Original Article
Zoledronic acid inhibits cell growth of multiple myeloma cells and shows synergistic anti-myeloma effects with Bortezomib via downregulation of pim-2 through NF-κB pathway

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Abstract: Objective: To explore the possible mechanism of the third-generation biphosphonate zoledronic acid (ZOL) in antitumour activity. Methods: In this study, RPMI-8226 cell line was treated with ZOL alone at various concentrations and combined with Bortezomib in vitro, then Cell proliferation, cell apoptosis, downstream signaling pathway were detected by qRT-PCR and Western blot. Results: ZOL alone strongly inhibited proliferation of myeloma cell in vitro and induced apoptosis. The same results were obtained in ZOL combined with Bortezomib after 24 h. Furthermore, Ras, pAKT and NF-κB were suppressed in the group of ZOL alone and combined with Bortezomib at 24 h. We demonstrated NF-κb/pim-2 pathway was inhibited in the group of 24 h. However, it did not show the same result in the group of 48 h. Additionally, NF-κB played a pivotal role in regulating of pim-2 which was proved by the downregulation of pim-2 after the NF-κB inhibitor (Ro 106-9920) was used in RPMI-8226 cell line. Conclusion: ZOL and the combination of Zol with Bortezomib inhibit proliferation of myeloma cell via inhibiting NF-κb/pim-2 pathway. Combination of Zol with Bortezomib within 24 hours maybe more beneficial to alleviate tumor load in MM patients.

Keywords: Zoledronic acid, Bortezomib, pim-2, NF-κb, myeloma

Introduction

Multiple myeloma (MM) is a hematological malignancy of monoclonal plasma cells that grow in the bone marrow (BM) and produce various end-organ damage including anemia, hypercalcemia, renal insufficiency and osteolytic bone disease [1, 2]. Bortezomib and zoledronic acid (ZOL) are widely used in the management of MM. Bortezomib prevents plasma cell growth by inhibiting the activation of nuclear factor κB (NF-κB) [1, 2].

ZOL is widely used for the treatment of both osteoporosis and skeletal metastasis. Besides its main bone antiresorptive activity, ZOL displays antitumor properties. A randomized controlled trial (MRC Myeloma IX) indicated that ZOL reduced the mortality by 16% and also significantly improved progression-free survival by 12%. ZOL shows potential anticancer effects in MM cell [1, 2]. The main mechanism of anticancer effect of ZOL is the inhibition of mevalonate pathway leading the decrease of the small GTP-binding proteins (eg. RAS, Rap1A, Rac and Rho) [3]. ZOL also inhibits the activity of NF-κB which is a transcriptional factor that regulates multiple genes associated with inflammatory responses, cell growth control, and apoptosis. ZOL reverses epithelial-mesenchymal transition (EMT), which leads to a decrease in self-renewal through inactivation of NF-κB [4].

The small GTP-binding protein p21^{ras} (ras) activation which is known to stimulate, in particular, PI3K and Akt [5, 6]. At the same time, PI3K/Akt pathway also stimulates the transcriptional activity of NF-κB [7].

Pim-2 is a member of Pim kinases family that play an essential role in cell development and differentiation. Three members have been identified so far: Pim-1, Pim-2 and Pim-3. Among them, pim-2 may have a pivotal role in tumor
progression and bone loss in MM, Bone marrow stromal cells (BMSCs) and osteoclasts (OCs) upregulate pim-2 expression in MM cells largely via the IL-6/STAT3 and NF-κB pathway respectively and acts as an important pro-survival mediator [8].

However, the links between Ras, AKT, NF-κB and pim-2 is indistinct after ZOL treatment. In the present study, the effects of ZOL on MM cells were examined. Further, we investigated the probable mechanisms of ZOL antitumor effect by which NF-κB/pim-2 pathway. At the same time, the effect of ZOL combined with Bortezomib and the underlying molecular mechanisms were researched.

Materials and methods

Cell culture

RPMI-8226 cell line was obtained from the American Type Culture Collection (Rockville, Maryland, USA), grown in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 10% heat-inactivated fetal calf serum (Boehringer), 100 μg/mL penicillin (Gibco), 100 U/mL streptomycin (Gibco), in a humidified atmosphere (37.5°C; 5% CO₂).

Proliferation assay (CCK-8)

Cell viability was measured using the tetrazolium salt-based CCK-8 assay (Dojindo Molecular Technologies, Inc). Zoledronic acid (Novartis Pharma, Basel, Switzerland) and Bortezomib (Ortho Biotech, Janssen Cilag) dissolved in PBS were diluted with medium to high concentration stored solvent. RPMI-8226 cells were seeded into 96-well plates at 1.5×10⁵ cell/mL in 150 µL complete medium. After 24 hours, Zoledronic acid and Bortezomib were added to a final volume of 200 µL at 1.5×10⁵ cell/mL in 150 µL complete medium. After 24 hours, Zoledronic acid and Bortezomib were added to a final volume of 200 µL. A control reaction received PBS medium (Sigma, St. Louis, MO, USA) 50 µM. Plates were incubated for 24 h or 48 h at 37°C in 5% CO₂, then 20 µL of CCK-8 reagent was added to the wells and incubated for 1.5 h. And the OD was read at 450 nm within 15 minutes. The experiment was repeated 3 times and each sample had 3 duplications. Cell viability was calculated using the following equation: cytotoxicity (%) = (1-OD₄₅₀ of isogarcinol group/OD₄₅₀ of control group) ×100% [9]. The final concentrations of Zoledronic acid were 25, 50, 100, 500, and 1000 µM, and Bortezomib were 10 nM.

Apoptosis analysis

Cell apoptosis [10] was detected by flow cytometry. Cells were stained with fluorescein-conjugated annexin V and PI. After washing with buffer PBS two times, cells were resuspended in binding buffer (10 mmol/L N-2-hydroxyl pipera-zine-N0-2-ehane sulfonic acid/NaOH, pH 7.4, 140 mmol/L NaCl, 2.5 mmol/L CaCl₂). One hundred microliters of cell suspension were incubated with 10 µL annexin V-FITC and 10 µl PI for 20 min at room temperature in the dark. Then, cells were detected with flow cytometry within 30 min, and the percentage of apoptosis in the total number of cells in each group was compared.

Quantitative real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using TRizol reagent (Invitrogen, Carlsbad, CA). One microgram of purified total RNA was used for the real-time PCR analysis with the SuperScript First-Strand Synthesis System (Invitrogen). The quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara Biomedical, Siga, Japan) and the Thermal Cycler Dice Real Time system (Takara Biomedical) in a 96-well plate according to the manufacturer’s instructions. The amplification utilized 40 cycles at 94°C for 10 s and 56.7°C for 30 s with the extension at 72°C for 30 s. The primers used for RT-PCR were as follows: human pim-2, sense 5’-TTGGGAAGGAATGGAAGATG-3’ and anti-sense 5’-CAGGAGAACAAACAGCAAGC-3’, AKT, sense 5’-GCCCAACACCTTCATCATC-3’ and anti-sense 5’-CTCCTCCTCCGGCTGCTTCA3’, human NF-κB, sense 5’-CTGAGTCCTGCTCCTTCCA3’ and anti-sense 5’-CTTGGGTAGCCATTCGTATG-3’. Human β-actin, used as a housekeeping gene for quantity normalization, sense 5’-CTGGAACGGTGAAGGTTGACA-3’, antisense 5’AAGGGACTTCCTGTAAACAATGCA-3’. The pm-2, AKT, and NF-κB levels were calculated using the 2⁻ΔΔCt method [(Ct, target gene Ct, β-actin)sample-(Ct, target gene Ct, β-actin)control] after normalizing the data according to the β-actin mRNA expression [11].

Western blotting

Western blot [12] evaluated the content of Ras, AKT, pAKT, NF-κB and Pim-2 in cell extracts without (medium) and with exposure to ZOL, Bortezomib alone or together in RPMI-8226.
Cells were cultured with NF-κB inhibitor (Ro 106-9920) (TOCRIS) for 48 h before harvesting for analysis. Cells were lysed with a lysis buffer (20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 2 mM EDTA, 100 mM NaF, 1% NP40, 1 μg/mL leupeptin, 1 μg/mL anti-pain, and 1 mM phenylmethylsulfonyl fluoride), and the protein concentrations were determined with BCA protein
assay kit (Pierce, Rockford, IL, USA). Proteins (30 µg) were separated using 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the SDS-PAGE gels were transferred electronically to polyvinylidene difluoride (PVDF) membrane (Bio-Rad Laboratories, Hercules, CA, USA). PVDF membranes were blocked with a solution containing 5% skim milk and then incubated overnight at 4°C with the following antibodies: anti-Ras (#3965), anti-AKT (#9272), anti-pAKT (#4051), anti-NF-κB (#8242), anti-β-actin antibody (#3700) (Cell Signaling Technology, Beverly, MA, USA), antibody anti-pim-2 (ab97475) (Abcam, Cambridge, UK). After washing with Tris-buffered saline with Tween-20 (TBST), the membranes were incubated for 1 h at room temperature with anti-rabbit IgG sheep antibody or anti-mouse IgG sheep antibody coupled to horseradish peroxidase (Amer-sham). Reactive proteins were visualized using a chemiluminescence kit (Millipore, Bedford, MA, USA).

**Statistical analysis**

All results are expressed as means and S.D. of several independent experiments. Pairwise comparison of apoptosis results were done by Student’s t-test. Multiple comparisons of the CCK-8 and PCR data was done by ANOVA test to determine statistical significance of detected differences. A value of P<0.05 was considered statistically significant.

**Results**

**ZOL induced cytotoxicity in RPMI-8226 cells with enhancing effects of Bortezomib**

To investigate whether ZOL can inhibit MM cell directly, cytotoxic effect of ZOL was demonstrated with the cck-8 assay after 24 h, 48 h at the concentrations of 25 µM, 50 µM, 100 µM, 500 µM, and 1000 µM. The results showed that the cytotoxic effects increased with ZOL concentration- and time-dependence. The cytotoxicity of ZOL in different concentrations in 24 h was 19.842±10.425%, 21.857±6.958%, 30.5378±4.65108%, 42.287±5.965% and 47.149±9.939% respectively. At 48 h, they were 31.767±6.04%, 33.168±5.909%, 34.596±4.716%, 41.212±5.427% and 45.032±3.5% respectively (Figure 1A). In 25 µM and 50 µM concentrations of ZOL, the cytotoxicities at 48 h were significantly higher than those at 24 h. However, there was no time dependence from 100 µM to 1000 µM (P>0.05) (Figure 1B). That was the reason why we selected the concentrations of ZOL (125 µM and 250 µM) at 24 h which could avoid time difference in different group experiment.

The combination cytotoxicity of ZOL and Bortezomib (10 nM) on MM cells was detected further. The combination of ZOL at 500 µM, 1000 µM and Bortezomib (10 nM) after 24 h showed synergistic antitumor effects (44.367±7.124%, 49.87±11.797%) when compared with Bortezomib (10 nM) (30.264±4.363%) (Figure 1D). The combination at 48 h had no significant differences within each united group with Bortezomib (10 nM) (Figure 1E). There was no time dependence between the combination groups of ZOL 500 µM-1000 µM (Figure 1F). We finally decided the concentration of ZOL at 125 µM and 250 µM.

**ZOL increased apoptosis of RPMI-8226 cells in the concentration of 250 µM at 24 h and 48 h, while only enhanced with Bortezomib at 24 h**

To clarify the mechanisms of ZOL-induced cytotoxicity, we performed apoptotic assays on RPMI-8226 cells exposed to Bortezomib (10 nM), ZOL (125 µM, 250 µM) and their combination.

At 24 hours, the percentages of apoptosis detected by Annexin-V and PI staining in the concentration of ZOL 250 µM (14.43±3.983%) was significantly higher than that in controls (P<0.05); The percentages of apoptosis in combination groups (B+Zol 125 nM, B+Zol 250 µM) were 31.07±3.675%, 49.00±13.952% respectively, which were significantly higher than those in Bortezomib 10 nM (19.50±2.685%) (P<0.05) (Figure 1A).

While at 48 hours, the percentages of apoptosis in the concentration of ZOL 250 µM (35.33±7.059%) was the only one which had significantly difference compared with blank controls (P<0.05). In both of 24 h and 48 h at the concentration ZOL (125 µM) alone had no significant apoptosis effect compared with blank controls (P>0.05), suggesting that induc-
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![Figure 2.](image)

**Table 1.** The mRNA expression of signaling pathway member AKT, NF-κb, Pim-2. Data are representative of 3 independent experiments.

<table>
<thead>
<tr>
<th></th>
<th>Blank</th>
<th>B (10 nM)</th>
<th>B (10 nM)+ZOL (125 µM)</th>
<th>B (10 nM)+ZOL (250 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AKT</td>
<td>1.00±0.06</td>
<td>1.00±0.16</td>
<td>1.10±0.20</td>
<td>1.00±0.15</td>
</tr>
<tr>
<td>NF-κb</td>
<td>1.05±0.02</td>
<td>0.47±0.09</td>
<td>0.96±0.08</td>
<td>0.66±0.04</td>
</tr>
<tr>
<td>Pim-2</td>
<td>1.00±0.05</td>
<td>0.63±0.05</td>
<td>0.93±0.67</td>
<td>0.81±0.04</td>
</tr>
</tbody>
</table>

*compared with Blank indicate *P<0.05, *compared with B (10 nM) indicate the *P<0.05.

The percentages of apoptosis in combination groups (B+Zol 125 µM, B+Zol 250 µM) were 44.07±2.706%, 52.27±11.100% respectively, which had no significant differences compared with Bortezomib group. However, there was no significant reduction in the apoptosis effect of combination was significantly higher at 24 h instead of 48 h. Interestingly, the apoptosis effect of combination was significantly higher at 24 h instead of 48 h.

**ZOL suppressed mRNA expression of NF-κb and Pim-2**

To investigate downstream signaling pathway of ZOL, we analyzed the mRNA levels of AKT, NF-κb, Pim-2 by quantitative real-time PCR (qRT-PCR). The results revealed that the mRNA levels of NF-κb, Pim-2 were markedly downregulated following treatment with 250 µM ZOL (P=0.0001, P=0.002) instead of 125 µM ZOL (P=0.129, P=0.161) compared with the blank control. But there was no significant differences on AKT in different concentrations of Zol (125 µM and 250 µM) (Table 1A). In Synergistic group, the results showed that the mRNA levels of NF-κb, Pim-2 decreased significantly in both 125 µM ZOL with Bortezomib (P=0.001, P=0.015) and 250 µM ZOL with Bortezomib at 24 h compared with Bortezomib group (P=0.0001, P=0.001) and the blank control (P=0.0001, P=0.0001) (Table 1B, 1C).
two groups at 48 h compared with Bortezomib group (P>0.05). In addition, each synergistic group also showed no significant variations of AKT, indicating that ZOL and the combination with Bortezomib did not inhibit the mRNA expression of AKT.

**ZOL and the combination with Bortezomib suppressed protein expressions of Ras, pAKT, NF-kb and pim-2**

We further investigated whether ZOL and the combination with Bortezomib can inhibit the downstream signal transduction of Ras. The expression of Ras, NF-kb p65 subunit, pim-2 as well as activated AKT (pAKT) tested by western blot decreased significantly in the concentration of 250 µM compared with the control (Figure 3A). The same results could not find in the concentration of 125 µM.

Furthermore, we also detected the signal transduction in the combination of ZOL and Bortezomib. The results showed that the expression of Ras, NF-kb p65 subunit, pim-2 and activated AKT decreased significantly compared with Bortezomib 24 h, both in the two concentrations of ZOL (125 µM and 250 µM) combined with Bortezomib (Figure 3B). However, at 48 h, there were no substantial changes in the level of this signal transduction, such as phosphorylated AKT, Ras, NF-kb, pim-2 (Figure 3C). Therefore, the inhibition of pAKT activity and Ras, NF-kb p65 subunit, pim-2 proteins expression may be responsible for the induction of cell apoptosis by ZOL (250 µM) and ZOL (125 µM and 250 µM) combined with Bortezomib at 24 h.

**NF-kB plays a pivotal role in regulating of pim-2 in RPMI-8226 cell line**

To investigate whether NF-kB regulates pim-2 expression in RPMI-8226 cell line. Cells were cultured with NF-kB inhibitor (Ro 106-9920) for 48 h at various concentration 0 µM, 1 µM, 2.5 µM, 5 µM, 10 µM. The reduction of pim-2 was followed by the inhibition of NF-kB without concentration-dependence (Figure 4).

**Discussion**

In this study, we identified antitumour effects of ZOL on multiple myeloma cell and synergistic antmyeloma effects with Bortezomib. The result accorded with previously published data. Ural [13] have observed decreased metabolic activity (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-
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Figure 4. NF-κB regulated the expression of Pim-2 in MM cells. RPMI-8226 cells were cultured with NF-κB inhibitor (Ro 106-9920) by increasing doses. PIM-2 decreased along with the inhibiting of NF-κB.

ZOL inhibited NF-κB and NF-κB activity in RPMI-8226 cell line with a concentration of 50 µM. ZOL significantly reduced the expression of NF-κB (Figure 4). It has been confirmed that ZOL induced apoptosis of multiple myeloma cells by decreasing prenylation of the small GTP-binding protein Ras which leads inhibition of mevalonate pathway. It may be probable that ZOL (via Ras/Raf/Mek/Erk MAP kinase signalling) and Bortezomib (directly) simultaneously inhibit NF-κB activity in a synergistic way [22]. Ras can lead to the activation of PI3K/Akt pathway and PI3K/mTOR pathway [22]. The PI3K/Akt pathway has been reported to regulate NF-κB expression [23]. Our results clearly demonstrate that ZOL induce decrease in pAKT and NF-κb (Figure 3). Furthermore, we observed that ZOL inhibited NF-κb mRNA expression in RPMI-8226 cell line. Nuclear factor (NF)-kappa B activation as a downstream target of PI3K/AKT signaling [23]. Previous research showed that bisphosphonates and statins inhibit MIP-1α expression by suppressing Ras/PI3K/Akt pathways [23]. However, it’s arbitrary to say Ras/pAkt/NF-κB is inevitable pathway in ZOL induced apoptosis of multiple myeloma cells by suppressing Ras, pAKT and NF-κB. Further studies need to uncover the connection between Ras, AKT and NF-κB. The only certainty is that Ras, AKT and NF-κB molecules take part in the process of ZOL antitumor effect.

Pim-2 is a novel anti-apoptosis kinase in multiple myeloma cells which is regulated via NF-κb [8]. In our study, we observed that NF-κb plays a pivotal role in regulating of pim-2 in RPMI-8226 cell line (Figure 4) which consistent with...
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previous research that Pim-2 is regulated via NF-κB [8]. Moreover, we find not only Ras, the activation of AKT (pAKT) and NF-κB, but also the mRNA and protein levels of Pim-2 decreased with ZOL or ZOL combined with Bortezomib. Therefore, our present findings indicate that ZOL inhibit pim-2 by suppressing NF-κB. Recently, several study revealed that the downstream of pim-2, such as the translational repressor 4E-BP1, the BH3 protein BAD and TSC2 [24, 25]. Whether there has connection between the ZOL antitumor pathway and the downstream of pim-2 still need more studies.

In conclusion, these results demonstrated ZOL and the combination of ZOL with Bortezomib inhibited proliferation of myeloma cell via inhibiting NF-κb/pim-2 pathway. Similar effects were found on Ras and pAkt. Overall these data suggest that these proteins may be considered as a target of ZOL in MM patients. The result of synergistic antimyeloma effects combined with Bortezomib at 24 h supported that the combination of ZOL with Bortezomib could help patient gain more benefits in clinical therapy. However, the probable concentration in vivo is unclear. Further in-depth in vivo studies are presently under investigation in our laboratory. To find out a concentration of ZOL clinically relevant antimyeloma effects, more work should be done in future. Only the results of randomized phase III studies can answer this question.

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Disclosure of conflict of interest

None.

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