ORIGINAL

International Journal of Myeloma 6(1): 7–11, 2016

Susceptibility to bendamustine considerably varies among myeloma cells, but is enhanced in acidic conditions

Shingen NAKAMURA[1], Hirokazu MIKI[2], Asuka ODA[1], Ryota AMACHI[3], Jumpei TERAMACHI[4], Kimiko SOGABE[1], Hikaru FUJINO[1], Tomoko MARUHASHI[1], Shiro FUJII[1], Kumiko KAGAWA[1] and Masahiro ABE[1]

Myeloma (MM) cells and osteoclasts create a highly acidic milieu in MM bone lesions by proton produced by osteoclasts and lactate by MM cells. Although bendamustine exerts clinical activity against MM, the potential mechanisms of action of bendamustine remain largely unknown. In the present study, we therefore explored anti-MM activity of bendamustine with special reference to its effects on MM cells in acidic conditions. Bendamustine at the range of 25 to 100 μM dose-dependently induced death in INA6 and TSPC-1 MM cells. However, bendamustine did not impair the viability of RPMI8226, U266 and OPC cells even at 100 μM. In acidic conditions at pH 6.8 or lower, the bendamustine's cytotoxic effects were further enhanced in INA6 and TSPC-1 cells, and triggered in RPMI8226 and MM.1S cells which were resistant to bendamustine at pH 7.4. Intriguingly, bendamustine reduced Pim-2 protein levels in MM cells and enhanced its anti-MM activity in combination with the Pim inhibitor SMI-16a, preferentially in acidic conditions. These results demonstrate that anti-MM effects of bendamustine are augmented in acidic conditions, but considerably vary among MM cells, and that Pim inhibition further enhances the bendamustine's anti-MM activity in acidic conditions.

Key words: bendamusutine, myeloma bone lesion, acidic condition, Pim-2

Introduction

Multiple myeloma (MM) is characterized by the accumulation of neoplastic plasma cells in the bone marrow, and generates bone destruction through enhancement of osteoclastogenesis and concomitant suppression of osteoblastic differentiation from bone marrow stromal cells. Along with the progression of bone disease, the bone marrow microenvironment is skewed by MM cells, which underlies the unique pathophysiology of MM and confers aggressiveness and drug resistance in MM cells.

We previously demonstrated that osteoclasts are not only bone-resorbing cells but also potently support MM cell growth and survival [1,2]. Osteoclasts produce multiple growth and survival factors for MM cells, including TNF family cytokines, BAFF and APRIL, thereby forming a vicious cycle between osteoclastogenesis and myeloma tumor progression [3]. Osteoclasts produce a large amount of protons, and MM cells produce lactate through enhanced glycolysis; therefore, bone lesions with an interaction between MM cells and acid-producing osteoclasts create a highly acidic microenvironment. Tumor acidity has been known to confer drug resistance in cancers [4]; therefore, the development of novel therapies targeting MM cells in the pathologically skewed tumor microenvironment in acidic bone lesions is needed to further improve the outcome of treatment for MM.

Bendamustine, a purine analogue/alkylator hybrid agent, exerts anti-tumor activity against various human cancers, including MM. However, the potential mechanisms of action of

Received: August 28, 2015, accepted: October 8, 2015
[1]Department of Hematology, Endocrinology and Metabolism, Tokushima University Graduate School, Tokushima, Japan
[2]Division of Transfusion Medicine and Cell Therapy, Tokushima University hospital, Tokushima, Japan
[3]Department of Orthodontics and Dentofacial Orthopedics, Tokushima University Graduate School, Tokushima, Japan
[4]Department of Histology and Oral Histology, Tokushima University Graduate School, Tokushima, Japan

Corresponding author: Masahiro ABE
Department of Hematology, Endocrinology and Metabolism, Tokushima University Graduate School, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan
E-mail: masabe@tokushima-u.ac.jp

bendamustine remain largely unknown. In the present study, we explored the mechanisms of anti-MM activity by bendamustine with special reference to its effects on MM cells in acidic bone lesions, which confer drug resistance.

Materials and Methods

Reagents

The following reagents were purchased from the indicated manufacturers: bendamusutine and mouse polyclonal anti-β-actin antibody from Sigma (St. Louis, MO); rabbit anti-Pim-2 antibody from Cell Signaling Technology (Beverly, MA, USA); horseradish-peroxidase-conjugated goat anti-rabbit IgG and horse anti-mouse IgG from Cell Signaling Technology (Beverly, MA).

Cells and cultures

Human MM cell lines, RPMI8226 and U266, were obtained from American Type Culture Collection (ATCC) (Rockville, MD). INA6 and MM.1S MM cell lines were kindly provided by Dr. Renate Burger (University of Kiel, Kiel, Germany) and Dr. Steven Rosen (Northwestern University, Chicago, IL), respectively. TSPC-1 and OPC MM cell lines were established in our laboratory [1]. MM cells were cultured in RPMI1640 supplemented with 5% fetal bovine serum, 2 mM of L-glutamine (Sigma), 100 U/mL of penicillin G and 100 mg/mL of streptomycin (Sigma). pH values in culture media were adjusted by adding lactic acid (Wako, Osaka, Japan) or hydrochloride (Wako). IL-6-dependent INA-6, TSPC-1 and OPC cells were cultured in the presence of 1 ng/ml of rhIL-6 (PEPROTECH EC, London, UK). Primary bone marrow stromal cells were isolated from fresh bone marrow aspirates from patients with MM, and cultured as previously described [1]. All procedures involving human specimens were performed with written informed consent according to the Declaration of Helsinki and using a protocol approved by the Institutional Review Board for human protection.

Western blot analysis

Whole cell lysate was lysed in RIPA buffer. These lysates were supplemented with 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail solution (Sigma). Cell lysates and conditioned media were electrophoresed in 10% SDS-PAGE gel and blotted onto polyvinylidene difluoride membranes (Millipore, Bedford, MA). After blocking with 5% non-fat dry milk, the membranes were incubated with primary antibodies overnight at 4°C, followed by washing and addition of a horseradish-conjugated secondary antibody for 1 hour. The protein bands were visualized with an Enhanced Chemiluminescence Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ).

Cytotoxicity assay (WST assay)

Cell viability was determined by Cell Counting Kit-8 assay (DOJINDO, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, cells were incubated in culture plates with 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-2H-tetrazolium monosodium salt (WST-8) for 4 hours. The absorbance of each well was measured at 450 nm with a microtiter plate reader (Model 450 micro plate reader; Bio-Rad Laboratories, Hercules, CA).

Results and Discussion

Cytotoxic activity of bendamustine on MM cells

We first looked at the cytotoxic activity of bendamustine at 25 to 100 μM against different MM cell lines. Bendamustine dose-dependently induced cell death in INA6 and TSPC-1 cells, and weakly in MM.1S cells (Fig. 1A). However, bendamustine did not impair the viability of RPMI8226, U266, and OPC cells even at 100 μM. These results suggest that susceptibility to bendamustine significantly varied among MM cells.

Although bendamustine marginally induced death in U266 cells in vitro (Fig. 1A), bendamustine has been reported to suppress U266 cell growth in bone lesions in NOD/SCID/IL-2Rγc (null) (NOG) mice [5], suggesting dependence of bendamustine's cytotoxic activity on the microenvironment in bone lesions in MM. Because MM creates an acidic microenvironment in bone lesions, we hypothesized that acidic conditions may affect bendamustine's anti-MM effects. To verify the hypothesis, we next examined the cytotoxic effects of bendamustine on MM cells in acidic conditions. INA6 and TSPC-1 cells susceptible to bendamustine at 50 μM albeit at pH 7.4 underwent cell death more preferentially in acidified conditions (Fig. 1B). Interestingly, acidic conditions at pH 6.8 or lower were able to trigger bendamustine's cytotoxic activity against RPMI8226 and MM.1S cells which were resistant to bendamustine at 50 μM at pH 7.4. In contrast to bendamustine, the alkylator melphalan exerted the cytotoxic activity in a manner independent of ambient pH values against INA6 and RPMI8226 cells which were susceptible and resistant to bendamustine, respectively (Fig. 1C). Cytotoxic activity of bendamustine was similarly enhanced in acidic conditions created by hydrochloride compared to those by lactic acid, which can negate the possibility that lactic acid itself affects the activity of bendamustine (Fig. 1D). Thus, anti-MM effects of bendamustine appear to be uniquely induced and increased in acidic conditions.

Bendamustine reduces Pim-2 protein expression in MM cells

The serine/threonine kinase Pim-2 has been demonstrated to be constitutively over-expressed in MM cells [6–8], and further up-regulated in MM cells by IL-6 and/or TNF family cytokines

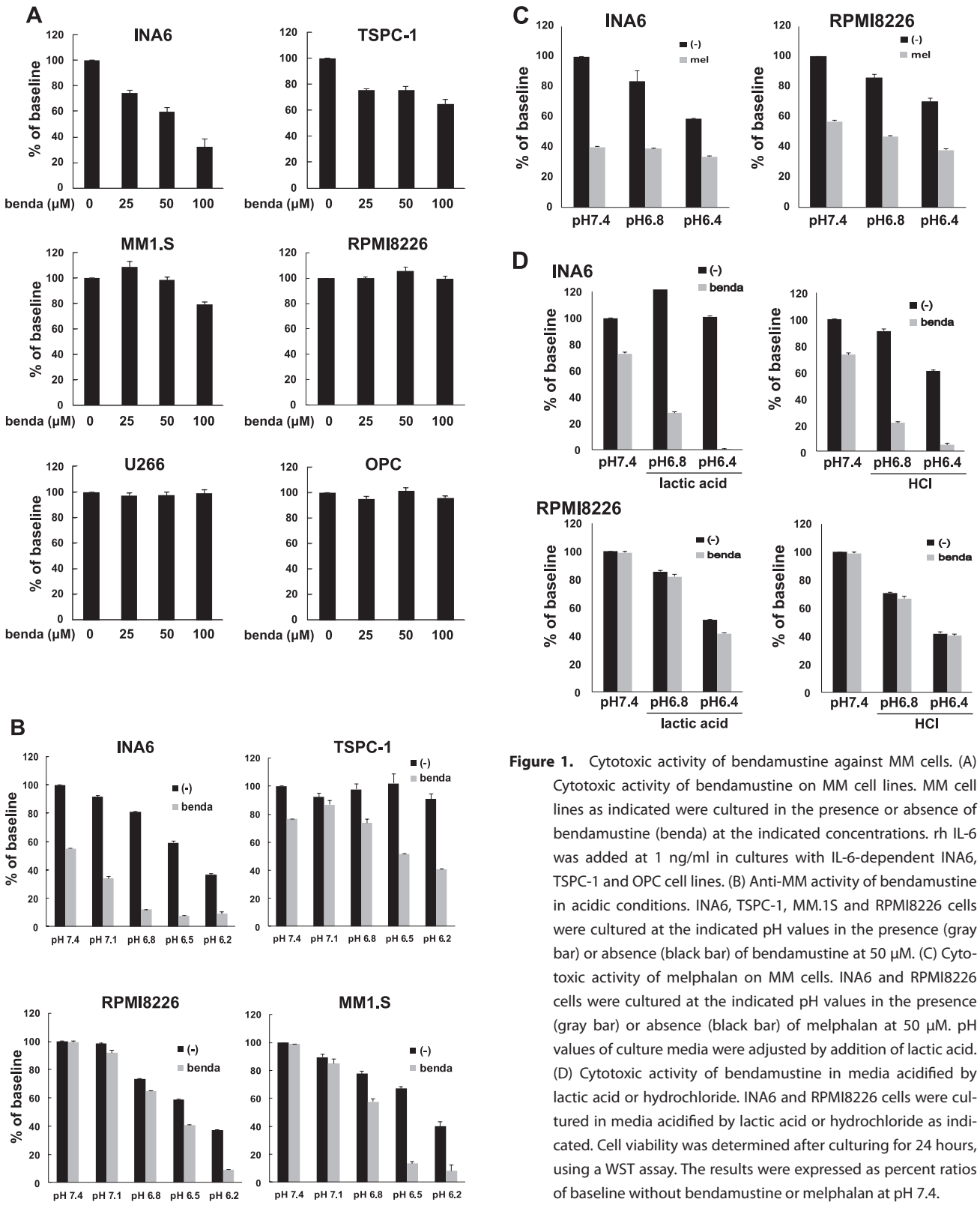

Figure 1. Cytotoxic activity of bendamustine against MM cells. (A) Cytotoxic activity of bendamustine on MM cell lines. MM cell lines as indicated were cultured in the presence or absence of bendamustine (benda) at the indicated concentrations. rh IL-6 was added at 1 ng/ml in cultures with IL-6-dependent INA6, TSPC-1 and OPC cell lines. (B) Anti-MM activity of bendamustine in acidic conditions. INA6, TSPC-1, MM.1S and RPMI8226 cells were cultured at the indicated pH values in the presence (gray bar) or absence (black bar) of bendamustine at 50 μM. (C) Cytotoxic activity of melphalan on MM cells. INA6 and RPMI8226 cells were cultured at the indicated pH values in the presence (gray bar) or absence (black bar) of melphalan at 50 μM. pH values of culture media were adjusted by addition of lactic acid. (D) Cytotoxic activity of bendamustine in media acidified by lactic acid or hydrochloride. INA6 and RPMI8226 cells were cultured in media acidified by lactic acid or hydrochloride as indicated. Cell viability was determined after culturing for 24 hours, using a WST assay. The results were expressed as percent ratios of baseline without bendamustine or melphalan at pH 7.4.

such as TNF-α, BAFF and APRIL produced by bone marrow stromal cells as well as osteoclasts[6]. Pim-2 plays a critical role in MM cell survival and is regarded as an important therapeutic target in MM[6–9]. Pim-2 is autophosphorylated and constitutively active without post-translational modification; its activity is dependent on its protein levels[10]. IL-6 is among predominant enhancers of Pim-2 expression in MM cells elaborated by bone marrow stromal cells[6].

We found that treatment with bendamustine at 50 μM, the concentration to induce their cell death as shown in Figure 1B,

suppressed Pim-2 expression preferentially in acidic conditions in INA6 and TSPC-1cells which were susceptible to bendamustine. The suppressive effects by bendamustine under acidic conditions were more clearly observed in cocultures with bone marrow stromal cells or in the presence of IL-6 in these MM cells whose Pim-2 expression was potently up-regulated (Fig. 2A). RPMI8226 cells were resistant to bendamustine at pH 7.4, but became susceptible in acidic conditions (Fig. 1B). Bendamustine at 50 and 100 μM was also able to dose-dependently decrease Pim-2 expression upregulated in RPMI8226 cells under cocultures with bone marrow stromal cells in acidic conditions (Fig. 2A). Therefore, acidic conditions are suggested to trigger or induce bendamustine's cytotoxic activity in parallel with suppression of Pim-2 expression in MM cells. However, although the expression of Pim-2 was weak in INA6 and TSPC-1 MM cells in the absence of BMSCs or IL-6 (Fig. 2A), acidic conditions still enhanced the killing effect of bendamustine on these MM cells (Fig. 1B). There may be mechanisms of acid-induced cytotoxic effects of bendamustine other than Pim-2 reduction.

Combinatory effects of bendamustine and Pim inhibition

Because bendamustine suppresses Pim-2 expression in MM cells in acidic conditions, and because Pim-2 is a critical anti-apoptotic mediator in MM, we next examined the role of additional inhibition of Pim-2 activity in acid-triggered bendamustine's anti-MM activity. Inhibition of Pim-2 activity by the Pim inhibitor SMI-16a even at suboptimal concentrations enhanced bendamustine's cytotoxic effects at pH 6.4 on RPMI8226 and U266 cells which were resistant to bendamustine at pH 7.4 (Fig. 2B). These results suggest acid-triggered bendamustine's anti-MM activity via curtailing Pim-2 expression and potentiation of bendamustine's therapeutic efficacy against MM in combination with Pim inhibitors in acidic MM bone lesions.

The present study demonstrates that sensitivity to bendamustine considerably varies among MM cells, but anti-MM activity of bendamustine is augmented in acidic conditions at least in part through down-regulation of Pim-2, which is essential for MM cell survival, and that Pim inhibition further enhances the bendamustine's anti-MM activity in acidic conditions. Acidic microenvironments are generally accepted to confer drug resistance and immune evasion capability in cancers [4]. However, bendamustine appears to rather preferentially kill MM cells in acidic microenvironments, which can hardly be targeted with other currently available anti-MM agents. From the present study, combinatory treatment of bendamustine and Pim inhibitors may become a novel therapeutic option targeting MM cells in acidic bone lesions. However, we need to address precise mechanisms responsible for determining susceptibility of MM cells to bendamustine for better use of this interesting anti-tumor agent.

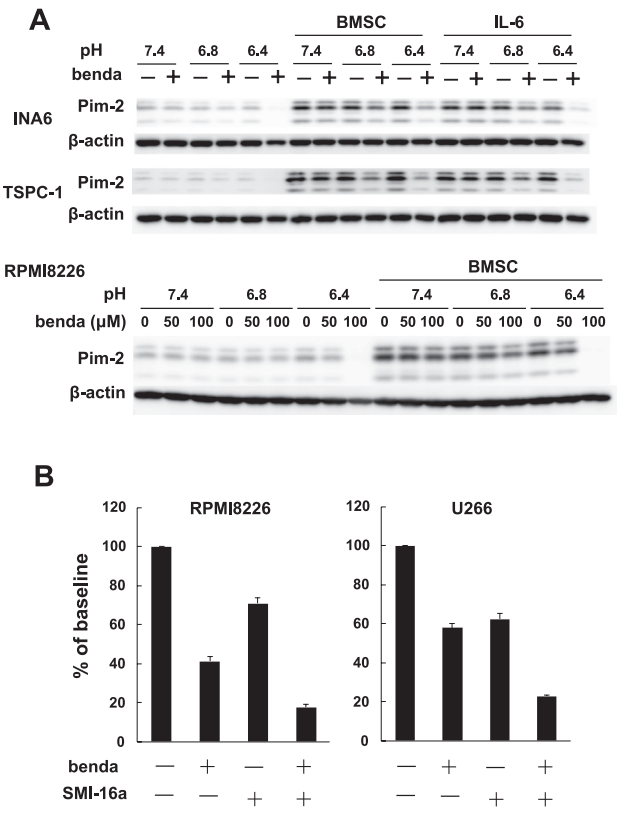

Figure 2. Bendamustine's anti-MM activity via curtailing Pim-2 expression. (A) Pim-2 protein expression in MM cells upon treatment with bendamustine. INA6 and TSPC-1 cells were cultured alone or cocultured with bone marrow stromal cells (BMSC), or cultured in the presence of rh IL-6 at 1 ng/ml. Bendamustine (benda) was added at 50 μM. RPMI8226 cells were cultured alone or cocultured with bone marrow stromal cells without or with bendamustine at 50 or 100 μM. pH values of culture media were adjusted by addition of lactic acid. After culturing for 24 hours, cell lysates were harvested and subjected to Western blotting to determine Pim-2 protein levels. (B) Combinatory treatment with bendamustine and Pim inhibition in an acidic condition. RPMI8226 and U266 cells resistant to bendamustine (benda) were cultured at pH 6.4 with bendamustine at 50 μM or the Pim inhibitor SMI-16a at suboptimal concentrations, 12.5 and 25 μM in RPMI8226 and U266 cells, respectively, alone or both in combination. Cell viability was determined after culturing for 48 hours, using a WST assay. The results were expressed as per cent ratios from baseline.

Acknowledgments

This work was supported in part by Grants-in-aid for Scientific Research (C) to M.A., Grant-in-Aid for Young Scientists (B) to R.A., S.N. and H.M. and a National Cancer Center Research and Development Fund (21-8-5) to M.A. from the Ministry of

Health, Labor and Welfare of Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Conflicts of Interest Disclosures

The authors declare no competing financial interests related to this work.

References

1) Abe M, Hiura K, Wilde J, Shioyasono A, Moriyama K, Hashimoto T, et al. Osteoclasts enhance myeloma cell growth and survival via cell-cell contact: a vicious cycle between bone destruction and myeloma expansion. Blood. 2004; 104: 2484–91.
2) Abe M. Targeting the interplay between myeloma cells and the bone marrow microenvironment in myeloma. Int J Hematol. 2011; 94: 334–43.
3) Abe M, Kido S, Hiasa M, Nakano A, Oda A, Amou H, et al. BAFF and APRIL as osteoclast-derived survival factors for myeloma cells: a rationale for TACI-Fc treatment in patients with multiple myeloma. Leukemia. 2006; 20: 1313–5.
4) Webb BA, Chimenti M, Jacobson MP, Barber DL. Dysregulated pH: a perfect storm for cancer progression. Nat Rev Cancer. 2011; 11: 671–7.
5) Iriuchishima H, Takubo K, Miyakawa Y, Nakamura-Ishizu A, Miyauchi Y, Fujita N, et al. Neovascular niche for human myeloma cells in immunodeficient mouse bone. PLoS One. 2012; 7: e30557.
6) Asano J, Nakano A, Oda A, Amou H, Hiasa M, Takeuchi K, et al. The serine/threonine kinase Pim-2 is a novel anti-apoptotic mediator in myeloma cells. Leukemia. 2011; 25: 1182–8.
7) Lu J, Zavorotinskaya T, Dai Y, Niu XH, Castillo J, Sim J, et al. Pim2 is required for maintaining multiple myeloma cell growth through modulating TSC2 phosphorylation. Blood. 2013; 122: 1610–20.
8) Johrer K, Obkircher M, Neureiter D, Parteli J, Zelle-Rieser C, Maizner E, et al. Antimyeloma activity of the sesquiterpene lactone cnicin: impact on Pim-2 kinase as a novel therapeutic target. J Mol Med (Berl). 2012; 90: 681–93.
9) Hiasa M, Teramachi J, Oda A, Amachi R, Harada T, Nakamura S, et al. Pim-2 kinase is an important target of treatment for tumor progression and bone loss in myeloma. Leukemia. 2015; 29: 207–17.
10) Warfel NA, Kraft AS. PIM kinase (and Akt) biology and signaling in tumors. Pharmacol Ther. 2015; 151: 41–9.