

ORIGINAL ARTICLE

Bromodomain-containing protein 4 inhibitor JQ1 promotes melanoma cell apoptosis by regulating mitochondrial dynamics

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Funding information

The Science and Technology Natural Foundation of Jilin Provincial, Grant/Award Number: 20200201125JC; The National Natural Science Foundation of China, Grant/Award Number: 81370240; Research Foundation of the Education Department of Jilin province (JJKH20211171KJ).

Abstract

Although the role of bromodomain-containing protein 4 (BRD4) in ovarian cancer, pancreatic cancer, lymphoma, and many other diseases is well known, its function in cutaneous melanoma is only partially understood. The results of the present study show that the BRD4 inhibitor JQ1 promotes the apoptosis of B16 melanoma cells by altering mitochondrial dynamics, thereby inducing mitochondrial dysfunction and increasing oxidative stress. We found that treatment of B16 cells with different concentrations of JQ1 (125 nmol/L or 250 nmol/L) significantly downregulated the expression of protein subunits involved in mitochondrial respiratory chain complexes I, III, IV, and V, increased reactive oxygen species, induced energy metabolism dysfunction, significantly enhanced apoptosis, and activated the mitochondrial apoptosis pathway. At the same time, JQ1 inhibited the activation of AMP-activated protein kinase, a metabolic energy sensor. In addition, we found that the mRNA and protein levels of mitochondrial dynamin-related protein 1 increased, whereas the levels of mitochondrial fusion protein 1 and optic atrophy protein 1 decreased. Mechanistically, we determined that JQ1 inhibited the expression of c-Myc and altered mitochondrial dynamics, eventually leading to changes in the mitochondrial function, metabolism, and apoptosis of B16 melanoma cells.

KEYWORDS

apoptosis, BRD4, energy metabolism, melanoma, mitochondrial dynamics

1 | INTRODUCTION

Melanoma is one of the most malignant skin tumors. The SEER database (<http://seer.cancer.gov/statfacts/ht>) reports that there were more than 192 000 new invasive melanoma skin cancer cases in 2019, making it the fifth most common cancer. Although early melanoma can be effectively treated by surgery and targeted therapies and small molecule inhibitors for melanoma have been successfully developed,¹ the prognosis of the disease remains poor. Therefore, a

better understanding of the pathogenesis of melanoma is urgently needed to enable new ideas for the treatment of these patients.

Bromodomain-containing protein 4 (BRD4) is a member of the bromodomain and extra terminal domain (BET) protein family. As a transcriptional and epigenetic regulatory factor, BRD4 plays a key role in embryogenesis and cancer development.^{2,3} JQ1 is a small molecule inhibitor targeting BRD4 that displaces the protein from chromatin by mimicking acetyl groups and binding to the acetyl-lysine pocket. JQ1 exerts its antitumor effects by regulating the

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transcription of *c-Myc* and other genes downstream of BRD4.^{4,5} Recent studies have shown that changes in mitochondrial dynamics can regulate the progression of melanoma cells.⁶ In prostate cancer stem cells (CSC), BRD4 inhibition decreases *Mff* transcription, leading to progressive mitochondrial dysfunction and aging.^{7,8} We analyzed the OncoPrint database and found that BRD4 is highly expressed in the melanoma dataset. Similarly, the GEPIA database showed that BRD4 expression in melanoma was negatively correlated with the overall survival rate of patients. However, the relationship between mitochondrial dynamics and BRD4 expression in melanoma remains unknown and requires further research.

As the “energy factory” of the cells, mitochondria provides energy for cellular life activities.⁹ Under normal circumstances, the balance between mitochondrial fission and fusion is maintained, but when this balance is disturbed, mitochondrial apoptosis pathways can be activated.^{10,11} During apoptosis, mitochondrial dynamin-related protein 1 (Drp1) is recruited to mitochondria, where it colocalizes with Bax.¹² However, the relationship between mitochondrial dynamics and apoptosis in melanoma cells has not been well studied. Furthermore, metabolic pathways are significantly different between cancer cells and normal cells.¹³ The dynamic regulation of glycolysis, oxidative phosphorylation (OXPHOS), and fatty acid oxidation by mitochondria plays an important role in the development of tumor cells.¹⁴ Mitochondria can regulate the survival and apoptosis of tumor cells by modulating cell metabolism. On the one hand, mitochondria activates caspase-dependent pathways by modifying the permeability of the inner and outer membranes to induce apoptosis.¹⁵ On the other hand, changes in mitochondrial morphology and function induce alterations in fatty acid oxidation (FAO), the tricarboxylic acid (TCA) cycle, the electron transport chain (ETC), reactive oxygen species (ROS), mitochondrial respiration, and energy metabolism,¹⁶ which eventually results in cell death.

In this study, we found that B16 cells treated with JQ1 showed mitochondrial respiratory chain dysfunction, changes in energy metabolism, and activated mitochondrial apoptosis. JQ1 may change mitochondrial dynamics by inhibiting the expression of *c-myc*, a downstream target of BRD4, and promoting B16 melanoma cell apoptosis.

2 | MATERIALS AND METHODS

2.1 | Cell culture

B16 melanoma cells were purchased from the Chinese Academy of Sciences (Shanghai) Cell Bank. B16 cells were cultured in DMEM medium supplemented with 10% FBS (Clark) and 1% penicillin and streptomycin sulfate (Hyclone). The cells were cultured in an incubator at 37°C with 5% CO₂.

TABLE 1 Primer sequences

Primer name	Forward and Reverse primer	Sequence (5'-3')
GAPDH	Forward	GGTTGTCTCCTGCGACTTCA
	Reverse	TGGTCCAGGGTTTCTTACTCC
DRP1	Forward	ACTGATTCATCCGTGATGAGT
	Reverse	GTAACCTATTCCAGGGTCTAGC
Fis1	Forward	CCTGGTTCGAAGCAAATACAAT
	Reverse	CTTTTCATATTCTTGAGCCGG
OPA1	Forward	CTTACATGCAGAATCCTAACGC
	Reverse	CCAAGTCTGTAACAATACTGCG
Mfn1	Forward	CCATCTTTCAGGTCCTAGATC
	Reverse	GCTCCGTACATACTTAAGGTGA
Mfn2	Forward	GCATTCTGTGGTCCGAGGAGTG
	Reverse	TGGTCCAGGTCAGTCGCTCATAG
BRD4	Forward	CCAAGATGCCTGATGAGCCTGAAG
	Reverse	GCTGTGCTGTCGGAAGAAGCTG
<i>c-Myc</i>	Forward	TTCTATCACCAGCAACAGCAGAGC
	Reverse	CGCAACATAGGATGGAGAGCAGAG
siBRD4	Forward	GCCUGAGAUGAAGCCUGUATT
	Reverse	UACAGGCUUCAUCUCAGGCTT
ND1	Forward	CGGCCCATTCGCGTTATTCT
	Reverse	CGGAAGCGTGGATAGGATGC
Rn18s	Forward	GGCGGCTTGGTGACTCTAGATAAC
	Reverse	CCTGCTGCCTTCCTTGGATGTG

2.2 | Quantitative PCR analysis

Total RNA was extracted using TRIzol (Invitrogen), and cDNA synthesis was performed using the Super RT Kit (BioTeke). Quantitative PCR (qPCR) experiments were conducted using a 2× plus SYBR real-time PCR mixture (BioTeke). The relative expression levels were calculated with the formula $2^{-\Delta\Delta CT}$. The results were normalized to the expression level of GAPDH and expressed relative to the control group (CON, B16 cells treated without drugs). The primer sequences are presented in Table 1.

2.3 | MTT assay

B16 cells were seeded into 96-well plates, and different concentrations of JQ1 or mitochondrial division inhibitor 1 (Mdivi-1) were added the next day. The Mdivi-1 and JQ1+Mdivi-1 groups were pretreated with 2 μmol/L Mdivi-1 1 hour before treatment with DMSO or JQ1. At 24, 48, and 72 hours, 20 μL MTT (5 mg/mL) were added and incubated for 4 hours in the dark. The medium was discarded from each well, and 150 μL of DMSO was added. The absorbance was measured at 570 nm (FLUOstar Omega).

2.4 | Cell counts

B16 cells were seeded in a 24-well plate at a concentration of 1×10^5 cells/well. Cells were collected at 24, 36, 48, and 72 hours after treatment. The number of cells was counted under a microscope, and growth curves of cells under different concentrations and time points were generated according to the number of cells.

2.5 | Assessment of cell apoptosis

In a 6-well plate, cells were collected after 48 hours of drug treatment and washed twice with PBS. Then, 100 μ L 1 \times buffer and Annexin V-FITC probe (5 μ L) was added, mixed, and incubated in the dark at room temperature for 10 minutes. Next, 5 μ L propidium iodide was added, mixed, and incubated for 5 minutes in the dark. Finally, 300 μ L 1 \times buffer was added, and cells were examined by flow cytometry (BD FACSCalibur) within 1 hour.

2.6 | Detection of mitochondrial membrane potential ($\Delta\Psi$) and reactive oxygen species by flow cytometry

After 48 hours of drug treatment in 6-well plates, the cells were collected and serum-free DMEM medium containing the JC-1 probe or DCFH-DA probe was added and incubated in the dark at 37°C for 20-30 minutes. Then, the cells were centrifuged at 700 g for 5 minutes, washed twice with serum-free culture medium, centrifuged at 200 g for 5 minutes, resuspended in serum-free medium, and immediately analyzed using a flow cytometer (BD FACSCalibur) to detect the fluorescence intensity.

2.7 | Hoechst 33258 staining assay

B16 cells were plated in a 12-well plate at a density of 1×10^4 cells/well. After incubation with the drug for 48 hours, the cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and carefully washed with PBS three times. Then, 400 μ L Hoechst 33258 dye (1 μ g/mL) was added for 2 minutes at room temperature in the dark, and the cell nuclei were analyzed under a fluorescence microscope.

2.8 | MitoTracker Green staining assay

B16 cells were incubated with 200 nmol/L MitoTracker Green (Beyotime) working solution for 30 minutes in an incubator and washed once with serum-free medium pre-warmed to 37°C. Next, 400 μ L of Hoechst 33258 dye was added to the cells and incubated in the dark at room temperature for 2 minutes. The cells were observed with a fluorescence or laser confocal microscope.

2.9 | Western blot

Total protein was prepared using RIPA Lysis Buffer (BostonBioProducts) containing PMSF (Sigma), quantified with a BCA Protein Assay Kit (Beyotime) and subjected to immunoblotting following standard protocols. Electrochemiluminescence assays were used to detect protein content. The antibodies used in this study are presented in Table S1. β -actin was used as an endogenous control and COX-IV as the mitochondrial control. All antibodies were diluted 1:1000 for the working solution. The density of the bands was analyzed using Image J software (NIH Image).

2.10 | Cell mitochondrial isolation

Cells were collected (5×10^7), and the mitochondrial protein was extracted using a Cell Mitochondrial Isolation Kit (Beyotime) according to the manufacturer's instructions.

2.11 | Determination of glucose and lactic acid contents

B16 cells were seeded into 6-well plates at a density of 3×10^5 cells/well and treated with drugs for 24 hours. Cell culture medium was collected, cells were centrifuged for 10 minutes at 200 g, and the supernatant was collected for future use. The protein concentration was quantified using the BCA Protein Assay Kit (Beyotime). A glucose detection reagent or lactic acid detection reagent was added to the supernatant according to the instructions, mixed, and incubated at 37°C for 10 minutes. The optical density of each well at 505 nm was measured, and the glucose and lactic acid contents were calculated according to the formula provided.

2.12 | Determination of triglyceride content

Cells were collected with a cell scraper and centrifuged at 168 g for 10 minutes at room temperature. The cell pellet was washed twice with 0.1 mol/L pH 7.4 PBS, centrifuged at 1000 rpm for 10 minutes, and then 200 μ L of 0.1 mol/L pH 7.4 PBS was added to the cell pellet. Finally, the cells were sonicated for 10-15 seconds in an ice-water mixture, and the uncentrifuged lysed liquid was directly measured according to the instructions.

2.13 | Measurement of mtDNA content

mtDNA was extracted from cells according to the instructions of the TIANamp Genomic DNA Kit (Tiangen Biotech). The DNA concentration was measured using a BioPhotometer (Eppendorf). The relative mtDNA content was measured by qPCR and the $2^{-\Delta\Delta CT}$ method. ND1

primers were used for mtDNA detection, and Rn18s primers were used for normalization. The primer sequences were presented in Table 1.

2.14 | Transmission electron microscopy

The cells were collected with a cell scraper and centrifuged at 700 g for 5 minutes. A 2.5% glutaraldehyde electron microscope fixing solution was added to the cell pellet for more than 4 hours. The samples were then processed and imaged by Wuhan Sevier Biological.

2.15 | Statistical analysis

All presented data were confirmed in a minimum of three independent experiments and expressed as mean \pm SD. All statistical analyses were performed with GraphPad Prism 7 (GraphPad Software). A one-way ANOVA followed by Student's *t*-test and Tukey's tests were used to compare differences between groups. $P < .05$ was considered statistically significant.

3 | RESULTS

3.1 | Bromodomain-containing protein 4 inhibition JQ1 attenuates B16 melanoma cell proliferation in vitro

To verify the effect of BRD4 inhibition on melanoma proliferation, we treated B16 melanoma cells with JQ1. The MTT and cell counts indicated that compared with the CON group, JQ1 can induced rapid growth inhibition of B16 cells at 48 hours (Figure 1A-B). At 48 hours, 125 nmol/L and 250 nmol/L JQ1 had the most obvious effect, so these concentrations were selected as the experimental doses for subsequent experiments. The transcription factor *c-Myc* is one of the primary targets of BRD4 inhibitors. After 48 hours of JQ1 treatment, compared with the CON group, we observed no significant changes in BRD4 protein expression and a slight increase in its mRNA level. In contrast, both the protein and mRNA levels of *c-Myc* were significantly decreased (Figure 1C-E), as determined by western blot and qPCR. These results show that the effect of JQ1 on B16 cells may be related to *c-Myc*.

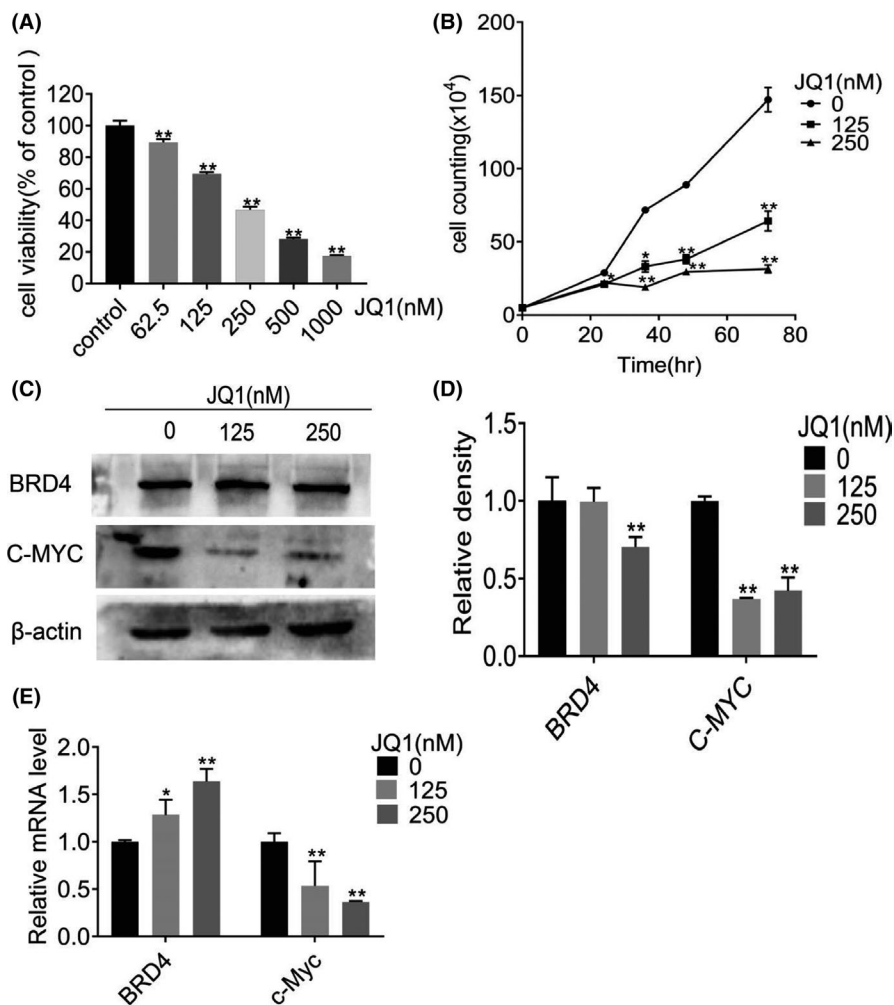


FIGURE 1 Bromodomain-containing protein 4 (BRD4) inhibition of JQ1 inhibits B16 cell proliferation. (A) Cell viability of B16 cells detected by MTT. (B) Growth counts showing the proliferation of B16 cells after JQ1 treatment. (C,D) BRD4 and C-MYC protein levels after JQ1 treatment for 48 h. (E) mRNA levels detected by quantitative PCR. * $P < .05$ vs CON, ** $P < .01$ vs CON

3.2 | Bromodomain-containing protein 4 inhibitor JQ1 promotes B16 cell apoptosis through the mitochondrial pathway

The mitochondrial apoptotic pathway is located upstream of caspase activation and is mediated by the Bcl-2 family proteins.¹⁷ To determine whether melanoma cell growth inhibition was mediated by the mitochondrial apoptotic pathway, we evaluated the biological markers of cell death at 48 hours by flow cytometry and western blot. The results showed that JQ1 increased the proportion of Annexin-V positive apoptotic cells in a dose-dependent manner (Figure 2A,B). The Hoechst staining assay results demonstrated that the nuclear morphology in B16 cells was irregular after JQ1 treatment: specifically, the nucleus shrank and parts of the nuclei were fragmented. In addition, dense staining around the nucleus and an uneven distribution of nuclear chromatin were observed. The above phenomena were more prominent in the 250 nmol/L treatment group than in the 125 nmol/L group (Figure 2C). Furthermore, increased expression of the pro-apoptotic proteins BAX and cleaved-caspase3 and decreased expression of the anti-apoptotic protein BCL-2 (Figure 2D,E) were observed by western blot. JQ1 reduced the mitochondrial membrane potential and increased ROS production in B16 melanoma cells (Figure 3A-F). At the same time, cleaved-caspase 9 and cleaved-PARP protein expression levels and the release of Cyt-C were increased (Figure 3G-I), suggesting that BRD4 inhibition may promote apoptosis through the mitochondrial pathway.

3.3 | Bromodomain-containing protein 4 inhibitor JQ1 impairs the mitochondrial function of B16 cells and increases oxidative stress

Mitochondrial dysfunction is a hallmark of many diseases, and mitochondria play a central role in the regulation of apoptosis.¹⁸ To understand the changes in mitochondrial function after BRD4 inhibition, we analyzed the expression of the mitochondrial respiratory chain complex I, III, IV, and V, proteins NDUV1, CYC1, COX7C, and ATP5F1, as well as the mitochondrial folding protein HSP60 and superoxide dismutase 2 (SOD2) by western blot. The results showed that after JQ1 treatment, the mitochondrial complex subunits protein expression levels were downregulated, with CYC1 and COX7C showing the most significant decrease (Figure 4A and B). HSP60 and SOD2 expression were also downregulated (Figure 4C and D). These results indicated that after JQ1 treatment, B16 cells had reduced expression of mitochondrial complex subunits and exhibited disordered electron transfer, which may explain the increase in ROS and the decrease in mitochondrial membrane potential ($\Delta\Psi$).

When the number of mitochondria decreases, the function of mitochondria can also be reduced.^{19,20} Therefore, we tested the changes in mtDNA copy number and the results showed that the mtDNA copy number gradually decreased with the increase in drug concentration (Figure 4E), which partially explained how BRD4 inhibition induced mitochondrial dysfunction.

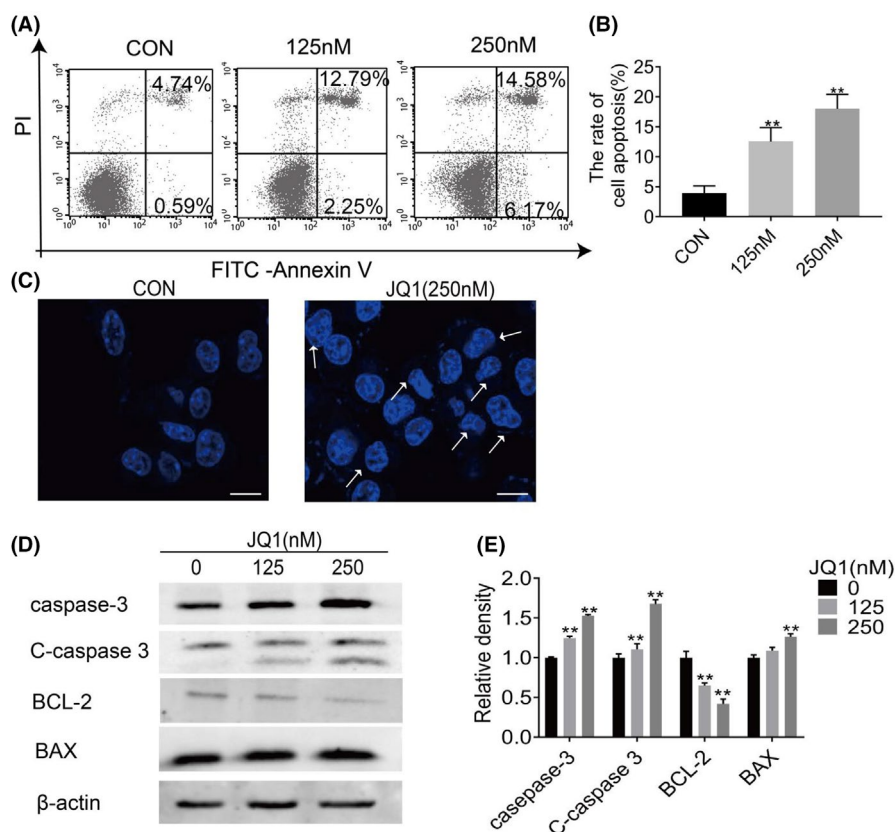


FIGURE 2 JQ1 promotes B16 cell apoptosis. (A, B) Apoptosis of B16 cells after JQ1 treatment for 48 h. (C) Hoechst staining assay showing changes in the nucleus of B16 cells. Scale bar = 20 μ m. (D, E) Western blot analysis of apoptosis-related proteins. * $P < .05$ vs CON, ** $P < .01$ vs CON

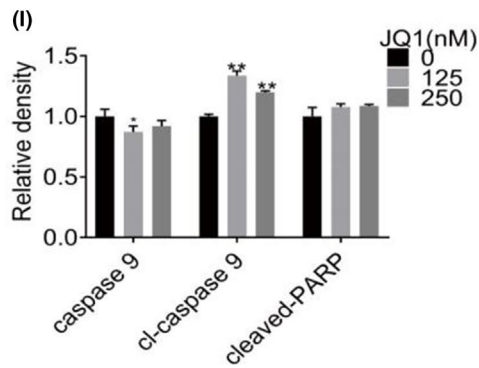
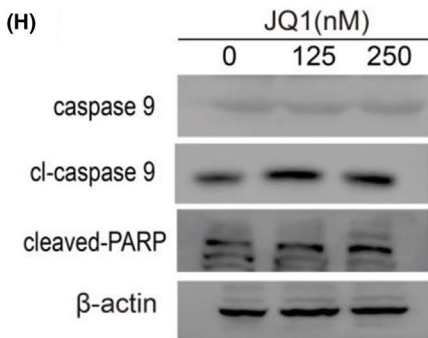
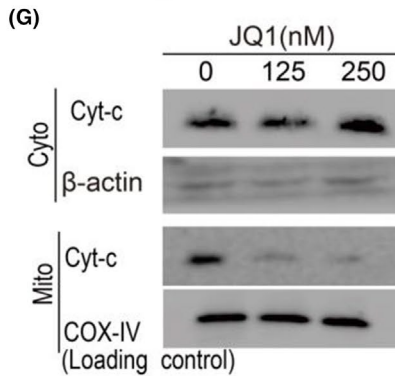
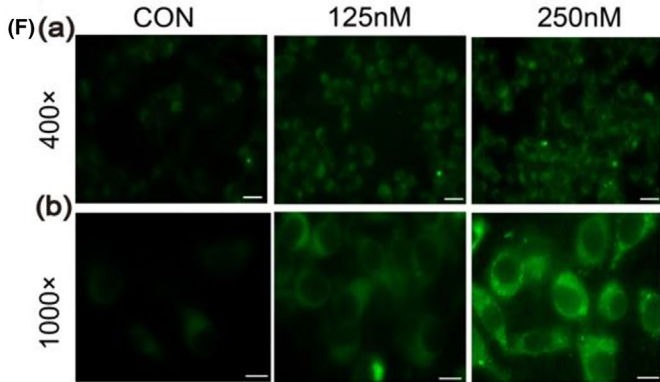
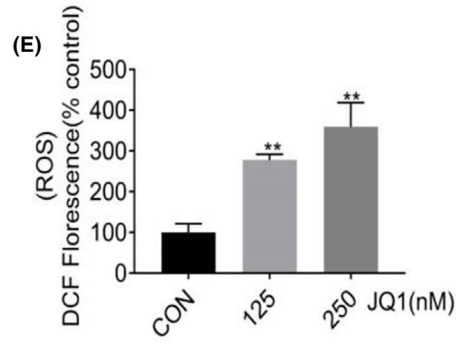
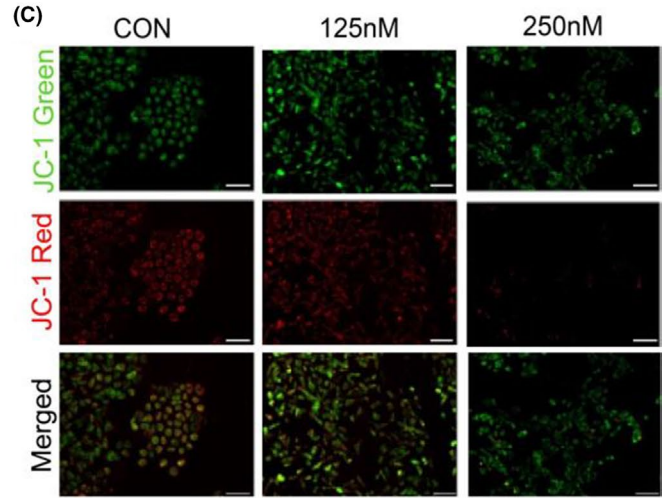
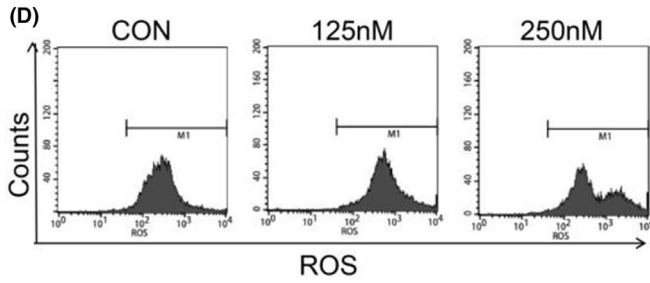
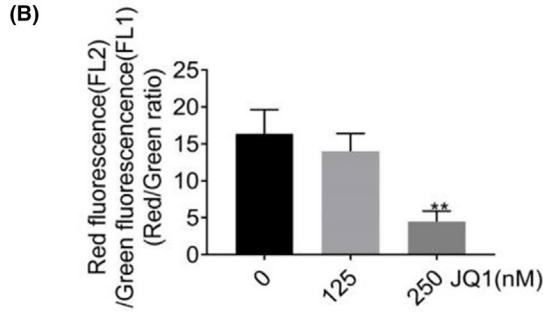
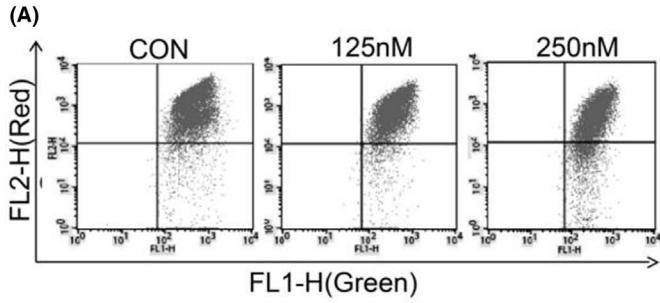


FIGURE 3 JQ1 promotes B16 cell apoptosis through the mitochondrial pathway. (A, B) The mitochondrial membrane potential of B16 cells detected by flow cytometry. (C) JC-1 immunofluorescence staining showing the fluorescence intensity in B16 cells. Scale bar = 50 μm . (D) Reactive oxygen species (ROS) levels detected by flow cytometry. (E) Quantification of ROS levels. (F) DCFH-DA immunofluorescence staining showing the ROS fluorescence intensity in B16 cells. Figure 3F(a), magnification = 400 \times , scale bar = 20 μm ; Figure 3F(b), magnification = 1000 \times , scale bar = 10 μm . (G) Protein levels of mitochondrial cytochrome C. (H, I) Levels of mitochondrial apoptosis-related proteins. * $P < .05$ vs CON, ** $P < .01$ vs CON

3.4 | Bromodomain-containing protein 4 inhibitor JQ1 inhibits glucose and lipid metabolism and alters energy production in B16 cells

The Warburg effect is closely related to increased glucose uptake, and melanoma cells are more sensitive than other tumors to the inhibition of glycolysis.^{21,22} Therefore, we examined changes in glycolysis and lactic acid production in B16 cells after JQ1 treatment for 24 hours. The results showed that after JQ1 treatment, the expression of proteins involved in glucose metabolism was downregulated (Figure 5A-D), and glucose uptake and lactic acid production were reduced (Figure 5G,H). Our results show that inhibition of BRD4 may rapidly reduce glucose production.

Increasing studies show that OXPHOS and fatty acid synthesis play important roles in tumor cell proliferation and metastasis.²³ We found that the BRD4 inhibitor JQ1 reduced isocitrate

dehydrogenase 2 (IDH2) protein expression, indicating that the TCA cycle and mitochondrial oxidative phosphorylation may be impaired. At the same time, the protein expression of the key enzyme required for lipid synthesis ATP citrate lyase (ACLY) was also downregulated (Figure 5E,F), and the triglyceride content in B16 cells after JQ1 treatment was decreased (Figure 5I), suggesting that fat synthesis was reduced.

3.5 | Bromodomain-containing protein 4 inhibitor JQ1 affects mitochondrial dynamics in melanoma cells

The regulation of mitochondrial dynamics is an important mechanism for mitochondrial quality control, and it is essential in maintaining normal mitochondrial morphology and function.²⁴ To determine whether the effect of BRD4 inhibition on B16 cells is related to mitochondrial

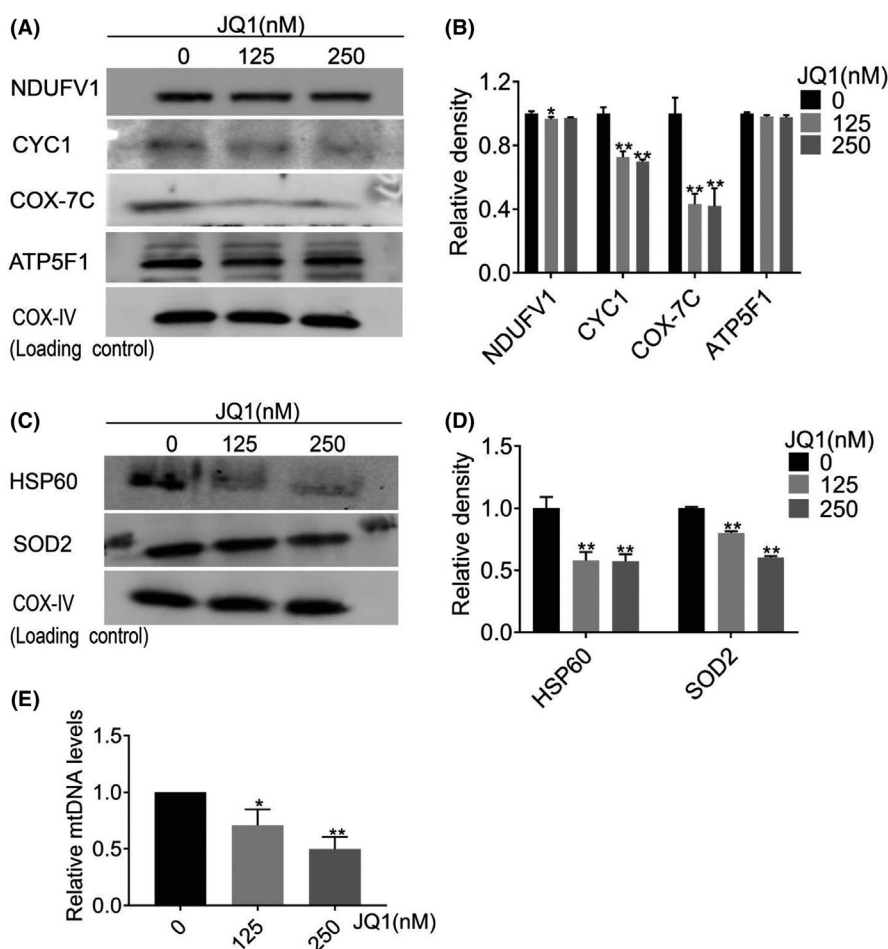


FIGURE 4 JQ1 inhibits mitochondrial function. (A, B) The levels of mitochondrial respiratory chain complex-related proteins. (C, D) Western blot detection of HSP60 and SOD2 protein contents. (E) Changes in the copy number of mitochondrial DNA (mtDNA). * $P < .05$ vs CON, ** $P < .01$ vs CON

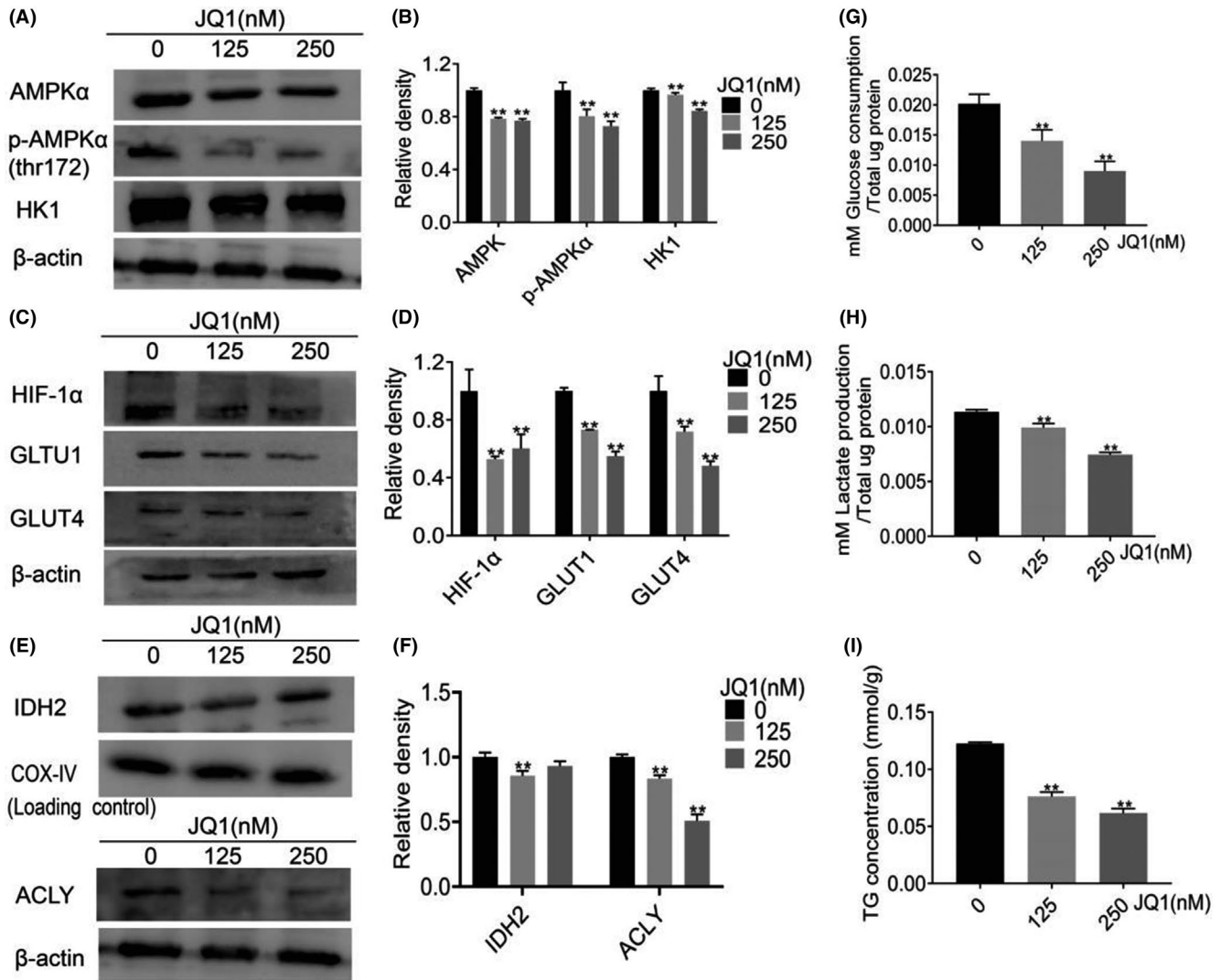


FIGURE 5 Alterations in glycolysis, oxidative phosphorylation, and fatty acid metabolism in B16 cells. (A-D) Western blot detection of glycolysis-related proteins. (E,F) The levels of tricarboxylic acid and fatty acid synthesis proteins. (G-I) Glucose, lactic acid, and triglyceride contents. * $P < .05$ vs CON, ** $P < .01$ vs CON

dynamics, we detected the protein and mRNA expression of factors involved in mitochondrial dynamics by western blot and qPCR, respectively. The mitochondrial-specific fluorescent probe Mito Tracker Green was used to stain B16 cells and identify changes in mitochondrial density. In addition, mitochondrial morphological changes were observed using transmission electron microscopy.

The results showed that the protein and mRNA level of mitochondrial fusion factors (mitochondrial fusion protein 1 [MFN1] and optic atrophy protein 1 [OPA1]) decreased, whereas the levels of DRP1 increased (Figure 6A-C). Mito Tracker Green staining showed that the mitochondria of a single cell were fragmented after JQ1 treatment, which was related to the increase of mitochondrial fission (Figure 6D). Mitochondrial division inhibitor 1 (Mdivi-1) acts as a mitochondrial division inhibitor, which can inhibit the fission of mitochondria. In the JQ1+Mdivi-1 combined treatment group, it was observed that the degree of mitochondrial fragmentation after JQ1 treatment was significantly reduced, and the connection of cells into a network increased

(Figure 6D). Transmission electron microscopy analysis confirmed that compared with untreated cells, B16 cells treated with JQ1 had smaller mitochondria but an increased number of mitochondria. Instead of the typical tubular mitochondrial phenotype of healthy cells, the mitochondria in treated cells were small and sometimes appeared as a spherical organelle. (Figure 6E). The number of the individual mitochondrion in these cells increased by 3.1 ± 0.29 -fold. The above results indicated that BRD4 regulates mitochondrial dynamics and that JQ1 treatment increases mitochondrial fission.

3.6 | Bromodomain-containing protein 4 inhibitor JQ1 promotes apoptosis by increasing mitochondrial fission

To verify the effect of BRD4 inhibition on the mitochondrial dynamics in B16 melanoma cells, we observed the changes in mitochondrial

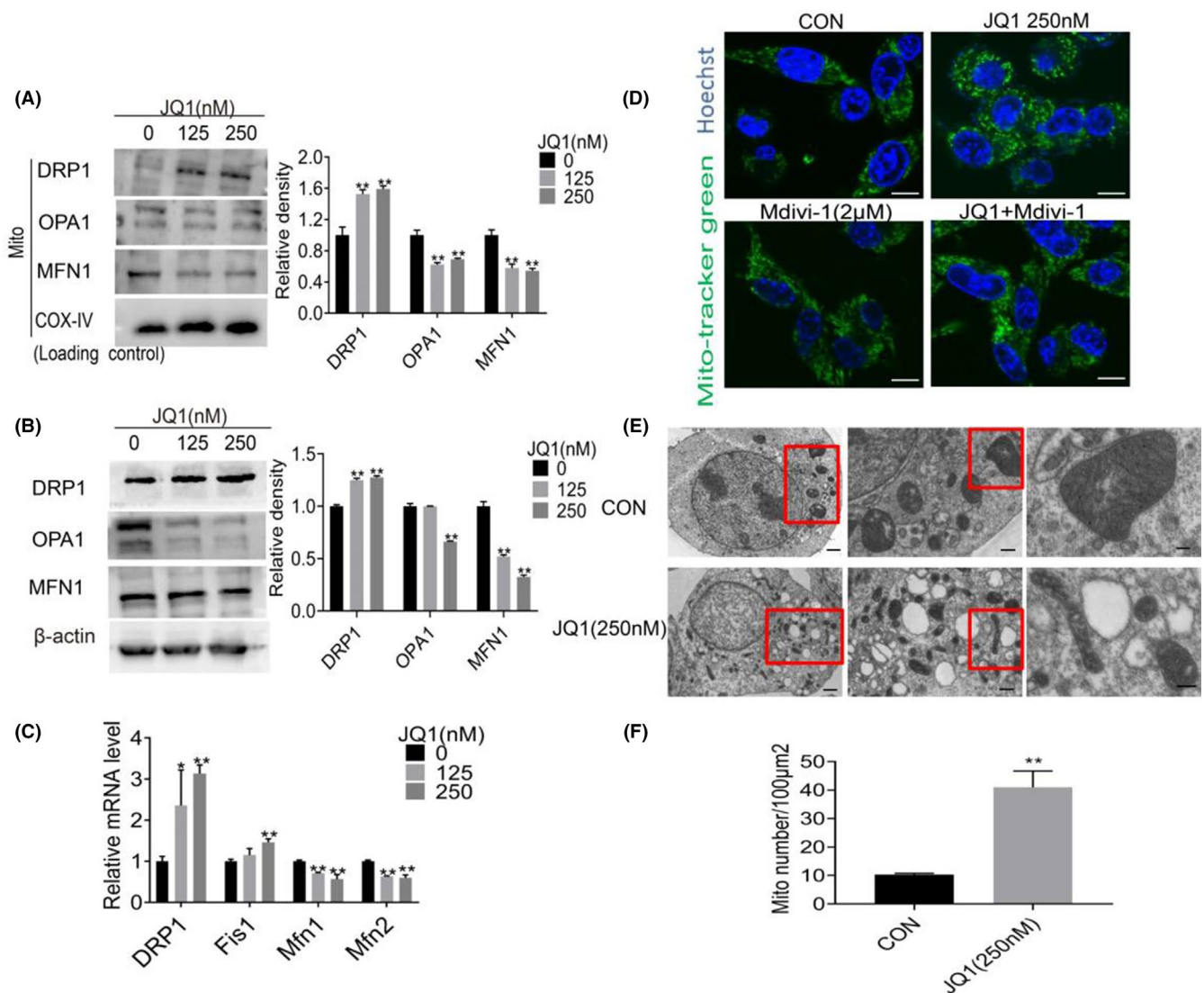


FIGURE 6 JQ1 induces changes in mitochondrial dynamics in B16 cells. (A, B) The levels of mitochondrial dynamics-related proteins in mitochondrial and total cell extracts. (C) The levels of mitochondrial dynamics-related mRNAs. (D) MitoTracker Green staining showing changes in mitochondrial density. Scale bar = 10 μ m. (E) Electron microscope of B16 melanoma cells. Representative images at 2000 \times (bar = 5.0 μ m), 5000 \times (bar = 2.0 μ m), and 15 000 \times (bar = 1.0 μ m) magnifications are shown. (F) Transmission electron microscopy images demonstrated mitochondria number and size of B16 cells with or without JQ1 addition. Corresponding quantification demonstrates percentage of mitochondria area fraction, mitochondria number per 100 μ m² and single mitochondria area. * $P < .05$ vs CON, ** $P < .01$ vs CON

dynamics of B16 cells by using mitochondrial division inhibitor 1 (Mdivi-1) to inhibit mitochondrial fission or using siRNA to silence BRD4. The experimental results showed that siBRD4 can significantly inhibit the proliferation of B16 cells and reduce the expression of C-MYC protein (Figure 7A-B). The results of confocal laser microscopy and western blot confirmed that siBRD4 caused mitochondrial fragmentation, increased DRP1 protein expression, and significantly decreased the fusion-related protein MFN1, which was consistent with the experimental results of JQ1 treatment of B16 cells (Figure 7C-D). At the same time, the addition of Mdivi-1 can improve the degree of mitochondrial fragmentation caused by siBRD4 (Figure 7C). The

experimental results in Figure 8 showed that JQ1 treatment groups pretreated with Mdivi-1 (2 μ mol/L) exhibited significantly reduced apoptosis, and the decreased mitochondrial membrane potential ($\Delta\Psi$ m) was improved. In contrast, Mdivi-1 treatment alone had no significant effect on apoptosis or the mitochondrial membrane potential (Figure 8A-D). Furthermore, we found that following the addition of Mdivi-1, the increased ROS and decreased glucose intake and lactic acid production induced by JQ1 treatment were recovered (Figure 8E-H). Western blot analysis showed that the combination of Mdivi-1 and JQ1 inhibited the expression of DRP1 protein and increased the expression of the fusion protein MFN1 (Figure 8I,J).

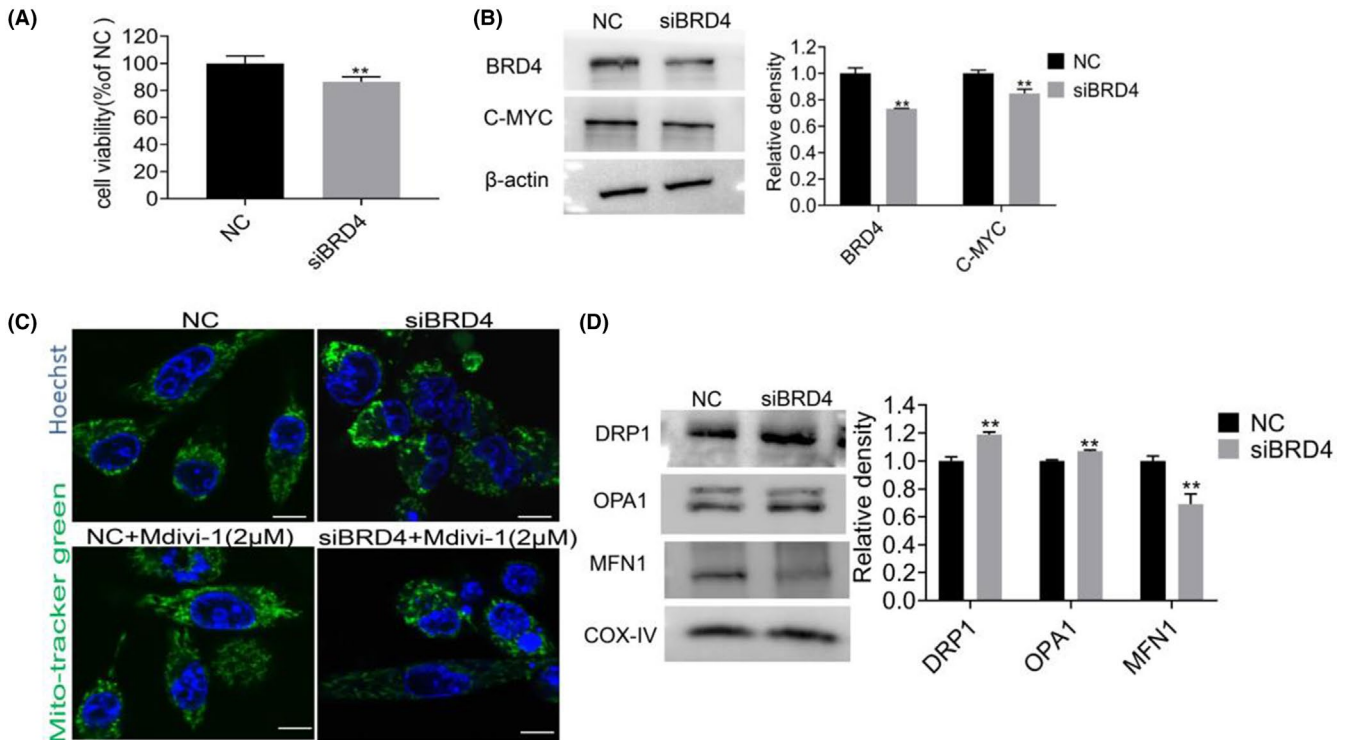


FIGURE 7 The effect of siBRD4 on the proliferation and mitochondrial dynamics of B16 melanoma cells. (A) Cell viability of B16 cells after siBRD4. (B) BRD4 and C-MYC protein levels after siBRD4. (C) Confocal laser microscope showed mitochondrial morphology changes after siBRD4. Scale bar = 10 μm. (D) Western blot showed changes in mitochondrial dynamics related proteins. * $P < .05$ vs NC, ** $P < .01$ vs NC

4 | DISCUSSION

Epigenetic disorders have been identified to play a key role in the activation and maintenance of abnormal transcriptional programs in the pathogenesis of melanoma.²⁵ JQ1 is a small molecule inhibitor of BRD4 and studies have shown that JQ1 can effectively inhibit the transcription and expression of *c-Myc* and its downstream target molecules. In addition, JQ1 has been shown to exhibit effective inhibitory actions in several hematological tumors.²⁶ However, because of the complex mechanisms underlying the pathogenesis of melanoma, the role of BRD4 in melanoma has not been fully elucidated.

The mitochondrion is an organelle that is directly involved in energy metabolism, oxidative stress, and cell homeostasis.²⁷ It has become a research focus in the last five years. Our experimental results show that the inhibition of BRD4 can lead to significant decreases in mitochondrial respiratory chain complex protein expression and mtDNA copy number, increases in ROS production, and impaired mitochondrial function, eventually promoting the apoptosis of B16 cells through the mitochondrial pathway.

One of the characteristics of mitochondrial dysfunction is changes in mitochondrial dynamics.²⁸ Fusion can increase the mitochondrial hyperfused network in cells, promote oxidative respiratory chain electron transfer, increase energy metabolism, and inhibit cell metabolism. Mitochondrial fusion is also positively correlated with increased ATP. Oxidative phosphorylation disorders, mtDNA deletions, and increased ROS can inhibit fusion.^{29,30} In contrast, fission reduces the use of oxygen and promotes apoptosis.^{31,32} The study of BRD4 and

mitochondrial dynamics is still in its infancy. In human prostate cancer, BRD4 can modify the activity of genes and control the survival and expansion of prostate CSC by regulating the fission of mitochondria.³³ As a downstream target gene of BRD4, *c-Myc* may be related to the relationship between BRD4 and mitochondrial dynamics. The down-regulation of *Myc* leads to a gradual decrease in mitochondrial mass, reduced fusion, and faster loss of membrane potential.³⁴ Studies have shown that in breast epithelial cells, PLD6 induces the fusion of mitochondria located downstream of *Myc* and activates AMP-activated protein kinase (AMPK) to inhibit the target genes of the YAP/TAZ co-activator, thus playing a role in maintaining cell growth.³⁵

Mitochondrial remodeling during apoptosis has been shown to mobilize the release of cytochrome *c*.^{36,37} In melanoma, there is limited information regarding the role of mitochondrial dynamics in tumor progression and its effect on mitochondrial dysfunction.³⁸ Cerium oxide nanoparticles (CNP; nanoceria) have been reported to induce changes in dynamics that lead to mitochondrial dysfunction and eventually lead to melanoma cell death.³⁹ Soares et al demonstrated that increased MFN2 expression in melanoma is significantly positively correlated with lymph node involvement and distant metastasis.⁴⁰ In this study, we found that JQ1 treatment significantly increased the expression of mitochondrial fission proteins and decreased the expression of fusion proteins in B16 cells. Mitochondrial fragmentation was observed by transmission electron microscopy, which is consistent with the increase in mitochondrial fission phenotypic characteristics.^{37,41,42} These changes may be related to the induction of apoptosis following BRD4 inhibition.

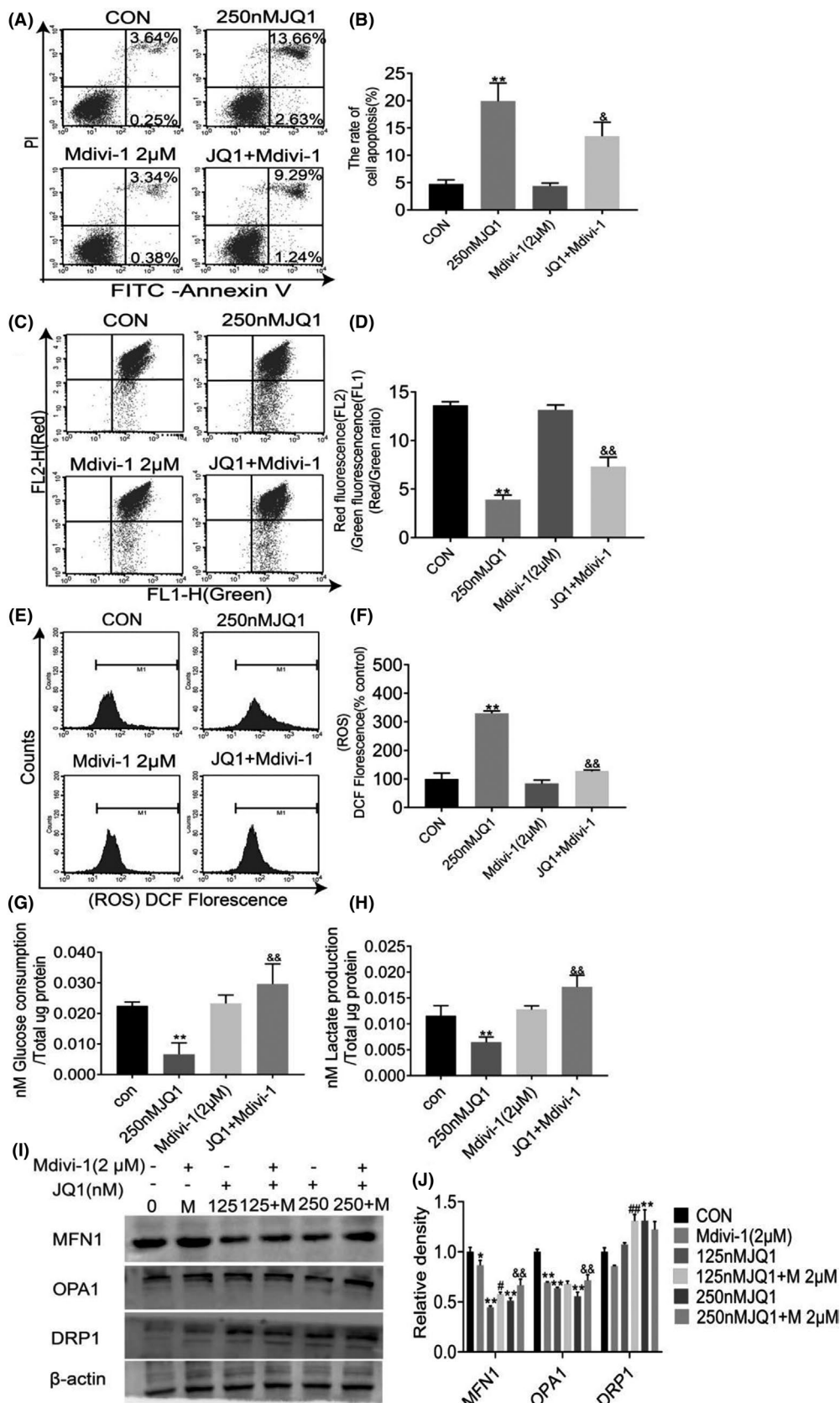


FIGURE 8 Mdivi-1 attenuates the effects of JQ1 on B16 melanoma cells. (A, B) B16 cell apoptosis after Mdivi-1 treatment. (C, D) Changes in the mitochondrial membrane potential ($\Delta\psi_m$) after treatment with Mdivi-1. (E, F) Changes in reactive oxygen species (ROS) after the addition of Mdivi-1. (G, H) Statistical analysis of the glucose and lactate contents in B16 cells after pretreatment with Mdivi-1 for 24 h. (I, J) The levels of mitochondrial dynamics-related proteins and c-myc after pretreatment with Mdivi-1. * $P < .05$ vs CON, ** $P < .01$ vs CON, # $P < .05$ vs 125 nmol/L JQ1, ## $P < .01$ vs 125 nmol/L JQ1, & $P < .05$ vs 250 nmol/L JQ1, && $P < .01$ vs 250 nmol/L JQ1

As the center of cell death regulation, mitochondria are also key regulators of cellular energy metabolism and energy changes may have an important effect on the growth of melanoma cells. Our research shows that BRD4 inhibition can attenuate glycolysis in B16 cells, leading to reduced glucose and lactic acid production. Although AMPK was originally thought to be related to tumor-suppressive processes, there is increasing evidence that AMPK activation can play a tumorigenic role in specific environments.⁴³ Inhibition of ARK5 (an upstream regulator of AMPK) signaling in cells expressing Myc results in a decrease in cellular ATP and an increase in reactive oxygen levels, eventually leading to apoptosis.⁴⁴ In a mouse myeloid leukemia model, AMPK protected leukemia-initiating cells from metabolic stress by inhibiting ROS.⁴⁵ Kfoury et al showed that the survival of c-Myc-positive melanoma cells depends on AMPK signaling to maintain ATP homeostasis and inhibit oxidative stress.⁴⁶ Our findings are consistent with the results of Kfoury et al as we found that JQ1 decreased AMPK protein levels in melanoma cells. The role of fatty acid metabolism in promoting tumor progression has received increasing attention. Studies have reported high ACLY expression in tumors and the inhibition of ACLY can attenuate cell proliferation and induce apoptosis.⁴⁷⁻⁵⁰ The TCA cycle can provide citric acid and acetyl-CoA for the synthesis of fatty acids. IDH2, as the rate-limiting enzyme for glycolysis and the TCA cycle, plays an important role in cellular metabolism. We found that the inhibition of BRD4 impaired fatty acid synthesis in melanoma cells, which was accompanied by mitochondrial dysfunction, reduced glycolysis, and decreased cell metabolism. These changes eventually resulted in cell death.

Mdivi-1 is a small molecule compound and potent inhibitor of DRP1.⁵¹ It can effectively inhibit DRP1-mediated mitochondrial fission. In various neurodegenerative diseases and cerebral ischemia models, Mdivi-1 blocking of DRP1 has a neuroprotective effect.⁵² Wieder et al showed that treatment of A375 cells with Mdivi-1 significantly reduced mitochondrial fission and apoptosis.⁶ Frank et al. demonstrated that inhibiting Drp1 activity before the induction of apoptosis not only reduced mitochondrial fission but also delayed caspase-3 activation and cell death.³⁷ In this study, we found that pretreatment with Mdivi-1 significantly reduced apoptosis and ROS production, partially restored mitochondrial function, and recovered glycolysis and lactic acid levels, which indicated that B16 cells had undergone energy metabolism reprogramming. Western blot analysis suggests that these changes may be related to mitochondrial dynamics. In other words, BRD4 can modulate mitochondrial fusion and fission to regulate melanoma cell apoptosis and tumor energy metabolism.

Although JQ1 has become a research hotspot in the last five years due to its highly specific recognition site, good cell permeability, and low toxic and side effects, its short half-life may lead to increased toxicity and side effects of the drug, which limits its clinical application.⁵³ In clinical trials, it has been found that thrombocytopenia is the most important toxicity of BET inhibitors; gastrointestinal diseases, anemia, and fatigue are the most common adverse events related to BET inhibitor treatment.⁵⁴ The combination of JQ1 and other drugs can not only enhance the efficacy but also effectively inhibit the side effects of drug treatment.^{55,56} Our research shows that BRD4 can regulate the growth of melanoma cells through the mitochondrial pathway and that

BRD4 inhibition impairs the mitochondrial function of B16 cells, leading to energy metabolism dysfunction. We speculate that BRD4 can alter mitochondrial morphology and function by modulating mitochondrial dynamics, ultimately affecting melanoma cell apoptosis and tumor growth. This study provides new insight for the future clinical application of JQ1 or other BRD4 inhibitors in combination with mitochondrial dynamics-related drugs in melanoma.

ACKNOWLEDGEMENTS

Not applicable

DISCLOSURE

The authors have no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Li L, Meng Y, Wu X, Li J, Sun Y.

Bromodomain-containing protein 4 inhibitor JQ1 promotes melanoma cell apoptosis by regulating mitochondrial dynamics. *Cancer Sci*. 2021;112:4013-4025. <https://doi.org/10.1111/cas.15061>