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ORIGINAL ARTICLE

Zoledronic acid induces apoptosis via stimulating the expressions of ERN1, TLR2, and IRF5 genes in glioma cells

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Abstract Glioblastoma multiforme (GBM) is the most common and aggressive brain tumor that affects older people. Although the current therapeutic approaches for GBM include surgical resection, radiotherapy, and chemotherapeutic agent temozolomide, the median survival of patients is 14.6 months because of its aggressiveness. Zoledronic acid (ZA) is a nitrogen-containing bisphosphonate that exhibited anticancer activity in different cancers. The purpose of this study was to assess the potential effect of ZA in distinct signal transduction pathways in U87-MG cells. In this study, experiments performed on U87-MG cell line (Human glioblastoma-astrocytoma, epithelial-like cell line) which is an in vitro model of human glioblastoma cells to examine the cytotoxic and apoptotic effects of ZA. IC_{50} dose of ZA, 25 μM, applied on U87-MG cells during 72 h. ApoDIRECT In Situ DNA Fragmentation Assay was used to investigate apoptosis of U87MG cells. The quantitative reverse transcription polymerase chain reaction (qRT-PCR) (LightCycler480 System) was carried out for 48 gene expression like NF-κB, Toll-like receptors, cytokines, and inteferons. Our results indicated that ZA (IC_{50} dose) increased apoptosis 1.27-fold in U87MG cells according to control cells. According to qRT-PCR data, expression levels of the endoplasmic reticulum-nuclei-1 (ERN1), Toll-like receptor 2 (TLR2), and human IFN regulatory factor 5 (IRF5) tumor suppressor genes elevated 2.05-, 2.08-, and 2.3-fold by ZA, respectively, in U87MG cells. Our recent results indicated that ZA have a key role in GBM progression and might be considered as a potential agent in glioma treatment.

Keywords Zoledronic acid · Gene expression · Glioma

Introduction

Glioblastoma multiforme (GBM) is the most common and aggressive brain tumor that affects older people. [1]. According to the World Health Organization (WHO) in 2007, GBM was determined as grade IV astrocytoma [2, 3]. Although the current therapy approaches for GBM are surgical resection, radiotherapy, and chemotherapeutic agent temozolomide, the median survival of patients is 14.6 months because of its aggressiveness [4, 5]. There is a need to investigate new therapeutic approaches for GBM due to the shortened survival of patients.

Synthetic analogues of pyrophosphate called bisphosphonates (BPs) act as efficient inhibitors of osteoclastic bone resorption [6, 7]. BPs consist of three generations that differ from their structure in R1 side chain. Both first and second generations do not include any nitrogen in the R1 chain, but third group of BPs has nitrogen in circular structure [7]. Zoledronic acid (ZA), a third-generation bisphosphonate, exhibited anticancer activity in various metastatic cancers including breast, prostate, lung cancer, and multiple myeloma with skeletal complications of bone metastasis [8, 9]. The possible mechanism of ZA in blocking osteoclast-mediate bone absorption is the inhibition of the farnesyl diphosphate synthase which is the key enzyme of the mevalonate pathway. Through this mechanism, farnesyl diphosphate and its

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downstream geranylgeranyl diphosphate are inhibited, and thus, small GTPases including Ras, Rho, and Rab which regulate osteoclast cannot be post-translated [10].

In recent years, direct or indirect effects of ZA on cancer cells have been shown in several studies. These studies show that ZA has anti-proliferative, proapoptotic, and anti-invasive activities and anti-angiogenic and immunomodulatory abilities [11]. Moreover, ZA has been used as a new therapeutic approach in combination with various agents such as cisplatin, etoposide, doxorubicin, paclitaxel, irinotecan, and imatinib, because of the synergistic effects of these combinations in various cancer cells [11].

The purpose of this study was to assess the potential effect of ZA on the expression of 48 genes in distinct signal transduction pathways in U87-MG cells.

Materials and methods

Chemicals

Zoledronic acid was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). ZA was diluted in distilled water and stored as 10-mM stock solution at −20 °C. Prior to experiments, different concentrations of ZA were prepared from stock solution.

Cell culture

U87-MG cell line was obtained from American Type Culture Collection (ATCC, Manassas, VA). U87-MG cells were seeded into 75-cm^2 tissue culture flask. They were maintained in BIO-AMF-1 basal medium containing 2 mM L-glutamine supplemented with 50 ml BIO-AMF-1 supplement and 1 % penicillin/streptomycin in a standard cell culture incubator at 37 °C, 95 % relative humidity, and 5 % CO_2 atmosphere.

Determination of cytotoxicity

Cytotoxicity of ZA in U87-MG cells was assessed by WST-1 [2-(4-iodofenil)-3-(4-nitrofenil)-5-(2,4-disülfofenil)-2H-tetrazolium sodium salt] assay (Roche). Cells were seeded in 96-well culture plate at a concentration of 5×10^3 cells/well and incubated overnight for their adhesion to plate surface. Cells were treated in the range of 10–100-μM doses of ZA for 72 h. After treatment, 10-μL WST-1 reagent was added per well. Formazan formation was quantified spectrophotometrically at 480 nm (reference wavelength 620 nM) by using a microplate reader (Bio-Rad, Coda, Richmond, CA).

Apoptotic DNA fragmentation assay

To detect the apoptotic effect of ZA to DNA Fragmentation on U87-MG cells, ApoDIRECT In Situ DNA Fragmentation Assay (BD Pharmingen) was performed by flow cytometry. For fixation procedure, $1–2\times10^6$ untreated cells and ZA-treated cells were suspended in 1 % paraformaldehyde in PBS. After centrifugation steps, according to kit protocol, cells were treated with 70 % ice-cold ethanol for 30 min. For staining protocol, cells were suspended in DNA Labeling Solution prepared as described in the kit for 60 min at 37 °C. At the end of the incubation time, cells were treated with rinse buffer followed by centrifugation. After removing the supernatant, cell were resuspended in PI/RNase staining buffer and incubated at room temperature for 30 min. Then, PI/RNase-treated cells were analyzed by flow cytometry.

RNA isolation and cDNA synthesis

Total RNA was isolated from U87-MG cells treated with IC_{50} dose (72 h) of ZA and untreated control cells (High Pure RNA Isolation Kit - Roche). Reverse transcription procedure was performed for complementary DNA (cDNA) synthesis by using Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Germany) according to the instructions of manufacturers.

Quantitative real-time PCR

Custom RT^2 PCR Array (Roche Diagnostics, Germany) was used to evaluate the quantitative gene expression analysis of 48 genes including housekeeping genes with LightCycler 480 quantitative reverse transcription polymerase chain reaction (qRT-PCR) system. The expression values of these genes were proportioned to housekeeping genes (*18S ribosomal RNA* and *GAPDH*) to calculate the relative expression ratios. List of the genes is shown in Table 1.

Statistical analysis

Cytotoxicity analysis was calculated by GraphPad Prism v5.0 Software. Data analysis was evaluated by ΔΔCT method and quantitated by "LightCycler 480 Quantification Software," and statistical analysis was evaluated by web-based RT^2 Profiler PCR Array Data Analysis version 3.5. The p value calculation was based on a Student's t test of the replicate $2^{-\Delta Ct}$ values for each gene in the control group and treatment groups. A value of $P<0.05$ was considered as significant.

Results

Cytotoxicity assay (WST-1 assay)

The cytotoxic effect of ZA on U87-MG cells was determined by WST-1 cytotoxicity assay as described in "Materials and

Table 1 List of the genes

Position	Gene symbol	Position	Gene symbol
A1	XBP1	E1	NFKB1
A2	ERN1	E2	NFKB2
A3	PAX5	E3	IKBKG
A4	AICDA	E4	IKBKB
A5	BCL6	E5	NFKBIA
A6	PRDM1	E6	REL
B1	MTA3	F1	RELA
B2	CYP27B1	F2	RELB
B3	TNFRSF11A	F3	IRF3
B4	TNFSF11	F4	IRF7
B5	TNFRSF11B	F5	IRF5
B6	TLR1	F6	ACTB
C1	TLR2	G1	IFNA1
C2	TLR3	G2	IFNG
C3	TLR4	G3	TNF
C4	TLR5	G4	MAP3K7
C5	TLR6	G5	MAP2K3
C6	TLR7	G6	GAPDH
D1	TLR8	H1	MAP2K6
D2	TLR9	H2	MAPK14
D3	FKBP1A	H3	MAP2K4
D4	MTOR	H4	MAPK8
D5	RPS6KB1	H5	MAP3K2
D6	AKT1	H6	18S RIBOSOMAL RNA

methods." Concentration doses of ZA were prepared in the range of 10–100 μM and treated 5×10^3 U87-MG cells per well. Data of the cytotoxic effect of ZA was assessed at 24th, 48th, and 72nd hours. The viability of U87-MG cells decreased in a time- and dose-dependent manner. In the present study, IC_{50} doses of ZA in U87-MG cells were calculated as 25 μM at 72nd hours via WST-1 assay (Fig. 1).

Determination of apoptotic DNA fragmentation assay by flow cytometry

Apoptotic effects of ZA on U87-MG cells were analyzed by a commercial kit according to the protocol of the manufacturer. Initially, U87-MG cells were cultured at $1–2\times10^6$ cells. After we had enough cell density, cells were fixed and stained, respectively, according to the protocol. At the end of all steps, apoptotic status of U87-MG cells treated with 25 μM of ZA was analyzed by flow cytometry. Our flow cytometry data indicated that apoptosis was increased 4.25-fold by ZA when compared to control cells untreated with ZA (Fig. 2). Also, we found that ZA induced apoptosis 1.14- and 2.33-fold at 24th and 48th hours, respectively.

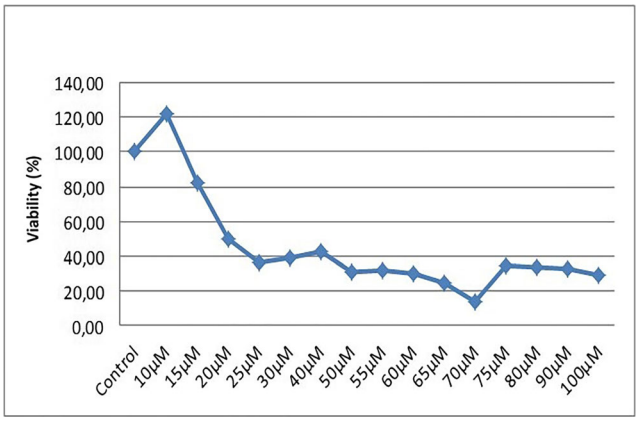

Fig. 1 Effect of zoledronic acid on the cell viability of U87-MG cells. The cells were treated with 0–100-μM zoledronic acid during 72 h. Cell viability was calculated by WST-1 cell viability assay. Data are the average results of three independent experiments (IC_{50} dose of ZA in U87-MG cells was determined as 25 μM at 72th hour

Real-time PCR

Afterwards total RNA isolation from control and ZA-treated cells by using commercial kit, cDNA synthesis was performed by Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Germany) as first step of reverse transcription. Custom RT^2-PCR array was performed to evaluate the quantitative gene expression analysis including housekeeping genes at LightCycler 480 qRT-PCR platform. The results of expression were proportioned to expression of *18S ribosomal RNA* and *GAPDH* (housekeeping genes) to calculate the relative expression ratios. We totally investigated the expression of 48 genes which serve in distinct signal transduction pathways in U87-MG cells. Gene expression results regarding IC_{50} dose of ZA showed that *ERN1*, *TLR2*, and *IRF5* tumor suppressor gene expressions were detected 2.05-, 2.08-, and 2.3-fold, respectively, according to untreated U87-MG control cells ($p<0.05$).

Discussion

Some of the cancers have ability to metastasize to their specific tissues by using lymphatic and blood-flow patterns, their cellular adhesion molecules and receptors and the cytokine signals which they can synthesize or they can react [12]. A dynamic and vascularized bone tissue provides a hematopoietic environment for cancer stem cells [12]. Bone can discharge some factors which cancer cells respond so that it can play role in metastasis development. Therefore, patients with metastatic cancers such as multiple myeloma (95–100 %), breast (75 %) or prostate, lung (40 %) and renal cell cancers metastasize to the bone [13]. As a result, researchers have aimed to develop new drug strategies to treat bone metastasis for many years.

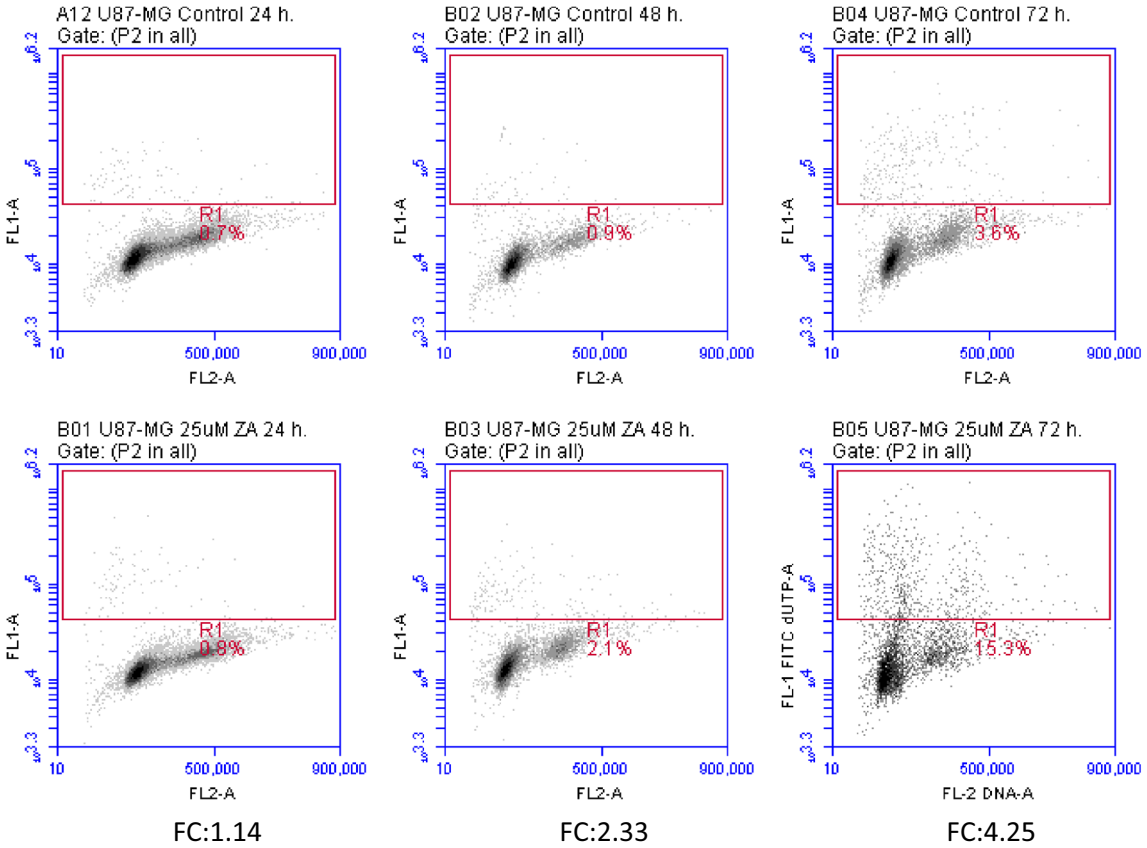

Fig. 2 Apoptotic effect of zoledronic acid depends on time and dosage. U87-MG cells were treated 25 μM ZA during 24, 48, and 72 h. Apoptotic effects of ZA were detected by commercial kit. Apoptotic status of U87-MG cell treated with 25 μM ZA was analyzed by flow cytometry (IC_{50} dose of ZA induced apoptosis 4.25-fold when compared to control cells that untreated with ZA at 72th hours)

Bisphosphonates (BPs) are the non-hydrolytic synthetic analogues of inorganic pyrophosphate by substitution of the oxygen atom with a carbon molecule that were used as a potential inhibitor of bone resorption over 40 years [14, 15]. BPs are primarily bound to the bone and absorbed by osteoclasts, so this absorption causes inhibition of bone resorption and osteoclast apoptosis [16]. BPs show whether their effectiveness includes nitrogen [17]. Non-nitrogen-containing group called as early-generation BPs that are ingested by osteoclasts into non-hydrolysable analogues of adenosine triphosphate leading to inhibition of cellular function ultimately results in cell death [15]. Nitrogen-containing BPs (N-BPs) show their effect by inhibiting mevalonate pathway via diminishing activity of farnesyl pyrophosphate synthase (FPP); thus, they reduce cellular protein prenylation which is vital for function and survival [15, 17, 18]. Berenson J. reviewed that BPs play an important role in induction of apoptosis, prevention of tumor cell proliferation, blocking of angiogenesis, inhibition of cell migration, prevention of tumor cell invasion, decrease in adhesion of tumor cells, reduction in tumor-infiltrating and angiogenic macrophages, and stimulation of gamma delta T cells [19]. In vitro studies revealed that BPs can induce apoptosis on a large scale of human cancers including melanoma, sarcoma, myeloma, leukemia, breast, colon, lung, prostate, and pancreas [18, 20–24]. Also, these studies showed that N-BPs induce apoptosis with low concentrations compared to early-generation BPs.

ZA, a heterocyclic and nitrogen-containing bisphosphonate, is an effective inhibitor of osteoclastic bone resorption, and it functions to decrease bone resorption in patients with osteoporosis in clinical practice [8, 25]. It is also used for the treatment of tumor-induced hypercalcemia and the decline of skeletal complications in solid tumors and multiple myeloma [25]. Initially, despite its usage as an inhibitor of osteoclastic bone resorption, current studies are focused on its direct anti-cancer effect independently of osteoclastic bone resorption effect. Direct anti-cancer effects of ZA were shown in some cancer types such as breast, pancreatic, prostate, and multiple myeloma [26, 27]. Nevertheless, its anti-cancer activity differentiates due to type and origin of cancer. On the other hand, the inhibitory effect of ZA and its molecular mechanism are not so clear in glioblastoma multiforme. In the present study, it

has been aimed to investigate the possible effects of the third-generation BPs, ZA on U87-MG glioblastoma cell line.

In this study, we first analyzed the effects of ZA on U87-MG glioblastoma cell line. We treated the cells in the range of 10–100 μM ZA for 24, 48, and 72 h. According to our WST-1 results, IC_{50} dose of ZA on U87-MG cells is 25 μM at 72nd hour in dose-dependent manner. Reports on lung, breast, prostate, osteosarcoma, and pancreatic cancers showed that 50 % inhibitory concentration (IC_{50}) of ZA was ranged from 1 to 100 μM depending on the cell viability assay [28–35]. Romani et al. reported that ZA inhibited cell growth in dose-dependent manner in EGI-1 and TFK-1 cholangiocarcinoma cell lines by using MTT assay with the IC_{50} range from 17 to 37 mM for cells, respectively [36]. Koto and colleagues showed that ZA inhibited the growth of HT1080 fibrosarcoma cells in a time- and dose-dependent manner and by using trypan blue dye exclusion method. The IC_{50} of ZA after 48 and 72 h of treatment was calculated as 1.66 and 1.26 μM, respectively [37]. In a previous study, in vitro treatment of HCT-116 colon carcinoma cells with the range of 0–50 μM ZA reduced cell growth dependently, after 5 days treatment with 50 μM ZA resulting in more than 90 % decrease in the number of viable cells as compared to untreated control groups [22]. By using MTS cell viability assay, Liu et al. reported that the range of 0–200 μM ZA inhibited NB4 acute promyelocytic leukemia cells in a dose-dependent manner at 48 and 72 h. The highest inhibition rate was observed at 200 μM on NB4 cells [38]. Karabulut et al. used XTT cell viability assay in order to analyze the effect of ZA in ovarian cancer, and they showed that IC_{50} values of ZA were calculated as 15.5 and 13 μM on OVCAR-3 and MDAH-2774 cells, respectively [39]. Porru et al. studied on pcDNA3-luc-transfected U373 (U373MG-LUC) cells, and the analysis of U373MG-LUC cell growth in terms of MTT assay demonstrated that IC_{50} dose of ZA was 46 μM [40].

Several previous in vitro studies showed that ZA can induce apoptosis of tumor cell in a wide range of human and murine cancer cell lines [41–43]. The former studies demonstrated that ZA-induced apoptosis occurred by activation of caspase-3/7 signaling pathway. A previous study on HCT-116 colon carcinoma cells suggested that ZA induced apoptosis through the activation of the mitochondrial pathway, including caspase-8-mediated Bid activation, Bax translocation, cytochrome c release, and eventually activation of the caspase-3 and caspase-7. This study also showed that ZA activated mitochondrial pathway simultaneously releases AIF into the cytosol [22]. Tamura et al. reported that treatment of ZA on HSC-3 oral carcinoma cells induced apoptosis via caspase-3, caspase-8, caspase-9 activation, PARP and Bid cleavage, and decreased Bcl-2 levels [7]. Liu and their colleagues performed ZA treatment on NB4 acute promyelocytic leukemia cells, and they observed that the rate of apoptosis was significantly higher in the ZA-treated cells. They also reported that ZA upregulated the expression of cleaved caspase-3 and cleaved PARP, downregulated the Bcl-2 and Bcl-xL expressions at lower levels, expressed the Bax and Puma, and cleaved caspase-9 at higher levels [38]. Wang and colleagues demonstrated that treatment of ZA on cervical cancer cells caused a dose- and time-dependent PARP cleavage and the activation of caspase-3 signaling pathway. In order to confirm mitochondrial apoptosis, they examined the Bcl-2/Bax ratio mRNA expression in three cervical cancer cell lines. ZA increased Bcl-2/Bax ratio, and they suggested that ZA leads to mitochondrial membrane disruption and so initiates the mitochondrial apoptosis [44]. Rachner et al. quantified the percentage of apoptosis with ZA treatment on MDA-MB-231 and MCF-7 breast cancer cells by using annexin V/PI technique. They showed that percentage of apoptosis increased from 3.1 to 26.3 % (by 8.5-fold) in MDA-MB-231 cells after 72 h of ZA exposure, whereas the annexin V-fraction of MCF-7 cells only increased from 4.2 to 7.8 %. They also indicated that ZA induced the cleavage of caspase-3, caspase-7, and PARP only in MDA-MB-231 cells [31]. On the other hand, treatment of ZA in pancreatic cancer cell leads to activation of caspase-9 pathway, but not caspase-3 cascade [35]. Salvatore et al. showed that ZA increased apoptosis (43 %) significantly on non-small cell lung cancer cells compared to untreated cells [28]. Mani et al. investigated the effect of ZA on PC-3, DU-145, and LNCaP prostate cancer cells, and they observed that the Annexin V/PI assay demonstrated early apoptosis in PC-3 and DU-145 and late apoptosis in LNCaP cells only at 100 mM ZA [32]. In line with the literature, we examined that IC_{50} dose of ZA induced apoptosis 4.25-fold in U87-MG glioblastoma cells when compared to control cells. Also, we saw that ZA induced apoptosis 1.14- and 2.33-fold at 24th and 48th hours, respectively. In the light of these data, it may be suggested that ZA exhibits different apoptotic pathways depending on cell types.

According to our knowledge from literature, in vitro studies for inhibitory effects of ZA and its mechanism on glioblastoma are limited and unsatisfying. Cimini et al. evaluated whether ZA treatment would make glioma cell lines more susceptible to lysis by in vitro expanded Vδ2 T-cells, improving their antitumor activity. They reported that ZA enhanced Vδ2 T-cell antitumor response to human glioma cell lines and ZA synergistically elevated the Vδ2 T-cell-mediated apoptosis of glioblastoma cell lines [44]. Nakazawa and colleagues demonstrated that ZA significantly enhanced the γδT cell-mediated killing of U87MG, U138MG, and A172 GBM cell lines, and they suggested that combination of the γδT cell-targeting therapy with ZA might be effective for GBM patients [45]. Fukai et al. investigated whether ZA can be an effective adjuvant to temozolomide (TMZ) in human malignant glioma cells which express MGMT. Combination of TMZ and ZA resulted in a significant decrease in cell growth, an increased apoptotic rate and significant activation of

caspase-3 and PARP. Beside this, they obtained decreased amounts of Ras-GTP, MAPK, and Akt phosphorylation and MGMT expression only in ZA-treated cells. They also showed that combined TMZ and ZA treatment lead to significant decrease in tumor growth in subcutaneous xenograft models [46]. In this study, we evaluated the expression of several genes including several signal transduction pathways such as cytokines and costimulator molecules, interferons, NF-κB and Toll-like receptors in U87-MG cells by using real-time PCR method. Our study showed that IC_{50} dose of 25 μM ZA significantly resulted in increased *ERN1*, *TLR2*, and *IRF5* genes expressions with fold changes of 2.05-, 2.08-, and 2.3-fold, respectively, compared with untreated U87-MG control cells. Our data suggest that ZA may induce apoptosis through enhancing the expressions of *ERN1*, *TLR2*, and *IRF* genes in glioma cells. To our knowledge, this is the first report of ZA that induced the expression of these genes in glioblastoma.

In conclusion, a distinct increase in apoptosis was observed following the treatment of glioblastoma cells with 25 μM of zoledronic acid. These novel findings showed that ZA might be important in prognosis of glioma, and it is aimed to question if it could be used as a target substance in glioma treatment with further research. Based on the mentioned information and results, due to its feature for crossing the blood–brain-barrier (BBB), ZA may be an effective and alternative therapy to treat glioblastoma. Further studies are needed to clarify its mechanism of ZA in different types of glioma cell lines.

Compliance with ethical standards

Conflicts of interest None

References

1. Parsons DW, Jones S. X Zhang et al. An Integrated Genomic Analysis of Human Glioblastoma Multiforme: Science; 2008. doi: 10.1126/science.1164382.
2. Bayin NS, Modrek AS, Placantonakis DG. Glioblastoma stem cells: molecular characteristics and therapeutic implications. World J Stem Cells. 2014;6(2):230–8.
3. Olar A, Aldape KD. Using the molecular classification of glioblastoma to inform personalized treatment. J Pathol. 2014;232:165–77.
4. Ramirez YP, Weatherbee JL, Wheelhouse RT, Ross AH. Glioblastoma multiforme therapy and mechanisms of resistance. Pharmaceuticals. 2013;6:1475–506.
5. Stupp R, Mason WP, van den Bent MJ, Weller M, Fisher B, Taphoorn MJ, et al. European organisation for research and treatment of cancer brain tumor and Radiotherapy groups, national cancer ınstitute of canada clinical trials group. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. NEngl J Med. 2005;352:987–96.
6. Okamoto S, Kawamura K, Quanhai L, Yamanaka M, Yang S, Fukamachi T, et al. Zoledronic acid produces antitumor effects on Mesothelioma through Apoptosis and S-phase arrest in p53-ındependent and Ras prenylation-ındependent manners. J Thorac Oncol. 2012;7:873–82.
7. Tamura T, Shomori K, Nakabayashi M, Fujii N, Ryoke K, Ito H. Zoledronic acid, a third-generation bisphosphonate, inhibits cellular growth and induces apoptosis in oral carcinoma cell lines. Oncol Rep. 2011;25:1139–43.
8. Verhulst A, Sun S, McKenna CE, D'Haese PC. Endocytotic uptake of Zoledronic acid by tubular cells may explain ıts renal effects in cancer patients receiving high doses of the compound. PLoS One. 2015;10(3):e0121861.
9. Morgan G, Lipton A. Antitumor effects and anticancer applications of bisphosphonates. Semin Oncol. 2010;37 Suppl 2:S30–40.
10. Gallo M, De Luca A, Lamura L, Normanno N. Zoledronic acid blocks the interaction between mesenchymal stem cells and breast cancer cells: implications for adjuvant therapy of breast cancer. Ann Oncol. 2012;23:597–604.
11. Stresing V, Daubine F, Benzaid I, Mönkkönen H, Clezardin P. Bisphosphonates in cancer therapy. Cancer Lett. 2007;257:16–35.
12. Gnant M, Clezardin P. Direct and indirect anticancer activity of bisphosphonates: a brief review of published literature. Cancer Treat Rev. 2012;38:407–15.
13. Coleman RE. Skeletal complications of malignancy. Cancer. 1997;80:1588–94.
14. Timothy MF. The role of Bisphosphonates in the management of patients that have cancer. Vet Clin Small Anim. 2007;37:1091–110.
15. Mahtani R, Jahanzeb M. Bisphosphonates as anticancer therapy for early breast cancer. Clinical Breast Cancer. 2010;10(5):359–66.
16. Neville-Webbe HL, Gnant M, Coleman RE. Potential anticancer properties of Bisphosphonates. Semin Oncol. 2010;37 Suppl 1: S53–65.
17. Singh T, Kaur V, Kumar M, Kaur P, Murthy RSR, Rawal RK. The critical role of bisphosphonates to target bone cancer metastasis: an overview. J Drug Target. 2015;23(1):1–15.
18. Chang J, Wang W, Zhang H, Hu Y, Yin Z. Bisphosphonates regulate cell proliferation, apoptosis and pro-osteoclastic expression in MG-63 human osteosarcoma cells. Oncol Lett. 2012;4:299–304.
19. Berenson JR. Antitumor effects of bisphosphonates: from the laboratory to the clinic. Curr Opin Support and Palliat Care. 2011;5: 233–40.
20. Senaratne SG, Pirianov G, Mansi JL, Arnett TR, Colston KW. Bisphosphonates induce apoptosis in human breast cancer cell lines. Br J Cancer. 2000;82:1459–68.
21. Nakajima H, Magae J, Tsuruga M, Sakaguchi K, Fujiwara I, Mizuta M, et al. Induction of mitochondria-dependent apoptosis through the inhibition of mevalonate pathway in human breast cancer cells by YM529, a new third generation bisphosphonate. Cancer Lett. 2007;253:89–96.
22. Sewing L, Steinberg F, Schmidt H, Goke R. The bisphosphonate zoledronic acid inhibits the growth of HCT-116 colon carcinoma cells and induces tumor cell apoptosis. Apoptosis. 2008;13:782–9.
23. Coxon JP, Oades GM, Kirby RS, Colston KW. Zoledronic acid induces apoptosis and inhibits adhesion to mineralized matrix in prostate cancer cells via inhibition of protein prenylation. B J U Int. 2004;94:164–70.
24. Shipman CM, Croucher PI, Russell RG, Helfrich MH, Rogers MJ. The bisphosphonate incadronate (YM175) causes apoptosis of human myeloma cells in vitro by inhibiting the mevalonate pathway. Cancer Res. 1998;58:5294–7.
25. Green J, Lipton A. Anticancer properties of zoledronic acid. Cancer Investig. 2010;28:944–57.
26. Marra M, Abbruzzese A, Addeo R, Del Prete S, Tassone P, Tonini G, et al. Cutting the limits of aminobisphosphonates: new strategies for the potentiation of their anti-tumour effects. Curr Cancer Drug Targets. 2009;9:791–800.

27. Yuasa T, Kimura S, Ashihara E, Habuchi T, Maekawa T. Zoledronic acid - a multiplicity of anti-cancer action. Curr Med Chem. 2007;14:2126–35.
28. Di Salvatore M, Orlandi A, Bagala C, Quirino M, Cassano A, Astone A, et al. Anti- tumour and anti-angiogenetic effects of zoledronic acid on human non-small-cell lung cancer cell line. Cell Prolif. 2011;44:139–46.
29. Li YY, Chang JW, Chou WC, Liaw CC, Wang HM, Huang JS, et al. Zoledronic acid is unable to induce apoptosis, but slows tumor growth and prolongs survivalfor non-small-cell lung cancers. Lung Cancer. 2008;59:180–91.
30. Ibrahim T, Mercatali L, Sacanna E, Tesei A, Carloni S, Ulivi P, et al. Inhibition of breast cancer cell proliferation in repeated and non-repeated treatment with zoledronic acid. Cancer Cell Int. 2012;12(1):48.
31. Rachner TD, Singh SK, Schoppet M, Benad P, Bornhauser M, Ellenrieder V, et al. Zoledronic acid induces apoptosis and changes the TRAIL/OPG ratio in breast cancer cells. Cancer Lett. 2010;28(1):109–16.
32. Mani J, Vallo S, Barth K, Makarević J, Juengel E, Bartsch G, et al. Zoledronic acid influences growth, migration and invasive activity of prostate cancer cells in vitro. Prostate Cancer Prostatic Dis. 2012;15(3):250–5.
33. Kubista B, Trieb K, Sevelda F, Toma C, Arrich F, Heffeter P, et al. Anticancer effects of zoledronic acid against human osteosarcoma cells. J Orthop Res. 2006;24(6):1145–52.
34. Iguchi T, Miyakawa Y, Saito K, Nakabayashi C, Nakanishi M, Saya H, et al. Zoledronate-induced S phase arrest and apoptosis accompanied by DNA damage and activation of the ATM/Chk1/cdc25 pathway in human osteosarcoma cells. Int J Oncol. 2007;31(2):285–91.
35. Tassone P, Tagliaferri P, Viscomi C, Palmieri C, Caraglia M, D'Alessandro A, et al. Zoledronic acid induces antiproliferative and apoptotic effects in human pancreatic cancer cells in vitro. Br J Cancer. 2003;88(12):1971–8.
36. Romani AA, Desenzani S, Morganti MM, La Monica S, Borghetti AF, Soliani P. Zoledronic acid determines S-phase arrest but fails to induce apoptosis in cholangiocarcinoma cells. Biochem Pharmacol. 2009;78(2):133–41.
37. Koto K, Murata H, Kimura S, Horie N, Matsui T, Nishigaki Y, et al. Zoledronic acid inhibits proliferation of human fibrosarcoma cells with induction of apoptosis, and shows combined effects with other anticancer agent. Oncol Rep. 2010;24(1):233–9.
38. Liu SS, Wang XP, Li XB, Liang JY, Liu LL, Lu Y, et al. Zoledronic acid exerts antitumor effects in NB4 acute promyelocytic leukemia cells by inducing apoptosis and S phase arrest. Biomed Pharmacother. 2014;68(8):1031–6.
39. Karabulut B, Karaca B, Varol U, Muslu U, Cakar B, Atmaca H, et al. Enhancing cytotoxic and apoptotic effect in OVCAR-3 and MDAH-2774 cells with all-trans retinoic acid and zoledronic acid: a paradigm of synergistic molecular targeting treatment for ovarian cancer. J Exp Clin Cancer Res. 2010;29:102.
40. Porru M, Zappavigna S, Salzano G, Luce A, Stoppacciaro A, Balestrieri ML, et al. Medical treatment of orthotopic glioblastoma with transferrin-conjugated nanoparticles encapsulating zoledronic acid. Oncotarget. 2014;5(21):10446–59.
41. Hiraga T, Williams PJ, Ueda A, Tamura D, Yoneda T. Zoledronic acid inhibits visceral metastases in the 4T1/luc Mouse breast cancer model. Clin Cancer Res. 2004;10:4559–67.
42. Fromigue O, Lagneaux L, Body JJ. Bisphosphonates induce breast cancer cell death in vitro. J Bone Miner Res. 2000;15:2211–21.
43. Verdijk R, Franke HR, Wolbers F, Vermes I. Differential effects of bisphosphonates on breast cancer cell lines. Cancer Lett. 2007;246:308–12.
44. Cimini E, Piacentini P, Sacchi A, Gioia C, Leone S, Lauro GM, et al. Zoledronic acid enhances Vδ2 T-lymphocyte antitumor response to human glioma cell lines. Int J Immunopathol Pharmacol. 2011;24(1):139–48.
45. Nakazawa T, Nakamura M, Park YS, Motoyama Y, Hironaka Y, Nishimura F, et al. Cytotoxic human peripheral blood-derived γδT cells kill glioblastoma cell lines: implications for cell-based immunotherapy for patients with glioblastoma. J Neurooncol. 2014;116(1):31–9.
46. Fukai J, Koizumi F, Nakao N. Enhanced anti-tumor effect of zoledronic acid combined with temozolomide against human malignant glioma cell expressing O6-methylguanine DNA methyltransferase. PLoS One. 2014;9(8):e104538.