INHIBITORY ACTION OF METHYLENE BISPHOSPHONIC ACID ON METABOLIC ACTIVITY AND VIABILITY OF J774A.1 CELLS

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Bisphosphonates (BPs) are primary agents in the current pharmacological arsenal against osteoclast-related bone loss due to osteoporosis, Paget disease, and bone tumours. Due to the lack of complete understanding of the molecular mechanism of their action in bone tissue and the overlap of key properties between BPs of different generations, integral studies of the BPs inhibitory and antiresorptive properties are relevant today. The present work was carried out to establish a comprehensive study of the inhibitory effects of methylene bisphosphonic acid (MBPA) on the mevalonate pathway, metabolic activity and cell death in vitro compared to zoledronic acid (Zol). Farnesylpyrophosphate synthase (FPPS) activity of MBPA-treated J774A.1 cells was inhibited by 80%, compared with a 79% reduction in Zol-treated samples. The ability of MBPA to decrease the percentage of viable cells in culture is slightly lower compared with zoledronic acid. After 24 h of incubation with lowest concentration, the percentage of inhibition of metabolic activity was 10.6 and 25% resp. After 48 h, these values were 34.8% and 55.6% respectively. The inhibitory effects of MBPA and Zol on the intensity of incorporation of radioactively labelled precursor [14C]-acetate to the cholesterol fraction were 76.2 and 59.1% resp. In the case of isoprenoid fraction, the inhibitory effects were 40.9 and 51.2% resp. MBPA and Zol differently induced apoptosis in the J774A.1 cells culture, increased count of apoptotic cells in 2.4 and 6.3 times, and also increased the number of PI-positive cells in 7.4 and 19 times respectively. MBPA and Zol also increased the number of TUNEL-positive cells in macrophage culture in 2.6 and 5 times resp. Zoledronate significantly reduced carbonic anhydrase 2 and NFATc1 gene expression levels compared to the MBPA action. Thus, the use of methylene bisphosphonic acid in future research and therapy of both cancer and osteoporosis looks promising due to lower cytotoxicity, high efficiency of mevalonate pathway inhibition and the possibility of dosage variation.

Keywords: methylene bisphosphonic acid, zoledronic acid, cell death, J774A.1 macrophages, FPPS enzymatic activity, cell viability, gene expression, radioisotopes.

Bisphosphonates (BPs) are synthetic stable analogues of inorganic pyrophosphate that have a high affinity with bone hydroxyapatites. BPs possess antiresorptive, antiproliferative, antiparasitic and immunomodulatory properties [1]. This gives a possibility for the clinical treatment of osteoporosis, bone metastasis, multiple myeloma, breast cancer and Paget’s disease, as they help prevent hypercalcemia, pain and pathological fractures [2]. Traditionally, BPs are divided into two large groups that are different in chemical structure and mechanism of effect on the cells of bone tissues [1, 2]. The first group includes BPs containing no nitrogen atoms (etidronate, clodronate). As assumed, the mechanism of their effect is the foremost formation of the adenosine triphosphate (ATP) analogues, which cannot hydrolize. They inhibit numerous ATP-dependent processes in the cell. As opposed to this type of BPs, the primary mechanism of the action of nitrogen-containing BPs is the specific inhibition of enzymes during the biosynthesis of cholesterol farnesyl pyrophosphate synthase (FPPS) and geranylgeranyl pyrophosphate synthase. It is a critical link for posttranslational modification (prenylation) of small signal G proteins that are important in the specific functions of osteoclasts. The most effective representative of this third-generation BPs group is zoledronic acid (Zol). Along with its expected influence, Zol may cause some side effects. If these side effects occur, they may need medical attention.
[3, 4]. The methylene bisphosphonic acid (MBPA) structure contains two hydrogen atoms, R1 and R2, and belongs to the first-generation BPs. MBPA has a wide range of biological effects, partly modulatory action on the bone tissues and immune system. Judging by previous studies, it does not have a toxic effect on the organism [5-7]. However, the exact mechanisms of MBPA's effect on bone tissue are not studied. Due to the lack of complete understanding of the molecular mechanism action in bone tissue and the overlap of key properties between BPs of different generations, integral studies of BPs inhibitory and antiresorptive properties are relevant today. The work aimed to comprehensively study the inhibitory effects of MBPA on the mevalonate pathway, metabolic activity and cell death in vitro compared with Zol.

Materials and Methods

Cell culture. The J774A.1 murine macrophage cell line was purchased from the American Type Culture Collection (ATCC) (Gaithersburg, Maryland, United States). Cells were cultured as recommended by the ATCC in Dulbecco’s Modified Eagle’s medium (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland), 4.5 g/L D-glucose supplemented with 10% v/v heat-inactivated fetal bovine serum (Sigma-Aldrich Ireland), 4.5 g/L D-glucose supplemented with 10% v/v heat-inactivated fetal bovine serum (Sigma-Aldrich Ireland Ltd., Wicklow, Ireland). Cells were maintained at 37°C in an incubator under 5% carbon dioxide and 95% relative humidity. Prior to the experiments, cells were detached from the flask using a Trypsin-EDTA solution, then centrifuged at 1,500 rpm for 4 min at room temperature (RT), and resuspended in antibiotic-free growth media. Cells were counted using Countess™ 3 FL Automated Cell Counter (Thermo Fisher Scientific) for 24 h before incubation with experimental BPs in different concentrations (allowed to grow to approximately 70–80% confluency). Control cells were also run in parallel and subjected to the same medium changes.

Inhibition of farnesyl pyrophosphate synthase activity assay. The FPPS enzyme for the experiment was obtained from the peroxisomal fraction of rat liver, as described previously [8]. Fresh perfused liver tissue was frozen with liquid nitrogen and homogenized in a cold buffer (pH 7.4) containing 0.3 M sucrose, 10 mM EDTA and 1 mM mercaptoethanol. After serial centrifugation of the homogenate at 900 g, 8700 g and 10000 g, the pellet was collected and resuspended in 0.2 ml of buffer cooled to 0°C (pH 7.4) containing 20 mM imidazole and 5 mM dithiothreitol. Protein was determined in an aliquot according to the Lowry assay. For the FPPS activity reaction, the amount of the diluted fraction containing 100 μg of protein in the sample was used.

Farnesyl pyrophosphate synthase activity. FPPS activity was measured with author modifications according to the method described by [9]. Briefly, FPPS activity was tested in 100 μl of a mixture containing 25 mM HEPES (pH 7.2), 10 mM sodium citrate, 5 mM MgCl₂, 1 mM dithiothreitol, 0.01% Triton X100, 5 μg/ml BSA, 0.05 U/ml pyrophosphatase enzyme, 20 μM geranyl pyrophosphate (GPP) and 20 μM isopentenyl pyrophosphate (IPP). The reaction started by adding 30 μl of the peroxisomal fraction and a mixture of substrates (GPP + IPP), then incubation for 60 min at 37°C. The reaction was stopped by heating for 10 min at 95°C, after which the mixture was cooled and centrifuged at 7000 rpm. In control samples, the peroxisome fraction was added immediately before the enzyme was inactivated by heating. Sixty μl of the supernatant was transferred to a 96-well plate and 140 μl of CytoPhos reagent (CytoPhos™ phosphate assay kit, Cytoskeleton) was added for terminal phosphate detection. After incubation for 10 min at RT, the optical density was measured at 650 nm on a microplate reader (ER500, MiBoxer).

Bisphosphonates inhibitory effect. BPs inhibitory effect on FPPS activity was assessed using the method described above. BPs were used in concentrations of 1–100 μM. The appropriate BP amount was pre-incubated with the peroxisomal fraction in the incubation medium for 30 min at RT in a total volume of reaction mix 70 μl, and then substrates were added.

[¹⁴C]-labeling and radioisotope assay. The J774A.1 cell culture was preincubated with the test BPs (10 μM) for 3 h before [¹⁴C]-labeled acetate (Sigma) was added, then incubation was continued for another 21 h. The labeled acetate was subsequently metabolized and accumulated, including in cholesterol and isoprenoid fractions of the mevalonate metabolic pathway. The method is based on determining the level of radiation in the fraction of isoprenoid alcohols (isopentenol + geraniol) obtained by chromatographic separation of the components of the hexane extract after acid hydrolysis of IPP and GPP. Extracts from cell lysates were prepared by the Folch method [10], and the methanol phase was treated with 2.5N HCl to hydrolyze pyrophosphates. After neutralization of this mixture, isoprenoid al-
cohols were extracted with hexane. To separate and identify isopentenol and geraniol in the samples, thin layer chromatography was used on Sorbfil analytical plates using the corresponding compounds (isopentenol, geraniol) (Sigma-Aldrich) as standards. A modified anisaldehyde reagent was used to visualize the fractions.

Cholesterol from macrophage lysates was extracted using the standard procedure described in [11].

The accumulation of [14C] in the isoprenoid and cholesterol fractions of macrophages was determined in solubilized aliquots using scintillation Delta 300 β-spectrometer (Tracor Analytic, USA) in scintillation liquid for non-aqueous samples (Ultima Gold, USA) (1.5 ml).

**MTT assay.** After 24 h and 48 h exposure with BPs in 10 μM – 1 mM concentrations, the control medium or test samples were removed, the J774A.1 cells were washed with phosphate-buffered saline (PBS) and 100 μl of freshly prepared thiazolyl blue tetrazolium bromide (MTT) in media (5 mg/ml of MTT in media without serum) was added to each well. After 3 h incubation, the medium was discarded, the cells were rinsed with PBS and 100 μl of dimethyl sulfoxide was added to each well to extract the dye. The plates were shaken at 240 rpm for 10 min, and the absorbance was measured at 595 nm in a microplate reader [12].

**Flow cytometry.** Apoptosis was determined using an FITC Annexin V/PI Apoptosis Detection Kit according to the manufacturer’s instructions (BMS306FI-300, Thermo Fisher). J774A.1 cells were incubated with 10 μM of BPs for 24 h. Then cells were collected and resuspended in a binding buffer (0.01 M Hepes (pH 7.4), 0.14M NaCl and 2.5 mM CaCl2). Subsequently, cells were incubated with Annexin V-FITC for 10 min at RT, washed with binding buffer and then 5 μl of propidium iodide (PI) was added before analysis. Cells were analyzed by the flow cytometer (DxFLEX Beckman Coulter, USA).

**TUNEL assay using confocal microscopy.** DNA fragmentation was tested using TUNEL Assay Kit - FITC (ab66108) (Abcam, GB), following guidelines as described [13]. J774A.1 macrophages were cultured on glass slides (poly-L-lysine used for adhesion) for 12 h and then treated with BPs (10 μM). The macrophages were fixed in 4% paraformaldehyde in PBS for 30 min, then permeabilized in 0.2% Triton X-100/PBS (Sigma-Aldrich, Germany) for 5 min. After washing the slides twice using PBS and adding 100 μl of the equilibration buffer for 10 min at 4°C, the samples were incubated in 50 μl of the terminal deoxynucleotidyl transferase reaction mixture for 1 h at 37°C in a humidified chamber in the dark. To stop the reaction, the glass + diamidino-2-phenylindole was added to the mounting medium and 5 μl of PI was added before analysis. TUNEL- and PI-positive cells were analyzed using the LSM 510 META confocal laser scanning microscope (Carl Zeiss, Germany). Pictures were quantified using an average optical density analysis using ImageJ (Fiji Software).

**RNA isolation and RT-qPCR.** Total RNA was extracted from the J774A.1 macrophages using TRIzol reagent according to the manufacturer’s instructions (Sigma-Aldrich, USA). The cDNA was synthesized from 1 μg of total RNA using random primers and Moloney murine leukemia virus reverse transcriptase (both from Life Technologies, USA) as previously described [14]. The forward and reverse primers for mice were as follows: for carbonic anhydrase 2 (Car2) mRNA – 5′-AAGGTCAAACTCCGCAGC-3′ and 5′-TTTTATAGGCTCCGGCACA-3′; for nuclear factor of activated T-cells 1 (NFATc1) mRNA – 5′-GTCAGAGGTAGACCGAGG-3′ and 5′-GACATGGGTTGTG-3′; for first reference gene 18S – 5′-CGGCCGCTAGAGGGAATTC-3′ and 5′-CATCTTGGCAAT-3′; and for second reference gene (as required for the normalization of gene expression levels) – GAPDH mRNA – 5′-TCAGAGGTAGACCGAGG-3′ and 5′-GACATGGGTTGTG-3′. Real-time quantitative polymerase chain reaction analyses were performed using the Mx3005P Real-Time PCR System (Stratagene, USA). For each condition, we quantified gene expression in duplicate, and 18S/GAPDH rRNAs were used to double the endogenous control in the comparative cycle threshold method. That data is shown as a relative expression ratio.

**Statistical analysis.** All the data were expressed as mean ± SEM deviation of at least three independent experiments. Statistical differences between the various groups were compared by using Student’s t-test and one-way ANOVA. P-values less than 0.05 were considered statistically significant.

**Results and Discussion**

Certainly, the use of Zol for treating bone tissue diseases has a high degree of therapeutic effectiveness. Still, it can cause significant side effects, including osteonecrosis of the jaw bones, tooth loss,
neuropathy and immunopathy, etc. [15, 16]. Moreover, modern research is focused on searching for new solutions in cancer therapy using the pronounced cytotoxic properties of zoledronate to create complex compounds with oncosuppressive effects [17]. Thus, a pressing issue for science and medicine today is to determine the cytotoxicity of BPs of different generations and to elucidate the molecular mechanisms behind this. This will open a possibility for more selective application of bisphosphonate drugs for treating bone tissue diseases in various cases, weighing the risks and accurately determining the therapeutic dosage and timing of treatment.

Since most studies about BPs inhibitory effect on the enzymatic activity of FPPS used a recombinant enzyme [9, 18], an important advantage in our work was the methodological modification by using the native enzyme from the rat liver peroxisomal fraction. Both zoledronate and MBPA, even at a concentration of 1 µM, strongly reduced FPPS enzymatic activity by 80% and 79%, respectively, compared with the control (Fig. 1). The exposure to these compounds at 10 µM concentration reduced enzymatic activity by 87% and 88.35%, respectively, compared with the control, Fig. 1. These results may indicate that both compounds have a high affinity for the active site of FPPS, but this requires clarification in subsequent comparative in silico experiment work.

The following important indicator that characterizes and compares the inhibitory effect of BPs on FPPS activity was the radioactive [14C]-label accumulation (in the composition of acetate) in the intermediate and final fractions of the mevalonate metabolic pathway – isoprenoids and cholesterol. The analytical determination of isoprenoids using gas chromatography-mass spectrometry or ultraperformance liquid chromatography-tandem mass spectrometry approaches is quite expensive and has a lot of methodological difficulties. The use of radioactive isotopes can essentially eliminate these disadvantages [19, 20]. A cell culture of mouse macrophage-like J774.1 cells was used to work with BPs in vitro because J774A.1, along with macrophages RAW264.7, are considered as potential precursors of osteoclasts – the main factor in bone tissue resorption [21]. Incubation with 10 µM BPs for 24 h demonstrated that zoledronate inhibited the incorporation of the radioactive mevalonate pathway precursor into the isoprenoid fraction in J774A.1 cells by 51.2%, while MBPA inhibited by 40.9% compared to control values (Fig. 2, B). Examination of the content of radioactive cholesterol in J774A.1 cell lysates as the final product of the mevalonate pathway after 24 h of incubation, showed that zoledronate reduced the incorporation of radiolabel into the fraction by 59.1%, while action of MBPA mediated a decrease in incorporation by 76.2% compared to control (Fig. 2, A). Thus, MBPA paradoxically inhibited incorporation of a radioactive label into the cholesterol fraction by 41.8% more strongly than samples with Zol.

As previous studies showed, Zol can inhibit metabolism and proliferation in a population of many cell lines in vitro, especially the monocytomacrophage lineage [22, 23]. At the same time, some experimental data indicates the high cytotoxicity of zoledronate for oncotransformed cells including lymphoid and myeloid lineages; therefore, research of Zol in potential use for cancer therapy is not limited exclusively to the treatment of bone-related types of cancer [24, 25]. Current research demonstrates the ability of MBPA to decrease the percentage of viable cells in potential osteoclast progenitors – the number of J774A.1 cells is slightly lower compared with Zol. After 24 h of incubation, the percentage of inhibition of metabolic activity at the lowest tested concentration (10 µM) was 10.6% and 24.9%, respectively (Fig. 3). After 48 h of incubation, these values were 34.8% and 55.6%, respectively (Fig. 3). The effect of MBPA on J774A.1 cells has less pronounced metabolic inhibitory properties
Fig. 2. The effect of tested BPs on the accumulation level of radioactively \([^{14}\text{C}}]\)-labeled acetate in the isoprenoids and cholesterol fractions in J774A.1 macrophages culture. (A) \([^{14}\text{C}}]\)-labeled cholesterol content after 24-hour incubation with 10 μM bisphosphonates. (B) Content of \([^{14}\text{C}}]\)-labelled isoprenoids after 24-hour incubation with 10 μM bisphosphonates. Results are shown as mean ± SEM and representative of three independent experiments done in triplicate; *P < 0.05 vs. Ctrl, #P < 0.05 vs. Zol.

Fig. 3. Viability of J774A.1 macrophages after exposure to various concentrations of tested bisphosphonates (10 μM, 100 μM and 1 mM); incubation time 24 h. M ± m, n = 10; *P < 0.05 compared to the control. Results are shown as mean ± SEM and representative of three independent experiments done in triplicate; *P < 0.05 vs. Zol 24 h, #P < 0.05 vs. MBP 24 h, $P < 0.05 vs. Zol 48 h.

and, therefore, less cytotoxicity than Zol. Despite this, MBPA is successfully used in clinical practice to treat bone-associated tumors [6]. The authors emphasize the absence of toxic effects on the patient’s body while showing selective cytotoxicity on tumor cells in bone tissue and their metastases [7, 26]. In this context, a relevant question arises about the effect of the studied BPs on the metabolism of monocyte-macrophage cells as potential precursors of osteoclasts considering their relative effects on bone tissue resorption and osteoporosis.

An important aspect of the BPs action is its ability to regulate and induce cell death (apoptosis, necrosis), mainly by suppressing of the mevalonate metabolic pathway in the cell. Our findings have shown that incubation of J774A.1 cells with 10 μM Zol led to a 6.3-fold elevation of Annexin V-FITC labeling of macrophages compared with the control, corresponding to the increased number of apoptotic cells in J774A.1 cell culture (Fig. 4, A, B). At the same time, exposure of macrophages to an identical concentration of MBPA led to a 2.4-fold increase...
Fig. 4. Apoptosis in J774.A.1 cells culture after bisphosphonates treatment (10 μM): (A) representative flow cytometry histograms of Annexin V – FITC accumulation (count – the number of events; FL1 LOG – fluorescence intensity); Gate – J774.A.1 population with the highest fluorescence along the FL1 channel; (B) quantification of Annexin V – FITC fluorescence. Results are shown as mean ± SEM and representative of three independent experiments done in triplicate; *P < 0.05 vs. Ctrl, #P < 0.05 vs. Zol

Besides necrotic cells, PI-positive macrophages may include cells at a late stage of apoptosis. Such a high level of apoptosis and necrosis of macrophages in the group exposed to zoledronate compared to MBPA correlates with experimental data from other studies.
that link the high cytotoxicity of zoledronate and its participation in the modulation of tissue inflammation in cases of osteonecrosis of the jaw [4, 27, 28]. This problem is common for third-generation BPs, while this is not the case for first-generation BPs.

Taking into account the small size of MBPA molecules, one of the options for the development of bisphosphonate chemistry may be the creation of supramolecular compounds – MBPA derivatives [29]. Often, integration onto supramolecular platforms...
Fig. 6. Effect of experimental bisphosphonates on apoptosis (DNA fragmentation) in J774A.1 cells using confocal microscopy. J774A.1 cells were exposed to BPs (10 µM) for 24 h. Representative pictures of TUNEL- and PI-positive cells (A) and its quantification by average optical density analysis (B). Arrows – Annexin V- and PI-merged cells. Results are shown as mean ± SEM and representative of three independent experiments done in triplicate; *P < 0.05 vs. Ctrl, #P < 0.05 vs. Zol.
impacts new useful properties to target molecules [30, 31].

Since the terminal phase of apoptosis is irreversible and manifested by cell degradation with cleavage of structural proteins, chromatin condensation and DNA fragmentation [32], we performed a TUNEL assay in addition to cytofluorometric studies. Adhered to glass, J774A.1 macrophages were exposed to 10 μM of Zol and MBPA, and the presence of TUNEL- and PI-positive cells were determined for various quantities. Thus, exposure to Zol increased the number of TUNEL-positive cells 5-fold, and with MBPA 2.6-fold compared with the control (Fig. 6, A, B). The percentage of PI-positive cells increased in 5.3 and 2.6 times, respectively, compared to the control (Fig. 6, A, B).

An important reason for this difference in cytotoxicity and induction of cell death among BPs of different generations may be their mechanisms of action. Growing evidence suggests that third-generation BPs, in addition to the canonical effect of the inhibiting of mevalonate pathway, also have other mechanisms of action on the cell molecular processes [33-35]. Zoledronate demonstrates a particularly interesting ability to directly regulate the expression of some vital macrophage genes, including Car2, NFATc1, interleukin 1 beta, etc. [36, 37]. Thus, it was decided to test the regulatory effect of the studied BPs on the expression of some of these genes in osteoclast progenitors J774A.1. After 24 h exposure to 10 μM Zol, the level of Car2 expression in J774A.1 macrophages decreased by 68% and NFATc1 by 48%, compared with the control (Fig. 7, A, B). Exposure to MBPA decreased the Car2 expression level by 18% but did not affect the expression of NFATc1 compared to the control (Fig. 7, A, B).

Thus, we assumed that zoledronate, as a third-generation bisphosphonate, has greater cytotoxicity due to the pleiotropic nature of its effect on molecular and biochemical processes in the macrophage cell. Apparently, this significantly impacts the higher occurrence of Zol side effects in therapy compared to first-generation BPs (MBPA, etc.) where side effects are observed much less often.

Conclusions. Overall, our findings suggest that MBPA inhibits FPPS activity in a cell-free system similarly to Zol and almost identically effectively inhibits the incorporation of a [14C]-radioactive label into both the isoprenoid and cholesterol fractions of the mevalonate pathway in J774A.1 macrophages. Zol inhibits metabolism and increases the number of Annexin V-, PI- and TUNEL-positive cells in the culture of J774A.1 macrophages significantly more intensively compared to MBPA. It was shown that Zol exhibits a pronounced regulatory effect on gene expression, significantly inhibiting the Car2 and NFATc1 gene expressions compared with MBPA, which can also determine the higher cytotoxicity of third-generation BPs. Thus, the use of MBPA in future research and therapy of both cancer and osteoporosis looks promising due to lower cytotoxicity, high efficiency of mevalonate pathway inhibition and the possibility of dosage variation.

Conflict of interest. The authors have filled the Unified Conflicts of Interest form at http://ukr-
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