Characterizing the Therapeutic Potential of a Potent BET Degrader in Merkel Cell Carcinoma

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Abstract

Studies on the efficacy of small molecule inhibitors in Merkel cell carcinoma (MCC) have been limited and largely inconclusive. In this study, we investigated the therapeutic potential of a potent BET degrader, BETd-246, in the treatment of MCC. We found that MCC cell lines were significantly more sensitive to BETd-246 than to BET inhibitor treatment. Therapeutic targeting of BET proteins resulted in a loss of “MCC signature” genes but not MYC expression as previously described irrespective of Merkel cell polyomavirus (MCPyV) status. In MCPyV+ MCC cells, BETd-246 alone suppressed downstream targets in the MCPyV-LT Ag axis. We also found enrichment of HOX and cell cycle genes in MCPyV− MCC cell lines that were intrinsically resistant to BETd-246. Our findings uncover a requirement for BET proteins in maintaining MCC lineage identity and point to the potential utility of BET degraders for treating MCC.

Neoplasia (2019) 21, 322–330

Abbreviations: MCPyV, Merkel cell polyomavirus; BET, bromodomain and extra terminal domain.

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1 Research reported in this publication was supported by the HHMI Medical Student Research Fellowship (J.E.C); and the National Cancer Institute of the National Institutes of Health under Award Numbers T32CA099676 (J.E.C.), R01 CA189352 (M.E.V, D. M., A.A.D.), and P30 CA046592 (A.A.D. and A.M.C.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Author contributions: J. E. C., M. E. V., R. M., A. A. D., and A. M. C. designed the project and directed the experimental studies. S. W. facilitated the experiments and provided the compounds. J. E. C., M. E. V., S. Y., M. Y., and J. G. performed the experimental studies. J. E. C., J. T., and K. J. performed the in vitro studies. X. J. performed the microarray experiment. X. C., F. S., and R. W. prepared cDNA libraries for RNA-sequencing. M. C. and Y. Z. processed the bioinformatic data. J. E. C. performed the microarray and RNA-sequencing analyses. X. C., P. W. H., D. M., and J. T. facilitated the experiments and interpreted the data. J. E. C., M. V, A. A. D, and A. M. C. interpreted the data and wrote the manuscript.

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1476-5586

https://doi.org/10.1016/j.neo.2019.01.003
**Introduction**

Patients presenting with metastatic Merkel cell carcinoma (MCC), an invasive cutaneous neuroendocrine cancer, have an overall 5-year survival rate of 13.5%-18% [1,2]. Despite FDA approval for immunotherapy in the treatment of metastatic MCC, 44%-68% of patients do not demonstrate durable response to treatment in clinical trials [3,4]. Since these patients are not candidates for surgical intervention, their options are limited to standard chemotherapy regimens which have only palliative benefit. Furthermore, targeted therapy, such as those against Bcl-2, PI3K/AKT, and tyrosine kinases, has failed in clinical trials [5] despite success in preclinical studies and individual clinical case reports [6–9].

Therapies inhibiting the bromodomain and extra terminal (BET) family of proteins, Brd2, Brd3, and Brd4, have shown promise in preclinical and early phase clinical studies [10–12]. The BET proteins have been demonstrated to drive transcription of oncogenes, such as MYC, by binding to acetylated lysine residues on histones present in super enhancer regions of chromatin [10,11,13]. A previous study identified MYC as a target of the BET inhibitor JQ1 in Merkel cell polyomavirus (MCPyV) negative MCC cell lines, nominating it as a clinical candidate drug [14]. More recently, compounds with the ability to degrade BET proteins have shown greater efficacy and a potentially distinct mechanism of action from BET inhibitors [15–17].

Here, we investigate the potential of BETd-246, a potent BET degrader, for the treatment of MCC [16,18]. We show that MCC cell lines undergo apoptosis at markedly lower concentrations of BET degrader when compared to BET inhibitors. Using microarray analysis, we found early downregulation of genes involved in MCC lineage specification [19–21]. Furthermore, apoptosis induced by BETd-246 was not coupled to MYC regulation in MCPyV+ or MCPyV- cell lines. Finally, we explored possible mechanisms of efficacy and resistance to BETd-246 by MCPyV status.

**Materials and Methods**

**Cell Lines**

The MCC cell lines used in this study, with the exception of the MKL-1 cell line, were established at the University of Michigan and cultured as previously described [6]. Briefly, University of Michigan MCC cell lines were cultured in a modified neural crest stem cell self-renewal medium supplemented with 15% chick embryo extract, while the MKL-1 MCC cell line was grown in RPMI medium with 10% FBS [6]. All cell lines were maintained within 6 months after thawing from liquid nitrogen stocks. They were tested biweekly for mycoplasma contamination and were confirmed by genotyping every 6-24 months.

**Reagents**

OTX-015, an in vivo grade BET inhibitor, was purchased from Active Biochem. BETi-211, BETd-246, and BETd-260 were developed and provided by Dr. Shaomeng Wang at the University of Michigan [16,18]. BETi-211 is a BET inhibitor. BETd-246 is a BET degrader synthesized from the conjugation of BETi-211 to thalidomide, which targets BET proteins for proteasomal degradation [16,18]. Dr. Wang then optimized BETd-246 for in vivo efficacy, which resulted in the new BET degrader BETd-260 [18].

**Dose-Response Curves**

Ninety-six–well plates were seeded (in triplicate) with 5 × 10³ MCC suspension cells per well. IC₅₀ curves were generated following treatment with serial dilutions of OTX-015, BETi-211, BETd-246, and thalidomide. DMSO-treated cells were used as a negative control. Cell viability was assessed on day five by a CellTiter-Glo luminescence assay (Promega Corporation).

**Immunoblot Analysis**

Cell lysates were collected in RIPA lysis buffer with 1% Halt Protease Inhibitor Cocktail (Thermo Fischer Scientific). Western blot was performed by standard protocols using NuPAGE 4%-12% Bis-Tris Protein Gels (Thermo Fischer Scientific). Protein signals were identified by enhanced chemiluminescence (Pierce ECL substrate, Thermo Fischer Scientific) using x-ray film.

Anti-ATOH1 antibody (1:1000–5000) was generously provided by Dr. Tom Coates and Dr. Matthew Kelley at NIDCD/NIH [22]. We purchased the following antibodies: Bethyl Laboratories: Brd4 (A700–004, 1:1000), Brd4 (A302-368A, 1:1000), and Brd2 (A700–008, 1:1000); Cell Signaling Technologies: cMyc (5605, 1:1000), cMyc (12,319, 1:1000), and GAPDH (2118, 1:1000).

**RNA Interference**

SiRNA knockdown experiments were performed using standard protocols for Lipofectamine RNAiMAX transfection reagent (Thermo Fischer Scientific). Cells were seeded at 1 × 10⁶ and 5 × 10³ cells in 6- and 96-well plates, respectively, followed by transfection with 25 nM of siRNA at 0 and 24 hours in complete media. Cells were collected for analysis 96 hours postseeding. The following siRNAs (Silencer Select, Thermo Fischer Scientific) were used: BRD4 (s23901, s23902), ATOH1 (s1714, s194299), MYB (s9108, 9110), and Negative Control #1 (AM6411).

**RNA Isolation and RT-qPCR**

Cell lysates were collected in QIAzol lysis reagent. RNA isolation was performed using Superscript III reverse transcriptase, and RT-qPCR was performed using SYBR Green dye (Thermo Fischer Scientific). The following primer pair sequences were used (Forward = F, Reverse = R): GAPDH-F: GTCTCTCTGTACCTCAACAGCG, GAPDH-R: ACCACCTCTTTGCTGTAGCCAA, BRD4-F: CGGTATGTCACCTCCTGTTTGC, BRD4-R: ACTCTAGGAACGAGAGGGCCCTT, MYC-F: CGTGCTCCTAGATCCCTGCT, MYC-R: GCTGTGTCTTCTGTTGTG, ATOH1-F: CCCTCCACAAACAGGTAATG, ATOH1-R: GAACACGCGATAAACATGCGGC, MYB-F: GGGAACACATGGGCGAATACTCG, MYB-R: GTCTGGCTTTAGGAAGCTC.

**Microarray**

Cells were treated with DMSO, thalidomide, BETd-246, or BET-211 for 3 and 24 hours, and RNA was isolated as described above. Expression data were captured using the Agilent Whole Human Genome Oligo Microarray (Santa Clara, CA) and were analyzed using the Bioconductor limma package in R as previously described [23–25]. Data are available on NCBI GEO database (19550104). All samples were run in duplicate with dye swap. Significantly differentially expressed genes between DMSO and each of the three treatments were identified as ≥0.6-fold change expression with a Bonferroni adjusted P value <.05.

**RNA Sequencing**

Un treated cells lysates were collected and processed as described previously. Expression data were captured using the Illumina Tru-Seq Stranded mRNA Library Prep Kit (San Diego, CA). Reads per kilobase of transcript per million mapped reads values were generated.
using the Bioconductor edgeR package in R as previously described [26]. Gene set enrichment analysis (GSEA) was performed to identify significantly enriched gene sets (FDR < 0.20). Data are available on NCBI SRA database (PRJNA503609).

In Vivo Xenograft Studies
All experimental studies utilizing mice were approved by the University Committee on Use and Care of Animals at the University of Michigan (PRO00006645) and conformed to all regulatory standards. BETd-260 was prepared in 10% PEG400: 3%Cremophor: 87% PBS [16]. OTX-015 was prepared in 40% PEG300 + 5% Tween 80 + ddH2O per manufacturer’s instructions. Five million MCC47 cells were injected subcutaneously into SCID mice (Charles River Laboratories) in 50% Matrigel solution. When tumors reached 100-150 mm3, mice were randomized and treated with BETd-260 (5 mg/kg, administered 3 times per week IV), OTX-015 (100 mg/kg administered 3 times per week PO), or vehicle for 16 days.

Statistical Analysis
All experiments included in this publication were performed in duplicate or triplicate. Two-sided Student’s t tests were used for comparison of continuous data. Significance was designated as follows: *P < .05, **P < .01, ***P < .001, and ****P < .0001.

Figure 1. Human MCC cell lines are sensitive to BET degrader BETd-246. (A) Heat map of IC50 values for OTX-015, BETi-211, and BETd-246 (nM). (B) MCC47 and MKL1+ cells were treated with DMSO, BETi-211 (250 nM), thalidomide (10 nM), or BETd-246 (10 nM) for 24 hours. Western blot shows on-target effect of BETd-246 by degradation of Brd4 and Brd2 protein and appearance of cleaved PARP with BETd-246 and BETi-211 treatment. (C) Cell viability following BRD4 siRNA transfection in MCC47 and MKL1+ cells. NT, nontargeting. Error bars represent mean ± SD. (D) Tumor volume in mm3 and weight in grams between treatment groups following 16 days of treatment. Mice were treated with OTX-015 (100 mg/kg, PO), BETd-260 (5 mg/kg, IV), or vehicle. Error bars represent mean ± SEM.
Results

**Human MCC Cell Lines are Sensitive to a BET Degrader**

To investigate the therapeutic potential of BET degraders in MCC, we screened 16 human MCC cell lines (5 MCPyV+ and 11 MCPyV−) for sensitivity to BET degrader, BETd-246, and BET inhibitors, BETi-211 and OTX-015. All cell lines were sensitive to BETd-246 (IC₅₀ [80 pM-91 nM]) (Figure 1A, Supp. Figure 1). Additionally, most cell lines were more sensitive to BETd-246 than to either BETi-211 (IC₅₀ [13 nM-5.9 μM]) or OTX-015 (IC₅₀ [67 nM-3.6 μM]).
Interestingly, MCPyV+ cell lines were more sensitive to BETd-246 when compared to MCPyV− cell lines. Complete degradation of Brd4, Brd3, and Brd2 proteins confirmed the on-target effect of BETd-246 at 24 hours posttreatment with BETi-211 and BETd-246 in MCC47 (MCPyV−) and MKL1 (MCPyV+) cell lines (Figure 1B). Furthermore, induction of apoptosis was indicated by the appearance of cleaved PARP at this time point. As expected, siRNA knockdown of Brd4 resulted in decreased cell viability (Figure 1C, Supp. Figure 1). To evaluate the in vivo efficacy of a BET inhibitor and degrader, we treated mice with MCC47 subcutaneous xenografts with OTX-015 (100 mg/kg) or BETd-260 (5 mg/kg), respectively. Despite being administered at a significantly lower dose, BETd-260 demonstrated degradation of Brd4 protein and comparable efficacy to OTX-015 (Figure 1D, Supp. Figure 1), resulting in significantly decreased tumor volume compared to vehicle-treated tumors after 16 days (P = .03). Additionally, changes in weight did not exceed 10% from pretreatment values and were comparable between OTX-015 and BETd-260 groups (Supp. Figure 1).

**Figure 3.** Efficacy of BET inhibitor and degrader in MCC is MYC-independent. (A) Left: Comparison of pretreatment MYC reads per kilobase of transcript per million mapped reads values across all MCC cell lines. Red bars indicate cell lines with high MYC expression nominated for further studies. Right: Western blot shows cMyc protein levels in a subset of MCPyV+ and MCPyV− cell lines. Asterisks indicate cell lines chosen nominated for further studies. (B-C) MCC47 and MCC29+ cells were treated with DMSO, BETi-211 (250 nM), thalidomide (10 nM), and BETd-246 (10 nM) for 3 and 24 hours. Untreated cells were included as an additional control. (B) RT-qPCR shows expression of MYC mRNA. (C) Western blot shows levels of cMyc protein with degradation of Brd4 by BETd-246. All error bars represent mean ± SD.
A recent study found that co-expression of lineage during normal development in mouse studies[22,27,28]. A been shown to be essential for specifying cells to the Merkel cell lineage, including and hypothesized to be important for defining cells to the MCC population of genes previously found to be overexpressed in MCC tumors at time points, BET protein modulation led to significant downregulation of expression at 24 hours compared to DMSO (Supp. Figure 2)[16]. At both increased number of differentially expressed genes at the 24-hour time point in the drug treatment groups (Supp. Figure 2). In particular, the ATOH1-Sox2-Is1 axis has been shown to be essential for specifying cells to the Merkel cell lineage during normal development in mouse studies [22,27,28]. A recent study found that co-expression of ATOH1 and McpYV small T antigen induced lesions expressing markers of MCC in mice [21]. We confirmed loss of Atoh1 and cMyb, a known oncogene and target of BET inhibition, by immunoblotting (Figure 2B). However, we found that siRNA knockdown of either ATOH1 or MYB alone did not result in reduction of cell viability in the MCC47 cell line (Figure 2C-D).

**Efficacy of BET Inhibitor and Degrader in MCC Is MYC-Independent**

We were surprised to find no significant change in MYC expression in our microarray data. A previous study had reported downregulation of MYC upon treatment with the BET inhibitor JQ1 in MCPyV– cell lines with MYC overexpression [14]. Although MCC47 is a MCPyV– cell line with significant overexpression of MYC, we found that treatment with high concentrations of JQ1 and OTX-015 to 1 μM did not significantly downregulate MYC expression (Supp. Figure 3). To further confirm this finding, we selected MCC29+, a MCPyV+ cell line with overexpression of MYC, for comparison (Figure 3A). In vitro treatment with apoptosis-inducing concentrations of BETi-211 or BETd-246 did not result in significant loss of MYC mRNA or cMyc protein in the MCC47 cell line at 3 or 24 hours (Figure 3, B-C). Despite a transient downregulation of MYC gene expression by BETd-246 at 3 hours in MCC29+ cells, this was rescued by 24 hours. Additionally, there was no significant loss of protein at either time point, indicating that MYC is not likely a direct target of BET inhibition or degradation.

**BET Degrader Alone Downregulates the MCPyV-LT Axis**

MCPyV+ cell lines were exceptionally sensitive to BETd-246 treatment, with IC50 values <500 pM (Figure 4A). MKL1+ and MCC29+ showed downregulation of McpYV Large T antigen (LT) following 24 hours of BETd-246 and BETi-211 treatment, indicating that it may be a common downstream target (Figure 4B). However, only BET protein degradation by BETd-246 downregulated additional downstream targets of MCPyV-mediated oncogenic transformation, such as Rb and E2f1. No changes were seen in the MCC47 cell line.

**Overexpression of HOX and Cell Cycle Genes is Associated with Reduced Sensitivity to BET Degrader**

Unlike the MCPyV+ cell lines, the MCPyV– cell lines exhibited a large range in sensitivity to BETd-246 treatment. Therefore, we conducted an exploratory analysis of possible intrinsic mechanisms of resistance to BETd-246. MCC32/MCC35 and MCC623/MCC624 are paired MCPyV– cell lines that were derived from matched primary and metastatic tumors from the same patient and were the most sensitive and resistant to BETd-246 treatment, respectively (Figure 4A). RNA-seq analysis between these matched pairs revealed that the family of Antp homeobox genes (HOXB2, HOXB3, HOXB4, HOBB5, HOXB6, HOXB8, HOB9) was the most highly upregulated genes in the resistant cell lines (Figure 5A). GSEA also demonstrated enrichment in G2M checkpoint pathway and E2f target genes (Figure 5B, Supp. Table 5).

**Discussion**

This is the first study to investigate the potential of a BET degrader for treatment of MCC. We found that MCC cell lines were more sensitive to the BET degrader BETd-246 when compared to the BET inhibitor BETi-211 or the widely used, commercially available BET inhibitor OTX-015.

We found both BET degrader and inhibitor induced early downregulation of MCC “signature” genes. Our results suggest
that, despite their potential role in lineage and cell fate determination in normal and cancerous cells, ATOH1 and MYB, individually, are not essential for MCC cell survival. Individual loss of gene candidates ATOH1 and MYB did not recapitulate the decrease in cell viability seen with Brd4 knockdown or death phenotype seen with treatment. Additionally, further studies are needed to determine whether simultaneous knockdown of multiple “signature” genes is required for loss of cell viability. Interestingly, previous studies have implicated BET proteins in the initiation, but not maintenance, of lineage-specific gene expression during adipogenesis and myogenesis[31,32], suggesting that any effects on cell fate will be highly dependent on cellular context.

Contrary to previous results, our data suggest that MYC is not a target of BET inhibitor or degrader in the MCC cell lines used in this study. MYC expression was not downregulated following high concentrations of BET inhibitor treatment with JQ1, OTX-15, and BETi-211 and was sustained following degradation of Brd4 protein in MCPyV+ and MCPyV− cell lines. Despite evidence of Brd4-dependent regulation of MYC transcription in MCPyV− MCC and other solid tumors, additional studies have shown that this regulation may be specific to individual cell lines even within a single cancer type [29–32].

We further characterized the response of MCPyV+ cells to BETd-246 as they were all exceptionally sensitive to treatment. Even though there was loss of MCPyV-LT with both BET inhibitor and degrader treatment, only BETd-246 showed loss of downstream targets. As oncogenic transformation and “hijacking” of the Rb/E2F pathway by MCPyV antigens is thought to be a driver of MCPyV+ MCC, complete “shutdown” of this pathway could explain the superior potency of BETd246. However, it remains to be seen if this difference is due to a direct mechanistic interaction between bromodomain proteins and LT antigen or if this a downstream effect of larger effect such as DNA damage. Further studies are required to determine the mechanism by which MCPyV-LT expression is modulated by BET proteins.

In contrast to MCPyV+ cell lines, MCPyV− cell lines had varying sensitivity to BETd-246, suggesting that the mechanism of action may differ by virus status. In addition to enrichment in genes involved.

Figure 5. Overexpression of HOX and cell cycle genes attenuates sensitivity to BETd-246. RNA-seq analysis was formed between matched primary and metastatic cell lines MCC32/MCC35 and MCC623/MCC624. (A) Heat map of top differentially expressed genes (lfc > 5, P < .0005). (B) Enrichment plots of GSEA analysis of the MSigDB hallmark gene set collection.
in the cell cycle, we found upregulation of the Anpt homeobox family of genes in MCPyV− cell lines most resistant to treatment. These genes are known to be involved in maintaining stem cells and have been associated with various malignancies, including pancreatic cancer and leukemia.

When combined with our observations of the loss of MCC lineage genes with drugs targeting the BET proteins, this suggests that factors influencing cell identity, such as stemness, may be an important marker of intrinsic resistance. Ultimately, our data demonstrate the potential of epigenetic modification through degradation of BET proteins as a promising strategy for treatment of MCC.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neop.2019.01.003.

Acknowledgements
We would like to acknowledge Abhijit Parolia, Sundanshu Shukla, and Steve Kregel for their advice and Sisi Gao for her assistance in preparing the manuscript.

Potential Conflict of Interests
The University of Michigan has filed a number of patent applications on BET degraders, including the BET degrader used in this research for which S. W. and B. Z. are co-inventors. These patents have been licensed by Oncopia Therapeutics LLC for clinical development. S. W. and A. M. C. are cofounders of Oncopia Therapeutics LLC. S. W. serves on the board of directors and A. M. C. serves on the SAB of Oncopia. Both S. W. and A. M. C. are paid consultants of Oncopia. S. W. receives a research contract from Oncopia, which did not support this research. Oncopia was not involved in the design, funding, or approval of this study.

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