BET inhibitors combined with chemotherapy synergistically inhibit the growth of NSCLC cells

XIAO ZHOU[1*], TONG SUN[2*], YUTING MENG[3*], JUAN LUO[1], JUN CHEN[1], BO XIA[1], ZUHAO ZHANG[3], ZHIXIANG CHENG[1,4] and XUERONG WANG[2,3]

[1]Department of Pain Management, The Second Affiliated Hospital, Nanjing Medical University, Nanjing, Jiangsu 210011; [2]Key Laboratory of Human Functional Genomics of Jiangsu Province, Nanjing Medical University, Nanjing, Jiangsu 211100; [3]Department of Pharmacology, Nanjing Medical University, Nanjing, Jiangsu 210029; [4]Department of Pain Management, Sir Run Run Hospital, Nanjing Medical University, Nanjing, Jiangsu 211100, P.R. China

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Abstract. The bromodomain and extra-terminal domain (BET) family proteins are essential epigenetic regulators in lung cancer. However, BET inhibitors have not had the anticipated therapeutic efficacy. Combined treatment using BET inhibitors along with other drugs had favorable therapeutic effects but the underlying molecular mechanisms remain elusive. The aim of the present study was to investigate the antineoplastic effects and mechanisms of a combination of a BET inhibitor and paclitaxel or cisplatin in non-small cell lung cancer (NSCLC). By using the online Kaplan-Meier plotter, it was revealed that increased mRNA levels of several BET protein-coding genes were associated with poor prognosis in NSCLC. SRB assay results revealed that pharmaceutical or genetic targeting of BET proteins suppressed the growth of NSCLC cells. Inhibition of BET protein expression, in combination with the use of chemotherapeutic drugs such as paclitaxel and cisplatin, further restrained NSCLC cell growth in a synergistic manner. Mechanistically, this combination of suppression of BET expression and chemotherapeutic treatment blocked NSCLC cell growth by inhibiting autophagy and promoting apoptosis, which were revealed by both western blot and ELISA results. The present findings revealed a new rationale for using a combination of BET inhibitors with chemotherapy in NSCLC treatment.

Correspondence to: Professor Zhixiang Cheng, Department of Pain Management, The Second Affiliated Hospital, Nanjing Medical University, 121 Jiang Jia Yuan, Nanjing, Jiangsu 210011, P.R. China
E-mail: zhixiangcheng@njmu.edu.cn

Professor Xuerong Wang, Department of Pharmacology, Nanjing Medical University, 140 Hanzhong Road, Nanjing, Jiangsu 210029, P.R. China
E-mail: wangxrwn@hotmail.com

*Contributed equally

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Introduction

Lung cancer is one of most common types of cancer worldwide in terms of both incidence and mortality. In 2018, approximately 2.1 million new cases of lung cancer were reported and 1.8 million deaths were predicted (1,2). In China in particular, recent statistics provided by the National Central Cancer Registry of China (NCCRC) indicated that lung cancer had the highest incidence rate in males as well as in the whole population. Lung cancer was also the top contributor to cancer-related deaths in China (3,4).

Over 80% of lung cancers are non-small cell lung cancer (NSCLC), which is treated primarily by chemotherapy, immunotherapy, surgery, targeted therapy, and radiation therapy (5-7). Some significant advances have been made in NSCLC treatment; however, the survival rate remains poor due to the large number of late-stage diagnoses (8). The call for precision medicine in cancer therapy has also raised an urgent need for the discovery of new drug targets for NSCLC (9).

One promising approach for identifying new therapeutics is epigenetic editing, which is now undergoing extensive studies in cancer biology. Epigenetic editing mediates gene expression by changing the patterns of chromatin accessibility (10). Epigenetic changes are an important feature of NSCLC, making this a valuable approach for lung cancer therapy (11,12). One family of epigenetic regulators is represented by the bromodomain and extra-terminal domain (BET) family proteins. These proteins bind to acetylated histones or transcription factors and serve as scaffolds for the recruitment of mediator complexes to promote protein transcription (13). Members of the BET family, such as BRD2, BRD3, BRD4, and BRDT, are all structured with two highly conserved N-terminal bromodomains, an extra-terminal domain, and a divergent C-terminal recruitment domain (14,15). Numerous studies have confirmed that BET proteins are highly expressed in benign tumors and in malignancies, such as lung, prostate, breast, colon, intestine, pancreas, liver, and brain tumors. The roles and mechanisms of BRD4 have been extensively

studied (16-18); whereas studies on BRD2 and BRD3 are extremely limited, especially in lung cancer. Moreover, the correlations of BRD2, BRD3, and BRD4 expression and the progression of lung cancer remain elusive. Nevertheless, inhibition of BET protein activity may represent a promising therapeutic strategy for treating NSCLC.

The first potent and selective BET family protein inhibitor ('BET inhibitor', henceforth) was JQ1, a thienotriazolodiazepine. Since its discovery, numerous other BET inhibitors, such as I-BET151 and OTX015, have been used in clinical trials and some have exhibited clear therapeutic effects (19). However, BET inhibitors have not exhibited the anticipated therapeutic efficacy in solid tumors, such as lung cancer, for reasons that remain unknown. One important strategy for enhancing the anticancer effect of BET inhibitors could therefore be to combine these inhibitors with other drugs.

In small-cell lung cancer, both *in vitro* and *in vivo* experiments have revealed a strong synergistic apoptosis-promoting effect when cells are co-treated with the BCL2 inhibitor venetoclax (ABT-199) and the BET inhibitor ABBV-075 (20). In NSCLC, combined treatments using the BET inhibitor JQ1 and the DDR2 inhibitor dasatinib inhibited tumor growth both *in vivo* and *in vitro*; however, the mechanism remains unclear (21). Therefore, identifying the underlying molecular mechanism could aid in the development of more effective combination therapies for NSCLC.

NSCLC is currently treated with chemotherapy as the standard treatment for late-stage (III and IV) patients with NSCLC. Doublet chemotherapy, such as a combination of platinum (cisplatin or carboplatin) with taxanes (paclitaxel, docetaxel or vinorelbine), is a common first-line therapy. Paclitaxel promotes the assembly of microtubules and stabilizes the microtubules, and paclitaxel-induced apoptosis in lung cancer has been confirmed in numerous studies (22-24). The anticancer effect of cisplatin largely relies on the formation of platinum-DNA adducts by interactions between platinum and the nucleotide bases (25). Some patients benefit from doublet chemotherapy; however, others undergo no benefits or experience severe adverse drug reactions (ADRs) (5,26).

In the present study, the association between the expression of BET and patient survival was analyzed according to online databases. Henceforth, BRD2, BRD3, and BRD4 are indicated as 'BET' and their genes or mRNAs are designated as '*BET*.' The growth-inhibitory effects of BET silencing were evaluated using siRNAs and BET inhibitors, such as JQ1 and I-BET151. The effects of BET inhibitors on each BET member at both the mRNA and protein levels were also revealed. Lastly, the growth-inhibitory effect of a combined therapy of a BET inhibitor and a chemotherapy drug on NSCLC cells was assessed. The effects of combined therapy on cell autophagy and apoptosis were also explored. The present findings provide new strategies for enhancing the anticancer effects of BET inhibitors.

Materials and methods

UALCAN analysis. UALCAN is a comprehensive online tool for analyzing cancer transcriptome data (27). We utilized this tool to explore the correlation between *BET* expression and two types of NSCLC, lung squamous cell carcinoma (LUSC) and lung adenocarcinoma (LUAD).

Cell lines and culture conditions. All cell lines [human bronchial epithelial (HBE), A549, H157, H1299] were purchased from the American Type Culture Collection (ATCC). Cells were maintained in RPMI-1640 medium (Hyclone; Cytiva) supplemented with 5% fetal bovine serum (Gibco BRL; Thermo Fisher Scientific, Inc.) in a 5% humidified incubator at 37˚C. All cell lines were authenticated via short tandem repeat (STR) profiling of 9 genomic loci with the Powerplex 16 HS system (Promega Corporation).

Antibodies and reagents. Antibodies were purchased as follows: Anti-BRD4 (E2A7X) (product no. 13440), anti-BRD2 (D89B4) (product no. 5848), anti-PARP (product no. 9542), and anti-caspase-3 (product no. 9665) were purchased from Cell Signaling Technology, Inc.. Anti-BRD3 (2088C3a; product code ab50818) was purchased from Abcam, anti-LC3I/II (cat. no. NB100-2220) was purchased from Novus Biologicals, Ltd., anti-GAPDH (cat. no. AP0063) was purchased from Bioworld Technology, Inc.. All antibodies were utilized at a 1:1,000 dilution except for GAPDH (1:10,000). JQ1 (cat. no. HY-13030) was purchased from Haoyuan Chemexpress Co., Ltd. I-BET151 (cat. no. SML0666) and chloroquine (CQ) (product no. C6628) were purchased from Sigma-Aldrich; Merck KGaA. Reagents were dissolved in DMSO at 20 mmol/l, stored at -20˚C, and diluted just before use. Paclitaxel (PTX) (cat. no. HY-B0015) was purchased from Taiji industry (Group) Co., Ltd., cisplatin (cat. no. HY-173940) was purchased from MedChemexpress Co., Ltd., and diluted to 1 mg/ml, and stored at 4˚C.

Sulforhodamine B (SRB) assays. Cells were seeded into 96-well plates at 3,000 cells/well, and treated with either 2, 5, 10 ng/ml of paclitaxel or 1, 2, 4 μl/ml of cisplatin along with respective controls on the second day of cell culture. After 3 days, cells were subjected to SRB staining assay as previously described (28). The IC_{50} and the combination index (CI) were determined using Compusyn software (29). CI <1 was defined as a synergistic effect.

siRNAs and transfection. All siRNAs were obtained from Shanghai GenePharma Co., Ltd.. Cells were seeded in 6 well plates and transfected with either 2 μg of targeting siRNAs or scrambled control to obtain a final concentration of 100 nmol/l using Lipofectamine 2000 (cat. no. 11668019; Invitrogen; Thermo Fisher Scientific, Inc.) for 24 h at 37˚C followed by subsequent experiments such as being cultured another 24 h for whole-cell protein preparation or RNA purification, or reseeded to 96-well plates in an equal amount for SRB assay. The sequences of siRNAs were as follows: siRNA#1 (for BRD4),5'-CUCCCUGAUUACUAUAAGATT-3',5'-GCACAAUCAAGUCUAAACUTT-3' and 5'-GGAGAUGACAUAGUCUUAATT-3'; siRNA#2 (for BRD3), 5'-GUGCAAGCGAAUGUAUGCATT-3', 5'-CGGAUGUUCUCGAAUUGCUTT-3' and 5'-GUAGUGCACAUCAUCCAAUTT-3'; siRNA#3 (for BRD2), 5'-CAGCUGCAAUACCUACACATT-3', 5'-GACUUCUCAAGUCCUUGCATT-3' and 5'-GGACAGCUCAAUUCUACUATT-3'.

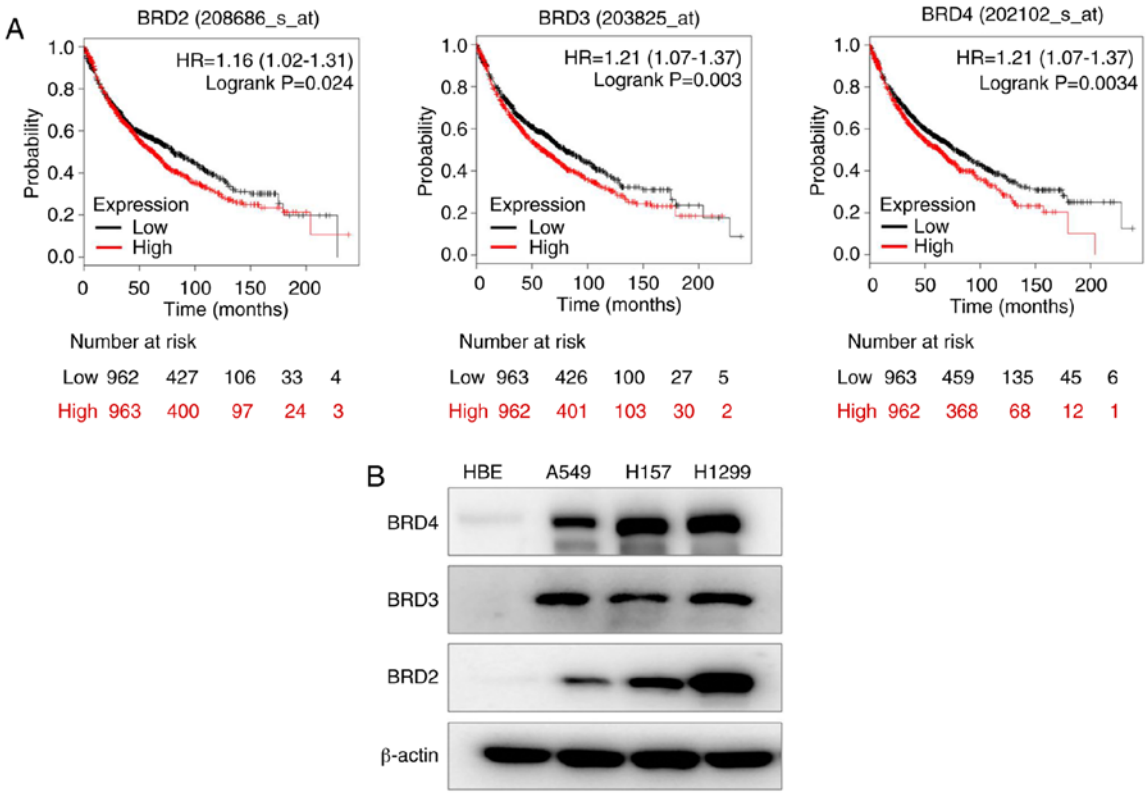

Figure 1. Kaplan-Meier plot of overall survival probability among NSCLC patients with high or low expression of BET. (A) Association between the expression of BET and the survival of patients with NSCLC. Analysis was performed by the Kaplan-Meier method. The log-rank test was used to compare survival curves. (B) Indicated cells were cultured for 24 h, and whole-cell lysates were prepared and subjected to western blotting. NSCLC, non-small cell lung cancer; BET, bromodomain and extra-terminal domains.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Cells were harvested for RT-qPCR as previously described (30). The primer sequences used for the present study are as follows: BRD4 forward (F), 5'-AGCAGCAACAGCAATGTGAG-3' and reverse (R), 5'-GCTTGCACTTGTCCTCTTCC-3'; BRD3 F, 5'-CGGAAGCTCCAGGACGTGTT-3' and R, 5'-GGAGCCACCTTGGCCTTCTT-3'; BRD2 F, 5'-CAGGAACAGCTTCGGGCAGT-3' and R, 5'-TCATGGGCCTGCTCTCTTCC-3'; GAPDH F, 5'-ATGGGGAAGGTGAAGGTCG-3' and R, 5'-GGGGTCATTGGCAACAACAATA-3'.

Western blots analysis. Cells were subjected to western blot analysis as previously described (31). The chemiluminescent signal was collected and analyzed using the ChemiScope 3400 Mini by Clinx Science Instruments Co., Ltd..

ELISA assay. Cells were treated with either 4 μmol/l of JQ1 or 10 ng/ml of PTX for 24 h. For the JQ1 and PTX co-treatment, cells were pre-treated with 4 μmol/l of JQ1 for 2 h before PTX was added to the medium at a final concentration of 10 ng/ml. Cells were then cultured for another 24 h. The concentration of caspase-3 in the cells was determined using a human caspase-3 ELISA kit (product no. E-EL-H0017c; Elabscience) according to the manufacturer's instructions.

Statistical analysis. All results are expressed as the mean ± SD of at least three independent experiments. For statistical analysis, one-way analysis of variance (ANOVA) with Sidak's correction for multiple comparisons or two-tailed unpaired t-test with GraphPad Prism 7.0 (GraphPad Software, Inc.) were used. $P<0.05$ was considered to indicate a statistically significant difference. Kaplan-Meier database (www.kmplot.com) was used to analyze the association between the expression of BET and overall patient survival using a hazard ratio (HR) with 95% confidence intervals (CI) and log rank P-value. Parameters were set as follows: Set 'Survival' as 'Overall Survival (OS)', set 'Follow up threshold' as 'all' and set all of the 'Restrict analysis to subtypes' as 'all'.

Results

Association between BET and NSCLC. Increased expression of BRD4 has been reported in the cancerous tissues from patients with NSCLC when compared to peripheral normal tissues. A negative association has also been revealed between BRD4 expression and the 5-year survival rate of patients with NSCLC (16). However, the expression and the role of BRD2 and BRD3 in NSCLC has not been investigated. Therefore, the association between BET mRNA expression levels and NSCLC was evaluated according to the datasets in the TCGA database using the UALCAN website (http://ualcan.path.uab.edu), and significant increases in BRD2, BRD3, and BRD4 in lung squamous cell carcinoma, but not in adenocarcinoma (Fig. S1) were observed.

The association between the expression of BET and overall patient survival based on datasets in the Kaplan-Meier database (www.kmplot.com) (32) were then analyzed. The patients

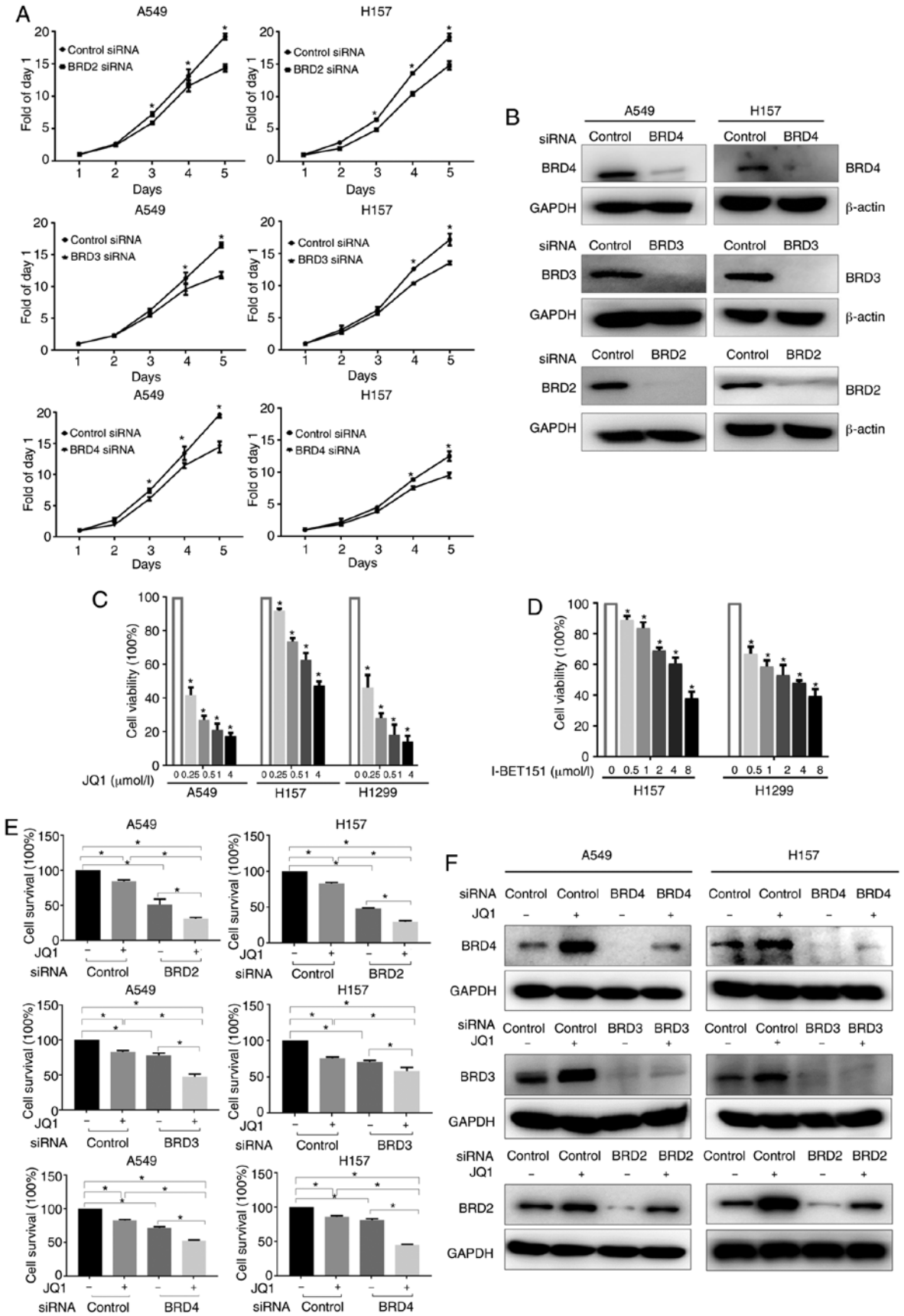

Figure 2. Targeting BET inhibits the growth of NSCLC cells. (A and B) NSCLC cells were transfected with siRNAs (a pool containing 3 sequences of siRNA) targeting BRD2, BRD3, BRD4, or control siRNA for 24 h, cells were then re-seeded into 96-well plates, cultured for 5 days, and subjected to either (A) SRB assay or (B) western blot analysis. (C and D) NSCLC cells were treated with either JQ1 or I-BET151 for 3 days and subjected to SRB assay. Data are presented as the mean ± SD (n=4). (E) NSCLC cells were transfected with BRD2, BRD3, BRD4 or control siRNAs for 24 h, re-seeded into 96-well plates, and treated with JQ1 (2 μmol/l) on the second day for another 72 h. The cell survival rate was evaluated by SRB assay. (F) Twenty-four hours after BET siRNA-transfection, cells were treated with or without JQ1 for another 24 h, and subjected to western blot analysis. *P<0.05. BET, bromodomain and extra-terminal domains; NSCLC, non-small cell lung cancer; SRB, sulforhodamine B.

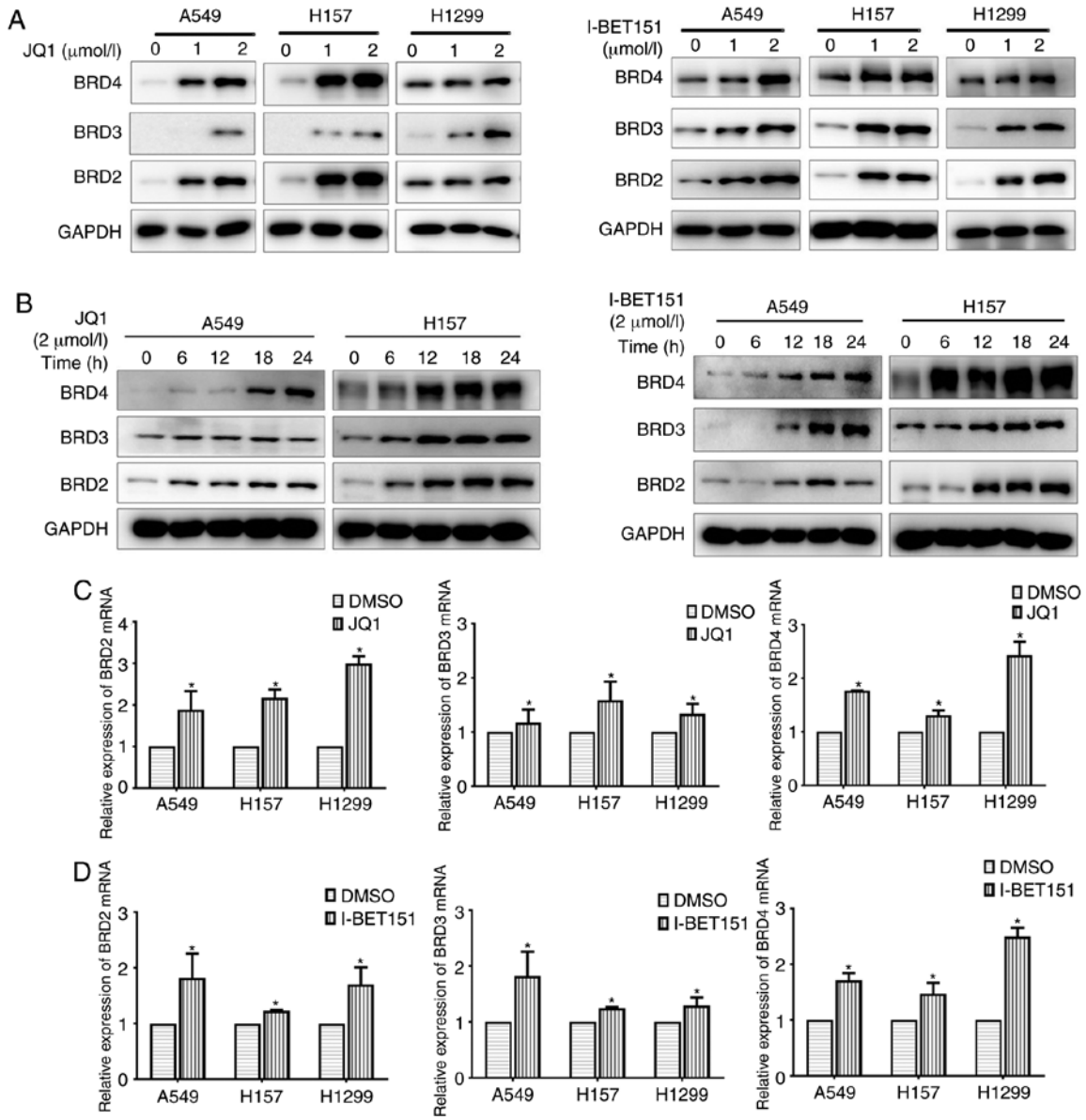

Figure 3. BET inhibitors upregulate the expression of BET. (A) NSCLC cells were treated with different concentrations of JQ1 or I-BET151 as indicated for 24 h and subjected to western blot analysis. (B) NSCLC cells were treated with JQ1 or I-BET151 (2 μmol/l) for various time-points as indicated and subjected to western blot analysis. (C and D) NSCLC cells were treated with JQ1 (2 μmol/l) or I-BET151 (2 μmol/l) for 6 h, and subjected to RT-qPCR analysis. Data are presented as the mean ± SD (n=3). *P<0.05. BET, bromodomain and extra-terminal domains; NSCLC, non-small cell lung cancer; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

were divided into two groups based on their median mRNA expression levels (high vs. low expression) and assessed by a Kaplan-Meier survival plot using a hazard ratio (HR) with 95% confidence intervals (CI) and log rank P-value. The expression of BRD2, BRD3, and BRD4 was negatively associated with the overall survival (OS) probability of the patients with NSCLC (Fig. 1A). Then, the BET protein levels in four NSCLC cell lines were determined, and it was revealed that BET expression was higher in A549, H157, and H1299 cells than in normal HBE cells (Fig. 1B). Therefore, all three BET may have important roles in NSCLC.

Targeting BET inhibits the growth of NSCLC. The function of BET (especially of BRD2 and BRD3, as these have not been evaluated previously) was explored by knocking down their expression in A549 and H157 cells using siRNAs. The SRB assay results revealed that silencing of each individual *BET* gene significantly inhibited cell growth compared to control cells (Fig. 2A). Western blotting confirmed successful knockdown of all three proteins in both A549 and H157 cell lines (Fig. 2B). The effect of BET inhibitors on the growth of NSCLC cells was then evaluated. Treatment with JQ1, a BET family protein inhibitor that mainly blocks BRD4, dose-dependently suppressed the growth of A549, H157, and H1299 cells, with IC_{50} values of 0.06, 4.625, and 0.11 μmol/l, respectively (Fig. 2C). Another BET family protein inhibitor, I-BET151, also dose-dependently inhibited the growth of NSCLC cells (Fig. 2D).

The effect of BET silencing on cell survival in combination with JQ1 treatment was also assessed. A549 and H157 cells were transfected with BRD2, BRD3, or BRD4 siRNAs and then treated with or without JQ1 for another 24 h. As

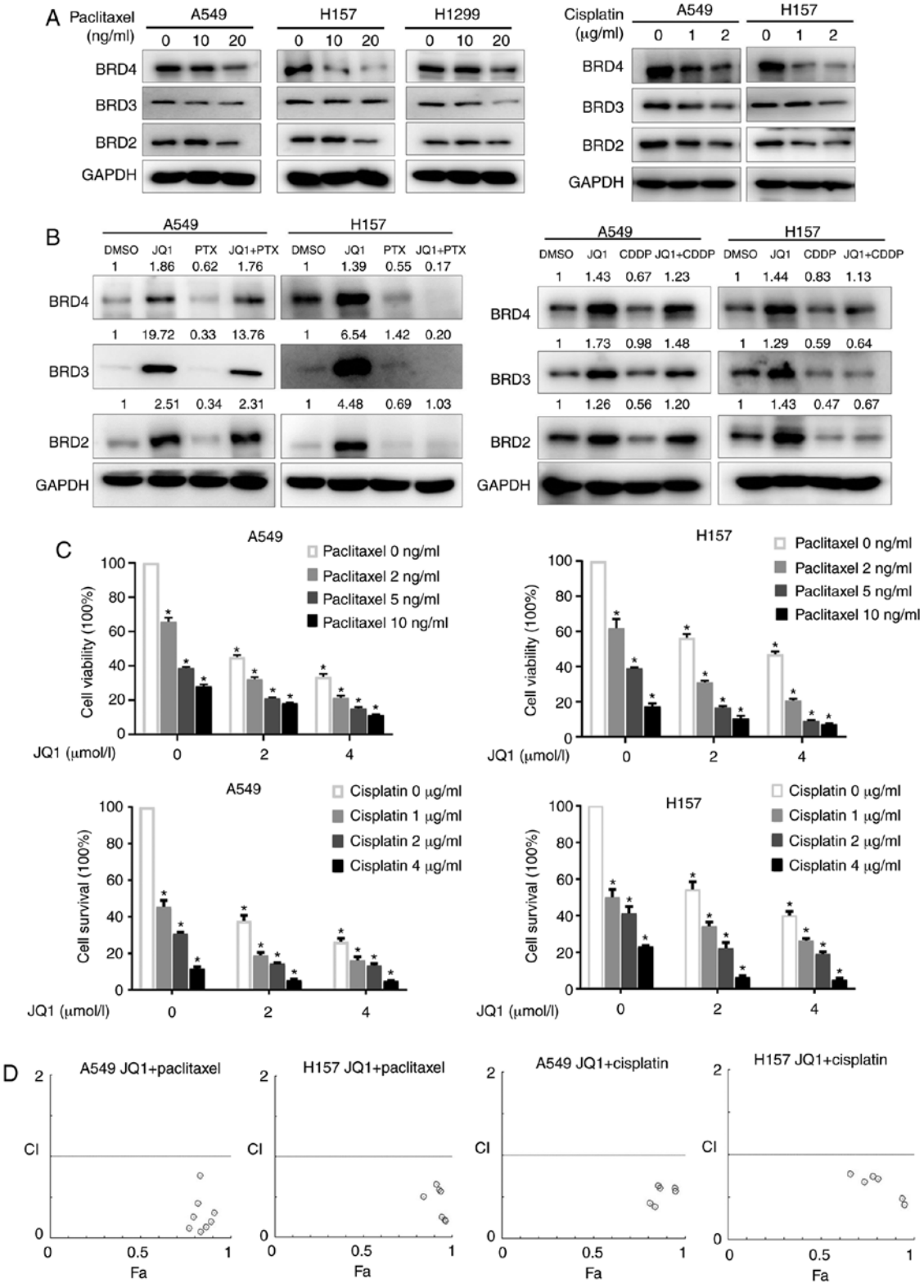

Figure 4. PTX and CDDP reduce the expression of BET and enhance the growth inhibitory effect of JQ1. (A) NSCLC cells were treated with different concentrations of paclitaxel or cisplatin as indicated for 24 h, followed by western blot analysis. (B and C) NSCLC cells were treated with JQ1, PTX or CDDP, or their combination as indicated for 24 h, and subjected to either (B) western blot analysis or (C) SRB assay after 72 h treatment. Data are presented as the mean ± SD (n=4). *P<0.05. Relative densitometric quantitation numbers are presented above the bands. (D) Combination index of JQ1 with PTX or CDDP in C was calculated using CompuSyn software. PTX, paclitaxel; CDDP, cisplatin; BET, bromodomain and extra-terminal domains; NSCLC, non-small cell lung cancer; SRB, sulforhodamine B.

revealed in Fig. 2E, JQ1 treatment enhanced the growth inhibitory effects triggered by silencing of each individual *BET* gene. Western blotting confirmed the successful knockdown of each individual BET (Fig. 2F). It was also observed that knockdown of one BET had negligible effects on the expression level of the other two BET, indicating an absence of any

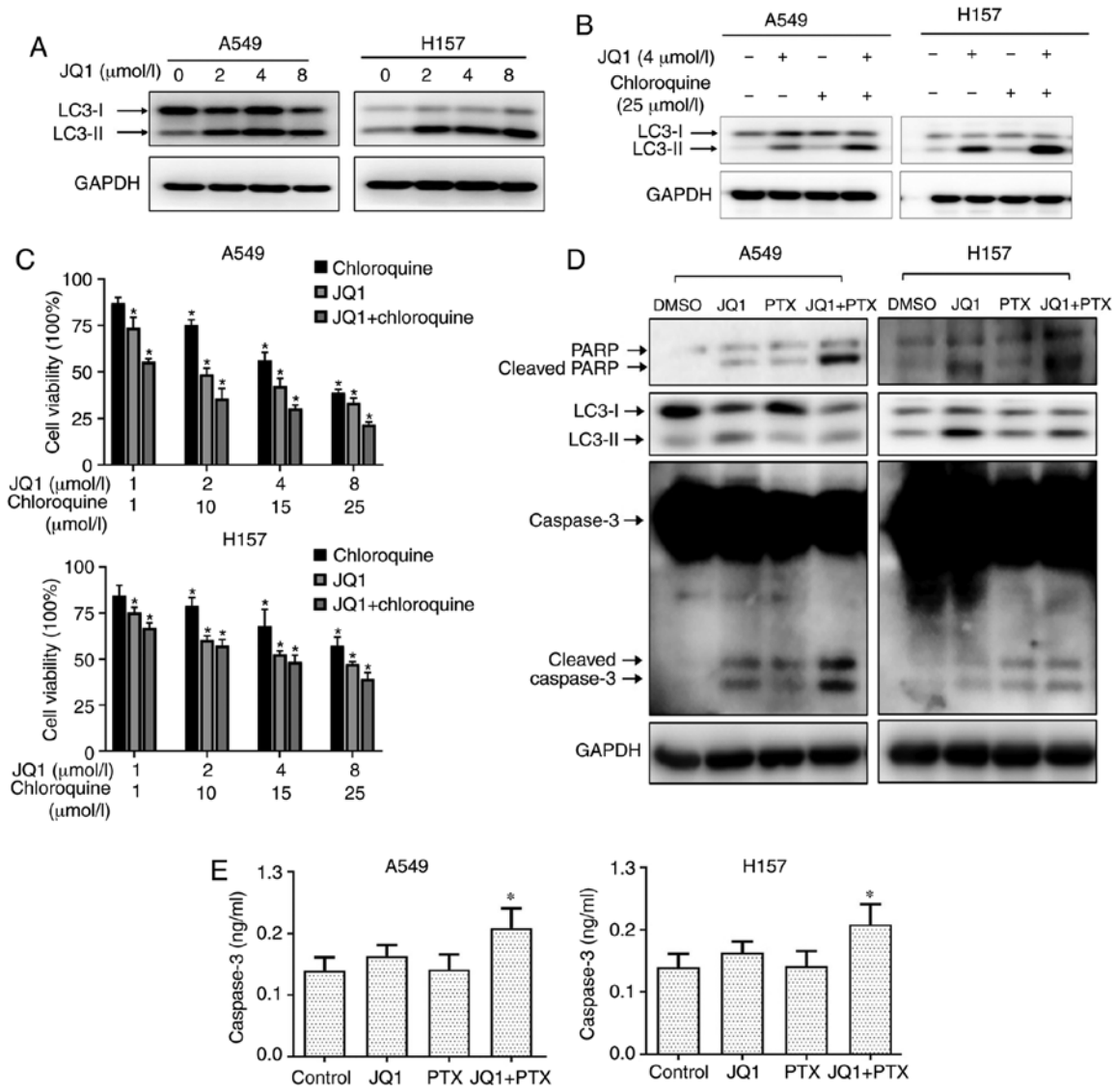

Figure 5. JQ1 combined with paclitaxel inhibits protective autophagy and induces apoptosis. (A) NSCLC cells were treated with different concentrations of JQ1 as indicated and subjected to western blot analysis. (B) NSCLC cells were pre-treated with CQ (25 μmol/l) for 2 h, then co-treated with JQ1 for 24 h. Cells were then subjected to western blot analysis. (C) NSCLC cells were treated with JQ1, CQ, or their combination with the indicated concentrations for 3 days followed by SRB assay. Data are presented as the mean ± SD (n=4). *P<0.05. (D and E) NSCLC cells were pretreated with PTX for 2 h followed by co-treatment with or without JQ1 for 24 h. Cells were then subjected to either (D) western blot analysis or (E) ELISA assay. *P<0.05. NSCLC, non-small cell lung cancer; CQ, chloroquine; SRB, sulforhodamine B; PTX, paclitaxel.

compensatory expression among the BET (data not shown). It was also observed that JQ1 treatment alone induced a significant increase in the expression of BET compared with that in the control group without JQ1 treatment (Fig. 2F). Therefore, BET inhibitor treatments inhibited the protein function of BET by binding competitively to the BET bromodomains, as previously reported (33), but they also concurrently upregulated BET expression.

BET inhibitors upregulate both the mRNA and protein expression of BET. The regulation of BET was examined by the BET inhibitors, JQ1 and I-BET151. Both JQ1 and I-BET151 upregulated BET expression in a dose- and time-dependent manner (Fig. 3A and B). A significant increase in BET expression was also observed after a 6-h JQ1 treatment, indicating a relatively rapid regulation (Fig. 3B). The regulatory pattern of I-BET151 on BET was similar to that of JQ1, except that a significant increase in BRD2 and BRD3 expression was only observed after a 12-h treatment, rather than 6-h (Fig. 3B). Both BET inhibitors upregulated the mRNA expression levels of *BET* (Fig. 3C and D). The upregulation of BET expression by BET inhibitors could therefore contribute to the limited efficacy of BET inhibitors observed for NSCLC compared to leukemia or glioblastoma (34).

Chemotherapeutic medicines paclitaxel and cisplatin downregulate BET protein levels and enhance the growth inhibitory effect of JQ1. Paclitaxel and cisplatin are classical anticancer medicines that are recommended as first-line chemotherapies for NSCLC. Their effects on the expression of BET were explored. Western blotting revealed a dose-dependent downregulation of BET by either paclitaxel or cisplatin (Fig. 4A). Both paclitaxel and cisplatin partially reversed the JQ1-induced increase in BET expression in A549 and H157 cells (Fig. 4B).

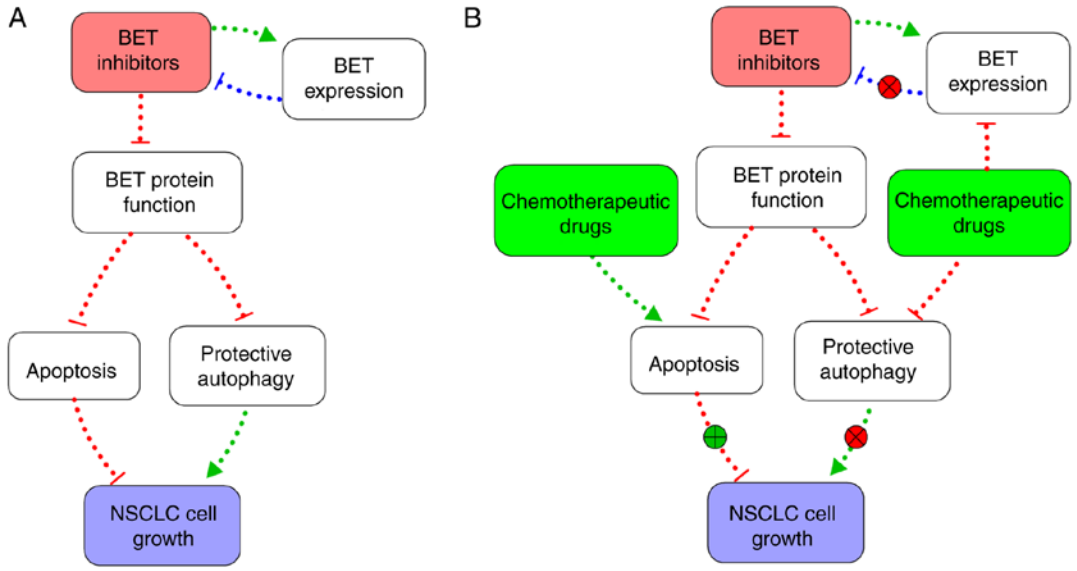

Figure 6. Working model of both single treatment and combined treatment that could affect the growth of NSCLC cells. (A) When NSCLC cells were treated with BET inhibitors, NSCLC cell growth was inhibited, which mainly resulted from the up-regulation of apoptosis. The growth-inhibitory effect of BET inhibitors could be attenuated by the stimulation of protective autophagy as well as the compensatory up-regulation of BET expression. (B) When cells were treated with chemotherapeutic drugs in addition to BET inhibitors, NSCLC cell growth was further inhibited due to the reinforcement of apoptosis, suppression of protective autophagy as well as inhibition of the compensatory increment of BET expression. Green arrow-headed line represents positive regulation; red dash-headed line represents negative regulation; blue dash-headed line represents drug resistance. Green color-filled circle with a '+' inside indicates a stimulatory effect; red color-filled circle with 'X' inside represents an inhibitory effect. BET, bromodomain and extra-terminal domains; NSCLC, non-small cell lung cancer.

The combination of JQ1 with paclitaxel or cisplatin revealed synergistic growth-inhibitory effects on A549 and H157 cells in SRB assays, with CIs <1 (Fig. 4C and D). The combination of JQ1 and chemotherapeutic medicine therefore synergistically inhibited the growth of NSCLC cells. This synergistic effect may involve the downregulation of BET by chemotherapeutic drugs.

Combination of JQ1 and paclitaxel inhibits protective autophagy and promotes apoptosis. Autophagy and apoptosis both play important roles in lung cancer progression. JQ1 induces both autophagy and apoptosis in acute myeloid leukemia (AML), and protective autophagy is known to promote cancer cell survival by allowing escape from chemotherapy-induced apoptosis (35-37). Microtubule-associated protein light chain 3 (LC3 II) is a standard marker for autophagosomes and is generated by the conjugation of cytosolic LC3 I to phosphatidylethanolamine (PE) on the surface of nascent autophagosomes (38).

The effect of JQ1 on cell autophagy was evaluated by examining LC3 II expression in NSCLCs. JQ1 dose-dependently increased the expression of LC3 II, indicating an induction of autophagy (Fig. 5A). The combined use of JQ1 and CQ, a late-stage autophagy inhibitor, further upregulated LC3 II levels compared to either JQ1 or CQ treatment alone, suggesting an inhibition of autophagic flux (Fig. 5B). Furthermore, CQ significantly enhanced the growth-inhibitory effects of JQ1 in A549 and H157 cells, suggesting that the protective autophagy induced by JQ1 impeded its inhibitory efficacy (Fig. 5C). JQ1 treatment also increased the levels of cleaved PARP and cleaved caspase-3 in both A549 and H157 cells, suggesting an induction of apoptosis. The combination of PTX and JQ1 partially attenuated the JQ1-induced increase in LC3 II and further increased the levels of cleaved PARP and cleaved caspase-3, suggesting an inhibition of autophagy and enhancement of apoptosis (Fig. 5D).

Enzyme-linked immunoassays of caspase-3 confirmed the enhancement of apoptosis observed with the combination of JQ1 and PTX (Fig. 5E). Collectively, the results of combined treatments using a BET inhibitor and a chemotherapeutic drug indicate a synergistic suppression of NSCLC cell growth through the promotion of apoptosis and suppression of autophagy (Fig. 6).

Discussion

The antitumor effects of BET inhibitors have been reported in benign tumors and in malignant diseases, such as glioblastoma (39), castration-resistant prostate (40), breast (41), epithelial ovarian (42) and lung cancers (43). In a previous study, we revealed that targeting BRD4 inhibited the growth of NSCLC by downregulating eIF4E expression (17). Liao *et al* reported that BRD4 expression was increased in tissue samples from patients with NSCLC and negatively correlated with the 5-year survival rate (16). In the present study, higher expression of BET was observed in several lung cancer cell lines than in normal bronchial epithelial cells. Bioinformatics analysis of the BET expression revealed that individual *BRD2*, *BRD3*, and *BRD4* expression was significantly increased in squamous cell carcinoma but not adenocarcinoma. Our KM-plot analysis also revealed that increased expression of *BRD2, BRD3,* and *BRD4* was individually associated with low overall survival of patients with lung cancer, indicating an oncogenic function of these genes.

Targeting these three BET, either by administering the BET family protein inhibitors (JQ1 and I-BET151) or by silencing

BET expression, suppressed the growth of NSCLC cells. The inhibitory effect on cell growth in different cell lines using BET inhibitors varied, which may due to the different basal expression levels of BET among cell lines. JQ1 and I-BET151 were more selective for BRD4, but they also affected BRD2 and BRD3. The use of a BET inhibitor along with silencing the expression of *BET* revealed more potent inhibitory effects than was achieved with a single agent treatment. These findings indicated that inhibiting the activity of BET proteins using small molecular inhibitors or suppressing protein expression by gene silencing could suppress the growth of NSCLC. Conversely, silencing the expression of all three BET spontaneously using techniques such as CRISPR-Cas9 system or shRNAs would be a promising research direction in the future.

In the present study, it was also revealed that I-BET151 or JQ1 treatment upregulated the expression of BET at both the mRNA and protein levels, which suggested that inhibition of the BET function in cancer cells triggered a compensatory upregulation of BET expression. The mechanisms underlying this increase in BET expression are unknown, however it is theorized that they may involve either epigenetic regulation or a decrease in protein degradation through E3 ligase-mediated ubiquitination signaling (44). BET inhibitors may bind to E3 ligase, thereby preventing the binding of BET with E3 ligase due to steric hindrance. It was also observed that JQ1-induced expression pattern of BET varied between different NSCLC cell lines. This could be caused by the heterogeneity of different cell lines, and deserves future investigation.

ubiBrowser (45) was used to predict the possible E3 ligase associated with BET and E3 ubiquitin-protein ligase Mdm2 (MDM2), which is reported to facilitate BRD4 degradation, was identified to be a potential BET binding partner (18). However, proteolysis-targeting chimaeras (PROTACs) are known to bridge one target protein with an E3-ubiquitin ligase and subsequently trigger protein degradation (46). The BET-protein PROTACs, ARV-771 and ARV-825, can degrade BET effectively by recruiting an E3-ubiquitin ligase (47). Therefore, induction of BET expression by BET inhibitors may attenuate BET anticancer efficacy. Clarifying the mechanism underlying BET inhibitor effects may therefore help to enhance the efficacy of BET inhibitors as treatment for NSCLC, and would be an interesting future research direction.

The efficacy of numerous anticancer drugs is limited by drug resistance, one of the substantial challenges in cancer therapeutics. The resistance to epigenetic-targeting drugs is now attracting great attention, however the mechanism for this resistance remains poorly understood (48). Iniguez *et al* revealed that forced expression of PI3K promoted the resistance to BET inhibitors in neuroblastoma and that PI3K inhibitors synergized with BET inhibitors to suppress tumor progression (49). Similarly, Hishiki *et al* reported that resistance to I-BET151 in the U937R histiocytic lymphoma cell line was related to the constitutive activation of NF-κB signaling that increased both BRD2 and BRD4 expression (50). Zong *et al* revealed that BRD4 expression levels determined the sensitivity of the BET degraders, ZBC260, but they did not correlate this with a response to JQ1 (51). In the present study, it was determined that BET inhibitors induced the expression of BET, which may weaken the efficiency and confer resistance to BET inhibitors in NSCLC. Whether this induction of expression of BET by BET inhibitors is specific for NSCLC cells warrants further study. The effects of combination therapies using BET inhibitors along with the chemotherapeutic drugs paclitaxel and cisplatin in two of the NSCLC cell lines were assessed. Both drugs downregulated BET expression and they both reversed the upregulation of BET expression caused by JQ1. Notably, in certain cell lines, such as in A549, the reversal of the expression of BET induced by inhibitors using chemotherapeutic drugs was not markedly evident. The effect of knockdown of BET in parallel with chemotherapeutics would be one of the goals for next-step research. Furthermore, the detailed mechanism through which the chemotherapeutic agents induce the inhibition of the expression of BET also warrants further study. The combination of JQ1 with either paclitaxel or cisplatin significantly delayed the growth of cancer cells when compared with treatment with a single agent. Consistent with our study, JQ1 was revealed to synergize with a PD-1 blockade to promote an antitumor response in lung cancer (52). Overall, the combination of chemotherapy and BET inhibitors could reveal synergistic anticancer effects that could represent a potent NSCLC therapy.

The anticancer effects of most drug treatments rely on effects on autophagy, a self-degradative system that represents a double-edged sword in the cancer research field. Interventions that stimulate or inhibit autophagy have been suggested in different cancer therapies (53,54). Drug-activated autophagy can protect cancer cells against apoptosis, and this contributes to the multi-drug resistance of cancer cells. Therefore, the effect of chemotherapeutic drugs could be improved by suppressing autophagy in certain cases (55). Li *et al*, using both *in vivo* and *in vitro* experiments, reported a link between osimertinib resistance and enhanced autophagy in lung cancer (56). In the present study, we found that JQ1 induced a protective autophagy, as inhibition of autophagy by CQ suppressed NSCLC cell growth. This JQ1-induced autophagy may in turn confer resistance to JQ1 in NSCLC. JQ1 treatment stimulated cell apoptosis, whereas paclitaxel treatment suppressed JQ1-induced autophagy and promoted cell apoptosis.

Mechanistically, it was reported recently that knockdown of BRD4 could inhibit NSCLC cell growth by inhibiting eIF4E expression. JQ1 treatment greatly reduced the binding of BRD4 with eIF4E promoter, which led to the abatement of the mRNA level of eIF4E (17). Inhibition of BET by BET degraders, particularly ZBC260, promoted apoptosis in sensitive NSCLC cells in parallel with the reduction of c-FLIP and Mcl-1 levels (51). In other types of cancer, studies revealed that suppression of BET could lead to the downregulation of *MYC* expression (57,58). Inhibition of BET by JQ1 was revealed to be more effective in *MYC*-amplified medulloblastoma cell lines (57). In glioblastoma, p21 and Bcl-xL were revealed to play key roles in BET-mediated cell growth and apoptosis, while $p21^{CIP1/WAF1}$ partially contributed to JQ1-induced cell growth inhibition (33). Tumor cell growth could be influenced by chromatin accessibility (57). A recent study reported that BET inhibitor JQ1 could reduce chromatin accessibility, which resulted in the suppression of RUNX2/NID1 signaling (59).

βIII-tubulin (TUBB3) has been revealed to play an important role in breast cancer metastasis. BET inhibition could increase *TUBB3* expression by suppressing the expression of transcription factor myeloid zinc finger-1 (*MZF-1*), the upstream regulator of TUBB3 (60). BET bromodomain inhibitors promote apoptosis and cell cycle arrest resulting from the reduction of the KIT (a receptor tyrosine kinase) enhancer domain and decreased *KIT* expression in gastrointestinal stromal tumor (61). While in multiple myeloma, the function of BET inhibitor lies in its ability to inhibit myeloid ecotropic insertion site 2 (MEIS2) expression (62). All these mechanisms, where the efficacy of BET and BET inhibitors lies, deserve further exploration in our combined treatment system described in this study.

In a clinic-related study, Liao *et al* revealed increased expression of BRD4 in human NSCLC tissues and increased BRD4 expression was correlated with the poor prognosis of NSCLC patients (16). In the present study, bioinformatics analysis of the datasets in TCGA database was performed. Further validation is required *in vivo* and in the clinic in the future. Another aspect that requires consideration are the side effects of BET inhibitors, as summarized by Doroshow *et al* (15). It was reported that the side effects for MK-8628/OTX015, a BET inhibitor, included thrombocytopenia, anemia and fatigue in NSCLC (15), indicating that the choice of a specific type of inhibitor for each particular type of cancer varies and requires individual assessment.

The aforementioned results indicated that a combination of chemotherapy and BET inhibitors could give rise to synergistic anticancer effects and could represent a promising therapeutic strategy for treating NSCLC in the clinic. Additionally, these synergistic anticancer effects may be even more evident in BET-overexpressing cell lines and NSCLC cases compared to those in BET medium or low-expressing cells and cases. Further studies are required to verify this point.

In conclusion, the present study revealed that targeting BET with small molecular inhibitors or gene silencing can suppress NSCLC cell growth. However, BET inhibitors also increased the expression of BET and induced a protective autophagy, which in turn compromised the efficacy of BET inhibitors as anticancer agents. Chemotherapeutic medicines, such as paclitaxel and cisplatin, can enhance the effects of JQ1 by downregulating BET expression, thereby inhibiting this autophagy while also promoting apoptosis. The present findings indicated a potential novel combination therapy for NSCLC.

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Availability of data and materials

The datasets analyzed during the present study are available from the corresponding authors upon reasonable request.

Authors' contributions

XZ, YM, JL, JC, BX, and ZZ conducted the experiments. XZ, TS, and ZC were involved in study design and data analysis. XW and ZC were responsible for study design, data analysis, and manuscript writing. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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