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AK-968 efficiently inhibits proliferation and induces cell apoptosis in human multiple myeloma cells

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AK-968 is a small molecular weight compound. In this study, we evaluated its anti-tumor effects on human multiple myeloma (MM) cells. Our results demonstrated that AK-968 markedly inhibited proliferation of MM cell lines in a dose- and time-dependent manner. Additionally, our flow cytometry data showed that MM cell apoptosis was significantly induced by AK-968. As expected, caspase-3 cleavage and alternation of mitochondrial membrane potential (MMP) in the MM cells treated by AK-968 was detected or quantified by Western Blot or Flow cytometry technique, respectively. In conclusion, our results suggested that AK-968 may act as a potential drug to treat human multiple myeloma.

1. Introduction

Multiple myeloma (MM) is a kind of malignant hyperplasias, which originates from B cell line and can produce monoclonal immunoglobulin. It is characterized by malignant plasma cell infiltration of the bone marrow and results in increased level of immunoglobulin in the blood (Kumar et al. 2017). Myeloma cells growing out of control cause serious consequences, including osteoclasia, bone marrow failure, decrease of normal immunoglobulin, and renal insufficiency (Kyle et al. 2007). Although multiple myeloma remains incurable (Soekojo et al. 2018), prognosis of MM patients has gotten a measurable improvement in the past few years because of advanced treatment (Kumar et al. 2014), such as autologous stem cell transplantation (ASCT) and the application of new drugs, as well as improvements of nursing concept. (Rajkumar 2016).

Multiple myeloma is the second most frequent hematological cancer in the United States with estimated 30,770 newly diagnosed cases and 12,770 deaths in 2018, accounting for 1.8 % and 2.1 %, respectively (Issa et al. 2017). The age-specific incidence was highest in 70-79 and about 61.8 % of newly diagnosed cases cannot survive more than five years (Turesson et al. 2018).

Current therapies for MM include two or three kinds of chemotherapy drugs union treatments and autologous stem cell transplantation (ASCT) (Hideshima et al. 2000; Stewart et al. 2013). Patients with recurrent multiple myeloma are still facing a severe challenge to new therapeutic treatments, such as immunotherapy and molecular-targeted chemotherapy. The incidence of new myeloma cases has increased by an average of 0.9 % per year over the past 10 years. Between 2006 and 2015, the death rate fell by an average of 0.5 % a year. The five-year survival rate increased to 53.2 % in 2010.

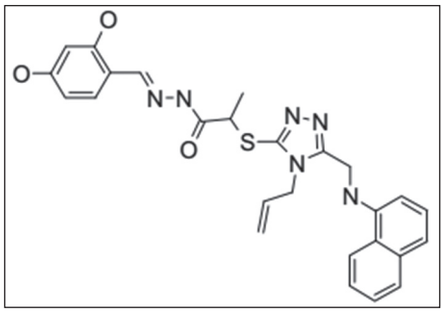

Fig. 1: Structure of AK-968.

In order to elevate the survival rate of patients with MM, we selected the newly synthesised drug AK-968 (Fig. 1) which shows effects on myeloma cell lines. It has never been studied for its anti-tumor effect. In the present study, we evaluated that AK-968 can efficiently suppress the proliferation of myeloma cells and induce the cell apoptosis. Furthermore, the underlying molecular mechanisms were confirmed by caspase-3 signaling pathway.

2. Investigations and results

2.1. AK-968 inhibited the growth of human myeloma cell lines

Cell viability was measured by an MTT assay. We used two time points and two different concentrations (Fig. 2). These results demonstrated that AK-968 inhibited the growth of human myeloma cell lines MM.1S and RPMI-8226 in a dose-and time-dependent manner. With the action time of the drug extending, AK-968 had a more obvious effect especially at concentrations of 10 and 20 μM. MM.1S were more vulnerable than RPMI-8226 when treated with AK-968 for 72 h.

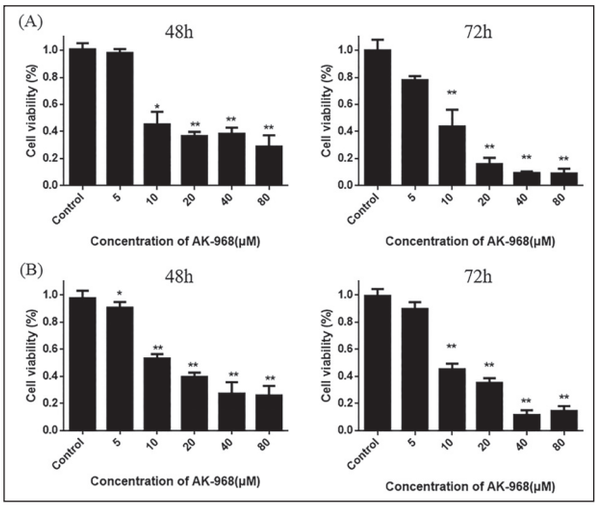

Fig. 2: AK-968 inhibited cell proliferation in human MM cell lines. (A) MM.1S and (B) PRMI-8226 human MM cells were treated with different concentrations of AK-968 for 48h and 72h, then an MTT assay was performed. Each was repeated in 3 independent experiments. **P<0.05, **P<0.01.*

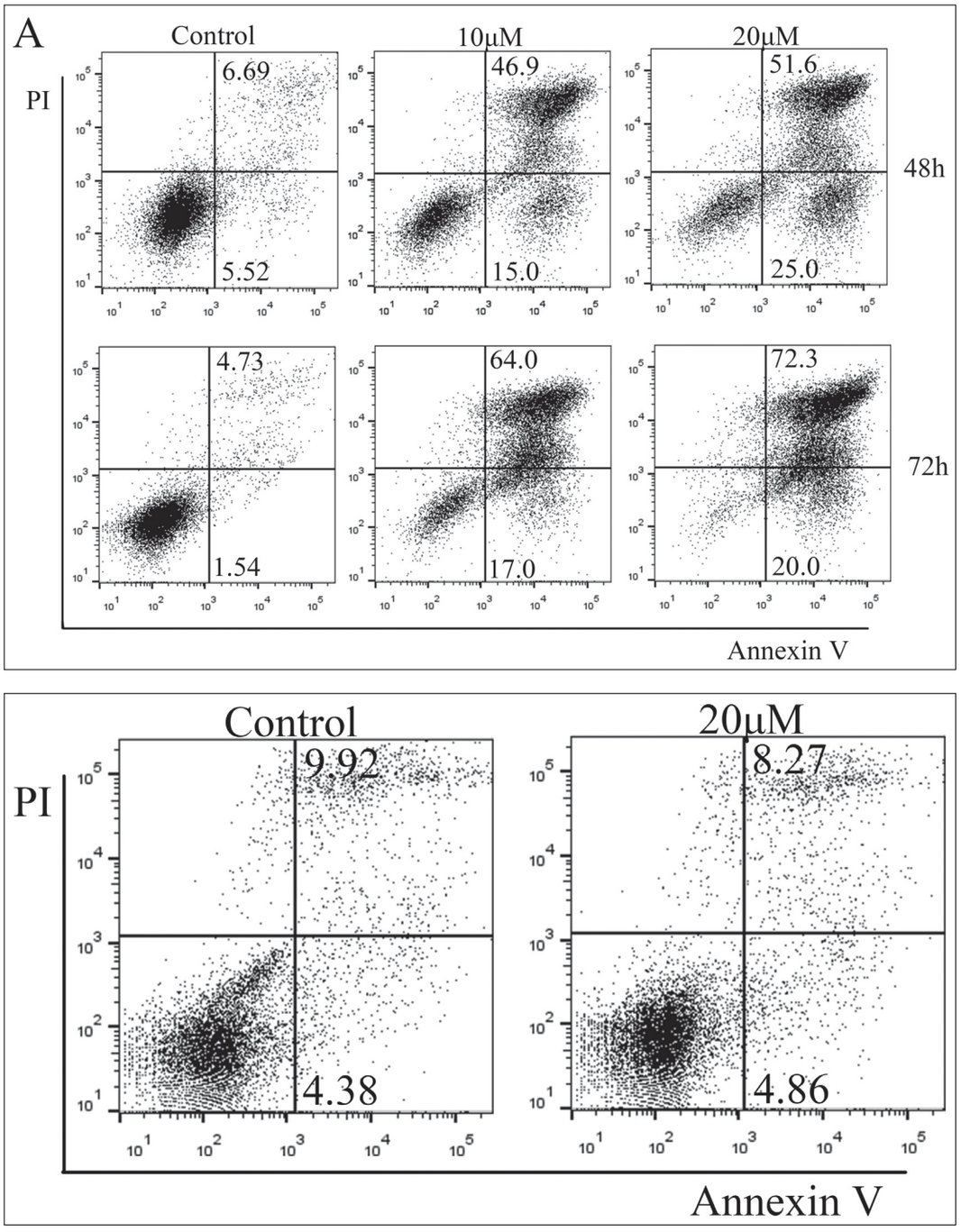

Fig. 3: AK-968 induced apoptosis in MM.1S cells but had no effect on PBMCs. Apoptosis were analyzed by flow cytometry following Annexin V FITC and PI staining. (A) MM.1S cells were treated with AK-968 at the indicated doses for 48h and72h, labeled with Annexin V and PI, then analyzed by flow cytometer. (B) Treat PBMCs with AK-968 for 48h, and detect it in the same way. The fold changes were greater than 2.

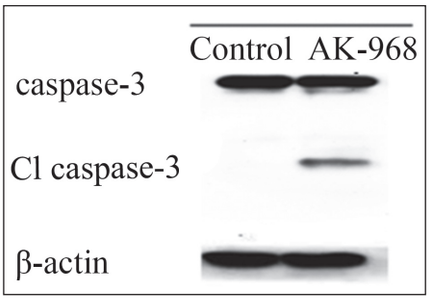

Fig. 4: AK-968 activated caspase-3. MM.1S cells were treated with AK-968 at 48h. Cell lysates were prepared and the levels of cleaved caspase-3 (CL caspase-3) were determined by western blot analysis.

2.2. AK-968 induced apoptosis of myeloma cell lines

In order to understand whether AK-968 induces the apoptosis of MM cells, we used an Annexin V-PI Apoptosis Detection Kit to detect MM cells apoptosis through a flow cytometer when treated with AK-968. Results are shown in Figs. 3A and B. Compared with DMSO treated cells, AK-968 led to a significant increase in the percentage of apoptotic cells in a dose-dependent and time-dependent manner. AK-968 had no effect on PBMCs but increased the proportion of apoptotic cells by 50 % and 65 % at the concentrations of 10 and 20 μM, respectively. Likewise, the proportions were 75 % and 85 % with the concentrations of 10 μM and 20 μM, respectively.

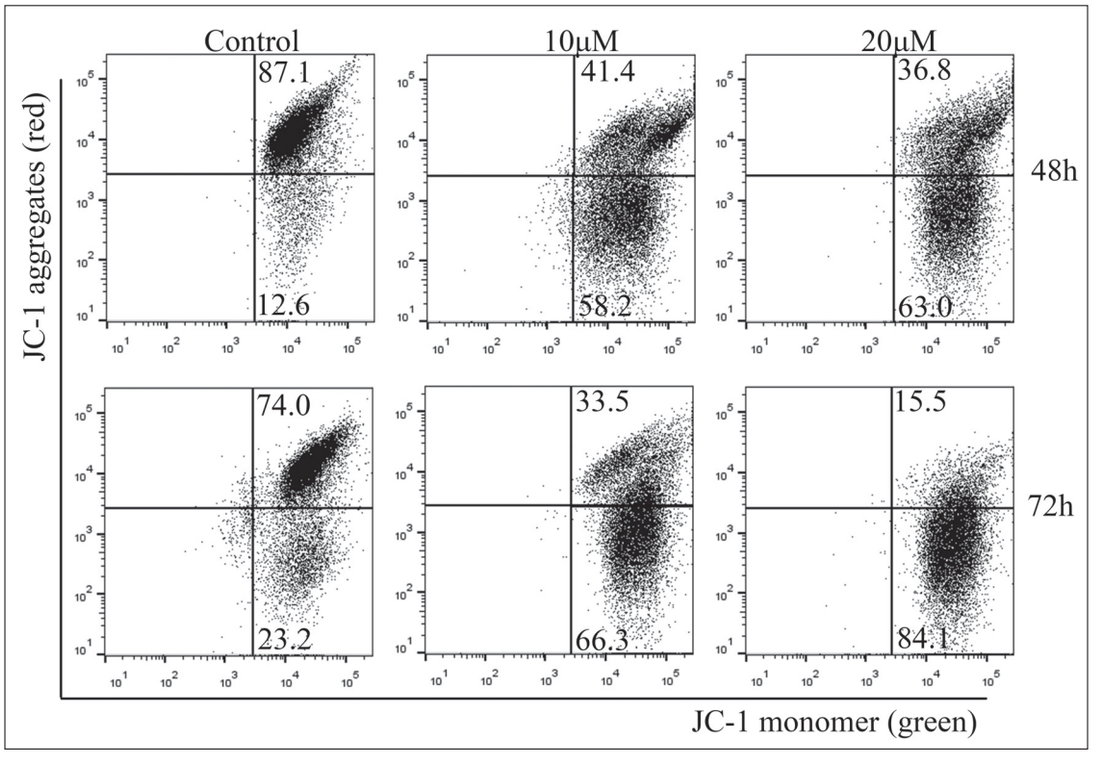

Fig. 5: AK-968 caused a membrane potential change in MM cells. Human myeloma cells MM.1S were treated with concentrations of AK-968 for 48h and 72h. The cells were collected, stained with JC-1 and the percentage of cell fluorescence distribution was analyzed by flow cytometry. The fold changes were greater than 2.

2.3. AK-968 activated caspase-3

Caspase-3 is an important part of CTL killing mechanism and plays an irreplaceable role in apoptosis. (Jänicke et al. 1998, Porter and Jänicke 1999) After treatment with AK-968, the expression of caspase-3 was decreased and the expression of cleaved caspase-3 was increased, as shown in Fig. 4. These results clearly indicate that AK-968 activates the caspase-3 then leads to apoptosis.

2.4. AK-968 affected the mitochondrial membrane potential of apoptotic celles.

Previous studies have suggested that there are remarkable changes in the early period of apoptosis, including mitochondrial membrane potential (Koya et al. 2000). Once the mitochondrial membrane potential decreases, the cell will enter an irreversible apoptosis process. JC-1 gathers in the matrix of mitochondria to form polymeraggregates, which can produce red fluorescence, when the mitochondrial membrane potential is high. When the mitochondrial membrane potential is low, JC-1 cannot accumulate in the matrix of mitochondria and forms a monomer, producing green fluorescence. As shown in Fig. 5, compared with DMSO-treated cells, AK-968 resulted in a marked alteration of MMP in the AK-968 treated cells in a dose-dependent and time-dependent manner.

3. Discussion

Multiple myeloma is the second most common hematological malignancy accounting for approximately 13 % of all hematologic cancers. Current therapies include proteasome inhibitors, immunomodulatory drugs, and monoclonal antibodies which have obvious implications for median overall survival (Chauhan et al. 2008; Mitsiades et al. 2009; van Zaanen et al. 2015). We plan to find a new anti-myeloma drug that can improve the survival rate of MM patients through our experiment. AK-968, a synthesized small molecular weight compound, is proved to have significant effects on the proliferation of myeloma cells line MM.1S in our screening experiment. In the present study, we evaluated if AK-968 can efficiently suppress proliferation and induce the apoptosis of myeloma cells. Furthermore, the underlying molecular mechanisms were discussed in view of caspase signaling pathways. Mitochondria can release apoptosis-inducing factor after mitochondrial membrane depolarization and cause apoptosis (Ozaki and Yamashita 2009). It plays a crucial role in caspase-dependent apoptosis that caspase activation leads to produce the phenotypic changes characteristic of apoptosis, such as cytoskeletal disintegration, DNA fragmentation, and disruption of cellular and DNA repair processes, which result in cell death (Wang et al. 2014). In our study, we demonstrated that AK-968 can induce apoptosis in human multiple myeloma MM.1S cells. Moreover, results from western blotting suggested that cleaved caspase-3 was significantly increased after treatment with AK-968. Besides, we stained cells with JC-1 and used flow cytometry to identify the change of mitochondrial membrane potential (MMP) (Cossarizza and Salvioli 2001). JC-1 staining showed that red fluorescence significantly decreased in AK-968-treated cells, whereas green fluorescence significantly increased simultaneously. AK-968-induced alteration in MMP, an early marker of apoptosis, suggested the occurrence of apoptosis, which was confirmed by Annexin V/PI staining.

In conclusion, our study demonstrates that AK-968 has significant effects on human multiple myeloma cells. We demonstrated that AK-968 could inhibit cell proliferation by changing MMP, and causes cell apoptosis. In addition, we also demonstrated that AK-968 activates the caspase signaling pathway and induces cell apoptosis. In the following experiments, it is necessary to make the molecular mechanism AK-968 more certain, which may give us a deeper understanding of AK-968 working on MM. In a word, the experimental results suggest that AK-968 could be a new effective antineoplastic agent which is likely to provide more options to MM patients for their therapy.

4. Experimental

4.1. Chemicals and reagents

AK-968 is a synthesized small molecule weight compound, purchased from Specs, Netherlands, a company providing chemistry and chemistry related services that are

required in drug discovery. The chemical name of AK-968 is 2-({4-allyl-5-[(1-naphthylamino) methyl]-4*H*-1,2,4-triazol-3-yl}sulfan. Its formula is $C_{26}H_{26}N_6O_3S$ and its mol. weight is 502.60.

4.2. Cell culture

The human myeloma cell lines MM.1S and RPMI-8226 were obtained from the Department of Cancer Immunology, Institute of Translational Medicine, The First Hospital of Jilin University, Changchun, China. Peripheral blood mononuclear cells (PBMCs) were obtained from healthy adult volunteers. Cells were cultivated in RPMI-1640 medium (Corning and Media tech) supplied with 10% fetal bovine serum (Gibco) and 0.5 % penicillin/streptomycin solution. Cells were cultured at 37 °C with 5 % CO_2, 95–98 % humidity.

4.3. Cell proliferation assay

The MM cells were plated into 96-well plates at a density of $1x10^4$/well and treated with different concentrations (5, 10, 20, 40, and 80 μM) of AK-968 for 48 h and 72 h and incubated at 5 % CO_2, 37 ˚C. Subsequently, 5 mg/ml MTT was added into 96-well plates and the cells were incubated at 37 °C for 4 h. The plates were centrifuged at 2000 rpm and the supernatant was removed. Then 200 μl dimethyl sulfoxide (DMSO) was added to each well, following which the optical density was measured at 490 nm using a microplate reader (Thermo Fisher). The experiments were performed in triplicate and repeated at least three times. Data are represented as the percentage of the control.

4.4. Flow cytometric analysis of apoptosis

The apoptotic rate of MM cells treated with AK-968 was measured using FCM (flow cytometry) through staining with the Annexin V-PI Apoptosis Detection Kit (KeyGEN BioTECH). Cells were cultured in 6-well plates and treated with different concentrations of AK-968 for 48 h and 72 h, then collected for staining with Annexin V and PI according to the manufacturer's protocol. To put it simply, cells were washed with PBS 2 times and resuspended in 200 μl binding buffer. To each tube was added 2 μl Annexin V/FITC solution and 2 μl propidium iodide, put in a dark place at 37 °C for 10 min. The samples were analyzed using the BD FACSAria II flow cytometer.

4.5. Flow cytometry detection of JC-1 fluorescence

To detect JC-1 fluorescence, a Mitochondrial Membrane Potential Detection Kit (KeyGEN BioTECH) was used. Cells were cultured in 6-well plates and treated with different concentrations of AK-968. The action time was set to 48 h and 72 h. Cells were collected and stained with a JC-1 kit after completion of treatment according to the manufacturer's protocol. To put it simply, cells were washed with PBS 2 times and resuspended in 100 μl binding buffer. To each tube was added 500 l JC-1 solution, put in a dark place at 37 °C for 20 min. The samples were analyzed using a BD FACSAria II flow cytometer.

4.6. Western blot analysis

Total cellular protein was extracted using a protein extraction kit and protein concentration was measured using a BCA protein kit. The devitalized protein samples of 20 μg each were added into SDS-PAGE (Beyotime) and transferred onto a polyvinylidene difluoride membrane. Following this, the membrane was blocked in 5 % skim milk (Solabio) for 1 h and then put in primary antibodies solution including anti-β-actin and anti-caspase-3 (both 1:1000) at 4 °C overnight. The second day, wash the membrane in PBS-Tween20 10 min at a time for 3 times, and then put in secondary antibody (1:5000) at the room temperature for 1 h. Wash it 3 times with PBS-Tween20. Add ECL and digitalize by scanning.

4.7. Statistical analysis

All MTT assays were performed in at least three independent experiments and expressed as mean±SE. The statistical differences were calculated by one-way ANOVA analysis of variance with unpaired Student's t-test. P values < 0.05 is considered significant. All flow cytometry detections were performed in two independent experiments. Fold changes of mean values > 2 were considered significant.

Conflicts of interest: None declared.

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