ORIGINAL RESEARCH

DCZ0814 induces apoptosis and G0/G1 phase cell cycle arrest in myeloma by dual inhibition of mTORC1/2

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Purpose: The present study investigates the effect of DCZ0814 in multiple myeloma (MM) cells, and determines the molecular mechanism of its antitumor activity against MM.

Methods: The effects of DCZ0814 were evaluated in vitro using human MM cell lines (ARP1 and OCI-MY5) and in vivo in a murine xenograft MM model. Cell viability was measured with the CCK-8 assay and mitochondrial membrane potential (MMP) was assessed with the JC-1 dye. Apoptosis and cell cycle distribution were examined by flow cytometry. Inhibition of mTORC1 and mTORC2 was assessed by western blot analysis, and the synergistic effect of DCZ0814 and known MM drugs was assessed by calculating the combination index value, using the CalcuSyn software.

Results: DCZ0814 effectively inhibited proliferation in MM cells, an effect that was associated with the induction of apoptosis, G0/G1 cell cycle arrest, MMP reduction and reactive oxygen species (ROS) generation. Meanwhile, DCZ0814 repressed the mTOR signaling via dual mTORC1/C2 inhibition and overcame the protective effect of the bone marrow (BM) microenvironment in myeloma cells. In addition, co-treatment with DCZ0814 and other anti-MM agents induced synergistic effects. Finally, the efficacy of the DCZ0814 treatment was confirmed in an MM xenograft mouse model.

Conclusion: DCZ0814 exhibits potent anti-MM activity and abrogates the activation of the mTOR/Akt signaling pathway mediated by the BM stroma-derived cytokines. Our results provide a theoretical basis for the development of novel therapeutic strategies in MM using DCZ0814 as a natural product combination compound.

Keywords: DCZ0814, multiple myeloma, mTOR, bone marrow microenvironment, apoptosis

Introduction

Multiple myeloma (MM) is a heterogeneous B-cell malignancy characterized by the accumulation of clonal plasma cells in the bone marrow.[1] The incidence rate has increased significantly along with the aging world population and MM has now become the second most common hematological malignancy.[2] Over the past decade, the introduction of novel treatments, including proteasome inhibitors (bortezomib, carfilzomib and ixazomib), immunomodulators (thalidomide, lenalidomide and pomalidomide), and autologous stem cell transplantation has remarkably improved the survival of patients with MM.[3,4] Nevertheless, this disease remains incurable in many cases, and the development of novel and effective therapeutics is urgently needed.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that exists in two distinct protein complexes, mTORC1 and mTORC2, and functions as a key

regulator of cell differentiation, proliferation, and survival.[5,6] mTORC1 is composed of mTOR and three associated proteins, raptor, mLST8, and PRAS40 and regulates p70S6K and 4E-BP1, which activate protein translation and ultimately cell growth and proliferation.[7,8] mTORC2, which contains mTOR, mLST8, mSin1 and rictor is required for the phosphorylation of Akt at Ser473 and modulates cell survival and cytoskeletal dynamics.[9,10]

MM is a complex multi-step process, in which the BM microenvironment supports the survival of myeloma cells and affects the response to therapeutic agents.[11] The dynamic interaction between tumor cells and the BM microenvironment, through signaling cascades activated by cytokines and growth factors, triggers the activation of downstream signaling including the PI3K/Akt, Ras/Raf/MEK/MAPK-, JAK/Stat-, NFκB- and Wnt pathways.[12] Although no mutations have been identified in the *PI3K/Akt* genes, this pathway is highly activated in the majority of patients with MM and regulates, through mTORC1/C2, protein expression and cytoskeletal organization, which contribute to cell survival and resistance to apoptosis in MM cells.[13] Therefore, novel anti-MM therapeutic regimens aim to target not only myeloma cells but also the interactions between MM and stromal cells.

In previous studies, osalmide has been reported to potently suppress ribonucleotide reductase activity in treating drug-resistant chronic hepatitis B virus infection, and pterostilbene has been demonstrated in both solid and non-solid tumors.[14–16] In the present study, we investigated the effect of the novel natural product combination DCZ0814 (osalmide, pterostilbene and proline) on MM cells, and found that it has potential antitumor activity in MM cells. DCZ0814 effectively induced cytotoxicity in MM cells, at doses that were not cytotoxic to normal cells, and inhibited tumor growth in an MM xenograft model. In addition, we showed that simultaneous dual inhibition of mTORC1/C2 overcomes the protective effect of the BM niche, with a synergistic effect between DCZ0814 and bortezomib/panobinostat/dexamethasone, indicating a novel multi-target mechanism for DCZ0814.

Materials and methods
Cells and cell culture
The human MM cell lines ARP1, OCI-MY5, the bortezomib-sensitive MM cell line RPMI-8226 and the bortezomib-resistant cell line RPMI-8226/R5 were kindly provided by Fenghuang Zhan (Department of Internal Medicine, University of Iowa, Iowa City, IA, USA). NCI-H929, OPM2, WIL2-S and the bone marrow stroma cell (BMSC) line HS-5 were purchased from the American Type Culture Collection (Manassas, VA, USA). The bortezomib-resistant cell line NCI-H929/bortezomib was cultured in the presence of 40 nM bortezomib. Primary cells were obtained from MM patient BM samples separated by Ficoll-Hypaque density gradient centrifugation, and the bone marrow mononuclear cells (BMMCs) were then distinguished using human APC conjugated anti-CD138 microbeads (BioLegend, SanDiego, CA, USA). Peripheral blood mononuclear cells (PBMCs) were obtained from peripheral blood samples of healthy donors using lymphoprep (Stemcell Technologies, Vancouver, BC, Canada) by Ficoll-Hypaque density gradient centrifugation. Written informed consent was obtained from MM patients and healthy donors and conducted in compliance with the Declaration of Helsinki. This study was approved by the institutional review board of the Shanghai Tenth People's Hospital, Tongji University.

The human MM cell lines, CD138$^+$ MM cells, PBMCs and WIL2-S were cultured in RPMI-1640 medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS; Gibco, BRL, USA) and 1% penicillin-streptomycin (PS; Gibco, Carlsbad, CA, USA) at 37 °C, 5% carbondioxide. Human BMSC line HS-5 was cultured in DMEM/HIGH GLUCOSE medium (Gibco, Carlsbad, CA, USA) containing 10% FBS and 1% PS at 37 °C, 5% carbondioxide. Cell lines were authenticated by Short Tandem Repeat profiling (Shanghai Biowing Applied Biotechnology Co., Ltd., Shanghai, China).

Reagents
DCZ0814 (methyl ((4-(3,5-dimethoxystyryl)phenoxy) (4-(2-hydroxybenzamido)phenoxy)phosphoryl)-L-prolinate) was synthesized by the Shanghai Institute of Materia Medica (Chinese Academy of Sciences, Shanghai, China). Bortezomib, panobinostat and dexamethasone were purchased from Sigma–Aldrich (St. Louis, MO, USA). IL-6 and IGF-1 were obtained from R&D Systems (Minneapolis, MN, USA).

Cell viability
Cell viability was determined using a Cell Counting Kit-8 (CCK-8) colorimetric assay (Yeasen Biotechnology Co., Ltd, Shanghai, China). To detect whether DCZ0814 can

overcome the protective influence of the BM niche, MM cells were cultured with DCZ0814 alone or in the presence of HS-5 or cytokines (IL-6 or IGF-1) for 48 h. Half maximal inhibitory concentration (IC_{50}) values and combination index (CI) were measured by using CalcuSyn software, Version

2.0. The CI was calculated by using the Chou-Talalay equation: $CI=(D)1/(Dx)1+(D)2/(Dx)2+(D)1(D)2/(Dx)1(Dx)2$, in which $(Dx)1$ and $(Dx)2$ are the doses of drug 1 and drug 2 alone, and $(D1)$ is the dose of drug 1 in combination, and $(D2)$ the dose of drug 2 in combination.[17] Where CI values <1 indicated synergism; CI values equal to 1 indicated an additive effect; and CI values >1 indicated antagonism.

Apoptosis analysis

MM cells were treated with DCZ0814 and/or Z-VAD-FMK (50 μM, Selleckchem, Houston, TX, USA) for 48 h and stained with Annexin V-FITC/PI (BD Pharmingen, Franklin Lakes, NJ, USA). Apoptotic cells were identified as Annexin V^+/PI^- (early apoptotic) and Annexin V^+/PI^+ (late apoptotic) cells.

Cell cycle analysis

MM cells were treated with DCZ0814 for indicated time, harvested and fixed with 70% ice-cold ethanol overnight at −20 °C. After washing twice with cold PBS, the cells were incubated with 300 μL of staining solution (10 μg/mL PI and 5 U/mL RNaseA, BD Pharmingen, Franklin Lakes, NJ, USA) at 4 °C for 30 min and analyzed by the BD FASCanto II flow cytometry (BD Biosciences, San Jose, CA, USA). Results were analyzed with the ModFitLT 3.2 software (Verity Software House, Inc., Topsham, ME, USA).

Western blotting

MM cells were treated, harvested, and lysed on ice for 30 min in lysis buffer (100 mM Tris-HCl [pH 6.8], 4% SDS, 20% glycerol). The protein samples were separated by SDS-PAGE in 8–12% polyacrylamide gels, transferred to nitrocellulose membranes and blocked with 5% non-fat dry milk or 5% bovine serum albumin at room temperature for 1 h, and then probed with the relevant primary antibodies overnight at 4 °C . Subsequently, the membranes were probed with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h at room temperature. Protein bands were visualized using the Odyssey two-color infrared laser imaging system (LI-COR Biosciences, Lincoln, USA).

Primary antibodies for specific detection of cleaved caspase-3, cleaved caspase-8, caspase-9, AIF, Cyto C, B-cell lymphoma 2 (Bcl-2)-associated X protein (Bax), Bcl-2, Bcl-extra large (Bcl-xL), Akt, phosphorylated (p)-Akt (Ser473), p-p70S6K (Thr389), p70S6K, p-4E-BP1 (Ser65), 4E-BP1, p-mTOR (Ser2481), mTOR and β -actin were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Antibodies against Cyclin D1, cyclin-dependent kinase 4 (CDK4), CDK6, p-Chk2 and cdc25A were from Abcam (Cambridge, UK).

Reactive oxygen species (ROS) levels

MM cells were treated with DCZ0814 (15 μM) and/or pre-treated with the active oxygen scavenger NAC (10 mM) for 2 h, and then incubated for 48 h. After treatment, the cells were incubated with 10 μM carboxy-2′7′-dichlorodihydrofluorescein diacetate (H_2DCFDA; Thermo Fisher Scientific, Lnc.) in serum-free medium at 37 °C for 30 min using Rosup (50 μg/ml) as a positive control, and analyzed by flow cytometry.

Mitochondrial membrane potential (MMP) analysis

The mitochondrial depolarization occurring during apoptosis was detected by flow cytometry using a JC-1 kit (Beyotime Institute of Biotechnology, Shanghai, China). ARP1 cells were treated with DCZ0814 (5, 10, 15 or 20 μM) for 48 h, and then collected, washed, and incubated at 37 °C for 15 to 30 mins in the presence of the JC-1 working solution. Finally, cells were suspended in warm PBS and analyzed on a flow cytometer with 488 nm excitation.

Xenograft

BALB/c nude mice (4–6 week of age and weighting 17–20 g) were purchased from the Shanghai Laboratory Animal Center (SLAC, Shanghai, China). All mice were maintained under a 12h light/dark cycle in an air-conditional room at 24 °C and 45% relative humidity, provided water and food, and allowed to acclimatize for a minimum of one week. Human ARP1 cells ($2.5×10^6$) in 100 μL serum-free culture medium were subcutaneously injected into the upper flank of nude mice. When the tumors were measurable, mice were randomly divided into control (vehicles [5% DMSO and saline only]-treated) group and treatment (10 mg/kg DCZ0814 in 5% DMSO and saline) group. Mice were injected

intraperitoneally with vehicles or DCZ0814 for 19 days. The tumor volume was monitored every two days and calculated as (length×width^2) ×0.5. At the end of the treatment, the mice were sacrificed by CO_2 asphyxiation and the tumors were obtained and imaged. Hematoxylin-eosin (HE), Ki-67, TUNEL and cleaved-caspase 3 staining were performed. The study protocols for animal experiments were approved by the Animal Care and Use Committee of Tongji University (Shanghai, China) and the institutional review board of the Shanghai Tenth People's Hospital (ID: SYXK 2011–0111). All animal-related procedures in vivo were complied with Regulations for the Administration of Affairs Concerning Experimental Animals (Order No. 2; Ministry of Science and Technology 1988).

Statistical analysis

Data are presented as the mean ± standard deviation (SD). Statistical analysis was conducted using the unpaired two-tailed Student's t-test or one-way analysis of variance followed by least-significant difference test for multiple comparisons. All statistical analyses were performed using the SPSS version 20.0 software (IBM Corp., Armonk, NY, USA). $p < 0.05$ was considered to reflect a statistically significant difference.

Results

DCZ0814 exerts potent cytotoxicity against MM cells and overcomes the protective effect of the BM microenvironment

DCZ0814 is a natural small-molecular compound with molecular weight of 658.6 (Figure 1A). To examine whether DCZ0814 has anti-MM activity, we evaluated its effect on MM cell growths using a CCK-8 assay. As shown in Figure 1B, a dose-dependent growth inhibition was observed in all MM cell lines after the treatment with DCZ0814 (2.5–40 μM) for 48 h. The calculated IC_{50} values were as follows: 11.2 μM (ARP1), 7.92 μM (OCI-MY5), 13 μM (NCI-H929), 9.5 μM (NCI-H929/bortezomib), 16.03 μM (RPMI-8226), 26.8 (RPMI-8226/R5) and 12.64 μM (OPM2). The IC_{50} for bortezomib-sensitive and –resistant cell lines indicated that treatment with DCZ0814 was able to overcome bortezomib resistance. Additionally, we observed that DCZ0814 induced a dose- and time- dependent cytotoxicity in ARP1 and OCI-MY5

cells (Figure 1C). These findings suggested that DCZ0814 inhibits the proliferation of myeloma cells.

Because the BM supports myeloma cells survival and chemoresistance, we investigated whether DCZ0814 could overcome the protective effect of the BM microenvironment on MM cells. The BM microenvironment was mimicked by culturing myeloma cells in the presence of cytokines (IL-6 and IGF-1) or by direct co-culture with BMSCs (HS-5), using RPMI-1640 medium as a control. We found that cytokines and HS-5 effectively promoted the growth of MM cells but did not counteract the potent cytotoxicity of DCZ0814 in both ARP1 and OCI-MY5 cells (Figure 1D and E). As shown in Figure 1F and G, DCZ0814 could induce myeloma cells apoptosis in the presence of BMSCs. In contrast, DCZ0814 treatment did not cause cytotoxicity and induce apoptosis in BMSCs (Figure 1E–G).

DCZ0814 induces apoptosis in MM cells

To explore the mechanism of DCZ0814 cytotoxicity, MM cells treated with DCZ0814 at the indicated concentrations were analyzed by Annexin-V/PI staining and flow cytometry. In accordance with the data of the CCK-8 assays, ARP1 and OCI-MY5 cells showed a dose-dependent increase in apoptosis after treatment with DCZ0814 (Figure 2A and B). In addition, we also found that treatment of ARP1 cells with DCZ0814 significantly decreased MMP in a dose-dependent manner (Figure 2C and D), indicating that DCZ0814 was capable of activating the intrinsic apoptosis pathway.

Next, we evaluated ROS generation following DCZ0814 treatment in MM cells by flow cytometry. In ARP1 cells, ROS levels in the DCZ0814 group were significantly increased (3392±181.8) compared with the control group (1343.67±122.21), and Rosup (1562.67±83.7) was used as a positive control. Treatment with DCZ0814 (15 μM) for 48 h markedly increased ROS production in OCI-MY5 cells as well. Meanwhile, the ROS scavenger NAC could partially abrogate DCZ0814-induced apoptosis in ARP1 and OCI-MY5 cell lines (Figure 2E and F) and the pan-caspase inhibitor Z-VAD-FMK significantly blocked DCZ0814-induced apoptosis as well (Figure 2G and H). The effect of DCZ0814 on human B lymphocytes WIL2-S cells and PBMCs from healthy donors were also analyzed. All concentrations showed no apparent apoptosis in WIL2-S and no cytotoxic effect on PBMCs (Figure 2I and J), while all patients were sensitive to DCZ0814 treatment (Figure 2K), suggesting that DCZ0814 has a selective cytotoxicity in MM and normal
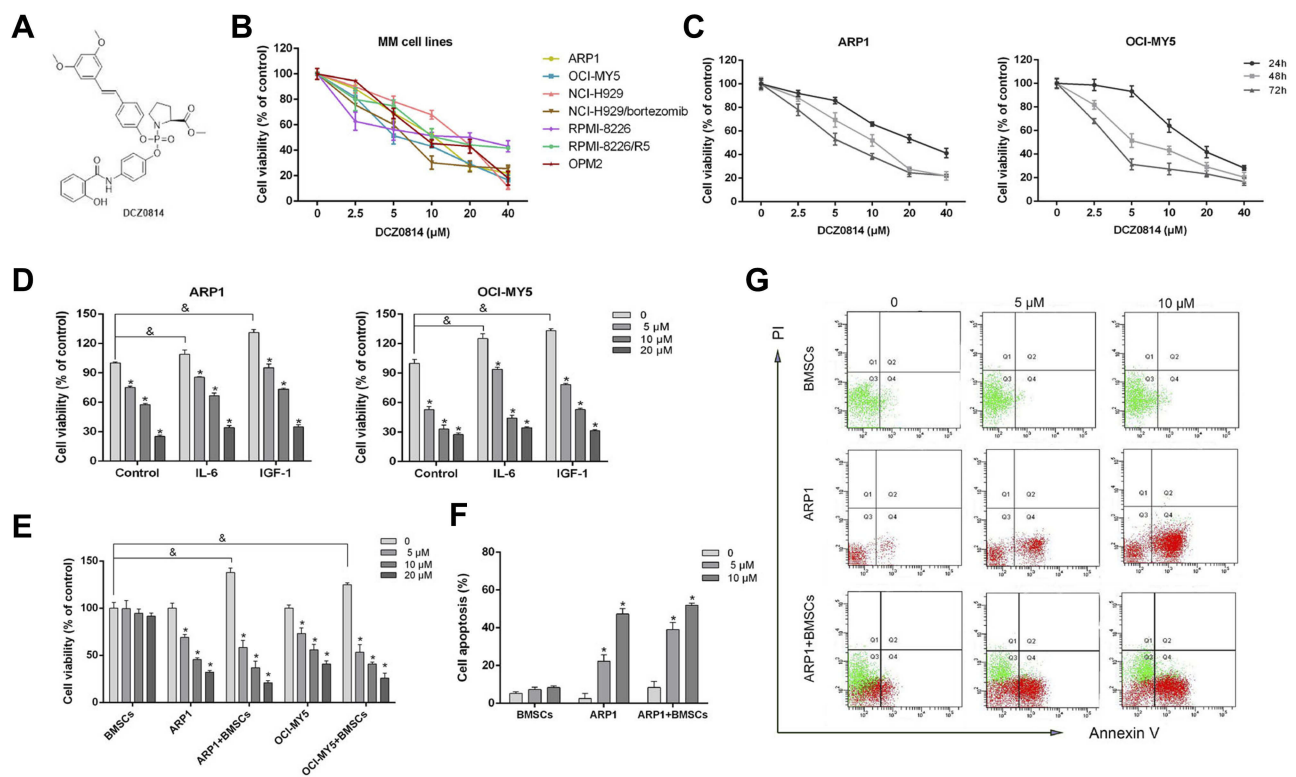
Figure 1 DCZ0814 induced cytotoxicity in MM cells and overcome the protective effect of the BM microenvironment. (**A**) Molecular structure of DCZ0814. (**B**) MM cells treated with DCZ0814 for 48 h and cell viability determined by CCK-8 assay. (**C**) MM cells treated with DCZ0814 for the indicated time and cell viability determined by CCK-8 assay. (**D** and **E**) Cell viability of ARP1 and OCI-MY5 cells treated with DCZ0814 alone or in the presence of IL-6 (25 ng/mL), IGF-1 (25 ng/mL) or BMSCs (HS-5) for 48 h. *$p<0.05$ compared with the 0 μM group, $^{\&}p<0.05$. (**F**) The percentage of Annexin-V positive cells. *$p<0.05$ compared with the 0 μM group. (**G**) BMSCs, ARP1, or both ARP1 and BMSCs cells were treated with DCZ0814 for 48 h, and apoptosis was detected by Annexin-V/PI staining followed by flow cytometry. Green represents BMSCs HS-5 cells, and red represents ARP1 cells. In each case, data are presented as the mean ± SD of triplicate treatments.
Abbreviations: MM, multiple myeoma; BM, bone marrow; CCK-8, cell counting kit-8; IL-6, interleukin-6; IGF-1, insulin-like growth factor-1; BMSC, bone marrow stromal cell.

cells. In addition, western blot analysis further confirmed that DCZ0814 dose-dependently activated the caspase pathway, as indicated by the increasing cleavage of caspase 3, 8, 9, as well as the upregulation of Bax, AIF, Cyto C and down-regulation of Bcl-2 and BCL-XL (Figures 2L and 4A).

Treatment of MM cells with DCZ0814 results in G0/G1 phase arrest

The anti-proliferative activity of DCZ0814 was further investigated by evaluating the cell cycle distribution of MM cells treated with DCZ0814 (5, 10, or 20 μM). DNA content analyses revealed that DCZ0814 caused a time- and dose-dependent accumulation of cells in the G0/G1 phase, while the fraction of cells in the S and G2/M phase decreased (Figure 3A and B). Western blot analysis of the expression of the relevant cell cycle regulatory proteins following DCZ0814 treatment of ARP1 and OCI-MY5 cells for 24 h clearly showed downregulation of cyclinD1, CDK4, CDK6 and cdc25A, while the phosphorylation of Chk2 was upregulated (Figure 3C).

DCZ0814 inhibits both the mTORC1 and mTORC2 signaling pathways

To identify the potential target(s) of DCZ0814 and explore the underlying molecular mechanism in DCZ0814-induced apoptosis, we first examined the effects of DCZ0814 on mTORC1 and the phosphorylation of its downstream effectors 4E-BP1 and p70S6K by western blot analysis. Additionally, the investigation of the mTORC2 signaling pathway showed that the levels of mTOR phosphorylated at Ser2481 (a marker of mTORC2 activation) decreased, leading to the decrease of the phosphorylation of Akt (Ser473) (Figure 4A). In the present study, DCZ0814 was able to suppress the phosphorylation of mTOR (Ser2481) and Akt (Ser473), and the downstream Bcl-2, Bcl-xL and Bax were also observed, indicating that DCZ0814 may exhibit potent apoptotic effect on MM cells due to the suppression of mTORC2/Akt activity. Meanwhile, it was also demonstrated that treatment of MM cells with DCZ0814 reduced p70S6K and 4E-BP1 phosphorylation, indicating that DCZ0814 inhibits the proliferation and growth
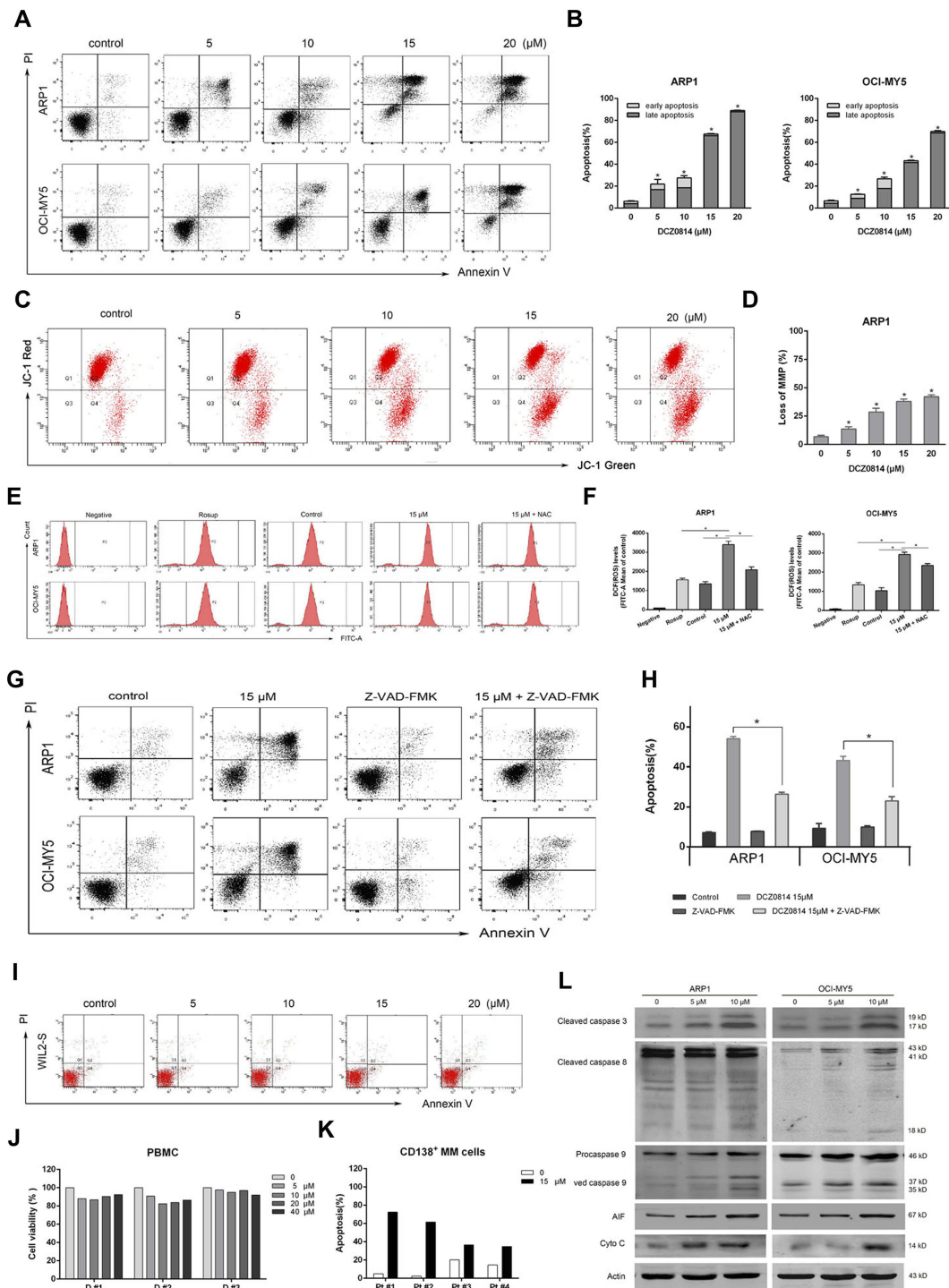
Figure 2 DCZ0814 treatment resulted in apoptosis of MM cells. (**A** and **B**) Apoptosis analyzed using Annexin V/PI staining and flow cytometry. Apoptotic cells include early apoptotic cells (Annexin V^+/PI^-) and late apoptotic cells (Annexin V^+/PI^+). (**C**) ARP1 cells were treated with DCZ0814 (0–20 µM) for 48 h. MMP was analyzed by flow cytometry using JC-1 dye. Only JC-1 green positive (lower right quadrant) cells were analyzed for the loss of MMP. (**D**) Columns represent the average percent of only JC-1 green positive cells from three independent experiments. (**E**) MM cells were pre-incubated and/or NAC (10 mM) for 2 h and then treated with DCZ0814 (15 µM) for 48 h, stained with AnnexinV-FITC/PI and analyzed by flow cytometry; and (**F**) the percentage of FITC positive cells treated with 15 µM of DCZ0814 that pre-incubated and/or NAC, while Rosup (50 µg/ml) was used as a positive control. (**G**) MM cells exposed to DCZ0814 (15 µM) and/or pan-caspase inhibitor Z-VAD-FMK (50 µM) for 48 h and analyzed by Annexin V/PI staining and flow cytometry. (**H**) Statistical analysis of Annexin-V positive cells in (**G**). (**I**) Human B lymphocytes WIL2-S cells were treated with DCZ0814 (0–20 µM) for 48 h and analyzed by flow cytometry. (**J**) PBMCs obtained from three healthy volunteers were treated with DCZ0814 for 48 h, and cell viability was then measured. (**K**) Primary CD138$^+$ MM cells from patients were treated with DCZ0814 for 48 h, and apoptosis was then detected. (**L**) Western blot of the expression of apoptosis-related proteins. In each case, the untreated cells cultured in their growth medium were used as a control. Data are presented as the mean ± SD of three independent experiments and *p<0.05 compared with the 0 µM group.
Abbreviations: MMP, mitochondrial membrane potential; NAC, N-acetyl- L –cysteine; PBMCs, peripheal blood mononuclear cells; PI, propidium iodide; D, healthy donor; Pt, patient.
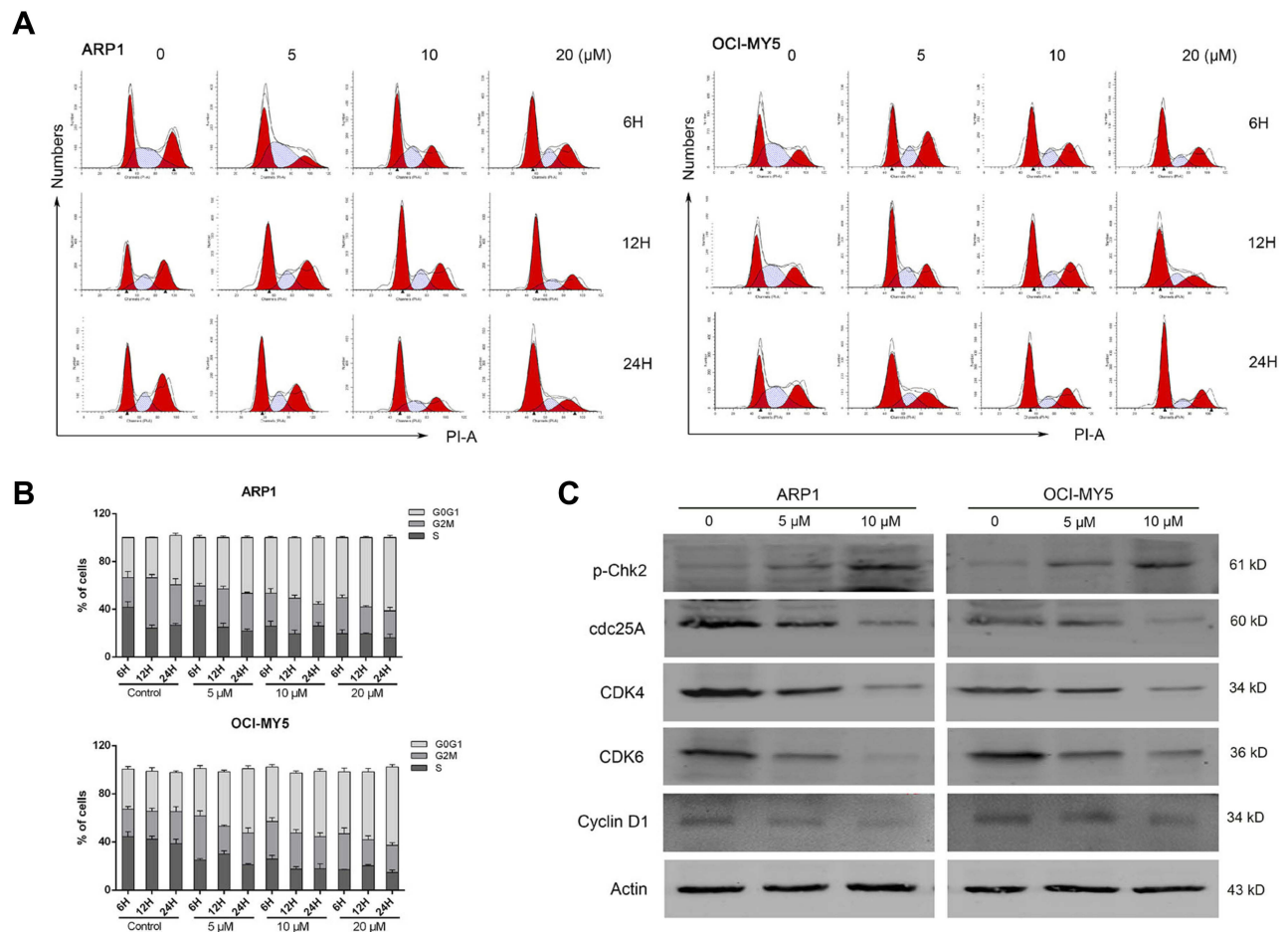
A

B

C

Figure 3 DCZ0814 caused G0/G1 arrest in MM cells. (**A**) Cell cycle analysis of MM cells treated with DCZ0814. The representative result of three repetitions is presented. (**B**) Cell cycle distribution in MM cells. Bar graphs show the percentage of cells in the G0/G1, S and G2/M phases of the cell cycle. (**C**) Western blot of the expression of G0/G1 phase-related proteins in MM cells treated with DCZ0814 for 24 h. In each case, the untreated cells cultured in their growth medium were used as a control. Data are presented as the mean ± SD (n=3).

of MM cells via mTORC1/p70S6K/4E-BP pathway (Figure 4B). In addition, western blot analysis showed that co-culturing BMSCs directly with myeloma cells activated the expression of mTORC1 and mTORC2, however, abrogated following by DCZ0814 treatment, including the phosphorylation of mTOR, Akt, p70S6K, and 4E-BP1 were decreased (Figure 4C).

DCZ0814 inhibits tumor growth in vivo

We further examined the growth-inhibitory effect of DCZ0814 in a MM xenograft model. DCZ0814 or vehicle were administered daily to mice by intraperitoneal injection for 19 days. DCZ0814 was found to effectively inhibit the growth of ARP1 xenografts: tumor size was significantly lower and tumor growth was obviously suppressed in the DCZ0814-treated group compared with vehicle controls (Figure 5A and B). The body weight of the mice was monitored during the treatment to evaluate potential side-effects caused by DCZ0814. As

shown in Figure 5C, DCZ0814 treatment had no significant effect on body weight, indicating that it was well tolerated. Immunohistochemical staining of resected tumors from mice was also performed (Figure 5D). HE staining showed that cell shrinkage and fragmentation increased upon DCZ0814 treatment, while, immunohistochemical staining showed that Ki-67 expression was downregulated compared with that in the vehicle group, whereas the number of TUNEL-positive cells and the cleavage of caspase-3 was upregulated by DCZ0814 treatment. These data demonstrated that DCZ0814 has a potent anti-MM activity in vivo.

Combination of DCZ0814 with bortezomib, panobinostat, or dexamethasone results in synergistic cytotoxicity in MM cells

To investigate whether DCZ0814 has a synergistic effect with known anti-MM agents, we treated MM cells (ARP1 and OCI-MY5) with both DCZ0814 and bortezomib, panobinostat or
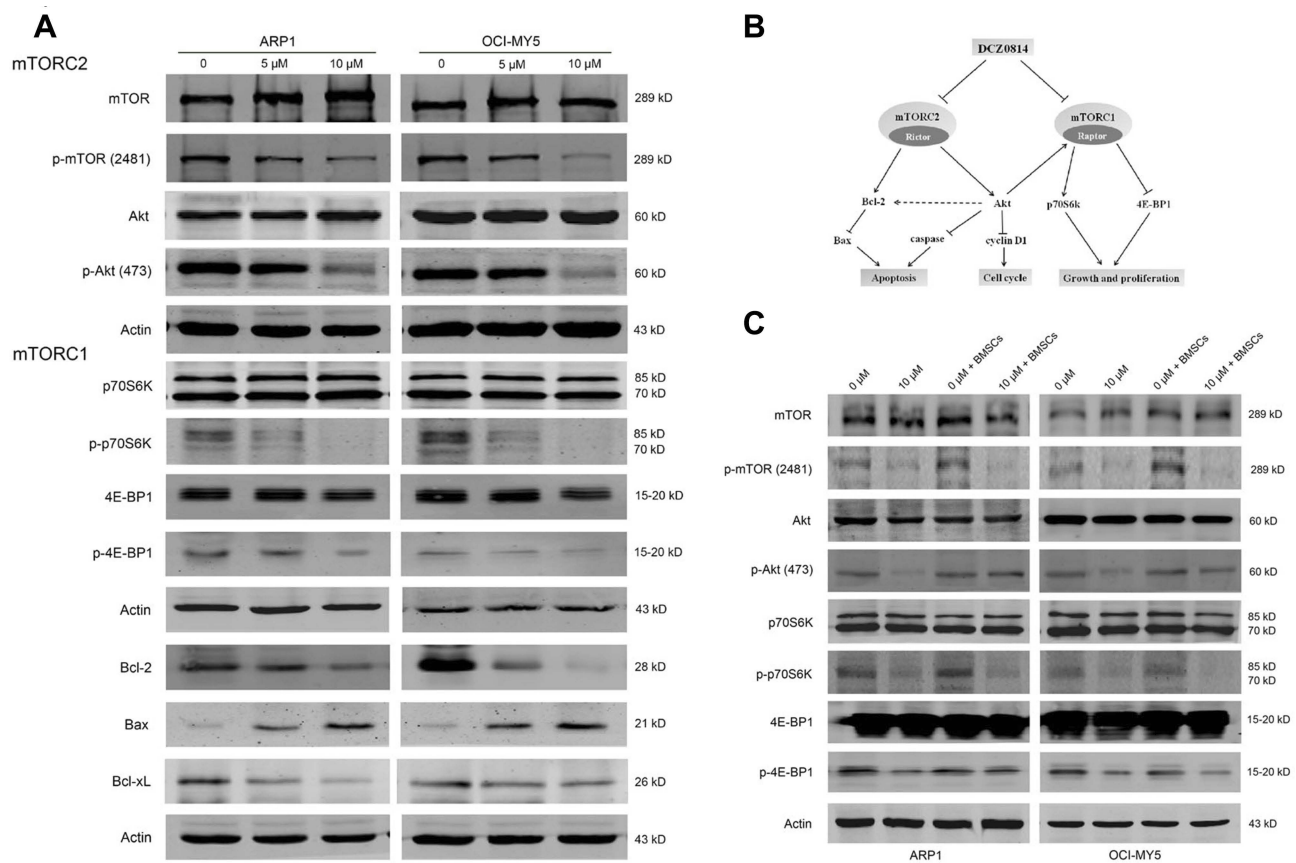
Figure 4 DCZ0814 inhibited mTOR kinase activity. (**A**) Expression of mTOR kinase activity-related proteins in ARP1 and OCI-MY5 cells treated or not with DCZ0814 for 48 h from three independent experiments. (**B**) Diagram illustrating the effects of DCZ0814 on mTORC1 and mTORC2. (**C**) Expression of mTOR kinase activity-related proteins in myeloma cells treated with DCZ0814 alone or in the presence of BMSCs (HS-5) for 48 h from three independent experiments, with Actin used as an internal control.

dexamethasone. As shown in Figure 5E, G and I, the combination of DCZ0814 with these agents synergistically induced cytotxicity in MM cells. Additionally, median dose-effect analysis revealed that the CI values were all <1.0, indicating indeed a synergistic effect (Figure 5F, H and J).

Discussion

Osalmide is a traditional cholagogue in clinical practice, and pterostilbene, isolated from blueberries, is a dimethylether analogue to resveratrol and has been reported to have anti-inflammatory, antioxidant, antimicrobial, and cardiovascular protective effects.[18] In previous studies, we have shown that pterostilbene has anti-tumor activity in hematologic malignancies, including myeloma and lymphoma.[19,20] Osalmide has a similar effect (data not published). Following up on our previous work, we synthesized a novel compound, DCZ0814, which is composed of osalmide, pterostilbene and proline, and investigated its activity on myeloma cells, both in vitro and in vivo.

We found that DCZ0814 represses both mTORC1 and mTORC2 signaling pathways by inhibiting the activation of Akt and related proteins in myeloma cells, and inducing apoptosis and cell cycle arrest. Our data showed that DCZ0814 has a potent inhibitory effect on cultured MM cells, as well as on a MM xenograft model. Importantly, DCZ0814 effectively overcame the protective effect of the BM microenvironment. In addition, we tested the cytotoxicity of DCZ0814: in normal cells, it did not affect normal PBMCs at concentrations up to 40 μM; and in a mouse xenograft model, it did not reduce the body weight. Therefore, our in vitro and in vivo findings preliminarily support the favorable therapeutic effect of DCZ0814 in MM treatment.

The first generation of mTOR inhibitors, such as rapamycin and its analogs, inhibit mTORC1 activity, but the presence of a negative feedback loop (the mTORC1/S6K1/IRS/PI3K axis) modulates the upregulation of Akt, which further attenuates the BM microenvironment and the anti-
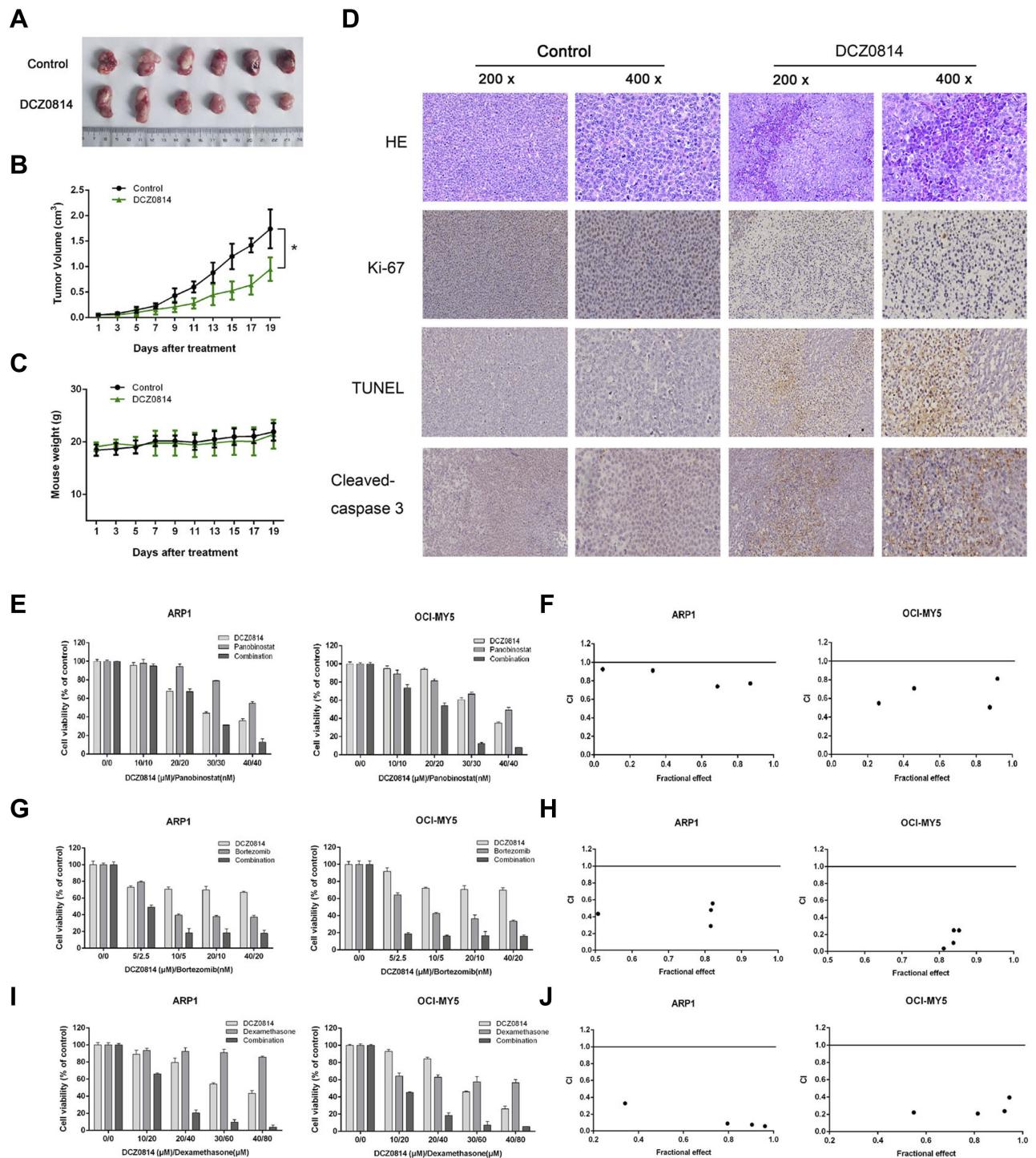
Figure 5 DCZ0814 exhibited anti-MM activity in vivo and synergized with novel and conventional anti-MM agents. (**A**) Tumors on day 19. (**B**) Tumor growth curve (n=6 tumors/group). Treatment with control (vehicles [5% DMSO and saline only]-treated) and DCZ0814 (10 mg/kg DCZ0814 in 5% DMSO and saline) began on day 0. (**C**) Body weight was measured daily for 19 days. At day 19 the difference between groups was not significant (p>0.05). Data are presented as the mean ± SD. *p<0.05. (**D**) Tumor sections were stained with HE, Ki-67, TUNEL or cleaved caspase-3. The positive cells in tumor sections stained with Ki-67, cleaved caspase-3 or TUNEL are the dark brown ones. (**E–J**) MM cells were treated with DCZ0814 plus bortezomib, panobinostat or dexamethasone for 48h. Cell viability was assessed by CCK-8 assay. Data are presented as mean ± SD. CI <1 indicates synergistic effects.
Abbreviations: HE, hematoxylin eosin; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end-labeling.

proliferative effect of rapamycin.[21–23] Increasing evidence indicates that targeting the mTOR signaling may provide an efficient strategy against MM.[24–26] It has been shown that

a dual mTORC1/C2 inhibitor has a more effective anti-MM activity than mTORC1 inhibition alone (through rapamycin), due to the feedback activation of Akt at Ser473, which

is a direct downstream target of mTORC2.[13,27,28] However, one of the weakness of this inhibitor is that it induces the upregulation of IGF-1 receptor phosphorylation in MM cell lines that leads to rescue myeloma cells from apoptosis despite mTOR kinase inhibition and TORC2/Akt blockage.[29] Given the fact that exogenous cytokines (IL-6, IGF-1, and VEGF) do reverse AZD8055 (a dual mTORC1/C2 inhibitor) -induced cell death and AZD8055 does not inhibit mTORC2 activity, there is a need to identify a novel dual mTORC1/C2 inhibitor that can target myeloma and overcome the BM microenvironment. In this study, we showed that DCZ0814 effectively inhibits the mTORC1 pathway, with downregulated expression of p-P70S6K and p-4E-BP1, which results in the inhibition of protein synthesis and cell proliferation.[30] Additionally, we showed that DCZ0814 inhibits also the mTORC2 signaling: our data show that DCZ0814 treatment markedly reduces the expression of p-mTOR (Ser2481) and p-Akt (Ser473) and prevents feedback loops by repressing mTORC2, and inhibiting cell proliferation. Thus, DCZ0814 is a potent candidate inhibitor whose use might be beneficial in the treatment of myeloma.

It is well established that the BM environment is crucial in myeloma pathogenesis, and its inter-relation with MM cells contributes to uncontrolled proliferation and chemoresistance.[12,13,31] Based on the functional importance of the MM-BM microenvironment, novel molecular targets and derived treatment regimens have been investigated in recent years.[32–35] In this study, DCZ0814 effectively exerted anti-MM activity by inhibiting cell proliferation and inducing apoptosis in cultured MM cells and in vivo tumors. More importantly, DCZ0814 repressed p70S6K/4E-BP1/Akt/mTOR phosphorylation simultaneously and induced myeloma cytotoxicity in the presence of IL-6, IGF-1, and HS-5, indicating that DCZ0814 regulates mTOR kinase activity in the BM microenvironment. Taken together, these data support ongoing studies on the molecular mechanism of mTORC1/2 dual inhibition between tumor cells and the BM microenvironment.

Combinational therapy is often required for relapsed and/or refractory patients because it can increase the effectiveness and optimization of standard therapy treatment and overcome chemoresistance.[36,37] Therefore, we detected whether DCZ0814 could enhance the cytotoxicity of several agents frequently used to treat hematological tumors. Our data indicated that the combination of DCZ0814 with bortezomib, panobinostat or dexamethasone induces synergistic cytotoxicity in MM cells. Thus, this study provides the rationale for a promising therapeutic strategy combing DCZ0814 and conventional anti-MM agents in clinical practice.

Toxicity should be also considered when developing therapeutic regimens. We tested the cytotoxicity of DCZ0814 in PBMCs, and found no apparent toxicity in normal cells. Additionally, our data showed that a 10 mg/kg dose of DCZ0814 inhibited tumor growth but did not affect the mice body weight. Consistent with our in vitro data, DCZ0814 has the advantage of safety in MM treatment. However, since the most common treatment-related adverse event concerning both first and second generation mTOR inhibitors is hyperglycemia, it is important to explore whether DCZ0814 has an effective hypoglycemia treatment in the future.

In summary, our study demonstrates that DCZ0814 exerts a potent antitumor activity by targeting mTORC1/C2 in vitro and in vivo. Interestingly, our study showed that the simultaneous repression of mTORC1 and mTORC2 overcomes the protective effect of the BM microenvironment. Together, our data provide a theoretical basis for the use of this inhibitor, either alone or in combination with other drugs, in MM treatment.

Abbreviation list

MM, multiple myeoma; BM, bone marrow; CCK-8, cell counting kit-8; IL-6, interleukin-6; IGF-1, insulin-like growth factor-1; BMSC, bone marrow stromal cell; MMP, mitochondrial membrane potential; NAC, N-acetyl- L – cysteine; PBMCs, peripheal blood mononuclear cells; PI, propidium iodide; HE, hematoxylin eosin; TUNEL, terminal deoxynucleotidy1 transferase dUTP nick end-labeling.

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Disclosure

The authors report no conflicts of interest in this work.

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