ORIGINAL ARTICLES

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Therapeutic effect and mechanism of ibrutinib combined with dexamethasone on multiple myeloma

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Ibrutinib is an irreversible inhibitor of Bruton's tyrosine kinase and has proven to be an effective agent for B-cell-mediated hematological malignancies, including multiple myeloma (MM). Several clinical trials of ibrutinib treatment combined with dexamethasone (DXMS) for relapsed MM have demonstrated high response rates, however, the mechanism still remains unclear. In this study, we explored the therapeutic effect and mechanism of ibrutinib combined with DXMS on MM *in vitro* and *vivo*. The apoptosis of MM cell lines and mononuclear cells from MM patients' bone marrow induced by ibrutinib combined with DXMS was detected by flow cytometry and the expression of apoptosis-related proteins were detected by Western blot. A mice MM model was established to verify the therapeutic effect of ibrutinib combined with DXMS on MM. We found that ibrutinib combined with DXMS increased the apoptosis of MM cell lines through the PI3K/PARP pathway, significantly reduced CD38 expression in MM cells from patients *in vitro*, and reduced tumor size and increased the survival time in mice model. This study provides a theoretical basis for the treatment of relapsed refractory MM with ibrutinib combined with DXMS, and a potential therapeutic target for MM clinical treatment.

1. Introduction

Multiple myeloma (MM), a B-cell hematologic malignancy characterized by abnormal infiltration of terminally differentiated plasma cells in bone marrow, is the second most common hematological malignancy after non-Hodgkin's lymphoma (Anderson and Carrasco 2011; Siegel et al. 2018). Although numerous therapeutic options have improved outcomes, relapse is frequent, and MM remains incurable with a 5-year survival rate of 40%. Therefore, novel treatments producing optimal outcomes are urgently needed (Dimopoulos et al. 2012).

Bruton's tyrosine kinase (BTK) is a B-cell receptor (BCR) signaling kinase expressed by various hematopoietic cells, including B-cell lymphomas and leukemias. Recent studies have shown that BTK is overexpressed on MM cells and implicated in their growth and survival (Liu et al. 2014). Moreover, BTK expression is correlated with poor prognosis and overexpression may contribute to the development of drug resistance in MM cells (Yang et al. 2015). Ibrutinib, an irreversible BTK inhibitor with excellent pharmacodynamics, is approved for the treatment of various B-cell malignancies in both the United States and the European Union. Clinical trials of ibrutinib treatment for relapsed mantle cell lymphoma, non-Hodgkin's lymphoma, and chronic lymphocytic leukemia have achieved high response rates. Ibrutinib has been found to inhibit tumor growth and improve MM-induced osteolysis in a murine model (Tai et al. 2012), and to be cytotoxic in malignant plasma cells of MM patients *in vitro* and to synergize with bortezomib and lenalidomide (Rushworth et al. 2013). Therefore, ibrutinib shows potential as a novel therapeutic approach for MM, targeting MM cells and the bone marrow microenvironment (Tai and Anderson 2012).

Corticosteroids are standard initial treatments in multiple diseases, with many of these showing rapid efficacy. Dexamethasone (DXMS), an important corticosteroid, has been reported to provide an initial rapid response in several days to weeks (Stasi et al. 1995). However, corticosteroid-induced complications have limited its efficacy as well as long-term and high-dose utility. Consequently, more and more studies have combined other drugs with DXMS to improve efficacy and achieve long-term applications (David et al. 2014; Bussel et al. 2014). In patients with relapsed/refractory MM, ibrutinib combined with DXMS has demonstrated encouraging activity (Richardson et al. 2018); however, the mechanism of action of this combination is unclear. In the present study, we explored the therapeutic effect and mechanism of ibrutinib combined with DXMS on MM *in vitro and in vivo*.

2. Investigations and results

2.1. Ibrutinib/DXMS complex induced MM cell apoptosis

To confirm the killing effect of ibrutinib combined with DXMS on MM cells, RPMI-8226 and U266 cells were treated with ibrutinib with or without DXMS. The apoptosis of cells was evaluated by Annexin V/PI using flow cytometry. As shown in Fig. 1, ibrutinib induced RPMI-8226 apoptosis (Fig. 1a and 1b) and U266 apoptosis (Fig. 1c and 1d) in a dose-dependent manner (in comparison with the control group). Although, DXMS did not induce apoptosis in either cell line, the combination of ibrutinib and DXMS significantly increased (compared with ibrutinib alone) RPMI-8226 apoptosis (Fig. 1a and 1b) and U266 apoptosis (Fig. 1c and 1d). These data indicate that ibrutinib-induced apoptosis in MM cell lines was significantly elevated by the addition of DXMS.

2.2. Ibrutinib/DXMS complex reduced the percentage of CD38⁺ cells and increased the apoptosis of CD38⁺ cells in bone marrow MNCs from MM patients

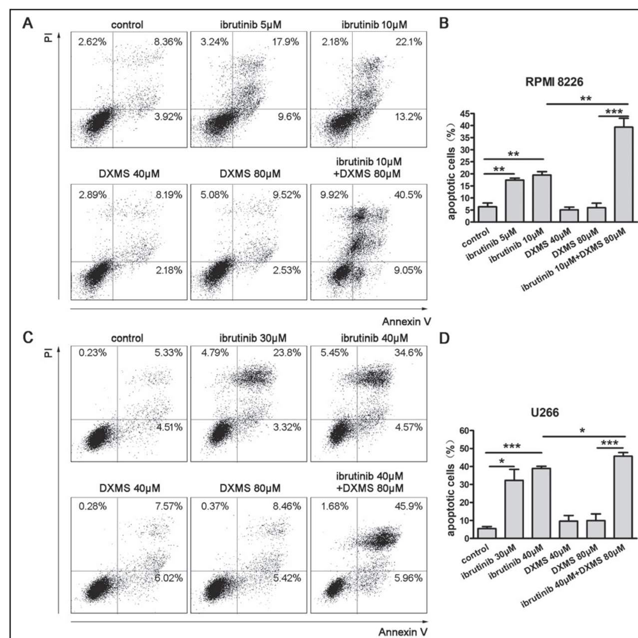

Fig. 1: Synergistic effects of ibrutinib and dexamethasone (DXMS) on the induction of cell apoptosis in MM cell lines. (A) The proportion of apoptotic RPMI-8226 cells was assessed by Annexin V-PI staining and flow cytometry after treatment with ibrutinib and/or DXMS. (B) The apoptotic RPMI-8226 cell proportion in different groups was statistically analyzed by *t* test. (C) The proportion of apoptotic U266 cells was assessed by Annexin V-PI staining and flow cytometry after treatment with ibrutinib and/or DXMS. (D) The apoptotic U266 cell proportion in different groups was statistically analyzed by *t* test. All experiments were performed three times independently. Annexin V and PI-positive cells were considered as apoptotic cells. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

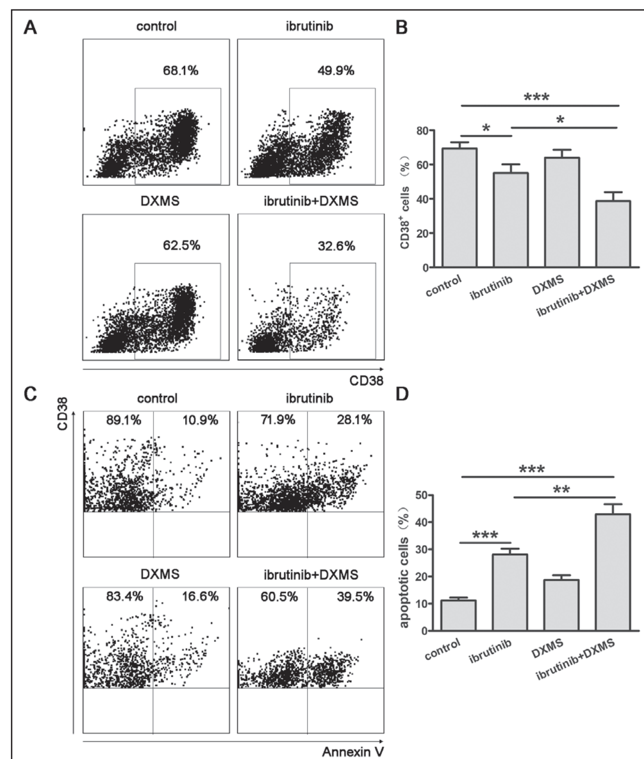

Fig. 2: Combining ibrutinib and dexamethasone (DXMS) decreased the percentage of $CD38^+$ cells and increased the apoptosis of $CD38^+$ cells in mononuclear cells (MNCs) from the bone marrow of patients with multiple myeloma (MM). (A) The percentage of $CD38^+$ cells in MNCs was detected by flow cytometry after treatment with ibrutinib and/or DXMS. (B) The proportion of $CD38^+$ cells in different groups was statistically analyzed by *t* test. (C) The percentage of $CD38^+$ cell apoptosis was assessed by Annexin V staining and flow cytometry after treatment with ibrutinib and/or DXMS. (D) The apoptotic proportion of $CD38^+$ cells in different groups was statistically analyzed by *t* test. The results shown are representative of three independent experiments. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

To confirm the effect of ibrutinib combined with DXMS on MM patient's primary cells, the bone marrow MNCs of MM patients were separated and treated with ibrutinib with or without DXMS. Following ibrutinib treatment, the percentage of $CD38^+$ cells in MNCs was obviously decreased compared with control cells, and the ibrutinib-induced decrease was further potentiated by the addition of DXMS (Fig. 2a and 2b).

Compared with the control group, the proportion of apoptotic cells was significantly increased after ibrutinib treatment of $CD38^+$ MNCs (Fig. 2c and 2d). Although DXMS did not induce apoptosis of $CD38^+$ cells, DXMS combined with ibrutinib resulted in a marked increase in apoptosis compared with ibrutinib alone or control group (Fig. 2c and 2d). These data indicate that ibrutinib-induced apoptosis of $CD38^+$ cells from bone marrow MNCs of MM patients was significantly elevated by the additional of DXMS.

2.3. Ibrutinib/DXMS complex suppressed PI3K and Bcl2 expression, and enhanced Bax and PARP expression

To investigate the mechanism of ibrutinib combined with DXSM to induce apoptosis of MM cells, RPMI-8226 and U266 cells were treated with ibrutinib with or without DXMS. After 24 h, total cell protein was extracted, and the expression of signal pathway proteins was assessed by western blot analysis. As shown in Fig. 3, ibrutinib, a known BTK inhibitor, can significantly inhibit the expression of BTK in both cell lines. Moreover, inhibition of BTK expression was more obvious following DXMS co-treatment. Although ibrutinib/DXMS co-treatment significantly suppressed Bcl-2 expression, co-treatment promoted Bax expression (compared with ibrutinib alone). These results are consistent with the trend showing increased apoptosis in both RPMI-8226 and U266 cells. In addition, although ibrutinib/DXMS co-treatment significantly suppressed PI3K expression, co-treatment promoted PARP cleavage (compared with ibrutinib alone). These data suggest that ibrutinib/DXMS co-treatment may regulate the apoptosis of MM cells through the PI3K/PARP pathways.

2.4. Ibrutinib and DXMS demonstrated synergistic anti-tumor activity in vivo

To test the anti-tumor effect of ibrutinib combined with DXMS *in vivo*, NOD/SCID mice were injected RMPI-8226 cells to build a MM mice model and treated with ibrutinib with or without DXMS. As shown in Fig. 4a, tumor size in the ibrutinib-treated group was

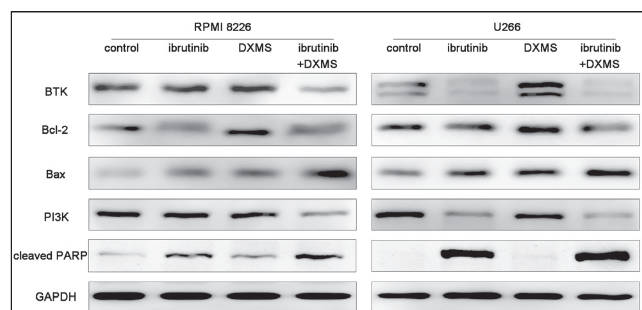

Fig. 3: The effect of ibrutinib and dexamethasone (DXMS) combination on the protein level of apoptosis-related genes. The expression of BTK, Bcl-2, Bax, PI3K, and cleaved PARP in RMPI 8226 and U266 cells was detected by western blot after treatment with ibrutinib and/or DXMS.

smaller than in the control group. Moreover, tumor size in the ibrutinib combined with DXMS group was considerably lower than that in the ibrutinib or DXMS monotherapy group, demonstrating the synergistic anti-myeloma effect of DXMS. In addition, ibrutinib combined with DXMS markedly prolonged the survival of MM mice (Fig. 4b). These results confirm that ibrutinib combined with DXMS demonstrates synergistic anti-tumor activity *in vivo*.

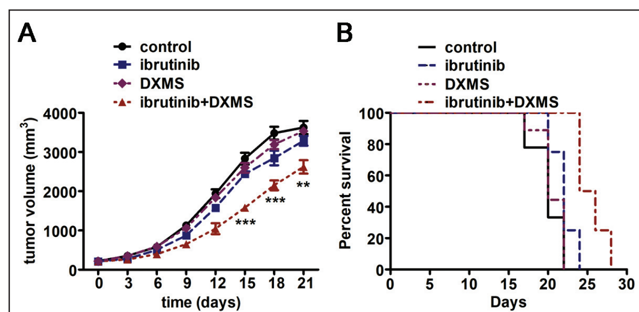

Fig. 4: Treatment of multiple myeloma (MM) mouse model with ibrutinib and dexamethasone (DXMS) combination. (A) Tumor diameters were measured and tumor volume in mm^3 was calculated in four groups. n=5, **P*<0.05, ***P*<0.01, ****P*<0.001. (B) A survival curve of MM mice treated with ibrutinib combined with DXMS obtained at the end of the experiment.

3. Discussion

In the present study, the results provide evidence of a synergistic effect of ibrutinib and dexamethasone (DXMS) on apoptosis in myeloma cells *in vitro* and *in vivo*. We found that ibrutinib induced apoptosis in RPMI-8226 and U266 MM cell lines, and that the apoptotic effect was enhanced by the addition of DXMS in both cell lines (even though DXMS only had no effect). The synergistic apoptotic effect of ibrutinib with DXMS combination was also demonstrated using bone marrow $CD38^+$ cells from MM patients. The additive inhibition effect of ibrutinib combined with DXMS was also observed in a mouse model of MM. Mechanistically, ibrutinib combined with DXMS markedly reduced the expression of PI3K, Bcl-2, and BTK, and significantly increased the expression of Bax, thus activating the caspase-3/PARP apoptotic pathway.

BTK is a key molecule in the BCR signaling pathway and plays an essential role in the development and differentiation of normal B cells. BTK deactivation or abnormal expression plays a major role in the development of various B-cell tumors, including chronic lymphocytic leukemia (CLL), mantle cell lymphoma, and MM. In the classical BCR signaling pathway, BTK activates PLCy2 resulting in the activation of the Ras/ERK, PKC/NF-κB, and IP3 signaling pathways, which are involved in proliferation, anti-apoptosis, and survival (Bhatt et al. 2014). It has previously been reported that BTK is expressed in MM cell lines and malignant plasma cells from MM patients (Bose et al. 2014). Moreover, upregulation of BTK in MM cells yields DXMS- or bortezomib-resistant cells (Bose et al. 2014; Murray et al. 2015) and may augment the expression of stem cell genes (Richardson et al. 2018). Consistent with previous studies, we found that BTK is highly expressed in the RPMI-8226 and U266 MM cell lines, both of which are resistant to DXMS-induced apoptosis.

Ibrutinib is an oral BTK inhibitor, and it can inhibit many downstream activities of BTK. Ibrutinib demonstrated a cell cytotoxic effect on malignant plasma cells from MM patients and suppressed the development of MM in the mouse model (Rushworth et al. 2013; Bam et al. 2013). As previously reported, our results show that ibrutinib can induce apoptosis in MM cell lines and bone marrow $CD38^+$ MNCs from MM patients *in vitro*. Moreover, ibrutinib inhibited tumor growth in the MM mouse model *in vivo*.

DXMS is an essential drug for the treatment of MM. DXMS-induced apoptosis has been shown in primary MM cells and many cell lines (Sharma and Lichtenstein 2018). The mechanism underlying DXMS-induced apoptosis is the transactivation of proapoptotic genes resulting from DXMS binding to its glucocorticoids receptor (GR) (Sharma and Lichtenstein 2018). However, MM cell lines such as RPMI-8226 and U266 were resistant to DXMS-induced apoptosis (Sharma and Lichtenstein 2018; Salem et al. 2013). Consistent with these previous studies, our results showed that DXMS induced slight apoptosis in primary cells but not in RPMI-8226 or U266 cell lines. DXMS-resistant MM cells have been demonstrated to overexpress BTK (Chauhan et al. 2002) or PI3K (Yang et al. 2008), while DXMS resistance in RPMI-8226 or U266 cells may be mediated by activation of BTK (Bose et al. 2014) or PI3K (Jiang et al. 2018). In the present study, our results show that RPMI-8226 and U266 cells highly express BTK or PI3K. DXMS combined with ibrutinib have been shown to display synergistic anti-tumor effects in CLL and Burkitt lymphoma (Manzoni et al. 2016; Chu et al. 2019). In myeloma, combined use of ibrutinib and DXMS has achieved good effects in clinical trials, although the mechanism underlying the combined action of ibrutinib and DXMS on MM cell apoptosis is not clear. Here, we demonstrate that DXMS potentiated the apoptotic role of ibrutinib in MM cell lines and bone marrow $CD38^+$ MNCs from MM patients. Surprisingly, the ibrutinib with DXMS combination not only suppressed the growth of tumor, but also prolonged survival in MM mouse models.

DXMS was observed to decrease the expression of spleen tyrosine kinase (Syk) which is upstream of BTK in the BCR pathway (Manzoni et al. 2016). The DXMS and ibrutinib combination has also been reported to synergistically inhibit the expression of BTK in stimulated normal B lymphocyte cells (Manzoni et al. 2016). In the present study, the ibrutinib/DXMS combination was also observed to significantly inhibit BTK expression in MM cell lines. Ibrutinib-induced apoptosis may be mediated by blocking the intranuclear transport of NF-κB and downregulation of anti-apoptotic genes such as Bcl-xL and surviving (Rushworth et al. 2013). Apoptosis is usually regulated by the Bcl-2 family proteins, including anti-apoptotic Bcl-2, and proapoptotic Bax. An unbalance in the Bcl-2/Bax ratio was shown to induce cell apoptosis *via* the mitochondrial-dependent pathway (Bai et al. 2019). In our study, the ibrutinib/DXMS combination downregulated the expression of the anti-apoptotic gene Bcl-2 and upregulated the expression of the pro-apoptotic gene Bax. A consequent decrease in the Bcl-2/Bax ratio may account for ibrutinib/DXMS combination induced apoptosis.

PI3K is an intracellular enzyme that catalyzes the phosphorylation of inositol lipids on the cell membrane and participates in cell proliferation and apoptosis. Previous tumor research has indicated a central role for the PI3K pathway in promoting proliferation and restraining apoptosis in tumor cells (Zhu et al. 2020). The PI3K/Akt signaling pathway has been reported to inhibit apoptosis in myeloma cells, and blockage of the PI3K/Akt pathway results in decreased cell proliferation, migration, and clonogenicity in MM cells, and increased apoptosis (Jiang et al. 2018). Metformin also demonstrated synergistic activity with DXMS, inhibited proliferation, and induced apoptosis of MM cells through inhibition of the PI3K/Akt signaling pathway (Zi et al. 2015). Our data demonstrate that the ibrutinib and DXMS combination markedly suppresses the expression of PI3K. Therefore, the additive effect of ibrutinib/DXMS may result from their synergistic suppression of PI3K. Cleavage of poly (ADP-ribose) polymerase (PARP) by caspase-3 is an early biochemical marker of cell apoptosis (Oliver et al. 1998). Inactivation of PARP prevents its consumption of NAD and ATP, and this is important in later apoptosis processes (Virag et al. 2013). In our study, treatment of MM cells with ibrutinib/DXMS combination leads to an increase in levels of PARP cleavage. Therefore, the ibrutinib/DXMS combination induced apoptosis may be mediated by PI3K/PARP pathways. But the mechanism of ibrutinib regulating PI3K/PARP pathway needs further study.

In summary, our research demonstrates that the ibrutinib/DXMS combination has an additive effect on MM cell apoptosis *in vitro*, can reduce tumor load, and can prolong the survival time

of MM mice *in vivo*. The synergistic effect of the ibrutinib/DXMS combination in inhibiting BTK and inducing apoptosis in MM cells may be mediated by inhibition of the expression of PI3K, a decrease in the ratio of Bcl-2/Bax, and may facilitate the cleavage of PARP. Ibrutinib/DXMS combination may be a better choice for MM patients especially those with dangerous risk factors.

4. Experimental

4.1. MM Cell lines and mononuclear cells from MM patient bone marrow

RPMI-8226 and U266 cells were purchased from the American Type Culture Collection (CRM-CCL-155 and TIB-196, ATCC, Manassas, VA, USA). Bone marrow (4 ml) of patients with MM who underwent initial treatment and plasma cells more than 20% was acquired. Ficoll (P8900, Solarbio, Beijing, China) density gradient centrifugation was used to separate mononuclear cells (MNCs) from bone morrow. Cells were cultured in RPMI-1640 medium (CF0001, Sparkjade Science Co., Ltd., China) supplemented with 10% fetal bovine serum (FBS, 04-001-1ACS, Biological Industries, Kibbutz Beit Haemek, Israel) and penicillin-streptomycin solution (SV30010, HyClone, Logan, UT, USA) in a 5% CO_2 humidified atmosphere at 37 °C. Cells that had reached the logarithmic growth period were used for experiments.

4.2. The treatment of MM cells in vitro

The cells were seeded in 12-well plates (at a density of 2×10^5 cells/well) and treated with ibrutinib combined with or without DXMS for 24 h. Ibrutinib (S2680, Selleck Chemicals, Houston, TX, USA) was dissolved in dimethyl sulfoxide (DMSO). RPMI-8226 cells were treated with 5 µM or 10 µM ibrutinib, 40 µM or 80 µM DXMS, or 10 µM ibrutinib combined with 80 µM DXMS. U266 cells were treated with 30 µM or 40 µM ibrutinib, 40 µM or 80 µM DXMS, or 40 µM ibrutinib combined with 80 µM DXMS. MNCs were treated with 40 µM ibrutinib, 80 µM DXMS, or 40 µM ibrutinib combined with 80 µM DXMS. An identical volume of DMSO was substituted in the control group. After 24 h, cells were collected and prepared for flow cytometry and western blot analysis.

4.3. Apoptotic analysis

The treated cells were collected and washed with cold Phosphate Buffered Saline (PBS, SH30256.01B, Hyclone). After resuspension in 100 µl PBS, cells were stained with 5 µl FITC-conjugated Annexin V and 5 µl PI for 15 min in the dark. After incubation, 400 µl PBS was added and the cells were analyzed by BD FACSAria III (BD Biosciences, Franklin Lakes, NJ, USA) and FlowJo VX10 software.

4.4. Western blot

After treatment with ibrutinib combined with or without DXMS for 24 h, RPMI-8226 and U266 cells were collected and lysed by RIPA lysis buffer (R0020, Solarbio) containing protease inhibitor cocktail (5871, Cell Signaling Technology (CST), Danvers, MA, USA). After adding 5× loading buffer (P013, ABP Biosciences, Beltsville, MD, USA), the protein was denatured in boiling water for 5 min. Next, total protein (30 µg) was subjected to electrophoresis in an SDS-polyacrylamide gel. The separated proteins were then transferred to a membrane for subsequent western blot analysis. The primary antibodies used for western blotting were rabbit anti-human PARP (5625, CST), Bcl-2 (15071, CST), Bax (5023, CST), PI3K (4292, CST), BTK (8547, CST), and GAPDH (5174, CST). All primary antibodies were used at a concentration of 1:1000. The secondary antibody was HRP-conjugated goat anti-rabbit IgG (7074, CST). The blots were developed by incubation with Chemiluminescent HRP substrate (WBKLS0500, Millipore), and then visualized and analyzed using the AlphaView-FluorChem Q system (ProteinSimple, San Francisco, CA, USA).

4.5. Mouse models

Female NOD/SCID mice (8 weeks old) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). Mice were housed in independent ventilation cages with constant temperature (23 ºC), humidity, and a 12 h light/dark cycle. All experiments were approved by the Animal Ethics Research Committee of the Second Hospital of Shandong University. The mouse model was implemented according to a previous report (Xu et al. 2020). Briefly, 1×10^7 RPMI-8226 cells suspended in 200 µl normal saline (NS) were subcutaneously injected under the right armpit of mice. After the tumor volume reached approximately 200 mm^3 (around 2 weeks), mice were randomly separated into four groups (6 mice per group). The ibrutinib alone group was injected intraperitoneally every other day with 6 mg/kg ibrutinib dissolved in 100 µl NS (containing 10% DMSO and 10% Cremophor EL). The DXMS alone group was injected intraperitoneally with 8 mg/kg DXMS dissolved in 100 µl NS (containing 10% DMSO and 10% Cremophor EL). The group of ibrutinib combined with DXMS was injected intraperitoneally with 6 mg/kg ibrutinib and 8 mg/kg DXMS in the same solvent. The control group was injected intraperitoneally with 100 µl NS (containing 10% DMSO and 10% Cremophor EL) only. All mice were humanely euthanized after the endpoint was reached (when tumor size exceeded 2 cm in any direction). The tumor diameters were measured with a digital caliper every three days until the last mouse reached the endpoint. Tumor volume in mm^3 was calculated by the formula: volume = length × $(\text{width})^2/2$ (Daneshmandi et al. 2019).

4.6. Statistical analyses

All statistical analyses and survival curve analysis were performed using the GraphPad Prism 6 software (La Jolla, CA, USA). Unpaired Student's *t* tests were used to compare differences between two groups. Data are shown as mean±SEM. A p value<0.05 was considered to be statistically significant.

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Conflicts of interest: None declared.

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