Clinical Cancer Research

AMPK–ULK1-Mediated Autophagy Confers Resistance to BET Inhibitor JQ1 in Acute Myeloid Leukemia Stem Cells

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Abstract

Purpose: Bromodomain and extraterminal domain (BET) inhibitors are promising epigenetic agents for the treatment of various subsets of acute myeloid leukemia (AML). However, the resistance of leukemia stem cells (LSC) to BET inhibitors remains a major challenge. In this study, we evaluated the mechanisms underlying LSC resistance to the BET inhibitor JQ1.

Experimental Design: We evaluated the levels of apoptosis and autophagy induced by JQ1 in LSC-like leukemia cell lines and primary $CD34^+CD38^-$ leukemic blasts obtained from AML cases with normal karyotype without recurrent mutations.

Results: JQ1 effectively induced apoptosis in a concentration-dependent manner in JQ1-sensitive AML cells. However, in JQ1-resistant AML LSCs, JQ1 induced little apoptosis and led to upregulation of beclin-1, increased LC3-II lipidation, formation of autophagosomes, and downregulation of p62/SQSTM1. Inhibition of autophagy by pharmacologic inhibitors or knockdown of beclin-1 using specific siRNA enhanced JQ1-induced apoptosis in resistant cells, indicating that prosurvival autophagy occurred in these cells. Independent of mTOR signaling, activation of the AMPK (pThr172)/ULK1 (pSer555) pathway was found to be associated with JQ1-induced autophagy in resistant cells. AMPK inhibition using the pharmacologic inhibitor compound C or by knockdown of AMPKα suppressed autophagy and promoted JQ1-induced apoptosis in AML LSCs.

Conclusions: These findings revealed that prosurvival autophagy was one of the mechanisms involved in the resistance AML LSCs to JQ1. Targeting the AMPK/ULK1 pathway or inhibition of autophagy could be an effective therapeutic strategy for combating resistance to BET inhibitors in AML and other types of cancer. *Clin Cancer Res; 23(11); 2781–94. ©2016 AACR.*

Introduction

Despite advances in our understanding of the molecular pathogenesis of acute myeloid leukemia (AML), the prognosis remains poor, primarily because of high relapse rates (1). Cytotoxic therapy has shown limited effectiveness in eradicating the functionally distinct leukemia stem cells (LSC; ref.2), which may contribute to chemotherapy resistance and relapse (3, 4). Therefore, novel treatment strategies with acceptable toxicity that target LSCs are urgently needed to improve therapeutic outcomes for patients with AML (5).

Chromatin regulators have recently been evaluated as potential therapeutic targets in LSCs because altered chromatin modification affects cell fate determination, resulting in generation of LSCs that aberrantly self-renew and propagate the disease (6, 7). Bromodomain-containing protein 4 (BRD4), a member of the bromodomain and extraterminal domain (BET) family, is a chromatin reader that recognizes epigenetic modifications and regulates the transcription of survival- and growth-related genes (8, 9). BRD4 preferentially localizes to "super-enhancer" regions upstream of a variety of oncogenes, including c-Myc, to regulate their expression (9). In addition, accumulating evidence has suggested that BRD4 is critically required for the maintenance of AML and LSCs characterized by $CD34^+CD38^-$ populations (10–12). Therefore, BRD4 inhibition may be an effective alternative strategy for targeting c-Myc, which is considered undruggable and deregulated in many human cancers, including AML, thereby leading to synthetic lethality of these c-Myc-dependent tumor cells (11).

Selective inhibitors of BET, such as JQ1, have been shown to be highly effective against different subtypes of AML (13–15). Because transcriptional regulation by c-Myc plays an important role in LSC self-renewal (16), JQ1 may inhibit the self-renewal activities of LSCs (10). Recently, JQ1 was shown to induce growth arrest and apoptosis in AML LSCs while sparing normal bone marrow progenitor cells, independent of the AML subtype or disease status (12). Thus, JQ1 appears to be a promising therapeutic option for targeting LSCs of various AML subtypes (17).

However, leukemia cells appear to have differences in apoptotic responses to JQ1 (18). Furthermore, the resistance of some AML cells to JQ1 cannot be explained by changes in the levels of c-Myc and BRD4 (19). These findings suggested that primary resistance to JQ1 may not be due to its inability to suppress c-Myc, but may be driven by compensatory mechanisms triggered by c-Myc

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Translational Relevance

The prognosis of patients with acute myeloid leukemia (AML) is poor. Bromodomain and extraterminal domain (BET) inhibitors are promising epigenetic agents for the treatment of various tumor types, including AML. However, the resistance mechanisms underlying differential sensitivity among leukemia stem cells (LSC) and various human cancers to BET inhibitors remain elusive. In this study, we found that the BET inhibitor JQ1 induced cytoprotective autophagy in JQ1-resistant LSCs. The AMPK/ULK1 pathway played a key role in autophagy of LSCs, conferring resistance to JQ1-induced apoptosis. Moreover, treatment with inhibitors of autophagy or AMPK could overcome JQ1 resistance and enhance the apoptotic response, suggesting the potential clinical utility of these unique targeted therapies against AML LSCs and possibly in other types of cancer.

inhibition. A recently published study suggested that resistance to BET inhibitors in AML is not mediated through abnormal drug efflux or metabolism but emerges from LSCs both *in vitro* and *in vivo* (20). However, the mechanisms underlying differences among LSCs in sensitivity to BET inhibitors remain elusive. A better understanding of the mechanisms involved in AML LSC resistance to JQ1 is needed to combat resistance to BET inhibitors in AML.

In the present study, we investigated the mechanisms underlying the resistance of LSCs to JQ1. We showed, for the first time, that JQ1-resistant LSC-like cell lines and primary $CD34^+CD38^-$ LSCs acquire resistance to JQ1 through induction of autophagy. Autophagy in JQ1-resistant cells was mediated by activation of the 5′ AMP-activated protein kinase (AMPK)/Unc-51 like autophagy activating kinase 1 (ULK1) pathway, independent of mammalian target of rapamycin (mTOR) signaling.

Materials and Methods

Patients and isolation of AML cells

This study adhered to the tenets of the Declaration of Helsinki and was approved by the institutional review of board of Severance Hospital. Study participants provided written informed consent, and all patient samples were coded and linked anonymously. Identification of samples was possible using a code, and anonymized clinical information of linked samples was provided for researchers. Human leukemia cells were obtained from diagnostic bone marrow aspiration of patients with *de novo* AML diagnosed at Yonsei University Severance Hospital between 2006 and 2015. Mononuclear cells were isolated by Ficoll–Hypaque (GE Healthcare Bio-Sciences) density gradient centrifugation and then cryopreserved.

Because AML is a heterogeneous disease, patients are stratified into risk groups based on cytogenetic and molecular abnormalities (21, 22). In particular, mutations in *FLT3-ITD* and *NPM1* have prognostic value in cytogenetically normal AML (23, 24). Thus, to minimize the effects of a heterogeneous response to treatment, we selected 21 samples from patients with cytogenetically normal AML and without *FLT3-ITD* or *NPM1* mutations. After thawing the cryopreserved primary AML cells, $CD34^+$ leukemia cells were enumerated by flow cytometry (FACSVerse; BD Biosciences) using APC-labeled antibodies targeting the myeloid stem cell marker CD34 (BD Biosciences). Samples from 13 patients with abundant $CD34^+$ leukemia cells (>60% mononuclear cells) were finally chosen for experiments (Supplementary Table S1).

Cell lines and culture conditions

The AML cell lines KG1, KG1a, and Kasumi-1, which are enriched in cells expressing an LSC phenotype ($CD34^+CD38^-$), were obtained from the American Type Culture Collection. KG1 and Kasumi-1 cells were cultured in RPMI-1640 medium (Gibco Life Technologies), and KG1a cells were cultured in Iscove's modified Dulbecco's medium (Gibco Life Technologies) supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in a 5% CO_2 humidified incubator at 37°C.

Reagents

Stock solutions of the following reagents were prepared by dissolving in dimethyl sulfoxide (Biosesang; D1022): (+)-JQ1 [Tocris Bioscience; 4499; 98.9% purity by high-performance liquid chromatography (HPLC), 99.9% purity by chiral HPLC, and structural confirmation by mass spectrometry], bafilomycin A1 from *Streptomyces griseus* (Sigma-Aldrich; B1793), hydroxychloroquine (Myung-in Pharmaceuticals), rapamycin (Calbiochem; 553210), and compound C (Sigma-Aldrich; P5499). A stock solution of 3-methyladenine (3-MA; Sigma-Aldrich; M9281) was prepared by dissolving in distilled water. Control cells were treated with equal amounts of the solvent.

Cell viability assay

Cells were seeded in 96-well plates and incubated overnight before treatment. After 48 hours of treatment with the indicated concentrations of JQ1, 10 μL of the Cell Counting Kit-8 solution (DOJINDO Laboratories) was added to each well, and the cells were incubated for 4 hours. Absorbance at 450 nm was measured in a microplate reader (VersaMax, Molecular Devices).

Apoptosis and cell-cycle analyses

The annexin V assays were performed as previously described (25) using a FACSVerse flow cytometer (BD Biosciences). To study apoptosis in LSCs, cells were grouped into $CD34^+CD38^-$ and $CD34^+CD38^+$ cell fractions by staining with anti-CD34-APC (BD Biosciences), anti-CD38-PE (BD Biosciences), and 7-AAD (Beckman Coulter) for 30 minutes. The labeled cells were subsequently resuspended in annexin V binding buffer and incubated with annexin V–FITC (BD Pharmingen) for 15 minutes before flow cytometry analysis. Data were analyzed using FACSuite software (BD Biosciences).

Transmission electron microscopy

Transmission electron microscopy was performed using standard procedures. Briefly, cells were fixed with 2% glutaraldehyde/paraformaldehyde, pelleted, and treated with 1% osmium tetroxide (Polysciences). After embedding samples in pure, fresh resin, and polymerizing, thin sections were double stained with 6% uranyl acetate and lead citrate. Sections were analyzed by transmission electron microscopy (JEM-1011, JEOL, Japan) at 80 kV.

Transfection of green fluorescent protein (GFP)-microtubule-associated protein 1 light chain 3 (LC3)

The GFP-LC3 construct was kindly provided by Dr. Young Sam Kim (Division of Pulmonology, Yonsei University College of Medicine). A suspension of 2×10^6 leukemia cells was immediately transfected with GFP-LC3 cDNA (5 μg) using program V-01 of the Amaxa Nucleofector 2b device (Lonza Cologne GmbH) according to the manufacturer's instructions. Immediately after electroporation, the cells were resuspended in complete medium and incubated for 24 hours at 37°C in a humidified atmosphere containing 5% CO_2. Cells expressing GFP-tagged LC3 were used to evaluate autophagy induction.

Immunostaining of primary $CD34^+$ AML cells obtained from bone marrow aspirates

Primary AML bone marrow cells with higher CD34 positivity (>88%) were incubated with JQ1 for 24 hours, after which the cells were fixed with 4% formaldehyde diluted in warm Dulbecco's phosphate-buffered saline (DPBS; Gibco Life Technologies). The cells were permeabilized by incubation for 10 minutes at −20°C and then blocked for 1 hour in DPBS with 5% bovine serum albumin (BSA; Santa Cruz Biotechnology) and 0.3% Triton X-100 (Sigma-Aldrich). After removal of the blocking buffer, the cells were incubated with anti-LC3B antibodies (Cell Signaling Technology) and purified anti-human CD34 antibodies (BioLegend) in DPBS with 1% BSA and 0.3% Triton X-100 overnight at 4°C. The cells were then harvested and incubated with Alexa Fluor 488 F(ab′)2 fragments of goat anti-rabbit IgG (H+L) (Invitrogen) and R-PE goat anti-mouse IgG1 (Invitrogen) secondary antibodies for 2 hours at room temperature in the dark.

Confocal microscopy

Cells were centrifuged at $800 \times g$ onto glass slides, and coverslips were mounted with aqueous mounting medium (Dako) with DAPI (Sigma-Aldrich). Fluorescent signals were analyzed using a Zeiss LSM 700 laser-scanning confocal microscope (Göettingen). The number of LC3 puncta per cell was quantified as described elsewhere (26). To estimate the average number of LC3 puncta per cell in each treatment group, 20 cells were randomly selected, and puncta in each cell were manually counted. Results are expressed as the mean of at least three independent experiments.

Western blot analysis

Total cell lysates were prepared and analyzed by Western blotting, as described previously (27). Rabbit polyclonal antibodies against LC3 (NB100-2220) and beclin-1 (NB500-249) were obtained from Novus Biologicals. Rabbit polyclonal antibodies against c-Myc (9402), poly(ADP-ribose) polymerase (PARP; 9542), caspase-9 (9502), cleaved caspase-3 (9661), phospho (p)-$mTOR_{S2448}$ (2971), mTOR (2972), p-$p70S6K_{T389}$ (9205), p70S6K (9202), p-$AMPK\alpha_{T172}$ (9282), AMPKα (2532), p-$ULK1_{S555}$ (5869), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (7074), and HRP-conjugated goat anti-mouse IgG (7072) were purchased from Cell Signaling Technology. Mouse anti-α-tubulin monoclonal antibodies were obtained from Merck Millipore (05-829). Secondary antibodies were coupled to horseradish peroxidase and visualized by enhanced chemiluminescence (GE Healthcare Bio-Sciences; RPN2232).

Small-interfering RNA (siRNA) transfection

Beclin-1- and AMPKα-specific siRNA were purchased from Santa Cruz Biotechnology and Qiagen, respectively. A cell suspension of 2×10^6 leukemia cells was immediately transfected with siRNA (1 μmol/L) using program V-01 of the Amaxa Nucleofector2b device (Lonza Cologne GmbH) according to the manufacturer's instructions. After electroporation, the cells were resuspended in complete medium and incubated at 37°C in a humidified atmosphere containing 5% CO_2. Control cells were transfected with scrambled siRNA. The cells were then treated with the indicated concentrations of JQ1 and collected for Western blot and apoptosis analyses.

Statistical analysis

Results are expressed as the mean ± standard deviation (SD) of at least three independent experiments. Comparison of two groups was performed using the two-tailed Student *t* test. Statistical analysis was performed using GraphPad Prism 4.0 (GraphPad Software Inc). Differences with *P* values of less than 0.05 were considered statistically significant.

Results

Effects of JQ1 on AML LSCs

We initially examined the effects of JQ1 on cell proliferation in the AML LSC candidate cell lines KG1, KG1a, and Kasumi-1. These cell lines originated from early myeloid stem cells with over 70% $CD34^+$ cells (28–30). As shown in Fig. 1A, JQ1 inhibited the proliferation of these cells in a concentration-dependent manner. Next, we evaluated the effects of JQ1 on $CD34^+$-enriched blasts obtained from 13 patients with AML having a normal karyotype. AML cases with recurrent genetic mutations, such as internal tandem duplication mutations in *FLT3*, were excluded (Supplementary Table S1). After JQ1 treatment, cell viability uniformly decreased in primary $CD34^+$-enriched AML cells in a concentration-dependent manner (Fig. 1B). However, the apoptotic response to JQ1 differed depending on the cell line. In Kasumi-1 cells, cell death was induced in a concentration-dependent manner. In contrast, apoptotic cell death was minimal in KG1 and KG1a cells (Fig. 1C). Similarly, an increase in the frequency of sub-G_1 cells was observed only in Kasumi-1 cells (Fig. 1D). Differences in the levels of apoptotic cell death were also observed in the patient samples (Fig. 1E). For analysis, AML cases were classified into JQ1-sensitive ($n = 7$) and JQ1-resistant ($n = 6$) groups based on the median value of apoptosis (median 41.7%, range, 5.7%–69.5%) after induction by treatment with 5 μmol/L JQ1 for 48 hours in all patients samples. There was a significant difference in the level of apoptotic cell death between the sensitive (median 60.3%, range, 41.7%–69.5%) and resistant groups (median, 15.6%, range, 5.7%–22.1%; $P < 0.0001$). No significant clinical differences were observed between patients with JQ1-sensitive versus JQ1-resistant AML cells (Supplementary Table S2).

Because JQ1 effects are known to be mediated through c-Myc downregulation, we evaluated JQ1-induced changes in the level of c-Myc protein in the LSC candidate cell lines. Regardless of whether the cell lines were sensitive or resistant to JQ1, c-Myc protein was markedly decreased after treatment with 5 μmol/L JQ1 for 24 hours (Fig. 1F, top). Similarly, c-Myc protein was

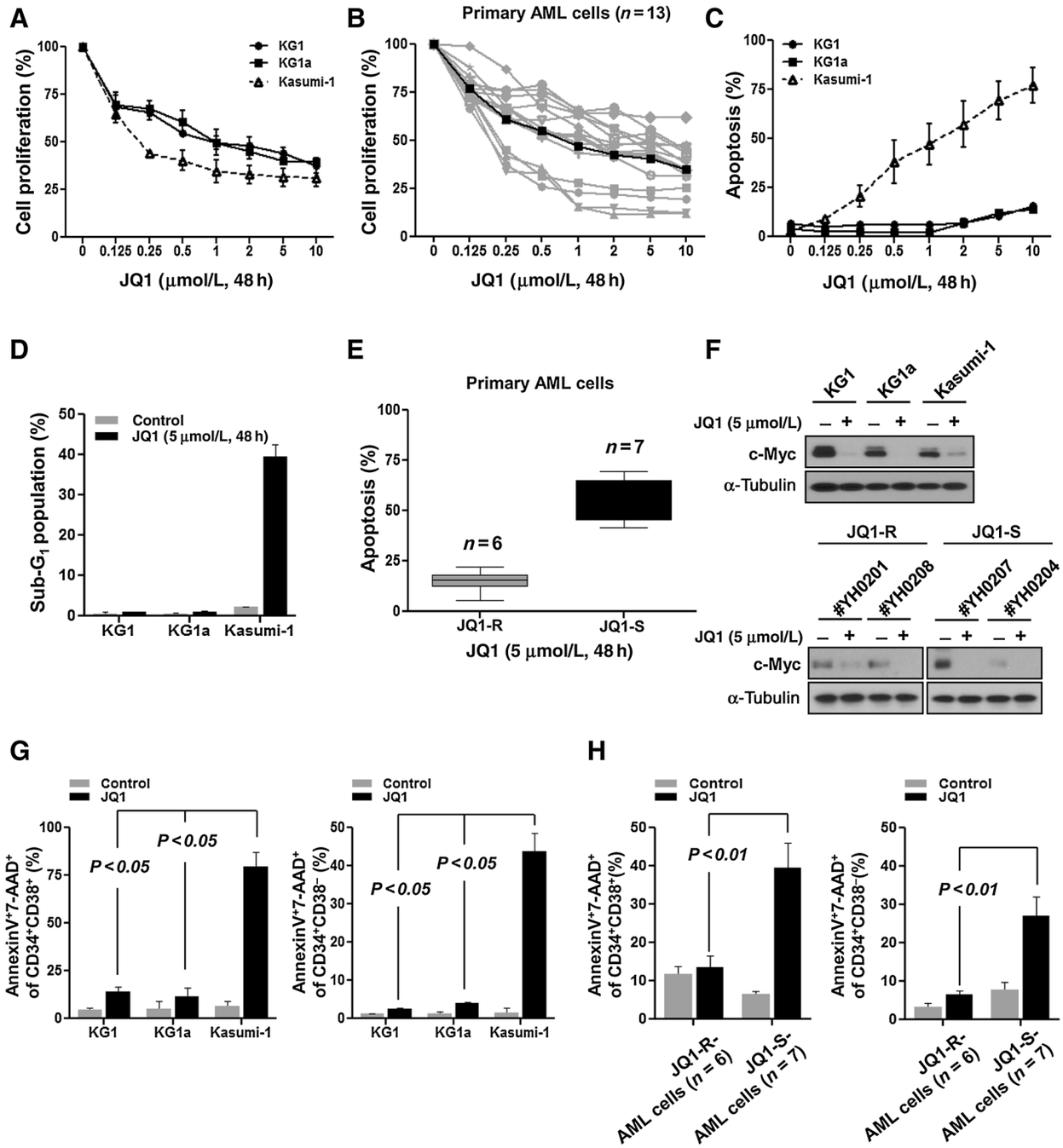


Figure 1.

Differential sensitivity of human AML LSCs to JQ1. **A–E,** LSC lines KG1, KG1a, and Kasumi-1 and primary leukemia cells obtained from patients with AML were incubated with various concentrations of JQ1 for 48 hours. **A** and **B,** After treatment, cell proliferation was measured for LSC lines (**A**) and primary AML cells (**B**) using Cell Counting Kit-8 assays. Gray lines, values of independent experiments for 13 samples from patients with AML; black line, average value of independent experiments for 13 samples from patients with AML. **C,** The fraction of apoptotic cells was analyzed for cell lines using Annexin V/PI exclusion and flow cytometric analysis. **D,** The sub-G_1 cell population was measured in LSC lines using flow cytometric analysis as described in Materials and Methods. **E,** The fraction of apoptotic cells was analyzed for primary AML cells using Annexin V/PI exclusion and flow cytometric analysis. **F,** After treatment of the cell lines and representative samples of primary AML with 5 μmol/L JQ1 for 24 hours, cell lysates were subjected to Western blotting for c-Myc expression. α-Tubulin was used as a protein-loading control. **G** and **H,** The apoptotic cell fractions (Annexin V^+7-AAD^+) of $CD34^+CD38^+$ and $CD34^+CD38^-$ cells were compared after 48 h of 5 μmol/L JQ1 treatment in leukemia cell lines (**G**) and primary leukemia cells (**H**) according to JQ1 sensitivity using flow cytometric analysis as described in Materials and Methods. Columns, mean values of three independent experiments for cell lines and data obtained from 13 primary samples; bars, standard deviations (SDs); JQ1-S, sensitive to JQ1-induced apoptosis; JQ1-R, resistant to JQ1-induced apoptosis.

strongly decreased in primary $CD34^+$-enriched AML cells, regardless JQ1 sensitivity (Fig. 1F, top).

Because $CD34^+CD38^-$ LSCs are thought to be more resistant to antileukemia therapy than progenitor $CD34^+CD38^+$ cells (31), we analyzed the comparative effects of JQ1 (5 μmol/L) on the induction of apoptosis in $CD34^+CD38^+$ and $CD34^+CD38^-$ leukemia cell fractions. In JQ1-resistant KG1 and KG1a cells, the apoptotic fraction (annexin V^+7–aminoactinomycin [7-AAD]$^+$) was not increased in $CD34^+CD38^+$ cells (Fig. 1G, left) or $CD34^+CD38^-$ cells (Fig. 1G, right). In contrast, the apoptotic fraction of both $CD34^+CD38^+$ cells (Fig. 1G, left) and $CD34^+CD38^-$ cells (Fig. 1G, right) was markedly increased in the JQ1-sensitive Kasumi-1 cell line. Similarly, in JQ1-sensitive primary AML cells, the apoptotic fractions of $CD34^+CD38^-$ AML LSCs (Fig. 1H, right; 6.4% ± 3.0% for JQ1-resistant cells and 27.1% ± 12.9% for JQ1-sensitive cells; $P < 0.01$) and $CD34^+CD38^+$ progenitor cells (Fig. 1H, left; 13.5% ± 7.6% for JQ1-resistant cells and 39.4% ± 17.3% for JQ1-sensitive cells; $P < 0.01$) showed higher increases upon JQ1 treatment than those in JQ1-resistant primary AML cells (Supplementary Fig. S1).

JQ1 induced autophagy in JQ1-resistant LSCs

Next, we examined whether JQ1 induced autophagy in AML LSCs. Because cytosolic LC3-I is converted to its active form LC3-II through lipidation by a ubiquitin-like system during autophagy (32), we first evaluated the effects of JQ1 treatment on LC3 conversion in KG1, KG1a, and Kasumi-1 cells. As shown in Fig. 2A, LC3 conversion was not observed in Kasumi-1 cells. In contrast, the level of LC3-II increased after treatment with JQ1 in the resistant LSC candidate cell lines (Fig. 2A; Supplementary Fig. S2A). Microscopic images indicated that GFP-LC3 puncta, which represent autophagosomes, were markedly increased in KG1 and KG1a cells, but not in Kasumi-1 cells (Fig. 2B). JQ1 treatment induced a significant increase (greater than 10-fold) in the number of GFP-LC3 puncta in KG1 and KG1a cells ($P < 0.00001$, both), whereas no increase was observed in Kasumi-1 cells (Fig. 2C). The degradation of p62/SQSTM1 during JQ1-induced autophagy was also observed in KG1 and KG1a cells (Supplementary Fig. S3A).

Transmission electron microscopy (TEM) micrographs showed that autophagic vesicles were greatly increased in KG1 and KG1a cells (Fig. 2D). In contrast, in Kasumi-1 cells, only chromosome condensation and nuclear fragmentation into apoptotic bodies were observed. As shown in Fig. 2E, the number of total autophagosomes significantly increased with JQ1 treatment in KG1 and KG1a cells ($P < 0.00001$, both). Autophagy activation was also observed preferentially in JQ1-resistant primary AML cells. LC3-II conversion was evident in JQ1-resistant primary AML cells (#YH0201 and #YH0208) but not in JQ1-sensitive primary AML cells (#YH0207 and #YH0204) after treatment with 5 μmol/L JQ1 for 24 hours (Fig. 3A, right; Supplementary Fig. S2B). Similarly, the numbers of GFP-LC3 puncta (Fig. 3B and C) and autophagosomes per cell (Fig. 3D and E) were markedly increased in the representative JQ1-resistant primary AML sample (#YH0201) as compared with that in JQ1-sensitive primary AML cells (#YH0207).

Autophagy inhibition enhanced LSC sensitivity to JQ1-induced apoptosis

Next, we examined whether autophagy induction contributed to survival in JQ1-resistant LSCs by autophagy inhibition using pharmacological inhibitors or RNA interference of beclin-1, an essential mediator of cell autophagy (33, 34). The addition of bafilomycin A1, 3-MA, or hydroxychloroquine to JQ1 treatment resulted in a significant increase in the level of apoptosis in KG1 cells (Fig. 4A; bafilomycin A1, $P < 0.01$; 3-MA, $P < 0.001$; hydroxychloroquine, $P < 0.001$) and KG1a cells (Fig. 4B; bafilomycin A1, $P < 0.01$; 3-MA, $P < 0.05$; hydroxychloroquine, $P < 0.001$) as compared with that of JQ1 treatment alone. Because cleavage of PARP by activated (cleaved) caspase-3 is a hallmark of apoptosis (35), we next assessed changes in the levels of these molecules after treatment with JQ1 in the presence or absence of autophagy inhibitors. As expected, cleaved caspase-3 and PARP were not detected after treatment with either JQ1 (5 μmol/L) or 3-MA (5 mmol/L) for 24 hours in KG1 and KG1a cells (Fig. 4C). However, the combined use of JQ1 and 3-MA led to increases in the levels of cleaved caspase-3 and PARP in both KG1 and KG1a cells, indicating that JQ1-dependent activation of autophagy was responsible for resistance to apoptotic cell death. When 3-MA was added to JQ1, an increase in the number of apoptotic bodies, with notable decreases in the numbers of GFP-LC3 puncta (Fig. 4D) and autophagosomes (Fig. 4E), was observed in KG1 and KG1a cells. In primary AML cells ($n = 6$) resistant to JQ1-induced apoptosis, the addition of 3-MA to JQ1 resulted in a significant increase in the extent of apoptosis (Fig. 4F; $P < 0.01$). The apoptotic fraction (Annexin V^+7-AAD^+) of $CD34^+CD38^-$LSCs from primary AML was significantly increased by combined treatment with JQ1 and 3-MA (Fig. 4G, $P < 0.001$). There were no significant differences in JQ1-induced apoptosis following cotreatment with JQ1 and bafilomycin A1 in JQ1-sensitive Kasumi-1 cells (Supplementary Fig. S3B).

To elucidate the associated mechanisms, we downregulated autophagy by targeting beclin-1 in KG1 and KG1a cells. Total protein expression of becin-1 was stable in the presence of JQ1 but was significantly decreased after treatment with targeted siRNA. Moreover, knockdown of beclin-1 blocked the accumulation of LC3-II in JQ1-treated cells, indicating that autophagy was suppressed under physiological conditions (Fig. 4H). The number of GFP-LC3 puncta was reduced after silencing of beclin-1 in JQ1-treated cells (Fig. 4I). Beclin-1 knockdown also enhanced JQ1-induced apoptosis in KG1 and KG1a cells (Fig. 4J).

Activation of the AMPK/ULK1 pathway in JQ1 resistance

Because AMPK can activate autophagy through the activation of ULK1 or by inhibiting mTOR signaling (36, 37), we next examined alterations in the AMPK/ULK1 and mTOR pathways induced by JQ1 by evaluating autophagy-related proteins, i.e., LC3-II and beclin-1, in relation to JQ1 sensitivity and resistance. ULK1 induces autophagy by phosphorylating beclin-1 (38). In KG1 and KG1a cells, beclin-1 increased in parallel with elevated LC3-II in a concentration-dependent manner; however, in Kasumi-1 cells, JQ1-induced increases in LC3-II and beclin-1 were not observed (Fig. 5A; Supplementary Fig. S2A). JQ1 inhibition of c-Myc transcription increases adenosine triphosphate (ATP) consumption and leads to AMPK activation via phosphorylation at Thr172 (39). Consistent with this, we found that JQ1 induced phosphorylation of AMPK at Thr172 in all LSC candidates (Fig. 5A). Total AMPK protein levels were not changed. Interestingly, phosphorylation of

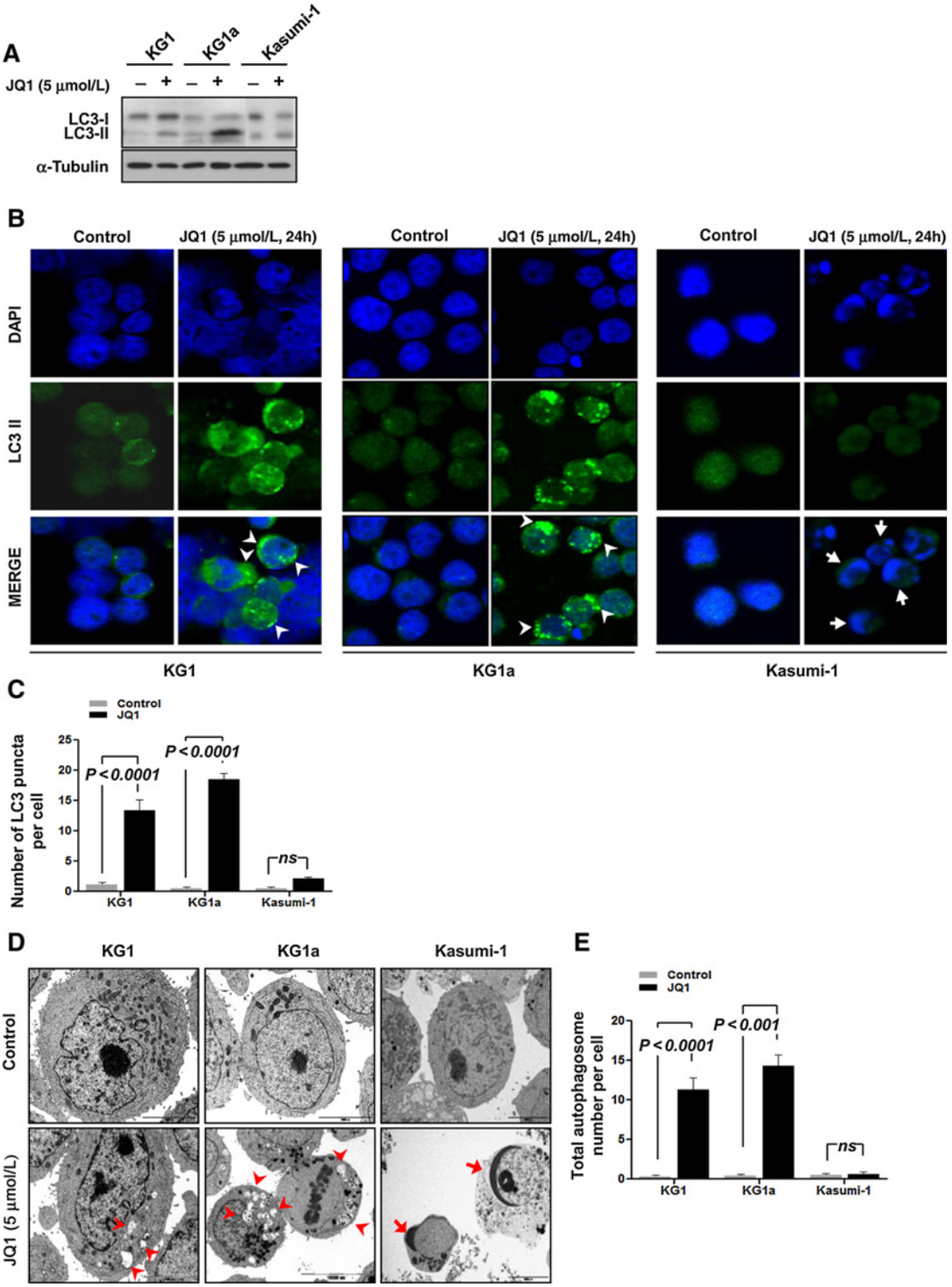


Figure 2.

Autophagy induction in JQ1-resistant LSC lines. **A,** After treatment of KG1, KG1a, and Kasumi-1 cells with 5 μmol/L JQ1 for 24 hours, cell lysates were subjected to Western blotting for LC3-I/II. α-Tubulin was used as a protein loading control. **B,** GFP-LC3-transfected cell lines were treated with 5 μmol/L JQ1 for 24 hours followed by confocal microscopic observations, as described in Materials and Methods. The green punctuate fluorescence indicates the cytoplasmic localization of the LC3 proteins. Representative micrographs demonstrated characteristic punctuate staining indicative of autophagosome formation. Nuclei were stained with DAPI. The arrowheads indicate LC3 puncta, and the arrows denote condensed nuclei. **C,** GFP-LC3 dots in each cell were enumerated in at least three independent visual fields. Columns, the mean number of GFP-LC3 dots per cell; bars, SDs. **D,** TEM ultrastructural examination of leukemia cell lines treated with 5 μmol/L JQ1 for 24 hours. The arrowheads and arrows indicate autophagosomes and condensed chromatin, respectively. **E,** Autophagosomes were counted in at least three independent visual fields. Columns, mean number of autophagosomes; bars, SDs; NS, not significant.

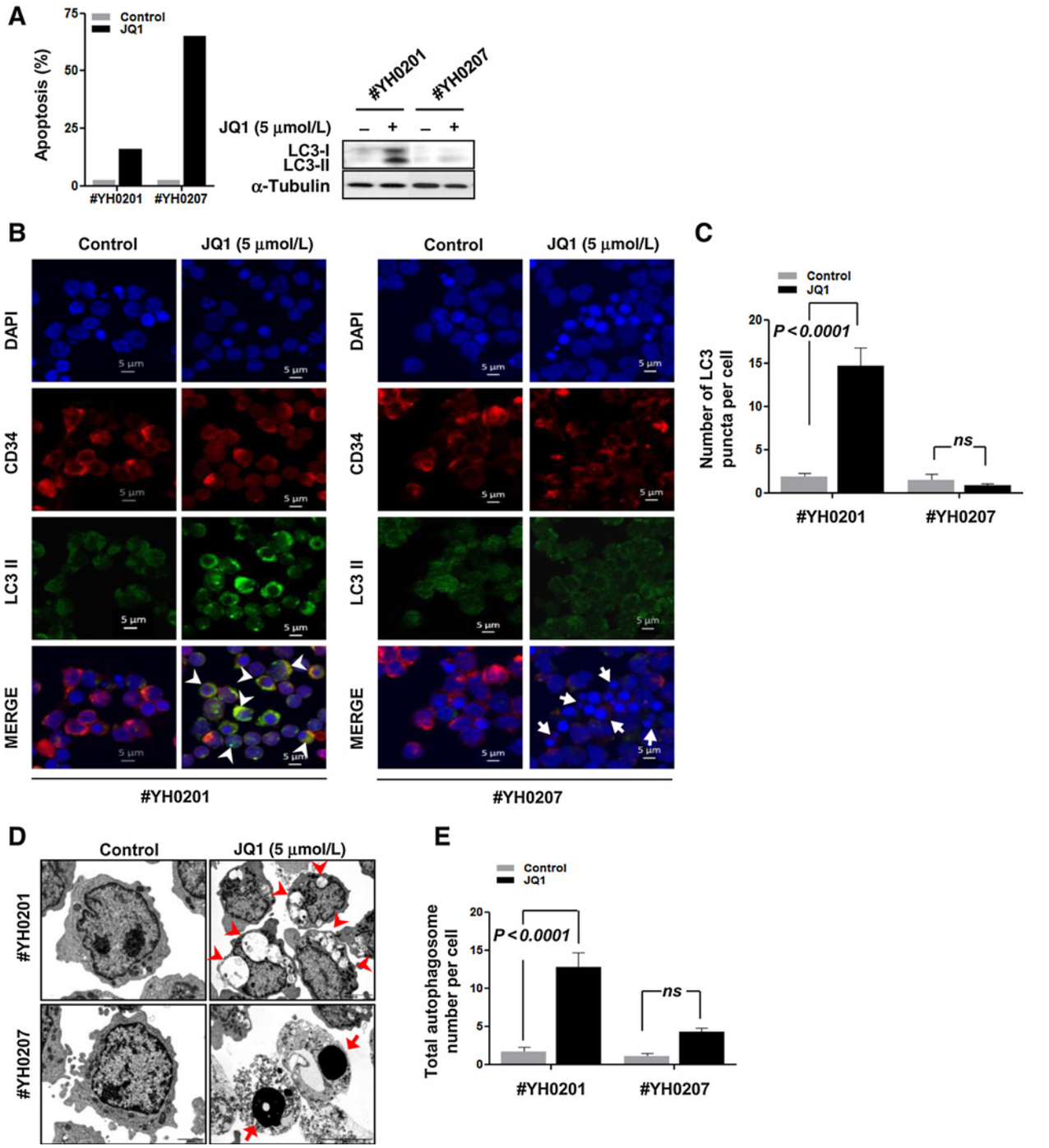
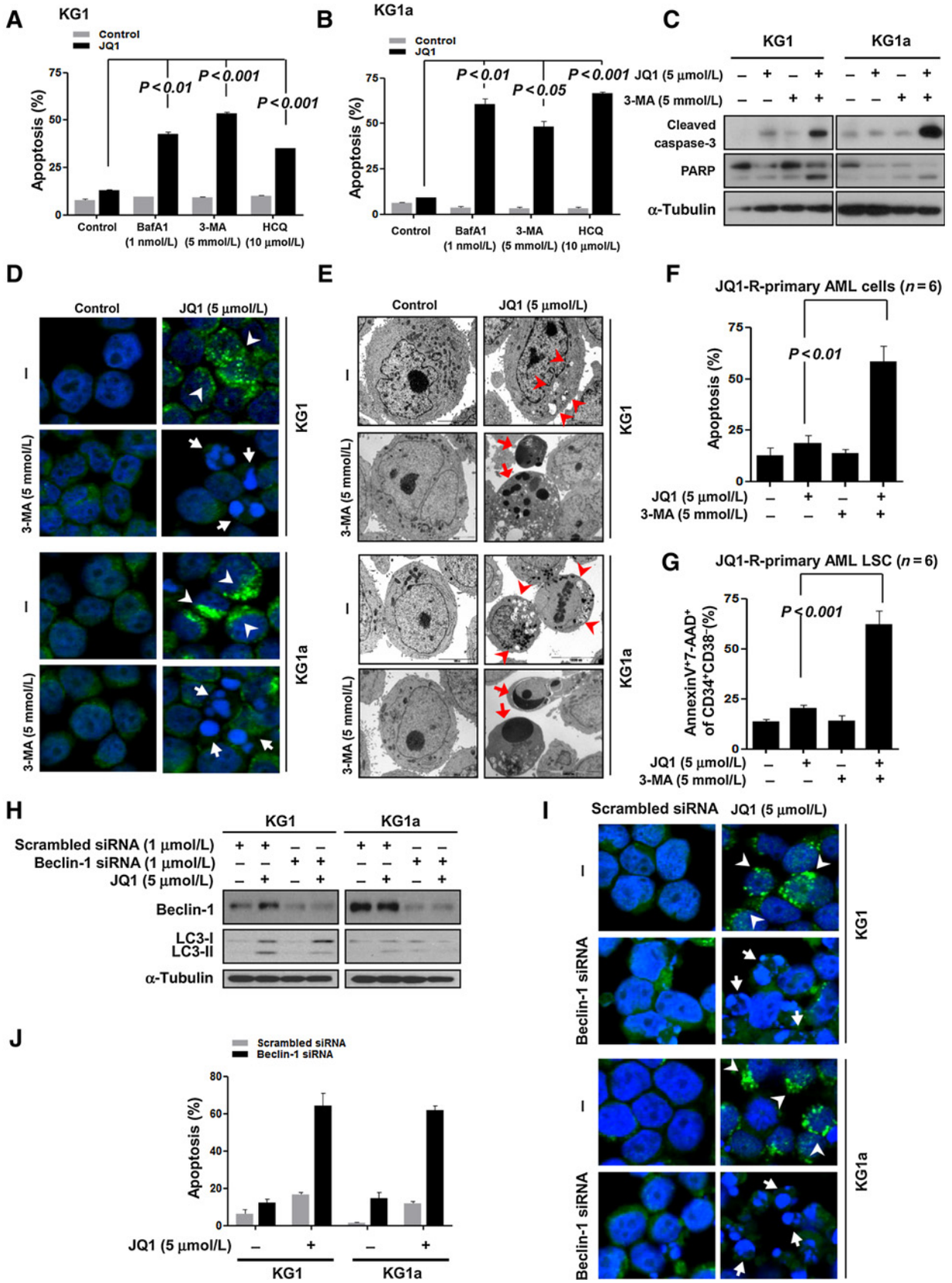


Figure 3.

Autophagy induction according to JQ1 sensitivity in CD34$^+$-enriched primary AML cells. (**A**, left) Apoptotic cell fractions in the CD34$^+$-enriched primary AML cells from #YH0201 and #YH0207 after treatment with 5 μmol/L JQ1 for 48 hours. (**A**, right), After treatment of representative CD34$^+$-enriched primary AML cells from #YH0201 and #YH0207 with 5 μmol/L JQ1 for 24 hours, cell lysates were subjected to Western blotting for LC3-I/II. α-Tubulin was used as a protein loading control. **B,** Primary AML cells (#YH0201 and #YH0207) were treated with 5 μmol/L JQ1 for 24 hours and fixed. Cells were stained for LC3B (green) and CD34 (red) followed by confocal microscopic observation. Representative micrographs demonstrated characteristic punctuate staining, indicative of autophagosome formation. Nuclei were stained with DAPI. The arrowheads indicate LC3 puncta, and the arrows denote condensed nuclei. **C,** LC3 puncta in each cell were enumerated in at least three independent visual fields. Columns, mean number of LC3 puncta per cell; bars, SDs. **D,** TEM ultrastructural examination of CD34$^+$-enriched primary AML cells (#YH0201 and #YH0207) treated with 5 μmol/L JQ1 for 24 hours. The arrowheads and arrows indicate autophagosomes and condensed chromatin, respectively. **E,** Autophagosomes were counted in at least three independent visual fields. Columns, mean number of autophagosomes; bars, SDs. NS, not significant.



ULK1 at Ser555 was increased only in KG1 and KG1a cells (Fig. 5A). With regard to the mTOR pathway, we found that Ser2448-phosphorylated mTOR protein and its target protein p70S6K were decreased in Kasumi but not in KG1 and KG1a cells. Similar results were obtained in the $CD34^+$-enriched primary blasts obtained from JQ1-resistant (#YH0201 and #YH0208) and JQ1-sensitive (#YH0207 and #YH0204) AML cases (Fig. 5B). The increase in LC3-II protein paralleled the increased phosphorylation of AMPK (Thr172) and ULK1 (Ser555) in JQ1-resistant $CD34^+$-enriched primary AML cells (Fig. 5B; Supplementary Fig. S2B). At the same time, modest increases in phosphorylated mTOR (Ser2448) and p70S6K (Thr389) proteins were observed. In JQ1-sensitive $CD34^+$-enriched primary AML cells, phosphorylation of AMPK (Thr172) was enhanced by JQ1 treatment. However, phosphorylation of ULK1 at S555 was not increased (Fig. 5B; Supplementary Fig. S2B). Phosphorylated mTOR (Ser2448) and p70S6K (Thr389) decreased with JQ1 treatment. These findings suggested that AMPK-mediated ULK1 activation, independent of the mTOR pathway, induced autophagy, thereby conferring resistance to JQ1-induced apoptosis. In Kasumi-1 cells and JQ1-sensitive primary AML cells, which failed to acquire ULK1 (Ser555) phosphorylation, JQ1 increased the levels of cleaved caspase-9, cleaved caspase-3, and PARP (Supplementary Fig. S4).

To further clarify the mechanisms involved in JQ1 resistance in LSCs, we evaluated the effects of AMPK inhibition using siRNA or specific inhibitors on JQ1-induced AMPK/ULK1 activation and apoptotic cell death in JQ1-resistant cells. As shown in Fig. 5C, siRNA against AMPKα effectively inhibited JQ1-mediated phosphorylation of AMPK and ULK1 (Ser555) in KG1 and KG1a cells. Concurrently, JQ1-induced LC3 conversion markedly decreased with AMPKα siRNA treatment. Similarly, compound C, a specific AMPK inhibitor, effectively inhibited JQ1-induced LC3 conversion and JQ1-induced phosphorylation of AMPK and ULK1 (Ser555; Fig. 5D). Although the level of phospho-mTOR (Ser2448) was increased by JQ1 in KG1 and KG1a cells, mTOR inhibitor rapamycin did not inhibit JQ1-induced LC3 conversion (Fig. 5E), indicating that autophagy activation occurred independently of mTOR regulation in LSCs.

AMPK inhibition overcame resistance to JQ1 in LSCs

Next, we investigated whether inhibition of AMPK-mediated autophagy could increase the sensitivity of JQ1-resistant LSCs to JQ1-induced apoptotic cell death. To our surprise, the addition of AMPKα siRNA (Fig. 6A) or compound C (Fig. 6B) led to a significant increase in the extent of JQ1-induced apoptotic cell death in KG1 ($P < 0.005$ for siRNA, $P < 0.005$ for compound C) and KG1a cells ($P < 0.005$ for siRNA, $P < 0.0001$ for compound C). Rapamycin did not enhance JQ1-induced apoptosis in KG1 and KG1a cells (Fig. 6C). Subsequently, we analyzed the effects of the pharmacologic AMPK inhibitor on JQ1-induced apoptosis in $CD34^+$ primary AML cells. Compound C enhanced the sensitivity of JQ1-resistant $CD34^+$-enriched primary AML cells and significantly increased JQ1-induced apoptotic cell death in these cells (19.0% ± 6.9% for JQ1 alone, 59.4% ± 7.0% for JQ1 plus compound C, $P < 0.0001$; Fig. 6D). The apoptotic cell fraction (Annexin V^+7-AAD^+) of $CD34^+CD38^-$ primary AML LSCs markedly increased with the addition of compound C to JQ1 (20.8% ± 2.7% for JQ1 alone, 66.4% ± 9.7% for JQ1 plus compound C, $P < 0.0001$; Fig. 6E). The $CD34^+CD38^-$ cell frequency was significantly decreased after cotreatment with JQ1 and compound C, while treatment with JQ1 alone did not affect the $CD34^+CD38^-$ cell frequency, suggesting that the AMPK-mediated resistance mechanism may act specifically in $CD34^+CD38^-$ LSCs (Fig. 6F). These findings indicated that AMPK inhibition could effectively overcome JQ1 resistance and enhance the sensitivity of AML LSCs to JQ1-induced apoptotic cell death.

Discussion

Although BET inhibitors such as JQ1 have shown early success in AML (10), drug resistance limits their clinical application. However, our knowledge of the molecular mechanisms underlying resistance to BET inhibitors, which is crucial to optimize the clinical efficacy of these drugs, remains incomplete. This study showed, for the first time, that AMPK/ULK1-mediated autophagy contributed to JQ1 resistance in LSCs.

Previous studies have shown that JQ1 induces cell death in leukemia bulk cells and AML LSCs (10, 12). Recent studies have shown that resistance to BET inhibitors is not mediated through increased drug efflux or metabolism but emerges in LSCs with increased expression of Wnt/β-catenin pathway components (20, 40). These data suggested that compensatory activation of transcriptional pathways sustaining the expression of Myc (e.g., Wnt-Myc) may underlie BET inhibitor resistance in AML and LSCs; however, not all LSCs are intrinsically resistant to BET inhibitors (20). Our current findings confirmed that the

Figure 4.

Effects of autophagy inhibition on JQ1-induced autophagy and apoptosis in LSCs. **A** and **B,** JQ1-resistant KG1 and KG1a cells were treated with 5 μmol/L JQ1 in the presence or absence of autophagy inhibitors bafilomycin-A1 (BafA1, 1 nmol/L), 3-methyladenine (3-MA, 5 mmol/L), and hydroxychloroquine (HCQ, 10 μmol/L). After incubation for 48 hours, the apoptotic fraction was measured using Annexin V/PI-based flow cytometric analysis. Columns, mean value of three independent experiments; bars, SDs. **C,** Cleaved caspase-3 and PARP were examined after treatment of KG1 and KG1a cells with JQ1 in the presence or absence of 3-MA for 24 hours using Western blot analysis. α-Tubulin served as a loading control. **D,** GFP-LC3-transfected KG1 and KG1a cells were treated with 5 μmol/L JQ1 with or without 5 mmol/L 3-MA for 24 hours, followed by confocal microscopic observations. The arrowheads and arrows indicate LC3 puncta and condensed nuclei, respectively. **E,** TEM ultrastructural examination of KG1 and KG1a cell lines treated with 5 μmol/L JQ1 in the presence or absence of 3-MA for 24 hours. The arrowheads and arrows indicate autophagosomes and apoptosomes, respectively. **F,** JQ1-resistant primary AML cells from six cases were treated with 5 μmol/L JQ1 in the absence or presence of 5 mmol/L 3-MA for 48 hours, after which the level of apoptosis was measured using Annexin V/PI flow-cytometric analysis. **G,** The apoptotic cell fractions (Annexin V^+7-AAD^+) of $CD34^+CD38^-$ primary LSCs were analyzed after treatment with JQ1 in the absence or presence of 5 mmol/L 3-MA for 48 hours using flow-cytometric analysis. Columns and bars represent the means ± SDs. **H–J,** For beclin-1 inhibition experiments, KG1 and KG1a cells were transfected with siRNA directed at beclin-1 as described in Materials and Methods. **H,** KG1 and KG1a cells were treated with 5 μmol/L JQ1 for 24 hours in the absence or presence of beclin-1 siRNA. Scrambled siRNA was used as a control. After incubation, cell lysates were subjected to Western blotting using the indicated antibodies. α-Tubulin was used as a protein loading control. **I,** GFP-LC3-transfected KG1 and KG1a cells were treated with 5 μmol/L JQ1 for 24 hours in the absence or presence of beclin-1 siRNA, followed by confocal microscopic observations. The arrowheads and arrows indicate LC3 puncta and condensed nuclei, respectively. **J,** KG1 and KG1a cells were treated with 5 μmol/L JQ1 for 48 hours in the absence or presence of beclin-1 siRNA. Scrambled siRNA was used as a control. The levels of apoptosis were determined using flow-cytometric analyses. Columns, mean value of three independent experiments; bars, SDs.

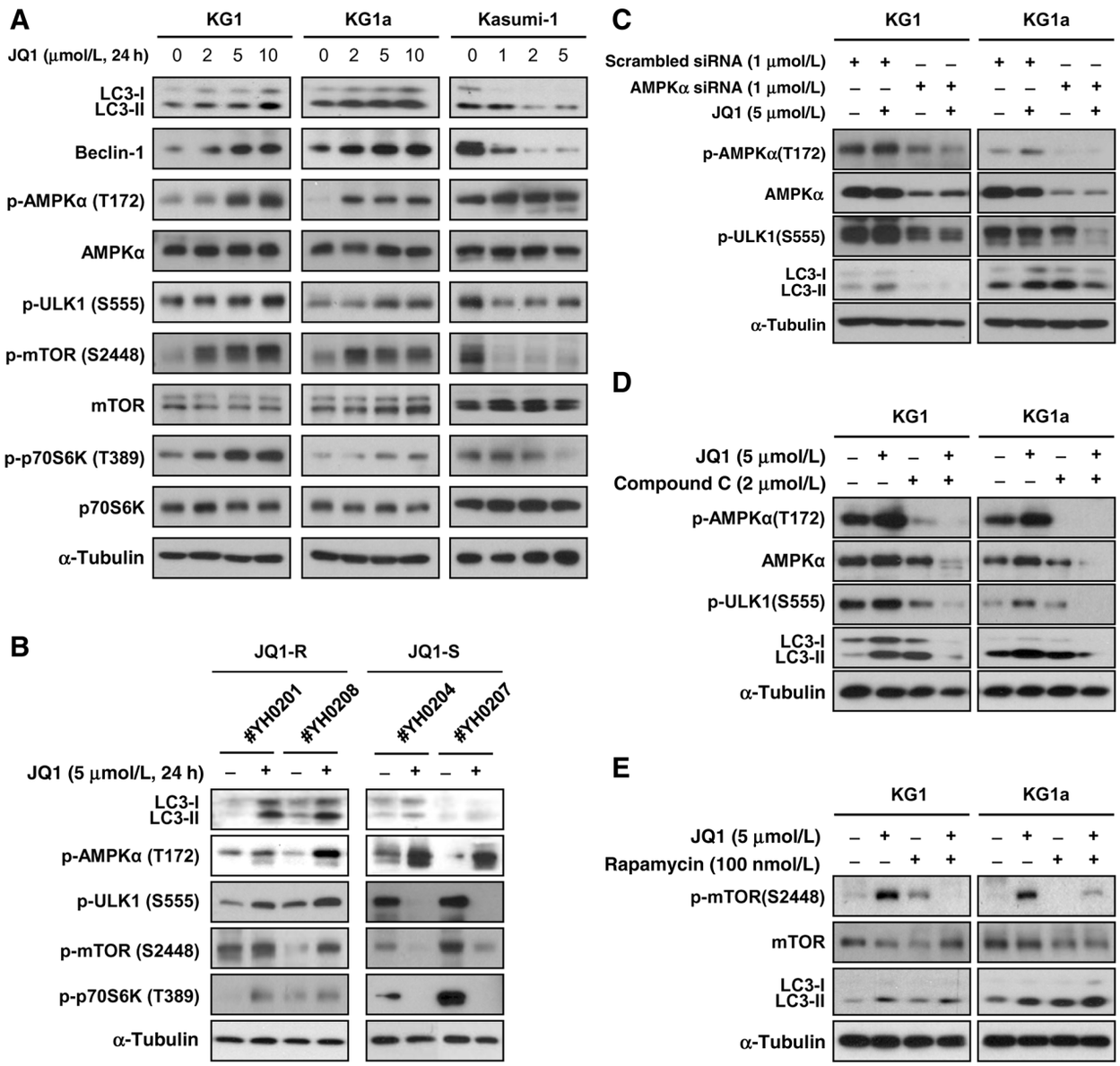


Figure 5.

Activation of AMPKα/ULK1 in JQ1-resistant LSCs. **A,** LSC candidate lines were incubated with various concentrations of JQ1 for 24 hours. After incubation, cell lysates were subjected to Western blotting using antibodies against the indicated molecules. **B,** After $CD34^+$-enriched primary AML cells (#YH0201 and #YH0208 for the JQ1-resistant group; #YH0207 and #YH0204 for the JQ1-sensitive group) were incubated with 5 μmol/L JQ1 for 24 hours, cell lysates were subjected to Western blotting using the indicated antibodies. **C,** For AMPK inhibition experiments, KG1 and KG1a cells were transfected with siRNA directed at AMPKα as described in Materials and Methods. Cells were treated with 5 μmol/L JQ1 for 24 hours in the absence or presence of AMPKα siRNA. Scrambled siRNA was used as a control. After incubation, cell lysates were subjected to Western blotting using the indicated antibodies. **D** and **E,** After KG1 and KG1a cells were treated with JQ1 for 24 hours in the absence or presence of the specific AMPKα inhibitor compound C (**D**) or mTOR inhibitor rapamycin (**E**), cell lysates were subjected to Western blotting with the indicated antibodies. α-Tubulin was used as a protein loading control.

response differs among LSC-like cells, although a substantial proportion of primary AML LSCs was resistant to JQ1. However, we also observed considerable c-Myc downregulation in both JQ1-resistant and JQ1-sensitive LSCs. Previous studies have reported contradictory results with regard to the correlation between c-Myc levels and JQ1 sensitivity in AML (19, 20, 40). Although c-Myc transcription appears to be dependent on BRD4, accumulating evidence indicates that the level of c-Myc suppression does not directly predict sensitivity to JQ1, suggesting that other mechanisms contribute to JQ1 resistance (17). Furthermore, the concentrations of JQ1 used in previous studies (i.e., 50–200 nmol/L) may be too low to induce LSC apoptosis. Similar to other studies, we found that the concentration of JQ1 required for 50% growth inhibition (GI_{50}) for

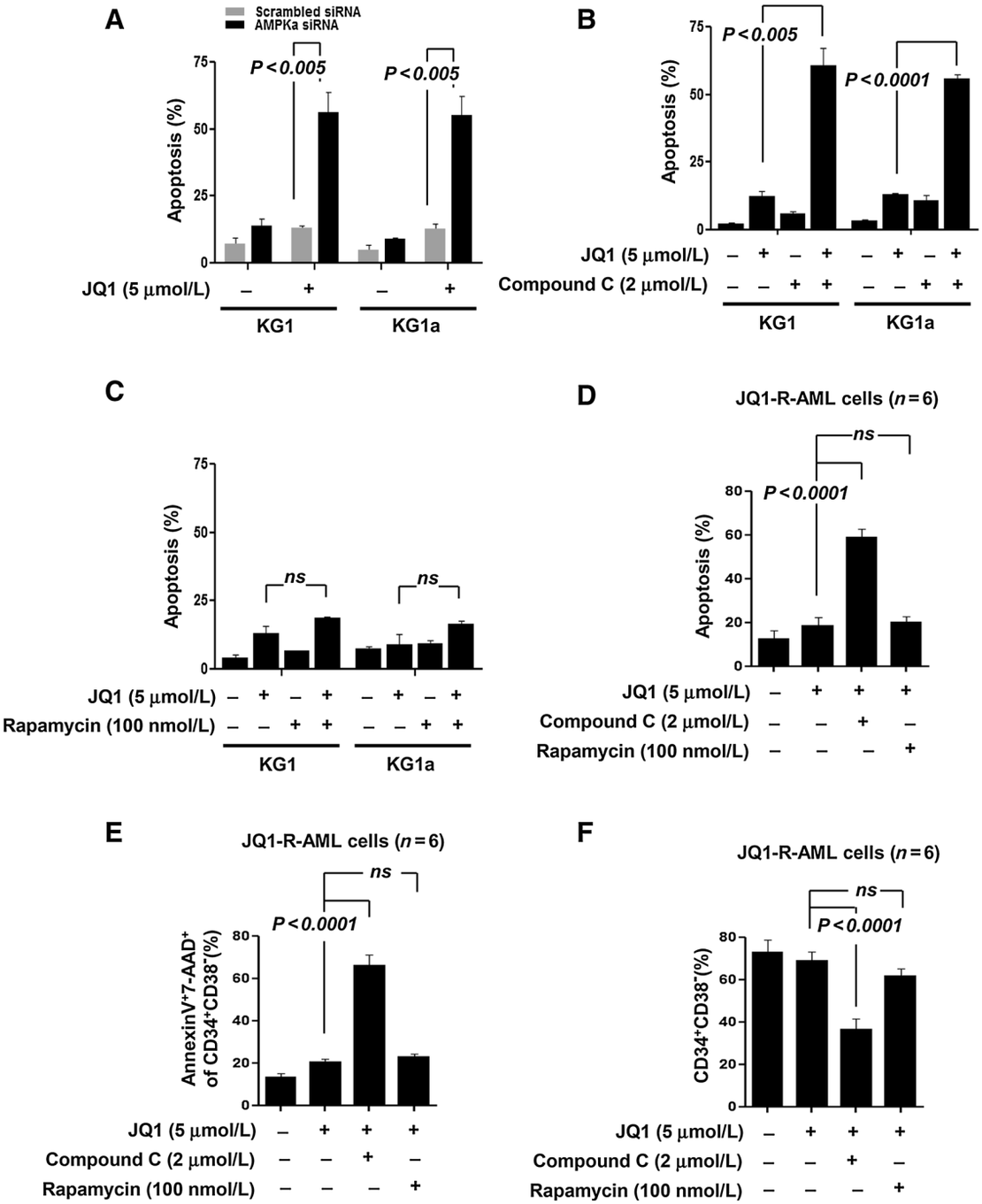


Figure 6.

Enhanced JQ1-induced apoptosis in AML LSCs with AMPK inhibition. **A,** KG1 and KG1a cells were transfected with AMPKα siRNA or scrambled siRNA. After incubation of transfected cell lines with 5 μmol/L JQ1 for 48 hours, the levels of apoptosis were determined using flow-cytometric analyses. Columns, mean value of three independent experiments; bars, SDs. **B** and **C,** KG1 and KG1a cells were treated with 5 μmol/L JQ1 in the absence or presence of the specific AMPKα inhibitor compound C (2 μmol/L, **B**) or mTOR inhibitor rapamycin (100 nmol/L, **C**). After 48 hours of incubation, cells were harvested and evaluated for the fraction of apoptotic cells using flow-cytometric analysis. **D–F,** $CD34^+$-enriched primary AML cells obtained from six JQ1-resistant cases were treated with JQ1 for 48 hours in the absence or presence of compound C (2 μmol/L) or rapamycin (100 nmol/L). **D,** After the cells were treated, the apoptotic fraction was measured using Annexin V/PI flow-cytometric analysis. **E,** After treatment, the apoptotic fraction (Annexin V^+7-AAD^+) of primary $CD34^+CD38^-$ LSCs was determined by flow cytometric analysis, as described in Materials and Methods. **F,** After treatment, the frequency of $CD34^+CD38^-$ LSCs among viable cells was determined by flow-cytometric analysis. Columns and bars represent the means ± SDs from six specimens.

JQ1 in $CD34^+$-enriched primary AML cells was 196.1 nmol/L (data not shown). Apoptosis was not induced in JQ1-resistant LSCs, even at a concentration 10-fold higher than the GI_{50}, although c-MYC transcription was inhibited (Fig. 1C, E, and F). These findings support that targeting BET is not effective for inducing c-Myc-related synthetic lethality in JQ1-resistant LSCs. Activation of LSC-relevant alternative signaling pathways or epigenetic changes may play important roles in the response to JQ1. To elucidate the mechanisms underlying cell survival despite c-Myc inhibition in JQ1-resistant LSCs, most experiments have used high concentrations of JQ1 (e.g., 5 μmol/L). Notably, in this study, we did exclude the effects of gene mutations on the JQ1 response by avoiding the use of cells from patients with AML having recurrent mutations.

Anticancer therapies commonly activate prosurvival autophagy, allowing cancer cells to overcome cytotoxic or other stresses induced by the treatment (41). We previously described the involvement of autophagy induction in the resistance of myeloid leukemia cells to the cytosine arabinoside (42). Paradoxically, previous studies have reported that c-Myc activates the unfolded protein response, enhancing cell survival by inducing cytoprotective autophagy; conversely, c-Myc inhibition decreases autophagy in cancer cells (43). Consistent with this finding, we showed that autophagy was attenuated in JQ1-sensitive LSCs after JQ1 treatment, resulting in apoptosis. Autophagy induced by c-Myc or BET inhibitors has not been examined in cancer cells. Interestingly, we found that a prosurvival autophagic response was associated with JQ1 resistance in AML LSCs; JQ1 induced high levels of autophagy, and autophagy inhibition using knockdown of beclin-1 or pharmacological inhibitors resulted in synergistic enhancement of JQ1-induced apoptosis in these LSCs. Furthermore, we observed degradation of p62/SQSTM1 during JQ1-induced autophagy in JQ1-resistant cells, and did not observe decreased JQ1-induced apoptosis following cotreatment with JQ1 and the autophagic degradation inhibitor bafilomycin A1 in JQ1-sensitive cells. These results showed that accumulation of autophagosomes was not associated with JQ1 sensitivity. It is possible that evidence of autophagy could not be observed in the eliminated sensitive cells. We did not further examine the effects of enforcing autophagy in JQ1-sensitive cells. However, our findings clearly showed that cytoprotective autophagy increased the ability of LSCs to resist apoptosis by JQ1. Taken together, our results indicated that autophagy induction by JQ1 exposure was a critical mechanism involved in JQ1 resistance in AML LSCs.

Cells treated with a c-Myc inhibitor respond to the loss of ATP by activating AMPK, which normally replenishes ATP by promoting glycolysis and oxidative phosphorylation (39). However, AMPK activation following c-Myc inhibition by JQ1 is futile because of the lack of functional Myc to support the requisite anabolic response; thus, AMPK activation cannot stop the onset of apoptosis (39). Accordingly, JQ1-sensitive LSCs underwent apoptosis in our study, despite AMPK activation. Notably, AMPK activation is one of the critical pathways involved in autophagy induction (37). AMPK interacts with ULK1 and mTOR in the regulation of autophagy (44); AMPK inhibits the mTOR pathway, which inactivates ULK1-induced initiation of autophagy. AMPK also directly phosphorylates and activates ULK1 at Ser317, Ser555, and Ser777 to initiate autophagy. However, recent studies have reported that inactivation of mTOR is not always necessary for autophagy (45). Consistent with this finding, we found that AMPK activation after JQ1 treatment induced autophagy through ULK1 (Ser555) phosphorylation in JQ1-resistant LSCs, independent of mTOR inhibition. The role of AMPK/ULK1 activation in JQ1-induced autophagy was confirmed by targeting of AMPK with a pharmacologic inhibitor and siRNA. AMPK inhibition decreased JQ1-induced autophagy in LSCs, leading to an increase in JQ1-induced apoptosis. AMPK activation did not lead to JQ1 resistance when autophagy was blocked, suggesting that autophagy was activated downstream of AMPK to promote resistance. Based on our findings, we propose a model to explain resistance to JQ1 in LSCs (Supplementary Fig. S5). In JQ1-sensitive LSCs, ULK1 was not activated, despite AMPK activation and mTOR inhibition. It remains unclear why ULK1 phosphorylation was induced with AMPK activation preferentially in JQ1-resistant cells. Phosphorylation of ULK1 could be modulated by AMPK/mTOR activity and by conformational changes, alterations in allosteric coupling, and ULK1 mutations (37, 46). One of these mechanisms or other unknown causes could explain the differential phosphorylation of ULK1 in response to AMPK activation in AML LSCs. Further investigation of the interactions and regulatory mechanisms of the AMPK/ULK1 axis will improve our knowledge of the mechanisms of autophagy-associated drug resistance in AML LSCs.

A recent study reported that AMPK protects AML LSCs from metabolic stress (47). Our data showed that AMPK inhibition markedly enhanced JQ1-induced apoptosis in LSCs. These results suggest that combining AMPK inhibition with JQ1 treatment may be a useful approach for elimination of LSCs, resulting in therapeutic cures for AML. Based on our study, differential expression or mutations in genes associated with AMPK-mediated autophagy induction may serve as a prognostic marker in AML. However, future studies are needed to determine how these cellular processes are differentially regulated between JQ1-sensitive and JQ1-resistant AML LSCs. Additionally, *in vivo* studies are needed to confirm the effects of the combination of JQ1 and autophagy inhibitors. JQ1 displays antitumor activities in a variety of human cancers with differential sensitivity (48, 49). One of the major challenges for the further development and clinical testing of JQ1 is the lack of reliable biomarkers to predict sensitivity to this promising compound. Our findings may provide valuable information on the molecular mechanisms involved in resistance to the BET inhibitor JQ1 in various tumor types, including AML, and suggest treatment strategies for overcoming resistance.

Although all of the events responsible for AMPK-induced ULK1 activation in JQ1-resistant LSCs have not yet been elucidated, we have provided new insights into the role of AMPK/ULK1 activation-mediated autophagy as a critical mechanism underlying JQ1 resistance in AML LSCs. Our finding that treatment with inhibitors of autophagy or AMPK can overcome JQ1 resistance and enhance the apoptotic response suggests the clinical usefulness of these unique targeted therapies against AML LSCs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Disclaimer

The funders had no role in the study design, data collection and analysis, decision to publish, or manuscript preparation.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H.-K. Jeung
Study supervision: J.S. Kim, Y.H. Min

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