Original Article
A novel silicone derivative of natural osalmid (DCZ0858) exerts anti-multiple myeloma activity by promoting cell apoptosis and inhibiting cell cycle and mTOR signaling

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Abstract: Multiple myeloma (MM) is a malignant disease characterized by abnormal proliferation of clonal plasma cells. Based on the organic drug osalmid, the novel small molecule compound DCZ0858 was designed and synthesized for treating MM. DCZ0858 inhibited the proliferation and activity of MM cells and reduced colony formation. It also promoted the apoptosis of primary cells from patients with MM and cultured MM cell lines but had little effect on peripheral blood mononuclear cells in healthy people. Simultaneously, DCZ0858 activated caspase family proteins, blocked MM cells in G0/G1 phase, and reduced the expression of related cyclins CDK4/6 and CyclinD1. Moreover, DCZ0858 overcame the protective effect of the bone marrow microenvironment and effectively inhibited the activity of mTORC1 and mTORC2. Further, xenograft model experiments in mice showed that DCZ0858 significantly inhibited the proliferation and growth of tumors, with low drug toxicity. These results indicate that DCZ0858 has marked anti-MM activity and little effect on normal cells and tissues, making it a new candidate clinical drug for the treatment of MM.

Keywords: Multiple myeloma, DCZ0858, mTOR, bone marrow microenvironment, cell proliferation, cell apoptosis

Introduction

Multiple myeloma (MM) is a malignant disease characterized by abnormal proliferation of clonal plasma cells. It is the second most common malignant tumor of the blood system [1], accounting for approximately 13% of hematologic malignancies and 1% of all tumors [2]. It is most common in middle-aged and elderly people and no curative treatments are available.

Traditional treatment for MM includes chemotherapy and hematopoietic stem cell transplantation. In the last 10 years, with the emergence of novel drugs such as the proteasome inhibitor bortezomib and immunomodulators thalidomide and lenalidomide, the complete response rate and overall survival rate of patients with MM have significantly improved. However, some limitations remain, such as low single drug efficiency, disease relapse, drug resistance, and side effects. The median survival time of patients with MM is only 6-7 years [3-5]. Therefore, new therapeutic drugs should be developed and tested for treating MM.

Previous studies showed that the interaction between MM cells and the bone marrow microenvironment induces the activation of multiple cell signaling pathways, such as JAK2/STAT3, MEK/ERK, and PI3K/AKT/mTOR, and promotes uncontrolled proliferation and drug resistance of MM cells [6-8]. Among these pathways, the PI3K/AKT/mTOR signaling pathway, activated by signaling factors such as interleukin (IL)-6, insulin-like growth factor (IGF)-1, and vascular endothelial growth factor (VEGF) in the bone marrow microenvironment, plays the most

important role in the survival and anti-apoptotic activity of MM cells [9-12].

To address these issues, we designed and synthesized a small molecule compound named as DCZ0858, a novel silicone derivative of the natural drug osalmid. The effect of DCZ0858 on the proliferation of MM cell lines was first assessed using a Cell Counting Kit-8 (CCK-8). Next, the effect of DCZ0858 on the proliferation of MM cells was evaluated by colony formation assay. The effect on the bone marrow microenvironment was verified by evaluating apoptosis. More specifically, flow cytometry was used to detect apoptotic cells and assess the cell cycle of MM cells, as well as to compare apoptosis induction between peripheral blood mononuclear cells (PBMCs) from healthy people and primary cells from patients with MM. The expression of apoptotic, periodic, and signaling pathway-related proteins was detected by western blotting. Finally, a xenograft tumor mouse model was established, and the weight of the mice was measured along with the volume and weight of the transplanted tumor. Hematoxylin-eosin (H&E) staining and immunohistochemistry were used to assess the effect of DCZ0858 on nude mice *in vivo*. Our findings will provide a foundation for evaluating the treatment potential of DCZ0858 for MM in future clinical trials.

Materials and methods

Cell culture

The ARP-1, U266, and OCI-MY5 cell lines were provided by Fenghuang Zhan (Department of Internal Medicine, University of Iowa, Iowa City, IA, USA). NCI-H929 and RPMI-8226 were purchased from the American Type Culture Collection (Manassas, VA, USA). Bone marrow samples from four patients with MM were isolated by lymphoid separation (Stemcell Technologies, Vancouver, BC, Canada), and primary CD138+ MM cells were obtained from the bone marrow by magnetic bead selection (Miltenyi Biotech, Gladbach Bergisch, Germany). Normal PBMCs were isolated from the peripheral blood of healthy people. Specimens were collected with the informed consent of each patient and healthy donor. This study was approved by the examination committee of Shanghai Tenth People's Hospital, Shanghai, China. The cells were cultured in RPMI1640

(Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% penicillin-streptomycin (Gibco). All cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

Reagents and antibodies

The Cell Counting kit-8 (CCK-8) was obtained from Shanghai Yeasen Biotechnology Co., Ltd. (Shanghai, China), and the BD Pharmingen™ Annexin V/propidium iodide (PI) Apoptosis Detection kit was obtained from BD Biosciences (Franklin Lakes, NJ, USA). The pan-caspase inhibitor Z-VAD-FMK was purchased from Selleck Chemicals (Houston, TX, USA). IL-6 and IGF-1 were obtained from R&D Systems (Minneapolis, MN, USA). The primary antibodies against cleaved caspase 3 (#9661, 1:1000), cleaved caspase 8 (#9496, 1:1000), p70S6K (#2708, 1:1000), p-p70S6K (#9205, 1:1000), 4E-BP1 (#9644, 1:1000), p-4E-BP1 (#9456, 1:1000), AKT (#2920, 1:2000), p-AKT (473; #4060, 1:2000), and mTOR (#2983, 1:3000) were obtained from Cell Signaling Technology (Danvers, MA, USA). Antibodies against caspase 9 (#ab202068, 1:1000), CDK4 (#ab-108357, 1:1000), CDK6 (#ab124821, 1:1000), Cyclin D1 (#ab134175, 1:1000), and p-mTOR (2481; #ab137133, 1:3000) were obtained from Abcam (Cambridge, UK).

Cell viability

MM cells were inoculated into 96-well plates and treated with DCZ0858 in the presence of established factors (IL-6 or IGF-1) for 48 h to investigate whether DCZ0858 could overcome the protective effect of the bone marrow microenvironment. Cell viability was determined using the CCK-8 kit according to the manufacturer's instructions.

Apoptosis detection

Cells were treated with DCZ0858 and/or Z-VAD-FMK (50 μM, Selleck Chemicals) for 48 h, and then stained with Annexin V/PI (BD Pharmingen) as previously described. Apoptotic cells included Annexin V +/PI + and Annexin V +/PI - cells.

Cell cycle analysis

MM cells were treated with DCZ0858 and collected at a predetermined time. The cells were

fixed with 75% ethanol overnight at -20°C. The next day, cells were treated with 300 µL PI/RNase staining buffer (BD Pharmingen) at 4°C for 30 min, and then analyzed by flow cytometry.

Western blotting

Total protein was extracted from the cells using radioimmunoprecipitation assay buffer, followed by separation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred onto nitrocellulose membranes and blocked with 5% fat-free powdered milk at room temperature for 1 h. The membranes were incubated with primary antibodies overnight at 4°C, followed by incubation with the appropriate fluorophore-conjugated secondary antibodies (1:2000) at room temperature for 1 h. The blots were probed using an Odyssey two-color infrared laser imaging system (LI-COR Biosciences, Lincoln, NE, USA).

Tumor xenograft model

Six BALB/C nude mice (age: 6 weeks; weight: 17-20 g) were purchased from the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). The mice were maintained in an air-conditioned room at 24°C with a 12 h light/dark cycle and 45% relative humidity. Mice had free access to water and food. After the mice acclimatized for 3-5 days, H929 human MM cells (2.5×10^6 cells) were suspended in 100 µL serum-free culture medium and then subcutaneously injected into the right subscapular region, near the armpit. When tumors were measurable, the mice were randomly and equally divided into control and DCZ0858 groups. In the DCZ0858 group, mice were administered 30 mg/kg DCZ0858 (dissolved in 10% dimethyl sulfoxide, 20% Tween-80, and saline), whereas in the control group, mice were administered 100 µL vehicle (10% dimethyl sulfoxide, 20% Tween-80 and saline) each day for 16 consecutive days. Tumor size and mouse body weight were measured every day, and the tumor volume was calculated as $4\pi/3 \times (width/2)^2 \times (length/2)$. At the end of treatment, mice were sacrificed by cervical dislocation. H&E, TUNEL, and Ki67 staining of the tumor tissue sections were performed. The animal studies were approved by the institutional review board of the Shanghai Tenth People's Hospital (ID: SYXK 2018-0034).

Statistical analysis

All experiments were repeated three times. Differences were evaluated using a Student's t-test for two samples, or one-way analysis of variance for multiple samples, using SPSS v 20.0 (SPSS, Inc., Chicago, IL, USA). The threshold for significance was $P < 0.05$.

Results

DCZ0858 inhibits the proliferation of MM cells

The chemical structure of DCZ0858 is shown in **Figure 1A**. First, we treated MM cell lines (H929, ARP-1, OCI-MY5, RPMI-8226, U266) with DCZ0858 at different concentrations (0, 5, 10, 20, 40 µM) for 48 h (**Figure 1B**). With increasing concentrations of DCZ0858, the inhibition of cell proliferation was enhanced. The half-maximal inhibitory concentrations (IC_{50}) were 11.2 µM (H929), 2.9 µM (ARP-1), 2.8 µM (OCI-MY5), 6.3 µM (RPMI-8226), and 4.2 µM (U266). H929 and ARP-1 were selected for the next series of experiments. H929 cells were treated with DCZ0858 (0, 5, 10, 20, 40 µM) for 24, 48, and 72 h (**Figure 1C**). The results demonstrated that the inhibitory effect of DCZ0858 on cell proliferation was time- and concentration-dependent. **Figure 1D** was obtained using the same method as used for the ARP-1 cell line. Subsequently, we used the control and DCZ0858 (15 µM) groups to evaluate the effects of DCZ0858 on colony formation of H929 and ARP-1 cells. The colony formation of MM cells in the DCZ0858 group was significantly inhibited (**Figure 1E**), which is consistent with the previous experimental results (**Figure 1C**, **1D**).

DCZ0858 promotes the apoptosis of MM cells

Induction of apoptosis in tumor cells can disrupt their dynamic balance and is effective for anti-tumor therapy. To determine whether DCZ0858's inhibitory effect on the proliferation of MM cells was caused by the induction of apoptosis, we examined the effect of DCZ0858 on the apoptosis of MM cells. After treating H929 and ARP-1 cells with DCZ0858 at different concentrations (0, 10, 20, and 40 µM) for
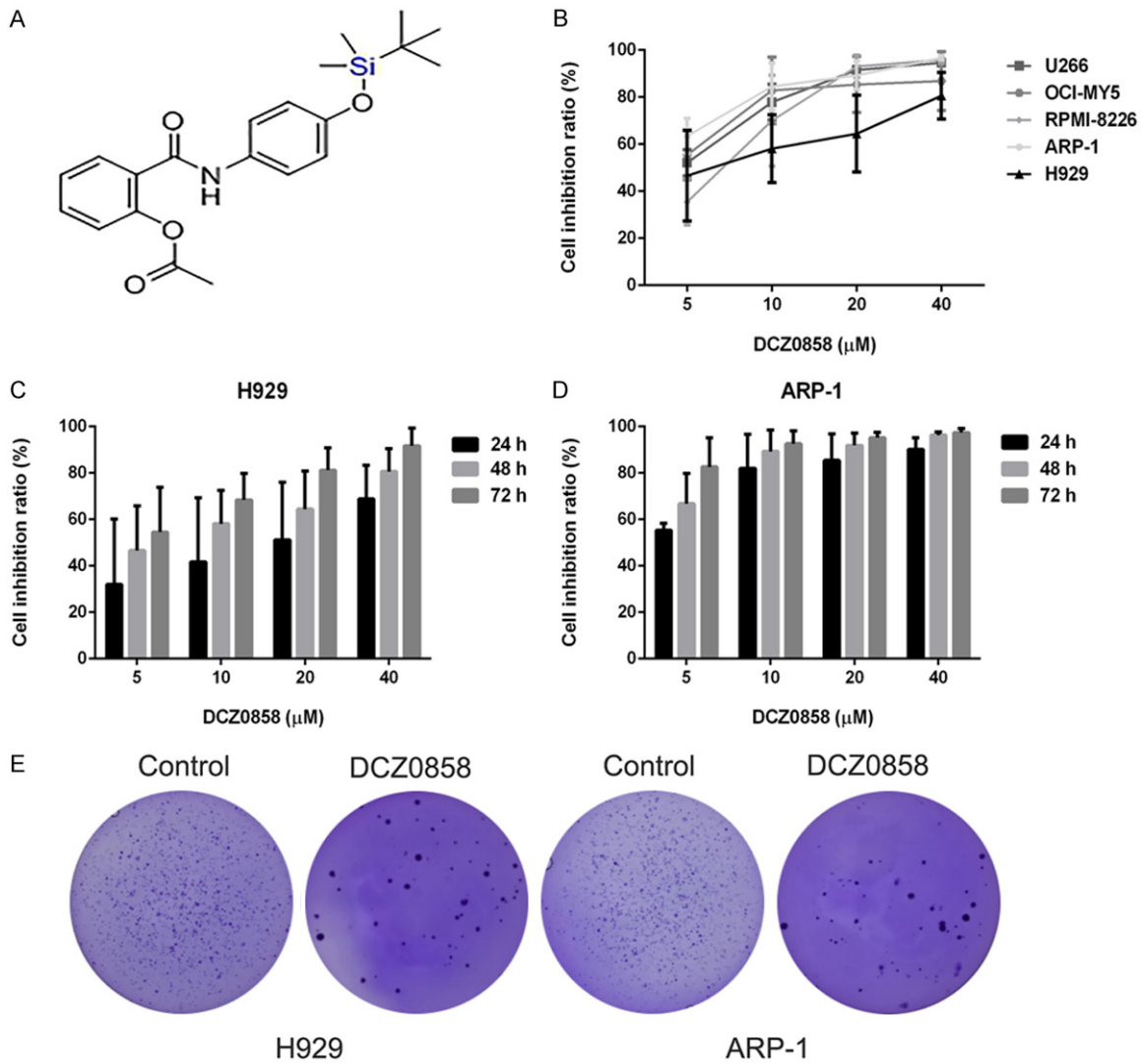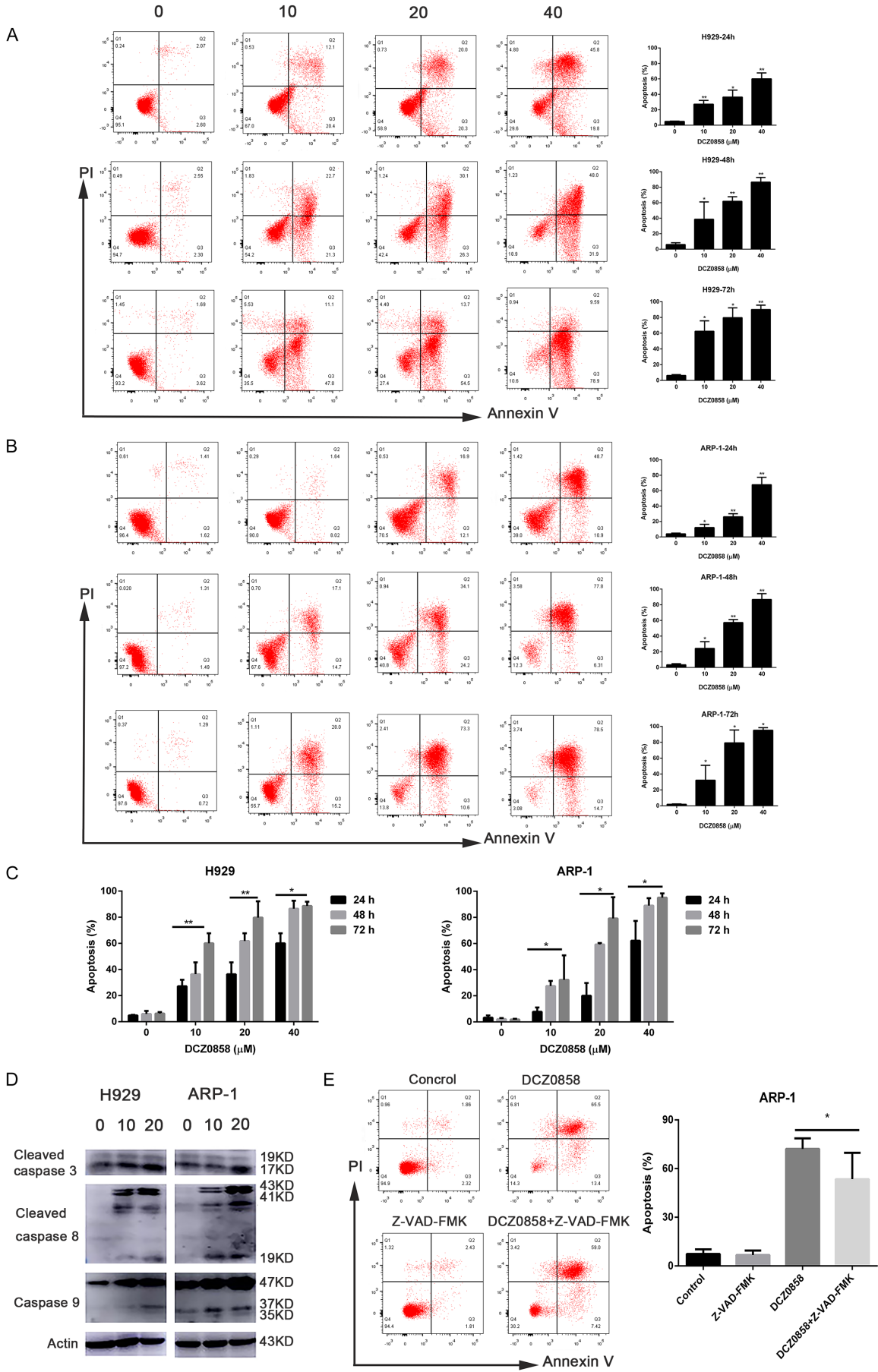
Figure 1. DCZ0858 inhibits the proliferation of multiple myeloma (MM) cells. A. Synthesis and chemical structure of DCZ0858. B. MM cells (H929, ARP-1, RPMI-8226, OCI-MY5, and U266) were treated with DCZ0858 at doses of 0, 5, 10, 20, and 40 µM for 48 h. C. H929 cells were treated with DCZ0858 (5, 10, 20, and 40 µM) for 24, 48, and 72 h. D. ARP-1 cells were treated with DCZ0858 (5, 10, 20, and 40 µM) for 24, 48, and 72 h. E. Representative images of overall colony formation of H929 and ARP-1 cells treated with DCZ0858.

24, 48, and 72 h, flow cytometry analysis revealed that DCZ0858 significantly induced the apoptosis of H929 and ARP-1 cells. This effect was enhanced with increasing time and concentration (**Figure 2A**, **2B**). The effect of DCZ0858 on the apoptosis of MM cells was time- and concentration-dependent (**Figure 2C**). In addition, western blot analysis was performed to detect changes in the protein levels of cleaved caspase-3, cleaved caspase-8, and caspase-9 after treatment with DCZ0858 at different concentrations (0, 10, 20 µM) for 48 h. The results showed that DCZ0858 activated

the caspase family proteins (**Figure 2D**). Additionally, the pan-caspase inhibitor Z-VAD-FMK inhibited the effect of DCZ0858 on ARP-1 apoptosis (**Figure 2E**). These results suggest that the pro-apoptotic effect of DCZ0858 is at least partially dependent on the caspase pathway. CD138+ cells isolated from four bone marrow samples of patients with MM were divided into a control group and DCZ0858 group (0 and 15 µM) and treated for 48 h. PBMCs isolated from four healthy volunteers were treated in the same manner. The results revealed that DCZ0858 induced apoptosis of primary MM
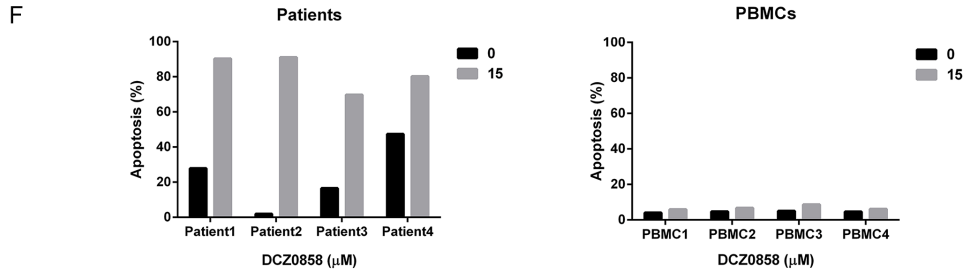
DCZ0858 inhibits multiple myeloma

Figure 2. DCZ0858 promotes the apoptosis of multiple myeloma (MM) cells. (A) H929 and (B) ARP-1 cells were treated with DCZ0858 (0, 10, 20, and 40 μM) for 24, 48, and 72 h and analyzed by flow cytometry after double-staining with Annexin-V/propidium iodide (PI). The results of the three replicates were consistent. (C) Correlation analysis of apoptotic MM cells from (A) and (B). (D) Western blot analysis of apoptosis-related proteins. (E) ARP-1 cells incubated with or without pan-caspase inhibitor Z-VAD-FMK (50 μM) were treated with DCZ0858 (40 μM) for 48 h, followed by Annexin V/propidium iodide (PI) analysis by flow cytometry. (F) Apoptosis in CD138$^+$ primary MM cells, separated and collected from four patients with MM, and PBMCs from four healthy volunteers, both treated with DCZ0858 for 48 h. Values are the means ± S.D., based on triplicate measurements; *$P < 0.05$ and **$P < 0.01$.

cells but caused little damage to normal cells (**Figure 2F**).

DCZ0858 blocks the G0/G1 phase of the MM cell cycle

Considering the close relationship between the cell cycle and cell proliferation, we conducted the following relevant experiments. H929 and ARP-1 cells were divided into a DCZ0858 blank group and DCZ0858 dosing group (0 and 15 μM). The cells were treated for 6 h and then analyzed by flow cytometry. DCZ0858 blocked the MM cell cycle in G0/G1 phase (**Figure 3A**). The cells were collected and subjected to western blot analysis. The results revealed that DCZ0858 reduced the levels of G0/G1 phase-related proteins CDK4, CDK6, and Cyclin D1 (**Figure 3B**), which is consistent with the results of flow cytometry detection.

DCZ0858 inhibits the mTOR signaling pathway and overcomes the protective effect of the bone marrow microenvironment

Next, we evaluated the effect of DCZ0858 on signaling pathways known to influence the survival of MM cells, such as the PI3K/AKT/mTOR signaling pathway [13]. After treatment with different concentrations of DCZ0858 for 48 h, the expression of p-p70S6k, p-4E-BP1, p-AKT (S473), and p-mTOR (S2481) in H929 and ARP-1 cells was significantly reduced with increasing doses (**Figure 4A**). As the mTOR pathway is

known to be inhibited by DCZ0858, we further evaluated whether DCZ0858 could overcome the protective effect of the bone marrow microenvironment on MM cells. MM cells were cultured with cytokines (IL-6 and IGF-1) to simulate the bone marrow microenvironment and then treated with DCZ0858. Although both cytokines effectively improved the viability of MM cells, DCZ0858 was significantly cytotoxic towards H929 and ARP-1 cells (**Figure 4B**).

DCZ0858 shows anti-MM activity in xenograft mice

To investigate whether DCZ0858 is also effective *in vivo*, we established a subcutaneous xenograft nude mouse model. Compared with the control group, the transplanted tumor was significantly smaller in the 30 mg/kg drug treatment group, whereas there was no significant difference in body weight between the two groups during the treatment period (**Figure 5A-D**). This confirms that DCZ0858 had a significant inhibitory effect on tumor growth in the nude mouse MM xenograft model. Further, the drug was well-tolerated by the nude mice.

After 16 days of treatment, the mice were sacrificed by cervical decompression, and tumor tissues and vital organs were stained with H&E. Compared with the control group, tumor necrosis was clearly observed in the drug treatment group (**Figure 5E**). In addition, there was no significant difference in H&E-stained sections of
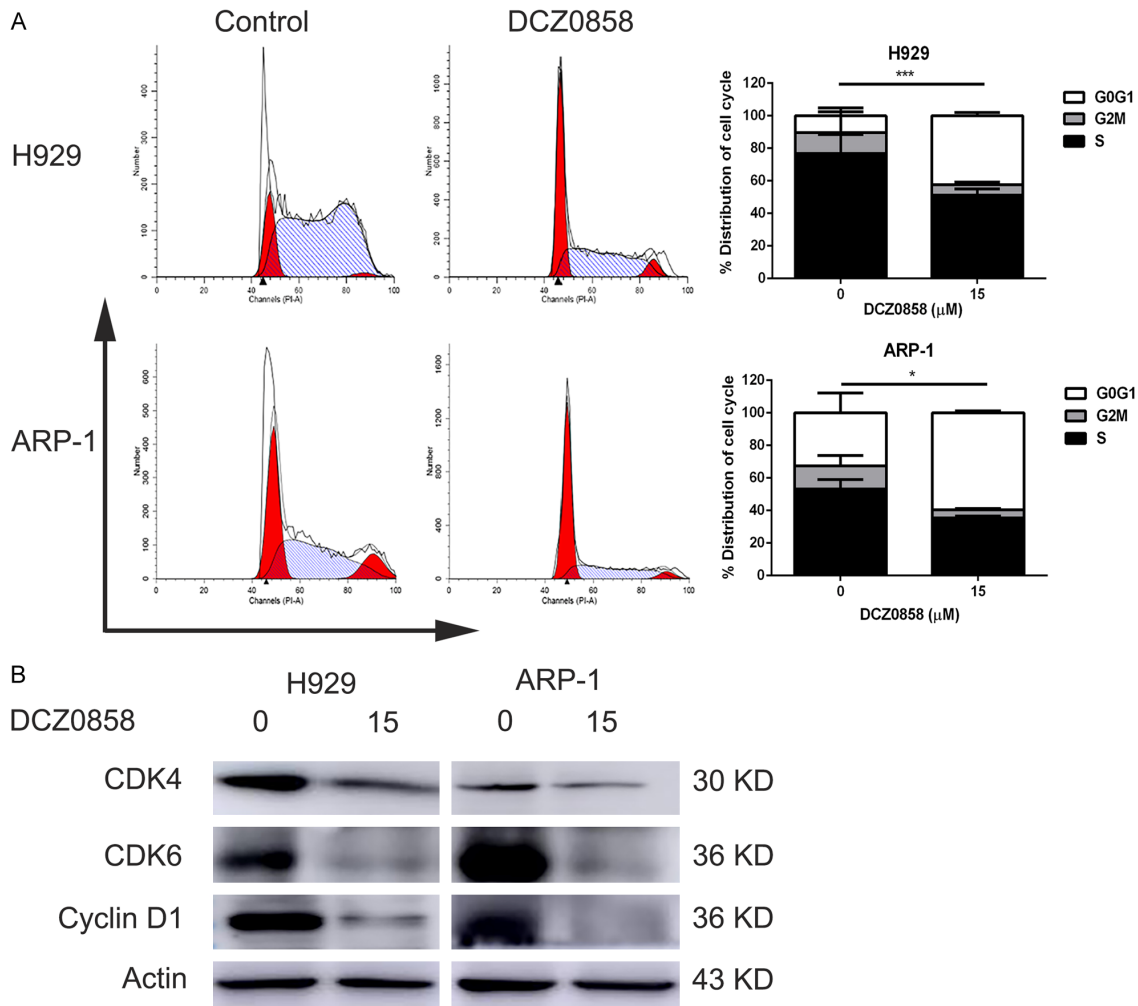
Figure 3. DCZ0858 blocks the G0/G1 phase of the multiple myeloma (MM) cell cycle. A. H929 cells and ARP-1 cells were treated with DCZ0858 (0 and 15 μM) for 6 h and analyzed with flow cytometry. B. Western blot of G0/G1 phase-related proteins in MM cells treated with DCZ0858 for 4 h. Values are the means ± S.D. of three replicates; *P < 0.05, ***P < 0.001.

the liver and kidney tissues between the two groups (**Figure 5F**), indicating that DCZ0858 did not cause damage to normal tissues.

Immunohistochemical analysis of tumor specimens was then performed. Compared with the control group, the expression of TUNEL in tumor tissues from the DCZ0858 treatment group was significantly higher, suggesting that DCZ0858 promotes the apoptosis of subcutaneously inoculated MM tumor cells. Further, we found that expression of the proliferation marker Ki67 in tumor tissues from the DCZ0858 treatment group was significantly reduced, indicating that DCZ0858 inhibited the proliferation of subcutaneously inoculated MM tumor cells (**Figure 5E**).

Discussion

MM is a B-cell malignancy and the second most common hematologic malignancy [1], accounting for 13% of all hematologic malignancies and 1% of all tumors [2]. The disease is often accompanied by typical clinical complications, including bone destruction, renal impairment, anemia, and hypercalcemia [14]. In a search for an alternative treatment, we synthesized the new compound DCZ0858 based on the natural drug osalmid using drug design technology. We then explored its application for treating MM.

We found that DCZ0858 significantly inhibited the proliferation of MM cells and reduced their clonal colony formation. We investigated wheth-
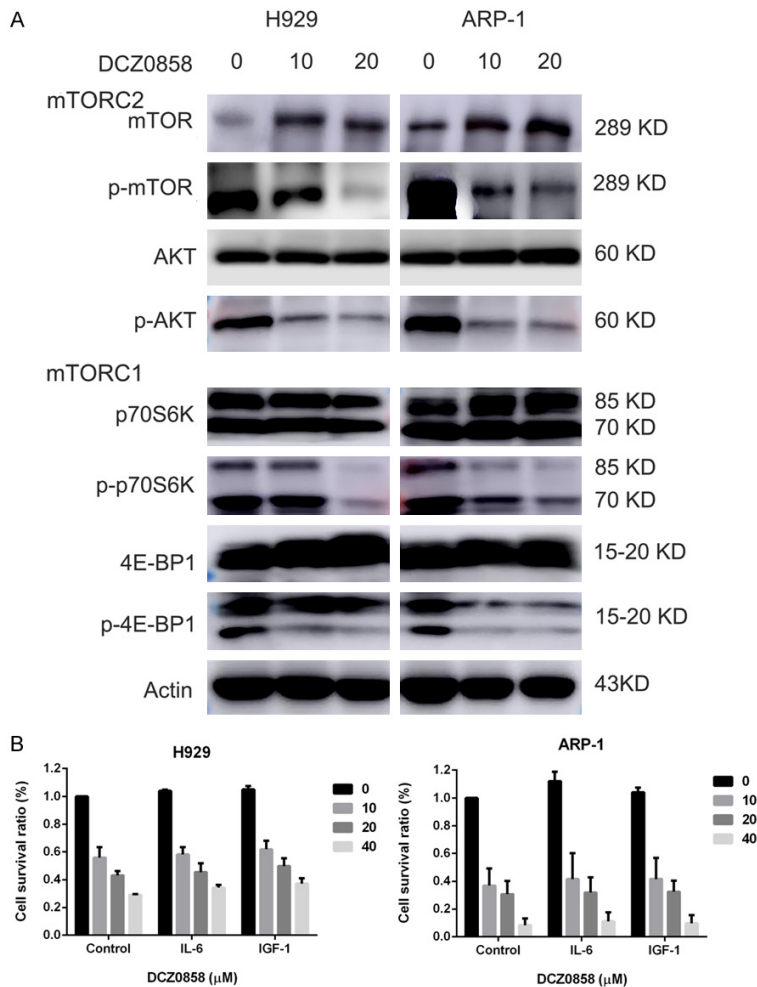
Figure 4. DCZ0858 inhibits the mTOR signaling pathway and overcomes the protective effect of the bone marrow microenvironment. A. Expression of mTOR signaling pathway-related proteins in H929 and ARP-1 cells. B. H929 and ARP-1 cells were treated with DCZ0858, DCZ0858+IL-6 (25 ng/mL), and DCZ0858+IGF-1 (25 ng/mL) for 48 h. Values are the means ± S.D., based on triplicate measurements.

ation is inhibited by blocking the cell cycle, thus hindering the occurrence and development of tumors [15, 16]. Here, cell cycle experiments revealed that DCZ0858 inhibited the expression of cell cycle regulatory proteins (Cyclin D1, CDK4, CDK6), which was consistent with the flow cytometry results showing that DCZ0858 blocked the cell cycle in G0/G1 phase.

Among the pathways involved in the proliferation and drug resistance of MM cells, the PI3K/AKT/mTOR signaling pathway, activated by signaling factors such as IL-6, IGF-1, and VEGF in the bone marrow microenvironment, plays the most important role in promoting survival and anti-apoptotic activity of MM cells [9-12]. mTOR is a key component of the pathway, forming two different protein complexes, namely mTOR complex 1 (mTORC1) and 2 (mTORC2) [17, 18]. The best markers of mTORC1 activity are phosphorylated p70S6K and 4E-BP1, and the best marker of mTORC2 activity is phosphorylated AKT (Ser473) [19]. Recent studies have shown that the activity of mTORC2 can also be monitored by evaluating phosphorylated mTOR (Ser2481) [20].

In this study, we found that DCZ0858 effectively downregulated the expression of proteins related to the mTOR signaling pathway [16]. In addition, DCZ0858, which blocked mTOR signaling, showed anti-MM activity in cultured MM cells and primary MM cells, as well as in the MM xenograft model. By adding IL-6 or IGF-1 to simulate the bone marrow microenvironment, followed by CCK-8 detection, DCZ0858 overcame the protective effect of the bone marrow microenvironment on MM cells. This further verified the effectiveness of the anti-MM activity of DCZ0858.

er DCZ0858 can induce the apoptosis of tumor cells, thereby disrupting their dynamic balance. The apoptosis-inducing effect of DCZ0858 on MM cells was time- and concentration-dependent. Enhanced expression of the apoptotic proteins cleaved caspase-3, cleaved caspase-8, and caspase-9 also demonstrated activation of the apoptotic signaling pathway. These observations suggest that the anti-tumor effect of DCZ0858 was at least partially dependent on the caspase signaling pathway. The results of the pan-caspase inhibitor Z-VAD-FMK rescue experiment supported this conclusion.

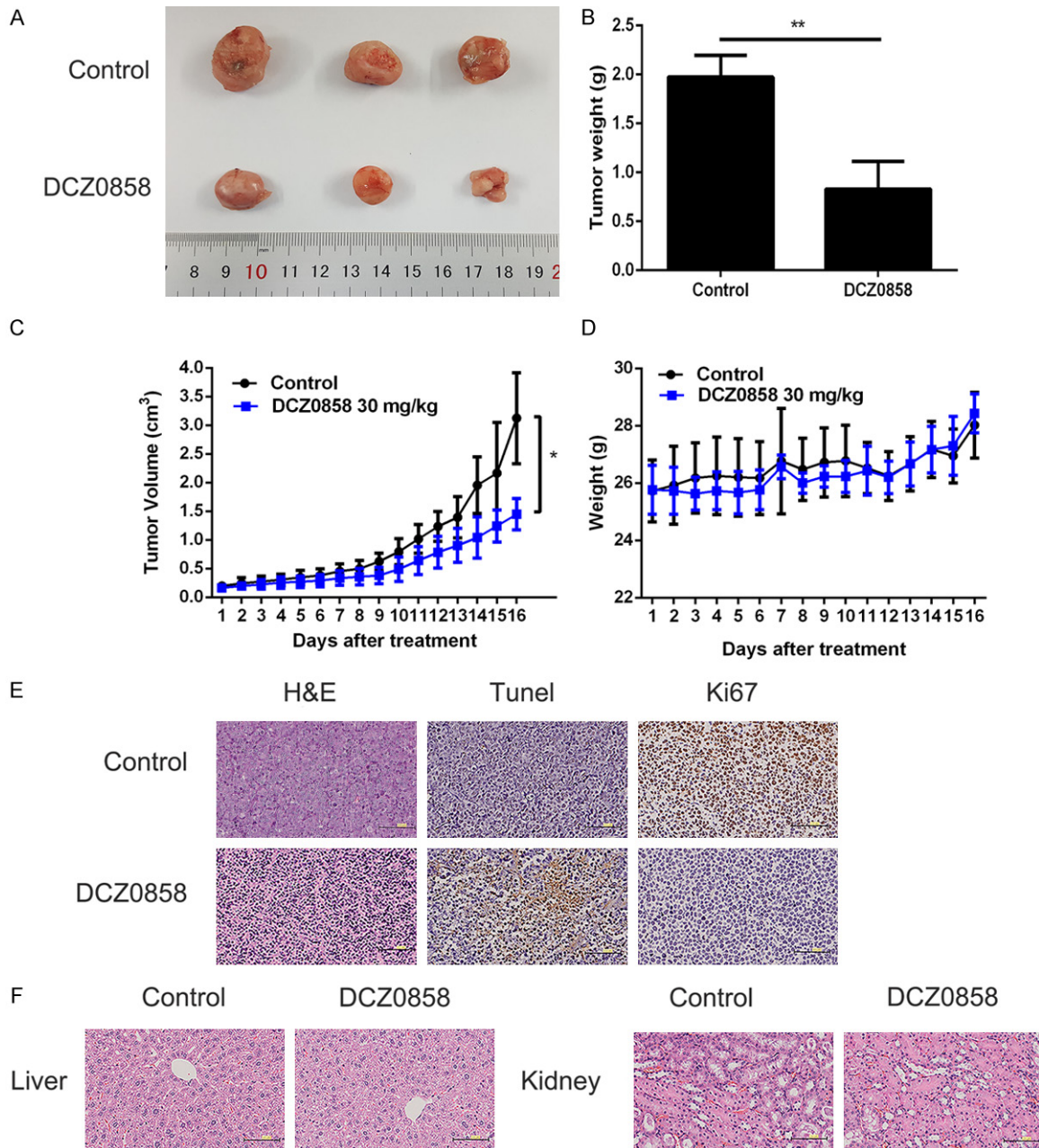Anticancer drugs currently in use often target the cell cycle of tumor cells. Tumor cell prolifer-

Figure 5. DCZ0858 shows anti-multiple myeloma (MM) cell activity in xenograft mice. A. Tumor samples were collected and imaged using a digital camera. B. Weights of tumors were measured after the photographs were taken. C. Tumor volume was measured each day for 16 days. D. Weight of mice was measured each day for 16 days. E. H&E, TUNEL, and Ki67 antibody staining of tumor tissues from control or DCZ0858-treated mice (100 ×). F. Representative H&E staining of liver and kidney (100 ×). *$P < 0.05$ and **$P < 0.01$.

Low toxicity is very important in drug development [21]. Therefore, we tested the cytotoxicity of DCZ0858 in normal cells and tissues. We found that 15 µM DCZ0858 induced significant apoptosis in MM cells from patients but did not affect the proliferation of normal cells. DCZ0858 at 30 mg/kg effectively inhibited tumor growth but did not cause weight loss or liver/kidney damage in nude mice. Therefore, our *in vitro* and *in vivo* data also preliminarily demonstrate the safety of DCZ0858 in MM treatment.

In summary, we found that DCZ0858 can overcome the protective effect of the bone marrow microenvironment and block mTOR signal

transduction, and that it has anti-MM activity in cultured MM cells, primary MM cells in human patients, and a mouse MM xenograft model. Further, the effective dose had no effect on normal cells, and mice showed good tolerance to this drug. Based on these observations, clinical testing and application of DCZ0858 for treating MM should be evaluated.

Disclosure of conflict of interest

None.

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