Therapeutic effects of the novel subtype-selective histone deacetylase inhibitor chidamide on myeloma-associated bone disease

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 doi:10.3324/haematol.2017.181172

Received: November 8, 2017.

Accepted: April 27, 2018.

Pre-published: May 17, 2018.

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Supplemental materials

Methods and Reagents

Reagents

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich Corp (St. Louis, MO, USA). Human RANKL, M-CSF and IL-6 were purchased from Peprotech, USA. Protease inhibitor, phosphatase inhibitor and Q-VD-Oph was purchase from Selleck Chemicals LLC, Shanghai, China. Primary antibodies against Caspase-3 and Caspase-9 were obtained from Proteintech (Rosemont, IL 60018, USA); antibodies against Caspase-7, Caspase-8, PARP-1, Myc, Mcl-1, p21, p27, CDK4, CDK6, Bcl-xl, Bcl-2, STAT3, p-STAT3, JAK2, p-JAK2, p-AKT, AKT, p-JNK, JNK and SOCS3 were purchased from Cell Signaling Technology (MA, USA); antibodies against HDAC1, HDAC2, HDAC3, HDAC10, cathepsin K, c-fos and DUSP-1 were purchased from Abcam (Cambridge, UK). Cell counting kit (CCK-8) and an apoptosis kit for flow cytometry were obtained from Dojindo, Kumamoto, Japan. The HDAC activity colorimetric assay kit (Catalog #K331-100) was obtained from BioVision (Milpitas, CA 95035 USA).Polyvinylidene difluoride membranes obtained from Merck Millipore, Germany. Primary antibodies of Caspase-3 and Caspase-9 for flow cytometry were obtained from Thermo Fisher Scientific. Sodium dodecyl sulfate (SDS)–polyacrylamide gels (4-20%) for western blotting were purchased from Genescript, China. Enhanced chemiluminescence detection kit and 5 × SDS loading buffer was purchase from Hangzhou Fude Biology Science Company, Ltd. RPMI-1640 and MEM-α were obtained from Shanghai BasalMedia Technologies Co., Ltd. Fetal bovine serum was purchased from Biological Industries Israel Beit Haemek Ltd., Israel. In vivo D-luciferin was purchased from Promega Corporation, an affiliate of Promega (Beijing) Biotech Co., Ltd. Human Ig lambda ELISA kit was purchased from Bethyl Laboratories, Inc., USA. Mouse serum CTX-I and the PINP ELISA kit was obtained from Immunodiagnostic Systems, UK. Calcium-coated plates were purchased from Corning Incorporated, Kennebunk, ME 04043 USA.

Cell proliferation, HDAC activity colorimetric assay, **cell cycle, and cell apoptosis assays**

CCK-8 assays were used to detect MM cell viability. Briefly, MM cells were seeded in 96-well plates and treated with chidamide. After 24, 48, and 72 h, CCK-8 was incubated with MM cells for 2 h, and the subsequent absorbance was measured at 450 nm using a microplate reader (Bio-Rad, Model 680). For measuring the HDAC activity, ARP-1 and RPMI-8226 were incubated with chidamide for 24 h, then cells were harvested and cell lysate was collected. Then, HDAC activity was measured according to the protocol provided in the kit. To assess the impact of chidamide on apoptosis and cell cycle of MM cells cultured alone or co-cultured with BMSCs and OCs, MM cells were seeded

in 6-well plates and treated with chidamide for 48 h. Then, flow cytometry was used to assess the cell cycle distribution and apoptosis, and the results were analyzed with FlowJo7.6.1 software.

Semi-quantification polymerase chain reaction

The total RNA from BMSCs and osteoblasts were extracted fron day 0, day 7 and day 21 by using the Trizol reagent (Takara, Ostu, Japan). Reverse transcription was performed by using a Prime Script RT reagent kit from Takara (Otsu, Japan). The target gene expression was analyzed by qRT-PCR using the iTaq universal SYBR Green Supermix (SYBR® Premix Ex TaqTM II, Bio-Rad, USA) with the Bio-Rad CFX96 real-time system, according to the manufacturer's instruction, and normalized to GAPDH RNA levels and plotted as relative quantification. Each sample was run in triplicate. The primers of the target genes were designed and synthesized by Tsingke (Beijing, China).

Immunoblotting

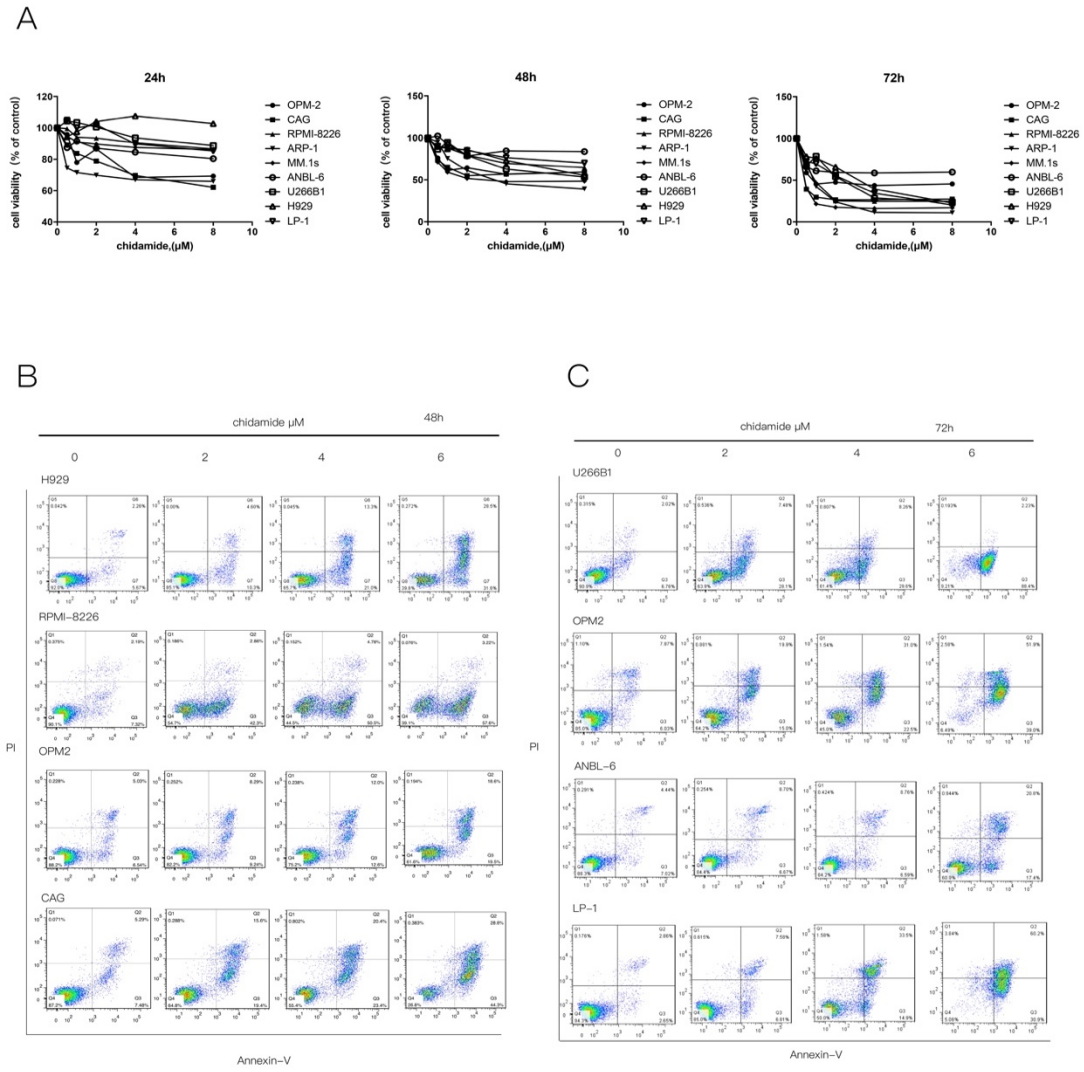
Western blotting was performed as previously reported. Briefly, MM cells and OCs were treated with chidamide at different concentrations for specific times. Cells were collected and washed twice with PBS, and then, the proteins were extracted with RIPA buffer containing protease and phosphatase inhibitors, and the supernatants were used for western blotting. Immunoblots were incubated overnight with specific primary antibodies, washed with TBST and then incubated with horseradish peroxidase-conjugated secondary antibodies. Autoradiography was performed using a ChemiDoc MP Imaging System (Bio-Rad) and an enhanced chemiluminescence detection kit to visualize the protein bands.

Animal models and micro-CT

To investigate the anti-tumor effect of chidamide, two murine models were employed. The first one was a subcutaneous xenograft model. Briefly, 4 - to 6-week-old female NOD–SCID mice were purchased from Vital River Laboratory Animal Technology Co. Ltd. (Beijing, China) and were injected with 2×10^6 RPMI-8226-luc cells subcutaneously in the left flank. After 10 days, when the tumor reached 60-100 mm^3, gavage with chidamide 35 mg/kg started and continued for 14 days. The tumor volume was calculated as 1/2 $a\times b^2$. The second model was a disseminated myeloma model, in which 2×10^6 RPMI-8226-luc cells were injected intravenously into 4- to 6-week-old female NCG mice purchased from Nanjing Biomedical Research Institute of Nanjing University. Two weeks after tumor injection, serum human Ig lambda was assessed and non-invasive bioluminescence imaging with an IVIS 100 system was used to assess the tumor burden. We also used a non-tumor bearing mouse model to clarify the direct effect of chidamide on bone metabolism in vivo. Briefly, 4- to 6-week-old female C57Bl/6 mice were administered chidamide 25 mg/kg for 3 weeks via gavage, and then, 1 mg/kg RANKL was given intraperitoneally every 24h (a total of three doses) as

previously described. Serum was collected within 90 minutes after the last injection. All experiments followed the procedures and protocols of the Animal Ethics Committee of the First Affiliated Hospital, Zhejiang University School of Medicine. Serum levels of Ig lambda, CTX-I and PINP were quantified by ELISA and were measured according to the manufacturer's instructions. Distal femur metaphyses were scanned with micro-CT (μCT-100, Scanco Medical, Bassersdorf, Switzerland) as previously described. Briefly, the evaluation of trabecular bone structure parameters was starting at the begnning point of the growth plate and moving upward. A three-dimensional reconstruction was built, and the trabecular number (Tb.N), trabecular space (Tb.sp, seperation) and relative bone volume over total bone volume were calculated.

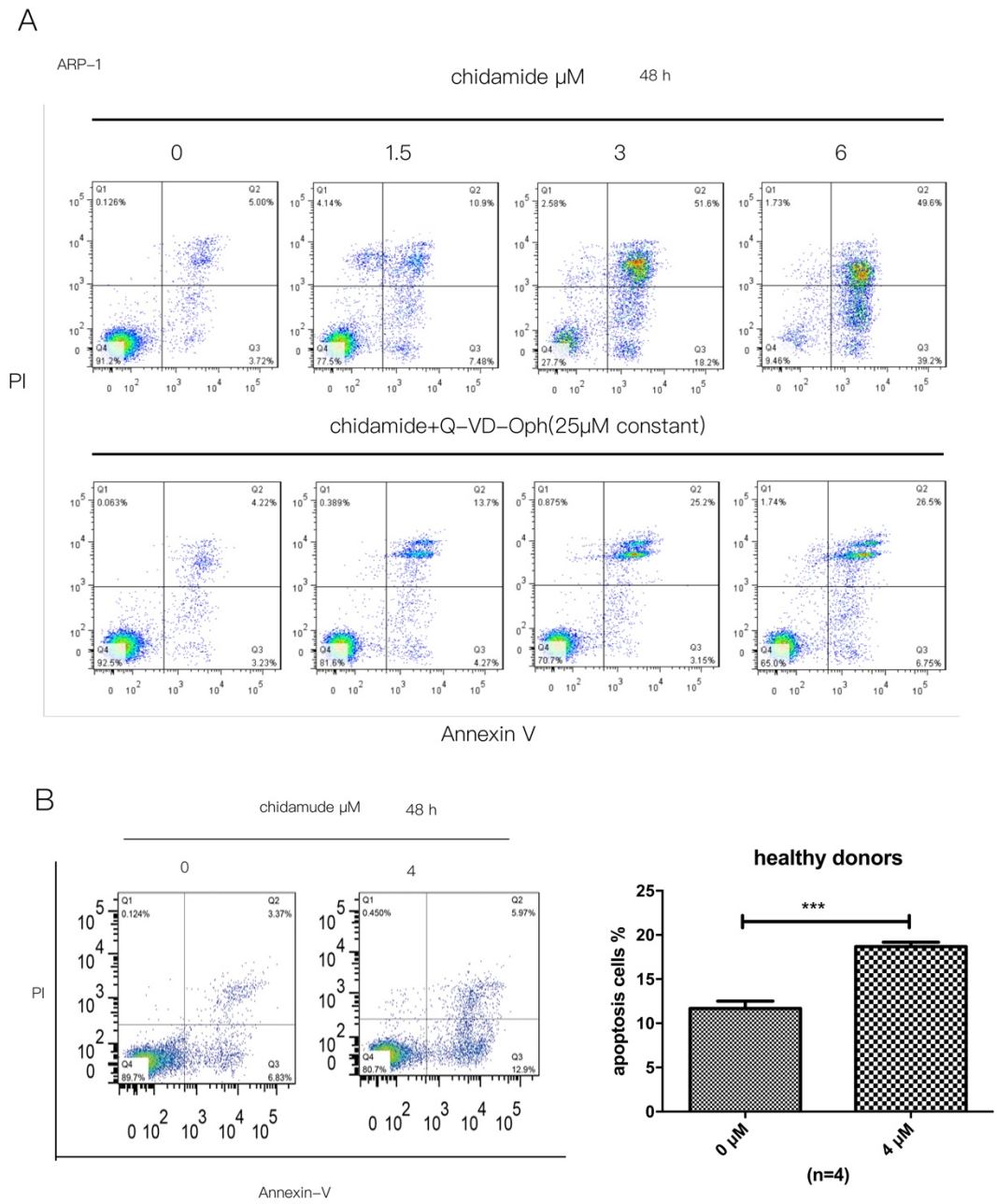
Results



s figure 1

Supplementary figure 1. Chidamide exerted anti-myeloma effect and induced MM cells apoptosis. (A) The cytotoxicity of chidamde toward MM cell lines was examined using CCK8 assays. Viabilities of untreated cells were set to 100%, and all data are summarized as means±SEM (n=3). (B) Sensitive MM cell lines H929, RPMI-8226 and

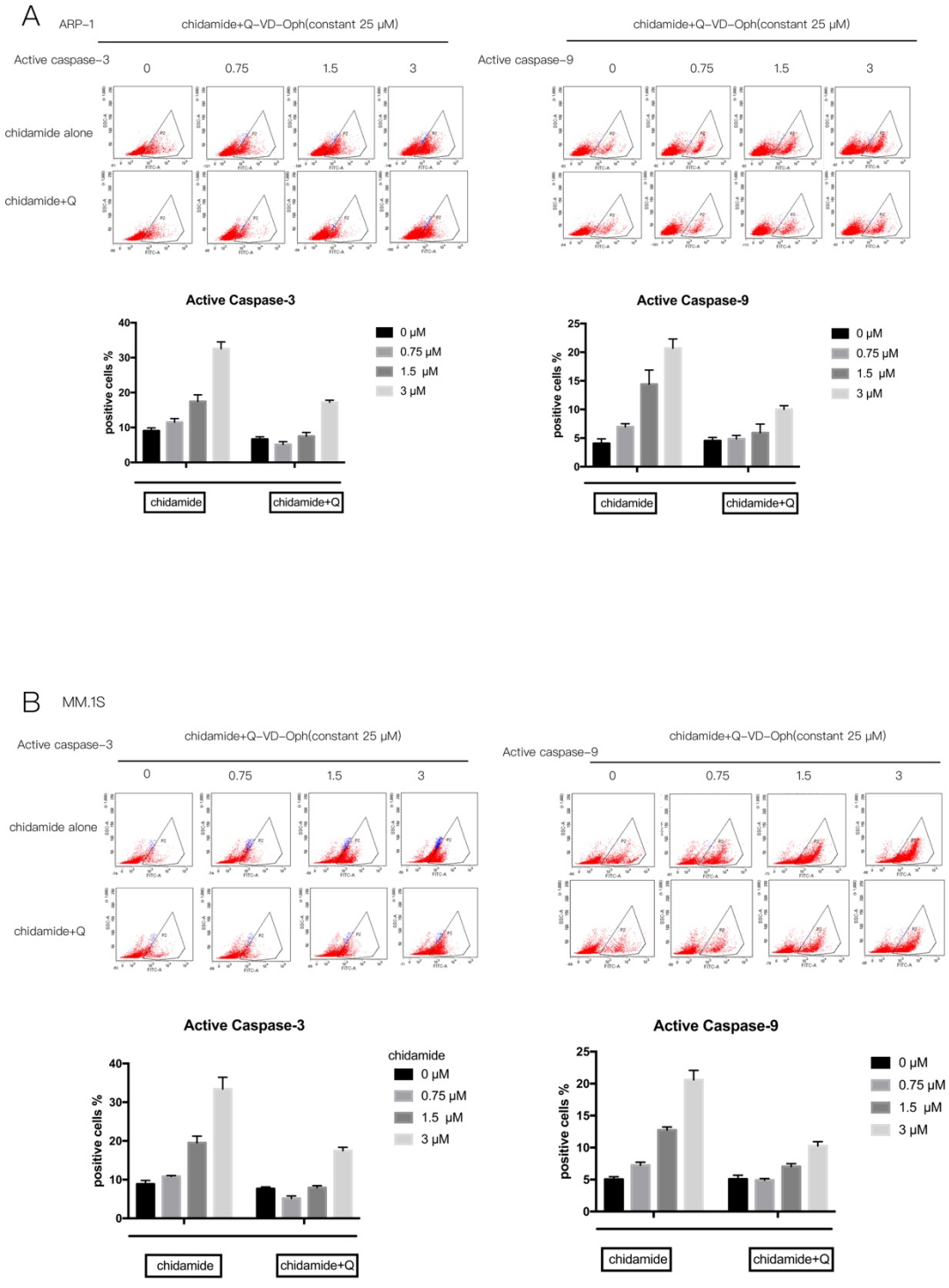
CAG were incubated with chidamide at different concentrations (0-6μM) for 48h, cell apoptosis were detected by flow cytometry, all data are summarized as means±SEM (n=3). (C) Less sensitive MM cell lines U266B1, OPM2, ANBL-6 and LP-1 were incubated with chidamide at different concentrations (0-6μM) for 72 h, all data are summarized as means±SEM (n=3).



s figure 2

Supplementary figure 2. Apoptosis effect of chidamide on ARP-1 and BM derived mononuclear cells from healthy donors. (A) Pan-caspase inhibitor Q-VD-Oph could

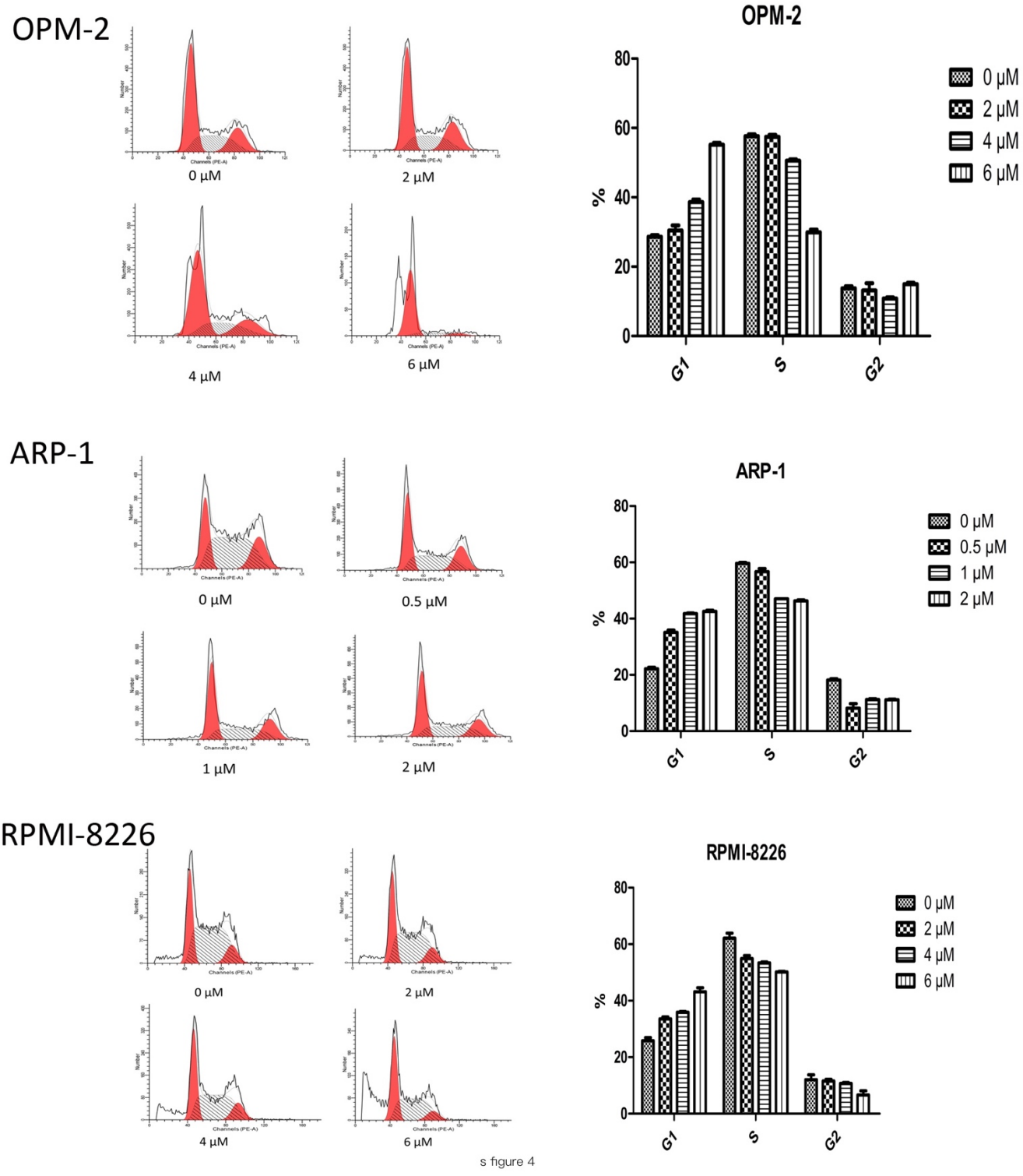
prevent the apoptosis induced by Chidamide in ARP-1. (B) BM derived mononuclear cells from 4 healthy donors were incubated with chidamide 4μM for 48h, then cell apoptosis was detected by flow cytometry.



s figure 3

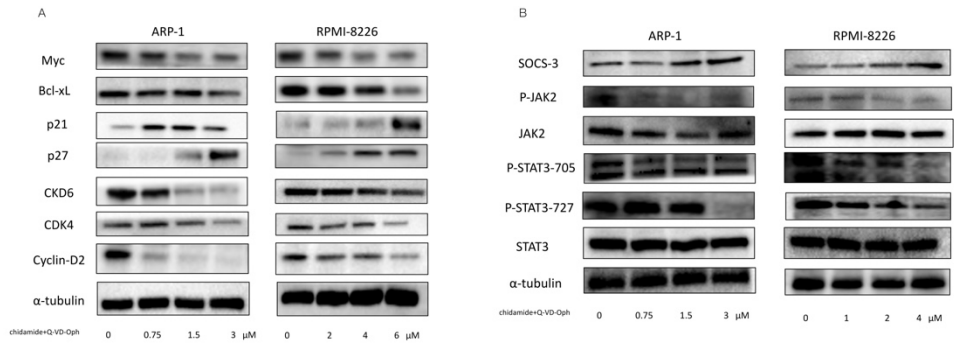
Supplementary figure 3. Pan-caspase inhibitor Q-VD-Oph could reduce the expression of active caspase-3 and active caspase-9 induced by chidamide. (A) ARP-1 cell line. (B) MM.1s cell lines. ARP-1 and MM.1s were treated with chidamide (0-3μM) with or without 25 μM Q-VD-Oph (abbreviated as Q) co-incubated for 48 h, then the

expression of active caspase-3 and caspase-9 were detected by flow cytometry.



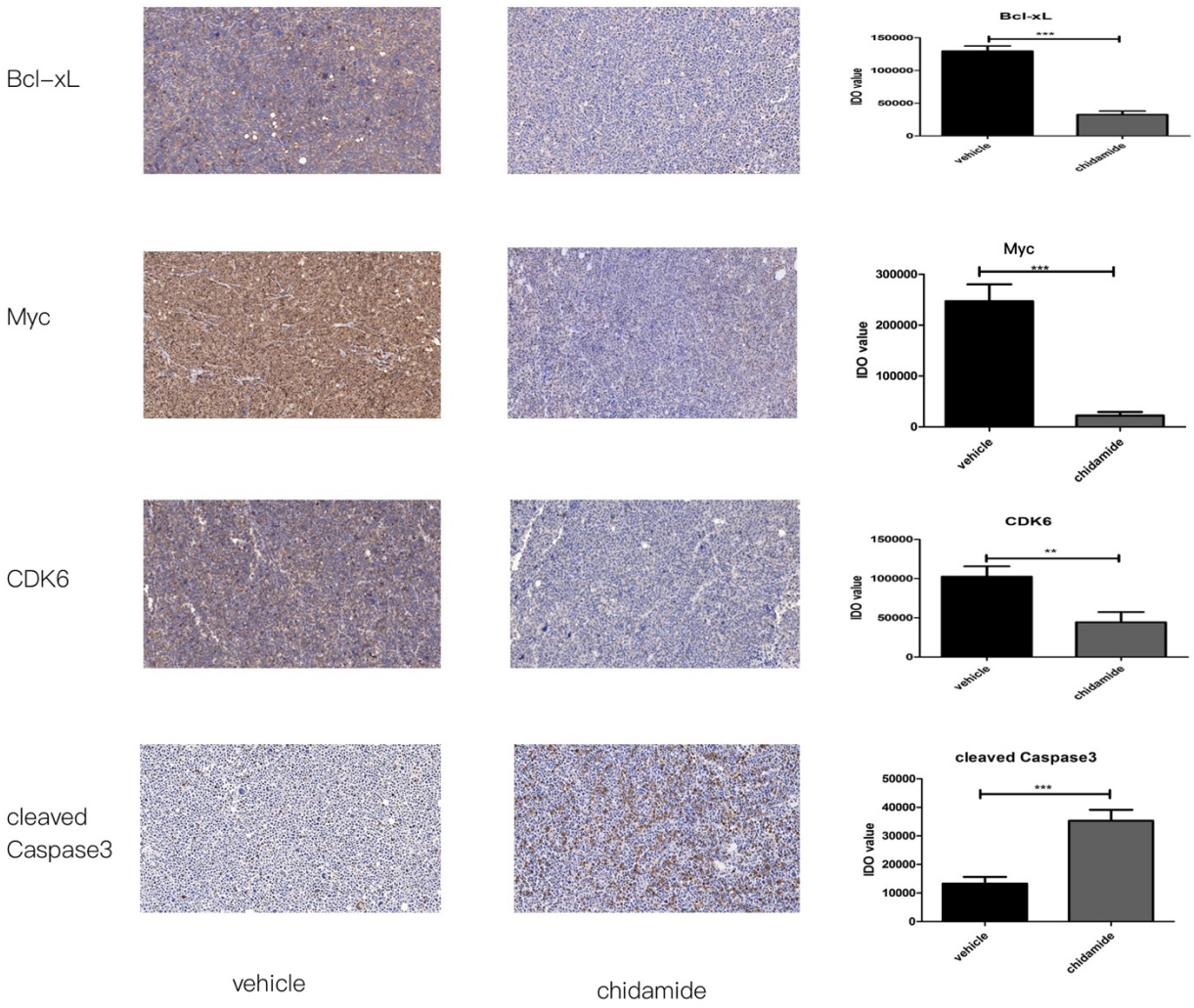
s figure 4

Supplementary figure 4. Chidamide induced MM cell lines OPM-2, LP-1 and RPMI-8226 cell cycle arrest in G0/G1 phase.



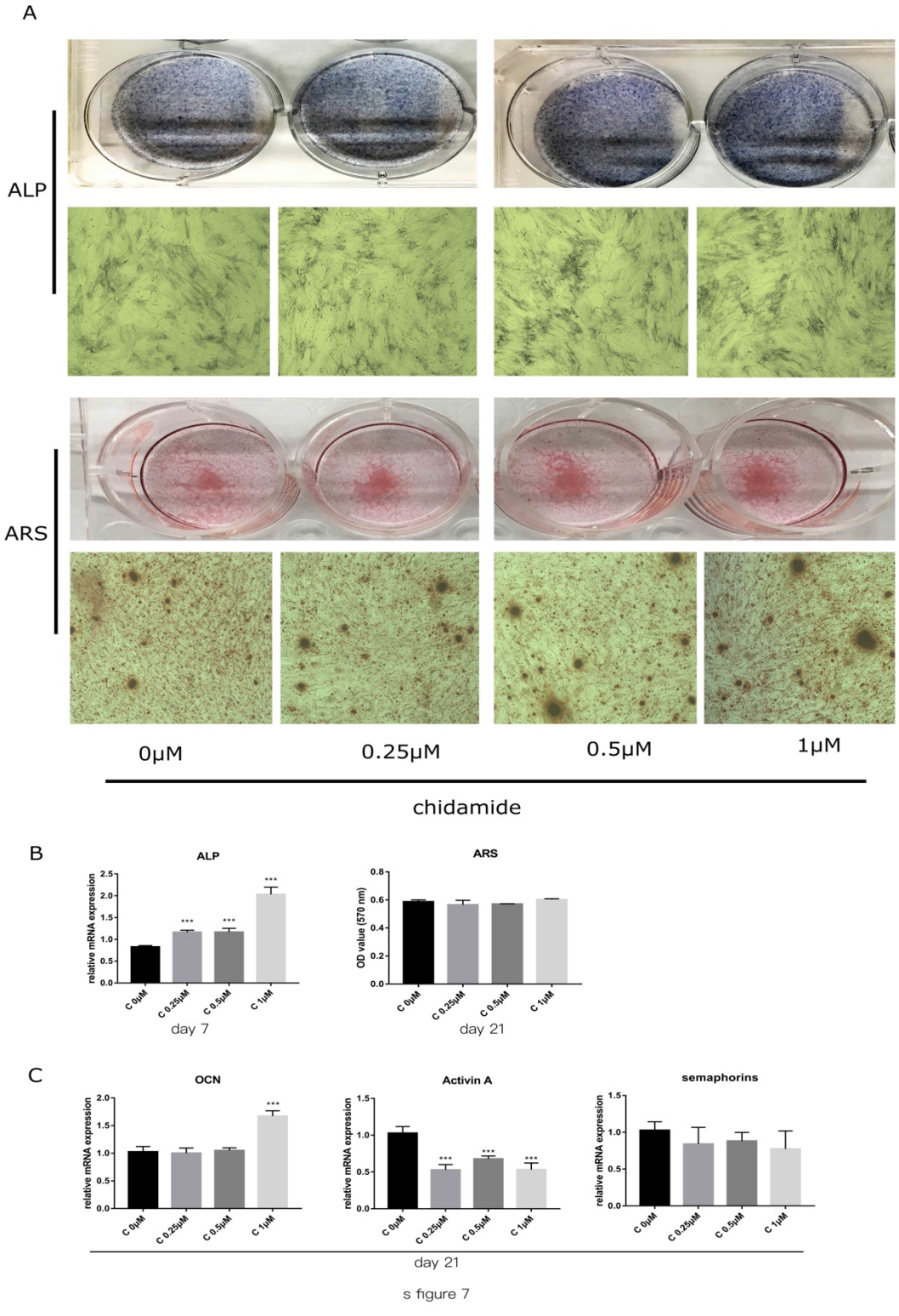
s figure 5

Supplementary figure 5. Pan-caspase inhibitor Q-VD-Oph did not affect the expression of key molecules and pathways proteins in MM cells after chidamide treated. Western blot analysis of Myc, Bcl-xL, Bcl-2, p21, p27, CDK4, CDK6 and Cyclin-D2 levels; α-tubulin was used as the loading control. (E) SOCS-3, p-JAK2, JAK2, p-STAT3-727, p-STAT3-705 and STAT3 levels were analyzed by Western blotting; α-tubulin was used as the loading control.



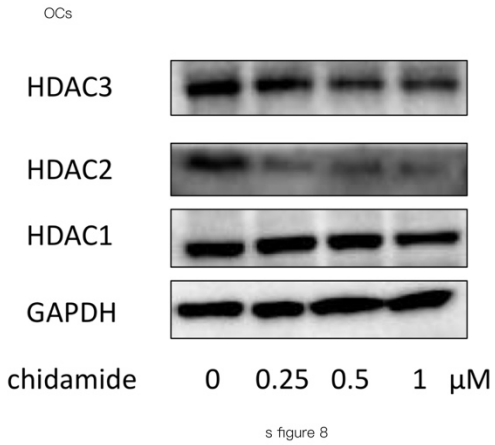
s figure 6

Supplementary figure 6. The immunohistochemistry results of tumor xenografts. Chidamide reduced the expression of Bcl-xL, Myc and CDK6 and increased cleaved Caspase3 expression in the xenografts, detected by immunohistochemistry; **P<0.01.



Supplementary figure 7. Effect of chidamide on osteoblasts. (A) ALP staining (day 7) showed that chidamide slightly up-regulated the expression of ALP; Alizarin Red staining (ARS) of calcium deposits (day 21) showed that chidamide had no promotion nor inhibitory effect on osteoblast differentiation. (B) Q-PCR showed that chidamide could increased ALP gene expression (day 7); Alizarin Red staining (day 21, absorbance was measured at 570 nm) showed no significant difference between each group,

revealed that chidamide had no inhibitory effect on osteoblast. (C) The mRNA expression levels of OCN, Activin A and semaphorin were measured by q-PCR. OCN (day 21) was increased while Activin A was down-regulated (***P<0.001); semaphorin only showed a decreased trend (P>0.05).



Supplementary figure 8. Effect of chidamide on HDAC1, 2 and 3 expression. We performed western blot to checked the protein expression levels of HDAC1, HDAC2 and HDAC3 in chidamide treated OCs. HDAC3 and HDAC2 were decreased after incubated with chidamide while HDAC1 was not changed obviously.