Therapeutics, Targets, and Chemical Biology

Cancer Research

Targeted Degradation of BET Proteins in Triple-Negative Breast Cancer

Longchuan Bai[1,2], Bing Zhou[1,2], Chao-Yie Yang[1,2], Jiao Ji[1,2], Donna McEachern[1,2], Sally Przybranowski[1,2], Hui Jiang[1,3], Jiantao Hu[1,2], Fuming Xu[1,2], Yujun Zhao[1,2], Liu Liu[1,2], Ester Fernandez-Salas[1,2,4], Jing Xu[1,4], Yali Dou[1,4], Bo Wen[1,5], Duxin Sun[1,5], Jennifer Meagher[6], Jeanne Stuckey[6], Daniel F. Hayes[1,2], Shunqiang Li[7], Matthew J. Ellis[8], and Shaomeng Wang[1,2,9,10]

Abstract

Triple-negative breast cancers (TNBC) remain clinically challenging with a lack of options for targeted therapy. In this study, we report the development of a second-generation BET protein degrader, BETd-246, which exhibits superior selectivity, potency, and antitumor activity. In human TNBC cells, BETd-246 induced degradation of BET proteins at low nanomolar concentrations within 1 hour of exposure, resulting in robust growth inhibition and apoptosis. BETd-246 was more potent and effective in TNBC cells than its parental BET inhibitor compound BETi-211. RNA-seq analysis revealed predominant downregulation of a large number of genes involved in proliferation and apoptosis in cells treated with BETd-246, as compared with BETi-211 treatment that upregulated and downregulated a similar number of genes. Functional investigations identified the *MCL1* gene as a critical downstream effector for BET degraders, which synergized with small-molecule inhibitors of BCL-xL in triggering apoptosis. In multiple murine xenograft models of human breast cancer, BETd-246 and a further optimized analogue BETd-260 effectively depleted BET proteins in tumors and exhibited strong antitumor activities at well-tolerated dosing schedules. Overall, our findings show that targeting BET proteins for degradation represents an effective therapeutic strategy for TNBC treatment. *Cancer Res; 77(9); 2476–87. ©2017 AACR.*

Introduction

Triple-negative breast cancer (TNBC) is characterized by the lack of expression of estrogen receptor (ER) α and progesterone receptor, and absence of HER2 overexpression. Although aggressive chemotherapy can achieve a high response rate in TNBC patients, the risk of recurrence is substantially higher than those with ER^+ or $HER2^+$ breast cancers. There is an urgent need to develop effective targeted therapies for TNBC.

Bromodomain and Extra Terminal (BET) proteins, including ubiquitously expressed BRD2, BRD3, BRD4, and testis-specific BRDT, are epigenetic "readers" and play a major role in epigenetic regulation of gene transcription. BET proteins have emerged as new therapeutic targets for human cancer and other diseases. Major breakthroughs in the discovery and development of potent and selective small-molecule BET inhibitors led to several such compounds now in clinical development. Early clinical trials have provided evidence that inhibition of BET proteins is effective against some human cancers, including NUT midline carcinoma, multiple myeloma, and acute myelogenous leukemia (1–7). Recent preclinical studies further suggested that BET proteins are exciting targets for breast cancer (8–11).

Theoretically, depletion of key oncogenic proteins in tumor cells could achieve much better clinical efficacy than inhibition of the same proteins. Fifteen years ago, the concept to design Proteolysis Targeting Chimeric (PROTAC) molecules to recruit targeted proteins for degradation was proposed (12) and major progress has been made in this field in recent years (reviewed in ref. 13). This strategy has recently been used to design PROTAC small-molecule degraders of BET proteins (14–16). dBET1 (14) and ARV-825 (15) were designed using JQ-1 for the BET inhibitor portion and thalidomide as the ligand for the Cullin–4A ligase complex. dBET1 efficiently induces degradation of BET proteins in leukemia cells and is more effective than JQ-1 in inhibiting tumor growth in a xenograft model of human acute leukemia cell line in mice (14). ARV-771 uses OTX-015 for the BET inhibitor portion and a ligand for the von Hippel–Landau (VHL) E3 ligase (16). ARV-771 was shown to be a highly effective BET degrader in castration-resistant prostate cancer (CRPC) models and to induce partial tumor regression in a CRPC xenograft model (16). These

[1]University of Michigan Comprehensive Cancer Center, University of Michigan, Ann Arbor, Michigan. [2]Department of Internal Medicine, University of Michigan, Ann Arbor, Michigan. [3]Department of Biostatistics, University of Michigan, Ann Arbor, Michigan. [4]Department of Pathology, University of Michigan, Ann Arbor, Michigan. [5]Department of Pharmaceutical Sciences, University of Michigan, Ann Arbor, Michigan. [6]Life Sciences Institute, University of Michigan, Ann Arbor, Michigan. [7]Division of Oncology, Department of Internal Medicine, Section of Breast Oncology, Washington University in St. Louis, St. Louis, Missouri. [8]Lester and Sue Smith Breast Center, Baylor College of Medicine, Houston, Texas. [9]Department of Pharmacology, University of Michigan, Ann Arbor, Michigan. [10]Department of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

L. Bai and B. Zhou contributed equally to this article.

Corresponding Author: Shaomeng Wang, University of Michigan, NCRC/Building 520, Room 1245, 1600 Huron Parkways, Ann Arbor, MI 48109-0934. Phone: 734-615-0362; Fax: 734-734-2532; E-mail: shaomeng@umich.edu

doi: 10.1158/0008-5472.CAN-16-2622

AACR

studies suggest that small-molecule BET degraders may be much more effective for cancer treatment than BET inhibitors. To date, the therapeutic potential of BET degraders and their mechanism of action in TNBC have not been reported.

Based upon our optimized, potent small-molecule BET inhibitor BETi-211, we have developed BETd-246 as a highly potent BET degrader and investigated its therapeutic potential and mechanisms of action in TNBC *in vitro* and *in vivo*. Our study shows that BET inhibition and degradation are distinctly different in their elicited biological responses, and targeting BET degradation represents a promising therapeutic approach for TNBC.

Materials and Methods

Chemicals

Detailed procedures for the synthesis of BETi-211, BETd-246, and BETd-260 are in Supplementary Information Scheme SI–SIII. BM-1197 was synthesized in our laboratory according to the previously published procedure (18). ABT-263 (navitoclax) and ABT-199 (venetoclax) were from Selleck Chemicals. A-1155463 was from ChemieTek.

Cell lines

The SUM human breast cancer cell lines were developed by Dr. Steve Ethier at the University of Michigan Comprehensive Cancer Center (17) and authenticated at the University of Michigan Comprehensive Cancer Center. All the other breast cancer cell lines were purchased from the ATCC during the course of this study and used within 2 months after initiating from original stocks. The authentication of ATCC breast cancer cell lines was performed by the Promega-ATCC Cell Line Authentication Service by Short Tandem Repeat profiling. All cell lines were cultured as recommended.

Antibodies and reagents

A detailed list of antibodies and reagents is in Supplementary Materials and Methods.

Cell viability, apoptosis, cell-cycle, and immunoblot analyses

Conventional cell viability, Annexin V-propidium iodide apoptosis, cell-cycle, and immunoblotting analyses were performed as described previously (18, 19).

Lentiviral constructs and siRNAs

Human MCL1 lentiviral construct was from Applied Biological Materials and transduced according to the manufacturer's instructions. Lentiviral vectors for human MCL1 shRNA was described previously (19). ON-TARGETplus CRBN and siCONTROL siRNAs were from Dharmacon. Cells were transfected using Lipofectamine RNAiMAX (Thermo Fisher Scientific) following the manufacturer's instructions.

Transcriptomic profiling

Total RNA was purified using the RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. The rRNA depleted total RNA was reversely transcribed into first strand cDNA using random primers. The cDNA libraries were clustered and sequenced with Illumina RNA sequencing flow. RNA-seq data are deposited at GEO as GSE95375. Details of transcriptomic profiling are in Supplementary Materials and Methods.

Quantitative PCR

Real-time PCR was done using a QuantStudio 7 Flex Real-Time PCR System. The relative abundance of gene expression was calculated using the comparative C_t method, which compares the C_t value of target gene to GAPDH ($2^{\Delta\Delta C_t}$). Details of qRT-PCR analysis are in Supplementary Materials and Methods.

Proteomic profiling

Cell lysis was proteolyzed and labeled with TMT 10-plex (Thermo Fisher Scientific) following the manufacturer's protocol. Details of proteomic profiling are in Supplementary Materials and Methods.

In vivo pharmacodynamic and efficacy studies

WHIM24 PDX model, initially developed at the Washington University School of Medicine, St. Louis, and tumors were passaged in SCID mice. To develop breast cancer cell line xenografts, five million cells in 50% Matrigel were injected subcutaneously into SCID mice. For pharmacodynamics studies, when tumors reached 100–200 mm^3, mice were treated with vehicle control or a single dose of the drug, sacrificed at indicated time-points, and tumor tissue was harvested for analyses. For *in vivo* efficacy experiments, when tumors reached 80–200 mm^3, mice were randomized into groups. BETi-211, BETd-246, BETd-260 or vehicle control (10% PEG400: 3% Cremophor: 87% PBS, or 2% TPGS:98% PEG200) was given at the dose and with the duration indicated. Tumor sizes and animal weights were measured 2–3 times per week. Tumor volume (mm^3) = (length × width^2)/2. Tumor growth inhibition was calculated as TGI (%) = $(V_c - V_t)/(V_c - V_o) \times 100$, where V_c, V_t are the median of control and treated groups at the end of the study and V_o at the start. All the *in vivo* studies were performed under an animal protocol (PRO00005315) approved by the University Committee on Use and Care of Animals of the University of Michigan, in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Statistical analyses

For the cell viability and apoptosis analyses, data were presented as mean ± SEM. For dose-dependent cell viability assay, data were plotted as mean ± SD and sigmoid fitted (variable slope). Differences in mean values of cell growth inhibition among different groups were analyzed by two-way analysis of variance. For *in vivo* studies, the significance (P) was calculated by the Student t test. All statistical tests were two-sided. All statistical analyses were performed using GraphPad Prism 6. P values less than 0.05 were considered statistically significant.

Results

Design of BETd-246 as a potent small-molecule degrader of BET proteins

We have optimized our BET inhibitor RX-37 (20) and discovered UM-BETi-211 (BETi-211; Fig. 1A; Supplementary Information Scheme SI). BETi-211 binds to BET proteins with K_i values of <1 nmol/L and is >10–100 times more potent than RX-37, JQ-1, OTX-015 or IBET-762 (Supplementary Table S1). Based upon the modeled structure of BRD4 in complex with BETi-211 (Fig. 1B), we identified an appropriate site for tethering BETi-211 to thalidomide for the design of BET degraders (Fig. 1B). We designed and synthesized a series of compounds containing linkers with different lengths and physiochemical properties and discovered UM-BETd-246 (BETd-246, Fig. 1A; Supplementary Information Scheme SII) as a potent BET protein degrader.

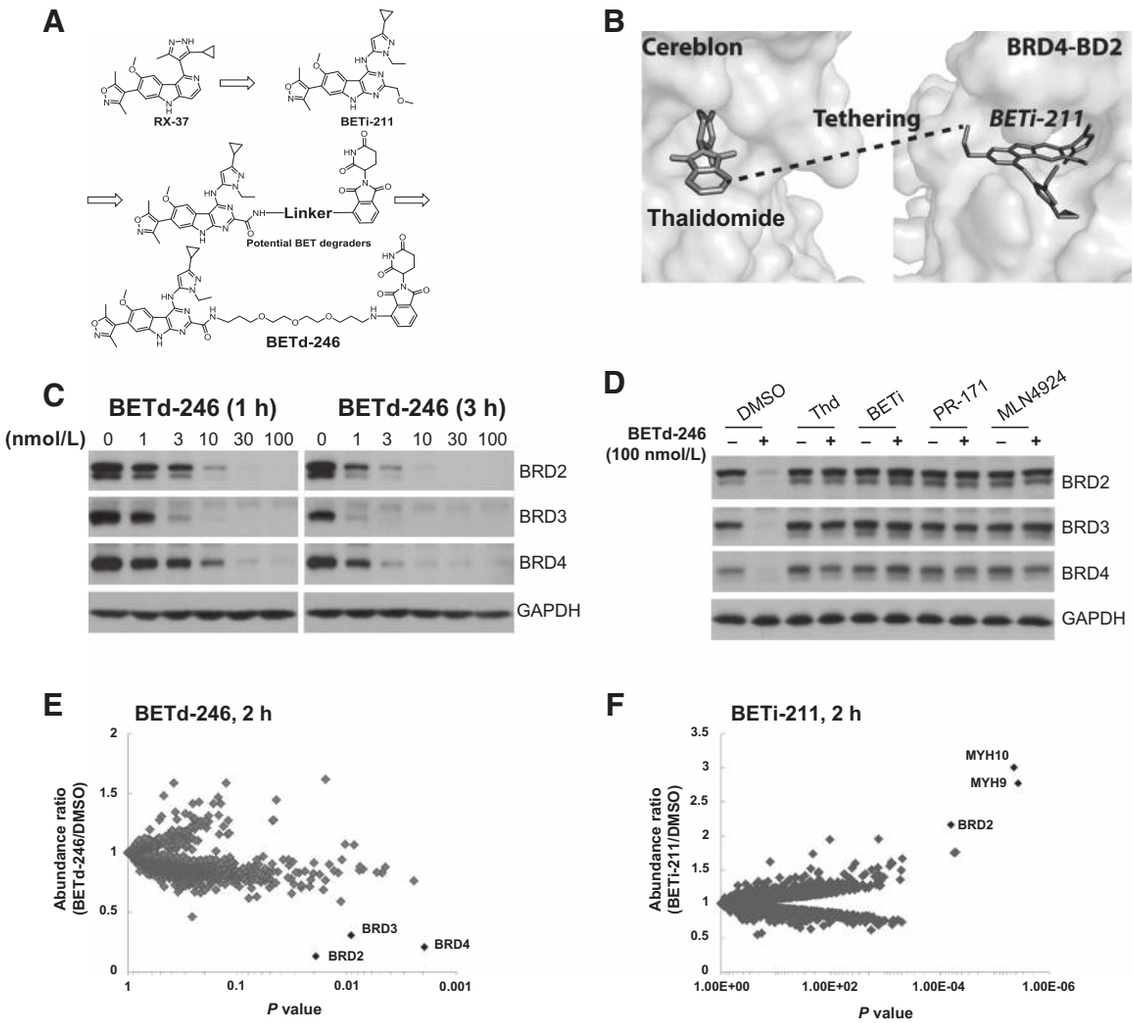


Figure 1.

BETd-246 is a potent and highly selective degrader of BET proteins. **A,** Design and development of BETd-246 as a BET protein degrader based upon the potent and selective BET inhibitor BETi-211 and thalidomide. **B,** Identification of an appropriate site in BETi-211 based upon its modeled structure with BRD4 and an appropriate site in thalidomide based upon its crystal structure with cereblon. **C,** MDA-MB-468 cells were treated with BETd-246 at the indicated doses for 1 and 3 hours for immunoblotting. GAPDH was included as a loading control. **D,** MDA-MB-468 cells were pretreated with thalidomide (2,000 nmol/L), BETi-211 (2,000 nmol/L), PR-171 (1,000 nmol/L), or MNL4924 (500 nmol/L) for 1 hour, followed by treatment with BETd-246 (100 nmol/L) for 1 hour. **E** and **F,** MDA-MB-468 cells were treated with BETd-246 (**E**; 100 nmol/L) and (**F**) BETi-211 (1,000 nmol/L) for 2 hours. Whole-cell lysates were labeled with the Tandem Mass Tag 10-plex reagent for LC/MS-MS analysis.

BETd-246 potently and selectively depletes BET proteins in TNBC cells

We first evaluated BETd-246 for its ability to degrade BET proteins in representative TNBC cell lines. BETd-246 treatment caused a dose-dependent depletion of BRD2, BRD3 and BRD4 in these cell lines (Fig. 1C; Supplementary Fig. S1A and S1B). A near-complete depletion of BRD2-4 proteins was observed with 30–100 nmol/L of BETd-246 for 1 hour or with 10–30 nmol/L of BETd-246 for 3 hours. In contrast, the BET inhibitor BETi-211 did not degrade BET proteins in any cell line.

Hetero-bifunctional BETd-246 was designed to bind concurrently to BET proteins and cereblon (CRBN) through its BET inhibitor and thalidomide moiety, respectively, bringing the CUL4–RBX1–DDB1–CRBN E3 ubiquitin ligase ($CRL4^{CRBN}$) and BET proteins into close proximity for subsequent ubiquitination and proteasomal degradation of BET proteins. We thus examined the mechanism of BET protein degradation by BETd-246. Thalidomide alone had no significant effect on the levels of BET proteins in all TNBC cell lines evaluated (Fig. 1D; Supplementary Fig. S1C) and pretreatment either with excess thalidomide or BETi-211 effectively blocked BETd-246-induced BET protein depletion (Fig. 1D; Supplementary Fig. S1C). Proteasome inhibitor PR-171 (Carfilzomib; ref. 21) blocked depletion of BET proteins by BETd-246 (Fig. 1D; Supplementary Fig. S1C). NEDD8 activating E1 enzyme inhibitor MLN4924 (22) also effectively blunted BETd-246-induced depletion of BET proteins (Fig. 1D; Supplementary Fig. S1C), consistent with the notion that activation of $CRL4^{CRBN}$ requires NEDD8 neddylation (23).

We next performed proteomic analysis to assess the global effect of BETd-246 and BETi-211 on cellular protein levels in MDA-MB-468 cells. Out of approximately 5,500 proteins quantified, BRD2, BRD3, and BRD4 were the only proteins whose levels were significantly decreased by ≥2-fold ($P < 0.05$) with 100 nmol/L of BETd-246 for 2 hours. No protein was increased by ≥2-fold (Fig. 1E; Supplementary Table S2). In contrast, BETi-211 increased the BRD2 protein level by 2-fold (Fig. 1F; Supplementary Table S3).

Collectively, our data demonstrate that BETd-246 is a highly potent and selective BET protein degrader in TNBC cells.

BETd-246 displays strong growth inhibition and apoptosis induction activity in TNBC cell lines

We next evaluated BETi-211 and BETd-246 for their antiproliferative activity in a panel of 13 TNBC cell lines.

Consistent with a recent study using JQ1 (8), BETi-211 displayed potent growth-inhibitory activity in the TNBC cell lines (Fig. 2A; Supplementary Fig. S2A), with $IC_{50} < 1$ μmol/L in 9 of the 13 cell lines. However, BETi-211 at 2.5 μmol/L only achieved ≥90% growth inhibition in 2 of the 13 cell lines (Fig. 2B), suggesting the predominantly cytostatic effect of BETi-211. Flow-cytometry analysis confirmed that while BETi-211 at 1,000 nmol/L induced a pronounced decrease in S-phase cells after 24 hours treatment, it elicited only modest apoptosis induction (3%–25%) after 48 hours treatment (Fig. 2C and D; Supplementary Fig. S3A).

BETd-246 on the other hand exhibited strong growth-inhibitory activity with $IC_{50} < 10$ nmol/L in 9 of the 13 cell lines (Fig. 2A). Based upon the IC_{50} values, BETd-246 is >50 times more potent than BETi-211 in a majority of these cell lines. Significantly, BETd-246 achieved $IC_{90} < 100$ nmol/L in 7 of the 13 cell lines (Fig. 2B),

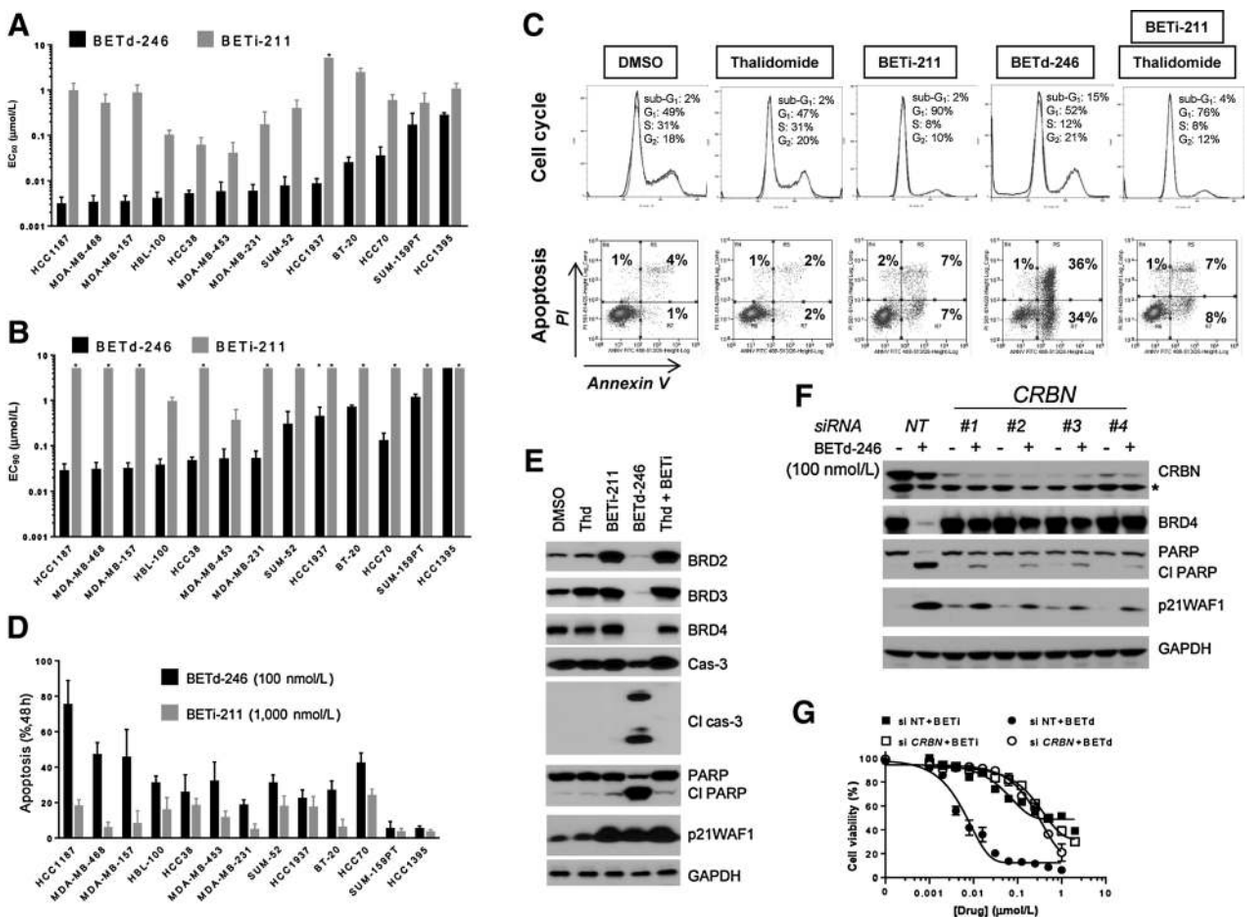


Figure 2.

BETd-246 displays potent antiproliferative and apoptosis induction activities in TNBC cell lines. **A** and **B,** Cells were treated with serially diluted BETi-211 or BETd-246 for 4 days for CellTiter-Glo cell viability assay. Data are mean ± SEM ($n = 4$). **C,** MDA-MB-468 cells were treated with DMSO, thalidomide (1,000 nmol/L), BETi-211 (1,000 nmol/L), BETd-246 (100 nmol/L), or combination of thalidomide and BETi-211 (1,000 nmol/L at 1:1 molar ratio) for 24 hours (top) or 48 hours (lower) for flow cytometry analyses. Data are representative of three independent experiments. PI, propidium iodide. **D,** Cells were treated with BETd-246 (100 nmol/L) or BETi-211 (1,000 nmol/L) for 48 hours for Annexin V-PI apoptosis analysis. Data are mean ± SEM ($n = 3$). **E,** MDA-MB-468 cells were treated with DMSO, thalidomide (1,000 nmol/L), BETi-211 (1,000 nmol/L), BETd-246 (100 nmol/L), or thalidomide plus BETi-211 (1,000 nmol/L at 1:1 molar ratio) for 8 hours for immunoblotting. **F** and **G,** MDA-MB-468 cells were transfected with nontargeting or CRBN siRNAs for 40 hours, then treated with BETd-246 (100 nmol/L) for 8 hours for immunoblotting (**F**) or serially diluted BETi-211 or BETd-246 for 3 days (**G**) for cell viability assay. Data are mean ± SD.

suggesting strong cell killing effects in TNBC cells. Flow cytometry and immunoblotting confirmed robust apoptosis induction by BETd-246 in the majority of these cell lines (Fig. 2C–E; Supplementary Fig. S3B). Treatment with BETd-246 at as low as 10 nmol/L led to profound cleavage of caspase-3 and PARP (Fig. 2E; Supplementary Fig. S3B), and caspase-3/7 activation (Supplementary Fig. S4). In comparison, BETi-211 was much less potent in activating caspase-3/7 in these cell lines (Supplementary Fig. S4). Profiling of initiator caspases showed that BETd-246 treatment led to the activation of caspase-2, -8 and -9 in MDA-MB-468 cells (Supplementary Fig. S5A), suggesting the activation of multiple apoptotic pathways by BETd-246. Immunoblotting confirmed the cleavage of caspase-8, -9 and -3 within 5 hours of BETd-246 treatment in MDA-MB-468 cells (Supplementary Fig. S5B). Thalidomide alone had no significant effect on apoptosis induction and cell proliferation (Fig. 2C and E; Supplementary Fig. S2A and S3B). BETi-211 in combination with thalidomide had an effect similar to that of BETi-211 alone (Fig. 2C and E; Supplementary Figs. S2A and S3B). These data demonstrate that BET protein degradation by BETd-246, as opposed to BET-BRD inhibition by BETi-211, leads to strong apoptosis induction in the majority of the TNBC cell lines.

BET degradation by BETd-246 requires its binding to cereblon. Consistently, silencing *CRBN* (encoding cereblon) by siRNAs blocked BETd-246-induced BET degradation and PARP cleavage in MDA-MB-231 and MDA-MB-468 cells (Fig. 2F; Supplementary Fig. S6), and reduced the growth-inhibitory activity of BETd-246 to a level comparable with that of BETi-211 (Fig. 2G; Supplementary Fig. S6). Silencing *CRBN* had no significant effect on the growth-inhibitory activity of BETi-211 (Fig. 2G; Supplementary Fig. S6).

BETd-246 and BETi-211 elicit distinct transcriptional responses in TNBC cells

Although genetically and epigenetically heterogeneous, the majority of the 13 TNBC cell lines are highly sensitive to BETd-246, suggesting the presence of certain commonalities in their transcriptomic responses to BET degradation. The greater potency and effectiveness in cell growth inhibition and apoptosis induction by BETd-246 than by BETi-211 also indicate critical differences between these two different BET targeting approaches in their regulation of gene transcription. To understand the common and distinct actions of BETi-211 and BETd-246, we performed RNA-seq analysis on MDA-MB-157, MDA-MB-231 and MDA-MB-468. These cell lines were treated for 3 hours to capture the early transcriptional responses to BETi-211 or BETd-246.

Not surprising, thalidomide had a minimal effect on global transcriptome in all three cell lines (Fig. 3A). An approximately equal number of genes were up- and downregulated by BETi-211 in each cell line (Fig. 3A). In contrast, BETd-246 caused predominant downregulation of gene expression (Fig. 3A).

Given the pervasive transcriptomic responses to BET protein perturbation by BETi-211 and BETd-246, we focused on the genes that were significantly altered ($\geq$2-fold, $P < 0.01$) by BETi-211 or BETd-246 (Fig. 3B–D). This analysis revealed that the expressions of 248–470 genes were significantly affected by BETi-211 with a similar number of genes being up- or downregulated in each cell line (Fig. 3B). In contrast, BETd-246 caused overwhelmingly downregulation of gene expression in these cell lines (Fig. 3B). Despite the distinct transcriptional responses caused by BETi-211 and BETd-246, a set of genes was commonly downregulated by both BETi-211 and BETd-246 in each cell line (Fig. 3B). Of note, 23 of the 46 genes that were commonly downregulated by BETi-211 in all three TNBC cell lines were also downregulated by BETd-246 (Fig. 3B–D).

We then selected 45 representative genes related to gene transcription, cell-cycle transition, proliferation, and survival for quantitative (q) RT-PCR validation (Fig. 3E; Supplementary Fig. S7A). High Pearson correlations between the RNA-seq and qRT-PCR data were found in both MDA-MB-231 and MDA-MB-468 (Supplementary Fig. S7B–S7E). The differential regulation of these 45 genes was confirmed by qRT-PCR at 3 and 8 hours after treatment in MDA-MB-468 and MDA-MB-231 (Fig. 3E; Supplementary Fig. S7A).

The antitumor activities of BET inhibitors such as JQ1 in a variety of preclinical tumor models have often been associated with the downregulation of c-MYC and upregulation of cyclin kinase inhibitors such as p21WAF1 (24–28). *MYC* and *CDKN1A* (encoding p21WAF1) were indeed down- and upregulated, respectively, by both BETi-211 and BETd-246, albeit at different levels and with different kinetics (Fig. 3E; Supplementary Figs. S7A and S9).

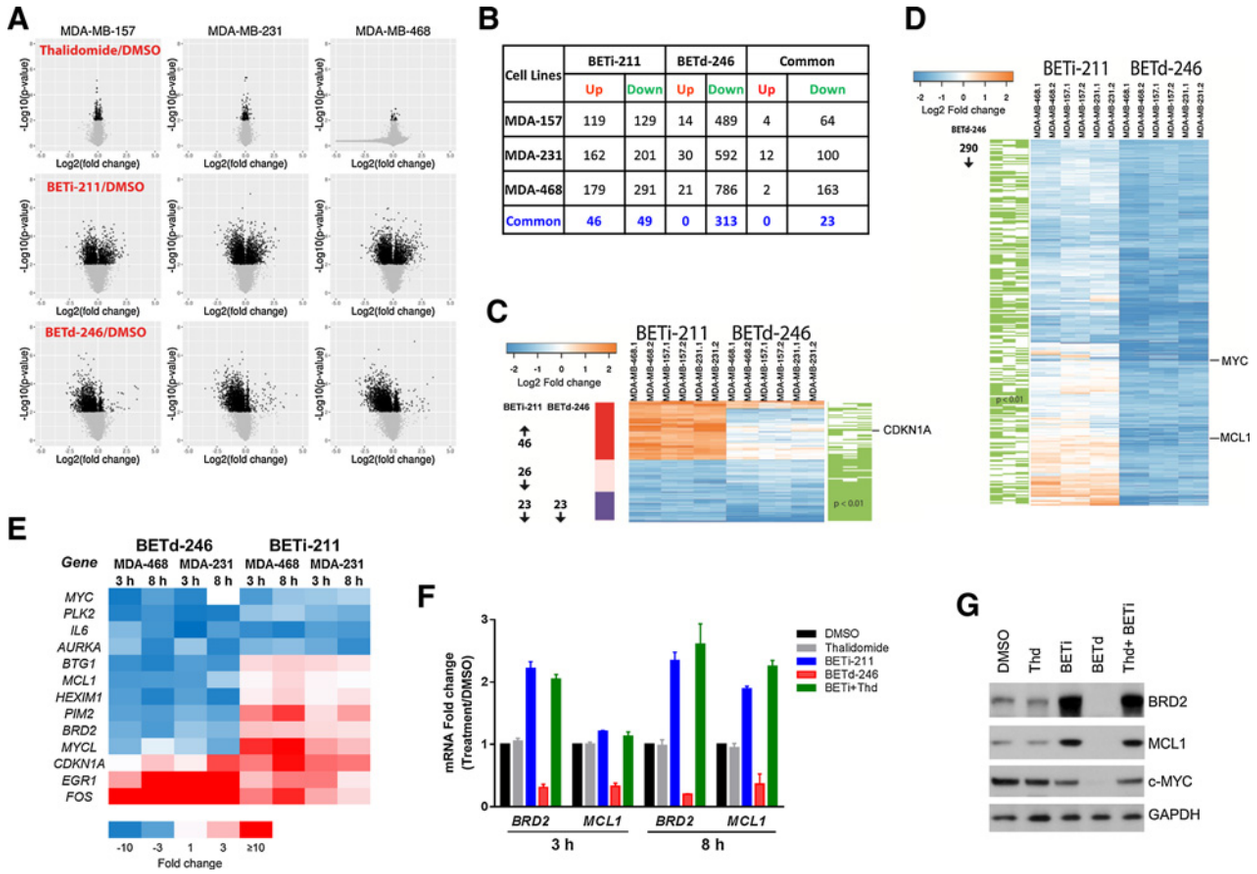
Our transcriptomic analyses also revealed that a set of proliferation and survival-related genes, including *BRD2* and *MCL1*, were oppositely regulated by BETi-211 and BETd-246 (Fig. 3E; Supplementary Fig. S7A). BRD2 is a direct target for both BETi-211 and BETd-246 but its mRNA level was increased by BETi-211 at 3 and 8 hours after BETi-211 treatment in multiple TNBC cell lines (Fig. 3F; Supplementary Fig. S8A–S8C). BRD2 protein increase by BETi-211 was confirmed by immunoblotting (Fig. 3G; Supplementary Fig. S9), consistent with our proteomic analysis (Fig. 1E). The mRNA level of *MCL1*, an antiapoptotic BCL-2 family member, was markedly downregulated by BETd-246 in TNBC cell lines (Fig. 3E and F; Supplementary Fig. S8A–S8C) but was significantly upregulated by BETi-211 at 8 hours in MDA-MB-468 (Fig. 3F). Opposite regulation of MCL1 protein expression by BETd-246 and BETi-211 was confirmed by immunoblotting in MDA-MB-468 cells (Fig. 3G). Downregulation of the mRNA levels of *BRD2* and *MCL1* by BETd-246 was reversed by excess thalidomide and BETi-211 (Supplementary Fig. S8D), confirming its dependence on BET protein degradation.

Hence, BET BRD inhibition by BETi-211 and BET protein degradation by BETd-246 result in distinct transcriptional responses in TNBC cells. Several proliferation and survival-related genes, such as *BRD2* and *MCL1*, are strongly downregulated by BETd-246, but upregulated by BETi-211 in TNBC cells.

MCL1 is a key target of BET degrader-induced apoptosis in TNBC

In the majority of the TNBC cell lines, BETd-246 induces much stronger apoptosis than BETi-211 (Fig. 2D). Because MCL1 expression is effectively downregulated by BETd-246 but not BETi-211 and MCL1 is a key apoptosis regulator, we investigated its role in apoptosis induction by BETd-246 and BETi-211 in TNBC cells.

BETd-246 induced a rapid and time-dependent downregulation of MCL1 protein in all the TNBC cell lines evaluated (Fig. 4A; Supplementary Fig. S9). Significant downregulation of *MCL1* mRNA by BETd-246 was observed at as low as 10 nmol/L, similar to that observed for *MYC* (Fig. 4B). In contrast, MCL1 protein was



Cell Lines	BETi-211		BETd-246		Common	
	Up	Down	Up	Down	Up	Down
MDA-157	119	129	14	489	4	64
MDA-231	162	201	30	592	12	100
MDA-468	179	291	21	786	2	163
Common	46	49	0	313	0	23

Figure 3.

BET inhibitor and degrader elicit extensive but different transcriptome changes in TNBC cells. **A,** MDA-MB-157, MDA-MB-231, and MDA-MB-468 cells were treated with DMSO, thalidomide (1,000 nmol/L), BETi-211 (1,000 nmol/L), and BETd-246 (100 nmol/L) for 3 hours for RNA-seq analysis. **B,** Identification of genes that are up- or downregulated by BETi-211 or BETd-246 by ≥2-fold ($P < 0.01$) over the DMSO control treatment in each cell line and numbers of overlapped genes that are commonly up- or downregulated by BETi-211 or BETd-246 in all three cell lines. **C,** Heatmap of up- or downregulated genes by BETi-211 (≥2-fold, $P < 0.01$) shared by all three TNBC cell lines by RNA-seq and their alterations by BETd-246 (green lines, $P < 0.01$) in each cell line. **D,** Heatmap of up- or downregulated genes by BETd-246 (≥2-fold, $P < 0.01$) shared by all three TNBC cell lines by RNA-seq and their alterations by BETi-211 (green lines, $P < 0.01$) in each cell line. **E,** Heatmap of relative mRNA levels for selected genes in MDA-MB-231 and MDA-MB-468 cells after 3 and 8 hours treatment with DMSO, BETi-211 (1,000 nmol/L), and BETd-246 (100 nmol/L) by qRT-PCR. Data are average of biological triplicates from one representative experiment. **F,** qRT-PCR of relative mRNA levels in MDA-MB-468 cells after 3 and 8 hours treatment with DMSO, thalidomide (1,000 nmol/L), BETi-211 (1,000 nmol/L), and BETd-246 (100 nmol/L), or thalidomide plus BETi-211 (1,000 nmol/L at 1:1 molar ratio). Data are mean ± SD. **G,** MDA-MB-468 cells were treated with DMSO, thalidomide (1,000 nmol/L), BETi-211 (1,000 nmol/L), BETd-246 (100 nmol/L), or thalidomide plus BETi-211 (1,000 nmol/L at 1:1 molar ratio) for 8 hours for immunobloting.

not downregulated by BETi-211 in any of the cell lines but instead was upregulated in MDA-MB-157, MDA-MB-231 and MDA-MB-468 (Fig. 4A; Supplementary Fig. S9). The expression of antiapoptotic BCL-2 and BCL-XL in these cell lines was not significantly altered by either BETi-211 or BETd-246 (Fig. 4A; Supplementary Fig. S9).

We next examined the functionality of MCL1 in cell growth inhibition and apoptosis induction by BETd-246 in TNBC cells. Silencing MCL1 promoted apoptosis and inhibited cell proliferation in MDA-MB-468 and MDA-MB-157 (Fig. 4C and D; Supplementary Fig. S10). Knockdown of MCL1 significantly enhanced the growth-inhibitory activity of BETi-211 but not BETd-246 in these cell lines (Fig. 4D; Supplementary Fig. S10). Conversely, ectopic expression of MCL1 attenuated BETd-246-elicited apoptosis induction and cell growth inhibition (Fig. 4E and F; Supplementary Fig. S10).

Of note, the opposite regulation of MCL1 by BETi-211 and BETd-246 was also observed in non-TNBC cell lines, such as HER2-amplified HCC1954 (Supplementary Fig. S11).

Taken together, these data suggest that downregulation of MCL1 by BETd-246 plays a key role in its robust apoptosis induction in TNBC cells.

Small-molecule BCL-XL inhibitors potentiate BET degrader–induced apoptosis in TNBC cells

Though a much more potent and effective apoptosis inducer than BETi-211, BETd-246 was not uniformly effective in apoptosis induction across the cell lines (Fig. 2D). Recent studies have demonstrated that MCL1 and BCL-XL are key but independent determinants of cell survival in TNBC (29–31). Therefore, we investigated whether small-molecule BCL-XL/BCL-2 inhibitors could enhance apoptosis induction by BETd-246 in TNBC cells.

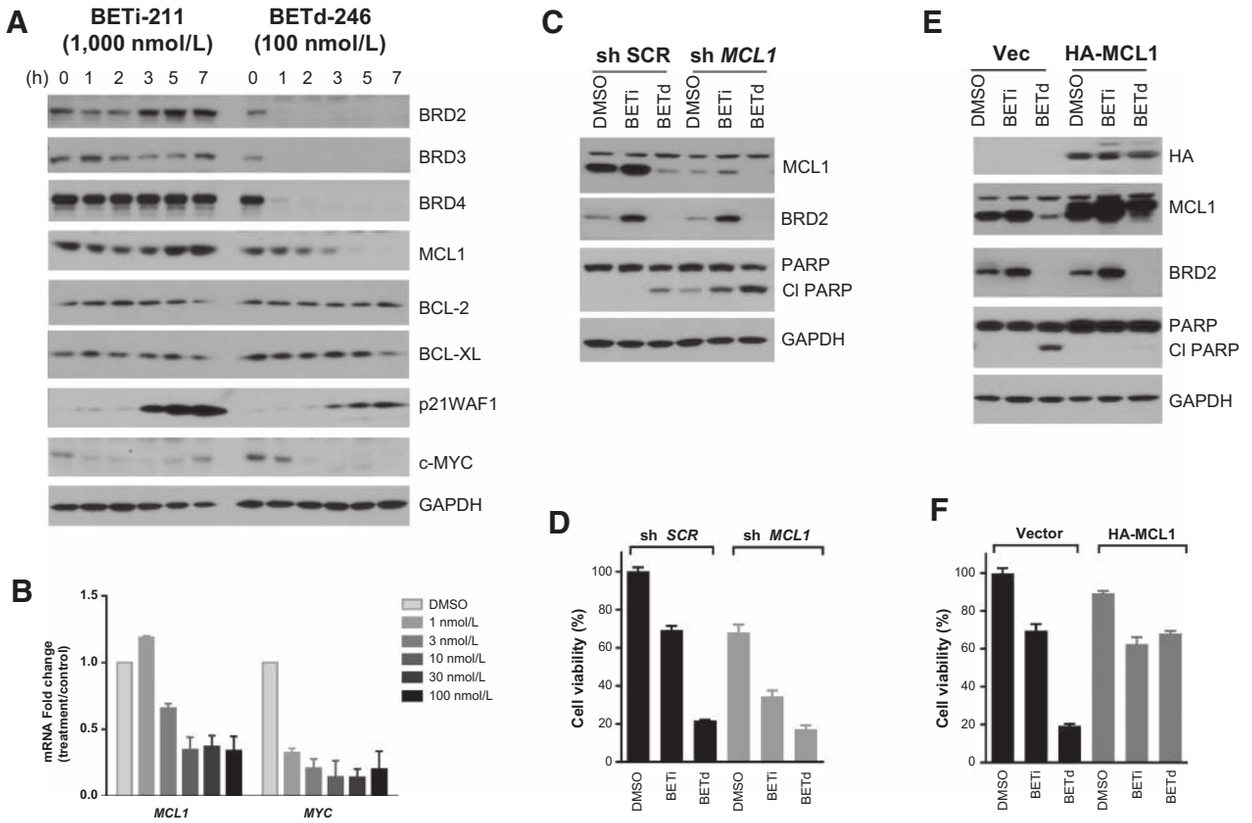


Figure 4.

Downregulation of MCL1 by BETd-246 plays a major role in its robust apoptosis induction in TNBC cells. **A,** MDA-MB-468 cells were treated with BETi-211 (1,000 nmol/L) and BETd-246 (100 nmol/L) at indicated time points for immunoblotting. **B,** qRT-PCR of relative mRNA levels in MDA-MB-468 cells after an 8 hour treatment with indicated concentrations of BETd-246 or DMSO. Data are mean ± SD. **C** and **D,** MDA-MB-468 cells were transduced with scrambled (SCR) or MCL1 shRNA lentivirus for 48 hours, followed by 8 hours treatment with DMSO, BETi-211 (1,000 nmol/L), or BETd-246 (100 nmol/L) for immunoblotting (**C**) or 2 days for cell viability assay (**D**). Data are mean ± SD. **E** and **F,** MDA-MB-468 cells were transduced with lentiviral vector or HA-MCL1-expressing lentivirus for 48 hour followed by an 8 hour treatment with DMSO, BETi-211 (1,000 nmol/L), and BETd-246 (100 nmol/L) for immunoblotting (**E**) or 2 days for cell viability assay (**F**). Data are mean ± SD.

ABT-263 (32) and BM-1197 (18) were selected as dual BCL-2/BCL-XL inhibitors, A-1153463 as a selective BCL-XL inhibitor (33) and ABT-199 as a selective BCL-2 inhibitor (34).

We treated a panel of 7 TNBC cell lines with dose–response matrix and examined the effects of BETd-246 in combination with BCL-2 and/or BCL-XL inhibitors by computing the excess growth inhibition over the Bliss independence model for each combination pairs (35). Modest to strong synergy between BETd-246 and BCL-XL-targeting BM-1197, ABT-263 and A-1155463 was observed in 6 of the 7 cell lines (Supplementary Fig. S12). Immunoblotting and/or Annexin-PI staining confirmed that BM-1197, ABT-263 or A-1155463 potentiated BETd-246-induced apoptosis in these cell lines (Fig. 5A and B; Supplementary Figs. S13 and S14). In contrast, strong synergistic induction of apoptosis by BETd-246 in combination with ABT-199 was only observed in MDA-MB-468 (Fig. 5A; Supplementary Figs. S13 and S14), indicating that BCL-XL, but not BCL2, is a prevalent resistance factor for BETd-246-induced apoptosis in these cell lines. No clear synergism was observed for BETi-211 with either BM-1197 or ABT-263 (Fig. 5B and C; Supplementary Fig. S14). The synergistic apoptosis induction by the combination of BETd-246 and BM-1197 was also readily detected in additional TNBC cell lines responsive to BETd-246–induced apoptosis (BT-20 and MDA-MB-157; Supplementary Fig. S15), further supporting that BCL-XL inhibitors potentiate BETd-246–induced apoptosis in TNBC.

We also extended our investigations to dBET1, the first-reported BET degrader designed upon JQ1 (14). dBET1 at 500 nmol/L effectively depleted BET proteins and downregulated MCL1 expression in MDA-MB-468 cells, concomitant with apoptosis induction (Fig. 5D). Apoptosis induction by dBET1 was also markedly enhanced by BM-1197 (Fig. 5C). Similar to BETi-211, JQ1 treatment led to increased expression of BRD2 and MCL1 and no obvious synergism between BM-1197 and JQ1 was observed (Fig. 5C and D).

Taken together, these data indicate that the mechanisms of action observed for BETd-246 and BETi-211 in TNBC cells are independent of their chemical classes and apply to other classes of BET inhibitors and degraders.

BET degraders decrease BET proteins in xenograft breast tumors and suppress tumor growth

To evaluate the *in vivo* antitumor activity of BETd-246, we first employed the "Washington Human in Mouse (WHIM)" 24

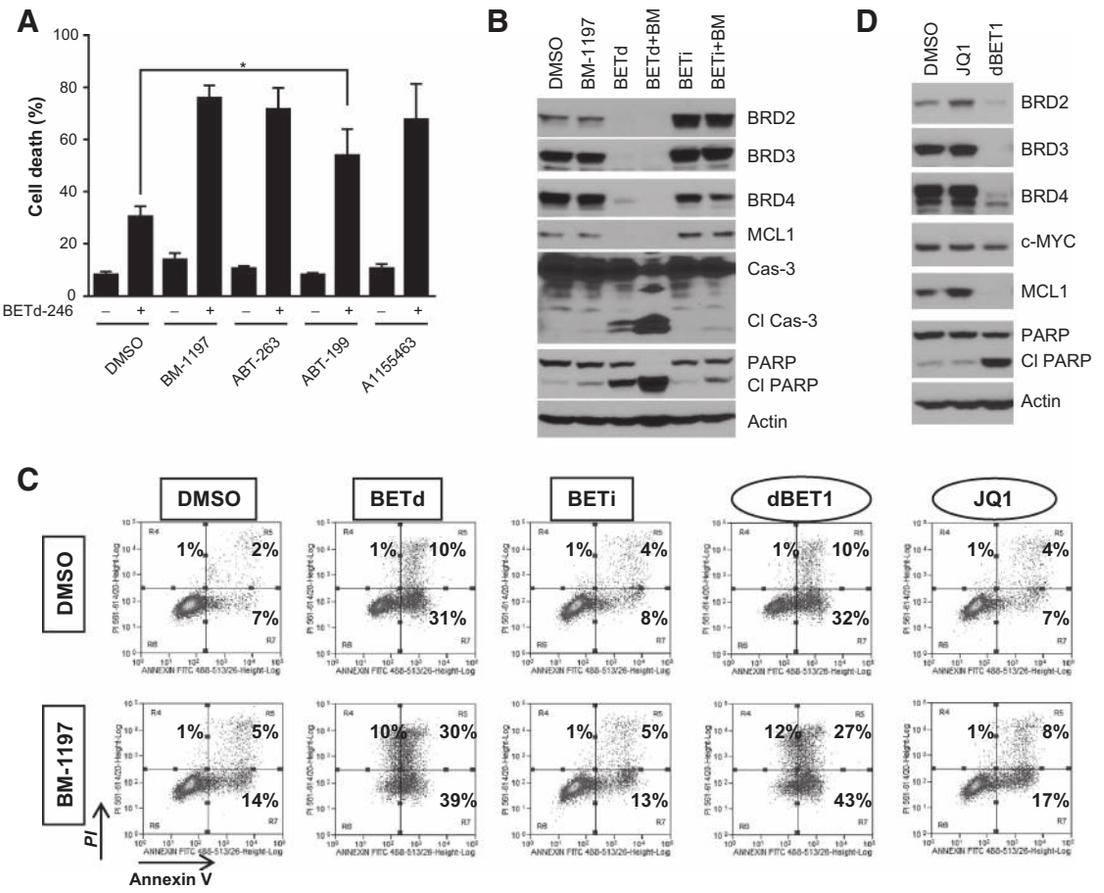


Figure 5.

Small-molecule BCL-XL inhibitors significantly enhance the apoptosis induction by BETd-246. **A,** MDA-MB-468 cells were treated with BETd-246 (50 nmol/L), BM-1197 (250 nmol/L), ABT-263 (250 nmol/L), ABT-199 (250 nmol/L), or A-1155463 (250 nmol/L) as indicated for 24 hours for Annexin V-PI apoptosis analysis. Data are the mean ± SEM ($n = 3$). *, $P < 0.05$. **B,** MDA-MB-468 cells were treated with BM-1197 (250 nmol/L), BETd-246 (50 nmol/L), or BETi-211 (1,000 nmol/L) as indicated for 6 hours for immunoblotting. **C,** MDA-MB-468 cells were treated with BM-1197 (250 nmol/L), BETd-246 (50 nmol/L), BETi-211 (1,000 nmol/L), JQ1 (2,000 nmol/L), or dBET1 (500 nmol/L) as indicated for 24 hours for apoptosis analysis. **D,** MDA-MB-468 cells were treated with JQ1 (2,000 nmol/L) or dBET1 (500 nmol/L) for 12 hours for immunoblotting.

(WHIM24), a patient derived xenograft (PDX) model developed from a patient with treatment-resistant breast cancer (ESR^{E380Q}, PR^{-} and $HER2^{-}$; ref. 36). BETd-246 at 5 mg/kg, i.v., 3 times per week for 3 weeks effectively inhibited WHIM24 tumor growth, similar to the antitumor activity of BETi-211 at 50 mg/kg, daily, oral dosing, 5 days a week for 3 weeks. BETd-246 at 10 mg/kg, 3 times per week for 3 weeks, induced partial tumor regression during treatment (Fig. 6A and B). Neither BETi-211 nor BETd-246 caused significant weight loss (Fig. 6C) or apparent toxicity in this model. PD analysis showed that a single intravenous dose of BETd-246 (10 mg/kg) reduced the levels of BET proteins by >80% at as early as 1 hour and this effect lasted for at least 9 hours in WHIM24 tumors (Supplementary Fig. S16). Notably, MCL1 protein levels in tumors were markedly reduced at as early as 3 hours after BETd-246 treatment (Supplementary Fig. S16). However, the protein levels of BRD2, BRD4, and MCL1 started to rebound at 12 hours, indicating that BET degradation by BETd-246 is reversible once the drug is cleared from the tumor tissue. Pharmacokinetic analysis revealed that, although a single dose of BETd-246 (10 mg/kg) in tumor-bearing mice achieved reasonable drug exposure in plasma and WHIM24 tumors at 1 and 3 hours, the drug concentrations diminished rapidly in both plasma and tumors (Supplementary Fig. S17).

We next tested the antitumor activity of BETd-246 in xenograft tumor models of TNBC cell lines. In MDA-MB-453 xenograft model, BETd-246 at 5 mg/kg significantly inhibited tumor growth with TGI (%) of 85% at the end of study (Fig. 6D and E). In sharp contrast, BETi-211 at 50 mg/kg, daily, 5 days a week for 2 weeks, failed to achieve tumor growth inhibition. No significant weight loss (Fig. 6F) or apparent toxicity was observed with BETi-211 or BETd-246. However, BETd-246 at 10 mg/kg, i.v., 3 times per week for 2–3 weeks had very limited or no antitumor activity in MDA-M-231 and MDA-MB-468 models, respectively (Fig. 6G and J). Our pharmacokinetic analysis revealed that BETd-246 had very limited drug exposure in the xenograft tumor tissue in these two models (Supplementary Figs. S18 and S19), in contrast with the good drug exposure in the WHIM24 xenograft tumor tissue.

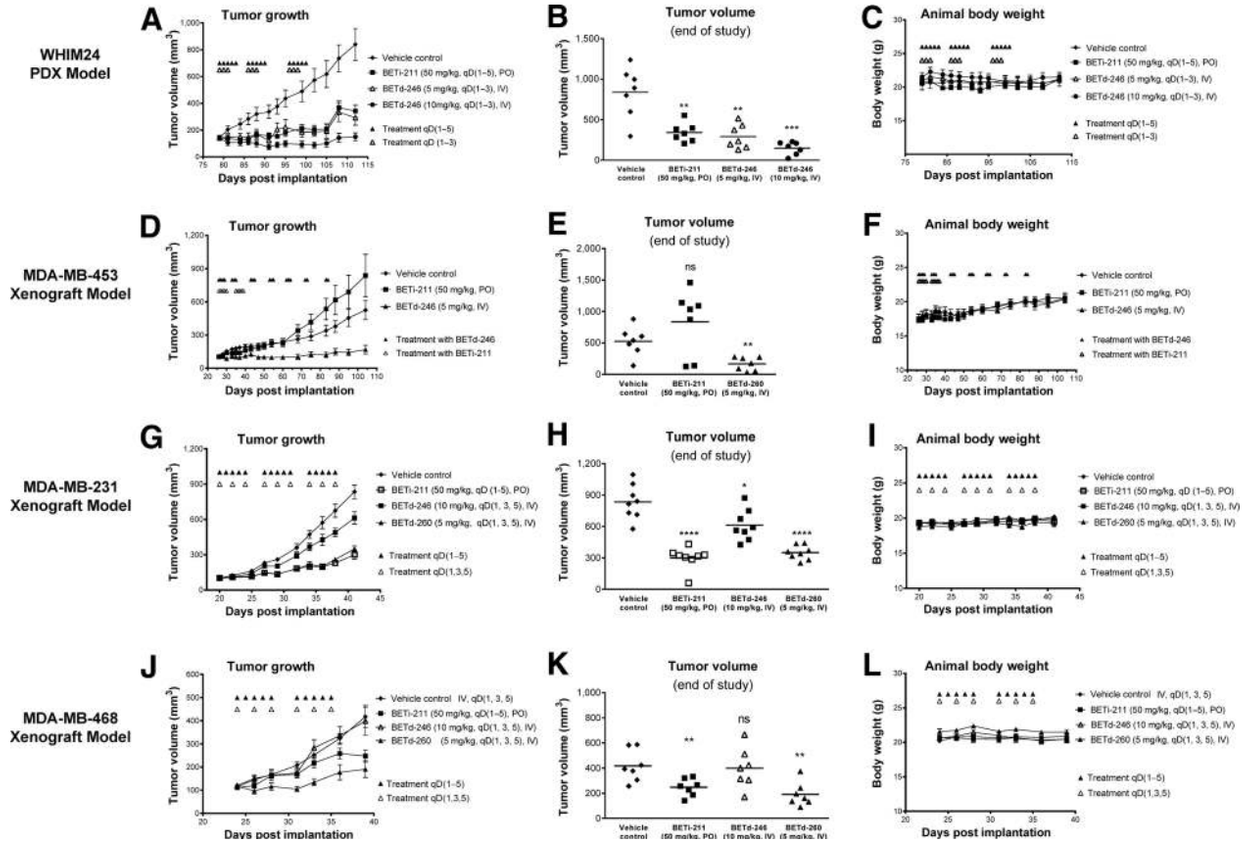


Figure 6.

Small-molecule BET degraders are efficacious in multiple xenograft models of breast cancer. SCID mice bearing xenograft tumors were treated as indicated. BETi-211 was given orally in 2% TPGS: 98% PEG200. BETd-246, BETd-260, and vehicle control were given intravenously in 10% PEG400: 3% Cremophor: 87% PBS. Tumor sizes and body weights were measured every 2–3 days. Data are mean ± SEM ($n = 7$–8). The study in MDA-MB-468 model was terminated early due to profound necrosis in the control tumors. Scatter plots represent the tumor volumes at the end of each study. *P* values between each treated and the vehicle control group were determined using two-tailed *t* test; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$. PO, orally.

To improve its pharmacokinetics, we performed extensive optimization of the linker and the cereblon-binding moiety of BETd-246 and identified UM-BETd-260 (BETd-260, Supplementary Fig. S20A, Supplementary Information Scheme SIII). Similar to BETd-246, BETd-260 potently depleted BET proteins at low nanomolar concentrations in TNBC cells. In fact, BETd-260 was 2–7 times more potent than BETd-246 in growth inhibition of TNBC cells (Supplementary Figs. S20B, S20C, and S21). Importantly, pharmacokinetic analysis showed that the exposure of BETd-260 in plasma and xenograft tumors was much higher than that of BETd-246 in xenograft models of MDA-MB-231 and MDA-MB-468 (Supplementary Figs. S18–S19). Accordingly, BETd-260 at 5 mg/kg, i.v., 3 times per week for 3 weeks exerted much stronger antitumor activity than BETd-246 at 10 mg/kg with the same dosing-schedule in both MDA-MB-231 and MDA-MB-468 xenograft models (Fig. 6G, H, J, and K). The antitumor activity of BETd-260 was comparable with or stronger than that of BETi-211 at 50 mg/kg, daily oral dosing, 5 days a week for 3 weeks in these models (Fig. 6G, H, J, and K). Studies in MDA-MB-468 model were terminated early due to profound necrosis in the control tumors. Neither BETd-246 nor BETd-260 caused significant weight loss (Fig. 6I and L) or overt toxicity in these tumor models. PD analysis confirmed that a single dose of BETd-260 at 5 mg/kg effectively reduced BET protein levels in the xenograft tumors of MDA-MB-231 and MDA-MB-468 at 1 to 3 hours after drug exposure (Supplementary Fig. S22), as well as upregulation of p21WAF1 and downregulation of MCL1 (Supplementary Fig. S22).

We also assessed BETd-246 for its potential toxicity in immune-competent Balb/c mice. Mice were treated with BETd-246 intravenously, daily, 5 days a week for 2 weeks, and then sacrificed at the end of the treatment to examine potential tissue damage. Although a single dose of BETd-246 (5 mg/kg) was able to effectively reduce BRD4 protein in mouse liver tissue, which has a high level of BRD4 protein expression but undetectable levels of BRD2 and BRD3 proteins (Supplementary Fig. S23), BETd-246 at 5 mg/kg exhibited no significant toxicity in normal mouse tissues (Supplementary Table S4–S6).

Collectively, our data demonstrated that small-molecule BET degraders effectively suppress tumor growth at well-tolerated dosing-schedules in xenograft breast tumor models.

Discussion

In this study, we investigated the therapeutic potential and mechanism of action of small-molecule BET degraders BETd-246 and its more potent and efficacious analogue BETd-260 in TNBC. BET degrader BETd-246 efficiently and selectively degrades BET proteins in TNBC cells at low nanomolar concentrations and exhibits exquisite growth-inhibitory activity in the majority of TNBC cell lines evaluated. In comparison, BETd-246 is much more potent than the corresponding BET inhibitor BETi-211 in growth inhibition and apoptosis induction *in vitro*. In xenograft mouse tumor models, BET degraders BETd-246 and/or BETd-260 effectively suppress breast tumor growth at well-tolerated dosing-schedules.

BET proteins participate in multiprotein complexes comprising of chromatin and transcription regulators to modulate transcription (37–41). In addition to acting in a BRD-dependent manner, BRD4 has BRD-independent functions in breast cancer cells (8, 11). Thus, the transcriptional responses to the BET BRD inhibition and BET protein degradation are distinctly different as evidenced by our transcriptome profiling using BETi-211 and BETd-246. Our data further reveal that BET BRD inhibitor and BET degrader disproportionately regulate the expression of a large set of genes, reinforcing a model in which BET proteins act collectively with chromatin and transcription regulators to modulate gene expression output. The distinct transcriptional responses to BET BRD inhibitor and BET protein degrader strongly support the notion that BET proteins modulate gene expression through both BRD-dependent and BRD-independent mechanisms. The detailed mechanisms of their differential effects of BET BRD inhibitor and BET protein degrader on gene expression warrant further investigation.

Previous studies have shown that the effects of BET BRD inhibitors such as JQ-1 are largely cytostatic, especially in solid tumor models, with apoptosis induction limited to certain blood cancer models (25, 42). Our data show that a key difference in the actions between BET BRD inhibitor and BET protein degrader in TNBC cells is the robust apoptosis induction by BET degrader and minimal to moderate apoptosis induction by BET inhibitor. Conery and colleagues (43) recently reported that the preclinical antitumor efficacy of BET inhibitors is determined by the magnitude of apoptotic, but not the cytostatic response in melanoma and leukemia models. Earlier studies have shown that BET BRD inhibitors suppress the expression of BCL-2 and/or BCL-XL while sparing MCL1 in multiple cell line models (1, 25). We found that MCL1, a key antiapoptotic BCL-2 member, is significantly downregulated by BET degraders in TNBC cells *in vitro* and *in vivo*. MCL1 silencing by siRNA greatly enhances apoptosis induction by BETi-211 but not by BETd-246, and overexpression of MCL1 significantly reduces apoptosis induction by BETd-246 in TNBC cells. These data suggest that MCL1 is a key mediator of BET degrader-induced apoptosis in TNBC.

MCL1 expression in breast tumors correlates with high tumor grade and a much lower survival rate in patients (44). Amplification of *MCL1* gene loci is prevalent in TNBC (~20%; refs. 45, 46). Although approximately 30% of TNBC patients receiving neoadjuvant chemotherapy achieve a pathologic complete response, the remaining patients with residual tumors exhibit high rates of metastatic disease and one of the most common genetic changes in the chemo-refractory tumors is the amplification of *MCL1* loci (54%; refs. 47–49). MCL1 has been identified as both an intrinsic and acquired resistance factor that limits the efficacy of a variety of anticancer agents (45, 50) and thus has been intensely pursued as a therapeutic target. Our finding here provides an exciting new strategy to target MCL1 for breast cancer and potentially other types of cancer. Our data also suggest that, with optimal pharmacokinetics, BET degraders are more likely to achieve more favorable clinical responses than BET inhibitors in breast cancer patients.

Although BETd-246 is very effective in suppressing MCL1 expression in TNBC cell lines, it is not uniformly effective in apoptosis induction in these cell lines, suggesting the existence of other apoptotic resistance factors. Using a set of selective BCL-2 and/or BCL-XL inhibitors, we have shown that BCL-XL is a key resistance factor for apoptosis induction by BETd-246 in TNBC. Thus, combination of a BCL-XL inhibitor and a BET degrader may provide an effective therapeutic strategy for breast cancer.

In summary, our study provides compelling preclinical data that targeting BET proteins for degradation is a promising therapeutic strategy for TNBC.

Disclosure of Potential Conflicts of Interest

J.L. Meagher has ownership interest in patent. D.F. Hayes reports receiving a commercial research grant from AstraZeneca, Pfizer, Janssen, and University of Michigan holds a patent regarding circulating tumor cell phenotyping in which he is the named inventor and receives royalties from Janssen. M.J. Ellis has ownership interest (including patents) in Bioclassifier LLC, is a consultant/advisory board member for Puma, Pfizer, AstraZeneca, and Nanostring/Prosigna. S. Wang reports receiving a commercial research grant and has ownership interest (including patents) in Medsyn Biopharma. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: L. Bai, B. Zhou, J. Hu, F. Xu, E. Fernandez-Salas, Y. Dou, D. Sun, D.F. Hayes, M.J. Ellis, S. Wang

Development of methodology: L. Bai, J. Ji, J. Hu, Y. Zhao, L. Liu, J. Xu, B. Wen, D.F. Hayes, S. Li, M.J. Ellis

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Bai, B. Zhou, H. Jiang, Y. Zhao, L. Liu, E. Fernandez-Salas, J. Meagher, J. Stuckey, S. Li

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Bai, B. Zhou, C.-Y. Yang, D. McEachern, S. Przybranowski, H. Jiang, Y. Zhao, L. Liu, E. Fernandez-Salas, D. Sun, J. Meagher, J. Stuckey, M.J. Ellis

Writing, review, and/or revision of the manuscript: L. Bai, B. Zhou, C.-Y. Yang, D. McEachern, J. Stuckey, D.F. Hayes, S. Li, M.J. Ellis, S. Wang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Bai, C.-Y. Yang, D. McEachern, S. Przybranowski, H. Jiang, F. Xu, S. Li

Study supervision: L. Bai, D. Sun, M.J. Ellis, S. Wang

Acknowledgments

The authors thank the University of Michigan Biomedical Research Core Facilities (DNA Sequencing, Flow Cytometry and Vector Cores), Unit for Laboratory Animal Medicine and the Proteomics Resource Facility of the Department of Pathology for their excellent support.

Grant Support

The Breast Cancer Research Foundation (to S. Wang), the University of Michigan Comprehensive Cancer Center Strategic Fund for Breast Cancer (to S. Wang), the University of Michigan Comprehensive Cancer Center Core grant (to S. Wang as a program leader) from the National Cancer Institute, NIH (P30CA046592), and the Susan G. Komen for the Cure Promise Grant (PG12220321 to S. Wang and M. J. Ellis).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received September 28, 2016; revised October 31, 2016; accepted January 25, 2017; published OnlineFirst February 16, 2017.

References

1. Dawson MA, Prinjha RK, Dittmann A, Giotopoulos G, Bantscheff M, Chan WI, et al. Inhibition of BET recruitment to chromatin as an effective treatment for MLL-fusion leukaemia. Nature 2011;478:529–33.
2. Odore E, Lokiec F, Cvitkovic E, Bekradda M, Herait P, Bourdel F, et al. Phase I population pharmacokinetic assessment of the oral bromodomain inhibitor OTX015 in patients with haematologic malignancies. Clin Pharmacokinet 2016;55:397–405.
3. Berthon C, Raffoux E, Thomas X, Vey N, Gomez-Roca C, Yee K, et al. Bromodomain inhibitor OTX015 in patients with acute leukaemia: a dose-escalation, phase 1 study. Lancet Haematol 2016;3:e186–e95.
4. Filippakopoulos P, Qi J, Picaud S, Shen Y, Smith WB, Fedorov O, et al. Selective inhibition of BET bromodomains. Nature 2010;468:1067–73.
5. Stathis A, Zucca E, Bekradda M, Gomez-Roca C, Delord J-P, de La Motte Rouge T, et al. Clinical response of carcinomas harboring the BRD4–NUT oncoprotein to the targeted bromodomain inhibitor OTX015/MK-8628. Cancer Discov 2016;6:492–500.
6. Amorim S, Stathis A, Gleeson M, Iyengar S, Magarotto V, Leleu X, et al. Bromodomain inhibitor OTX015 in patients with lymphoma or multiple myeloma: a dose-escalation, open-label, pharmacokinetic, phase 1 study. Lancet Haematol 2016;3:e196–e204.
7. Chaidos A, Caputo V, Gouvedenou K, Liu B, Marigo I, Chaudhry MS, et al. Potent antimyeloma activity of the novel bromodomain inhibitors I-BET151 and I-BET762. Blood 2014;123:697–705.
8. Shu S, Lin CY, He HH, Witwicki RM, Tabassum DP, Roberts JM, et al. Response and resistance to BET bromodomain inhibitors in triple-negative breast cancer. Nature 2016;529:413–7.
9. Sengupta S, Biarnes MC, Clarke R, Jordan VC. Inhibition of BET proteins impairs estrogen-mediated growth and transcription in breast cancers by pausing RNA polymerase advancement. Breast Cancer Res Treat 2015;150:265–78.
10. Nagarajan S, Hossan T, Alawi M, Najafova Z, Indenbirken D, Bedi U, et al. Bromodomain protein BRD4 is required for estrogen receptor-dependent enhancer activation and gene transcription. Cell Rep 2014;8:460–9.
11. Marcotte R, Sayad A, Brown KR, Sanchez-Garcia F, Reimand J, Haider M, et al. Functional genomic landscape of human breast cancer drivers, vulnerabilities, and resistance. Cell 2016;164:293–309.
12. Sakamoto KM, Kim KB, Kumagai A, Mercurio F, Crews CM, Deshaies RJ. Protacs: chimeric molecules that target proteins to the Skp1–Cullin–F box complex for ubiquitination and degradation. Proc Natl Acad Sci U S A 2001;98:8554–9.
13. Toure M, Crews CM. Small-molecule PROTACS: new approaches to protein degradation. Angew Chem Int Ed Engl 2016;55:1966–73.
14. Winter GE, Buckley DL, Paulk J, Roberts JM, Souza A, Dhe-Paganon S, et al. DRUG DEVELOPMENT. Phthalimide conjugation as a strategy for *in vivo* target protein degradation. Science 2015;348:1376–81.
15. Lu J, Qian Y, Altieri M, Dong H, Wang J, Raina K, et al. Hijacking the E3 ubiquitin ligase cereblon to efficiently target BRD4. Chem Biol 2015;22:755–63.
16. Raina K, Lu J, Qian Y, Altieri M, Gordon D, Rossi AM, et al. PROTAC-induced BET protein degradation as a therapy for castration-resistant prostate cancer. Proc Natl Acad Sci U S A 2016;113:7124–9.
17. Ethier SP, Mahacek ML, Gullick WJ, Frank TS, Weber BL. Differential isolation of normal luminal mammary epithelial cells and breast cancer cells from primary and metastatic sites using selective media. Cancer Res 1993;53:627–35.
18. Bai L, Chen J, McEachern D, Liu L, Zhou H, Aguilar A, et al. BM-1197: a novel and specific Bcl-2/Bcl-xL inhibitor inducing complete and long-lasting tumor regression *in vivo*. PLoS ONE 2014;9:e99404.
19. Hoffman-Luca CG, Ziazadeh D, McEachern D, Zhao Y, Sun W, Debussche L, et al. Elucidation of acquired resistance to Bcl-2 and MDM2 inhibitors in acute leukemia *in vitro* and *in vivo*. Clin Cancer Res 2015;21:2558–68.
20. Ran X, Zhao Y, Liu L, Bai L, Yang CY, Zhou B, et al. Structure-based design of gamma-carboline analogues as potent and specific BET bromodomain inhibitors. J Med Chem 2015;58:4927–39.
21. Demo SD, Kirk CJ, Aujay MA, Buchholz TJ, Dajee M, Ho MN, et al. Antitumor activity of PR-171, a novel irreversible inhibitor of the proteasome. Cancer Res 2007;67:6383–91.
22. Soucy TA, Smith PG, Milhollen MA, Berger AJ, Gavin JM, Adhikari S, et al. An inhibitor of NEDD8-activating enzyme as a new approach to treat cancer. Nature 2009;458:732–6.
23. Chiba T, Tanaka K. Cullin-based ubiquitin ligase and its control by NEDD8-conjugating system. Curr Protein Pept Sci 2004;5:177–84.
24. Zuber J, Shi J, Wang E, Rappaport AR, Herrmann H, Sison EA, et al. RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia. Nature 2011;478:524–8.
25. Delmore JE, Issa GC, Lemieux ME, Rahl PB, Shi J, Jacobs HM, et al. BET bromodomain inhibition as a therapeutic strategy to target c-Myc. Cell 2011;146:904–17.
26. Mertz JA, Conery AR, Bryant BM, Sandy P, Balasubramanian S, Mele DA, et al. Targeting MYC dependence in cancer by inhibiting BET bromodomains. Proc Natl Acad Sci U S A 2011;108:16669–74.
27. Cheng Z, Gong Y, Ma Y, Lu K, Lu X, Pierce LA, et al. Inhibition of BET bromodomain targets genetically diverse glioblastoma. Clin Cancer Res 2013;19:1748–59.
28. Jacques C, Lamoureux F, Baud'huin M, Calleja LR, Quillard T, Amiaud J, et al. Targeting the epigenetic readers in Ewing Sarcoma inhibits the oncogenic transcription factor EWS/Fli1. Oncotarget 2016;7:24125–40.
29. Goodwin CM, Rossanese OW, Olejniczak ET, Fesik SW. Myeloid cell leukemia-1 is an important apoptotic survival factor in triple-negative breast cancer. Cell Death Differ 2015;22:2098–106.
30. Xiao Y, Nimmer P, Sheppard GS, Bruncko M, Hessler P, Lu X, et al. MCL-1 is a key determinant of breast cancer cell survival: validation of MCL-1 dependency utilizing a highly selective small molecule inhibitor. Mol Cancer Ther 2015;14:1837–47.
31. Petrocca F, Altschuler G, Tan SM, Mendillo ML, Yan H, Jerry DJ, et al. A genome-wide siRNA screen identifies proteasome addiction as a vulnerability of basal-like triple-negative breast cancer cells. Cancer Cell 2013;24:182–96.
32. Tse C, Shoemaker AR, Adickes J, Anderson MG, Chen J, Jin S, et al. ABT-263: a potent and orally bioavailable Bcl-2 family inhibitor. Cancer Res 2008;68:3421–8.
33. Tao ZF, Hasvold L, Wang L, Wang X, Petros AM, Park CH, et al. Discovery of a potent and selective BCL-XL inhibitor with *in vivo* activity. ACS Med Chem Lett 2014;5:1088–93.
34. Souers AJ, Leverson JD, Boghaert ER, Ackler SL, Catron ND, Chen J, et al. ABT-199, a potent and selective BCL-2 inhibitor, achieves antitumor activity while sparing platelets. Nat Med 2013;19:202–8.
35. Berenbaum MC. Criteria for analyzing interactions between biologically active agents. Adv Cancer Res 1981;35:269–335.
36. Li S, Shen D, Shao J, Crowder R, Liu W, Prat A, et al. Endocrine-therapy-resistant ESR1 variants revealed by genomic characterization of breast-cancer-derived xenografts. Cell Rep 2013;4:1116–30.
37. Belkina AC, Denis GV. BET domain co-regulators in obesity, inflammation and cancer. Nat Rev Cancer 2012;12:465–77.
38. Shi J, Vakoc CR. The mechanisms behind the therapeutic activity of BET bromodomain inhibition. Mol Cell 2014;54:728–36.
39. Bhagwat AS, Roe JS, Mok BY, Hohmann AF, Shi J, Vakoc CR. BET bromodomain inhibition releases the mediator complex from select cis-regulatory elements. Cell Rep 2016;15:519–30.
40. Shen C, Ipsaro JJ, Shi J, Milazzo JP, Wang E, Roe JS, et al. NSD3-short is an adaptor protein that couples BRD4 to the CHD8 chromatin remodeler. Mol Cell 2015;60:847–59.
41. Roe JS, Mercan F, Rivera K, Pappin DJ, Vakoc CR. BET bromodomain inhibition suppresses the function of hematopoietic transcription factors in acute myeloid leukemia. Mol Cell 2015;58:1028–39.
42. Chapuy B, McKeown MR, Lin CY, Monti S, Roemer MG, Qi J, et al. Discovery and characterization of super-enhancer-associated dependencies in diffuse large B cell lymphoma. Cancer Cell 2013;24:777–90.
43. Conery AR, Centore RC, Spillane KL, Follmer NE, Bommi-Reddy A, Hatton C, et al. Preclinical anticancer efficacy of BET bromodomain inhibitors is determined by the apoptotic response. Cancer Res 2016;76:1313–9.
44. Ding Q, He X, Xia W, Hsu JM, Chen CT, Li LY, et al. Myeloid cell leukemia-1 inversely correlates with glycogen synthase kinase-3beta activity and associates with poor prognosis in human breast cancer. Cancer Res 2007;67:4564–71.
45. Balko JM, Giltnane JM, Wang K, Schwarz LJ, Young CD, Cook RS, et al. Molecular profiling of the residual disease of triple-negative breast cancers after neoadjuvant chemotherapy identifies actionable therapeutic targets. Cancer Discov 2014;4:232–45.

46. Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J, et al. The landscape of somatic copy-number alteration across human cancers. Nature 2010;463:899–905.
47. Guarneri V, Broglio K, Kau SW, Cristofanilli M, Buzdar AU, Valero V, et al. Prognostic value of pathologic complete response after primary chemotherapy in relation to hormone receptor status and other factors. J Clin Oncol 2006;24:1037–44.
48. Kuerer HM, Newman LA, Smith TL, Ames FC, Hunt KK, Dhingra K, et al. Clinical course of breast cancer patients with complete pathologic primary tumor and axillary lymph node response to doxorubicin-based neoadjuvant chemotherapy. J Clin Oncol 1999;17:460–9.
49. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, Mejia JA, et al. Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 2008;26:1275–81.
50. Wertz IE, Kusam S, Lam C, Okamoto T, Sandoval W, Anderson DJ, et al. Sensitivity to antitubulin chemotherapeutics is regulated by MCL1 and FBW7. Nature 2011;471:110–4.