Targeting MYCN-driven transcription by BET-bromodomain inhibition

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Abstract

Purpose: Targeting BET proteins was previously shown to have specific antitumoral efficacy against MYCN-amplified neuroblastoma. We here assess the therapeutic efficacy of the BET inhibitor, OTX015, in preclinical neuroblastoma models and extend the knowledge on the role of BRD4 in MYCN-driven neuroblastoma.

Experimental Design: The efficacy of OTX015 was assessed in in vitro and in vivo models of human and murine MYCN-driven neuroblastoma. To study the effects of BET inhibition in the context of high MYCN levels, MYCN was ectopically expressed in human and murine cells. The effect of OTX015 on BRD4-regulated transcriptional pause release was analyzed using BRD4 and H3K27Ac chromatin immunoprecipitation coupled with DNA sequencing (ChIP-Seq) and gene expression analysis in neuroblastoma cells treated with OTX015 compared to vehicle control.

Results: OTX015 showed therapeutic efficacy against preclinical MYCN-driven neuroblastoma models. Similar to previously described BET inhibitors, concurrent MYCN repression was observed in OTX015-treated samples. Ectopic MYCN expression, however, did not abrogate effects of OTX015, indicating that MYCN repression is not the only target of BET proteins in neuroblastoma. When MYCN was ectopically expressed, BET inhibition still disrupted MYCN target gene transcription without affecting MYCN expression. We found that BRD4 binds to super-enhancers and MYCN target genes, and that OTX015 specifically disrupts BRD4 binding and transcription of these genes.

Conclusion: We show that OTX015 is effective against mouse and human MYCN-driven tumor models and that BRD4 not only targets MYCN, but specifically occupies MYCN target gene enhancers as well as other genes associated with super-enhancers.
Statement of translational relevance: We provide preclinical data about the BET inhibitor, OTX015, and mechanistic insight into BRD4 functions in MYCN-amplified neuroblastoma. We show that BRD4 specifically occupies MYCN target genes as well as other genes associated with super-enhancers. OTX015 specifically disrupts BRD4 binding to chromatin and mouse models of MYCN-driven neuroblastoma treated with OTX015 show significant survival advantage compared to untreated controls. OTX015 therefore has the potential to generate a measurable response in patients with high-risk MYCN-driven neuroblastoma. This new insight should provide a translational framework for clinical trial development of OTX015 for pediatric patients with MYCN-amplified neuroblastoma.
**Introduction**

Targeting what has been recently coined as super-enhancers has emerged as a powerful therapeutic strategy in cancer treatment (1). Super-enhancers have been shown to epigenetically regulate the transcription of specific gene sets, and are most abundant upstream of oncogenes such as *MYC* (1). Epigenetic readers recognize and bind to covalent chromatin modifications in super-enhancer regions, influencing expression of associated genes (1). These regions are enriched with acetylated histone H3 at lysine K27 (H3K27Ac) (2). The bromodomain and extra-terminal domain (BET) protein family, composed of BRD2, BRD3, BRD4 and BRDT recognize and bind histone acetylation at super-enhancer sites (1, 3, 4). BRD4 interacts with the positive transcription elongation factor b (P-TEFb) (5). Through its interaction with BRD4, P-TEFb is recruited to promoters to phosphorylate RNA polymerase II. BRD4, thus, plays an important role in regulating gene expression by retaining P-TEFb at the promoters of genes associated with super-enhancer regions (6, 7).

Neuroblastomas are embryonal childhood tumors of neuroectodermal origin. They are the most common extracranial solid tumors in children, accounting for more than 15% of all childhood cancer-related deaths (8). Despite multimodal therapy, the relapse rate of patients with high-risk neuroblastoma exceeds 50% and is almost always fatal (9). Targeted therapies with low toxicity would be a clear improvement over the aggressive cytotoxic agents currently used to treat children with high-risk neuroblastomas. The MYCN transcription factor is a central oncogenic driver in neuroblastoma (8). *MYCN* amplification is an adverse prognostic factor used for patient stratification in most neuroblastoma trials. As MYCN-driven neuroblastomas are addicted to high levels of MYCN expression, development of targeted therapies functionally disrupting MYCN is expected to be of therapeutic value for patients with these tumors.

We and others have recently discovered that targeting BRD4 with JQ1 disrupts *MYCN* transcription leading to significant antitumoral effects in neuroblastoma models (10-12). Most compelling are previous observations that *MYCN* amplification appeared to be the strongest
positive predictor of sensitivity to BRD4 inhibition. However, the reason why MYCN-driven neuroblastoma models were more susceptible to BRD4 inhibition is still unclear. Considering recent observations describing the importance of BRD4 binding to super-enhancers, we sought to uncover if and how super-enhancer disruption contributes to the antitumoral effects of BRD4 inhibition in MYCN-driven neuroblastoma. OTX015 (Oncoethix, Lausanne, Switzerland) is a small molecule that prevents BRD2/3/4 from binding to acetylated histones (13). Recently, OTX015 was shown to be active in preclinical models of acute leukemia and B-cell lymphoma (14, 15). Cellular uptake of OTX015 was shown to be rapid (<5 min) in both sensitive and resistant cell lines (16). Extracellular levels of OTX015 remain stable up to 6 h after exposure, and downstream transcriptional changes are observed as soon as 2 h after exposure (16). OTX015 is orally bioavailable and clinical phase I/II trials in adults have shown promising pharmacological properties (17, 18) (NCT01713582, NCT02259114, NCT02296476). No dose-limiting toxicity was observed for up to 80 mg OTX015 once daily or 40 mg twice daily. These doses resulted in plasma trough concentrations >250 nM, thus, well in excess of previously reported in vitro IC50 concentrations. Adverse events were mainly hematologic and gastrointestinal, including aggravation of pre-existent diabetes, thrombocytopenia, neutropenia, diarrhea and elevated transaminases. No cumulative toxicity was observed (16). Encouraged by the promising antitumoral activity of BET inhibitors, we here evaluated whether OTX015 is suited for further clinical testing in pediatric patients with neuroblastoma.
**Material and Methods**

**Cell culture and reagents**

Reagents were obtained from Sigma-Aldrich Chemie GmbH (Taufkirchen, Germany), and materials from Carl Roth GmbH & Co.KG (Karlsruhe, Germany) unless otherwise specified. OTX015 (Oncoethix SA, Lausanne, Switzerland) was provided as a powder. For *in vitro* experiments OTX015 was dissolved in DMSO. The Chp-212, Chp-134, GI-M-EN, IMR-5, IMR-32, NB69, SK-N-AS, SK-N-BE and SK-N-BE (2) human neuroblastoma cell lines were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) or the American Type Culture Collection (ATCC, Manassas, Virginia, USA) within the last 3 years. Human foreskin fibroblasts were obtained from the ATCC (CRL-2522) in 2013. The mNB-A1 murine cell line was described previously (12). The identity of all cell lines was verified at the DSMZ using Short Tandem Repeats (STR) genotyping in 2014. *Mycoplasma sp.* contamination was excluded as described previously (13). Cell lines were cultured under standard conditions in RPMI-1640 supplemented with 10% FCS and antibiotics.

**Cell viability, death, proliferation and cycle analyses**

Cell lines were treated with 0.1 to 6,000 nM OTX015 for 72 h and cell viability was assessed using the MTT assay (Roche, Mannheim, Germany) as previously described (13). GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) was used to calculate 50% inhibition of growth (IC$_{50}$) and maximal effect of the highest OTX015 dose (6 μM). Apoptosis and cell proliferation were assessed after 72 h treatment with 250 and 500 nM OTX015 using the Cell Death Detection or BrdU ELISA (Roche) assays performed according to the manufacturer’s protocols, respectively. For cell cycle analysis, cell lines were treated for 72 h with 500 nM OTX015 or DMSO. Cells were harvested, incubated with propidium iodide (100 μg/ml) to stain DNA and RNase (1 mg/ml). Cellular DNA content was analyzed on a FC500 flow cytometer (Beckman Coulter, Jersey City, NJ, USA). Cell survival after treatment with 250, 500, and 1,000 nM OTX015 or 0.2 % DMSO was also evaluated in real-time using
the xCELLigence system (ACEA Biosciences Inc., San Diego, CA, USA) as described before (13). Adherence to the culture plates was continuously monitored for 120 h (or until cell growth reached a plateau). Proliferative capacity was calculated by means of the slope using RTCA Software version 2.0 (ACEA).

**Affymetrix microarray analysis**

IMR-5 and mNB-A1 cells were plated at 1 x 10^5 cells/well in 6-well plates, cultured overnight, then treated in triplicate with 0.2 % DMSO (control), 500 nM OTX015 or 500 nM JQ1 for 24 h. Total RNA was extracted using the RNeasyMini kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Human-derived samples were profiled using the HG-U133 Plus 2.0 Human Gene Expression Array (Affymetrix, Santa Clara, CA, USA), and murine-derived samples using the MG-MM4302 Murine Gene Expression Array (Affymetrix), using established protocols (12). Microarray CEL files were normalized and summarized to gene level using the Bioconductor R statistical language repository for gcRMA normalization (19). Probes for which the log2 expression was <4 in >10 % of samples were defined as "not expressed" and were not used in analyses. The strength of similarity between different samples was evaluated using Ward-Manhattan clustering performed using the 50 genes with the highest standard deviation over all analyzed samples. Differential expression analysis was performed using Rank Product Analysis in R (v 2.13, RankProd package) (20). Hierarchical clustering of the Manhattan distance of log2 expression values of the 50 most differentially expressed genes was applied to visualize differential gene expression after treatment. Gene set enrichment analysis (GSEA) was conducted using GSEA version 2.0 (www.broadinstitute.org/gsea) and the c2.cgp.v4.0.symbols.gmt and c6.all.v4.0.symbols.gmt gene set collections (21). Genes were ranked using a signal-to-noise ranking metric calculated using the difference of the comparator population means scaled by standard deviation. MYCN gene signature scores were calculated using a previously reported algorithm (22). The following gene signatures were used to analyze MYCN activity: MYCN-regulated genes as previously defined by Westermann et al. (23), JQ1-responsive genes as
previously defined by Puissant et al. (11) and MYCN signatures retrieved from curated gene
sets (c2) in MSigDB, version 4.0 (24). Microarray data are available in the ArrayExpress
database (www.ebi.ac.uk/arrayexpress) under accession number E-MTAB-3672 and are in a
format conforming to the Minimum Information About a Microarray Gene Experiment
(MIAME) guidelines of the Microarray Gene Expression Data society (MGED).

Chromatin immunoprecipitation-sequencing (ChIP-seq) and PCR (ChIP-PCR) of
H3K27Ac and BRD4

IMR-5 cells were seeded at 1 x 10^7 cells/175 cm^2 cell culture flask, and harvested either after
adherence (ChIP-seq for H3K27Ac) or after 24 h treatment with 500 nM OTX015 or DMSO
control medium (ChIP-PCR and ChIP-seq for Brd4). After harvesting, 5 x 10^6 cells were
cross-linked with 1 % formaldehyde in 1 ml PBS for 7 min at room temperature. Cross-linking
was stopped by adding 50 µl of 2.5 M glycine for 5 min and cell pellets were washed twice
with PBS then stored at -80°C. ChIP pull-downs were prepared by Zymo Research (Irvine,
CA, USA) using frozen cross-linked cells and the following antibodies: anti-BRD4 (A301-
985A50, Bethyl Laboratories, Montgomery, TX, USA), anti-H3K27ac (ab4729,
lot #GR144577-2; Abcam, Cambridge, UK) and normal rabbit IgG (12-370, lot #2295402;
Millipore, Billerica, MA, USA). H3K27Ac ChIP DNA enriched in three independent ChIP
assays was verified by qPCR using positive control primers for the human RPL10 promoter.
ChIP-seq libraries were sequenced on the HiSeq next-generation sequencing platform.
Samples had >40 million raw reads that were trimmed of low-quality and adapter sequences.
Read alignment to the human hg19 genome assembly was performed using bowtie2 with
default settings, which achieved >93 % sequence mapping. Genome browser maps were
created by extending reads to 200 bp and computing the density using BEDTools (25). All
ChIP-seq and input maps (H3K27Ac and BRD4) were normalized for sequencing depth by
scaling to 10 million reads. Previously published primers for the MYCN promoter were used
for BRD4 ChIP-PCR after OTX015 or JQ1 treatment (11, 26). Real-time PCR was used to
analyze BRD4 abundance at MYCN promoter regions before and after treatment with OTX015, JQ1 or the DMSO control as previously described (11). GSEA was performed using the pre-ranked option, whereby each promoter region (±2 kb of start site) was ranked by the log2 ratio of the normalized read counts after OTX015 versus DMSO treatment. MYCN target gene sets defined in Valentijn et al. and Westermann et al. were tested for enrichment in genes differentially bound by BRD4 (23, 27). For composite plots, the ChIP-seq signal from cells treated with OTX015, JQ1 or DMSO was averaged over 25 bp bins in promoters and putative enhancers containing BRD4 peaks in the DMSO-treated sample. MACS v1.4 with default parameters was used to identify genome-wide enrichment of the ChIP signal relative to input in chromosomal regions (28). Putative enhancers were defined as enriched segments located at least 5 kb from a transcriptional start site documented in RefSeq (http://www.ncbi.nlm.nih.gov/refseq/). Putative super-enhancers were identified as previously described (29). Briefly, enriched segments within 12.5 kb were merged, and a normalized ChIP signal calculated and plotted against signal rank. The threshold for putative super-enhancers was defined by the point at which the y=x line was tangent to the signal-rank curve. Genes were assigned to super-enhancers if they occurred within 100 kb of the enhancer.

**Real-time reverse transcriptase PCR**

Total RNA was isolated from cells using the RNeasyMini kit (Qiagen) and cDNA synthesis was generated using the SuperScript reverse transcription kit (Invitrogen, Darmstadt, Germany). Quantitative mRNA expression levels were monitored using Assays-on-Demand™ (Applied Biosystems, Foster City, CA, USA). Alternatively, fluorescence-based kinetic RT-PCR was performed using the StepOnePlus PCR platform (Applied Biosystems) in combination with the SYBR Green I intercalating fluorescent dye according to the standard protocol. Expression was calculated using the delta Ct method and GAPDH expression as internal reference. Data analysis was performed using the qbasePLUS software, version 1.5 (http://www.biogazelle.com).
Western blot analysis

Protein lysate preparation and blotting has been described previously (13). Primary antibodies against the following proteins were used: BRD4 (1:500; ab75898, Abcam), MYC (1:1,000; #9402, Cell Signaling, Danvers, MA, USA), Cyclin D1 (1:200; sc-753, Santa Cruz, Dallas, TX, USA), E2F1 (1:500; AF4825, R&D Systems, Minneapolis, MN, USA) and MYCN (1:1,000; #9405, Cell Signaling). β-actin (1:2,000; A3853, Sigma-Aldrich) or GAPDH (1:2,000; MA3374, Millipore) were used as loading controls. Horseradish peroxidase (HRP)-conjugated secondary antibodies, anti-mouse IgG (#NA9310V; GE Healthcare, Solingen, Germany), anti-rabbit IgG (#NA9340V; GE Healthcare) or anti-sheep IgG (#HAF016; R&D Systems), diluted 1:2,000 in 5% dry milk were used. Proteins were visualized using the ECLplus western blotting detection kit (GE Healthcare) and analyzed on the FusionFX7 detection device (Peqlab, Erlangen, Germany).

OTX015 treatment of IMR-5 neuroblastoma xenograft tumors in nude mice

IMR-5 cells (2 x 10^7) were suspended in 200 µl Matrigel™ (BD Bioscience, Heidelberg, Germany) and subcutaneously inoculated into the left flank of 6-week-old female athymic (nu/nu) mice. Mice were randomly assigned to vehicle control (100 µl water) or OTX015 (n=9 mice/group) after tumors reached 150 - 200 mm³ in size. OTX015 was administered by oral gavage at 25 or 50 mg/kg body weight daily, or 25 mg/kg body weight twice daily. Tumor growth was monitored using calipers, and tumor volume was calculated using the formula (width × length × height) / 2. Mice were sacrificed after 42 days of treatment or when tumor size exceeded 2,500 mm³. Six doses of 100 mg OTX015/kg body weight were administered twice daily over 3 days to mice whose xenograft tumors were to be examined for apoptosis (cleaved caspase 3) and proliferation (Ki-67). Mice were sacrificed by cervical dislocation 4 h after the final OTX015 dose. Xenograft tumors were excised, and divided in half. One half was snap-frozen in liquid nitrogen then stored at -80°C and the other half was formalin-fixed and paraffin-embedded for immunohistochemical analyses. All animal experiments were...
performed in accordance with the Council of Europe guidelines for accommodation and care of laboratory animals. Protocols were approved by the Ethics Commission for Animal Experimentation at the University Hospital Essen.

**Ectopic MYCN expression and OTX015 treatment of murine cells and tumors grown as grafts in nude mice**

Murine tumors (≈1,000 mm³) derived from the genetically engineered mouse model LSL-MYCN;Dbh-iCre (12) were minced manually with scissors and digested with 2 mg/ml collagenase in PBS for 30 min at 37°C. Tumor pieces were passed through a sieve (400 µm pore size) to obtain a cell suspension. Cells were washed with PBS and suspended in 2.5 ml Matrigel™ for subcutaneous inoculation (200 µl per mouse) into the left flank of 6-week-old female athymic (nu/nu) mice. OTX015 treatment was the same as described for xenografts, as was grafted tumor excision and processing. M-NB-A1 cells were used as a murine model of cells ectopically expressing MYCN (12). To model ectopic expression of MYCN in human neuroblastoma cells, we transfected IMR-5 cells with pMSCV-puro plasmids (#21654, Addgene, MA, USA) containing MYCN, GFP or empty negative control. Cells were selected with 2 µg/mL puromycin for two days. After selection cells were expanded and treated with OTX015 as described above. Cells treated with 500 nM OTX015 were harvested for RT-PCR and western blot analysis of MYCN expression.

**Tumor tissue immunohistochemistry**

Samples of xenograft and murine tumors treated with vehicle or OTX015 were fixed and embedded in paraffin and stained as previously described (30). Primary antibodies against Ki-67 (1:50, pH 6; #275R-16, Cell Marque, Rocklin, CA, USA) and cleaved caspase 3 (1:100, pH 6; #9661, Cell Signaling) were used with corresponding secondary antibody detection kits for reduced background (Histofine Simple Stain MAX PO, Medac, Hamburg, Germany) and the automated LabVision Autostainer 480S (Thermo Scientific, Waltham, MA, USA). Slides were scanned with a Pannoramic 250 slide scanning program (3D Histech.com).
Statistical analysis

Expression data from IMR-5 cells treated with DMSO, JQ1 or OTX015 were analyzed with the R2 visualization and analysis platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi). SPSS, version 18.0 (IBM, New York, NY, USA) and R, version 2.13, were used for further statistical analyses. Interval variables were compared using the Student's two-sided t-test and categorical variables compared by chi-square test. Graph Pad Prism 5.0 was used to perform Kaplan-Meier survival analysis with log-rank statistics on OTX015-treated and control mouse cohorts.
**Results**

OTX015 is a BET protein inhibitor with no significant effect on normal human cells.

OTX015 is a synthetic small molecule targeting the BET bromodomain proteins, BRD2/3/4 (Fig.1a)(13). To assess putative effects of OTX015 on non-malignant primary human cells, we measured the cell viability of human fibroblasts after 72 h of OTX015 treatment. Although OTX015 concentrations above 1 µM slightly reduced fibroblast viability (maximal reduction <50 % at 4 µM), this was not significant (Fig. 1b and Suppl. Fig. 1a and b).

OTX015-induced reductions in neuroblastoma cell viability correlate with MYCN status and expression.

OTX015 significantly reduced viability of neuroblastoma cells (Fig. 1c and Suppl. Fig. 2a and b) with IC₅₀ values ranging from 37 nM to >1 µM (Suppl. Fig. 2a). Maximal reduction of cell viability was almost 100% in IMR-5, CHP-134, CHP-212, NB69 and GI-M-EN cell lines (Suppl. Fig. 2b). MYCN-amplified neuroblastoma cell lines (red curves, Fig. 1c) were more sensitive to OTX015 treatment than cell lines lacking MYCN amplification (p=0.003, Fig. 1d). These results are in line with those previously reported for the BET inhibitor, JQ1 (11). The cell lines IMR-5, IMR-32, CHP-134 and CHP-212, which express higher levels of MYCN at both mRNA and protein levels, were more sensitive than the SK-N-AS, GI-M-EN and NB-69 cells lines with low-level expression (Suppl. Figs. 2c-e), however, there was a poor correlation between IC₅₀ values and both MYCN mRNA and protein levels. Notably, SK-N-AS cells did not respond to OTX015 even though expressing high MYC levels. Taken together, OTX015 treatment in vitro strongly reduces viability of neuroblastoma cell lines with predominant effects on MYCN-amplified cells.

Oral OTX015 shows therapeutic efficacy against human MYCN-amplified neuroblastoma xenografts.

To further investigate antitumoral effects of OTX015 in vivo, we utilized a mouse xenograft model of MYCN-amplified human neuroblastoma, with the human IMR-5 cell line. Average
tumor volume was significantly smaller in the OTX015 groups compared to control mice after day 9 of treatment (p < 0.05, Fig. 1e). Kaplan-Meier analysis showed that tumor-free survival was delayed significantly in all treatment groups compared to the control group (p < 0.02, Fig. 1f). Thus, OTX015 treatment significantly improved survival in mice harboring MYCN-amplified neuroblastoma xenografts. Two of nine mice from the group treated with 25 mg OTX015/kg body weight twice a day died because of treatment misapplication on days 8 and 9. No considerable side-effects were observed in mice treated with OTX015. Oral administration of OTX015 achieved antitumoral effects in a MYCN-driven neuroblastoma xenograft model.

**OTX015 reduces proliferation and induces apoptosis and cell cycle arrest in human neuroblastoma cell lines and in vivo neuroblastoma models.**

When investigating the impact of OTX015 on typical tumorigenic properties, MYCN-amplified cells most predominantly showed significantly reduced cell growth over time (Suppl. Fig. 3b and 3c). The percentage of MYCN-amplified cells in G1 increased after OTX015 treatment compared to DMSO-treated control cells. This was accompanied by fewer cells in the S phase. The percentage of cells in sub-G1 also increased in the IMR-5 cell line after treatment (Suppl. Fig. 3d). OTX015 treatment of SK-N-AS cells, lacking MYCN amplification, did not alter the distribution of cells in the cell cycle. These data suggest that OTX015 can cause a shift towards either G1 arrest or apoptosis in MYCN-amplified cells.

The fraction of apoptotic cells was significantly higher in IMR-5, Chp-134 and Chp-212 MYCN-amplified cell lines after OTX015 treatment compared to the DMSO control, but there was no detectable induction of apoptosis in SK-N-BE (MYCN-amplified) and SK-N-AS (single-copy MYCN) cells (Suppl. Fig. 3e). BrdU incorporation showed significantly reduced proliferative capacity in all cell lines except Chp-212 after OTX015 compared to the DMSO control (Suppl. Fig. 3f).

Tumors from OTX015-treated IMR-5 xenografts also showed a significant increase in the proportion of apoptotic cells (Suppl. Fig. 4), and the proportion of cells staining positively for
Ki-67 was significantly reduced (Suppl. Fig. 4b and 4c). Taken together, OTX015 induced cell cycle arrest and apoptosis with a range of sensitivity across a panel of neuroblastoma cells, most strongly dependent on cellular MYCN status.

**OTX015 disrupts the BRD4-chromatin interaction and represses MYCN expression in neuroblastoma cell lines.**

BRD4 inhibition has been shown to suppress transcription of MYCN in neuroblastoma, thus, the phenotypic effects were previously primarily attributed to this observation (11). Consistent with this previous observation, treatment with OTX015 or JQ1 significantly reduced BRD4 binding to the MYCN promoter (Fig. 2a). We next examined the effect of OTX015 treatment on MYCN expression in several genetic backgrounds relevant for human neuroblastoma in vitro and in vivo. OTX015 treatment reduced MYCN transcript levels in all MYCN-amplified cell lines after 4 h and up to 72 h of treatment (Fig. 2b). This reduction in gene expression in OTX015-treated cell lines was recapitulated at the protein level for MYCN and other BRD4 targets (Fig. 2c). In vivo OTX015 treatment of IMR-5 xenografts reduced MYCN expression at both the mRNA and protein levels as well as E2F1 and cyclin D1 protein expression (Fig. 2d and 2e). Together these observations support the previous observations of MYCN being a target of BRD4 in MYCN-driven neuroblastoma cells, and provide evidence for this activity with OTX015.

**Ectopic MYCN expression does not abrogate the effects of BRD4 inhibition.**

The effect of BRD4 inhibition in neuroblastoma cells has previously been attributed to MYCN repression (11). We set out to test the existing hypothesis that BET protein inhibition mainly acts through transcriptional suppression of MYCN. To do this, we ectopically expressed MYCN in IMR-5 cells in order to stabilize its high expression level (Fig. 3a) then treated cells with OTX015. Interestingly, there was no difference in OTX015 dose-response between IMR-5 cells ectopically expressing MYCN or control cells (Fig. 3a). As an orthogonal model we used the genetically engineered mouse model (GEMM), LSL-MYCN;Dbh-iCre, and the mNB-
A1 cell line derived from a neuroblastoma tumor arising in this GEMM (12). In this murine
system, MYCN transcription is strongly driven by a chicken actin promotor. We hypothesized
that MYCN would not be under the epigenetic control of endogenous elements such as
enhancers in this model. It follows that MYCN expression should not be affected by BRD4
inhibition if our hypothesis is correct, and that MYCN expression from the transgene should
not be affected to the same extent as endogenous MYCN. Any effects of BET bromodomain
inhibition observed in this context would not be due to MYCN transcriptional silencing.
Indeed, in LSL-MYC;Dbh-iCre derived mNB-A1 cells, MYCN protein levels remained stable
even after treatment with OTX015 (Fig. 3d). Interestingly, in vitro treatment of the mNB-A1
cell line with OTX015 significantly reduced cell viability as did JQ1 treatment (Fig. 3c). Mice
harboring regrafted LSL-MYC;Dbh-iCre tumors were orally treated with OTX015 or control
(n=6). Consistent with in vitro treatment of LSL-MYC;Dbh-iCre derived mNB-A1 cells, we
observed a delay in tumor progression in OTX015-treated mice harboring regrafted LSL-
MYC;Dbh-iCre tumors, resulting in significantly prolonged mouse survival (Figs. 3e and 3f).
Again, MYCN expression was not affected by OTX015 treatment (Fig. 3g). Concurrently, the
fraction of apoptotic cells was significantly increased in the LSL-MYC;Dbh-iCre tumors,
while the fraction of proliferating cells was reduced (Fig. 3h and 3i). Together, OTX015
showed therapeutic efficacy in both murine neuroblastoma GEMM models and human cells
ectopically expressing MYCN without repressing MYCN transcription. These results suggest
that effects of BET protein inhibition are at least partly independent of MYCN repression.

**Inhibiting BRD4 by OTX015 disrupts transcription of MYCN target genes independently
of MYCN expression levels.**

Considering the interesting observation that OTX015 showed anti-tumoral effects on cells
ectopically expressing MYCN without repressing MYCN expression, we performed gene
expression profiling to explore the underlying mechanisms and alternative targets of the BET
proteins. OTX015 or JQ1 treatment of IMR-5 cells significantly altered gene expression
patterns compared to DMSO-treated control cells (Fig. 4a). Genes that were differentially expressed in OTX015- or JQ1-treated cells showed significant overlap. Treatment with either OTX015 or JQ1 commonly upregulated 63.7% (2534 of 3976) and downregulated 66.5% (3051 of 4590) of genes in IMR-5 cells. This similar action is consistent with the similar specificity of JQ1 and OTX015 for BET proteins. Gene sets enriched among the genes downregulated by OTX015 represented known oncogenic pathways, such as the KRAS, ALK and MYC pathways (Fig. 4b). The same gene sets were enriched among the genes downregulated by JQ1, supporting the high overlap in differentially expressed genes between the two BET inhibitors.

Interestingly, treatment of both IMR-5 and the LSL-MYCN;Dbh-iCre-derived mNB-A1 cell line with either BET inhibitor significantly decreased MYCN target gene signature scores (Fig. 4c and d). This indicates that MYCN target genes are repressed by BET inhibition even at unchanged MYCN levels (see MYCN expression in mNB-A1 in Fig. 3d). As this effect on MYCN target genes occurs without MYCN transcriptional suppression in mNB-A1 cells, this raises the question whether MYCN target genes are direct BRD4 targets. Our data suggests that BRD4 inhibition in non-engineered human neuroblastoma cells directly suppresses both MYCN and MYCN target genes.

OTX015 selectively disrupts BRD4 binding at MYCN target genes and represses super-enhancer associated gene transcription

Our observation that the therapeutic efficacy of BET inhibitors against MYCN-driven tumors was partly independent of MYCN repression raised the question about other therapeutically important BRD4 targets in neuroblastoma. Our current understanding of BRD4 function supports BRD4 binding to acetylated histones at what is referred to as super-enhancer sites that regulate transcriptional pause release of oncogenes such as MYC. We hypothesized that super-enhancers associated with genes other than MYCN might be important to mediate the effects of BET inhibition in neuroblastoma. Using H3K27Ac ChIP-seq we detected 1335 super-enhancers in the IMR-5 genome using an algorithm proposed by Whyte et al. (Fig. 5a)
Among the genes associated with the top 200 super-enhancers (SE-genes) we detected a large number of genes of known importance in cancer, including MYCN and GLI2 (Fig. 5a and Suppl. Fig. 5). We hypothesized that super-enhancer-associated genes would be most affected by BRD4 inhibition. Indeed, genes downregulated in IMR-5 cells after treatment with OTX015 or JQ1 were significantly enriched for super-enhancer-associated genes (Suppl. Fig. 5c). The cumulative distribution of gene expression changes was also significantly shifted towards a stronger repression of genes associated with super-enhancers (Fig. 5b). Expression of genes associated with super-enhancers was significantly lower than genes not associated with super-enhancers after treatment with either OTX015 or JQ1 (Fig. 5c). Consistent with this, ChIP-Seq detected a significant decrease in BRD4 binding to chromatin, particularly at super-enhancer regions (Fig. 5d-f). Since OTX015 significantly affected MYCN target gene expression, we searched for MYCN target genes in the BRD4 ChIP-seq data. MYCN target genes were significantly enriched in chromatin areas where BRD4 binding was strongly reduced. This was observed for both the Valentijn et al. and Westermann et al. independently developed MYCN target gene sets (Fig. 5g)(23, 27). This suggests a functional importance for BRD4 in MYCN-driven gene expression, and may explain our observations that BET inhibition disrupts MYCN target gene transcription even when MYCN is ectopically expressed (Fig. 5h). Our data show that pharmacological BRD4 inhibition specifically disrupts BRD4 binding not only to MYCN but also to MYCN target genes and preferentially leads to transcriptional repression of super-enhancer associated genes.
Discussion

The importance of super-enhancers for transcriptional regulation of oncogenes such as MYC family transcription factors has recently been brought to light, opening up new opportunities for drug development and raising new questions about their functions in cancer. Here we demonstrate antitumoral potency of the BET protein inhibitor, OTX015, against cell and animal models of MYCN-driven neuroblastoma, and provide mechanistic insights indicating that OTX015 selectively disrupts MYCN-driven transcription, preferentially affecting super-enhancer associated genes.

Neuroblastoma is the most common extracranial tumor in children (31). The majority of children with high-risk disease succumb to neuroblastoma and surviving patients often suffer from long-term sequelae due to cytotoxic chemotherapy (32). Novel targeted treatment approaches with lower toxicity are clearly needed for these patients. The MYCN transcription factor is a driving oncogene in neuroblastoma. Tumor cells are addicted to high levels of MYCN. The critical role of MYCN renders it a compelling target for drug development, and several strategies indirectly targeting MYCN are emerging (33-37) (reviewed in (38)). BRD4 inhibition was the first successful approach used to target MYCN transcription in neuroblastoma (10, 11, 39).

There has been widespread interest in developing drugs selectively targeting BET proteins that are well suited to clinical use. In past years, several BET inhibitors such as I-BET 762 (GSK525762), TEN-010 and CPI-0610 have been introduced into clinical phase I trials (NCT02308761, NCT01987362, NCT01587703, NCT01949883, NCT02157636, NCT02158858), but to our knowledge, none have yet entered pediatric clinical trials. With development for oral bioavailability, OTX015 entered adult clinical phase I/II trials in 2012 (16, 17). Here we explored the possibility of selectively targeting BRD4 with OTX015 in preclinical neuroblastoma models. Similar to JQ1, OTX015 effectively reduced neuroblastoma cell viability in vitro and in vivo. OTX015 potently inhibited growth in a MYCN-amplified neuroblastoma xenograft tumor model and significantly prolonged mouse survival.
Responsiveness to BRD4 inhibition has previously been attributed to the extent of endogenous MYCN expression (11). We observed similar correlations between MYCN status and sensitivity to OTX015 treatment. High-level MYCN expression and MYCN amplification were significantly correlated with the response to OTX015 treatment. SK-N-AS cells, which express high levels of MYC, were rather resistant to BET inhibition, indicating that sensitivity to BET inhibition might indeed be specific to MYCN status in the context of neuroblastoma biology. Together, our findings indicate OTX015 is a potent agent against preclinical models of MYCN-driven neuroblastoma.

Since neuroblastoma cells are addicted to high MYCN levels, the effect of BRD4 inhibition on cell viability was previously attributed to MYCN repression (3, 39). Supporting these findings, we observe a similar reduction of MYCN expression following OTX015 treatment. Previous reports showed that disrupting BRD4-chromatin binding was the mechanistic cause for these transcriptional changes. OTX015 treatment inhibited BRD4 binding to chromatin to a comparable or stronger extent as the known BRD4 inhibitor, JQ1, providing support that OTX015 robustly reduces BRD4 binding to acetylated histone tails at the MYCN promoter.

To further understand the role of MYCN as a known mediator of antitumoral effects of BRD4 inhibition in neuroblastoma, we investigated the effects of BRD4 inhibition on models ectopically expressing MYCN. MYCN expression was not affected by BET inhibition in our models. Interestingly, ectopic MYCN expression did not attenuate the antitumoral effects of OTX015 in this context. This is in line with our previous observations that JQ1 is effective against LSL-MYCN;Dbh-iCre neuroblastoma (12), but was intriguing since MYCN amplification and high-level MYCN expression are strong predictors of BRD4 inhibitor susceptibility. These findings suggested that mechanisms other than transcriptional repression of MYCN contribute to the effect of BRD4 inhibition in neuroblastoma.

Understanding these mechanisms is essential for the future clinical application of OTX015 and other BRD4 inhibitors. We observed that genes transcriptionally repressed after OTX015 treatment were enriched for MYCN target genes, in both MYCN-amplified IMR-5 cells and mNB-A1 cells ectopically expressing MYCN. This suggested that BRD4 inhibition suppresses
MYCN target gene transcription even in the presence of high MYCN levels. Gergano et al. described MYC recruitment of P-TEFB to its target genes (40) and recent findings indicate a similar recruitment of CDK7 to super-enhancer sites by MYCN (37). This recently published insight indicates that MYCN-driven transcription is dependent on recruitment of P-TEFB and, thus, BRD4 to MYCN target gene promoters and enhancers. Indeed, our BRD4 ChIP-Seq analysis showed that BRD4 binding was most significantly reduced at MYCN target genes. This further sustains our hypothesis that inhibiting BRD4 with OTX015 blocks BRD4 and P-TEFb-dependent MYCN-driven transcription. Considering, however, that Chipumuro et al. described MYCN as a general transcriptional amplifier (37) similar to MYC, our gene-expression results might be underestimating the effect of BET inhibition on MYCN-driven transcription. Conducting gene-expression analysis using spike-in normalization, as recently suggested by Loven et al. (38,39), should reveal MYCN-induced changes on global expression levels. Consistent with previous reports, we conclude that MYCN-driven transcription might be dependent on BRD4-mediated transcriptional pause release, which would explain why MYCN target genes were more susceptible to BET inhibition. This conclusion is further supported by (i) the strong correlation of MYCN amplification with susceptibility to OTX015 treatment, (ii) the widespread and robust inhibition of MYCN target gene transcription in cells ectopically expressing MYCN by OTX015 and (iii) the fact that ectopic MYCN expression in a murine neuroblastoma model did not significantly attenuate OTX015 effects.

Considering recent discoveries implicating BRD4 in super-enhancer regulation, we set out to understand the extent to which inhibiting BRD4-chromatin binding would specifically disrupt super-enhancer associated gene expression. Interestingly, we found that H3K27Ac-enriched areas were most abundant near genes of known importance for tumorigenesis. As expected, the expression of genes associated with super-enhancers was most significantly reduced after OTX015 treatment. MYCN was highly ranked in the list of super-enhancer-associated genes, indicating its regulation by a super-enhancer and further supporting its role as a BRD4 target. Building on published work implicating MYCN in BRD4 inhibition, we verified
and extended understanding of the mechanism of BRD4 inhibition in neuroblastoma, reporting that inhibiting BRD4 binding to the chromatin using OTX015 preferentially disrupts transcription of super-enhancer-associated genes, which include MYCN.

Previous studies have proposed MYCN amplification as a biomarker to predict tumor susceptibility to BET inhibitors (11). Considering our new findings, we propose that a MYCN activity score be evaluated as an improved biomarker for tumor susceptibility to BET protein inhibitors. A MYCN activity score based on the expression of MYCN target genes would also identify patients with tumors lacking MYCN amplifications, but otherwise activated MYCN, as described previously (27). Neuroblastomas with activated MYCN but lacking MYCN amplification are known to be similarly aggressive and result in poor patient outcome (27). Remarkably, such signature scores are also capable of identifying poor outcome in patients with tumors expressing low MYCN transcript levels but high nuclear MYCN protein levels (27). These patients would likely also benefit from BET protein inhibitor treatment and this could be applicable in other tumor types in which MYCN is the driving oncogenes.

The results we report here provide conclusive evidence that BET protein inhibition disrupts super-enhancer functions and preferentially suppresses MYCN-driven transcription. We show that OTX015 is active against both in vitro and in vivo preclinical neuroblastoma models. Based on the survival advantage observed in mouse models of MYCN-driven neuroblastoma treated with OTX015, this drug has the potential to generate a measurable response in patients with high-risk MYCN-driven neuroblastoma. To identify patients who would likely benefit from BET inhibitor treatment, we propose using the previously established MYCN activity score (27). Our data present a preclinical rationale that can feed into upcoming phase I/II trials of OTX015 in pediatric patients.
Acknowledgments

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**Funding and grant information**

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References


Figures

Figure 1. The BET inhibitor, OTX015, displays antitumoral activity against MYCN-amplified neuroblastoma in vitro and in vivo. (a) The molecular structure of OTX015, with the formula \((6S)-4-(4\text{-chlorophenyl})-N-(4\text{-hydroxyphenyl})-2,3,9\text{-trimethyl-6H-thieno}[3,2-f][1,2,4]triazolo[4,3-a][1,4]diazepine-6-acetamide\). (b) OTX015 had no significant effect on human fibroblast viability, as shown in a dose-response curve of fibroblasts treated with increasing doses of OTX015 for 72 h and measured with the MTT cell viability assay. (c) Cell viability dose-response curve for the IMR-5, Chp-134, Chp-212, SK-N-BE(2), IMR-32 SK-N-BE, NB69, SK-N-AS and GI-M-EN neuroblastoma cell lines treated with increasing OTX015 concentrations for 72 h. Red curves indicates cells harboring MYCN amplifications and blue curves are cell lines lacking amplification. (d) The IC\(_{50}\) values for the OTX015 impact on cell viability are plotted for cell lines harboring MYCN amplifications (red) and cell lines lacking MYCN amplification (blue). The difference between the two groups was analyzed using the Student’s t-test, supporting a significant correlation between MYCN status and OTX015 efficacy against neuroblastoma cell lines in vitro. (e) Nude mice harboring IMR-5 xenograft tumors with volumes of 200 mm\(^3\) were treated with OTX015 by oral gavage at the concentrations, 25 mg/kg body weight/day (light green, \(n=9\)), 2 \times 25 mg/kg body weight/day (green, \(n=9\)) and 50 mg/kg body weight/day (dark green, \(n=9\)), or with 200 \(\mu\)l H\(_2\)O/day (black, \(n=9\)) as a negative control. Tumor volume was measured using a caliper every 2 days during treatment, and the average tumor volume in each treatment group is plotted for the course of treatment. Statistical difference between the treatment and control groups was analyzed by Student’s t-test (all treatment groups have \(p < 0.05\) from day 9 to the end of treatment). (f) Kaplan-Meier analysis was conducted for the xenograft mouse model treated in panel (e), showing that OTX015 treatment significantly improved survival of mice with established IMR-5 xenograft tumors (Log-rank test, \(p < 0.02\)).
Figure 2. OTX015 treatment reduces BRD4 binding to chromatin and leads to decreased BRD4 target gene expression in vitro and in vivo. (a) Schematic H3K27Ac track (adapted from genome browser, http://genome.ucsc.edu/) showing PCR primer localization (region 1 and 2) relative to MYCN gene body (left). Quantitative chromatin immunoprecipitation (ChIP) and PCR (ChIP-PCR) of BRD4 from IMR-5 cells treated with OTX015, JQ1 or control, at the MYCN promotor regions 1 and 2. (b) Time course of MYCN mRNA expression (measured using real-time RT-PCR) after treatment of neuroblastoma cell lines with 500 nM OTX015 relative to DMSO control. Expression was normalized to GAPDH expression. MYCN expression was not assessed in SK-N-AS cells, since they did not express significant endogenous levels of MYCN. (c) Whole-cell protein lysates collected after 72 h of 500 nM OTX015 (+) or control DMSO (-) treatment of neuroblastoma cell lines in vitro, and known BRD4 targets were analyzed in Western blots. β-actin was used as loading control. (d) Real-time RT-PCR was used to assess MYCN expression in IMR5 xenograft tumors. Expression was normalized to GAPDH expression, and statistical difference between groups was assessed by Student’s t-test. (e) Protein lysates were prepared from IMR5 xenograft tumors, and BRD4 targets were analyzed in Western blots. β-actin was used as loading control.

Figure 3. Ectopic MYCN expression does not attenuate effects of BET inhibition. (a) Western blot of MYCN and V5 in IMR5 cells transfected with pMSCV-MYCN, V5-tagged MYCN, EGFP or empty control after 48 h of treatment with 500 nM OTX015 and DMSO control. (b) Viability (measured by MTT assay) of IMR5 cells ectopically expressing MYCN, V5-tagged MYCN, GFP or empty control. (c) Viability (measured by MTT assay) of the murine neuroblastoma cell line mNB-A1 ectopically expressing MYCN, over 96 h after treatment with either OTX015 (dark grey), JQ1 (light grey) or DMSO control (black). (d) MYCN protein expression in LSL-MYCN;Dbh-iCre murine neuroblastoma cell line mNB-A1 after treatment with OTX015. (e) Tumor volumes of LSL-MYCN;Dbh-iCre murine neuroblastoma tumors grown as grafts in nude mice and orally treated with 25 mg/kg body
weight/day OTX015 or H₂O alone (control). The average tumor volume was smaller in the OTX015-treated groups compared to the control group (Student’s t-test: p=0.07 on treatment day 13). (f) Kaplan-Meier analysis showing the survival of mice bearing tumors described in (c) treated with OTX015 or H₂O alone. Log-rank tests were used to calculate significance. (g) Relative MYCN mRNA expression in LSL-MYCNDbh;iCre tumors after treatment with OTX015 or vehicle control. (h) Representative pictures of sections of grafted LSL-MYCNDbh;iCre tumor stained with hematoxylin/eosin and immunohistochemical staining for apoptotic cells (cleaved caspase 3) and proliferating cells (Mib-1/Ki-67). Scale bar=250 µm. (i) The relative fraction of positively-stained cells for cleaved caspase 3 and Mib-1 were calculated from three representative images from each grafted LSL-MYCNDbh;iCre tumor and are shown as box plots. Statistical difference between groups was assessed by Student’s t-test.

Figure 4. BET inhibition causes distinct gene expression changes and affects MYCN target genes even at stable MYCN levels. (a) Heatmap of the top 50 differentially expressed genes after OTX015 treatment in IMR-5 cells compared to DMSO-treated control cells (left) and a heatmap of the top 50 commonly differentially expressed genes after OTX015 and JQ1 treatment (right). (b) GSEA (version 2.0, Broad Institute) of differentially expressed genes in IMR5 cells after treatment with OTX015 or JQ1 using C6 gene sets representing signatures of cellular pathways often dysregulated in cancer. (c) Enrichment of the published gene expression signature for MYCN target genes (24) in differentially expressed genes in IMR-5 cells after treatment with OTX015, JQ1 or DMSO control. (d) Enrichment of the published gene expression signature for MYCN target genes (24) in differentially expressed genes in LSL-MYCNDbh;iCre murine neuroblastoma cell line mNB-A1 cells after treatment with OTX015, JQ1 or DMSO control (* p < 0.05, ** p < 0.01, *** p < 0.001).
Figure 5. BET protein inhibition specifically disrupts BRD4 binding to MYCN target genes and preferentially represses super-enhancer driven transcription. (a) The H3K27Ac signal in MYCN-amplified IMR-5 cells is shown across enhancer regions for all enhancers. Super-enhancers were defined as surpassing the threshold defined by the point at which the y=x line was tangent to the signal-rank curve. In IMR-5 cells, 4.6% of the enhancers were classed as super-enhancers, including those genes named on the right. (b) Cumulative distribution plots of the top 200 super-enhancer-associated genes and their fold-change measured using genes which are differentially regulated in IMR-5 cells by OTX015 treatment. Plots show a shift towards negative logFC for super-enhancer associated genes (blue curves) compared to genes not associated with super-enhancers (black curves). (c) Box plots of log2-fold changes in the top 200 genes associated with super-enhancers (SE) and regular enhancers (non-SE) in genes differentially regulated (compared to DMSO-treated controls) in IMR-5 cells treated with OTX015. Statistical significance was determined using a two-sided Mann-Whitney test. (d-f) The normalized BRD4 ChIP-Seq signal across the genome (d), at enhancers and promoters (e) as well as at super-enhancers sites (f) in IMR5 cells after treatment with OTX015 or DMSO control. (g) GSEA of genes where BRD4 binding was most significantly reduced show significant enrichment for MYCN target genes (Valentijn et al. MYCN target genes (left), p<0.002; Westermann et al. MYCN target genes, p<0.001 (right)). (h) Schematic view of proposed mechanism of BRD4 inhibition in neuroblastoma. BRD4 is recruited to super-enhancer complexes of the MYCN gene (top). MYCN acts as a transcription factor at MYCN target gene promoter and enhancer sites where BRD4 is also recruited to (bottom). BRD4 inhibition by OTX015 therefore not only indirectly leads to decreased expression of MYCN target genes by inhibiting MYCN expression, but also suppressed MYCN activity at its target genes directly.
Supplementary Figures

Supplementary Figure 1. OTX015 has no significant effect on non-malignant human fibroblasts. (a) Representative pictures of human fibroblasts treated for 72 h with 500 nM OTX015 or DMSO control. Scale bar=100 µm. (b) Growth of fibroblasts treated with 500 nM OTX015 (black) or DMSO control (grey) monitored over 96 h using the xCELLigence system. The slope (1/h) of both curves was calculated, and did not show a significant difference (right) using the Student’s t-test.

Supplementary Figure 2. OTX015 effect on cell viability positively correlates with MYCN status. (a) IC₅₀ of the IMR-5, Chp-134, Chp-212, SK-N-BE(2), IMR-32, SK-N-BE, NB69, SK-N-AS and GI-M-EN neuroblastoma cell lines measured using MTT assays. (b) Maximum reduction of viability in IMR-5, Chp-134, Chp-212, SK-N-BE(2), IMR-32, SK-N-BE, NB69, SK-N-AS and GI-M-EN cells treated for 72 h with 6 µM OTX015 and measured using MTT assays. (c) Relative MYCN mRNA (top) and protein (bottom) expression in all analyzed neuroblastoma cell lines. (d/e) The IC₅₀ values of the cell lines shown in (a) were plotted against MYCN mRNA expression (d) or MYCN protein expression (e), which is shown in (c). Both graphs show weak anti-correlative tendencies in both cell lines (MYCN mRNA p=0.22, MYCN protein p=0.16).

Supplementary Figure 3. Mechanisms of reduced cell viability induced by OTX015 are cell line dependent. OTX015 reduces cell proliferation over time and induces cell cycle changes as well as apoptosis. (a) Representative photomicrographs of neuroblastoma cell lines treated with OTX015 for 72 h. Scale bar=200 µm. (b) The slope (1/h) of cell proliferation over time measured using xCELLigence of cell lines treated with OTX015. Significance was measured with the Student’s t-test. (c) Cell proliferation monitored using the xCELLigence system over time in neuroblastoma cell lines treated with 500 nM OTX015 (black) or DMSO control (grey). (d) Cell lines were treated 72 h with 500 nM OTX015 or DMSO, then fixed and stained with propidium iodide for flow cytometry. The distribution of OTX015-treated cells in

...
different stages of the cell cycle is shown. (e) Fraction of apoptotic cells measured in the cell death ELISA assay after 72 h treatment with 250 nM or 500 nM OTX015 or DMSO control. Significant differences between treated cells versus controls were calculated by Student’s t test. * p < 0.05, ** p < 0.01. (f) Cell proliferation measured in the BrdU ELISA after 72 h of treatment with 250 nM or 500 nM OTX015 or DMSO. Significant differences between treatment groups and the controls were calculated by Student’s t test.

Supplementary Figure 4. OTX015 reduces cell proliferation and induces apoptosis in MYCN amplified human xenografts. (a) Representative pictures of IMR5 xenograft tumor sections stained with hematoxylin/eosin, and immunohistochemical staining for apoptotic cells (cleaved caspase 3) and proliferating cells (Mib-1/Ki 67) are shown. Scale bar=250 µm. The relative fraction of positively stained cells for cleaved caspase 3 (b) and Mib-1 (c) were calculated from three representative images from each xenograft tumor and are shown as box plots. Statistical difference between groups was assessed by Student’s t test. * p < 0.05, ** p < 0.01, *** p < 0.001.

Supplementary Figure 5. BET bromodomain inhibition leads to disruption of MYCN driven gene-expression programs even at high levels of MYCN. (a) Enrichment of the published gene expression signature for JQ1-treated human MYCN-amplified neuroblastoma cell lines (11) in differentially expressed genes in IMR-5 cells after treatment with OTX015, JQ1 or DMSO control. (b) Enrichment of the published gene expression signature for MYC target genes (16) in differentially expressed genes in IMR-5 cells after treatment with OTX015, JQ1 or DMSO control. (c) Enrichment of the published gene expression signature for JQ1-treated human MYCN-amplified neuroblastoma cell lines (11) in differentially expressed genes in mNB-A1 cells after treatment of with OTX015, JQ1 or DMSO control. (d) Enrichment of the published gene expression signature for MYC target genes (16) in differentially expressed genes in mNB-A1 cells after treatment with OTX015, JQ1 or DMSO control (* p < 0.05, ** p < 0.01, *** p < 0.001). (e) GSEA (version 2.0, Broad Institute) of
differentially expressed genes in IMR5 cells after treatment with OTX015 or JQ1 using gene sets representing signatures of JQ1 as well as MYC target genes (11, 16).

**Supplementary Figure 6. BET inhibition preferentially disrupts expression of genes associated with super-enhancers.** (a) ChIP-seq profiles for H3K27Ac binding at representative super-enhancer-associated gene loci, including MYCN, NCOR2, BCOR and GLI2, in MYCN-amplified IMR-5 cells. The x-axis shows genomic position and the y-axis the histone mark signal in units of reads per million per base pair (rpm/bp). The gene model is depicted below and scale bars above the binding profiles. (b) The Fisher exact test shows enrichment of super-enhancer associated genes among the downregulated genes (logFC < 0 and q < 0.05) by OTX015 or JQ1 treatment. (c) Cumulative distribution plots of the top 200 super-enhancer-associated genes and their fold-change measured using genes which are differentially regulated in IMR-5 cells by OTX015 treatment, JQ1 treatment or the overlap between these datasets (left to right). Plots show a shift towards negative logFC for super-enhancer associated genes (blue curves) compared to genes not associated with super-enhancers (black curves). (d) Box plots of log2-fold changes in the top 200 genes associated with super-enhancers (SE) and regular enhancers (non-SE) in genes differentially regulated (compared to DMSO-treated controls) in IMR-5 cells treated with OTX015, JQ1 or the overlap from both datasets (left to right). Statistical significance was determined using a two-sided Mann-Whitney test.
## Supplementary Table 1: List of supplemental data files

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<th>Description</th>
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<tr>
<td>MYCN targets OTX015_BRD4 and gene expression.xlsx</td>
<td>Westermann et al. as well as Valentijn et al. MYCN target gene list and expression changes after OTX015 treatment as well as BRD4 binding</td>
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<td>Differentially expressed genes OTX015 JQ1.xlsx</td>
<td>Differentially expressed genes in IMR5 cells after OTX015 and JQ1 treatment respectively</td>
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<td>GSEA cancer gene sets OTX015 and JQ1.xlsx</td>
<td>GSEA of cancer gene sets after treatment with OTX015 and/or JQ1(GSEA, Broad institute)</td>
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<tr>
<td>GSEA general cell biology gene sets.xlsx</td>
<td>GSEA of gene sets describing general cellular processes after treatment with OTX015 and/or JQ1(GSEA, Broad institute)</td>
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<td>List of all Super-enhancers identified in IMR5 cells</td>
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<td>All genes Hg19_BRD4_OTX.xlsx</td>
<td>List of all genes from hg19 with changes in BRD4 binding using BRD4- ChIP-seq in IMR5 cells with changes after treatment with OTX015</td>
</tr>
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</table>
Figure 1

(a) OTX015

(b) Fibroblasts

Fibroblast viability vs. log [OTX015 (nM)]

(c) Number of viable cells (% control) vs. log [OTX015 (nM)]

(d) IC₅₀ (nM) vs. MYCN status

(e) Tumor volume (mm³) vs. time (days)

(f) Survival (%) vs. time (days)
Figure 2

(a) H3K27Ac and MYCN expression levels in PCR amplicon locations 1 and 2.

(b) MYCN expression over time in IMR5, Chp-134, Chp-212, SK-N-BE, and SK-N-AS cell lines treated with DMSO, JQ1, and OTX015.

(c) Western blot analysis of BRD4, E2F1, MYC, Cyclin D1, MYCN, and Actin expression in IMR5, Chp-134, Chp-212, SK-N-BE, and SK-N-AS cell lines treated with OTX015.

(d) Box plot showing relative MYCN expression in control and OTX015-treated samples, with p-value = 0.06.

(e) Western blot analysis of E2F1, Cyclin D1, MYCN, and Actin expression in H2O and OTX015-treated samples.
Figure 3

(a) 

(b) 

(c) mNB-A1 cells (LSL-MYC\textsuperscript{N};Dbh-iCre) 

(d) mNB-A1 cells (LSL-MYC\textsuperscript{N};Dbh-iCre) 

(e) LSL-MYC\textsuperscript{N};Dbh-iCre (tumors) 

(f) LSL-MYC\textsuperscript{N};Dbh-iCre (tumors) 

(g) 

(h) 

(i) Rel. Cl. Cas3

Rel. Ki-67

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Figure 4

(a) DMSO OTX015

(b) OTX015 JQ1

kras.kidney up.v1 dn
alk dn.v1 dn
kras.600.lung.breast up.v1 dn
myc up.v1 up
stk33 up
mtor up.n4.v1 dn
stk33 skm up

p-value
< 0.05
< 0.25

(c) IMR5 cells
Westermann - MYCN targets

max
min

(d) mNB-A1 cells (LSL-MYCn:Dbh-iCre)
Westermann - MYCN targets

max
min
Figure 5

(a) Enhancer ranking

(b) OTX015 and JQ1 treatment

(c) OTX015 and JQ1 treatment

(d) Total BRD4

(e) Enhancers

(f) Super-enhancers

(g) BRD4 binding after OTX015 treatment vs. MYCN target genes

(h) Super-enhancer complex
Targeting MYCN-driven transcription by BET-bromodomain inhibition

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