86% of apoptotic cells, respectively). In the case of WG8185B2 BP, incubation for 72 hrs resulted in a statistically significant increase in the percentage of apoptotic cells. None of the studied aminobisphosphonates evoked a

significant necrosis in J774E cells. The percentage of dead cells did not exceed 5% in any of the incubation time tested and did not differ as compared with control cells.

Effect of bisphosphonates on cell cycle progression

In order to find the mechanism how WG8185B2 and WG9001B BPs affect the cell cycle progression, J774E cells were incubated with the compounds at concentrations equal to their IC_{20} values for 24, 48, and 72 hrs. As shown before, treatment with either zoledronate or incadronate led to the arrest of cell cycle progression in S phase (T. Iguchi, Miyakawa, Yamamoto, Kizaki, & Ikeda, 2003; Okamoto et al., 2014). This was manifested most profoundly after 24 hrs of incubation by the increase in the number of cells in this phase from 37% (control) to 55% and 57% for incadronate- and zoledronate-treated cells, respectively (Fig. 7). Similarly, use of WG8185B2 compound resulted in significant accumulation of J774E cells in S phase. However, this effect was also observed after 24 and 48 hrs of incubation. Interestingly, the WG9001B compound showed different mode of action. Incubation with WG9001B BP led to slight and transitory accumulation of macrophages in G_2/M phase and tended to the arrest of cell cycle progression in S phase after 72 hrs of treatment (26% vs 41% of cells).

Discussion

Bisphosphonates are widely used for the prevention and treatment of osteoporosis, corticosteroid-induced bone loss, and Pagets's disease. BPs are also used in the management of cancer-related bone diseases, as an adjuvant therapy in patients with osteolytic bone metastases, as well as in patients treated with agents leading to bone loss. Numerous clinical

studies have shown that BPs can reduce the occurrence of bone fractures and pain, thus leading to improved quality of life. Recently, more and more attention has been paid to the possible anticancer activity of BPs. It is now postulated that BPs influence directly tumor growth and progression as well as indirectly by anti-angiogenic (Gao et al., 2017; Michailidou et al., 2010; Wood et al., 2002) and immunomodulatory effects (Fujimura, Kambayashi, Furudate, Kakizaki, & Aiba, 2013; Kunzmann et al., 2000; Rossini et al., 2012).

Osteoclasts and macrophages are endocytic cells that are derived from the same hematopoietic progenitors. They are both very sensitive to the biological effects of BPs. It was shown that a strong apoptotic activity in J774E macrophages reflects, in general, their potency for inhibiting bone resorption (M. J. Rogers et al., 1996). BPs were shown not only to exert pro-apoptotic effect in macrophages, but also to inhibit their migration and invasion (T. L. Rogers & Holen, 2011). Thus, murine macrophage cell lines, mainly J774E and RAW264.7, appeared to be an appropriate and convenient model for studies on pharmacological properties of BPs in vitro.

As we have previously demonstrated, both WG8185B2 and WG9001B BPs show anti-proliferative activity against J774E macrophages and MCF-7 cancer cells. In former studies WG8185B2 inhibited the proliferation of J774E and MCF-7, cells with IC_{50} values of 90.1 and 96.5 μM, respectively. The anti-proliferative activity of WG8185B2 against J774E cells was comparable to zoledronate and six times higher than in the case of incadronate. WG9001B exhibited a specificity of action against J77E cells manifested by 16 times stronger anti-proliferative activity in comparison to zoledronic acid (5.8 μM vs. 92.4 μM) (Goldeman & Nasulewicz-Goldeman, 2014). These observations point to potential benefits of the application of WG9001B and WG8185B2 BPs in combined anticancer treatment, not only as bone protecting agents, but also in the context of tumor–microenvironment

interactions. This approach is based on the Paget's hypothesis of "seed" and "soil." More than 100 years ago, Stephen Paget suggested that although therapies against the cancer cell (the "seed") are important, affecting the microenvironment that supports cancer cell growth (the "soil") may be equally valuable. The bone microenvironment provides a supportive niche for cancer cell survival and tumor growth (Langley & Fidler, 2011). Once metastatic breast cancer cells reach the bone microenvironment, they start interacting with osteoblast and osteoclasts to promote bone degradation. In turn, bone-derived growth factors, calcium ions, and cytokines released during bone resorption attract cancer cells to the bone surface and facilitate their growth and propagation. This relationship between tumor metastatic cells and bone resident cells is called "a vicious cycle". Our results show that the two investigated BPs may not only inhibit the proliferation of osteoclast precursors, but also influence beneficially the effects of antitumor treatment by breaking of vicious cycle in bone.

Many studies have reported that the combination of BPs with anticancer agents can significantly influence antitumor effects both in vitro and in vivo. Thus, based on preliminary data, we selected two compounds with the highest anti-proliferative activity to examine their potential influence on cytotoxic activity of cisplatin and doxorubicin, which are commonly used chemotherapeutics in patients with breast cancer. Our studies show that both simultaneous and subsequent treatment of J774E macrophages with cytostatics and WG9001B or WG8185B2 resulted in a synergistic anti-proliferative effect, which is most pronounced when cells were pretreated with BP. This raises the question of the possible mechanism of cell sensitization to cytostatics by BPs. Benassi et al. reported that zoledronic acid may enhance the effectiveness of cisplatin in osteosarcomas expressing wild-type p53 (Benassi et al., 2007). Similarly, zoledronic acid has been shown to augment significantly the anti-proliferative effects in docetaxel-treated fibrosarcoma (Koto et al., 2010), prostate (Ullén et al., 2005), or pancreatic cancer cells (M. Zhao et al., 2012) Furthermore, Matsumoto et al.

showed that zoledronate potentiates the anti-proliferative activity of paclitaxel, etoposide, cisplatin, irinotecan, and imatinib against SBC-3 human lung cancer cells (Matsumoto et al., 2005). Thus, BPs when used adjunctively with cytostatics in cancer patients with osteolysis may prevent skeletal-related events. In addition, many cancer treatments have detrimental effects on bone and can increase the risk of fracture. The increasing use of aromatase inhibitors, in particular, results in treatment-induced bone loss, which can be prevented with BP treatment (Langley & Fidler, 2011).

It is now well accepted that nitrogen-containing BPs exert biological effects through the inhibition of the mevalonate pathway (Luckman, Hughes, et al., 1998). This results in the loss of prenylation of small GTPases, which leads to the inhibition of osteoclast differentiation and apoptosis of cells (Luckman, Coxon, Ebetino, Russell, & Rogers, 1998). Apoptosis and caspase activation can be prevented by addition of GGOH. This intermediate of the mevalonate pathway restores protein prenylation and resorptive activity of osteoclasts (Benford et al., 1999; Halasy-Nagy, Rodan, & Reszka, 2001). However, the effect of FOH treatment on the mevalonate pathway is not as significant as that of GGOH. Tsubaki et al. reported that inhibition of osteoclast formation from C7 macrophage-like cells by treatment with minodronate or alendronate may be successfully re-established by the co-treatment with 20 μM GGOH. In contrast, a combination with FOH did not significantly reverse the effects of minodronate and alendronate on a generation of TRAP-positive multinucleated cells (Tsubaki et al., 2014). Here, we present that the addition of GGOH to the culture medium significantly decreased the anti-proliferative activity of zoledronate in J774E macrophages in a dose-dependent manner. The value of IC_{50} increased from 92.4 to 263.1 and 566.6 μM when cells were simultaneously treated with 10 and 20 μM GGOH for 72 hrs, respectively. The effect is less remarkable in incadronate-treated cells, with an increase in IC_{50} value from 380.8 to 682.2 and 840.7 μg/mL in co-culture with 10 and 20 μM GGOH, respectively. By

contrast, FOH did not significantly influence the anti-proliferative potential of reference BPs. This finding is in agreement with data previously reported by Iguchi which showed that pretreatment of myeloma cells with 20 μM GGOH but not with FOH resulted in a significant reduction of the anti-proliferative effect of incadronate (T. Iguchi et al., 2003). In our studies, both WG9001B and WG8185B2 compounds inhibited the proliferation of J774E cells, and WG9001B BP showed approximately fivefold higher activity as compared to reference zoledronate. Surprisingly, neither GGOH nor FOH affected significantly the anti-proliferative activity of the WG9001B and WG8185B2 bisphosphonates. These results may suggest that the mechanism of action of the studied aminobisphosphonates is different from the mechanism of the reference zoledronate and incadronate. One of the possible explanations are the differences in the chemical structure of reference and investigated compounds. This seems probable in the case of zoledronic acid, which belongs to the group of the nitrogen-containing hydroxybisphosphonates. However, the differences in the mechanism of action between incadronic acid and investigated BPs are surprising since both studied BPs and incadronate belong to the same group of BPs-aminomethylidenebisphosphonates. Owing to the chemical similarity between these compounds, such mechanism of action could be expected. However, there are two significant structural factors that differentiate the studied BPs from the control incadronate, which may affect their mechanism of action. WG9001B and WG8185B2 BPs have two bisphosphonic groups in their structures, and both of studied compounds contain aromatic ring (1,4-disubstituted phenyl and 1,5-disubstituted naphthyl, respectively) bounded directly to the nitrogen of the aminomethylidenebisphosphonic group. The planarity of the phenyl and naphthyl rings makes both molecules more rigid in comparison to incadronate-containing cycloheptyl ring. Furthermore, their aromatic character increases hydrophobicity of the investigated BPs and decreases the basicity of the nitrogen atom of the aminomethylidenebisphosphonate group.

Next, we examined the cellular effects of WG9001B and WG8185B2 BPs on J774E macrophages.

Osteoclast precursors migrate to bone resorption sites where they differentiate into mature osteoclasts. They cross blood vessels walls and migrate through the bone marrow cavity, which is triggered by the chemokine gradient. Once osteoclast precursors reach the osteoclast niche, they start to differentiate, fuse, and finally get activated in the resorption process. Thus, we investigated whether examined BPs influence the migration process in J774E cells. We showed that treatment with WG9001B and WG8185B2 compounds for 48 hrs led to a significant (90 and 80%, respectively) inhibition of J774E cell migration through Matrigel-coated pores. The effect was comparable to that observed in incadronate-treated cells and more pronounced as compared to zoledronate. This inhibitory effect seems to be related to biochemical changes rather than to anti-proliferative activity of tested BPs, which were applied at a dose not exceeding IC_{30}. Similarly, Kimachi et al. showed that zoledronic acid impairs not only the differentiation of RAW264.7 macrophages into osteoclasts, but also TNF-α-induced migration through collagen type I-coated chambers (Kimachi, Kajiya, Nakayama, Ikebe, & Okabe, 2011).

As a result of the preventing post-translational prenylation, nitrogen-containing BPs of the third generation induce apoptosis in a variety of cells, including osteoclasts, macrophages, and cancer cells (Karabulut et al., 2010; Koto et al., 2010; Luckman, Coxon, et al., 1998). Interestingly, data obtained from in vitro studies in J774E macrophages showed that the pro-apoptotic activity of BPs in vitro reflects their anti-resorptive potency in vivo. According to this, the highest pro-apoptotic activity has been found for zoledronate, risedronate, and ibandronate (Moreau et al., 2007). In our studies, WG8185B2 and WG9001B BPs showed a significant pro-apoptotic activity in J774E cells, which was manifested by a significant increase in caspase-3 activity and the percentage of apoptotic

cells. However, the pro-apoptotic activity of these aminobisphopshonates seems not to be related to inhibition of mevalonate pathway.

Cell cycle control is one of the key mechanisms of cell growth and proliferation. Many therapeutic agents, including BPs, cause cell cycle arrest before inducing apoptosis. However, the exact molecular mechanism underlying this phenomenon remains to be revealed, and experimental data concerning the influence of nitrogen-containing BPs on cell cycle progression are rather conflicting. Results of many studies indicate that nitrogen-containing BPs, like zoledronate, risedronate, or pamidronate, typically cause the arrest of cell cycle progression in S phase, with a corresponding decrease in the number of cells in other phases. This was demonstrated in both normal and cancer cells (Iguchi et al., 2003; Mansouri, Mirzaei, Lage, Mousavi, & Elahian, 2014; Okamoto et al., 2014; Zhao et al., 2018). On contrary, it has been also demonstrated that treatment with ibandronate leads to increased cell cycle arrest in S phase in U-2 osteosarcoma cells and in G2/M phase in MG-63 osteoblast-like cells (Karlic et al., 2017). In addition, Merrel et al. showed that treatment with zoledronate increased the percentage of MDA-MB-231 cells in G1 phase (Merrell, Wakchoure, Lehenkari, Harris, & Selander, 2007). The molecular or cellular background of these discrepancies is unclear. In our studies, the reference BPs, zoledronate and incadronate, induced the arrest of cell cycle progression of J774E cells in S phase even after 24 hrs of treatment. Similarly, treatment with WG8185B2 compound significantly increased the number of cells in S phase and decreased the number of cells in G0/G1 phase. However, the effect was the most pronounced after 48 hrs of incubation. By contrast, in WG9001B-treated cells a transient arrest in progression through G2/M-checkpoint followed by an increase in the percentage of cells in S phase was observed. One of the possible explanations of this phenomenon may be the differences in molecular structure.

In summary, BPs are organic compounds that are capable of inhibiting osteoclast-mediated bone resorption. Nitrogen-containing BPs are widely used for the prevention of age-related bone loss and as an adjuvant anticancer therapy in patients with bone lesions. Here, we report that two aminomethylidenebisphosphonates, WG9001B and WG8185B2, show remarkable anti-proliferative activity toward osteoclast precursors in vitro. They also significantly augment the anti-proliferative effect of cytostatics. Both BPs influence the motility of J774E cells. Moreover, they cause cell cycle arrest in osteoclast precursors and show a potent pro-apoptotic activity. Further studies regarding the molecular mechanism of action and in vivo activity profile are needed to characterize their therapeutic potential.

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Author contributions:

ANG conceived, planned and performed the experiments, analyzed the data, and wrote the paper. WG synthesized the studied bisphosphonates, and wrote the paper. EM performed the experiments with the support of ANG. JW critically read and improved the manuscript.

Conflict of interest:

The authors declare no conflict of interest.

Data Availability Statement

Author elects to not share data

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Figure legends

Fig. 1. Chemical structures of the studied bisphosphonates.

Fig. 2. Synthesis of benzene-1,4-bis[aminomethylidene(bisphosphonic)] acid (WG8185B2) and naphthalene-1,5-bis[aminomethylidene(bisphosphonic)] acid (WG9001B).

Fig. 3. The effect of geranylgeraniol (**GGOH) and farnesol (FOH) on the anti-proliferative activity of WG9001B, WG8185B2 bisphosphonates, zoledronate, and incadronate.** J774E cells were pretreated with 50 µL of either GGOH or FOH at the concentration 10 or 20 µM for 1 hr. Then, the cells were incubated with WG9001B or WG8185B2 bisphosphonate (at serial concentrations of 100 – 10 –1 – 0.1 µg/mL) for further 48 hrs. Cells treated with studied or reference bisphosphonates alone as well as non-treated cells served as controls. The experiments were repeated at least four times. Data are expressed as mean ± standard deviation, ***$p<0.001$ was assessed with Kruskal–Wallis test

Fig. 4. Influence of bisphosphonates on migratory properties of J774E cells through Matrigel- and collagen-coated membranes. Cells were treated with 3.4 µM WG9001B, 27.6 µM WG8185B2, 73.2 µM incadronate, or 20.7 µM zoledronate for 48 hrs. Untreated J774E cells served as controls. Cells were applied to the upper section of the invasion (a) or migration (b) chamber at the density of 5×10^4 cells/insert. The number of cells per membrane was determined and accumulated into groups, and the average is presented. Data are expressed as mean ± standard deviation. *$p<0.01$ and ***$p<0.01$ vs control cells were assessed with one-way analysis of variance

Fig. 5. Effect of bisphosphonates on caspase-3 activity in J774E cells treated with 3.4 µM WG9001B, 27.6 µM WG8185B2, 76.7 µM incadronate, or 41.4 µM zoledronate for 24, 48 or 72 hrs. Untreated J774E cells served as controls. The experiments were repeated at least three times. Results were normalized to the protein content using the SRB method and reported as mean relative caspase-3/7 activity compared to the untreated control sample ±

standard deviation. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 were assessed with Kruskal–Wallis test

Fig. 6. The pro-apoptotic effect of bisphosphonates in J774E cells treated with 3.4 μM WG9001B, 27.6 μM WG8185B2, 76.7 μM incadronate, or 41.4 μM zoledronate for 24, 48, or 72 hrs (panel A). Untreated J774E cells served as controls. The experiments were repeated at least three times. Cells were stained with DAPI and analyzed using Diva Software. Data are expressed as mean ± standard deviation. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 were assessed with one-way analysis of variance a – significant vs. control cells, b – significant vs WG9001B group. Panel B shows dotplots from one 72 hrs incubation when the most pronounced differences were observed.

Fig. 7. Effect of bisphosphonates on cell cycle progression in J774E cells treated with 2.5 μM WG9001B, 14.7 μM WG8185B2, 52.3 μM incadronate, or 27.6 μM zoledronate for 24, 48, or 72 hrs (panel A.). Untreated J774E cells served controls. The experiments were repeated at least three times. The cellular DNA content was determined by BD LSRFortessa instrument (Becton Dickinson) and ModFit LT 3.0 program I. Data are expressed as mean ± standard deviation. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001 were assessed with one-way analysis of variance. Panel B shows the most representative histograms for 24 hrs incubation when the most pronounced differences were observed.

Table 1. Combination Index (CI) for combined treatment of naphthalene-1,5-bis[aminomethylidene(bisphosphonic)] acid (WG9001B) and cytostatics against J774E cells

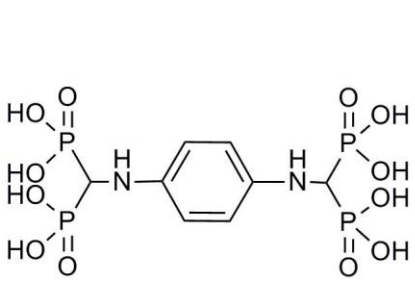
	Doxorubicin [ng/ml]			
	1	10	100	1000
WG9001B + doxorubicin simultaneously	**1.057**	**0.796**	**0.506**	**0.135**
Pretreatment with WG9001B + doxorubicin	**0.216**	**0.265**	**0.285**	**0.288**
Pretreatment with doxorubicin + WG9001B	4.383	1.323	**0.665**	**0.182**
	Cisplatin [µg/ml]			
	0.01	0.1	1	10
WG9001B + cisplatin simultaneously	2.943	**0.367**	**0.464**	**0.336**
Pretreatment with WG9001B + cisplatin	**0.213**	**0.284**	**0.176**	**0.151**
Pretreatment with cisplatin + WG9001B	2.753	**1.033**	1.142	**0.321**

WG9001B was applied at the dose 2 µg/mL (3.76 µM). CI values were calculated using CalcuSyn software as described in Materials and Methods. CI values <1 indicate synergism, whereas CI values = 1 and >1 indicate additivity and antagonism, respectively.

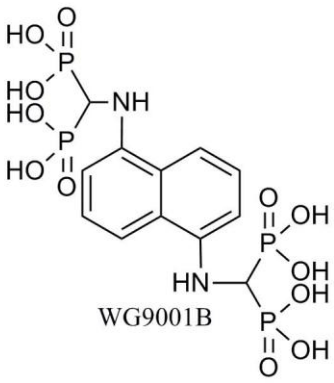
Table 2. Combination Index (CI) for combined treatment of benzene-1,4-bis[aminomethylidene(bisphosphonic)] acid (WG8185B2) and cytostatics against J774E cells

	Doxorubicin [ng/ml]			
	1	10	100	1000
WG8185B2 + doxorubicin simultaneously	1.540	3.170	**0.579**	**0.396**
Pretreatment with WG8185B2 + doxorubicin	**0.524**	**0.496**	**0.208**	**0.308**
Pretreatment with doxorubicin + WG8185B2	4.263	3.685	**0.233**	**0.174**
	Cisplatin [µg/ml]			
	0.01	0.1	1	10
WG8185B2 + cisplatin simultaneously	5.478	1.116	**0.607**	**0.811**
Pretreatment with WG8185B2 + cisplatin	**0.672**	**0.488**	**0.337**	**0.711**
Pretreatment with cisplatin + WG8185B2	5.381	2.102	**0.883**	**0.617**

WG8185B2 was applied at the dose 25 µg/mL (54 µM). CI values were calculated using CalcuSyn software as described in Materials and Methods. CI values < 1 indicate synergism, whereas CI values = 1 and >1 indicate additivity and antagonism, respectively.



WG8185B2



WG9001B

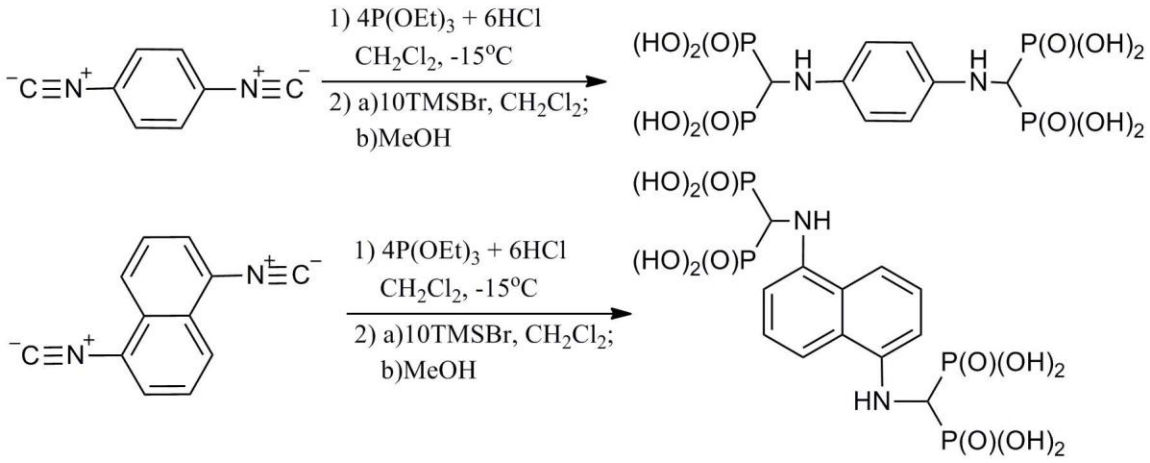


Figure 3.

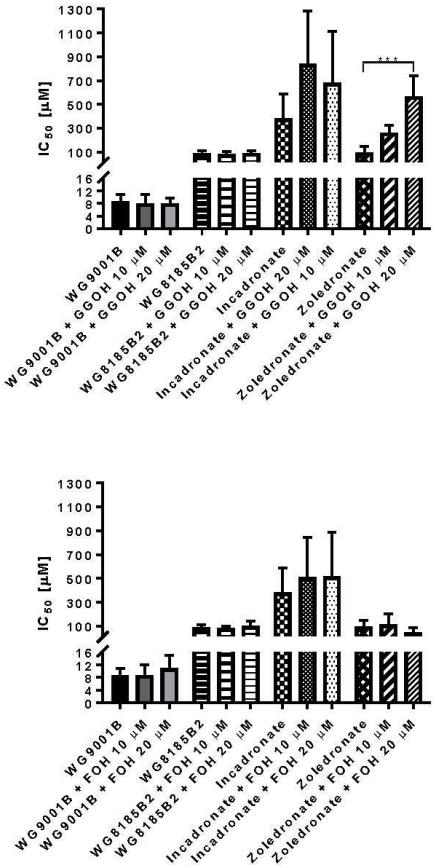


Figure 4.

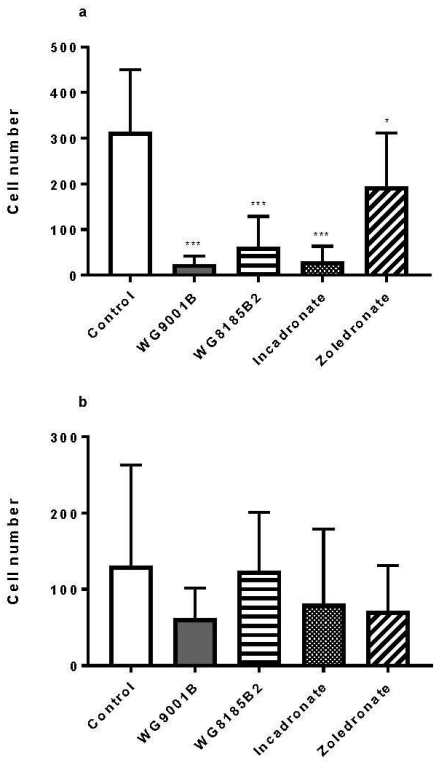


Figure 5.

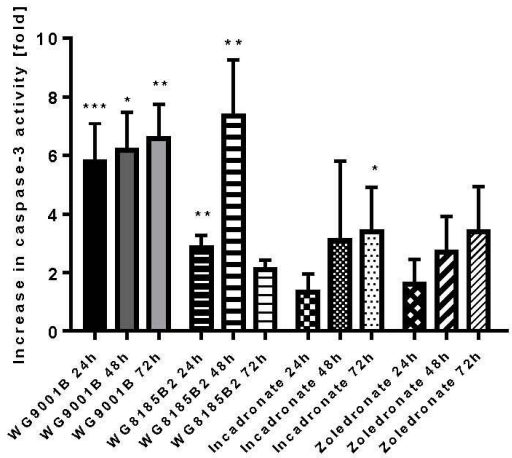


Figure 6.

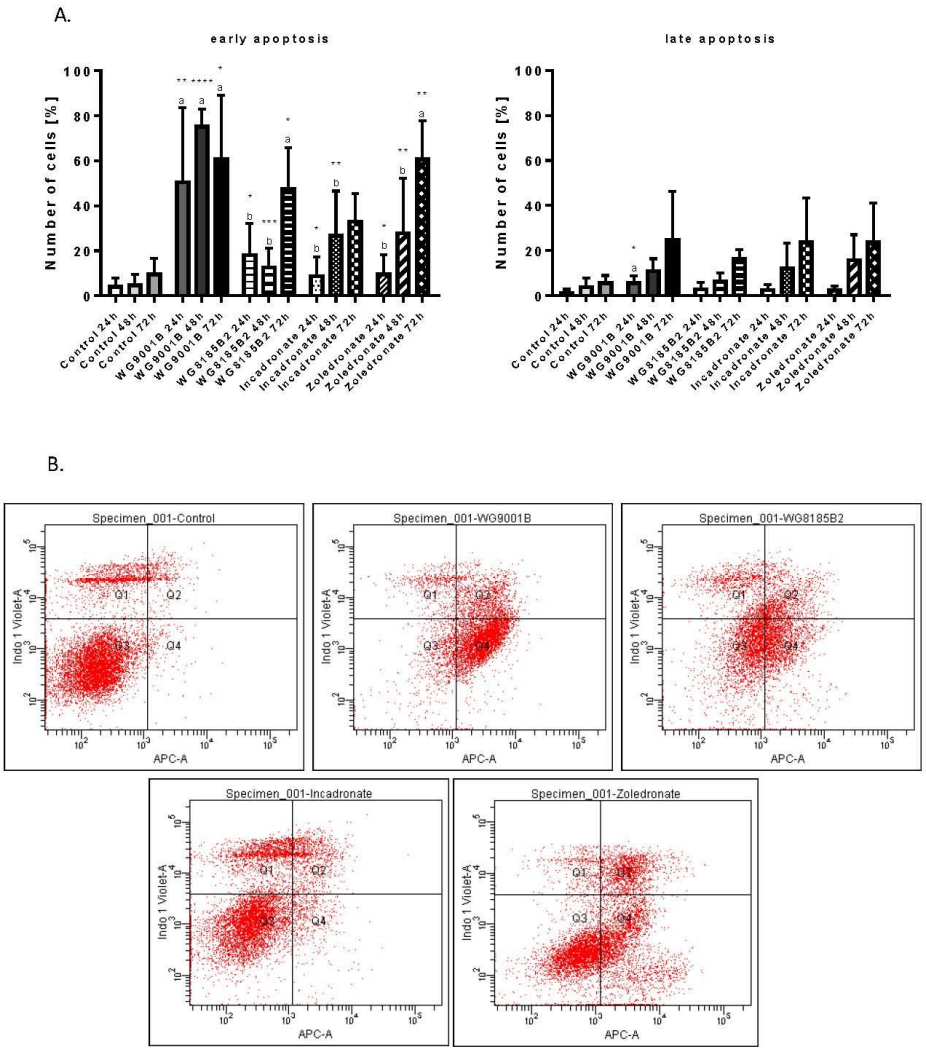


Figure 7.