The bromodomain inhibitor JQ1 and the histone deacetylase inhibitor panobinostat synergistically reduce N-Myc expression and induce anticancer effects

Jeyran Shahbazi1,2, Pei Y. Liu1, Bernard Atmadibrata1, James E. Bradner3,4,5, Glenn M. Marshall1,6, Richard B. Lock1,7, and Tao Liu1,7

1Children’s Cancer Institute Australia, Lowy Cancer Research Centre, UNSW Australia, Randwick NSW 2031, Australia. 2School of Biotechnology and Biomolecular Sciences, UNSW Science, UNSW Australia, Sydney, NSW 2052, Australia. 3The Broad Institute of MIT and Harvard, Cambridge, Massachusetts 02142, USA. 4Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115, USA. 5Department of Medicine, Harvard Medical School, Boston, Massachusetts 02115, USA. 6Kids Cancer Centre, Sydney Children's Hospital, Randwick NSW 2031, Australia. 7School of Women's & Children's Health, UNSW Australia, Randwick NSW 2031, Australia

Corresponding Author: Tao Liu, Children’s Cancer Institute Australia, Lowy Cancer Research Centre, UNSW Australia, Kensington, Sydney, NSW 2052, Australia. Phone: 61-2-93851935; Fax: 61-2-96626584; E-mail: tliu@ccia.unsw.edu.au

Running title: JQ1 and panobinostat combination therapy

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.
Translational Relevance

Neuroblastoma is the most common solid tumor in children under the age of five, and accounts for 15% of childhood cancer-related death. Amplification of the MYCN oncogene occurs in 20-30% of primary neuroblastoma tissues. BET bromodomain inhibitors are among the most promising novel anticancer agents by blocking BRD3 and BRD4 from activating oncogene transcription. However, treatment with BET bromodomain inhibitors alone does not result in cancer remission in many murine models. In this study, we have demonstrated that BRD3 and BRD4 directly activate LIN28B gene transcription, and that JQ1 and panobinostat synergistically reduce LIN28B gene and N-Myc protein expression. JQ1 and panobinostat synergistically induce neuroblastoma cell growth inhibition and apoptosis in vitro, reduce N-Myc protein expression in tumor tissues, and block tumor progression in neuroblastoma-bearing mice. Our findings therefore identify a novel strategy to reduce N-Myc oncoprotein expression and a novel therapy for aggressive neuroblastoma.
Abstract

Purpose: Patients with neuroblastoma associated with MYCN oncogene amplification experience a very poor prognosis. BET bromodomain inhibitors are among the most promising novel anticancer agents by blocking BRD3 and BRD4 from activating oncogene transcription. However, treatment with BET bromodomain inhibitors alone does not result in cancer remission in many murine models.

Experimental Design: MYCN-amplified neuroblastoma cells were treated with vehicle control, the BET bromodomain inhibitor JQ1, the histone deacetylase inhibitor panobinostat, or combination of JQ1 and panobinostat. Genes modulated by JQ1, panobinostat or the combination therapy were identified by Affymetrix microarray, and cell proliferation and apoptosis were examined by Alamar blue assays and flow cytometry analysis. Modulation of LIN28B promoter activity by BRD3 and BRD4 was examined by chromatin immunoprecipitation and luciferase assays. In addition, neuroblastoma-bearing mice were treated with vehicle control, JQ1 and/or panobinostat.

Results: LIN28B was one of the top genes synergistically reduced by JQ1 and panobinostat. BRD3 and BRD4 directly bound to the LIN28B gene promoter and activated LIN28B gene transcription, and knocking down LIN28B reduced the expression of N-Myc protein, but not N-Myc mRNA. JQ1 and panobinostat synergistically reduced LIN28B gene and N-Myc protein expression, and synergistically induced growth inhibition and apoptosis in neuroblastoma cells, but not normal non-malignant cells in vitro. In neuroblastoma-bearing mice, JQ1 and panobinostat synergistically and considerably reduced N-Myc protein expression in tumor tissues and blocked tumor progression.

Conclusions: Our findings have identified a novel strategy to reduce N-Myc oncoprotein expression and a novel therapeutic approach for the treatment of aggressive neuroblastoma.
Introduction

MYCN oncogene amplification occurs in approximately 25-33% of neuroblastoma cases, and results in MYCN gene and N-Myc protein overexpression (1, 2). Patients with neuroblastoma associated with MYCN amplification usually undergo intensive chemotherapy and have poor overall and event-free survival.

Similar to other Myc family homologs, N-Myc modulates gene transcription and macromolecular biogenesis, blocks cell differentiation, stimulates cell cycle progression as well as cell proliferation (2). To date, therapeutic strategies directly targeting the N-Myc or c-Myc protein have been elusive.

The BET bromodomain proteins, such as BRD3 and BRD4, bind to acetylated lysine residues on histone proteins as chromatin readers, and play essential roles in the transcription of oncogenes such as C-MYC, MYCN, BCL2 and FOSL1 (3-8). Small molecule BET bromodomain inhibitors, such as JQ1 and I-BET151, competitively bind to acetyl-lysine recognition pockets, displace BET bromodomain proteins from chromatin, and reduce the expression of oncogenes, leading to cancer cell growth inhibition and apoptosis. BET bromodomain inhibitors have shown promising in vitro and in vivo anticancer effects against NUT-midline carcinoma, multiple myeloma, lymphoma, leukemia, medulloblastoma and neuroblastoma (3-10). However, treatment with BET bromodomain inhibitors alone does not cause cancer remission in neuroblastoma-bearing mice.

Histone deacetylases (HDACs) remove the acetyl group from the lysine residue on histone tails, leading to chromatin compaction and transcriptional repression, mainly of tumor suppressor genes (11). HDAC inhibitors, such as panobinostat, induce cancer cell growth inhibition, differentiation and apoptosis in vitro and suppress tumour progression in mice (11). HDAC inhibitors have therefore emerged as promising anticancer agents, with more
than a dozen HDAC inhibitors (such as vorinostat and panobinostat) currently being evaluated in the clinic and/or clinical trials (11).

In this study, we investigated the therapeutic efficacy of combination therapy with the BET bromodomain inhibitor JQ1 and the HDAC inhibitor panobinostat against neuroblastoma. JQ1 and panobinostat synergistically reduced the transcription of the LIN28B oncogene, leading to synergistic post-transcriptional reduction in N-Myc oncoprotein expression, and exerted synergistic anticancer effects in vitro and in vivo.
Materials and Methods

Cell culture

Human SK-N-BE(2), SK-N-AS and Kelly neuroblastoma (from European Collection of Cell Cultures in 2010) and WI38 fetal lung fibroblast (from American Type Culture Collection in 2010) cells were cultured in Dulbecco’s modified Eagle’s, RPMI 1640 and Minimum Essential Medium respectively. All cell lines were authenticated by small tandem repeat profiling conducted at Garvan Institute or Cellbank Australia in 2015.

Small interfering RNA (siRNA) and plasmid transfection

Cells were transfected with 20 nM siRNAs using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) or transfected with 50 ng plasmid using Lipofectamine 2000 reagent, 100 µl opti-Minimum Essential Medium and 900 µl serum-free medium as we have described previously (18, 19). Validated control siRNA and siRNAs specifically targeting N-Myc, BRD3, BRD4, BRD2 or LIN28B were purchased from Qiagen, Hamburg, Germany.

Quantitative real-time RT–PCR

RNA was extracted from cells using PureLink RNA Mini kit (Invitrogen). After cDNA synthesis, real-time RT-PCR was performed as we have described (18, 19). Primer sequences used for PCR amplifications were as the following: 5’-CGACCACAAGGCCCTCAGTA-3’ (forward) and 5’-CAGCCTTGGTGTGGAGGAG-3’ (reverse) for N-Myc; 5’-GGAGCCCCTGTTTAGGAAGT-3’ (forward) and 5’-GCACTTCTTGGCTGAGGAG-3’ (reverse) for LIN28B; 5’-AGCCGTAGCAGTGAGGAGAG-3’ (forward) and 5’-GTTTGTTTACTGGGGCCTGA-3’ (reverse) for BRD3; 5’-ATGGCAGAAAGCTCTGGAAAA-3’ (forward) and 5’-
TGTGGTGTTGTTGGTACCGTGGAAA-3’ (reverse) for BRD4; 5’-GCACCTTGCGCTGAGCTAGAGCAGTGCA-3’ (forward) and 5’-TCTCCATCTTCCGCTTGACAGT-3’ (reverse) for BRD2; 5’-ATGTGTGGGAGACGGTCAA-3’ (forward) and 5’-ACAGTTCCACAAAGGCATCC-3’ (reverse) for BCL2; and 5’-CACCATGTACCCTGGGATT-3’ and 5’-ACGGAGTACTTGCGCTCAG-3’ for β-actin.

**Immunoblotting**

Cells were lysed, protein extracted and separated by gel electrophoresis. After western transfer, membranes were probed with an anti-N-Myc (1:1000, SC-53993, Santa Cruz Biotech, CA), anti-BRD3 (1:1000, catalog number 61489, Active Motif, Carlsbad, CA), anti-BRD4 (1:1000, catalog number 39909, Active Motif), anti-LIN28B (1:1000, 4196S, Cell Signaling, Danvers, MA) or anti-actin (1:10000, A1978, Sigma, St. Louis, MO) antibody.

**Alamar blue assays**

Cells were incubated with Alamar blue, and read on a microplate reader at 570/595 nm. Results were calculated according to optical density absorbance units, and expressed as percentage change in the number of cells relative to controls (18, 19).

**Flow cytometry studies**

Cells were harvested, washed and then stained with FITC-conjugated Annexin V and 7-AAD (BD Biosciences, San Jose, CA). Detection of the cells positively stained with Annexin V and/or 7-AAD were performed using a FACS Canto Flow Cytometer (BD Biosciences) and analyzed with FlowJo software (TreeStar Inc, Ashland, OR).

**Chromatin immunoprecipitation (ChIP) assays**
ChIP assays were performed with an anti-BRD3, anti-BRD4 or control antibody, and PCR carried out with primers targeting a negative control or the intron 1 region of the LIN28B gene promoter (15).

Luciferase reporter assays

A LIN28B gene promoter construct was kindly provided by Dr. Joshua T. Mendell (15). Kelly neuroblastoma cells were transfected with control siRNA, BRD3 siRNA-1 or BRD4 siRNA-2, followed by co-transfection with Cypridina TK control construct plus the pGL3 construct expressing empty vector or the LIN28B gene promoter (-1414bp to +1403bp) for 48 hours. Luciferase activity was measured, and expressed as percentage change relative to control siRNA transfected samples as we have described previously (19).

Differential gene expression study

SK-N-BE(2) cells were treated with control solvent, JQ1 (1 µM) (prepared in house by the Bradner Lab, Dana-Farber Cancer Institute), panobinostat (10 nM) (Selleckchem, Houston, TX), or JQ1 plus panobinostat. RNA was harvested at 6 hours after treatment, and subjected to Affymetrix microarray (Santa Clara, CA) analysis of differential gene expression. Gene expression was normalized with global normalization, the LimmaGP module (Gene Pattern, Broad Institute, Cambridge, MA) was used to identify genes differentially expressed between samples, and LimmaGP generated .rnx files were used for gene set enrichment analysis (GSEA). An enrichment score (ES) was calculated for each gene set, and enrichment plots were generated for each gene set. Gene expression data is available from Gene Expression Omnibus Expression (GEO) (accession number GSE68690).

Experimental therapy in neuroblastoma-bearing mice
Animal experiments were approved by the Animal Care and Ethics Committee of the University of New South Wales, Australia. Female Balb/c mice, aged 5-6 weeks, were injected subcutaneously with $5 \times 10^6$ SK-N-BE(2) cells into the flank. When the engrafted tumors reached 0.05 g, the mice were divided into 4 groups and treated via intraperitoneal injections with control solvent, JQ1 (50 mg/kg daily), panobinostat (7.5 mg/kg once every three days), or JQ1 plus panobinostat for 14 days. While we were not blinded to mouse group allocation, every effort was made to generate reliable data. Mice were euthanized at the end of the treatments. Tumor tissues were collected, formalin-fixed and paraffin-embedded.

**Immunohistochemistry analysis**

Mouse tissues were de-paraffinized, rehydrated, antigen retrieved, and incubated with rabbit anti-N-Myc (1:1,000) antibody overnight. The tissue sections were then incubated with biotinylated anti-rabbit (1:500) antibody, streptavidin peroxidase and diaminobenzidine. Slides were scanned, images taken, and positively stained cells quantified using ImageJ software (National Institutes of Health) (18).

**Statistical analysis**

All experiments were replicated at least three times for statistical analysis. Differences were tested for significance using ANOVA among groups or unpaired t-test for two groups. All statistical tests were two-sided. Synergy or additivity was calculated by combination index (CI) method for combinations of multiple doses of drugs (20), or by the fractional product (R) method for combinations of a single dose of two drugs (21, 22). Synergism is defined as a more than additive effect (CI < 1 or R < 1).
Results

The BET bromodomain proteins BRD3 and BRD4 induce N-Myc expression and neuroblastoma cell proliferation, but do not function synergistically

The BET bromodomain protein BRD4 has been shown to modulate MYC and MYCN gene transcription (3, 4, 7, 8, 13). We examined whether BRD3 also regulated MYCN gene expression, and whether BRD3 and BRD4 co-operatively modulated MYCN gene expression. RT-PCR and immunoblot analyses of N-Myc mRNA and protein expression were performed in MYCN-amplified SK-N-BE(2) and Kelly neuroblastoma cells after transfection with scrambled control siRNA, BRD3 siRNA (BRD3 siRNA-1 or BRD3 siRNA-2), BRD4 siRNA (BRD4 siRNA-1 or BRD4 siRNA-2) or a combination of BRD3 siRNA and BRD4 siRNA. As shown in Fig. 1A, Fig. 1B and Supplementary Fig. S1, both BRD4 siRNA-1 and BRD4 siRNA-2 significantly reduced BRD4 and N-Myc mRNA and protein expression, and both BRD3 siRNA-1 and BRD3 siRNA-2 significantly reduced BRD3 and N-Myc mRNA and protein expression in SK-N-BE(2) and Kelly cells. While simultaneously knocking down BRD3 and BRD4 mRNA and protein expression, co-transfection with BRD3 siRNA and BRD4 siRNA did not induce greater effects on N-Myc mRNA or protein reduction, compared with BRD3 siRNA or BRD4 siRNA alone, in SK-N-BE(2) and Kelly cells (Fig. 1A, Fig. 1B and Supplementary Fig. S1). In comparison, knocking down BRD2 gene expression with BRD2 siRNA showed no effect on N-Myc mRNA and protein expression in SK-N-BE(2) and Kelly cells (Supplementary Fig. S2), suggesting that BRD2 does not regulate N-Myc expression.

Next, we examined whether BRD3 and BRD4 co-operatively induce neuroblastoma cell proliferation. SK-N-BE(2) and Kelly cells were transfected with control siRNA, BRD3 siRNA-1 and/or BRD4 siRNA-1, BRD3 siRNA-2 and/or BRD4 siRNA-2, followed by
Alamar blue assays. As shown in Fig. 1C, knocking down BRD3 or BRD4 expression significantly reduced the numbers of SK-N-BE(2) and Kelly cells. However simultaneous knocking down BRD3 and BRD4, compared with knocking down BRD3 or BRD4 alone, did not cause any further reduction in the numbers of SK-N-BE(2) and Kelly cells. Therefore, it can be concluded that BRD3 and BRD4 induce N-Myc gene expression and neuroblastoma cell proliferation, but do not function synergistically.

Combination therapy with JQ1 and panobinostat synergistically induces apoptosis in neuroblastoma but not normal cells

As BET bromodomain inhibitors reduce the expression of oncogenes (3-8), and HDAC inhibitors activate the expression of tumor suppressor genes (11), we investigated whether the BET bromodomain inhibitor JQ1 and the HDAC inhibitor panobinostat exerted synergistic anticancer effects. Treatment with JQ1 in combination with panobinostat for 72 hours significantly decreased the numbers of SK-N-BE(2), Kelly, CHP134 and LAN1 cells, compared with treatment with JQ1 or panobinostat alone (Fig. 2A and Supplementary Fig. S3). The combination index (CI) method showed that JQ1 and panobinostat exerted synergistic anticancer effects (Supplementary Table S1 and Supplementary Fig. S3). Consistently, treatment with various concentrations of JQ1 in combination with 10 nM panobinostat synergistically reduced the numbers of SK-N-BE(2) and Kelly cells (Fig. 2B).

To determine whether JQ1 and panobinostat synergistically induce apoptosis, SK-N-BE(2) and Kelly cells were treated with control solvent, 1 µM JQ1, 10 nM panobinostat, or the combination of JQ1 and panobinostat for 48 hours, followed by staining with Annexin V and 7-AAD and flow cytometry analysis. While treatment with JQ1 or panobinostat alone slightly increased the percentage of cells positively stained with Annexin V alone or Annexin V plus 7-AAD, combination therapy with JQ1 and panobinostat synergistically increased the
percentage of cells positively stained with Annexin V alone or Annexin V plus 7-AAD to 70% for SK-N-BE(2) cells and 61% for Kelly cells, respectively (Fig. 2C and Fig. 2D), with combination R values of 0.15 and 0.41 respectively (fractional product method). Importantly, treatment of the normal embryonic fibroblast WI38 cells with JQ1 and panobinostat at different concentrations did not induce apoptosis (Fig. 2C, Fig. 2D and Supplementary Fig. S4). The data confirm that JQ1 and panobinostat synergistically induce apoptosis in neuroblastoma but not normal cells.

**JQ1 and panobinostat synergistically modulate target gene expression**

As BET bromodomain inhibitors and HDAC inhibitors exert anticancer effects by modulating gene expression, genome-wide differential gene expression studies were performed with Affymetrix microarray in SK-N-BE(2) cells 6 hours after treatment with control solvent, 1 µM JQ1, 10 nM panobinostat, or the combination of JQ1 and panobinostat. Using 2-fold change as the cut-off, analysis of the microarray data with GenePattern software showed that JQ1 up-regulated the expression of 14.5% (51/352) genes activated by panobinostat, and panobinostat up-regulated the expression of 29.8% (51/171) genes activated by JQ1 (Fig. 3A). Similarly, JQ1 down-regulated the expression of 27.5% (192/511) genes suppressed by panobinostat, and panobinostat down-regulated the expression of 54.9% (192/350) genes suppressed by JQ1 (Fig. 3A). The data suggest that JQ1 and panobinostat commonly activate, and more considerably commonly repress, target gene expression in neuroblastoma cells.

Importantly, the microarray data showed that the JQ1 and panobinostat combination synergistically modulated target gene expression (Fig. 3A). For example, using the fold change cut-off of 4-fold, LimmaGP analysis showed that JQ1 alone and panobinostat alone regulated the expression of 10 and 20 target genes respectively, and JQ1 and panobinostat
combination modulated the expression of 109 genes (Fig. 3A). In addition, GSEA analysis revealed significant enrichment of *MYC/MYCN* amplification and HDAC target gene sets as targets of JQ1 and panobinostat combination treatment (Fig. 3B and Supplementary Table S2).

Among the genes most significantly down-regulated by the JQ1 and panobinostat combination were the *LIN28B* and *BCL2* oncogenes (Fig. 3C and Supplementary Table S3). RT-PCR analysis confirmed that JQ1 and panobinostat synergistically reduced *LIN28B* and *BCL2* mRNA expression in SK-N-BE(2) cells 6 hours after treatment (R < 1.0 in both cases, fractional product method) (Fig. 3D). Taken together, the data suggest that JQ1 and panobinostat synergistically modulate target gene expression.

**JQ1 and panobinostat synergistically down-regulate *LIN28B* gene and N-Myc protein expression**

*LIN28B* has recently been shown to up-regulate N-Myc mRNA translation into N-Myc protein by suppressing the microRNA let-7 (14). As our microarray differential gene expression experiment showed that JQ1 and panobinostat co-operatively reduced *LIN28B* gene expression (Fig. 3C), we examined whether JQ1 and panobinostat synergistically reduced *LIN28B* and N-Myc expression.

SK-N-BE(2) and Kelly cells were treated with control solvent, 1 μM JQ1, 10 nM panobinostat, or the combination of JQ1 and panobinostat. As shown in Fig. 4A, while JQ1 or panobinostat alone reduced *LIN28B* mRNA expression, the combination synergistically reduced *LIN28B* mRNA expression with R values of 0.22 and 0.57 (fractional product method) in SK-N-BE(2) cells and R values of 0.51 and 0.52 in Kelly cells at 12 and 36 hours post-treatment respectively. Consistently, immunoblot analysis showed that JQ1 and panobinostat synergistically reduced the expression of the long as well as the short isoforms
of LIN28B protein, 36 and 48 hours post-treatment (Fig. 4B). In comparison, N-Myc mRNA expression was reduced by JQ1, and the JQ1/panobinostat combination did not synergize in reducing N-Myc mRNA expression (Fig. 4A). Importantly, combination treatment with JQ1 and panobinostat synergistically and significantly reduced N-Myc protein expression in both of the cell lines at 36 and 48 hours post-treatment (Fig. 4B).

GSK3β protein phosphorylation and Aurora A protein over-expression are well-known to promote and block N-Myc protein degradation respectively (2). We therefore examined whether JQ1 and panobinostat co-operatively increased GSK3β protein phosphorylation and reduced Aurora A protein expression. As shown in Supplementary Fig. S5A, combination therapy with JQ1 and panobinostat synergistically reduced N-Myc protein expression but did not show consistent effects on GSK3β protein phosphorylation and Aurora A protein expression. We then transfected SK-N-BE(2) and Kelly cells with control siRNA, LIN28B siRNA-1 or LIN28B siRNA-2. RT-PCR and immunoblot analyses showed that knocking down LIN28B did not affect N-Myc mRNA expression (Fig. 4C), but significantly down-regulated N-Myc protein expression (Fig. 4D). In addition, immunoblot analysis showed that transfection with LIN28B siRNAs did not have an effect on Aurora A protein expression in SK-N-BE(2) and Kelly cells (Supplementary Fig. S5B).

We next examined whether JQ1 and panobinostat co-operatively regulated LIN28B and N-Myc protein expression in other neuroblastoma cell lines. Immunoblot analysis confirmed that JQ1 and panobinostat synergistically reduced the expression of both LIN28B and N-Myc proteins in CHP134 and LAN1 neuroblastoma cells (Fig. 4E). Taken together, the data suggest that JQ1 and panobinostat synergistically reduce N-Myc protein expression by suppressing LIN28B gene expression.
BRD3 and BRD4 up-regulate LIN28B gene expression through direct binding to the LIN28B gene promoter

As the BET bromodomain inhibitor JQ1 significantly down-regulates LIN28B mRNA and protein expression (Fig. 4A and Fig. 4B), we investigated whether the BET bromodomain proteins, BRD3 and BRD4, modulate LIN28B expression. SK-N-BE(2) and Kelly neuroblastoma cells were transfected with control siRNA, BRD3 siRNA-1, BRD3 siRNA-2, BRD4 siRNA-1 or BRD4 siRNA-2. RT-PCR and immunoblot analyses showed that BRD3 siRNAs and BRD4 siRNAs reduced LIN28B mRNA and protein expression (Fig. 5A and Fig. 5B).

To determine whether LIN28B is a direct BRD3 and BRD4 target gene, ChIP assays were performed to assess whether BRD3 or BRD4 associate with the LIN28B gene promoter using PCR primers targeting a negative control and the intron 1 regions of the LIN28B gene (Fig. 5C), as described by Chang and colleagues (15). ChIP assays showed that immunoprecipitation with the anti-BRD3 and the anti-BRD4 antibodies significantly enriched the intron 1 region of the LIN28B gene, compared with the negative control region (Fig. 5D). Importantly, enrichment of the LIN28B gene promoter (intron 1) by the anti-BRD3 and the anti-BRD4 antibodies was dramatically reduced after the cells were treated with JQ1 (Fig. 5D).

To demonstrate activation of the LIN28B gene promoter by BRD3 or BRD4, we employed the pGL3 luciferase reporter construct containing empty vector or the LIN28B gene promoter (-1414 to +1403bp) (15). Kelly cells were transfected with control siRNA, BRD3 siRNA-1 or BRD4 siRNA-2, followed by co-transfection with Cypridina TK control construct plus the pGL3-empty vector construct or the pGL3-LIN28B promoter construct. In separate experiments, Kelly cells were co-transfected with Cypridina TK control construct plus the pGL3-empty vector construct or the pGL3-LIN28B promoter construct for 24 hours,
followed by treatment with control solvent, 1 µM JQ1, 10 nM panobinostat, or combination
of 1 µM JQ1 and 10 nM panobinostat for another 6 hours. Luciferase assays showed that
knocking down BRD3 or BRD4 expression with siRNA significantly reduced luciferase
activity of the LIN28B promoter construct (Fig. 5E); that treatment with JQ1 alone, but not
panobinostat alone, significantly reduced luciferase activity of the LIN28B promoter
construct; and that combination therapy with JQ1 and panobinostat synergistically reduced
luciferase activity of the LIN28B promoter construct (Fig. 5F). Taken together, the data
suggest that BRD3 and BRD4 up-regulate LIN28B gene expression by binding to its gene
promoter and activating its transcription, and that JQ1 and panobinostat synergistically
reduce LIN28B gene expression by reducing its promoter activity.

**JQ1 and panobinostat synergistically reduce N-Myc protein expression and block
tumor progression in neuroblastoma-bearing mice**

In order to evaluate the potential clinical application of JQ1 and panobinostat
combination therapy, it is essential to investigate the effect of the combination therapy on
neuroblastoma progression in vivo. Five week old nude mice were xenografted with SK-N-
BE(2) cells. When tumors reached 0.05 g, the mice were treated with vehicle control, JQ1,
panobinostat, or the combination of JQ1 and panobinostat for 14 days. As shown in Fig. 6A,
treatment with JQ1 or panobinostat alone suppressed tumor progression by approximately
40%, and treatment with JQ1 plus panobinostat substantially and synergistically blocked
tumor progression (R = 0.00043, fractional product method).

We next evaluated N-Myc protein expression in neuroblastoma tissues from the mice.
Immunohistochemistry analysis revealed that panobinostat did not have a significant effect
on N-Myc protein expression. While JQ1 reduced N-Myc protein expression, the
combination therapy with JQ1 and panobinostat synergistically reduced N-Myc protein
expression \((R = 0.27, \text{ fractional product method})\) (Fig. 6B and Fig. 6C). The data therefore demonstrate that JQ1 and panobinostat combination therapy synergistically reduces N-Myc protein expression and synergistically blocks tumor progression \textit{in vivo}. 
Discussion

The BET bromodomain protein BRD4 has been extensively shown to modulate MYC and MYCN gene transcription in leukemia, lymphoma, myeloma and neuroblastoma cells (3, 4, 7, 8, 13). While BRD3 has also been shown to regulate MYC gene transcription (8), it is unknown whether BRD3 modulates MYCN gene expression and whether BRD3 and BRD4 co-operatively modulate MYC and MYCN gene expression. In this study, we have found that knocking down BRD3 or BRD4 with siRNAs reduces N-Myc mRNA and protein expression in neuroblastoma cells. However, simultaneous suppression of BRD3 and BRD4 is only as effective as suppression of BRD3 or BRD4 alone in reducing N-Myc mRNA or protein expression. We propose that blocking BRD3 and BRD4 does not show synergy in modulating MYCN gene expression in neuroblastoma cells, because BRD3 and BRD4 induce MYCN gene transcription by recruiting the same transcriptional regulatory complexes to acetylated chromatin at the same sites.

Both BET bromodomain inhibitors and HDAC inhibitors are promising novel anti-cancer agents. Currently, several BET bromodomain inhibitors, including GSK525762, CPI-0610, OTX015 and TEN-010 are in multiple clinical trials in patients with solid tumors or hematological malignancies. Furthermore, the recent Food and Drug Authority-approval of panobinostat in combination with bortezomib in advanced multiple myeloma establishes the feasibility of clinical investigation of a BET bromodomain inhibitor-HDAC inhibitor combination strategy. In this study, we have confirmed that JQ1 and panobinostat synergistically reduce cell viability and induce apoptosis in neuroblastoma but not normal non-malignant cells. Importantly, in a mouse model of neuroblastoma, we have confirmed that treatment with JQ1 or panobinostat alone suppresses neuroblastoma progression, and that combination therapy with JQ1 and panobinostat substantially and synergistically blocks
neuroblastoma progression. Our study therefore suggests that when neuroblastoma patients are treated with BET bromodomain inhibitors in the future, they may be co-treated with the HDAC inhibitor panobinostat. The finding is consistent with recent reports that the BET bromodomain inhibitor RVX2135 and the HDAC inhibitor vorinostat synergistically kill Myc-induced murine lymphoma cells (12), and that JQ1 and panobinostat synergistically induce leukemia cell apoptosis (6).

BET bromodomain inhibitors exert biological effects by dislodging the acetylated histone readers BRD3 and BRD4 from chromatin, leading to transcriptional repression of oncogenes (7, 13, 16). In comparison, HDAC inhibitors exert biological effects by blocking the function of HDACs, leading to transcriptional activation of tumor suppressor genes (11). Surprisingly, a recent study showed that treatment with RVX2135, vorinostat or panobinostat for 24 hours commonly up-regulated the expression of a subset of genes, but did not commonly down-regulate target gene expression (12). In the current study, we have found that JQ1 up-regulates the expression of 14.5% genes activated by panobinostat, that panobinostat up-regulates the expression of 29.8% genes activated by JQ1, that JQ1 down-regulates the expression of 27.5% genes suppressed by panobinostat, and that panobinostat down-regulates the expression of 54.9% genes suppressed by JQ1. The data suggest that BET bromodomain inhibitors and HDAC inhibitors commonly activate, and more considerably commonly reduce, target gene expression in neuroblastoma cells. Importantly, while the majority of genes activated or suppressed by JQ1 or panobinostat, such as BCL2, are more considerably up- or down-regulated by JQ1 and panobinostat combination treatment, the combination also modulates the expression of a large number of genes not regulated by JQ1 or panobinostat alone. In addition, GSEA analysis of the microarray data reveals that Myc target gene sets are among the most significantly enriched by the combination therapy. Based on these data, it is concluded that JQ1 and panobinostat synergistically modulate gene
expression. We hypothesize that acetylation of histone lysine residues by panobinostat increases the affinity of BET bromodomain proteins to chromatin, therefore increasing transcription machinery accumulation at the promoters of target genes such as \( BCL2 \) and \( LIN28B \) and enhancing responses to the BET bromodomain inhibitor.

This study identified \( LIN28B \) as one of the genes most considerably and synergistically reduced by JQ1 and panobinostat combination therapy. In comparison, the JQ1 and panobinostat combination did not significantly synergize in reducing N-Myc mRNA expression. Importantly, immunoblot analysis showed that JQ1 and panobinostat considerably and synergistically reduced both LIN28B and N-Myc protein expression. In the literature, LIN28B has been demonstrated to reduce N-Myc mRNA translation into N-Myc protein via let-7 suppression (14). Consistent with this, our RT-PCR and immunoblot also show that knocking-down LIN28B reduces N-Myc protein but not N-Myc mRNA expression. We therefore conclude that JQ1 and panobinostat combination treatment synergistically reduces N-Myc protein expression due to JQ1 and panobinostat-mediated synergistic reduction in \( LIN28B \) gene transcription, leading to suppression in N-Myc mRNA translation to N-Myc protein.

BRD3 and BRD4 induce gene transcription by binding to target gene promoters or super-enhancers (4, 13, 17). In this study, we have found that knocking-down BRD3 or BRD4 expression reduces LIN28B mRNA and protein expression. ChIP assays demonstrate that BRD3 and BRD4 bind to the \( LIN28B \) gene promoter, and luciferase assays show that knocking-down BRD3 or BRD4 reduces \( LIN28B \) gene promoter activity, and that JQ1 and panobinostat synergistically reduce \( LIN28B \) gene promoter activity. We therefore conclude that BRD3 and BRD4 induce \( LIN28B \) gene expression by direct binding to its gene promoter, and that JQ1 and panobinostat synergistically reduce \( LIN28B \) gene expression by blocking its promoter activity.
In summary, this study has demonstrated that BRD3 and BRD4 directly activate \textit{LIN28B} gene transcription, and that JQ1 and panobinostat synergistically reduce \textit{LIN28B} gene and N-Myc protein expression. Importantly, JQ1 and panobinostat synergistically induce neuroblastoma cell growth inhibition and apoptosis \textit{in vitro}, reduce N-Myc protein expression in tumor tissues, and block tumor progression in neuroblastoma-bearing mice. Our findings have therefore identified a novel strategy to reduce N-Myc oncoprotein expression and a novel potential therapy for aggressive neuroblastoma.
Acknowledgments

We thank Dr. Mendell JT and Dr. Chang T-C for providing the LIN28B gene promoter construct. Children's Cancer Institute Australia is affiliated with UNSW Australia and Sydney Children’s Hospitals Network.

Grant Support

The authors were supported by National Health and Medical Research Council, Cancer Institute NSW and Cancer Council NSW. T Liu is the recipient of an Australian Research Council Future Fellowship. RB Lock is the recipient of a National Health and Medical Research Council Fellowship.
References


Figure Legends

Figure 1. The BET bromodomain proteins BRD3 and BRD4 induce N-Myc expression and neuroblastoma cell proliferation, but do not function synergistically. SK-N-BE(2) and Kelly cells were transfected with control (Cont) siRNA, BRD3 siRNA-1, BRD3 siRNA-2, BRD4 siRNA-1, BRD4 siRNA-2, a combination of BRD3 siRNA-1 and BRD4 siRNA-1, or a combination of BRD3 siRNA-2 and BRD4 siRNA-2. A and B, forty-eight hours after siRNA transfection, RNA and protein were extracted and subjected to RT-PCR (A) and immunoblot (B) analyses of BRD3, BRD4 and N-Myc mRNA and protein expression. C, seventy-two hours after siRNA transfection, cells were subjected to Alamar blue assays. Relative numbers of cells were expressed as a percentage change compared with samples transfected with control siRNA. Error bars represent standard error. **, *** and **** indicate $P < 0.01$, 0.001 and 0.0001, respectively, and NS indicates no significant difference.

Figure 2. Combination therapy with JQ1 and panobinostat synergistically induces apoptosis in neuroblastoma but not normal cells. A, SK-N-BE(2) and Kelly cells were treated with vehicle control, a range of doses of JQ1, panobinostat, or combination of JQ1 and panobinostat. Relative numbers of cells were analyzed by Alamar blue assays 72 hours post-treatment. B, SK-N-BE(2) and Kelly cells were treated with control solvent, different doses of JQ1, 10 nM panobinostat (Pano), or combination of JQ1 and panobinostat, followed by Alamar blue assays 72 hours later. C and D, SK-N-BE(2) and Kelly cells were treated with control solvent, 1 µM JQ1, 10 nM panobinostat, or combination of JQ1 and panobinostat for 48 hours. Normal fibroblast WI38 cells were treated with control solvent, 2 µM JQ1, 20 nM...
panobinostat, or combination of JQ1 and panobinostat for 48 hours. Cells were then stained with Annexin V and 7-AAD, followed by flow cytometry analyses. The graph showed one representative of three independent replicate experiments. D, the percentages of cells positively stained by Annexin V alone or Annexin V plus 7-AAD after treatment with JQ1 and/or panobinostat (Pano) were calculated. Error bars represented standard error. ** and **** indicated p < 0.01 and p < 0.0001 respectively.

**Figure 3.** JQ1 and panobinostat synergistically modulate gene expression. A, genome-wide differential gene expression studies were performed with Affymetrix microarray in SK-N-BE(2) cells 6 hours after treatment with control solvent, 1 µM JQ1, 10 nM panobinostat, or the combination of JQ1 and panobinostat. Venn diagrams showing numbers of genes commonly down or up-regulated by treatment with JQ1, panobinostat, or combination of JQ1 and panobinostat, compared with control solvent. B, GSEA generated histograms, confirming modulation of HDAC and Myc target genes by JQ1 and panobinostat combination therapy. FDR indicated false discovery rate. C, heatmap showing the top 35 genes most significantly down-regulated by JQ1 and panobinostat combination therapy. Pano represented panobinostat and Combo indicated combination therapy. D, RT-PCR analyses of LIN28B and BCL2mRNA expression in SK-N-BE(2) cells after treatment with control solvent, 1 µM JQ1, 10 nM panobinostat, or combination of JQ1 and panobinostat for 6 hours. Error bars represented standard error. *** indicated p < 0.001.

**Figure 4.** JQ1 and panobinostat synergistically down-regulate LIN28B gene and N-Myc protein expression. A and B, SK-N-BE(2) and Kelly cells were treated with control solvent, 1
µM JQ1, 10 nM panobinostat, or combination of JQ1 and panobinostat. RNA was extracted from the cells 12 or 36 hours post-treatment, and subjected to RT-PCR analysis of LIN28B and N-Myc mRNA expression (A). Protein was extracted from the cells 36 and 48 hours post-treatment, and subjected to immunoblot analysis of LIN28B and N-Myc protein expression (B). Both long and short isoforms of LIN28B protein were shown. C and D, SK-N-BE(2) and Kelly cells were transfected with control siRNA, LIN28B siRNA-1 or LIN28B siRNA-2 for 48 hours. RNA and protein were extracted for RT-PCR (C) and immunoblot (D) analyses of LIN28B and N-Myc mRNA and protein expression. E, CHP134 and LAN1 neuroblastoma cells were treated with control solvent, 1 µM JQ1, 10 nM panobinostat, or combination of JQ1 and panobinostat. Protein was extracted from the cells 48 hours post-treatment, and subjected to immunoblot analysis of LIN28B and N-Myc protein expression. Error bars represented standard error. ** and *** indicate $P < 0.01$ and $0.001$ respectively.

Figure 5. BRD3 and BRD4 up-regulate LIN28B gene expression through direct binding to the LIN28B gene promoter. A and B, SK-N-BE(2) and Kelly cells were transfected with control siRNA, BRD3 siRNA or BRD4 siRNA for 48 hours. RT-PCR (A) and immunoblot (B) were performed to examine LIN28B mRNA and protein expression. C, schematic representation of the LIN28B gene promoter region. D, ChIP assays were performed with a control, anti-BRD3 or anti-BRD4 antibody in Kelly cells after treatment with control solvent or 1 µM JQ1 for 24 hour, and PCR with primers targeting a negative control region or the intron 1 region. % of input of the different regions immunoprecipitated by the different antibodies was calculated. E, Kelly cells were transfected with control siRNA, BRD3 siRNA-1 or BRD4 siRNA-2, followed by co-transfection with Cypridina TK control construct plus pGL3-empty vector or pGL3-LIN28B promoter construct for 48 hours. Luciferase activity
was measured, normalized according to Cypridina TK control construct, and expressed as fold changes relative to control siRNA transfected samples. F, Kelly cells were co-transfected with Cypridina TK control construct plus pGL3-empty vector or pGL3-LIN28B promoter construct for 24 hours, followed by treatment with control solvent, 1 µM JQ1, 10 nM panobinostat, or JQ1 plus panobinostat for another 6 hours. Luciferase activity was measured, normalized according to Cypridina TK control construct, and expressed as fold changes relative to control solvent treated and empty vector transfected samples. Error bars represent standard error. *, ** and *** indicate $P < 0.05$, 0.01 and 0.001 respectively.

**Figure 6.** JQ1 and panobinostat synergistically reduce N-Myc protein expression and block tumor progression in neuroblastoma-bearing mice. Nude mice were xenografted with SK-N-BE(2) cells, and treated intra-peritoneally with control solvent, JQ1 at 50 mg/kg body weight (daily), panobinostat at 7.5 mg/kg body weight (once every three days), or JQ1 plus panobinostat for 14 days. A, graph showing tumor mass measured every other day. B, tumor tissues from the mice were immunostained with an anti-N-Myc antibody and visualized with diaminobenzidine (brown). The nuclei were counterstained with hematoxylin (blue). Scale bars represent 20 µm. C, N-Myc protein expression was semi-quantified and expressed as histology scores. Error bars represent standard error. **** indicate $P < 0.0001$. 
Figure 1
Figure 2
Figure 3

A

Down-regulated >2 fold Combination JQ1 Panobinostat

Up-regulated >2 fold Combination JQ1 Panobinostat

Down-regulated >4 fold Combination JQ1 Panobinostat

Up-regulated >4 fold Combination JQ1 Panobinostat

B

Heller HDAC targets down

Enrichment score (ES) 0.0

FDR = 0.0

p = 0.0

Kim MYC amplification targets up

Enrichment score (ES) 0.0

FDR = 0.246

p = 0.003

Kim MYCN amplification targets up

Enrichment score (ES) 0.0

FDR = 0.258

p = 0.028

C

D

LIN28A mRNA fold change

BCL2 mRNA fold change
Figure 4
Figure 5
Figure 6
The bromodomain inhibitor JQ1 and the histone deacetylase inhibitor panobinostat synergistically reduce N-Myc expression and induce anticancer effects

Jeyran Shahbazi, Pei Yan Liu, Bernard Atmadibrata, et al.

Clin Cancer Res  Published OnlineFirst January 5, 2016.

Updated version
Access the most recent version of this article at:

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2016/01/05/1078-0432.CCR-15-1666.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.