2-Methoxyestradiol at low dose induces differentiation of myeloma cells

Jian Hou *, Hong Xiong, Weiran Gao, Hua Jiang

Department of Hematology, Changzheng Hospital, 415 Fengyang Road, Shanghai 200003, China

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Abstract

Previous studies showed that 2-methoxyestradiol (2ME2) could suppress the proliferation of myeloma cells and induce their apoptosis. In the present study, we found that treatments with low-concentration of 2ME2 resulted in some maturing morphological changes of myeloma cells. Flow cytometric analysis showed that the expression of CD49e on the myeloma cells surface was significantly increased by 2ME2. Moreover, 2ME2 increased the secretion of light chain protein remarkably. Furthermore, the expressions of transcription factor XBP-1 mRNA and protein were also up-regulated. These results demonstrated that 2ME2 at low-concentration could induce differentiation of the myeloma cells, which would provide a new, safe strategy for myeloma therapy.

Keywords: 2-Methoxyestradiol (2ME2); Cell differentiation; Cell line; Myeloma

1. Introduction

Multiple myeloma (MM) is a malignancy of plasma cells, which are terminally differentiated cells of B cell lineage, generally regarded as incurable [1]. Up to now chemotherapy and stem cell transplantation have still been the most important therapies and improved some MM patients’ prognosis to a certain extent. It is well known that this disease mainly occurred in the elders. Since many old patients have little chance to undergo transplantation and could not endure high-dose chemotherapy because of the poor visceral function or age, it is of very important significance to explore novel, effective and safe therapy.

2-Methoxyestradiol (2ME2) once considered as an inactive end-metabolite of estradiol, has recently emerged as a very promising agent for cancer treatment [2]. Previous studies showed that it had a cytotoxic effect on various proliferating cells in vitro [3–5]. Despite being a natural derivative of estradiol (E2), 2ME2 binds poorly to the estrogen receptors (ERs); therefore its antiproliferative effects are not mediated by ERs [6]. Using xenografts and metastatic disease models in mice, it has been suggested that 2ME2 targets both the tumor cell and endothelial cell compartments by inducing apoptosis in rapidly proliferating cells and inhibiting blood vessel formation at several stages in the angiogenic cascade [7,8]. Other underlying anticancer mechanisms of 2ME2 have been suggested, including inhibition of tubulin polymerization [9,10], sulfonation of 2ME2 [11], and inhibition of superoxide dismutase [12].

Previous studies showed that 2ME2 could suppress the proliferation of myeloma cells and induce their apoptosis [13–15]. The mechanisms of anti-proliferation and inducing-apoptosis have partially been studied, but there’s little investigation about its effect on the differentiation of myeloma cells. It remained unknown whether 2ME2 exerts its effects on myeloma cells by inducing differentiation directly. In the present study, we demonstrated that 2ME2 at low concentration could induce differentiation of the myeloma cells.

2. Materials and methods

2.1. Cell Lines and cell culture

Human myeloma cell lines used in this study (CZ-1, LP-1, and NCI-H929) were stored in liquid nitrogen in our labo-
atory, and quickly recycled upon thawing before experiments. These cells were grown in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mmol/L L-glutamine at 37 °C in 5% CO2 humidified atmosphere. The cells in logarithmic growth phase were used in all experiments. And the medium was renewed every 3 days. CZ-1 cell line secreting λ light chain protein was established from the bone marrow of an advanced stage MM patient classified as λ light chain type in our laboratory[16]. LP-1 secreting IgG κ light chain was generously contributed by Dr. Hallek (Laboratorium für Molekulare Biologie, Genzentrum, Ludwig-Maximilians-Universität München, Germany), and NCI-H929 secreting IgA κ light chain by Dr. Margaret (Prince of Wales Hospital, Chinese University of Hongkong).

2.2. Reagents

2ME2, dimethyl sulfoxide (DMSO) and RPMI 1640 were purchased from Sigma (St. Louis, MO, USA). CD49e, the first mouse anti-human monoclonal antibodies, and PE-conjugated rabbit anti-mouse McAbs were purchased from Southern Biotechnology Associates Inc. (USA). Rabbit anti-human XBP-1 antibody was purchased from Biolegend Inc. (USA). Human Kappa-b&f ELISA Quantitation Kit and Human Lambda-b&f ELISA Quantitation Kit were purchased from Bethyl Laboratories Inc. (Montgomery, TX, USA). 1 mmol/L 2ME2 (molecular weight 302.4) in DMSO was stored at 4 °C, and was diluted with culture medium RPMI 1640 immediately before use. The final concentration of DMSO in the culture system was less than 0.1%, which had no significant effect on the cell growth.

2.3. Morphological evaluation and tunel assay

After exposure to 2ME2 at a series of concentrations, including 0–16 μmol/L for 72 h, the cells were sampled and analyzed for the morphological evaluation. The cells were assessed by cytospin, followed by Wright’s–Giemsa staining and examination under 100× microscopy. Photos were taken with Hunter Imaging System (China). Terminal deoxynucleotidyl transferase (TdT)-mediated X-DUTP nick end labeling (TUNEL)-assay was performed on the slices. After fixing, washing and labeling, added biotylation sheep anti-digoxigenin Fab fragments (Roche), 37 °C for 30 min, stained with alkaline phosphatase/tetrazolium(BCP/NBT) for 30 min. The number of TUNEL-positive cells was counted under high power lens. TUNEL-positive cells were expressed as a TUNEL index (TUNEL-positive cells/total cells).

2.4. Flow cytometric analysis

An indirect immunofluorescence staining technique was applied to analyze differentiation antigens on the cell surface. Single-cell suspensions (1 × 10^6/ml, 0.2% trypan blue staining showed the survival rate was more than 95%) were incubated with mouse anti-human monoclonal antibodies CD40e (5 μg/ml) for 30 min on ice and washed twice with PBS, labeled with PE-conjugated (20 μg/ml) for 30 min in dark at 4 °C. After washed twice with PBS, the labeled cells (a minimum of 10,000 cells per sample) were analyzed by flow cytometry and Cellquest 1.2 software. In each test, an irrelevant isotype-matched McAb was used as a negative control. The value of individual negative group was subtracted from that of each experiment group. All tests were performed in triplicate and were duplicated for three times.

2.5. Determination of light chain secretion in the Supernatant of Cells

After exposure to 2ME2 for 36 and 72 h, the supernatant was collected and stored at −20 °C. Kappa or Lambda ELISA Quantitation Kit was used to determine light chain levels in the supernatant. All tests were duplicated for three times. Briefly, 96-well plates were coated with either goat anti-human kappa or lambda antibodies overnight at 4 °C, aspirated the antibody solution from each well, washed three times with wash solution, and then blocked with 200 μl blocking solution (50 mmol/L Tris–Cl, 1% BSA, 0.14 mol/L NaCl, pH 8.0) incubated 30 min at room temperature. After incubation, removed the blocking solution and washed each well three times. Then, diluted standards or samples in sample diluent (50 mmol/L Tris–Cl, 1% BSA, 0.14 mol/L NaCl, and 0.05% Tween 20, pH 8.0) based on the expected concentration falling within the concentration range of the standards. Transferred 100 μl standards or samples to each well, incubated plate 60 min, removed samples and standards and washed five times. Added 100 μl HRP conjugate (1:50,000) to each well, incubated 60 min, removed HR conjugate and washed five times. Transferred 100 μl OPD substrate solution to each well, incubated plate 10 min, applied 100 μl 2 mol/L H2SO4 to stop the reaction, readed the plate at 492 nm wavelength with a microtiter plate reader. Created a standard curve by CurveExpert 1.3 software and calculated the results.

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

Using Trizol (BioBasic, CA) one-step method, total RNA was extracted from CZ-1, LP-1 and NCI-H929 cells, and transcribed into cDNA with random hexamers (Gibco BRL) as primer and M-MLV reverse transcriptase (Promega, USA). Resultant cDNA was then normalized for expression of the constitutively expressed housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH: 5' CCA CCC ATG GCA AAT TCC ATG GCA; 3' TCT AGA CCG GTC AGG TCC ACC). XBP-1 primers sequences were as follows: 5' GCT CAG ACT GCC AGA GAT CG; 3' GTC CAG AAT GCC CAA CAG G (Shenergy Biocolor Biotechnology co.
Samples were removed after 34 cycles, each cycle consisted of 1 min denaturation (94 °C), 1 min annealing (55 °C), and 1 min extension (72 °C) in a Thermal Cycler (PE2400, USA).

2.7. Western blotting

Total protein lysates and nuclear extracts were prepared according to the instruction of Nuclear and Cytoplasmic Extraction Kit (Cell Signaling Technology, USA). Briefly, collected cells (2 × 10^6) by centrifugation at 600 × g for 5 min at 4 °C, discarded supernatant completely. Added 200 μl ice-cold CER A to the cell pellet, vortexed the tube vigorously for 15 s. Incubated the tube on ice for 10 min. Added 11 μl ice-cold CER B, vortexed the tube for 5 s, incubated on ice for 1 min, vortex for 5 s, centrifuged for 5 min at 16,000 × g. Discarded the supernatant, and resuspended the pellet in 100 μl ice-cold NER, vortexed on the highest setting for 15 s; Returned the samples to ice and continued vortex for 15 s every 10 min, for a total of 40 min, centrifuged the tube at 16,000 × g for 10 min. Immediately transferred the supernatant fraction to a clean pre-chilled tube, placed on ice; determined the proteins concentrations using BCA Protein Assay Kit (Pierce Biotechnology, Inc., USA). 50 μg protein of each sample was resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane. The nitrocellulose membrane with transferred proteins was then blocked by incubation in 5% dry milk in TBST (0.1% Tween-20 in Tris buffered saline), and probed with anti-XBP-1, washed the membrane three times for 5 min. Blots were then developed by SuperSignal West Pico Chemiluminescent Substrate Kit (Pierce Biotechnology Inc., USA).

2.8. Isolation of primary CD\textsuperscript{+} (Syndecan-1) myeloma cells

Mononuclear cells were freshly isolated from bone marrow of seven patients with myeloma by Ficoll–Hypaque density gradient centrifugation. Informed consent was obtained from all patients in accordance with the Helsinki protocol. Tumor cells were purified with CD138 positive selection method using CD138 immunomagnetic beads and auto MACS magnetic cell sorter machine, according to the manufacturer’s instructions (Miltenyi Biotec, Auburn, CA). The primary CD\textsuperscript{+} myeloma cells were viable (95–97%) in vitro. The Cells treated with 0.5 mM 2ME2 for 36 and 72 h were used to analyze its differentiated antigens and the culture supernatants were performed to determine the light chain protein.

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2.9. Statistical analysis

All statistical calculations were performed with the statistical software program SPSS ver. 10.0. Differences between each group were evaluated by one-way analysis of variance (ANOVA) and Student’s t-test. The difference was of statistical significance, when \( p < 0.05 \).

3. Results

3.1. The general clinical data of seven patients with myeloma

These seven myeloma patients were inpatients of Changzheng Hospital from October 2004 to December 2004. There were two females, five males. The eldest was 71 years old, and the youngest was 37 years old. The median age was 55 years old. See Table 1.

3.2. Influence of 2ME2 on the morphology of myeloma cells

CZ-1 cells used in this study presented morphologic features of plasmablast, with a small quantity of cytoplasm and a high nuclearcytoplasmic ratio. Some of them had obvious nucleioli and fine nuclear chromatin. Following treatment with 0.1 μmol/L 2ME2 for 72 h, CZ-1 cells displayed some maturing morphological changes, including the ratio of nuclearcytoplasmic going down, nucleiolus reducing or disappearing, and chromatin getting rougher and more compacted. When the concentration of 2ME2 increased to 0.5 μmol/L, the cells morphologic changes were more remarkable, showing typical basic and abundant cytoplasm. Some had eccentric nucleus, rough and agglomerate chromatin arraying in wheels, some presented deep chromatosis. These maturing morphologic changes of plasma cells were obviously different from early apoptosis (Fig. 1). Meanwhile, the TUNEL index was not higher than 5% at these concentrations. When the concentration of 2ME2 increased to 0.5 μmol/L, the TUNEL index was 8%. With the concentration continuing to increase, the TUNEL index increased in a dose-dependent manner. After treatment with 16 μmol/L 2ME2, the TUNEL index increased to 27% (Fig. 2). These
results demonstrated that 2ME2 at low concentrations (less than 1.0 μmol/L) could induce morphologically differentiation of CZ-1 cells, but no apoptosis, otherwise the higher concentrations induced apoptosis of myeloma cells. Furthermore, treatment with 0.5 μmol/L 2ME2 for 72 h also induced the morphological maturation of LP-1 and NCI-H929 cells and primary CD138 cells from myeloma patients (Fig. 3).

3.3. Influence of 2ME2 on the CD49e expression on the myeloma cell lines

Treatments with 0.1–0.5 μmol/L 2ME2 for 72 h up-regulated the expression of CD49e on the CZ-1 cell surface in comparison with the control group (4.65 ± 0.73)% in a dose-dependent manner (Fig. 4). The expression of CD49e in 0.1 μmol/L 2ME2 group was (12.20 ± 1.70)%.
Fig. 3. The apoptotic effect of 2ME2 on the CZ-1 cells ($n=3$). Using TUNEL assay, the TUNEL index of CZ-1 cells was counted. When the concentration of 2ME2 was increased to 1 μM, the TUNEL-positive cells were increased obviously in comparison with control group ($p<0.05$). With the concentration increasing, the TUNEL index increased in a dose-dependent manner. $p<0.05$.

was different from the control significantly ($p<0.01$). In the 0.2 μM/2ME2 group, the expression of CD49e increased to $14.75 \pm 1.43\%$. With the concentration increasing to 0.5 μM/2ME2, the further up-regulation of the CD49e expression was observed ($24.47 \pm 1.34\%$ versus $4.65 \pm 0.73\%$, $p<0.001$). In order to confirm the influence of 2ME2 on the CD49e expression, CZ-1, LP-1 and NCI-H929 cells were exposed to 0.5 μM/2ME2 for 36 and 72 h. The result showed that the CD49e expression was increased obviously after exposure to 2ME2 for 72 h, but no obvious difference between the 36 h groups and its blank controls (Fig. 5).  

3.4. Treatment with 2ME2 increased the secretion of light chain protein of myeloma cell lines  

Following treatment with 0.5 μM/2ME2 for 36 h, the concentrations of κ light chain protein in the supernatant of CZ-1, LP-1 and κ light chain protein in NCI-H929 cells were different from the control significantly ($p<0.01$). But after exposure to 2ME2 for 72 h, the secretions of light chain proteins from these cells were increased obviously. The most obvious change was observed on CZ-1 cells, and subsequently was LP-1 and NCI-H929 cell lines (Fig. 6).

3.5. 2ME2 up-regulated the XBP-1 mRNA expression in myeloma cell lines  

After exposure to 0.5 μM/2ME2 for 72 h, the results of RT-PCR showed that 2ME2 increased (by 2.1–4-fold) XBP-1 transcripts in CZ-1, LP-1 and NCI-H929 cells, but exposure for 36 h, the changes of XBP-1 mRNA were not obvious (Fig. 7).

Fig. 4. The influence of 0.1–0.5 μM/2ME2 on the expression of CD49e on CZ-1 cells ($n=3$). Treatments with 0.1–0.5 μM/2ME2 up-regulated the expression of CD49e on the CZ-1 cell surface in comparison with the control group in a concentration-dependent manner. $p<0.01$.

Fig. 5. Influence of 0.5 μM/2ME2 on the CD49e expression on myeloma cell lines for 36 and 72 h ($n=3$). After exposure to 0.5 μM/2ME2 for 36 h, expression of CD49e on myeloma cells displayed some tendency of up-regulation, but statistical significance was not obvious in comparison with the control group. When the exposure time reached to 72 h, the statistical significance was obvious, especially CZ-1 cell line. $p<0.05$, $**p<0.01$.

Fig. 6. Secretion of light chain protein from CZ-1, LP-1 and NCI-H929 cell lines following treatment with 0.5 μM/2ME2 for 36 and 72 h ($n=3$). When treatment with 0.5 μM/2ME2 for 36 h, secretion of light chain protein from these three myeloma cell lines did not increase significantly, but treatment with 2ME2 for 72 h up-regulated the secretion of κ light chain from CZ-1 cells, the difference from its control at 72 h had obviously statistical significance ($p<0.01$). The secretions of κ light chain protein from LP-1 cells were up-regulated by 0.5 μM/2ME2 for 72 h also. $p<0.05$, $**p<0.01$.  

Fig. 7. XBP-1 mRNA expression in myeloma cell lines following treatment with 0.5 μM/2ME2 for 36 and 72 h ($n=3$). When treatment with 0.5 μM/2ME2 for 36 h, XBP-1 expression in CZ-1, LP-1 and NCI-H929 cells, but exposure for 36 h, the changes of XBP-1 mRNA were not obvious.
3.6. 2ME2 up-regulated XBP-1 protein expression in myeloma cell lines (Fig. 8)

2ME2 at the concentration of 0.5 μM/L induced the up-regulated protein levels of XBP-1 in CZ-1, LP-1 and NCI-H929 cells after treatment for 72 h, but for 36 h, the up-regulations were not obvious.

3.7. Influence of 2ME2 on the primary CD138 myeloma cells

In order to investigate whether 2ME2 could exert its effect of inducing differentiation on the primary myeloma cells, the CD138 cells were isolated by CD138 positive selection method using CD138 immunomagnetic beads from seven patients with myeloma. After exposure to 0.5 μM/L 2ME2 for 36 and 72 h, the morphological changes, CD49e expression and secretion of light chain proteins were detected. The results showed that after treatment for 72 h, obvious evidence of morphological differentiation was observed, such as the characteristic changes of eccentric nucleus, rough and agglomerate chromatin arraying in wheels (Fig. 2). At the same time, 0.5 μM/L 2ME2 for 72 h also obviously up-regulated the CD49e expression in 6/7 (85.7%) patients, although these changes were not remarkable after exposure to 2ME2 for 36 h (Table 3); and the secretions of light chain protein were significantly higher than that of the control in 5/7 (71.4%) patients following exposure to 2ME2 for 72 h. But after exposure for 36 h, these changes were not remarkable (p > 0.05) (Table 3).

4. Discussion

Inducing apoptosis and differentiation have become the new and important therapies in various tumors. Some medications or chemicals could have both of these effects, such as arsenic trioxide (As2O3) in acute promyelocytic leukemia (APL) cells. As2O3 had dose-dependent dual effects on APL cells: inducing preferentially apoptosis at relatively high concentrations and inducing partial differentiation at low concentrations [17]. In the present study, our results showed that 2ME2 at 1–16 μM/L could induce apoptosis of myeloma cells in a dose-dependent manner, corresponding to those results reported by Chauhan et al. [14]. In order to investigate whether 2ME2 could induce differentiation in myeloma cells, we explored the effect of 2ME2 at low concentration on myeloma cells.

Human myeloma cell lines CZ-1, LP-1 and NCI-H929 cells showed morphologic features of blast cells, high nucleocytoplasmic ratio, one to two nucleoli and non-condensed nuclear chromatin, abnormal nucleus, abundant and opaque cytoplasm stained to deep blue. In the present study, we found that these cells appeared to develop into mature cells, showing the ratio of nucleocytoplasmic going down, nucleolus reducing or disappearing, chromatin getting rougher and more compacted (Fig. 1). The similar regularities were observed on the CD138+ cells from myeloma patients. These results demonstrated that 2ME2 at these low concentrations induced morphological differentiation and maturation of myeloma cells.

When B cells differentiated into plasma cells under the antigenic stimuli, the related antigens of B cells such as HLA-DR, CD19, CD20 were lost and the special antigen of plasma cells such as CD138 were gained. During the further differentiation of plasma cells, CD38 was lost gradually, and MPC-1 and VLA-5 (very late antigen-5, i.e. CD49e) were gained gradually. Similar regularity of differentiation was found on myeloma cells [18].

Although multiple myeloma is a malignancy of the terminally differentiated B cells, the myeloma cells could further be classified into mature and immature groups. Using two-color fluorescence flow cytometry, Harada et al. [19] reported that the bone marrow plasma cells from 8/20 myeloma patients were CD138+CD49e+, which were classified to immature myeloma cells according to Greipp’s classification [20], the ones from the rest 12 myeloma...
patients were CD138+CD49e+, belonging to mature myeloma cells [19]. So the expression of CD49e could be regarded as a marker of more mature myeloma cells.

As for the mature of plasma cells, Ishikawa [21] proposed that B cells activated by antigens in the germinal center of follicular lymphatics differentiated into plasma cell precursors, which could emerge in the peripheral blood with the morph of lymphoid plasma cells of meager cytoplasm. The immunophenotype on the plasma cell precursors showed CD19−MPC−CD49e+. And then these cells homed to bone marrow and matured through the following 3 stages. First, the immunophenotype of the cells was CD19−MPC−CD49e−. The neoplastic cells of myeloma patients were a colonia of these three stages cells in a various proportion.

In the present study, following treatment with 0.1–0.5 μmol/L 2ME2, the expression of CD49e on CZ-1 cell surface increased in a dose-dependent manner. 2ME2 at the concentration of 0.5 μmol/L had the most obvious effect on CZ-1 cells. In order to confirm this effect, more myeloma cell lines (LP-1 and NCI-H929) and primary CD138 myeloma tumor cells from myeloma patients were studied at 0.5 μmol/L 2ME2 for 36 and 72 h. The results indicated that exposure to 0.5 μmol/L 2ME2 for 72 h, significant up-regulation of CD49e was observed on myeloma cell lines and CD138 cells from 6/7 (85.7%) myeloma patients.

It was reported [22] that during the development of myeloma cells, those cells with CD49e− were immature in the morphology, and had proliferative response to IL-6, and could be observed in bone marrow and peripheral blood. CD49e+ myeloma cells were mature cells and located in bone marrow with low proliferative activity, and had no proliferative response to IL-6. But they could secrete more quantity of M protein than CD49e− myeloma cells. B cell terminal differentiation is characteristically associated with the onset of high-level antibody secretion. It had been reported that more quantity of immunoglobulin protein was secreted when the myeloma cells differentiated into mature stage. When PD173074 (an inhibitor of fibroblast growth factor receptor 3) was investigated in myeloma cells, it was found that low-dose of PD173074 could induce MM cell lines KMS11 and KMS18 to present the morphologic changes of cell differentiation and maturation, such as nucleus decreasing, plasma increasing, the ratio of nucleocytoplasmic going down, nucleolus decreasing or disappearing, chromat in getting rougher and more pyknotic. Further investigation performed by ELISA was to assay the quantification of immunoglobulin in the culture supernatant. The results

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* p < 0.05.
** p < 0.01.

### Table 3

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* p < 0.05.
** p < 0.01.
indicated that light chain protein secreted by KSM11 and KMS18 cells increased from 10 to 20 μg/ml while the morphological changes of the differentiation appeared [23].

In the present study, we found that three myeloma cell lines used in our experiments and the CD(138)(+) cells from myeloma patients bone marrow secreted measurable amounts of kappa or lambda light chain proteins respectively with ELISA assay. Secreted light chain increased significantly after exposure to 0.5 μmol/L 2ME2 for 72 h. These data indicated that 2ME2 induced functional maturation of myeloma cells.

A recent study has demonstrated that XBP-1, a basic-region leucine zipper protein in the CREB/ATF family of transcription factors, mediates B-cell differentiation into plasma cells via UPR pathway [24]. Functional knockout of XBP-1 in mice leads to a number of defects associated with a plasma cells via UPR pathway [24]. A functional UPR system is therefore necessary both for the differentiations of B cells into plasma cells and to ensure that only properly folded antibodies are secreted. In the present study, RT-PCR and western blotting analysis showed that 2ME2 up-regulated the expression of XBP-1 mRNA and protein (Figs. 4 and 5) after exposure to 0.5 μmol/L 2ME2 for 72 h. These results confirmed that 2ME2 could induce myeloma cells differentiation, and the transcription factor XBP-1 may play an important role, which remains to be studied.

Taken together, we inferred that 2ME2 at low-concentration exerted obvious effect of inducing differentiation on the myeloma cells. This could be a novel anti-myeloma mechanism of 2ME2, which has not been demonstrated before. 2ME2 might have the potential to be used as a differentiation inducer applied to clinical patients. Further studies are needed to elucidate the mechanisms of the myeloma cell differentiation induced by low-concentration 2ME2.

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