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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).