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Antimyeloma activity of bromodomain inhibitors on the human myeloma cell line U266 by downregulation of MYCL

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Bromodomain and extraterminal protein (BET) inhibitors suppress the expression of c-MYC. U266, a human myeloma cell line, expresses the *MYCL* gene, but not the *c-MYC* gene. Our aim was to analyse the antimyeloma activity of BET inhibitors on U266 cells. Two BET inhibitors, I-BET151 and JQ1, were tested. U266 cell proliferation decreased to 61.5 and 54.0% of the control after incubation with 500 nmol/l I-BET151 for 72 and 96 h and to 53.5 and 56.4% of control after incubation with 500 nmol/l JQ1 for 72 and 96 h by MTS tetrazolium, respectively. BET inhibitors induced cell cycle arrest at the G1 phase in U266 cells, but did not induce apoptosis by flow cytometry. According to Gene Set Enrichment Analysis, *MYC*-related genes were significantly downregulated in U266 cells treated with I-BET151 similar to KMS11 cells that expressed *c-MYC*. The *MYCL1* was expressed in U266 cells, whereas *c-MYC* and *MYCN* were not by quantitative real-time reverse-transcription-PCR. Incubation with I-BET151 induced downregulation of *MYCL1* in U266 cells. BET inhibitors decreased the cell proliferation in U266 cells with overexpression of *MYCL* less than those without overexpression of *MYCL*. BET inhibitors induce G1 arrest without apoptosis and interfere with the proliferation of U266 myeloma cells, which express *MYCL,* but not *c-MYC*. BET inhibitors might be active in cancers that express *MYCL*, but not *c-MYC*. *Anti-Cancer Drugs* 27:756–765
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Introduction

Multiple myeloma is a clonal human plasma cell neoplasm that develops mainly in the bone marrow. Several novel agents, including bortezomib, thalidomide and lenalidomide, have improved patient outcomes in the last decade. Multiple myelomas are a heterogeneous group of plasma cell neoplasms in terms of morphology, phenotype, molecular biology and clinical behaviour. Thus, heterogeneity exists in the outcomes of patients with multiple myeloma, even with the availability of these novel agents.

Small-molecule inhibitors of the bromodomain and extraterminal (BET) family of bromodomain proteins, so-called BET inhibitors, suppress the expression of c-MYC, a master regulatory factor of cell proliferation in myeloma cells [1]. Translocation or MYC rearrangement is the common somatic event in myeloma patients [2] and transcriptional profiling identifies activation of the MYC pathway in myeloma cells in more than 60% of patients [3]. Members of the BET family, such as BRD2, BRD3, BRD4 and BRD-T, are associated with acetylated chromatin and facilitate transcriptional activation [4]. In particular, BRD4 marks M/G1 genes in mitotic chromatin for transcriptional memory and directs postmitotic transcription [5]. Inhibition of BRD4 leads to G1 arrest in human myeloma cell lines by downregulation of c-MYC [1].

Members of the MYC family are important oncogenes involved in the development of malignant cells, including multiple myeloma [6]. The activity of c-MYC in multiple myeloma increases with disease stage [3,7]. There are three kinds of genes, *c-MYC*, N-MYC and L-MYC, in the MYC family [8–11]. The *c-MYC* gene is expressed in the majority of human myeloma cell lines [12,13]. However, U266, one of the human myeloma cell lines, expresses the *MYCL* gene, but not the *c-MYC* gene [14,15].

In our study, the BET inhibitors, I-BET151 and JQ1, were found to be active not only against myeloma cell lines that express c-MYC but also against U266 cells. The

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aim of this study was to analyse the antimyeloma activity of BET inhibitors in U266 cells that do not express c-MYC.

Methods

Cell lines and drugs

Four human myeloma cell lines, U266, RPMI8226, MM1S and KMS11, were used in this study. U266, RPMI8226 and MM1S cell lines were obtained from the American Type Culture Collection (Rockville, Maryland, USA). KMS11 was obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Myeloma cells were grown in RPMI 1640 medium (Boehringer, Ingelheim, Germany) containing 10% heat-inactivated foetal calf serum (HyClone Laboratories, Logan, Utah, USA) in a humidified atmosphere (37°C; 5% CO_2). I-BET151 was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). JQ1 was purchased from BioVision Inc. (Milpitas, California, USA).

Cell count and Cell proliferation assay

Cell proliferation was calculated using an automated cell counter (Luna; Logos Biosystems, Anyang, Korea). Myeloma cells were seeded in 96-well flat-bottom microplates at a density of 5×10^3 cells/well for RPMI8226, 2.5×10^4 cells/well for MM1S, 5×10^3 cells/well for KMS11 and 2.5×10^4 cells/well for U266. The cells were incubated with or without drugs for 72 and 96 h at 37°C. After incubation, MTS terazolium compound (CellTiter 96 AQueous One Solution Cell Proliferation Assay; Promega, Madison, Wisconsin, USA) was added and the cells were incubated for 2–4 h. The absorbance was measured at a wavelength of 490 nm using a microplate reader (IMark Microplate Reader; Bio-Rad Laboratories, Hercules, California, USA) and expressed as a percentage of the value of the corresponding untreated cells.

Analysis of cell cycle

Myeloma cells (1×10^6) were incubated with or without BET inhibitors for 48, 72 or 96 h. The cells were then washed with PBS, permeabilized by 30-min exposure to 70% ethanol at − 20°C, incubated with propidium iodide (PI) (50 μg/ml in 0.5 ml PBS containing 20 units/ml RNase A) for 30 min at room temperature (20–25°C) and analysed by flow cytometry (MACSQuant Analyzer; Miltenyi Biotec, Bergisch Gladbach, Germany).

Analysis of apoptosis and cell death

Myeloma cells were stained with PI and annexin-V-fluorescein isothiocyanate (FITC) using an Apoptosis Kit (annexin V-FITC kit, MEBCYTO; Medical & Biological Laboratories, Nagoya, Japan). Annexin V-FITC (10 μl) and PI (5 μl) were added to 85 μl of a suspension of 2×10^5 myeloma cells washed with PBS and incubated at room temperature (20–25°C) for 15 min in the dark. Cells were analysed by flow cytometry. The apoptosis ratio was defined as the ratio of PI-positive cells : annexin-V-positive cells.

Gene expression analysis

U266 and KMS11 cells were cultured with 500 nmol/l I-BET151 or DSMO for 24 h. RNA was isolated from the cells using the RNeasy kit (Quiagen, Hilden, the Netherlands). The RNA samples were evaluated using an Affymetrix Prime View Human Gene Expression Array (Affymetrix, Santa Clara, California, USA) at Beth Israel Deaconess Medical Center (Boston, Massachusetts, USA). The Gene Set Enrichment Analysis (*http://www.broadinstitute.org/tools/software.html*. Broad Institute, Cambridge, Massachusetts, USA) was used to identify groups of functionally related oncogenic gene sets. Gene sets with afalse discovery rate of atleast 0.25 and a nominal *P*-value of at least 0.05 were defined as statistically significant.

Quantitative PCR for c-MYC expression

RNA from myeloma cells with or without BET inhibitors was isolated using the RNeasy kit (Quiagen). Reverse transcription of RNA to complementary DNA (cDNA) was performed using cDNA Synthesis Kits (Life Technologies, Carlsbad, California, USA). Quantitative real-time reverse-transcription-PCR (qRT-PCR) was performed using a 7500 Fast Real-Time PCR Systems (Life Technologies) with the SYBR Green PCR master mix (Roche Diagnostica, Risch-Rotkreuz, Switzerland). The sequences of forward and reverse primers, respectively, for *c-MYC*, *MYCN* and *MYCL1* were c-MYC 1295F (5′-TGCTCCATGAGGAGACACC-3′), c-MYC 1413R (5′-CTCTGACCTTTTGCCAGGAG-3′), MYCL1 F1 (5′-CGAGAGCCCAAGCGACTC-3′), MYCL1 R1 (5′-AG GGATCCAGGGGGTCTG-3′), MYCN F1 (5′-AGACAC CCGCGCAGAATC-3′) and MYCN R1 (5′-GTTTTAA TACCGGGGGTGCT-3′). The control sample was amplified under the same conditions with human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers. The sequence of the forward and reverse primer for GAPDH was 5′-ACAGTCAGCCGCATCTTCTT-3′ and 5′-AATTTGCCATGGGTGGAAT-3′, respectively.

Immunoblotting

MYCL and BCL-2, before and after treatment with transduction proteins, were detected by the western blotting methods. The samples were loaded onto a 5–20% gradient polyacrylamide (Wako Co. Ltd, Tokyo, Japan) and electrophoretically transferred to nitrocellulose membranes (GE Healthcare, Danbury, Connecticut, USA). The membranes were blocked with 10% skim milk in PBS. The primary antibodies were anti-MYCL mouse polyclonal antibody (ab167315; Abcam, Cambridge, UK), anti-MYCL rabbit polyclonal antibody (D01P; Abnova, Taipei, Taiwan), anti-BCL2 monoclonal mouse antibody (M0887; Dako, Glostrup, Denmark), anti-FLAG(TM) rabbit monoclonal antibody (F7425;

Sigma Aldrich, St Louis, Missouri, USA) and anti-GAPDH(FL-355) rabbit polyclonal antibody (sd-25778; Santa Cruz Biotechnology). The secondary antibodies were horseradish peroxidase-conjugated antirabbit IgG (GE Healthcare). Protein expression was detected using the clarity western ECL substrate (Bio-Rad).

Transfection of expression plasmids

The MYCL expression vector was constructed briefly. Sequences of *MYCL* were amplified from the cDNA of U266 cells using PCR primers and inserted into the HindIII/XhoI site of the pcDNA3.1 3xFLAG expression vector (Invitrogen, Carlsbad, California, USA). The primers were synthesized at a commercial laboratory (Invitrogen). The primers were as follows: MYCL vari1full EcoR1 F was GGAATTCCGACTACGACTCGTACC AGCACT and MYCL vari1-2full Xba1 R2 was GCTCTAGATGCACCGGCTGCAATGCATT, respectively. The MYCL construct was transfected into U266 cells (2×10^6 cells) using lipofectamin 3000 transfection reagent (Invitrogen). The controls were mock transfected with an empty vector.

Statistical analysis

Data was presented as mean (± SD) of three independent experiments. Comparisons between two groups of samples were evaluated using the *t*-test. All *P*-values reported were two tailed and statistical significance was defined as *P* less than 0.05. All statistical analyses were carried out using EZR (Saitama Medical Center, Jichi Medical University, Shimotsuke, Japan), which is a graphical user interface for R (The R Foundation for Statistical Computing, Vienna, Austria). More precisely, it is a modified version of R commander designed to add statistical functions used frequently in biostatistics.

Results

BET inhibitors decrease the proliferation of U266 myeloma cells

The BET inhibitors I-BET151 and JQ1 at concentrations ranging from 100 nmol/l to 4 μmol/l were applied to four myeloma cell lines. Both compounds inhibited the proliferation of U266, RPMI8226, MM1S and KMS11 cells in a concentration-dependent manner after incubation for 72 and 96 h (Fig. 1a–d). However, the antimyeloma activity of I-BET151 was not dependent on the duration of incubation. The proliferation of RPMI8226, MM1S and KMS11 cells was strongly inhibited by the BET inhibitors; the IC_{50} values for I-BET151 and JQ1 ranged from 250 to 750 nmol/l. Proliferation of U266 cells was only partially inhibited. Proliferation of U266 cells decreased to 61.5 and 54.0% of the control after incubation with 500 nmol/l I-BET151 for 72 and 96 h, respectively. U266 cell proliferation decreased to 53.5 and 56.4% of the control after incubation with 500 nmol/l JQ1 for 72 and 96 h, respectively (Fig. 1e and f). The antimyeloma activity of I-BET151 was dependent on the duration of incubation when 750, 1000 or 2000 nmol/l I-BET-151 was applied ($P = 0.0021$, 0.0361 and 0.0035, respectively). However, the antimyeloma activity of JQ1 was not dependent on the duration of incubation.

BET inhibitors induce G1 arrest

The analysis of cell cycle by flow cytometry showed that I-BET151 induced G1 arrest of U266 cells at 48, 72 and 96 h. The percentage of cells in the G1 phase increased to between 65.7 and 70.9% for U266 cells exposed to 500 nmol/l I-BET151 compared with 54.9 to 59.7% after exposure to dimethyl sulfoxide (DMSO) without the drug. I-BET151 treatment at 500 nmol/l decreased the percentage U266 cells in the S phase to between 8.4 and 10.5% compared with that observed after DMSO exposure (17.7–24.3%). In addition, JQ1 induced G1 arrest of U266 cells after 48, 72 and 96 h of incubation. JQ1 treatment at 500 nmol/l increased the percentage of U266 cells in the G1 phase to between 68.5 and 77.7% and decreased the percentage of S-phase cells to between 5.0 and 6.5% (Fig. 2).

The analysis of apoptosis with flow cytometry showed that DMSO induced apoptosis in 2.6% of U266 cells. I-BET151 and JQ1 did not induce apoptosis in U266 cells; the percentage of apoptotic cells after exposure to 500 nmol/l I-BET151 or JQ1 was 6.0 or 9.5%, respectively (Fig. 3).

I-BET151 decreased the expression of genes related to MYC

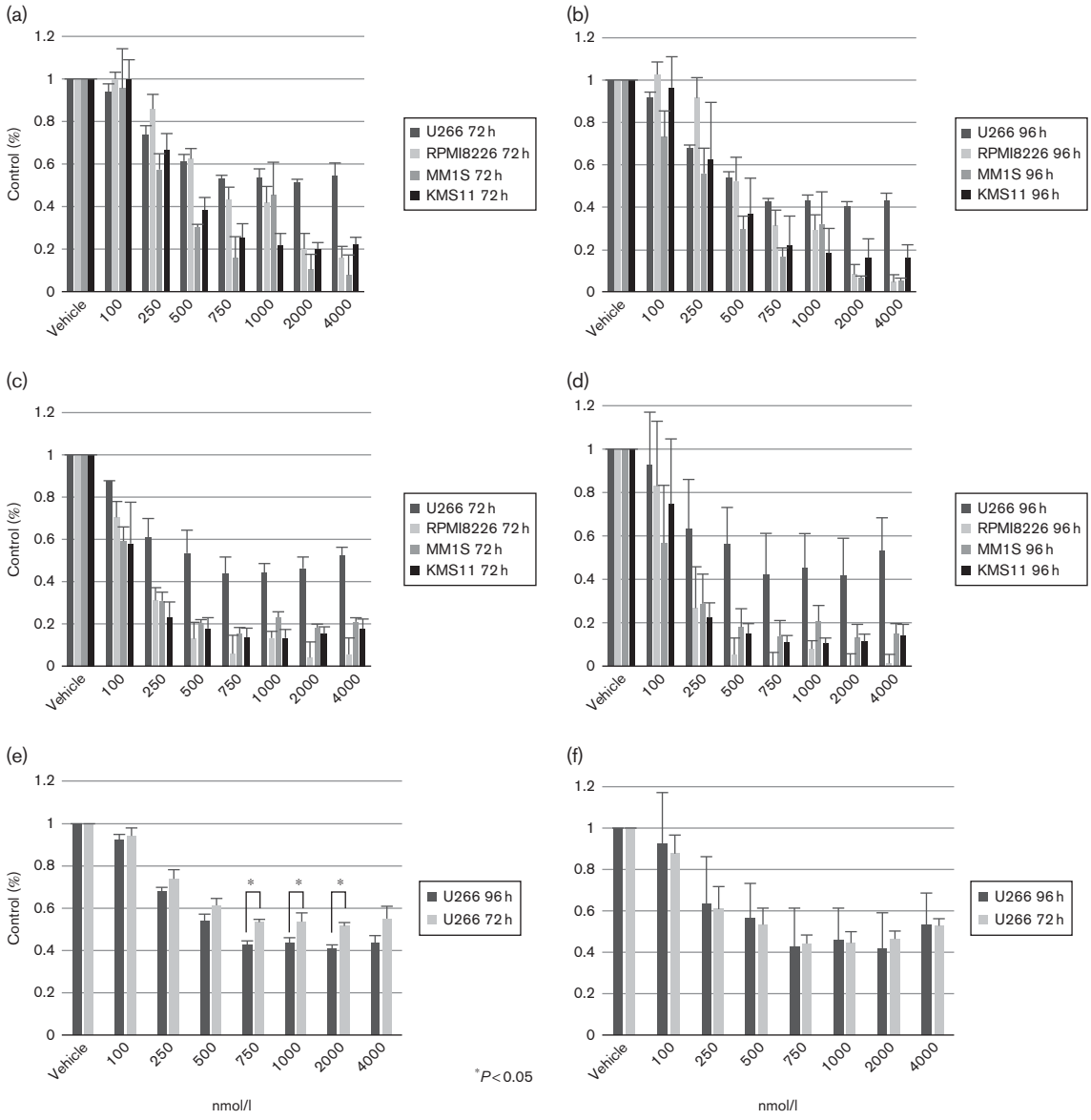
The microarray analysis showed that the expression of 97 genes decreased by 50% or more in U266 cells exposed to 500 nmol/l I-BET151 for 24 h compared with their expression in U266 cells exposed to DMSO. Notably, expression of the *MYCL1* gene decreased by 61.6% in response to I-BET151. The *c-MYC* and *MYCN* genes were not expressed in U266 cells treated with or without I-BET151. However, the expression of 500 genes decreased by 50% or more in KMS11 cells treated with 500 nmol/l I-BET151 for 24 h. Notably, the MYC gene was decreased by 65.9%. The MYCL1 and MYCN genes were not expressed in KMS11 cells with or without I-BET151.

According to the gene set enrichment analysis, *MYC*-related genes were significantly downregulated in U266 and KMS11 cells treated with 500 nmol/l I-BET151 for 24 h (Fig. 4a and b). The heat map showed that I-BET151 downregulated similar *MYC*-related genes in U266 KMS11 cells (Fig. 4c).

I-BET151 downregulated MYCL1, but not c-MYC or MYCN, in U266 cells

The qRT-PCR results showed that *MYCL1* was expressed in U266 cells treated with DMSO alone; *c-MYC* and *MYCN* were not expressed in these cells. I-BET151

Fig. 1



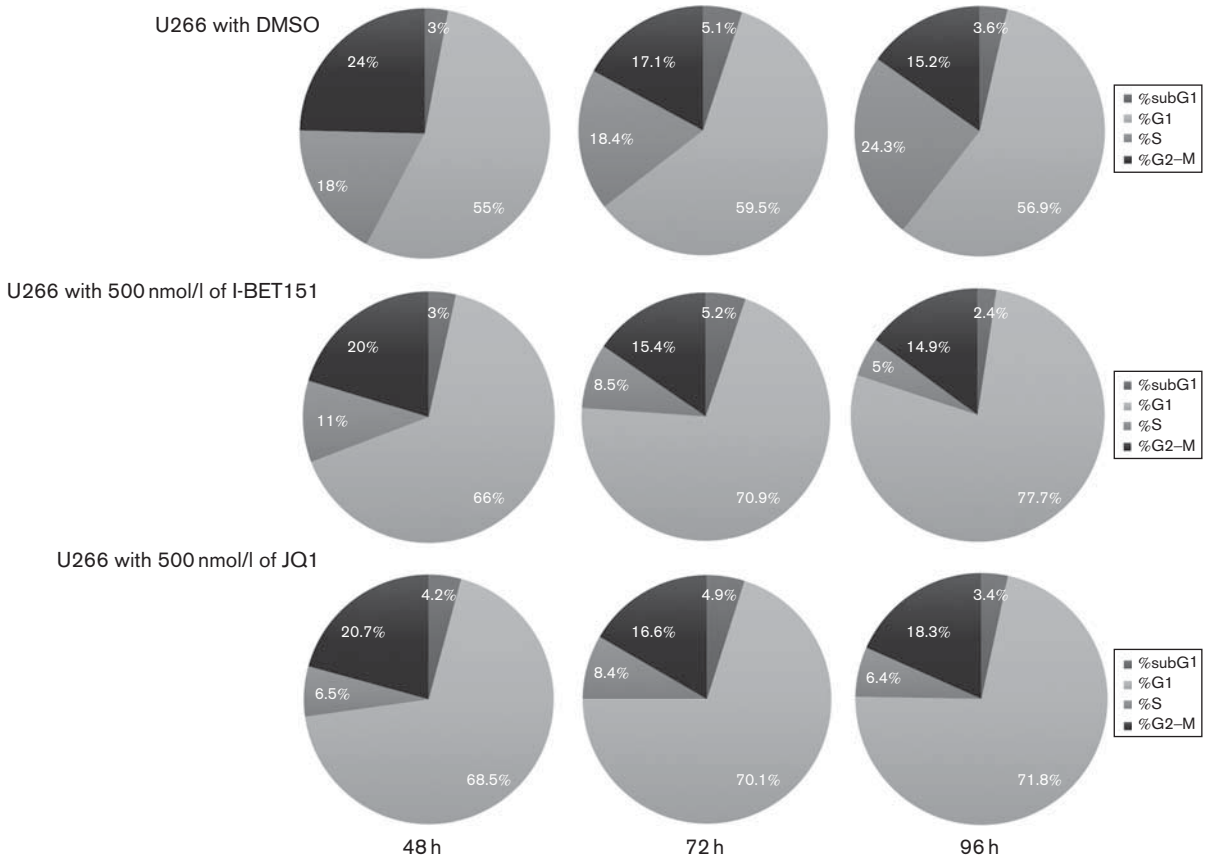
Proliferation of human myeloma cell lines treated with BET inhibitors. (a) Proliferation of U266, RPMI8226, MM1S and KMS11 cells treated with 500 nmol/l I-BET151 for 72 h. (b) Proliferation of U266, RPMI8226, MM1S and KMS11 cells treated with 500 nmol/l I-BET151 for 96 h. (c) Proliferation of U266, RPMI8226, MM1S and KMS11 cells treated with 500 nmol/l JQ1 for 72 h. (d) Proliferation of U266, RPMI8226, MM1S and KMS11 cells treated with 500 nmol/l JQ1 for 96 h. (e) Proliferation of U266 cells treated with 100 nmol/l to 4 μmol/l I-BET151 for 72 and 96 h. (f) Proliferation of U266 cells treated with 100 nmol/l to 4 μmol/l JQ1 for 72 and 96 h. BET, bromodomain and extraterminal protein.

induced downregulation of *MYCL1* in U266 cells after 24 h of incubation (Fig. 5a). In contrast, *c-MYC* was expressed in KMS11, RPMI8226 and MM1S cells treated with DMSO alone. I-BET151 induced downregulation of *c-MYC* in KMS11, RPMI8226 and MM1S cells after 24 h incubation (Fig. 5b). MYCL was not expressed in KMS11, RPMI8226 or MM1S cells. *MYCN* was not expressed in RPMI8226 and MM1S cells treated with DMSO. *MYCN* was expressed in KMS11 cells treated with DMSO; however, I-BET151 did not strongly downregulate the expression of *MYCN* in KMS11 cells after 24 h incubation (Fig. 5c).

BET inhibitors did not decrease the proliferation of U266 cells with overexpression of MYCL

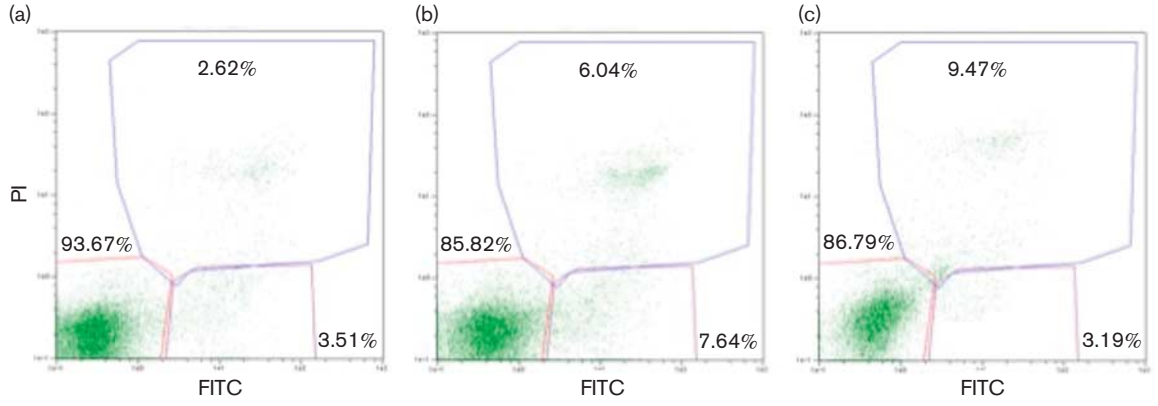
First, we produced the U266 cells with overexpression of *MYCL* using transfection of expression plasmids methods. The qRT-PCR results showed that *MYCL1* was highly expressed in U266 cells with overexpression of *MYCL* than those without (Fig. 6a). In contrast, the

Fig. 2



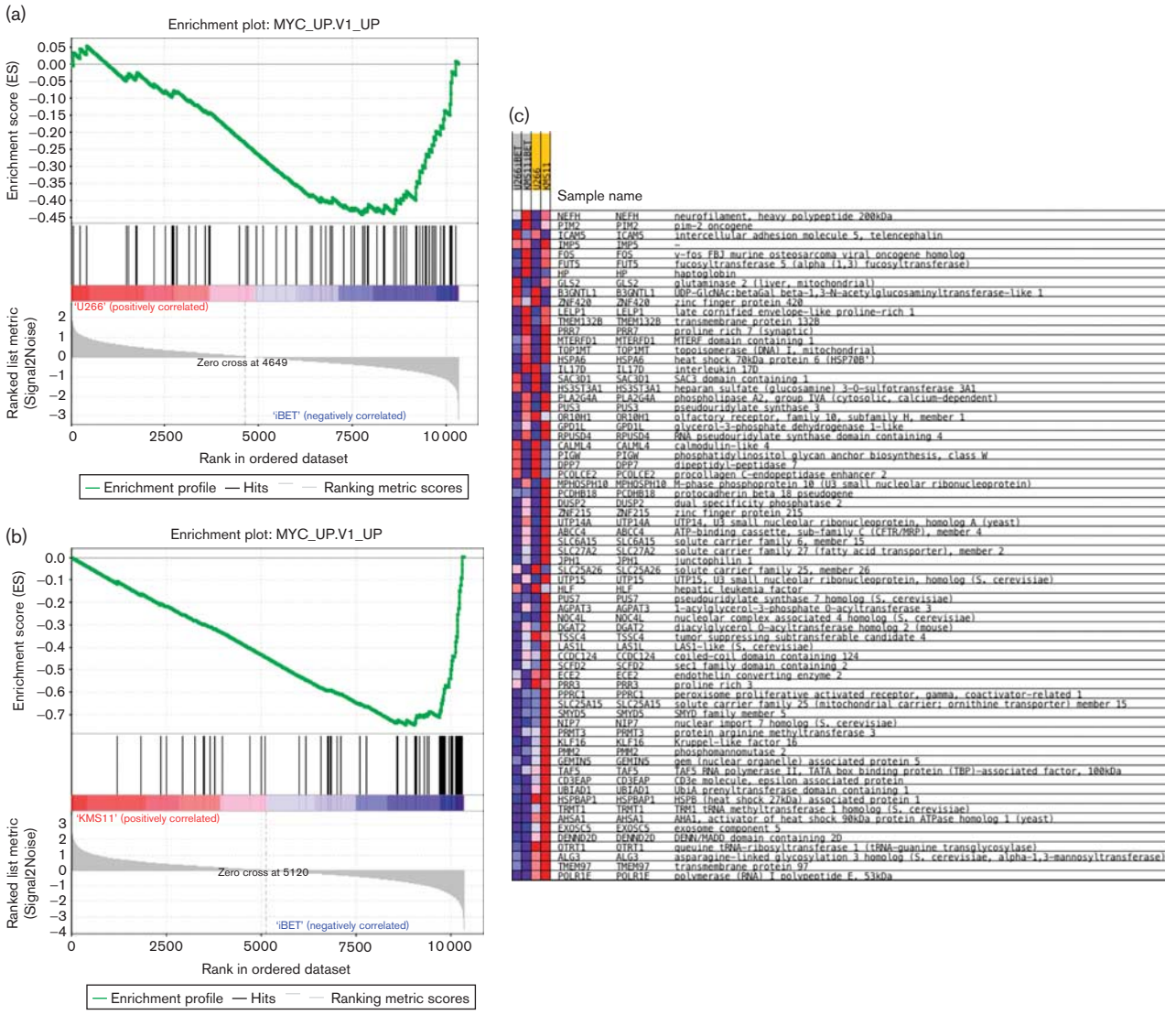
Cell-cycle analysis of U266 with BET inhibitors. Flow cytometry of PI staining for cell cycle analysis of U266 cells treated with DMSO, 500 nmol/l I-BET151 and 500 nmol/l JQ1 for 48, 72 and 96 h. BET, bromodomain and extraterminal protein; DMSO, dimethyl sulfoxide; PI, propidium iodide.

Fig. 3



Apoptosis of U266 cells treated with BET inhibitors. (a) Flow cytometry of PI and annexin-V staining for annexin-V-positive apoptosis analysis in U266 cells treated with DMSO for 72 h. (b) Flow cytometry of PI and annexin-V staining for annexin-V positive apoptosis analysis in U266 cells treated with 500 nmol/l I-BET151 for 72 h. (c) Flow cytometry of PI and annexin-V staining for annexin-V-positive apoptosis analysis in U266 cells treated with 500 nmol/l JQ1 for 72 h. BET, bromodomain and extraterminal protein; DMSO, dimethyl sulfoxide; FITC, fluorescein isothiocyanate; PI, propidium iodide.

Fig. 4



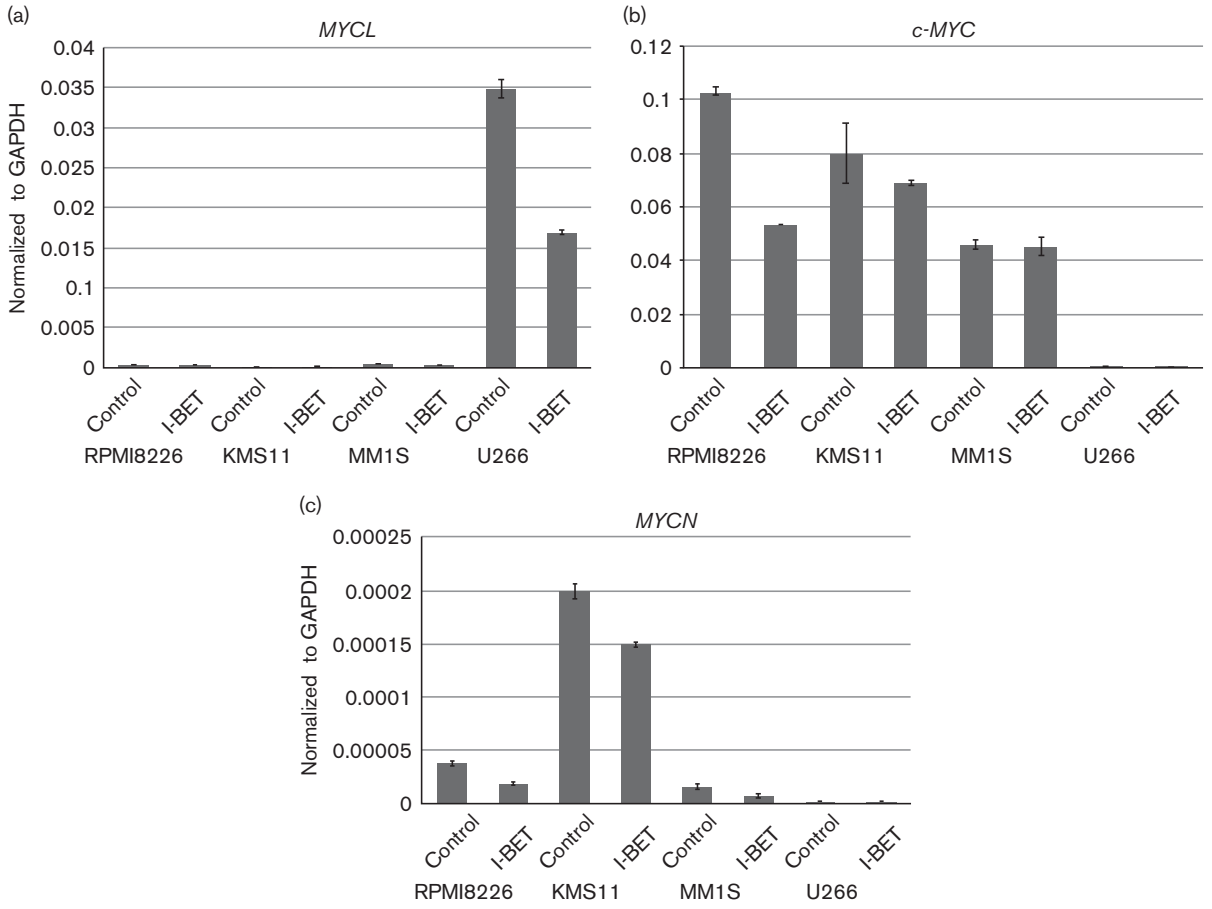
Gene set enrichment analysis of U266 and KMS11 cells treated with I-BET151. (a) Gene set enrichment analysis (GSEA) of the MYC-dependent gene set in RNA from U266 cells treated with 500 nmol/l I-BET151 for 24 h. (b) GESA of the MYC-dependent gene set in RNA from KMS11 cells treated with 500 nmol/l I-BET151 for 24 h. (c) Heat map of MYC-dependent genes in U266 and KMS11 cells treated with or without 500 nmol/l I-BET151. BET, bromodomain and extraterminal protein.

western blotting results showed that the signal of MYCL was weak in both U266 cells with and without overexpression of MYCL (Fig. 6b). Second, we treated U266 cells with overexpression of *MYCL* by 500 nmol/l I-BET151 for 24 h. The western blotting results showed that the signal of MYCL was slightly weak in the U266 cells after the administration of I-BET151 compared with those before the administration of I-BET151 (Fig. 6c). Finally, we analysed the cell count between U226 cells with or without overexpression of *MYCL* before and after treatment with I-BET151 500 nmol/l. I-BET151 reduced 37.1% of the U266 cells without overexpression of *MYCL*, whereas I-BET151 reduced 15.6% of the U266 cells with overexpression of *MYCL* (*t*-test, $P = 0.0689$; Fig. 6d).

Discussion

This study has shown that BET inhibitors reduce the proliferation of U266 cells, which do not express c-MYC, as well as that of RPMI8226, MM1S and KMS11 cells that express c-MYC. BET inhibitors induced G1 arrest, but not apoptosis. I-BET151 downregulated the *MYCL1* gene level in U266 cells. Similarly, *MYC*-related genes were downregulated in U266 and KMS11 cells. Finally, I-BET151 modulated the cell-cycle pathway of U266

Fig. 5



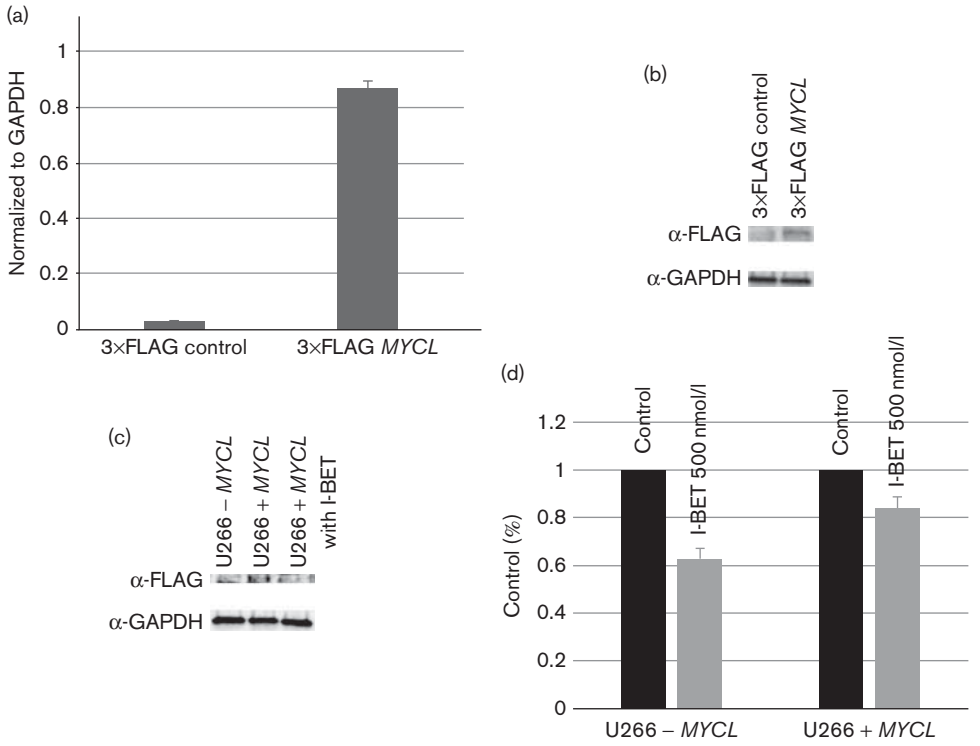
Quantitative RT-PCR analysis for *MYCL, c-MYC* and *MYCN* levels in four human myeloma cell lines. Quantitative RT-PCR was performed for *MYCL*, *c-MYC* and *MYCN* in U266, RPMI8226, MM1S and KMS11 cells with or without 500 nmol/l I-BET151 for 24 h. (a) I-BET151 downregulated the level of *MYCL* in U266 cells. *MYCL* was not expressed in RPMI8226, MM1S and KMS11 cells. (b) I-BET151 downregulated the level of *c-MYC* in RPMI8226, MM1S and KMS11 cells. *C-MYC* was not expressed in U266 cells. (c) I-BET151 slightly downregulated the level of *MYCN* in KMS11 cells. *MYCN* was not expressed in U266, RPMI8226 and MM1S cells. BET, bromodomain and extraterminal protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; I-BET, I-BET151; RT-PCR, real-time reverse-transcription-PCR.

cells independent of *c-MYC* expression and that of KMS11 cells by downregulation of *c-MYC*.

BET inhibitors interfere with bromodomains on the acetyl–lysine recognition domains of coactivator proteins that are implicated in the initiation and elongation of transcription. In myeloma cells overexpressing *c-MYC*, BET inhibitors inhibit the downregulation of *c-MYC* transcription by chromatin-dependent signal transduction to RNA polymerase [1]. Both I-BET151 and JQ1 are pan-BET inhibitors that inhibit cell proliferation in numerous types of malignancies, including acute myeloid leukaemia, malignant lymphoma, neuroblastoma, glioblastoma and osteosarcoma [16–21]. In addition, I-BET151 induces apoptosis *in vitro* and *in vivo* by transcriptional repression of MYC-dependent and MYC-dependent pathways by inhibiting recruitment to the transcriptional activator PTEFb [22]. In a previous study, BET inhibitors interfered with the proliferation of RPMI8226, KMS11 and MM1S cells because of overexpression of *c-MYC* in these human myeloma cell lines. In contrast, BET inhibitors did not affect U266 cells because L-MYC, and not c-MYC, is overexpressed in U266 cells. In our study, I-BET151 downregulated the level of *MYCL1* messenger RNA (mRNA) and induced G1 arrest in U266 cells without apoptosis. I-BET151 downregulated the level of *c-MYC* mRNA as determined by the qRT-PCR method in RPMI8226, KMS11 and MM1S cells.

The MYC proto-oncogene family comprises *c-MYC*, *MYCL* and *MYCN*. All three genes form dimers with MAX and bind to DNA [23]. N-MYC is similar to c-MYC in terms of domain structure and association with human malignancies [24]. In contrast, the MYCL protein is different in structure from c-MYC and N-MYC and, unlike c-MYC and N-MYC, was not active in human cell lines [9,25–28]. Aberrant expression of L-MYC is associated

Fig. 6



Analysis of U266 cells overexpressed *MYCL*. (a) The level of *MYCL* was higher in U266 cells with overexpression of *MYCL* than those without overexpression of *MYCL* by qRT-PCR. (b) The signal of MYCL protein was weak in U266 cells with overexpression of *MYCL* similar to those without overexpression of *MYCL* by the western blotting method. (c) The signal of *MYCL* was slightly weak in the U266 cells after the administration of I-BET151 compared with those before the administration of I-BET151 by the western blotting method. (d) I-BET151 reduced cell proliferation of U266 cells with overexpression of *MYCL* less than those without overexpression of *MYCL* (*t*-test, $P = 0.0689$). BET, bromodomain and extraterminal protein; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR, quantitative real-time reverse-transcription-PCR.

with only a small number of human malignancies. Dib and colleagues reported that 40 out of 43 human myeloma cell lines expressed c-MYC. U266 cells only expressed L-MYC. Similarly, L-MYC and N-MYC expression is rare in primary multiple myeloma cells (GEO accession GSE2658) [29].

The MYC gene was rearranged with the enhancer of the immunoglobulin gene in about 60% of patients with myeloma. In about 40% of patients with myeloma, the enhancer of the immunoglobulin gene was not observed [30]. Moreover, MYC rearrangements are rare or absent in monoclonal gammopathy of undetermined significance and smouldering multiple myeloma cells, but are present in about 15% of multiple myeloma cells, 45% of advanced myeloma cells and 90% of human myeloma cell lines [2,31,32].

The L-MYC proto-oncogene protein is a human protein that is encoded by the MYCL1 gene [33]. *MYCL1* was detected as a basic helix–loop–helix transcription factor implicated in lung cancer [34]. MYCL1 interacts with MAX as well as c-MYC and MYCN [35,36]. L-MYC was related to the pathogenesis of several cancers, such as lung cancer, gastric cancer, colorectal cancer, non-melanoma lip cancer and thyroid cancer [37–42]. Moreover, high expression of L-MYC was related to a poor prognosis in patients with lung cancer and gastric cancer [43,44]. Recently, L-MYC as well as c-MYC were found to be involved in the generation of human induced pluripotent stem cells [45]. Activity of BET inhibitors by downregulation of MYCL has never been reported in cancer cells. We considered that the activity of BET inhibitors for U266 was dependent on downregulation of MYCL because BET inhibitors did not decrease the proliferation of U266 cells with overexpression of MYCL and downregulated the mRNA level of *MYCL1*. We propose that BET inhibitors are active in several cancers, including multiple myeloma, in which L-MYC was overexpressed, but neither c-MYC nor N-MYC was expressed.

Catlett-Falcone *et al.* [46] reported that the BCL-2 gene is amplified and is responsible for its survival in U266 We analysed the signal of BCL-2 protein in U266 cells treated with or without BET inhibitors. The signal of BCL-2 in U266 treated with 500 nmol/l of I-BET151 and JQ1 for 24 h was similar to those without BET inhibitors using

western blotting methods (Supplementary material 1, Supplemental digital content 1, *http://links.lww.com/ACD/A163*). These results indicated that the activity of BET inhibitors for U266 was independent of the pathway related to BCL-2.

Our study has a limitation in terms of the analysis of the MYCL protein. The MYCL protein was not detected using the western blotting methods by two kinds of anti-MYCL antibodies, although the mRNA level of *MYCL* was sufficient for evaluation. To the best of our knowledge, studies on expression levels of MYCL protein in U266 have never been reported, although studies on mRNA levels of *MYCL* have been carried out previously. We considered that MYCL was unstable or metabolized constitutively in U266. The reason why the expression level of MYCL was difficult to evaluate remains unclear. In the future, we will attempt to analyse the significance of protein level and metabolism in U266.

In conclusion, BET inhibitors interfere with the proliferation of four human myeloma cell lines, including U266 cells that do not express c-MYC, as well as RPMI8226, MM1S and KMS11 cells that express c-MYC. BET inhibitors induced G1 arrest, but not apoptosis. I-BET151 downregulated *MYCL1* gene levels in U266 cells. Similar genes related to MYC were downregulated in U266 and KMS11 cell lines. Finally, I-BET151 modulated the cell-cycle pathway in U266 cells that do not express c-MYC and exerted similar effects on KMS11 cells by downregulation of c-MYC. Although the *MYCL1* gene is not expressed frequently in primary myeloma cells, it is related to pathogenesis and prognosis in several cancers. BET inhibitors might be active in cancer cells expressing L-MYC, but not c-MYC.

Acknowledgements

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Conflicts of interest

There are no conflicts of interest.

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