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 with 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.

Conclusions: 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. Clin Cancer Res; 22(10); 2470–81. ©2015 AACR.

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; ref. 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; ref. 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...
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 with 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.

Materials and Methods

Cell culture and reagents

Reagents were obtained from Sigma-Aldrich Chemie GmbH, and materials from Carl Roth GmbH & Co.KG unless otherwise specified. OTX015 (Oncoethix SA) 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) or the ATCC 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 RPMI1640 supplemented with 10% FCS and antibiotics.

Cell viability, death, proliferation, and cycle analyses

Cell lines were treated with 0.1 to 6,000 nmol/L OTX015 for 72 hours and cell viability was assessed using the MTT assay (Roche) as previously described (13). GraphPad Prism 5.0 (GraphPad Software Inc.) was used to calculate 50% inhibition of growth (IC_{50}) and maximal effect of the highest OTX015 dose (6 µmol/L). Apoptosis and cell proliferation were assessed after 72-hour treatment with 250 and 500 nmol/L 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 hours with 500 nmol/L OTX015 or DMSO. Cells were harvested and 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). Cell survival after treatment with 250, 500, and 1,000 nmol/L OTX015 or 0.2% DMSO was also evaluated in real-time using the xCELLigence system (ACEA Biosciences Inc.) as described before (13). Adherence to the culture plates was continuously monitored for 120 hours (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 × 10^5 cells/well in 6-well plates, cultured overnight, then treated in triplicate with 0.2% DMSO (control), 500 nmol/L OTX015 or 500 nmol/L IQ1 for 24 hours. Total RNA was extracted using the RNeasy Mini kit (Qiagen) according to the manufacturer’s instructions. Human-derived samples were profiled using the HG-U133 Plus 2.0 Human Gene Expression Array (Affymetrix), 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 SD over all analyzed samples. Differential expression analysis was performed using Rank Product Analysis in R (v 2.13, RankProd package; ref. 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.cp.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 SD. MYCN activity: MYCN-regulated genes as previously defined by Westermann and colleagues (23), JQ1-responsive genes 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). Quantitative mRNA expression levels were monitored using Assays-on-Demand (Applied Biosystems). 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 $\Delta C_{t}$ method and GAPDH expression as internal reference. Data analysis was performed using the qbase$^\text{PLUS}$ 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 Technology), Cyclin D1 (1:200; sc-753, Santa Cruz Biotechnology), E2F1 (1:500; AF4825, R&D Systems), and MYCN (1:1,000; #9405, Cell Signaling Technology). B-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), anti-rabbit IgG (#NA9340V; GE Healthcare), and 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 (Peglab).

OTX015 treatment of IMR-5 neuroblastoma xenograft tumors in nude mice
IMR-5 cells (2 x 10$^6$) were suspended in 200 mL Matrigel (BD Biosciences) and subcutaneously inoculated into the left flank of 6-week-old female athymic (nu/nu) mice. Mice were randomly assigned to vehicle control (100 mL water) or OTX015 (n = 9 mice/group) after tumors reached 150–200 mm$^3$ 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 callipers, and tumor volume was calculated using the formula (width x length x height)/2. Mice were sacrificed after 42 days of treatment or when tumor size exceeded 2,500 mm$^3$. 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 hours 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 for International Organizations of Medical Sciences (CIOMS) Committee.
of Europe guidelines for accommodation and care of laboratory animals. Protocols were approved by the Ethical 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-MYC:N;Dbh-iCre (12) were minced manually with scissors and digested with 2 mg/mL collagenase in PBS for 30 minutes 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 graft 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) containing MYCN, GFP, or empty negative control. Cells were selected with 2 μg/mL puromycin for 2 days. After selection, cells were expanded and treated with OTX015 as described above. Cells treated with 500 nmol/L 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 embedded in paraffin and stained as previously described (30). Primary antibodies against Ki-67 (1:50; pH 6; #2753R-16, Cell Marque) and cleaved caspase-3 (1:100; pH 6; #9661, Cell Signaling Technology) were used with corresponding secondary antibody detection kits for reduced background (Histofine Simple Stain MAX PO, Medac) and the automated LabVision Autostainer 4805 (Thermo Scientific). Slides were scanned with a Pannoramic 250 slide scanning program (3D Histonet.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) and R, version 2.13, were used for further statistical analyses. Interval variables were compared using the Student two-sided t test and categorical variables compared by χ² test. GraphPad Prism 5.0 was used to perform Kaplan–Meier 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; ref. 13). To assess putative effects of OTX015 on nonmalignant primary human cells, we measured the cell viability of human fibroblasts after 72 hours of OTX015 treatment. Although OTX015 concentrations above 1 μmol/L slightly reduced fibroblast viability (maximal reduction <50% at 4 μmol/L), this was not significant (Fig. 1B and Supplementary Fig. S1A and S1B).

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

OTX015 significantly reduced viability of neuroblastoma cells (Fig. 1C and Supplementary Fig. S2A and S2B) with IC₅₀ values ranging from 37 nmol/L to >1 μmol/L (Supplementary Fig. S2A). Maximal reduction of cell viability was almost 100% in IMR-5, CHP-134, CHP-212, NB69, and GI-M-EN cell lines (Supplementary Fig. S2B). MYCN-amplified neuroblastoma cell lines (red curves, Fig. 1C) were more sensitive to OTX015 treatment than cell lines lacking MYCN amplification (P = 0.0058, 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 cell lines with low-level expression (Supplementary Figs. S2c–S2e); 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 with 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 with the control group (P < 0.02, Fig. 1F). Thus, OTX015 treatment significantly improved survival in mice harboring MYCN-amplified neuroblastoma xenografts. Two of 9 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 (Supplementary Fig. S3B and S3C). The percentage of MYCN-amplified cells in G1 increased after OTX015 treatment compared with 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 (Supplementary Fig. S3D). 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 G0 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 with the DMSO control, but there was no detectable induction of apoptosis in SK-N-AS (MYCN-amplified) and SK-N-AS (single-copy MYCN) cells (Supplementary Fig. S3E). BedIrd incorporation showed significantly
reduced proliferative capacity in all cell lines except Chp-212 after OTX015 compared with the DMSO control (Supplementary Fig. S3F).

Tumors from OTX015-treated IMR-5 xenografts also showed a significant increase in the proportion of apoptotic cells (Supplementary Fig. S4), and the proportion of cells staining positively for Ki-67 was significantly reduced (Supplementary Fig. S4B and S4C). 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 IQ1 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
hours and up to 72 hours 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 E). 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 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 promoter. 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 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-MYCN;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-MYCN;Dbh-iCre tumors were orally treated with OTX015 or control (n = 6). Consistent with in vitro treatment of LSL-MYCN;Dbh-iCre–derived mNB-A1 cells, we observed a delay in tumor progression in OTX015-treated mice harboring regrafted LSL-MYCN;Dbh-iCre tumors, resulting in significantly prolonged mouse survival (Fig. 3E and F). Again, MYCN expression was not affected by OTX015 treatment (Fig. 3G). Concurrently, the fraction of apoptotic cells was significantly increased in the LSL-MYCN;Dbh-iCre tumors, while the fraction of proliferating cells was reduced (Fig. 3H and I). Together, OTX015 showed therapeutic efficacy in both murine neuroblastoma GEMM models and human cells ectopically expressing MYCN.
Ectopic MYCN expression does not attenuate effects of BET inhibition. A, Western blot analysis of MYCN and V5 in IMR-5 cells transfected with pMSCV-MYCN, V5-tagged MYCN, EGFP, or empty control after 48 hours of treatment with 500 nmol/L OTX015 and DMSO control. B, viability (measured by MTT assay) of IMR-5 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, V5-tagged MYCN, GFP, or empty control. D, MYCN protein expression in LSL-MYCNDbh-iCre murine neuroblastoma cell line mNB-A1 after treatment with OTX015. E, tumor volumes of LSL-MYCNDbh-iCre 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 with 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 (cl. cas3) 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.
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 antitumoral 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 with 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% (2,534/3,976) and downregulated 66.5% (3,051/4,590) 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-MYC/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 nonengineered 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 and colleagues (Fig. 5a) (29). 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 Supplementary Fig. SS). 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 (Supplementary. Fig. S5). 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). As 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 Valentiin and colleagues and Westermann and colleagues independently developed MYCN target gene sets (Fig. 5G, refs. 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 pharmacologic 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 (refs. 33–37; reviewed in ref. 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 1 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.
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 with 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 with 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-enhancer sites (F) in IMR-5 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 (Valentin and colleagues, MYCN target genes, P < 0.002; Westermann and colleagues 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.
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 and colleagues 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 and colleagues (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 oncogene.

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. On the basis of 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.

Disclosure of Potential Conflicts of Interest
E. Cvitkovic has ownership interest (including patents) in and is a consultant/advisory board member for Oncoethix SA. No potential conflicts of interest were disclosed by the other authors.

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References
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