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Research paper

NF-κB signaling activation via increases in BRD2 and BRD4 confers resistance to the bromodomain inhibitor I-BET151 in U937 cells

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ABSTRACT

Novel epigenetic therapies targeting bromodomain and extra-terminal (BET) family proteins have shown therapeutic efficacy in diverse hematologic malignancies and solid cancers. However, the mechanism of resistance remains poorly understood. In the present study, we evaluated the mechanism of resistance to the BET inhibitor I-BET151 and its signaling pathway to overcome resistance in U937 cells. Treatment with 10 μM I-BET151 significantly induced growth inhibition, apoptosis, and cell cycle modulation, including increases in sub-G1 and G1 phases and decreases in S and G2/M phases, in U937 cells. However, no significant changes in these factors were detected in I-BET151-resistant U937 (U937R) cells. Combined treatment with I-BET151 and IKK inhibitor VII synergistically induced apoptosis in U937 and U937R cells. Increased expression of bromodomain-containing protein (BRD) 2, BRD4, and nuclear NF-κBp65 proteins was detected in U937R cells. IKK inhibitor VII inhibited the activation of NF-κBp65 protein in the nuclear fraction of U937R cells. These findings suggest that resistance to I-BET151 in U937R cells is related to constitutive activation of the NF-κB signaling pathway via increased expression of both BRD2 and BRD4. Targeting the NF-κB signaling pathway may be an effective therapeutic strategy to enhance or restore the sensitivity to I-BET151 in U937 cells.

1. Introduction

Bromodomain, consisting of approximately 110 amino acid residues, is a key epigenetic factor that recognizes acetylated lysine residues in histones [1]. Proteins of the bromodomain and extra-terminal (BET) family, including bromodomain-containing protein (BRD) 2, BRD3, BRD4, and BRDT, have two tandem bromodomains and an extra-terminal domain with four alpha helices linked by two loops [1,2]. The BET family proteins play a pivotal role in the transcriptional regulation of genes through epigenetic interactions between bromodomains and acetylated histones [2]. Of these proteins, BRD4 modulates cell-cycle progression by controlling the release of active-type positive transcription elongation factor b from its inactive type complex with hexamethylene bisacetamide inducible protein 1 [3] and by interacting with the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway [4]. Small hairpin RNAs and the benzodiazepine class BET inhibitor JQ1, targeting BRD4, have been shown to inhibit cell growth and induce apoptosis in acute myeloid leukemia [5,6]. These findings suggest that BRD4 is required for proliferation of leukemia cells and could be a novel therapeutic target. Moreover, JQ1 has antitumor activity in multiple myeloma [7,8] and acute lymphoblastic leukemia [9]. Also showing antitumor activity is the quinolone-class BET inhibitor I-BET151 in *MLL*-related leukemia [10] and *JAK2V617F*-related myeloproliferative disorders [11]. However, BET inhibitors induce various molecular resistance responses resulting in clinical efficacy. The mechanism of resistance to BET inhibitors remains poorly understood. Therefore, in the present study, we evaluated the molecular mechanisms of resistance to BET inhibitors and attempted to overcome resistance in I-BET151-resistant U937 (U937R) cells.

2. Materials and methods

2.1. Cells, culture, and reagents

The human histiocytic lymphoma cell line U937 cells were cultured

Abbreviations: BRD, bromodomain-containing protein; BET, bromodomain and extra-terminal; IKK, inhibitor of κB kinase

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in RPMI 1640 (Wako Pure Chemical Industries, Osaka, Japan) with 10% fetal bovine serum (HyClone, GE Healthcare Japan, Tokyo, Japan), containing 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Wako Pure Chemical Industries) in a 5% CO_2 incubator with 100% humidity. The isoxazoloquinoline class BET inhibitor I-BET151 (Chemietek, Indianapolis, IN, USA) was dissolved in dimethyl sulfoxide (Wako Pure Chemical Industries). Cell numbers and viability were evaluated with an automatic cell counter (Luna, LMS, Tokyo, Japan) using trypan blue staining (Thermo Fisher Scientific, Yokohama, Japan). Three independent experiments were performed to determine means ± standard deviation.

2.2. Establishment of I-BET151-resistant U937 cells

U937R cells were established by culturing U937 cells in medium containing escalating I-BET151 concentrations, which were gradually increased from 0.5, 1.0, 2.0, 5.0, and 10 μM over a 6-month period. We confirmed with a polymerase chain reaction (PCR) amplification Kit (AmpFLSTR™ Identifiler™, Thermo Fisher Scientific) that the U937R cells had been derived from U937 cells (Supplemental Fig. 1). The cells were grown in medium containing 10 μM I-BET151 to maintain resistance. This medium was changed to medium without I-BET151 24 h before the following experiments.

2.3. Analyses of cell cycle

Cells (2.0×10^5/mL) that were cultured with I-BET151 were harvested, washed with phosphate-buffered saline, fixed with 70% ethanol, pretreated with 30 mg/mL RNase (Sigma-Aldrich, St. Louis, MO, USA), and stained with propidium iodide. The cell cycle profile was determined with a flow cytometric analyzer (MACSQuant, Miltenyi Biotec, Tokyo, Japan).

2.4. Cell proliferation assay

Cells were seeded on 96-well flat-bottomed tissue culture plates (Thermo Fisher Scientific) at a density of 3.5×10^3 cells/mL in complete culture medium and were incubated with I-BET151 or one of the following drugs: mitogen-activated protein kinase/extracellular signal-regulated kinase (MEK) inhibitor, PD98059 (Selleck Chemicals, Houston, TX, USA); inhibitor of κB (IκB) kinase (IKK) inhibitor, IKK inhibitor VII (Calbiochem, Tokyo, Japan); phosphoinositide 3-kinase (PI3K) inhibitor, LY294002 (Cell Signaling Technology, Danvers, MA, USA); JAK inhibitor, Ruxolitinib (Selleck Chemicals); glycogen synthase kinase-3 (GSK3) inhibitor, SB21673 (Selleck Chemicals); p38 inhibitor, SB202190 (Selleck Chemicals); c-jun-*N*-terminal kinase (JNK) inhibitor, SP600125 (Cayman Chemical Company, Ann Arbor, MI, USA); histone deacetylase inhibitor, Vorinostat (Selleck Chemicals); and aurora A kinase inhibitor, Alisertib (Selleck Chemicals). After treatment, 20 μL Cell Titer 96 AQueous One Solution (Promega, Tokyo, Japan) was added to each well. After incubation for 180 min at 37 °C, the cell samples were measured with a microplate absorbance reader (iMark, BIO-RAD, Hercules, CA, USA). Experiments were performed in triplicate, and each experiment was repeated three times independently.

2.5. Analyses of apoptotic cells

Cells (2.0×10^5/mL) that were cultured with I-BET151, IKK inhibitor VII, and combined treatment were harvested and washed with phosphate-buffered saline. The apoptotic cells were identified with the AnnexinV-FITC Kit (Beckman Coulter, Tokyo, Japan) and analyzed with MACSQuant (Miltenyi Biotec).

2.6. Reverse transcriptase-PCR and quantitative real-time PCR

Total RNA was extracted from U937 cells or U937R cells with ISOGEN reagent (Wako Pure Chemical Industries). Reverse transcription of RNA to complementary DNA (cDNA) was performed with reverse transcriptase (RT) (Strata Script II, Thermo Fisher Scientific). The expression of *c-MYC* and *β-actin* was determined with RT-PCR as follows: 25 cycles at 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 60 s. The primer sequences were as follows: for *c-MYC*, (sense) 5′-AAGACTCCA GCGCCTTCTCTC-3′ and (antisense) 5′-GTTTTCCAACTCCGGGAT CTG-3′ and for *β-actin*, (sense) 5′-GTGGGGCGCCCCACGCACCA-3′ and (antisense) 5′-CTCCTTAATGTCACGCACGATTTC-3′.

Quantitative analysis of *c-MYC* expression was performed using TaqMan Gene Expression Assays (Thermo Fisher Scientific; ID: Hs00153408_mL) on an Applied Biosystems Prism 7000 (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.7. Western blot analysis

Cells were harvested, washed with ice-cold phosphate-buffered saline, and lysed by sonication in the presence of RIPA buffer. The samples were separated on a sodium dodecylsulfate-polyacrylamide gel and then transferred onto PVDF membranes (Bio-Rad). The membranes were immunoblotted with antibodies against BRD2, BRD4, nuclear factor-κB (NF-κB) p65, IκBα, and β-actin, which were purchased from Thermo Fisher Scientific. The immunoblots were detected using enhanced chemiluminescence (Amersham Biosciences, Buckinghamshire, UK). The expression of BRD2 and BRD4 in U937 and U937R cells was compared to levels observed in the BET inhibitor-sensitive cell lines THP-1, MOLM13, and MV4-11, and the BET inhibitor-resistant cell line K562 [10,12].

Samples of nuclear and cytoplasmic fractions of U937 and U937R cells were separated by NE-PER™ Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific). The membranes were immunoblotted with antibodies against NF-κBp65 (Thermo Fisher Scientific), GAPDH (Thermo Fisher Scientific) as a loading control for the cytoplasmic fraction, and nucleolin (Thermo Fisher Scientific) as a loading control for the nuclear fraction.

2.8. Statistical analysis

The Student's *t*-test and Smirnov-Grubbs test were performed with the software program StatFlex version 5.0 (Artech Co., Ltd., Osaka, Japan). The level at which differences were considered significant was set at $p < 0.05$.

3. Results

3.1. Effect of I-BET151 on cell growth

Culturing U937 and to a lesser extent U937R cells in the presence of I-BET151 inhibited their proliferation in a time- and dose-dependent manner (Fig. 1). The difference in the sensitivity between U937 and U937R cells was significant at doses of 5, 10, and 20 μM for 48 h, and at doses of 0.5, 1, 5, 10, and 20 μM for 72 h. After 72 h, the cell proliferation assay showed 50% inhibitory concentration (IC_{50}) values of 0.96 μM in U937 cells and 19.7 μM in U937R cells. Treatment with 10 μM I-BET151 inhibited the growth of U937 cells but did not alter the growth of U937R cells (Fig. 2).

3.2. Effect of I-BET151 on the cell cycle

Treatment with I-BET151 significantly increased the proportion of U937 cells in the sub-G1 and G1 phases while consequently decreasing the proportion of cells in S and G2/M at 48 and 72 h, compared with untreated U937 cells, untreated U937R cells, and I-BET151-treated U937R cells (Fig. 3).

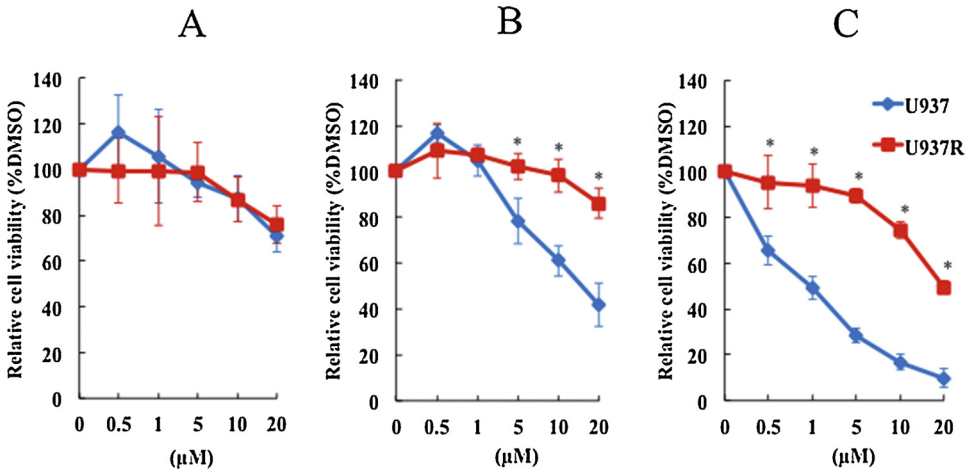

Fig. 1. The sensitivity of U937 and U937R cells to treatment with I-BET151 for (A) 24, (B) 48, and (C) 72 h. Values represent the mean ± standard deviation of three independent experiments performed in triplicate. *p < 0.05.

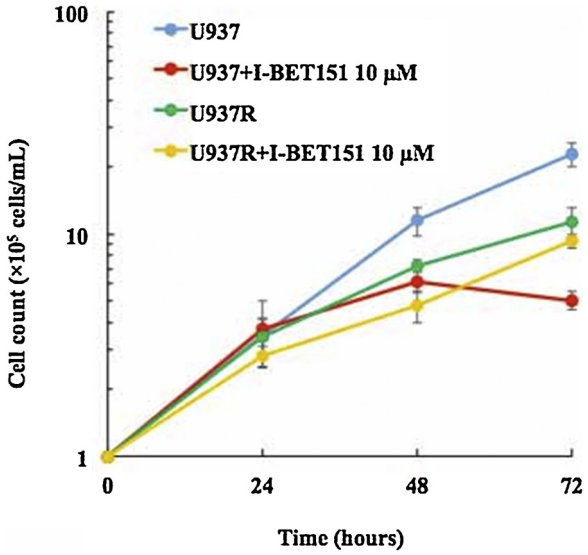

Fig. 2. Growth of U937 and U937R cells in the absence and presence of I-BET151 (10 μM). Viable cells were measured with an automatic cell counter. The cell counts shown are the mean ± standard deviation of three independent experiments.

3.3. Sensitivity to various signaling pathway inhibitors after acquisition of I-BET151 resistance

The drugs LY294002, SP600125, Vorinostat, and Alisertib produced the same levels of reduced cell viability in U937 and U937R cells, whereas Ruxolitinib, SB21673, SB202190, and PD98059 did not significantly modulate either cell line (Fig. 4). We found significant differences in the sensitivity at doses of 1, 2, 3, and 4 μM IKK inhibitor VII ($p < 0.05$) and at the dose of 0.5 μM Vorinostat ($p < 0.05$) between U937 and U937R cells (Fig. 4).

3.4. Effect of IKK inhibitor VII on sensitivity to I-BET151 in U937 and U937R cells

Treatment with 10 μM I-BET151 for 72 h significantly increased apoptosis in U937 cells but not in U937R cells (Fig. 5A and B). In U937 cells, the percentages of apoptosis were 2.5% ± 0.8% without treatment and 17.3% ± 4.5% with 10 μM I-BET151. In U937R cells, the percentages of apoptosis were 13.1% ± 1.5% without treatment and 12.8% ± 7.0% with 10 μM I-BET151.

Treatment with 3 μM IKK inhibitor VII for 72 h significantly increased apoptosis in U937R cells but not in U937 cells (Fig. 5A and B). In U937 cells, the percentages of apoptosis were 2.5% ± 0.8% without treatment and 2.4% ± 0.7% with 3 μM IKK inhibitor VII. In U937R cells, the percentages of apoptosis were 13.1% ± 1.5% without treatment and 36.8% ± 10.9% with 3 μM IKK inhibitor VII. Moreover, IKK inhibitor VII significantly enhanced I-BET151-induced apoptosis in

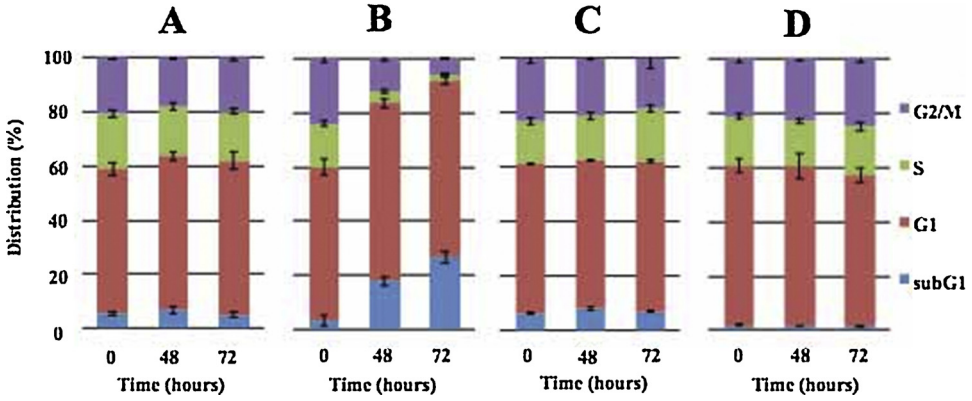

Fig. 3. Cell cycle analysis in (A) U937, (B) I-BET151 (10 μM)-treated U937, (C) U937R, and (D) I-BET151 (10 μM)-treated U937R cells. Values represent the mean ± standard deviation of three independent experiments.

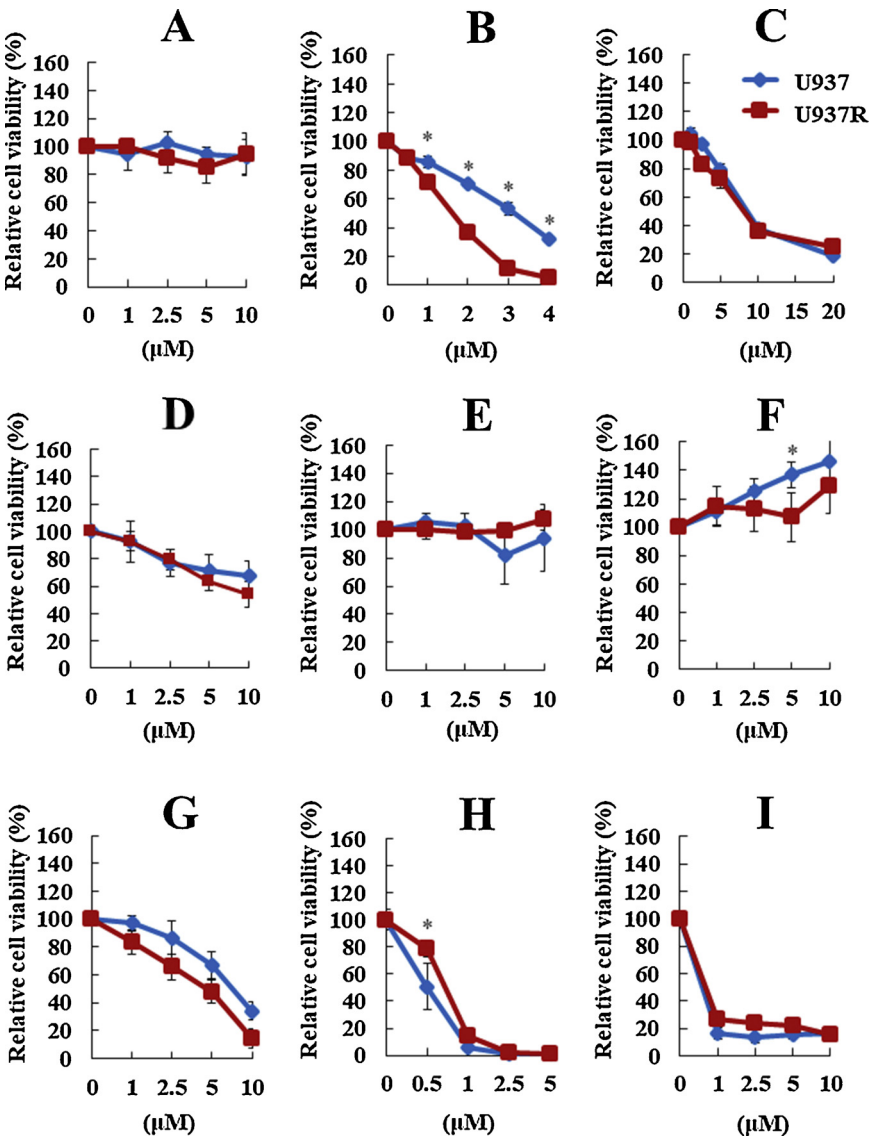

Fig. 4. The cell viability of U937 and U937R cells following exposure to inhibitors of signaling pathways. (A) PD98059 (MEK inhibitor), (B) IKK inhibitor VII (IKK inhibitor), (C) LY294002 (PI3K inhibitor), (D) Ruxolitinib (JAK inhibitor), (E) SB21673 (GSK3 inhibitor), (F) SB202190 (p38 inhibitor), (G) SP600125 (JNK inhibitor), (H) Vorinostat (histone deacetylase inhibitor), and (I) Alisertib (aurora A kinase inhibitor). Values represent the mean ± standard deviation of three independent experiments performed in triplicate. *$p < 0.05$.

both U937 and U937R cells: the percentages of apoptosis were 60.5% ± 6.6% and 72.1% ± 16.4%, respectively (Fig. 5A and B).

3.5. Effect of I-BET151 or IKK inhibitor VII on expression of c-MYC

A previous study showed that I-BET151 decreases cell proliferation and downregulates *c-MYC* expression in the human myeloma cell line U266 [8]. Similarly, treatment with 10 μM I-BET151 induced transient downregulation of *c-MYC* expression after 24 and 48 h in U937 cells, and the expression of *c-MYC* was restored after 72 h (Fig. 6). In contrast, the treatment induced less modulation of *c-MYC* in U937R cells (Fig. 6). The expression of *c-MYC* was decreased by treatment with 3 μM IKK inhibitor VII for 72 h in U937 cells but was stable in U937R cells. Combined treatment with I-BET151 and IKK inhibitor VII induced downregulation of *c-MYC* after 24, 48, and 72 h in U937 cells but only a slight modulation after 24 and 48 h in U937R cells. Moreover, quantitative analysis of *c-MYC* expression with TaqMan Gene Expression Assays produced data that are comparable to the above findings (Table 1).

3.6. Effects of I-BET151 resistance on expression of BET proteins and NF-κBp65

The expression of BRD2 and BRD4 in U937R cells was increased relative to U937 cells (Fig. 7). Moreover, IκBα and NF-κBp65 also exhibited elevated expression in U937R cells relative to U937 cells (Fig. 7). The BET inhibitor-resistant cell line K562 showed higher expression of BRD2, BRD4, NF-κBp65, and IκBα than the BET inhibitor-sensitive cell lines MV4-11, MOLM13, and THP1. The expression pattern of these molecules in U937R cells was similar to that in K562 cells.

Moreover, NF-κBp65 protein was expressed in the cytoplasmic fractions of both U937 and U937R cells, whereas it was detected in the nuclear fraction of U937R cells only (Fig. 8). In U937R cells, IKK inhibitor VII (3 μM) induced downregulation of nuclear NF-κBp65 protein levels after 24 and 48 h, whereas I-BET151 (10 μM) did not modulate the expression of nuclear NF-κBp65 protein levels after 48 h (Fig. 8).

4. Discussion

Although BET inhibitors targeting bromodomains have shown

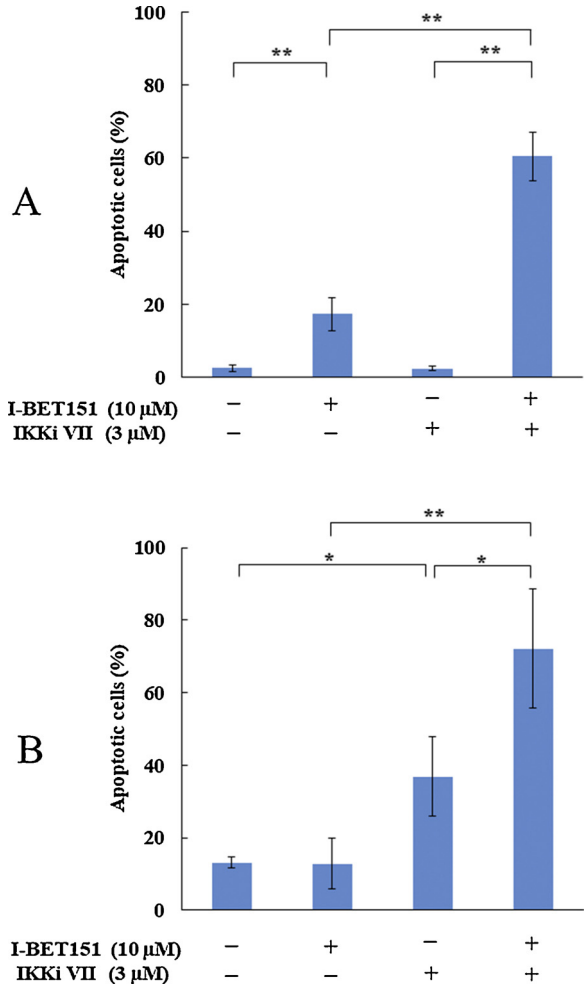

Fig. 5. Analysis of apoptotic cells induced by I-BET151, IKK inhibitor (IKKi) VII, or both I-BET151 and IKKi VII in (A) U937 and (B) U937R cells. U937 and U973R cells were treated with I-BET151, IKKi VII, or both I-BET151 and IKKi VII for 72 h. Values represent the mean ± standard deviation of 3–6 independent experiments. *p < 0.05; **p < 0.01.

Table 1
Quantitative analysis of *c-MYC* expression by TaqMan Gene Expression Assays.

U937				
Time (hours)	0	24	48	72
I-BET151 10 μM	1.00	0.43	0.06	1.46
IKKi VII 3 μM	1.00	5.40	0.89	0.07
I-BET151 10 mM + IKKi VII 3 mM	1.00	0.06	0.04	0.01
U937R				
Time (hours)	0	24	48	72
I-BET151 10 μM	1.00	0.73	0.51	0.76
IKKi VII 3 μM	1.00	0.70	0.68	2.98
I-BET151 10 mM + IKKi VII 3 mM	1.00	0.72	0.72	n.d.

n.d: no data.

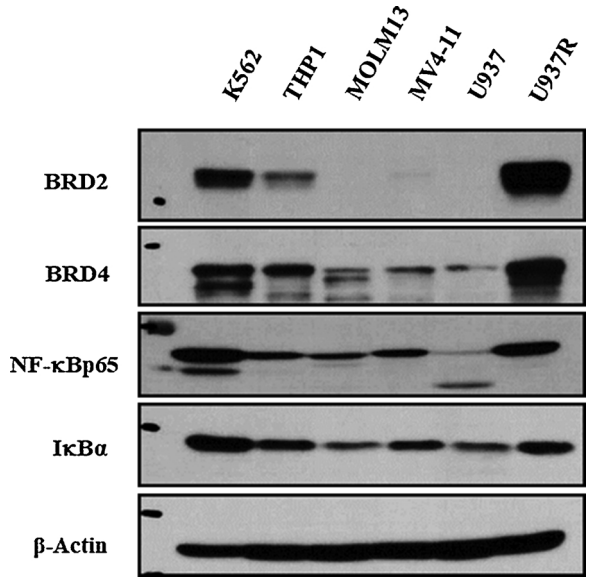

Fig. 7. Effect of I-BET151 resistance on the expression of BRD proteins and NF-κB signaling-related proteins. Effects of I-BET151 resistance on BRD2 and BRD4 proteins and NF-κBp65 and IκBα proteins were evaluated in the whole cell fraction of U937 cells and U937R cells, compared with the expression of these proteins in K562, THP-1, MOLM13, and MV4-11 cells.

strong antitumor activity in numerous solid tumors, including brain tumors [13–16], neuroblastoma [17], melanoma [18], and hematologic malignancies [19,20], resistance to BET inhibitors is a major problem limiting their antitumor activity. The molecular mechanism underlying this resistance must be elucidated to surmount this negative consequence. In the present study, we have demonstrated that resistance to I-BET151 is acquired in association with activation of the NF-κB pathway, as BET proteins are upregulated in U937R cells.

Resistance to BET inhibitors has recently been shown to be promoted when polycomb repressive complex 2, which regulates the cell cycle, stem cell self-renewal, and cell fate through epigenetic

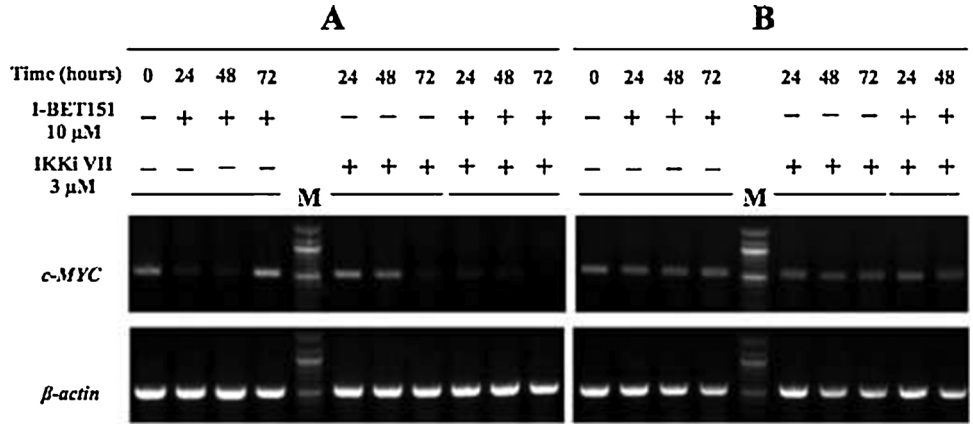

Fig. 6. The expression of *c-MYC* and *β-actin* in U937 and U937R cells treated with I-BET151, IKK inhibitor (IKKi) VII, or both I-BET151 and IKKi VII. M means a marker lane.

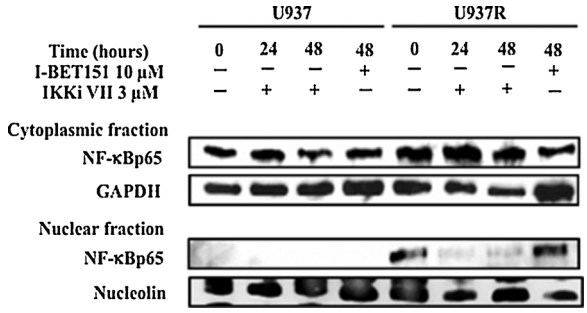

Fig. 8. Effect of I-BET151 or IKK inhibitor (IKKi) VII on NF-κBp65 protein levels in the cytoplasmic and nuclear fractions of U937 and U937R cells. The expression of NF-κBp65 in the cytoplasmic and nuclear fractions of U937 or U937R cells treated with I-BET151 or IKKi VII was evaluated by western blotting analysis. GAPDH was used as a loading control for the cytoplasmic fraction, and nucleolin was used as a loading control for the nuclear fraction.

modulation in cancer, is suppressed [21]. The study by Rathert et al. found that inhibition of BET proteins induces acute downregulation of *c-MYC* in human leukemia cells, and that resistant leukemia cells rapidly restore *c-MYC* expression [22]. Moreover, in I-BET151-resistant leukemia cell clones, WNT/β-catenin and transforming growth factor-β signaling are activated, whereas the NF-κB signaling pathway is inhibited [22].

In the present study, I-BET151 showed antitumor activity with reduced expression of *c-MYC* in U937 cells but less antitumor activity with stable expression of *c-MYC* in U937R cells. Interestingly, we found that U937R cells expressed higher levels of both BRD2 and BRD4 than did U937 cells. Proteins of the BET family regulate the transcription of *c-MYC*, *BCL2*, *CDK9*, and *IRF8* in acute leukemia and *c-MYC*, *E2F1*, *MYD88*, *TLR10*, *PAX5*, *NF-κB*, and *IRF8* in lymphoma [23]. However, *c-MYC* expression was not significantly increased in U937R cells compared with U937 cells. These findings suggest that U937R cells stabilize *c-MYC* expression by means of overexpressing BRD2 and BRD4 regardless of treatment with I-BET151.

We found that the NF-κB signaling pathway was activated in U937R cells: both IκBα and NF-κBp65 proteins were expressed at higher levels in the whole cell lysate of U937R cells relative to U937 cells. Moreover, we confirmed that NF-κBp65 was expressed in the nuclear fraction of U937R cells. The transcriptional activity of NF-κB has previously been shown to be regulated through the interaction between BRD4 and acetylated lysine-310 [23,24]. The BET protein inhibitors I-BET151 and JQ1 inhibit the binding of BRD4 to acetylated NF-κBp65 at lysine-310 and suppress NF-κB activation [24,25]. However, acetylation of NF-κBp65 is not affected by I-BET151 in either activated dendritic cells or T cells [25]. Depletion of BRD4 or treatment with JQ1 induces ubiquitination and degradation of the constitutively active nuclear form of NF-κBp65, suggesting that BRD4 stabilizes nuclear NF-κB by preventing ubiquitination of NF-κBp65 [24]. Our present findings and those of previous studies suggest that resistance to I-BET151 in U937R cells is related to constitutive activation of the NF-κB signaling pathway via increased expression of both BRD2 and BRD4. A study using small interfering RNA has suggested that BRD2 is the BET protein that plays the main role in NF-κB regulation, whereas BRD4 plays only a minor role in melanoma [26]. Additional studies are needed to determine whether BRD2 or BRD4 plays a pivotal role in regulating constitutive NF-κB activation in U937R cells.

Agents that synergistically enhance the antitumor activity of BET inhibitors have been identified: ibrutinib, a Bruton's tyrosine kinase inhibitor [27,28]; rapamycin and everolimus, mTOR inhibitors [29–31]; ponatinib and AC220, FLT3 kinase inhibitors [32]; Vorinostat, a histone deacetylase inhibitor [33,34]; and bortezomib, a proteasome inhibitor [35]. However, no NF-κB signaling inhibitors, including IKK inhibitors, have been reported to enhance the sensitivity to I-BET inhibitors. In the present study, IKK inhibitor VII restored the sensitivity to I-BET151 and synergistically enhanced the antitumor activity of I-BET151 in U937R cells; this finding supports the novel hypothesis that constitutive activation of NF-κB signaling plays a pivotal role in acquiring resistance to I-BET151 in U937 cells. IKK inhibitor VII is a benzamide-pyrimidine compound that acts as a potent, selective inhibitor of IKK, leading to inhibition of cellular IκBα degradation and NF-κB signaling [36]. In the present study, the antitumor activity of IKK inhibitor VII on U937R cells was associated with inhibition of nuclear NF-κBp65, but not with downregulation of *c-MYC* expression.

The BET inhibitor JQ-1 induces a decrease in phosphorylated IKKβ in the activated B-cell-like subtype of diffuse large B-cell lymphoma cell lines [27]. However, we failed to obtain western blot data showing that IKK inhibitor VII blocks the phosphorylation of IKKα/β.

Our results suggest that interference in the interaction between NF-κB and BRDs is a potential therapeutic approach for resistance to BET inhibitors in U937R cells. Further studies, including those of other cell lines and tumors from patients, are required to better understand the molecular mechanism of resistance to BET inhibitors and to provide a rationale for the development of novel therapies to overcome this resistance.

5. Conclusions

Our findings suggest that acquisition of resistance to I-BET151 in U937R cells is associated with increased expression of BRD2, BRD4, and nuclear NF-κBp65. Activation of nuclear NF-κBp65 may be stabilized by increased BRD4 through the prevention of NF-κBp65 ubiquitination. Moreover, IKK inhibitor VII can restore I-BET151 sensitivity in U937R cells and synergistically enhance the antitumor activity of I-BET151 in both U937 and U937R cells. Thus, interference in the interaction between NF-κB and BRDs is a potential therapeutic approach for resistance to BET inhibitors in U937R cells.

Conflict of interest

All authors have no conflicts of interest to report.

Acknowledgements

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.leukres.2018.09.016.

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