JaponiconeA Induces Apoptosis of Bortezomib-Sensitive and –Resistant Myeloma Cells In Vitro and In Vivo through Targeting IKKβ

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Research

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Abstract

Backgrounds: Multiple myeloma (MM) is a clonal proliferative disease of abnormal plasma cells. Relapse and drug resistance still remain to be solved, so new therapeutic drugs are needed to be adopted to further improve the prognosis of MM. JaponiconeA (JA) is a natural product isolated from Inula japonica Thunb, the anti-tumor effect and mechanism in MM has not been studied.

Methods: CCK8 and flow cytometry were used to detect the proliferation, apoptosis and cell cycle of MM cell lines with JA treatment. And the in vivo effects of JA were verified in the subcutaneous xenograft mice model of MM. In addition, we analyzed the possible targets and mechanism of JA through RNA-seq and c-Map databases, and we verified the specific target of JA in MM cell lines and bortezomib-resistant MM cell line through CETSA and rescue experiments. JA and bortezomib were used separately or together to treat MM cell lines to explore the synergetic effect.

Results: In vitro experiments, JA inhibited proliferation, induced apoptosis and G2/M phase arrest of MM cell lines, and JA selectively killed primary $CD138^+$ MM cells but spared normal human mononuclear cells. In vivo experiments, JA also showed good anti-tumor effect with no observable toxicity. In addition, JA achieved good synergetic effect in combination with bortezomib, and enhanced the anti-tumor effect of bortezomib in bortezomib-resistant cells. According to RNA-seq and c-Map data, the target protein of JA might be IKKβ. CETSA experiment confirmed that JA can bind IKKβ directly in vitro, and overexpression of IKKβ could partly rescue the apoptosis induced by JA.

Conclusion: JA exhibited strong anti-tumor effects in MM. It sensitized myeloma cells to bortezomib and overcame NF-κB induced drug resistance through inhibiting IKKβ, which providing a new treatment strategy for MM patients.

1. Background

Multiple myeloma (MM) is the second most common hematological malignancy, characterized by the proliferation of clonal plasma cells in bone marrow, which leads to typical symptoms including hypercalcemia, renal damage, anemia and bone diseases (1). Despite the application of proteasome inhibitors and immunomodulatory drug, multiple myeloma is still an incurable disease and almost all patients relapse eventually due to drug resistance (2). So it is still urgent to find new chemotherapeutic agents and strategies to improve prognosis.

Nuclear factor-kappa B (NF-κB) signaling pathway has been reported to be activated in MM and contributes to the progression and drug resistance (3–5). The activation of NF-κB pathway induces expression of several anti-apoptotic protein including Bcl-2, Bcl-XL,c-IAP1/2,c-FLIP, XAIP (6–9), which promotes the survival of MM cells. And it was also reported that bortezomib treatment induced the activation of NF-κB pathway which may limit the efficacy of bortezomib (10). And its stronger activation was also observed in bortezomib-resistant MM cells (11). In addition, a number of studies have point out that the activation of NF-κB pathway is strongly associated with bortezomib resistance (12, 13). Thus, targeting NF-κB pathway could be a feasible strategy to treat MM and overcome bortezomib resistance.

The active natural compounds, isolated from traditional herbal medicines, are likely to be the sources of developing new efficient anti-tumor drugs with minimal adverse effects (14, 15). JaponiconeA (JA) is a natural product isolated from *Inula japonica* Thunb(16). Previous study has demonstrated that JA inhibited breast cancer cell proliferation by inhibiting the expression of RAD54B(17).And JA could inhibit the growth of non-small cell lung cancer cells via

mitochondria-mediated pathways (18). In addition, JA was also effective in Burkitt lymphoma(19)Here, we found that JA showed potent anti-tumor effect in MM both in *vitro* and in *vivo* but spared the normal cells. It showed enhanced cytotoxicity when combined with bortezomib. Digging out the mechanism, our research showed that JA targeted IKKβ to inhibit the NF-κB pathway, inducing cell apoptosis and cell cycle arrest. These results showed the promising preclinical performance of JA for the therapy of MM.

2. Materials And Methods

2.1. Reagents and Compounds

JaponiconeA (JA), with a purify over 98%, was purified and kindly provided by Professor Weidong Zhang from Key Laboratory of Food Safety Research of Key Laboratory of Food Safety Research. Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich. Bortezomib and Cell Counting Kit-8(CCK-8) was obtained from MedChemExpress (New Jersey, USA).Antibodies including PARP-1, caspase3, caspase9, CDK1, CCNB1, β-Actin, p65, p-p65, IKBα and p-IKBα were purchased from Proteintech (Rosemont, IL, USA).

2.2. Cells culture methods

Myeloma cell lines NCI-H929, OPM2, LP-1, RPMI 8226, MM1.S were obtained from ATCC and they cultured and maintained in our own lab. NCI-H929 and MM1.S were cultured in RPMI-1640 medium, which was supplemented with 10% fetal bovine serum (FBS) and 100 IU/ml penicillin and 100ug/ml streptomycin. OPM2 and LP-1 cells were cultured in IMDM in Iscove's Modified Dubecco's(IMDM) medium supplemented with 15% FBS, penicillin (100 IU/ml) and streptomycin (100 μg/ml). RPMI 8226 was cultured in IMDM with a higher FBS concentration (20%).

2.3. Western Blotting assay

The whole cell lysates were extracted in 1 × SDS, equally loaded to 8–12% SDS–polyacrylamide gel, electrophoresed, transferred to nitrocellulose membrane (Bio-Rad, Hercules, CA, USA). After blocking with 5% nonfat milk in PBS, the membranes were incubated with antibodies overnight at 4 °C, followed by HRP-linked secondary antibody (Cell Signaling Technology, Beverly, MA, USA) for 1 h at room temperature. The signals were detected with a chemiluminescence phototope-HRP kit (Cell Signaling Technology), used according to manufacturer's instructions.

2.4. Detection of apoptosis

Apoptosis was detected with eBioscience™ Annexin V Apoptosis Detection Kit (Thermo Fisher Scientific, Waltham, MA, USA) according to the instructions from the manufacture. Briefly, MM cells were treated with JA for certain time, approximately 1×10^6 cells were harvested and washed with 1x binding buffer once and then suspended in 100 ul 1xloading buffer. After that, 5uL Propidium Iodide (PI) and 5 ul Annexin V-APC were added to cell suspension per sample then incubated at dark for 15 minutes. Then apoptotic cells were quantified by a flow cytometer (Fortessa, BD, USA) using the DIVA software. About 10000 cells were analyzed for each sample.

2.5. Cell cycle assay

The distribution of cell cycle was determined by measurement of DNA content with flow cytometry, as previously described (20).In brief, MM cells were fixed with 75% ethanol at least 12 hours at -20°C and then incubated with 50ug/ml RNase A and 100 μg/ml PI for 30 minutes respectively. The DNA content was determined by flow

cytometer (Foretassa, BD, USA). The distribution of each cell cycle was analyzed by Flowjo software. 20 000 cells were gated and analyzed for each sample.

2.6. Xenograft mouse model

The experiment was inspected and approved by the Shanghai Jiao Tong University School of Medicine Institutional Animal Care & Use Committee before implement. Female BALB/c nu/nu mice (5–6 weeks) were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and kept in specific pathogen-free (SPF) condition at Animal Center of Ruijin Hospital. Human myeloma xenograft model was established by subcutaneously inoculating 1×10^7 NCI-H929 cells into the region near armpit of forelimb of mice. When the tumor masses were visible, the mice were randomly divided into JA group and control group to receive treatment accordingly. JA was intraperitoneally administrated at 30 mg/kg once a day for 10 days. The body weight of the mice and length (L) and width (W) of tumor were monitored every day since the first treatment. The tumor growth was evaluated with the tumor volume (V) = $L \times W^2/2$. After 10 times treatment, the mice were sacrificed and tumor masses were stripped off and photographed.

2.7. CETSA

MM cell lines were harvested and diluted in PBS supplemented with protease inhibitor cocktail and phenylmethanesulfonyl fluoride (PMSF). The cell suspensions were freeze–thawed 3 times in liquid nitrogen. The soluble fraction was separated from the cell debris and divided into two aliquots. One aliquot was treated with DMSO and the other aliquot with JA. After a 30 min incubation at 37°C, the respective lysates were divided into separate aliquots (30 μl), Western Blotting experiment was taken with cell lysis.

2.8. Immunofluorescence analysis.

Cells were fixed with 4% paraformaldehyde and treated with 0.3% Triton X-100, then blocked with bovine serum albumin. Cells were incubated with antibody p65 overnight at 4 °C, and followed with FITC labeled anti-rabbit immunoglobulin G antibody (Invitrogen) and 4',6- diamidino-2-phenylindole. The stained cells were examined with immunofluorescence microscopy (Nikon, Tokyo, Japan).

2.9. Immunohistochemical analysis.

The tumors were formalin fixed, paraffin embedded, then sectioned into slices. Tissue sections were stained with hematoxylin and eosin, then performed the immunohistochemistry for TUNNEL and Ki67 on the sections.

2.10. Plasmid IKKβ *o*verexpression.

IKKβ overexpression plasmid was purchased from the DNA library of Shanghai Jiao Tong University School of Medicine. The OE-IKKβ or vector plasmids were transfected with lentivirus packaging vectors PSPAX2and PMD2G, introduced into HEK293T cells to produce lentivirus. The lentivirus was harvested to infect MM1.S cells.

2.11. Real-time fluorescence quantitative PCR (qPCR)

Real-time fluorescence quantitative PCR (qPCR) was performed with TransStart® Top Green qPCR SuperMix Kit (Transgen, Beijing ,China). And the primers were synthesized by Sangon Biotech (Shanghai, China) and listed as follows.

Bax, F:TCAGGATGCGTCCACCAAGAAG, R:TGTGTCCACGGCGGCAATCATC. Bcl-xl,F:GCCACTTACCTGAATGACCACC; R:AACCAGCGGTTGAAGCGTTCCT. c-Myc, F:CCTGGTGCTCCATGAGGAGAC; R: CAGACTCTGACCTTTTGCCAGG. ICAM1,F:AGCGGCTGACGTGTGCAGTAAT;R:TCTGAGACCTCTGGCTTCGTCA.IKKβ,F:ACAGCGAGCAAACCGAGTTTGG; R:CCTCTGTAAGTCCACAATGTCGG.

2.12. Statistical Analysis

Statistical significance of differences observed in drug-treated versus control cultures was determined using the Wilcoxon signed-rank test. The minimal level of significance was P value less than 0.05.

3. Results

3.1. JA inhibited proliferation, induced cell cycle arrest and led to apoptosis in MM cells

JaponiconeA (JA) is extracted from traditional herbal medicine, *Inula japonica* Thunb. The chemical construction of JA was shown in Fig. 1A. The anti-tumor of other Inula sesquiterpenoids has been extensively studied (21). To further assess the potential anti-tumor effect of JA, we screened the effect of JA in a variety of hematological malignances cell lines and found that myeloma cells were highly sensitive to JA with average IC_{50} (half-maximal inhibitory concentration) values ranging from 2.2 μM to 4.8 μM (Fig. 1B). To figure out the effect of JA on apoptosis in MM cells, MM1.S and NCI-H929 were exposed to different concentrations of JA for 24 hours with results showing that JA induced MM cells apoptosis in a dose-dependent manner (Fig. 1C). And apoptosis-related proteins also showed corresponding changes (Fig. 1E). In addition, the effect of JA on cell cycle distribution was also determined with the flow cytometer. Obviously, exposure to JA increased the proportion of the G2/M phase, indicating JA caused the G2/M phase cell cycle arrest in MM cells (Fig. 1D). And the G2/M related cell cycle checkpoint proteins were detected (Fig. 1E). In addition, primary myeloma cells from MM patients and bone marrow mononuclear cells from health donors (HD) were exposed to JA treatment for 48 hours. JA selectively killed the myeloma cells while spared the normal cells (Fig. 1F, G). To sum up, JA showed potent anti-tumor effect on myeloma cells but much less toxicity on normal cells, highlighting its therapeutic potential in treating MM patients.

3.2. JA inhibited MM cells proliferation in xenografts model

The anti-tumor activity of JA was also investigated in vivo. The myeloma xenografts model was established as is described in materials and methods, and the exact procedures was summarized in the schematic (Fig. 2A). Obviously, JA treatment significantly reduced tumor burden compared with the control group (Fig. 2B, C). Mice in JA treatment group showed no significant weight loss or other signs of poisoning during the administration (Fig. 2D). The representative pathological images of tumor in both JA treatment group and control group were shown in Fig. 2E. The percentage of ki67-positive cells were significantly decreased and TUNEL signal was slightly enhanced after treatment with JA, indicating JA strongly inhibited the growth of MM cells and partially induced apoptosis in MM cells in vivo. These results further proved that JA has an excellent anti-tumor effect.

3.3 JA potentiated cytotoxic effect of bortezomib and partially overcame drug resistance to bortezomib

Bortezomib is a first-line therapeutic agent and has significantly improved the prognosis of MM, but most patients eventually relapsed and drug resistance. Considering the good anti-MM effect of JA, we wonder if JA and bortezomib have synergistic effects in anti-myeloma activity. MM cells were treated with JA and bortezomib separately or together for 24 h, and apoptosis was detected subsequently. The proportion of apoptotic cells increased significantly in combination treatment group (Fig. 3A). In addition, combination index(CI) of JA and bortezomib was also calculated, further demonstrating the synergistic effect of JA and bortezomib(Fig. 3B,S1,Table 2,3). Furthermore, we verified the anti-MM activity of JA in bortezomib-resistant MM cells. Bortezomib-resistant H929 (H929-BR) cells were obtained after a long period of exposure to increased bortezomib with the capacity to bear more than 40 nM bortezomib, whose sensitivity to bortezomib is more than ten times lower than that of H929 cells (Fig. 3C). Surprisingly, we found JA was also effective in H929-BR with IC_{50} approximately 6.878 μM (Fig. 3D). JA induced obvious apoptosis in H929-BR cell in a dose-dependent manner, which was detected both by flow cytometry and western blotting (Fig. 3E, F). The synergetic effect of JA and bortezomib also calculated in H929-BR cells. As is shown in Fig. 3G and S2 (Table 4), strong synergism was observed between JA and bortezomib. JA and bortezomib also induced stronger apoptosis in H929-BR cells compared with single reagent treatment (Fig. 3H).

Table 2
The combination index of JA and bortezomib in MM1.S cells.

Bort(nM)	JA(μM)	Effect	CI
6	4	0.83296	0.35718
6	3	0.76542	0.32648
6	2	0.69791	0.27206
4	4	0.7939	0.38506
4	3	0.69771	0.3618
4	2	0.32335	0.59297
2	4	0.63334	0.50302
2	3	0.55884	0.43409
2	2	0.17082	0.78298

Table 3
The combination index of JA and bortezomib in H929 cells.

Bort(nM)	JA(μM)	Effect	CI
1	1	0.69	0.52271
1	1.5	0.9	0.31826
1	2	0.96	0.5498
1.5	1	0.56	0.92831
1.5	1.5	0.8	0.61115
1.5	2	0.92	0.40547
2.0	1	0.53	1.44047
2.0	1.5	0.765	0.92400
2.0	2	0.939	0.43554

Table 4
The combination index of JA and bortezomib in H929-BR cells.

Bort(nM)	JA(μM)	Effect	CI
100	6	0.82442	0.59389
100	4	0.69461	0.78560
100	2	0.52836	1.03257
80	6	0.80481	0.49955
80	4	0.67659	0.64910
80	2	0.50620	0.85486
60	6	0.7853	0.39232
60	4	0.62766	0.52914
60	2	0.49229	0.65508

3.4 JA inhibited the activation of NF-κB pathway

To further explore the mechanism of JA's anti-tumor effect on MM cells, MM1.S cells were treated with JA or DMSO for 24 hours, then cells were harvested and subjected to next-generation sequencing. Clustering heatmap showed good separation of samples in JA group and DMSO group (Fig. 4A). A total of 1001 differentially expressed genes (DEGs) were selected, including 498 up-regulated genes and 503 down-regulated genes, based on the criterion: adj.P.value < 0.05 and Fold change (FC) > 2 (Fig. 4B). As Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analysis was performed in down-regulated DEGs, the DEGs were predominately enriched in "signaling transduction" and cell cycle associated molecular functions in GO enrichment analysis (Fig. 4C). We noticed the NF-κB pathway, an activated pathway that contributes to the development and drug-resistance of MM, was enriched in the KEGG annotation of down-regulated DEGs, suggesting that JA may exert its

anti-MM effect through inhibiting the NF-κB pathway. Besides, the potential anti-tumor mechanism of JA was also analyzed by connective map database (c-MAP). Using the cMAP database, the expression characteristics of JA-treated cells were compared with that of cells treated with other compounds (22, 23). The compounds with similar expression signature may share similar pharmacological mechanism. A brief description of the operation mechanism of the database is shown in the Fig. 4D. Interestingly, 3 of the top 10 predictions are NF-κB Inhibitor Kinase Beta(IKKβ) inhibitor according to the c-MAP (Table 1). And we observed that phosphorylated p65 and IKBα were significantly downregulated in NCI-H929,MM1.S and H929-BR cells, indicating the strong inhibition of JA on NF-κB pathway(Fig. 4E). In addition, the mRNA level of NF-κB target genes were decreased after exposure to JA (Fig. 4F). These results fully demonstrated the inhibition of JA on NF-κB pathway.

Table 1
The targets of small molecule compounds predicted by cMAP.

Targets	Drugs
IKKβ	Withaferin A
	Parthenolide
	Geldanamycin
HDAC1/2	Vorinostat
	Trichostatin A
HSP90	Geldanamycin
Wnt	Pyrvinium
m-TOR	Resveratrol

It was reported that bortezomib could induce the activation of canonical NF-κB pathway in MM cells (10). As is shown in Fig. 5A, NF-κB pathway was indeed activated in both NCI-H929 and MM1.S after treated with bortezomib. And treatment with JA strongly inhibited the activation of NF-κB induced by bortezomib (Fig. 5B, C), which may help to explain the synergetic effect of JA and bortezomib. Besides, the activity of NF-κB was reported to be further enhanced in refractory MM patients to bortezomib therapy (11). So, we compared the activation of NF-κB pathway in both H929-S and H929-BR cells. Not surprisingly, an stronger activation of NF-κB pathway was observed in H929-BR cells(Fig. 5D). And JA could also effectively downregulated the activation of NF-κB in H929-BR cells(Fig. 5E,F). Considering the important role of activation of NF-κB in both sensitive and refractory cells, we hypothesize that JA may exert its effect via inhibition of NF-κB pathway.

3.5 JA directly binds with IKKβ to prevent the activation of downstream of NF-κB

In order to find direct target of JA on NF-κB pathway, an in vitro drug-target screening method cellular thermal shift assay (CETSA) experiment was conducted(24). Compared with DMSO, JA reduced thermal stability of IKKβ protein in NCI-H929 cells and MM1.S cells (Fig. 6A,B). No alteration of p65 and IκBα was observed (S3). These results preliminarily suggested that JA directly interacts with IKKβ in MM cells. Furthermore, to figure out if JA exerts its effect through inhibiting IKKβ, we over-expressed IKKβ in MM1.S and NCI-H929 cells. Remarkable increase of IKKβ both in mRNA and protein level was observed after stable transfection, with NF-κB-targeted genes increased

(Fig. 6C,E,S4). Then we treated the OE-IKKβ and control cells with same dose of JA, a significant decrease of apoptosis rate in OE-IKKβ group compare that of the control cells was detected (Fig. 6D, F). These results suggested that JA exerts anti-MM effect through inhibiting IKKβ to prevent the activation of NF-κB pathway.

4. Discussion

Although the therapeutic effect and prognosis of myeloma have been significantly improved with the clinical application of proteasome inhibitors, immunomodulatory drugs and monoclonal antibodies. However, due to evolving molecular changes, genetic mutations, and interaction with the bone marrow microenvironment, MM develops into drug resistance, which remains a huge challenge. It is urgent to explore novel agents to overcome the drug resistance. NF-κB signaling pathway was found to play a significant role in the promotion of MM and bortezomib resistance (11, 25). And inhibition of NF-κB pathway has been reported to be able to overcome bortezomib resistance. Knockdown of USP7 significantly enhanced the sensitivity to bortezomib via stabilizing IκBα to inhibit the NF-κB pathway (13). Inhibition of p65 activation by Ibrutinib or lenti-viral miRNA interference also restored sensitivity to bortezomib (12).NEK2 was found to contribute to bortezomib resistance through activation of NF-κB and destabilizing NEK2 kinase also help to overcome resistance to proteasome inhibitors(26). In addition, epidermal growth factor receptor pathway substrate 8 (EPS8), a downstream target of NF-κB was found to be assisted with bortezomib resistance in MM(27). These studies imply the feasibility to overcome bortezomib resistance via inhibition of NF-κB pathway.

The lack of identifiable hydrophobic pockets in NF-κB transcription factor dimers makes it a challenge to find an available small molecule inhibitor. So, it could be a promising strategy to perturb the druggable upstream factors that essential for the activation of NF-κB, such as IKKβ. IKKβ is the logical, first-choice target for the development of pharmacological inhibitors of the NF-κB pathway(28). IKKβ inhibitors have displayed significant therapeutic potential. For instance, MLN-120B inhibited MM cell growth in a clinically SCID-hu mouse model (29). In our study, we found another IKKβ inhibitor that can inhibit NF-κB pathway by interacting with IKKβ directly. Compared with MLN120B's modest anti-MM activity, lower dose of JA was able to exert stronger anti-MM effect. And it is possible to explore more potent and selective IKKβ inhibitors based on the chemical constitution of JA.

Natural products have been used to treat cancer for a long time, for example, harringtonine, camptothecin, paclitaxel, bleomycin, which are still the mainstay in treatment of malignances. In addition, tubulin inhibitors vinflunine, curcumin, resveratrol, apigenin, isothicyanates, still have broad and promising clinical application prospects in cancer treatment(15). Now phytochemicals are still regarded as economical, accessible, readily applicable, abundant sources for exploration of new drugs for cancer control and management (15). But one of the most difficult things in drug research is to decipher the underlying mechanism of action of drugs. To identify the direct targets, drugs are needed to be chemically modified in vitro to capture the physically binding proteins and re-verify the direct binding of drugs and targets through surface plasmon resonance equipment, which usually time-consuming and costly. Moreover, due to the limitation of funds and technology, not every researcher can study the specific mechanism of action of drugs in this way. In view of this, we screened the possible biological processes and pathways mostly impacted by the drugs of interest with biological information analysis. And then we identified the direct binding through CETSA, which was economic method to verify the direct binding of drugs and their targets(24, 30). In addition, knockdown or overexpression targets to observe the sensitivity to drugs further consolidate the relation between drugs and their targets. This strategy can provide economical and convenient method to preliminarily explore the mechanism of drugs.

In summary, we have shown that JA has potent and selective anti-myeloma activity by inducing cell apoptosis and G2/M phase arrest and partially overcame bortezomib resistance via inactivation of IKKβ. Considering that IKKβ is the most abundant and important IKK in MM, inhibition of IKKβ by JA is a promising strategy to inhibit NF-κB pathway in MM and JA may be a feasible drug in the treatment of MM.

5. Conclusion

Our findings elucidate that JA exhibited strong anti-tumor effects in MM. It sensitized myeloma cells to bortezomib and overcame NF-κB induced drug resistance through inhibiting IKKβ(Fig. 7), which providing a new treatment strategy for MM patients.

6. List Of Abbreviation

IKKb	NF-κB Inhibitor Kinase Beta
JA	JaponiconeA
MM	multiple myeloma
CCK8	Cell Counting Kit-8
CETSA	cellular thermal shift assay
EPS8	epidermal growth factor receptor pathway substrate 8
c-Map	connective map database
NF-κB	Nuclear Factor Kappa B
Bort	bortezomib
DMSO	Dimethyl sulfoxide
PI	Propidium Iodide
PMSF	phenylmethanesulfonyl fluoride
IC_{50}	half-maximal inhibitory concentration
HD	health donors
CI	combination index
H929-BR	Bortezomib-resistant H929
DEGs	Differentially expressed genes
FC	Fold change
KEGG	Kyoto Encyclopedia of Genes and Genomes
GO	Gene Ontology
q-PCR	Quantitative real-time polymerase chain reaction

Declarations

1. Ethics approval and consent to participate

Animal experiments were approved by the Ethics Committee of the Ruijin Hospital, Shanghai Jiao Tong University School of Medicine.

2. Consent for publication

Not applicable.

3. Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files]

4. Competing interests

The authors declare that they have no competing interests.

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6. Authors' contributions

ZLZ,JL and HY, YLW designed the project and experiments. ZLZ, CJY, XGX,XYZ,WBX,CW,QY conducted the experiments. HY and YLW analyzed the data and generated the figures. ZLZ and CJY wrote the manuscript, YLW and HY supervised all aspects of the work. All authors involved in the review and revision of the manuscript, and approved the final manuscript.

7. Acknowledgements

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Figures

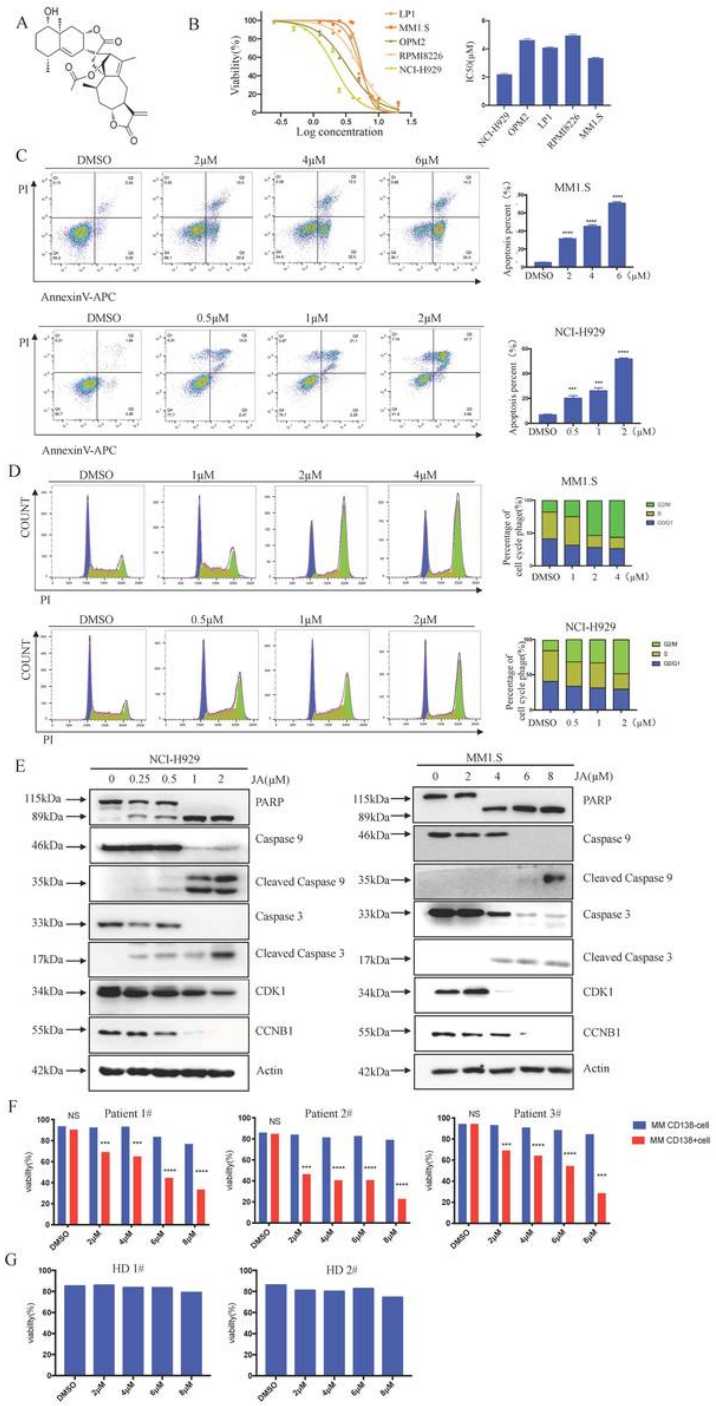

Figure 1

JA inhibited proliferation, induced cell cycle arrest and led to apoptosis in multiple myeloma cells. (A) Chemical structure of JA. (B) The inhibition of JA on MM cell lines for 24 h was detected with CCK8 and IC50 was calculated with Graphpad prism software. (C) (left) MM cells were exposed to various concentrations of JA for 24 h and apoptotic cells were analyzed by flow cytometry. (right)The data shown are statistic results of percentage of apoptotic cells from at least 3 independent experiments with similar results. (D) MM cells were treated with JA for 24 h, and cell cycle was analyzed by flow cytometry. (E) MM cells were treated with the indicated concentrations of JA for 24 h, followed by western blotting to detect the indicated proteins. (F) CD138+ myeloma cells and CD138- cells isolated from MM patients were treated with indicated concentration of JA and cell viability was determined

after 48 h. (G) Healthy donor(HD) BM mononuclear cells were treated with JA for 48 h and cell viability by detected with CCK8 reagent. (*p < 0.05, **p< 0.01; ***, p < 0.001 vs. the control)

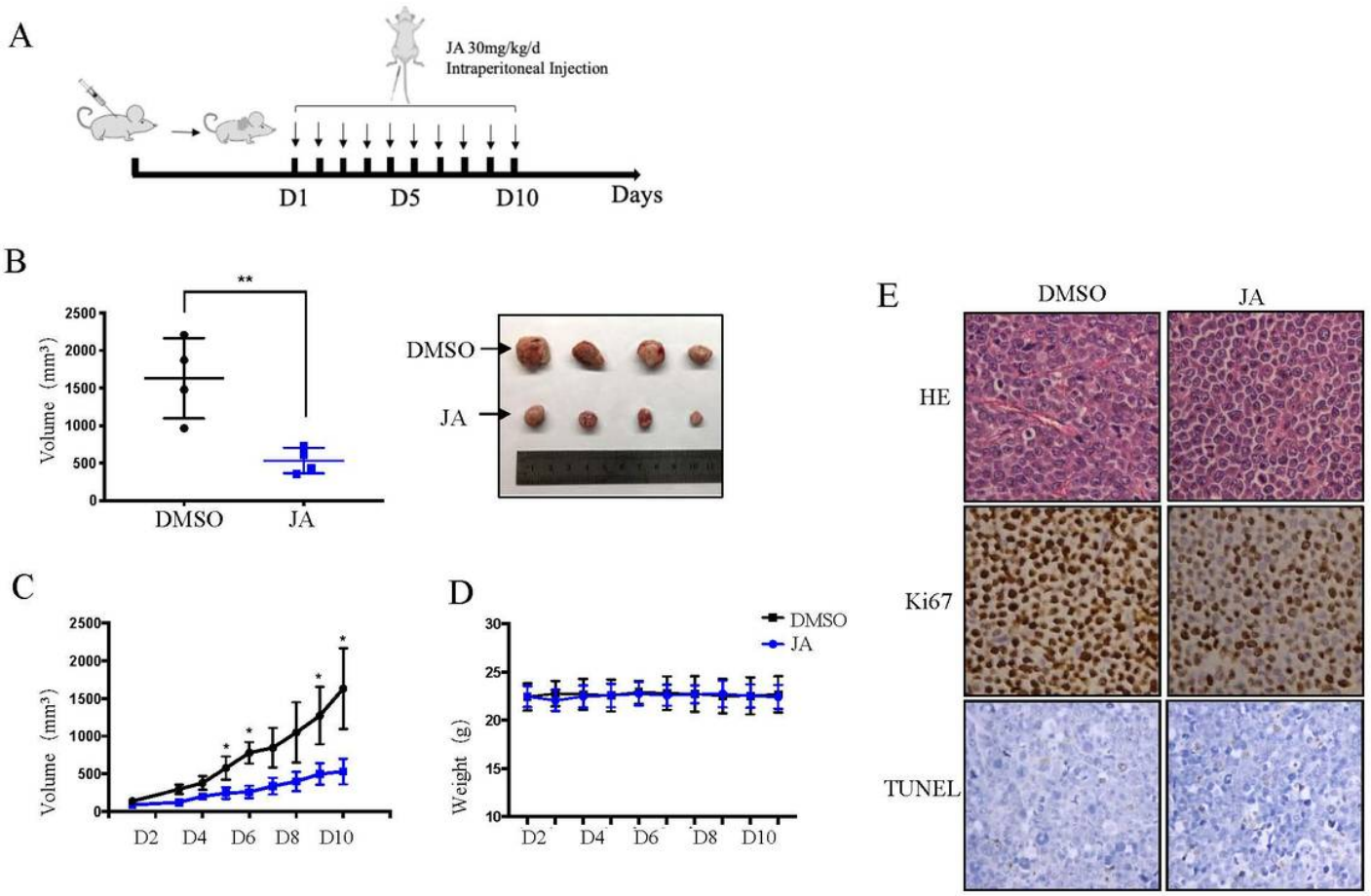

Figure 2

JA inhibited MM tumor growth in xenograft mice model. (A) Schematic plot of the process of the in vivo experiments. (B, C) JA (30 mg/kg) was intraperitoneally administrated daily for 10 days and tumor volumes were measured everyday during the administration. On the day 10, mice were sacrificed. Tumors were stripped off and tumors of each group and put together to take photos. (D) The body weight of mice was recorded everyday 2 days during the administration. (E) Ki67 and TUNEL were detected with immunohistochemistry in the xenograft tumors in each group. (*p < 0.05 vs. the control).

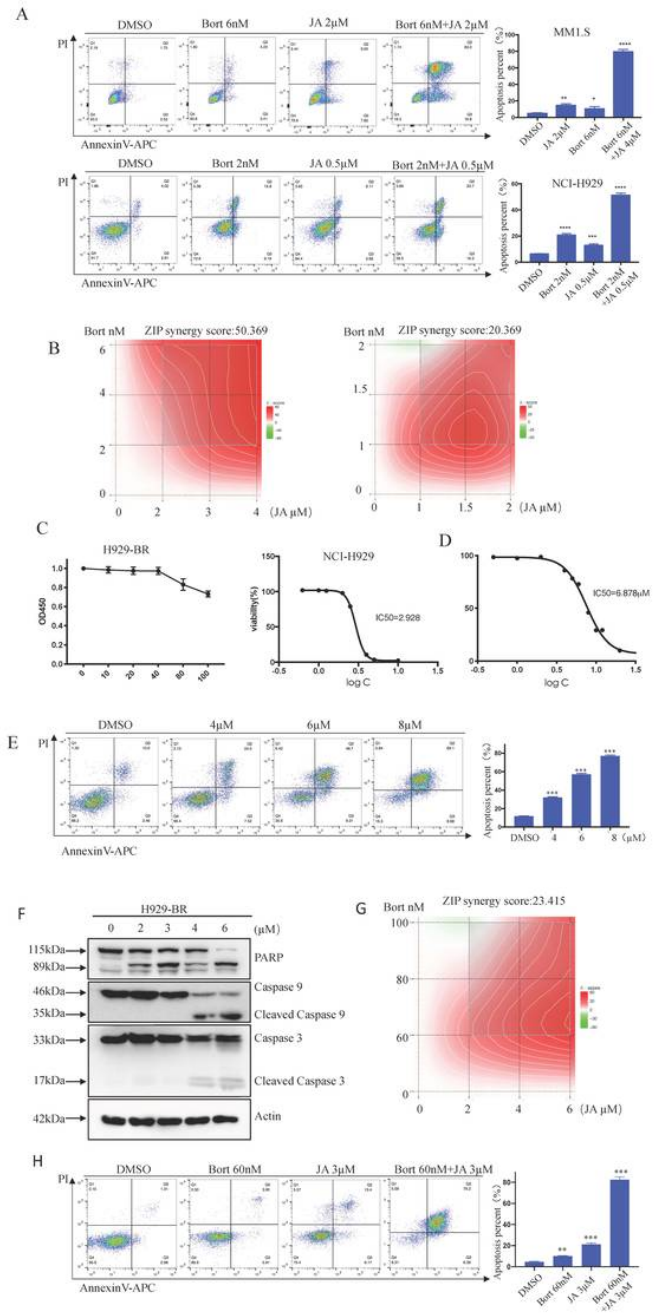

Figure 3

JA potentiated cytotoxic effect of bortezomib and partially overcame drug resistance to bortezomib. (A) MM cells were treated with bortezomib, JA separately, or together for 24 h and apoptotic cells were determined by flow cytometry with Annexin V/PI. (B) MM1.S (left) and NCI-H929 (right) were treated with bortezomib, JA separately or together for 24 h and inhibition rate was detected with CCK8. Then combination index (CI) was calculated in online network analysis (https://synergyfinder.fimm.fi). Red indicates synergy, green indicates antagonism.(C) H929-BR or NCI-H929 cells were treated with Bortezomib for 24h, IC50 was calculated with Graphpad prism software. (D) H929-BR cells were treated with JA for 24h, IC50 was calculated with Graphpad prism software. (E) H929-BR cells were treated with JA for 24h and apoptotic cells were detected by flow cytometry with Annexin V/PI. (F) H929-BR cells were treated with the indicated concentrations of JA for 24 h, followed by western blotting to detect the expression

of indicated proteins. (G) H929-BR cells were treated with bortezomib/JA for 24h to calculate the combination index (CI) of JA and bortezomib. (H) After exposure to JA/Bortezomib for 24 h, apoptotic cells were determined by flow cytometry with Annexin V/PI. (*p < 0.05, **p< 0.01; ***, p < 0.001; ****, p < 0.0001 vs. the control).

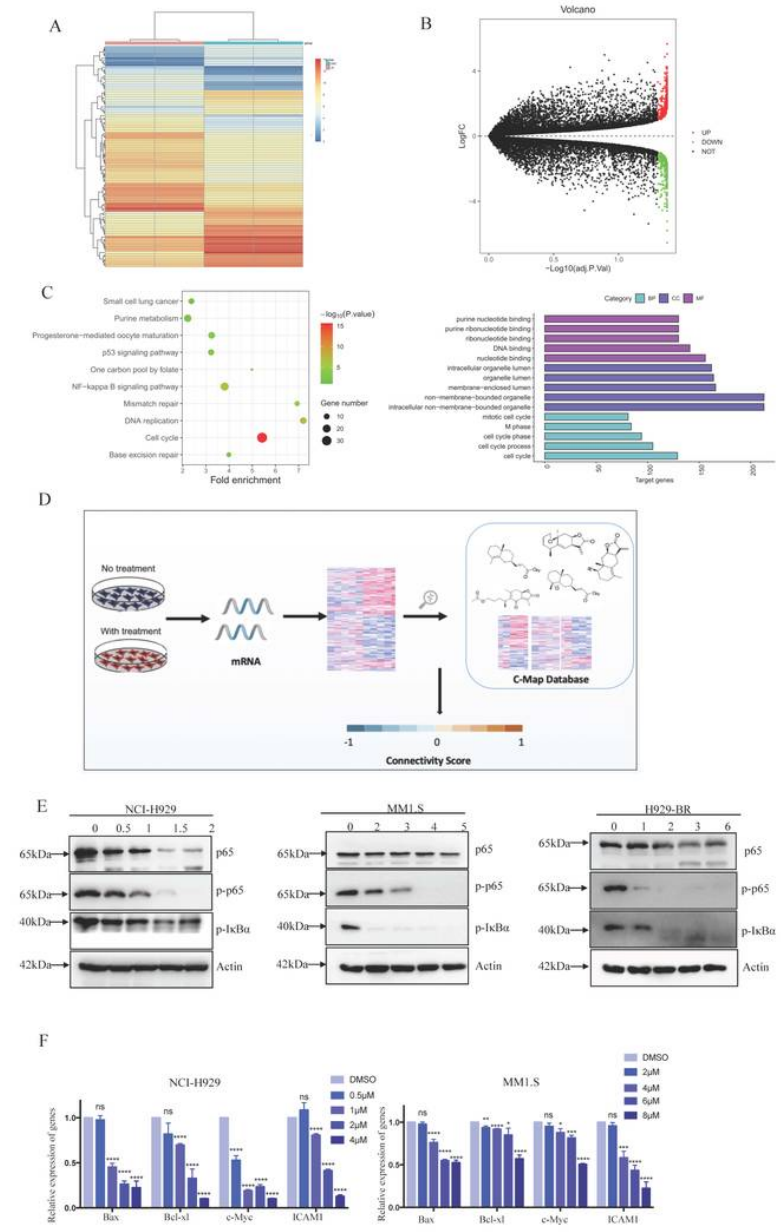

Figure 4

JA inhibited the activation of NF-κB pathway. (A) MM1.S cells were treated with JA and DMSO for 24 h, and then cells were harvested and subjected to next-generation sequencing. Clustering heatmap was showed as above. (B) Volcano map was used to show the differe differentially expressed genes(DEGs). A total of 1001 DEGs was selected based on the criterion: adj.P.value <0.05 and fold change (FC)> 2. (C) GO and KEGG enrichment analysis was performed in down-regulated DEGs. (D) A brief description of the operation mechanism of connective map (cMAP). (E) The components of NF-κB pathway of MM cells was detected after treated with bortezomib for 24 h. (F)

The mRNA level of NF-κB target genes including BAX, BCL-xl, c-Myc and ICAM1 were quantified by q-PCR after treated with JA for 12 h. (*p < 0.05, **p< 0.01; ***, p < 0.001; ****, p < 0.0001 vs. the control).

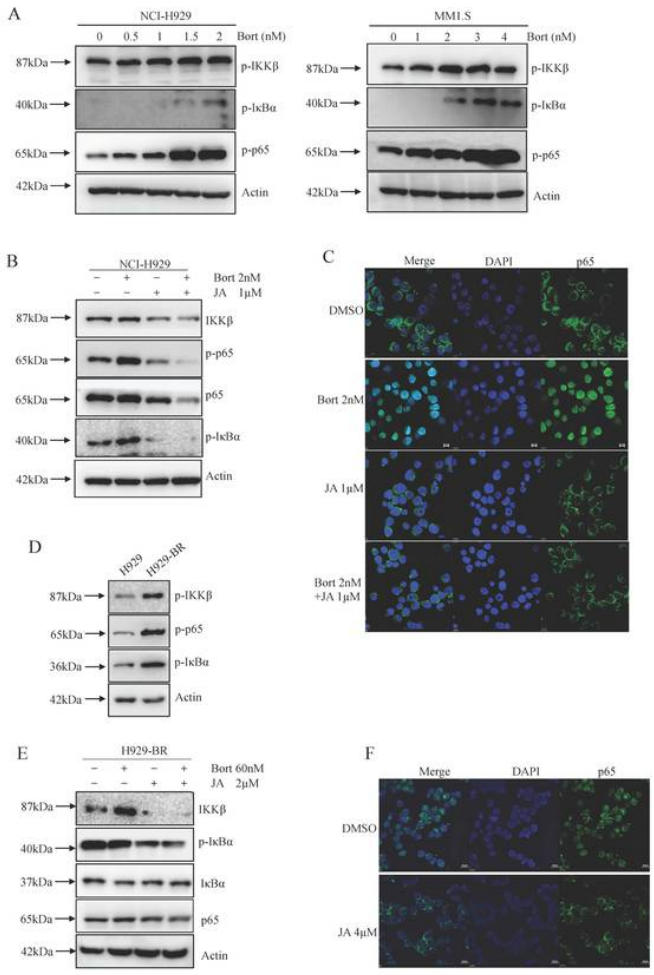

Figure 5

JA inhibited the activation of NF-κB pathway induced by bortezomib. (A) The components of NF-κB pathway of MM cells were detected after treated with bortezomib for 24h. (B) NCI-H929 treated by single or both of the drugs, followed by western blotting to determine the expression of the indicated proteins. (C) NCI-H929 cells were treated with indicated drugs for 24 h and p65 was detected by immunofluorescence. (D) The expression of p-IKKβ, p-p65 and p-IKBα in NCI-H929 and H929-BR cells were determined by western blotting. (E) H929-BR cells were treated by single or both of the drugs, followed by western blotting to detect the indicated proteins. (F) H929-BR cells were treated with JA for 24 h and p65 was detected by immunofluorescence.

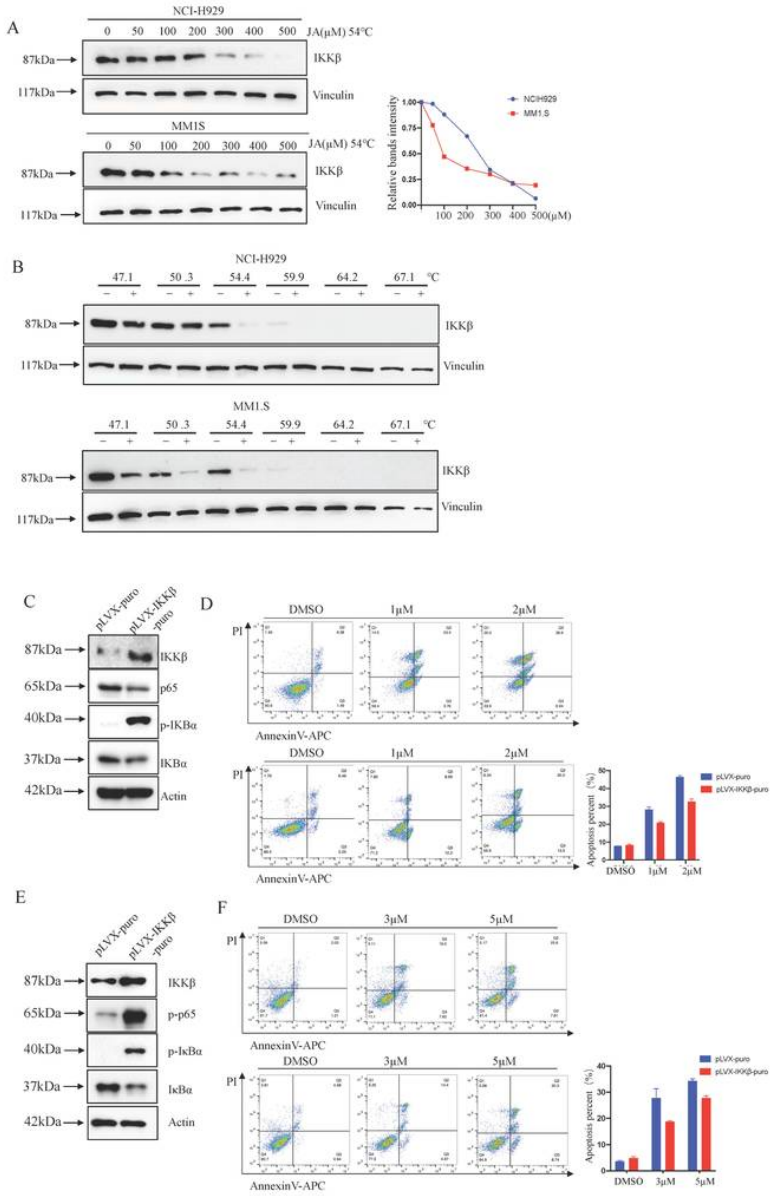

Figure 6

JA exerted function through binding IKKβ. (A, B) The thermal stabilization of IKKβ when incubated with JA on various dosages and various temperatures condition. (C, E) The overexpression of IKKβ in NCI-H929 or MM1.S cells and effect on downstream targets of NF-κB were verified by western blotting. (D, F) NCI-H929 or MM1.S cells transfected with IKKβ and control cells were treated with JA for 24 h, cell apoptosis were determined by flow cytometry. (*p < 0.05, **p< 0.01; ***, p < 0.001; ****, p < 0.0001 vs. the control).

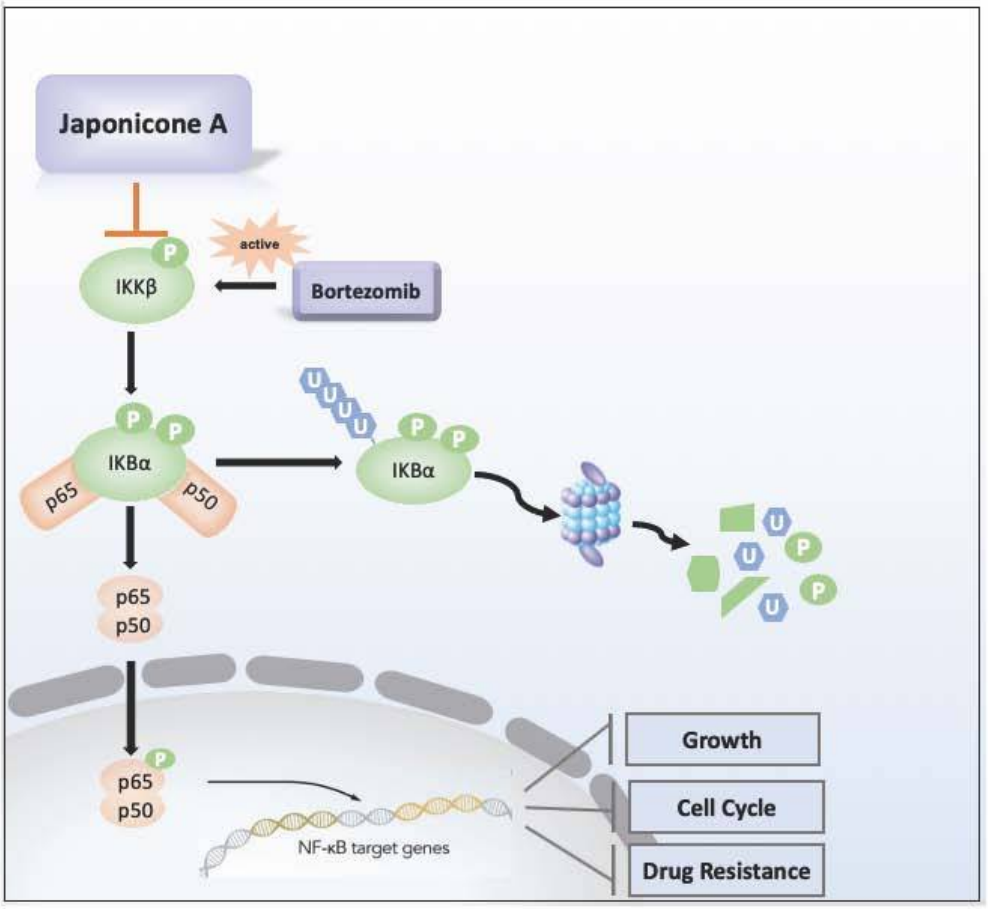

Figure 7

Mechanisms of the effect of JaponiconeA on MM cells. Bortezomib activates IKKβ, which subsequently phosphorylates IκBα. After proteasome degradation of IκBα, p50/p65 translocate to nucleus to exert their function. JA blocked the phosphorylation of IKKβ, and suppressed the IKKβ-IκBα-NF-κB axis, which enhances bortezomib-induced cytotoxicity.

Supplementary Files

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