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Targeting Bromodomain and Extraterminal Proteins in Breast Cancer

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Abstract

Breast cancer is a collection of distinct tumor subtypes that are driven by unique gene expression profiles. These transcriptomes are controlled by various epigenetic marks that dictate which genes are expressed and suppressed. During carcinogenesis, extensive restructuring of the epigenome occurs, including aberrant acetylation, alteration of methylation patterns, and accumulation of epigenetic readers at oncogenes. As epigenetic alterations are reversible, epigenome-modulating drugs could provide a mechanism to silence numerous oncogenes simultaneously. Here, we review the impact of inhibitors of the Bromodomain and Extraterminal (BET) family of epigenetic readers in breast cancer. These agents, including the prototypical BET inhibitor JQ1, have been shown to suppress a variety of oncogenic pathways while inducing minimal, if any, toxicity in models of several subtypes of breast cancer. BET inhibitors also synergize with multiple approved anti-cancer drugs, providing a greater response in breast cancer cell lines and mouse models than either single agent. The combined findings of the studies discussed here provide an excellent rationale for the continued investigation of the utility of BET inhibitors in breast cancer.

Graphical Abstract

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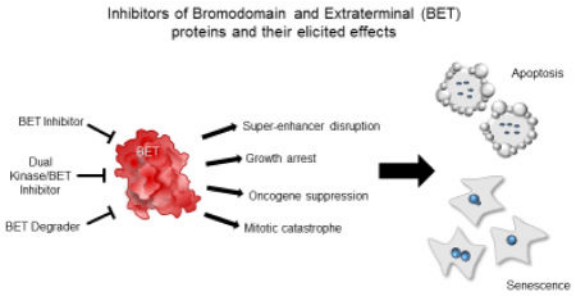

Keywords

Breast cancer; Bromodomain and Extraterminal Protein; BET inhibitor; anti-cancer drugs; transcriptional control; drug synergy

1. INTRODUCTION

Breast cancer is a heterogeneous disease, and multiple subtyping methods have been developed to group these diverse tumors. The most common and clinically relevant classification system is based on the expression of estrogen receptor (ER) and progesterone receptor (PR) and the amplification status of human epidermal growth factor receptor 2 (HER2). The status of these three receptors governs the first line of treatment for breast cancer patients. For example, patients with tumors expressing ER and/or PR are eligible for endocrine therapy while patients with tumors with HER2 amplification receive HER2-targeted therapies. Triple-negative breast cancer (TNBC) patients lack expression of ER/PR and amplification of HER2. There are currently no FDA-approved targeted therapies available for this disease, and the only treatment option is traditional cytotoxic chemotherapy.

Gene expression profiling led to the subdivision of breast cancers into six intrinsic molecular subtypes: luminal A, luminal B, HER2-enriched, basal-like, claudin-low, and normal-like (1–3). These subtypes vary in terms of phenotype, response to treatment, and clinical outcome (4–6). The vast majority of breast cancers fall into the luminal A and B subtypes. These tumors are characterized by expression of ER and/or PR with a low (luminal A) or high (luminal B) Ki67 index. They typically respond to ER-targeting agents including tamoxifen and are associated with good prognosis (7). HER2-enriched tumors overexpress the *ERBB2* gene and, as a result, can be treated with anti-HER2 agents such as trastuzumab (8). Most basal-like and claudin-low tumors can be categorized as TNBC, with basal-like tumors accounting for the majority of TNBCs. Basal-like tumors express a basal epithelial gene cluster which includes cytokeratins 5 and 17, laminin, and integrin-β4 (1). Claudin-low cancers express low levels of the tight junction proteins E-cadherin and claudins 3, 4, and 7; are poorly differentiated; have a large cancer stem cell population; are enriched in epithelial-to-mesenchymal transition (EMT) markers; and express high levels of immune response genes (9, 10). Tumors of both TNBC subtypes respond well to cytotoxic chemotherapies such as doxorubicin and taxanes (7). However, the incidence of metastatic recurrence for

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these cancers is high. Once metastasis occurs, the disease progresses quickly, with patients exhibiting a median survival of 13 months (4, 5, 11, 12).

Breast cancers are driven by numerous oncogenic pathways which can be subtype-specific. As such, the various subtypes must be treated with different agents. To target a diverse array of breast tumors and to prevent recurrence, it should also be useful to develop therapies that can target multiple pathways simultaneously and have broad implications for this group of diseases as a whole. Here, we discuss targeting Bromodomain and Extraterminal (BET) proteins, an approach already in clinical trials that has the potential to provide benefits across all subtypes of breast cancer.

2. BET PROTEIN STRUCTURE AND FUNCTION

2.1 BET protein structure

Various posttranslational modifications are added to nucleosomes that impact their association with chromatin and the recruitment of proteins to DNA. One such modification is lysine acetylation, which marks areas of chromatin for active transcription and is recognized by bromodomains (BRDs) in various proteins (13). The BRD is a conserved 110 amino acid structural motif composed of four α-helices (αZ, αA, αB, and αC) that comprise a left-handed bundle (14). Two loop regions (ZA and BC) connect the α-helices and form a surface that interacts with acetylated lysines in nucleosomal histones (15). In humans, there are 61 BRDs found within 42 multi-domain proteins that regulate transcription, including ATP-dependent chromatin remodeling complexes, transcriptional co-activators, histone acetyltransferases (HATs), and BET proteins (16).

The BET protein family consists of four members (BRD2, BRD3, BRD4, and BRDT) that reside in the nucleus and play critical roles in transcription (17). BET proteins act as epigenetic readers and are characterized by two tandem N-terminal BRD regions followed by an extraterminal domain. The BRD regions recognize and bind acetylated lysines in histone tails (histones H3 and H4) and transcription factors. The extraterminal domain is involved in protein-protein interactions with proteins such as E2Fs and latent nuclear antigen of Kaposi’s sarcoma-associated herpes virus (18, 19). BRD4 and BRDT have an additional C-terminal motif that links their reader function to transcriptional elongation: following the binding of BRD4/T to acetylated histones, the C-terminal motif interacts with P-TEFb (20), a complex of cyclin T and CDK9. This localizes P-TEFb to target promoters where it phosphorylates RNA Polymerase II (RNAPII) and releases it from pausing.

Alternative splicing generates three isoforms of BRD4: the long-form isoform A (13622 aa), isoform B (796 aa), and isoform C (722 aa) (21). Isoforms B and C lack the C-terminal domain and are distinguishable from each other by the presence of an additional 76 amino acid peptide at the C terminus of isoform B. Isoform B has only been identified in U2OS cells, and its activity is not well characterized (21). A separate set of studies identified two isoforms, a long-form (BRD4-LF) that corresponds to isoform A and a short-form (BRD4-SF) that most likely corresponds to isoform C (22, 23). BRD4-LF and BRD4-SF have differing effects on metastasis: BRD4-LF reduces metastasis while BRD4-SF promotes metastasis of the mouse mammary tumor Mvt-1 model of breast cancer (22, 24). It is

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possible that the ratio between the long and short isoforms of BRD4 dictate the oncogenic potential of BRD4, and differences in the ratio of these isoforms could explain why only a few studies have generated data suggesting that BRD4 acts as a breast tumor and metastasis suppressor (24–26) while the majority of studies demonstrate that BRD4 is an oncogenic driver.

Binding of BET proteins to acetylated histones recruits BET proteins to the enhancer and promoter regions of genes marked for active transcription. Here, they interact with co-activators/repressors, transcription factors, and the transcriptional machinery, forming protein complexes that influence target gene transcription (27). While they have a similar structure and usually enhance transcription, BET proteins regulate different processes based on their binding partners, which are often tissue-specific.

2.2 BET proteins and transcription

When BET proteins bind acetylated histones, they recruit several regulatory complexes that influence various aspects of chromatin structure and transcription. For example, BRD4 is important for transcription initiation. BRD4 regulates monoubiquitination of histone H2B (H2Bub1) and interacts with histone modifying proteins, such as the arginine demethylase JMJD6, and chromatin remodeling enzymes, such as CHD8 (28–31). This leads to a more open conformation of the chromatin and allows BRD4 to recruit key transcriptional regulators, such as Mediator and transcription elongation factors, to the DNA (32–35). BRD4 additionally enhances transcription of genes with ERα-bound estrogen response elements (EREs) that are also enriched for FOXA1 binding. BRD4 binds acetylated histones, including histone H4 acetylated at lysine 12 (H4K12ac) at these elements and recruits RNAPII which initiates the transcription of eRNAs (28, 36).

In addition, BET proteins are involved in transcriptional elongation. BRD4 directly promotes elongation by phosphorylating RNAPII at Ser2 of the C-terminal domain (37) and recruiting P-TEFb (30, 38, 39). P-TEFb in turn directly phosphorylates the carboxy-terminal heptad repeats of paused RNAPII and disrupts the interaction between RNAPII and the regulatory complexes DSIF and NELF (40, 41). Both events release RNAPII from the paused state at the promoter and thereby induce transcriptional elongation. During elongation, both BRD2 and BRD3 act as histone chaperones, remodeling histones in order for RNAPII to move along the DNA (42). BRD3 also recruits the polymerase-associated factor complex (PAFc), which coordinates several events during transcription, and the super elongation complex (SEC), which also regulates elongation (43). Based on the activity of BET proteins during transcription initiation and elongation, the disruption of BET protein function should have an enormous impact on the production of RNA transcripts in varied cell contexts.

2.3 BET proteins and the cell cycle

BET proteins play diverse roles in multiple phases of the cell cycle and control expression of cell cycle and proliferation genes (19, 42, 44–46). During mitosis, BRD4 ensures proper chromosomal segregation and cytokinesis by regulating expression of Aurora kinase B (47). Both BRD2 and BRD4 facilitate mitotic memory by remaining bound to M/G1 genes during

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mitosis and recruit P-TEFb to the DNA late in mitosis. This marks G1 genes for immediate transcription following mitotic exit, ensuring cell cycle progression (48–50). BRD4 also promotes G1-S and G2-M phase transitions (51, 52). As a result of their role in the regulation of the cell cycle, loss of BET expression induces cell cycle arrest (53–59).

2.4 BET proteins and inflammation

BET proteins also control inflammation. BRD2 binds to genes regulated by STAT5, an important mediator of cytokine signaling, and pan-BET inhibition suppresses expression of STAT5-target genes (60). Furthermore, mice that express half the amount of *Brd2* as wild-type mice (*brd2 lo*) develop severe obesity but are protected against insulin resistance and the obesity-induced inflammatory response (61). siRNA-mediated knockdown of *BRD2* suppresses NF-κB transcriptional activity (62). In addition to regulating pro-inflammatory gene expression though NF-κB, BRD2 directly binds the promoter regions of pro-inflammatory genes, especially following LPS stimulation, and macrophages from *brd2 lo* mice produce significantly less pro-inflammatory cytokines such as TNF-α, IL1β, and IL6 (62). NF-κB activity is also regulated by BRD4; BRD4 binds acetylated RelA, a subunit of NF-κB, and enhances transcription of NF-κB-dependent inflammatory genes (63). Suppression of BET proteins induces anti-inflammatory responses (62, 64), and inhibitors of BET proteins are currently being investigated as potential therapeutic options for the treatment of inflammatory diseases (65).

2.5 BET proteins and development

BET proteins are crucial during development, and loss of either BRD2 or BRD4 results in embryonic lethality. BRD2 controls neuronal differentiation (66), with *Brd2* null embryos displaying deficient neural tube formation and dying during mid-gestation (67). BRD4 maintains the self-renewal capability of stem cells by stimulating expression of genes involved in pluripotency. Over 20% of pluripotency genes, including *NANOG*, *OCT4*, *SOX2*, and *PRDM14*, are bound by BRD4 in embryonic stem cells (68–72). BRD4 also localizes to a number of stem cell genes in preimplantation embryos and maintains the inner cell mass, with BRD4 null embryos dying shortly after implantation (70, 73).

2.6 BRDT and spermatogenesis

Unlike the other three BET proteins which are ubiquitously expressed, BRDT expression is normally testis-specific (74). It is expressed in pachytene and diplotene spermatocytes and round spermatids, and its expression decreases during spermatid differentiation (75). BRDT is essential for spermatogenesis, and *BRDT* knockdown leads to sterility in male mice (75). Pharmacological inhibition of BRDT also confers reversible infertility in male mice without impacting testosterone levels, and offspring produced by these mice once treatment is removed are normal (76). As a result, BRDT is seen as a potential target for reversible male contraception.

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3. BET PROTEINS IN CANCER

3.1 BET proteins and super-enhancers

BET proteins are involved in various diseases, including inflammation, viral infection, heart failure, and cancer (77, 78). It is thought that BET proteins primarily mediate their effects in disease pathogenesis and progression primarily by localizing to super-enhancers (SEs) at pathology-associated genes and driving their expression (43, 79, 80). An SE is a large contiguous cluster of enhancers within a locus that is associated with increased gene expression, DNase I sensitivity, histone tail acetylation, and transcription factor and co-activator binding (80, 81). SEs are frequently identified by ChIP-seq analysis using antibodies against histone marks such as H3K27ac or the transcriptional regulators Mediator and BRD4, as these proteins are enriched at SEs (80–82). SEs vary by cell type and the different binding sites in distinct cells underlie the expression of cell identity genes that specify that cell type (81, 82). In addition, SEs drive expression of disease-associated genes in numerous diseases, including Alzheimer's disease, type 1 diabetes, and cancer (82, 83). In cancer, SEs are enriched at oncogenes known to play a role in specific cancer types, including *MYC* and *IRF4* in multiple myeloma, *RUNX1* and *FOSL2* in glioblastoma, and *CD79B* in diffuse large B cell lymphoma (79, 80). They are also associated with many oncogenes that are linked to general cancer pathogenesis, including *CCND1*, *MCL1*, and *BCL2L1* (80).

Only a fraction of enhancer regions are classified as SEs. For example, in multiple myeloma there are 308 putative SEs compared to nearly 8,000 typical enhancers (80). Despite the relatively small number of SEs, BRD4 disproportionately accumulates at these regions, with up to 40% of all bound BRD4 being localized to SEs (79, 80). BRD4, like other co-activators, exhibits cooperative binding. Thus, loss of BRD4 leads to a greater disruption of transcription at SE-associated genes compared to typical-enhancer associated genes, providing a mechanism to preferentially silence multiple SE-associated oncogenes at once (79, 80, 84).

3.2 Expression of the BET family in breast tumors and cell lines

BRD2, BRD3, and BRD4 are expressed in breast tumors while BRDT is rarely expressed. When examining all breast cancers, regardless of subtype, the genes encoding the BET proteins are amplified and/or overexpressed in less than 10% of tumors in the TCGA (85) and METABRIC (86, 87) datasets. However, when focusing on breast cancer subtypes, *BRD2* and *BRD4* are amplified and/or overexpressed in 12.1% and 20.6% of basal-like breast cancers, respectively (85). Analysis of copy number aberrations of 9445 tumors representing 20 cancer types in the TCGA dataset confirmed that *BRD4* is more commonly amplified in breast cancer as well as ovarian, liver, and endometrial cancers compared to cancers from other organs (88). In addition, *BRD4* was found to be amplified in a study evaluating focal amplification events in a panel of 10 DCIS and 151 invasive breast tumors representing all subtypes of breast cancer (89). Lastly, *BRD4* mRNA was more highly expressed in tumors compared to normal breast tissue (89). Together, these data suggest an association between *BRD4* levels and breast tumorigenesis.

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BRD4 is expressed in non-transformed breast epithelial, luminal breast cancer, and TNBC cell lines (90). By interrogating a microarray dataset of 477 breast cancer samples, Shi *et al.* found there was no difference in BRD4 mRNA expression between ER+ and ER- tumors (90). However, BET expression is less consistent at the protein level. When comparing BRD4 protein expression in two TNBC (MDA-MB-231 and BT549) and two ER+ (MCF7 and T47D) cell lines, T47D cells clearly express higher levels of BRD4 compared to the other three lines (91). In TNBC cell lines (MDA-MB-231, MDA-MB-468, and HCC1937), BRD4 is expressed at similar levels in all three lines while BRD2 and BRD3 expression was higher in HCC1937 cells (54). In addition, a larger study examining basal protein expression of BRD2, BRD3, and BRD4 in an 18 cell line panel representing non-transformed mammary, luminal, HER2+, and TNBC cell lines found that expression of BET proteins was variable and did not correlate with breast cancer subtype (53). Thus, despite relatively constant expression at the RNA level, BET protein expression varies by cell line, suggesting post-transcriptional or post-translational mechanisms are responsible for the observed differences in BET protein expression.

3.3 Essentiality of BET proteins in breast cancer

In an effort to identify subtype-specific and pan-disease essential genes in breast cancer, Marcotte, *et al.* utilized pooled lentiviral shRNA dropout screens in 78 breast cancer and four non-transformed mammary epithelial cell lines and analyzed the results using an algorithm they developed termed “the siRNA/shRNA mixed effect model” (siMEM) (92). This process successfully detected known drivers of breast cancer and breast cancer subtypes, affirming its utility. In addition, this study revealed new candidates for essential breast cancer genes, including *BRD4*. Silencing of *BRD4* gene expression using two shRNAs in SUM159, BT474, and T47D cells resulted in decreased proliferation, and this response was prevented by restoring BRD4 expression using an shRNA-resistant *BRD4* cDNA. However, sensitivity to *BRD4* depletion does not always translate into sensitivity to BET inhibitors, suggesting that BRD4 has both BRD-dependent and BRD-independent roles in breast cancer. This concept is supported by the finding that SUM159 cells that are resistant to BETi are still sensitive to genetic silencing of BRD4, indicating BRD4 may be recruited to the chromatin through a BRD-independent mechanism or that BRD4 has a role that is independent of chromatin binding (53).

Evidence that BRD4 may be essential for growth of estrogen-dependent breast cancer cells was first provided by a group that applied a novel triclustering algorithm to a publicly available microarray dataset corresponding to a time course of estrogen response of MCF7 cells (93). This approach revealed that *BRD4* may be a hub-gene in estrogen receptor-driven breast cancer (94). Later functional studies in four TNBC cell lines (SUM159, MDA-MB-231, MDA-MB-468, and MDA-MB-436) and one luminal line (ZR-75-1) using RNAi-mediated silencing of BET proteins demonstrated that suppression of either *BRD2* or *BRD4* reduces the growth of four of the cell lines with only siBRD4 inhibiting growth of MDA-MB-436 cells (53). Subsequently, we reported that simultaneously silencing *BRD2* and *BRD4* reduces expression of *BRD3* as well, suggesting a complex interplay in the regulation of these factors. We further showed that combined *BRD2/4* silencing reduces the expression of key mitotic regulators that play a crucial role in the BETi response of TNBC cells (44).

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Together, these findings further revealed a role for BET proteins in breast cancer growth and pathogenesis.

4. TARGETING BET PROTEINS IN BREAST CANCER

4.1 BET inhibitor structure and selectivity

The first BETi to be developed were JQ1 (95) and I-BET (64). Since then, multiple derivatives have been utilized in preclinical settings, including I-BET151 (43), I-BET762 (96), MS417 (97), and OTX015 (98), and at least 11 have moved on to be assessed in early phase clinical trials for a wide variety of hematologic cancers and solid tumors. BETi belong to varied chemical classes based on their core scaffolds, such as azepines (JQ1, OTX-015, CPI-0160), 3,5-dimethyl isoxazoles (I-BET151), pyridones (ABBV-075), and tetrahydroquinolones (I-BET762) (99).

While there are 42 BRD-containing proteins in humans (16), BETi selectively target the BET family of proteins (64, 95, 100). Most BETi inhibit all four members of this family, although BETi that selectively bind a specific BET protein, particularly BRD4, are currently being developed. BETi inhibit BET proteins by competing with acetylated lysines for binding to both BRD regions, preventing BET proteins from binding histones and thus from localizing to the chromatin (95). The specific BET protein(s) that must be suppressed for BETi to elicit their effects differs depending on the context. For example, in breast cancer models, we found that loss of BRD2 and BRD4 expression together was necessary for the induction of mitotic catastrophe in response to BETi in TNBC (44) while other studies have identified BRD4 as the sole critical target of BETi for controlling other phenotypic responses (53, 55, 101).

Multiple studies have examined the utility of BETi in various models of breast cancer (Table 1). Depending on the subtype studied and cell lines used, BETi can impact tumor formation, proliferation, the response to hypoxia, angiogenesis, cancer stem cells, metastasis, and metabolism by repressing the expression of genes that drive these critical oncogenic pathways (Figure 1). The effects of BETi in breast cancer reported thus far are reviewed below. It is important to keep in mind that the activity of BETi *in vivo* most likely depends on a convergence of several of these BETi-induced outcomes as opposed to a singular response.

4.2 Impact on breast cancer cell growth and tumor formation

Many cell lines representing the luminal (ER+), HER2+, and TNBC subtypes of breast cancer undergo growth inhibition in response to BETi treatment. Marcotte, *et al.* found luminal and HER2+ cell lines were more dependent on BRD4 expression than TNBC cells, as siRNA-mediated knockdown of *BRD4* led to a greater suppression of growth in these lines compared to basal cell lines (92). However, when comparing the IC_{50} of five BETi in a panel of 41 cell lines that represent luminal, HER2+, and TNBCs as well as non-transformed mammary epithelial cells, Polyak and colleagues found that TNBC cell lines were generally more sensitive to BETi than HER2+ and luminal cell lines (53). The discrepancy between

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these two studies could be due to the genetic silencing of a single BET protein compared to inhibition of the entire family with a small molecule.

Several studies have specifically assessed BETi activity in luminal breast cancer. BETi suppressed growth of MCF7, ZR75-1, and T47D cells with or without estrogen stimulation in 2D and 3D culture, and JQ1 inhibited expression of canonical estrogen-target genes (28, 56, 91, 102, 103). Tamoxifen-resistant (Tam-R) and estrogen-deprivation-resistant versions of ER+ cell lines were more sensitive to BETi than the parental lines (104). In addition, within two days, BETi treatment induced apoptosis of Tam-R MCF7 cells but not of parental MCF7 cells (104), suggesting BETi may be an effective treatment option for hormone therapy-resistant tumors. The difference in sensitivity of parental and Tam-R breast cancer cells to BETi likely stems from differences in the expression pattern of critical transcription factors. For example, parental MCF7 cells had higher expression of GATA3, which is necessary for sustained *ESR1* (ERα) gene expression, compared to Tam-R cells (105). Supporting this potential mechanism of BETi sensitization, silencing *GATA3* in parental cells rendered them more sensitive to BETi treatment (104).

Affirming the relative resistance of parental MCF-7 cells to BETi *in vitro,* JQ1 failed to impact MCF7-derived tumor growth in mice (102). MCF-7 cells are a model of luminal A breast cancer. In contrast to studies with these xenografts, JQ1 was reported to be effective in the MMTV-PyMT mouse model of luminal B breast cancer (106). Precursor lesions in this model are ER+ while established tumors are ER- but maintain a luminal gene expression signature (9, 107). Pérez-Salvia, *et al.* discovered that not only could JQ1 suppress growth of established tumors in MMTV-PyMT mice, but the drug could also slow the development of spontaneous mammary tumors when administered to four week old mice prior to the detection of palpable tumors (103). In addition, JQ1 improved overall survival in MMTV-PyMT mice. Based on these data, the authors suggested that BETi could be utilized as a preventative agent in women who have a high risk of developing breast cancer. BETi are quickly cleared from the target tissue, hence it would be necessary to treat these patients daily. Importantly, it is not yet known whether long-term exposure to BETi will lead to intolerable toxic side effects and increase the chances of developing BETi resistance.

Information on BETi treatment alone in HER2+ breast cancer growth is limited. In one study, both JQ1 and I-BET762 inhibited the *in vitro* growth of four HER2+ cell lines within five days in a dose-dependent manner (101). However, in a four-week clonogenic assay, BETi-resistant colonies still formed. Addition of lapatinib to BETi dramatically reduced the number of colonies formed, thereby inhibiting acquired BETi resistance.

The response of TNBC to BETi has been more thoroughly documented. When Shu, *et al.* treated 26 TNBC cell lines with BETi, the majority were highly sensitive to this drug class (53). Similarly, we found JQ1, I-BET151, and I-BET762 treatment suppressed growth of a panel of seven TNBC cell lines representing five of the six TNBC subtypes described by Lehmann, *et al.* (108) as well as both the claudin-low and basal subtypes in a dose-dependent manner (109). Multiple other studies have also shown that BETi suppressed 2D and 3D growth, wound-healing capacity, and colony formation of diverse TNBC cell lines (53, 54, 57, 58, 91, 110), indicating BETi could be an effective therapy across diverse TNBC

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tumor types. Our studies and others also revealed that sustained inhibition of BET proteins induced two terminal responses, apoptosis and senescence, and these responses did not correlate with the extent of BETi-induced growth inhibition, impact on c-Myc expression, or TNBC subtype (109). These effects were recapitulated *in vivo*. Tumors derived from MDA-MB-231 cells, which senesced *in vitro*, grew significantly slower when treated with JQ1, while MDA-MB-468 tumors, which died *in vitro*, partially regressed (109). In addition, JQ1 suppressed growth of tumors formed from a TNBC patient-derived xenograft (PDX). Three other studies confirmed the *in vivo* efficacy of BETi in TNBC: JQ1 and MS417 inhibited growth of MDA-MB-231, SUM1315, and SUM159 xenografted tumors as well as two PDX models (53, 57, 90). Together, these studies indicate that models of TNBC are highly responsive to BET inhibition both *in vitro* and *in vivo*.

It has been suggested that BETi induce subtype switching in TNBC, where TNBC cells lose basal markers and gain luminal markers, due to differential expression of luminal and basal cytokeratins following JQ1 treatment of the MDA-MB-231 and SUM159 cell lines (53). Differentiation of TNBC models following BETi treatment has also been assessed *in vivo*. Treatment of a PDX model with vehicle or JQ1 and staining for low molecular weight (luminal) and high molecular weight (basal) cytokeratins revealed that vehicle-treated tumors had very little expression of low molecular weight cytokeratins while their expression increased significantly with JQ1 treatment. These data, coupled with the *in vitro* analysis, suggested that BETi may induce differentiation of basal tumors to a more luminal phenotype. However, this conclusion was based on the restricted analysis of cytokeratin gene expression. In contrast, when vehicle- and JQ1-treated SUM159 xenografts were stained for diverse luminal (luminal cytokeratin, CK18, and CD24) and basal (basal cytokeratin, CK17, pSTAT, and CD44) markers, JQ1-treated tumors had variable responses, and there was no consistent loss of basal and simultaneous gain of luminal markers. Similarly, we performed GSEA using Neve (111) and Charafe-Jauffret (112) breast cancer subtype classifiers on gene expression array data from MDA-MB-231 and HCC70 cells treated with vehicle or JQ1 (44). While both cell lines lost expression of a subset of basal-signature genes, there was no consistent gain of expression of luminal genes, indicating TNBCs do not undergo extensive BETi-mediated differentiation to a characteristic luminal expression signature.

4.3 BET inhibitors and hypoxia

Severe intratumoral hypoxia is common in breast cancer (113). Hypoxia in solid tumors occurs due to increased metabolism and proliferation as well as poor vascular structure. It is linked to metastatic progression, resistance to radiation and chemotherapy, and poor prognosis (114). As in many other types of cancer, regions of hypoxia in breast tumors are associated with EMT and the acquisition of cancer stem cell properties via signaling through hypoxia-inducible factors (HIFs) which stimulate EMT, promote self-renewal, and inhibit differentiation (115). The phenotypes associated with hypoxia are regulated by HIF-1α and HIF-2α which heterodimerize with HIF-1β in low oxygen conditions. This complex then localizes to hypoxia response elements in the promoters of target genes to induce transcription. Compared to the other subtypes of breast cancer, TNBC is particularly associated with hypoxia, and HIF target genes are upregulated in TNBC patient tumors (116).

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OTX015 suppressed growth of three TNBC cell lines in both normoxic and hypoxic conditions, and GSEA following gene expression profiling revealed this drug downregulated hypoxia-responsive genes (54). In a second gene expression analysis study, when MCF7 and MDA-MB-231 cells were treated with JQ1 in normoxic and hypoxic conditions, JQ1 also altered expression of hypoxia-related genes and prevented the hypoxia-mediated upregulation of several gene sets, including those involved in angiogenesis and the hypoxic pathway (110). In MDA-MB-231 cells in particular, JQ1 altered expression of 44% of hypoxia-responsive genes, the majority of which were suppressed with drug exposure. While expression of HIF-1α and HIF-2α remained unchanged, JQ1 reduced expression of carbonic anhydrase 9 (*CA9*), a known hypoxia-responsive gene that helps to maintain a neutral intracellular pH (117, 118), in MCF7 cells, two TNBC cell lines (MDA-MB-231 and HCC1806), and HCC1806 xenografts. Notably, high expression of *CA9* has been associated with poor overall survival and a higher rate of distant metastases in a cohort of over 3600 breast cancer patients, and inhibition of CA9 suppresses metastasis (119). Thus, BETi inhibition of CA9 expression may provide an approach to limit metastatic progression. Mechanistically, the JQ1-induced reduction in CA9 expression was accompanied by the loss of HIF-1β binding at the *CA9* promoter following JQ1 treatment in hypoxic conditions (110). Thus, BETi prevented the localization of the HIF heterodimer to HIF target genes. Exposure to hypoxia can induce radio- and chemo-resistance, and inhibiting CA9 in combination with radiotherapy or chemotherapy is effective in preclinical models (120, 121). Together, these data suggest that BETi may be useful for sensitizing cancers to radiotherapy and/or chemotherapy, making it an effective approach for the treatment of solid tumors, including breast tumors.

4.4 BETi and angiogenesis

In addition to disrupting hypoxia-regulated pathways, BETi also appear to suppress angiogenesis, one of the hallmarks of cancer (122). Hypoxia and angiogenesis are inherently linked, with hypoxia inducing expression of VEGF that then stimulates the production of new blood vessels (123). Angiogenesis is critical for tumor growth and metastasis, making it a useful therapeutic target, particularly in renal cell carcinoma. Monoclonal antibodies against VEGFA or the VEGF receptor (VEGFR) and tyrosine kinase inhibitors that target VEGFR have been developed, but clinical trials in breast cancer have yielded mixed results, leading to the revocation of FDA approval of the anti-VEGFA antibody, bevacizumab, in breast cancer patients in 2011 (124). Therefore, it is essential to develop additional strategies to effectively disrupt angiogenesis. BETi have been shown to suppress angiogenesis in rhabdomyosarcoma, Ewing sarcoma, and testicular germ cell tumors (125, 126), suggesting they may also display anti-angiogenic activity in other tumor types, including breast cancer.

Only one study has directly assessed the impact of BETi on angiogenesis in breast cancer. In MCF7 and MDA-MB-231 cells, JQ1 prevented the upregulation of angiogenic signature genes under hypoxic conditions (110). BRD4 bound the promoter of *VEGFA* in MDA-MB-231 cells, and this binding increased in hypoxia. Both treatment with JQ1 and gene silencing of *BRD4* suppressed expression of VEGFA in hypoxia in MCF7, MDA-MB-231, and HCC1806 cells. In HCC1806 xenografted tumors, JQ1 also suppressed expression of *VEGFA*, as well as the *TIE2* and *NRP* genes that are critical for angiogenesis.

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Immunostaining revealed these tumors had lower expression of the blood vessel marker CD31. These data indicate BETi may impair angiogenesis. This could be due to a double hit: direct loss of BRD4 at the promoter regions of genes involved in angiogenesis and the suppression of the hypoxic response leading to the inhibition of hypoxia-induced angiogenic pathways.

Anti-angiogenic therapies are already used to treat numerous solid tumor types. However, drug-induced hypoxia occurs in the tumors of about half of these patients, leading to therapeutic resistance (114). Combining bevacizumab with CA9 knockdown suppressed colon cancer and glioblastoma xenografts growth better than bevacizumab alone (127). As mentioned above, BETi downregulated CA9 in breast cancer cell lines and xenografts, suggesting that combining BETi with anti-angiogenic agents could be a beneficial treatment strategy and revive the use of drugs such as bevacizumab in breast cancer.

4.5 BET inhibitors and cancer stem cells

Cancer stem cells (CSCs) are involved in numerous processes during tumor initiation and progression, are resistant to traditional cytotoxic chemotherapies, and play a role in metastasis and recurrence (128), making them a desirable target for anti-cancer therapies. A role for BET proteins in the maintenance of stem genes is now well established (68–71). In embryonic stem cells, inhibition of BET proteins suppress expression of critical stem cell factors and induce differentiation (71, 72). Extending to cancer, BETi induce apoptosis in progenitor and stem cells in acute myeloid leukemia (AML) and glioblastoma (129, 130). In MYC-driven medullablastoma, BETi reduce stem cell signaling and the self-renewal capacity of tumor cells (131).

The only subtype of breast cancer that has been investigated thus far for the impact of BETi on CSCs is TNBC. Expression of *WNT5A*, which plays crucial roles in maintaining stem cell pluripotency, was suppressed by JQ1 treatment due to reduced binding of BRD4 at its promoter (90). JQ1 also inhibited activity of the JAK/STAT pathway that promotes stem cell renewal as well as the pro-inflammatory response and EMT (53). Similarly, a more extensive study utilizing OTX015 found that BETi altered stem cell-related gene expression patterns. GSEA of OTX015-treated TNBC cells showed an overall loss of expression of CSC genes (54). In general, OTX015 downregulated CSC genes in three TNBC cell lines within 24 hours, although some of the genes that changed and the direction in which they were altered were cell line-specific. NANOG and OCT4, transcription factors that promote stemness, were suppressed as were two additional stem cell markers, CD133 and Musashi-1. Breast CSCs are often defined by high expression of CD44 and low expression of CD24. OTX015 treatment reduced CD44 expression in three cell lines while the impact on CD24 was variable. The suppression of stem cell markers by OTX015 was confirmed *in vivo* in mice bearing MDA-MB-231 tumors. However, expression of the epithelial marker EpCAM did not increase in any of the cell lines assessed. No other epithelial markers were examined, so it is unclear if OTX015 is capable of initiating differentiation in CSCs. As mentioned above, our studies indicated that TNBC cells do not undergo a basal to luminal transdifferentiation in response to JQ1 (44). Thus, while the loss of stem cell markers that occurs with BETi

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indicates a loss of the stem cell phenotype in TNBC, it is not accompanied by the acquisition of a luminal breast cancer profile.

In a separate study, JQ1 did not alter expression of three stem cell markers (CD44, CD49, and CD133) in MDA-MB-231 cells, yet it did significantly decrease the formation of primary and secondary tumorspheres (59). It will be important in the future to perform additional functional tests *in vitro* and *in vivo* to determine if and how BETi directly impact the population of stem cells in breast cancers.

4.6 BET inhibitors and metastasis

The vast majority of breast cancer patients do not die from their primary tumor. Instead, they succumb to metastatic lesions that develop in vital organs. Metastasis is a multi-step process, which includes invasion of surrounding tissue, intravasation and survival within the bloodstream, extravasation, and colonization of a distant organ (132). An early event during this cascade is EMT, a process that enables epithelial cells to adopt a more mesenchymal, motile phenotype (133). Not only do cells that undergo EMT become more migratory but they also acquire stem cell characteristics. BRD4 has been shown to regulate EMT in cancer. In several types of cancer, overexpression of BRD4 triggered metastasis while BET inhibition altered expression of key EMT genes, thereby preventing metastasis (134–137). Additionally, high expression of BRD4 correlated with lymph node metastasis in non-small cell lung cancer and renal cell carcinoma (138, 139).

In breast cancer, changes in BRD4 expression or treatment with BETi impact expression of genes linked to EMT and metastasis. Another set of proteins, those belonging to the extracellular matrix (ECM), can also modulate the EMT response. This has been demonstrated in mammary epithelial cells, with laminin inhibiting EMT and fibronectin promoting EMT following the addition of the EMT-inducing enzyme matrix metalloproteinase-3 (140). In addition, multiple mouse and human studies have revealed that expression of ECM genes are frequently dysregulated in tumors that are likely to metastasize (141–145). Modulating BRD4 levels by ectopic overexpression or gene silencing altered expression of ECM regulatory genes in breast cancer cell lines (24, 55). Overexpression of BRD4 also changed the activity of genes involved in other processes important for EMT and metastasis, including cytoskeletal remodeling and cellular adhesion (24). In addition, BRD4 regulates the expression of the HOX transcript anti-sense RNA (HOTAIR), a long non-coding RNA that promotes metastasis, regulates breast CSC properties, and is a biomarker for breast cancer diagnosis and metastasis (146–149). When claudin-low cells were grown in laminin rich ECM 3D cultures, BRD4 maintained *HOTAIR* expression by binding its promoter (150). As expected, treatment with JQ1 or gene silencing of *BRD4* decreased *HOTAIR* expression. Together, these data indicate that BET proteins, and specifically BRD4, control the production and sensing of the extracellular matrix, a key regulator of cellular motility.

In addition to modulating the ECM, BET proteins directly regulate EMT-modulating transcription factors. Inhibiting BET proteins with JQ1 reduces the binding of the transcription factor, activating enhancer binding protein 4 (AP4), to the *MYC* promoter, leading to the downregulation of *MYC* (58). AP4 expression is linked to EMT, metastasis,

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and poor prognosis in several cancers, including breast (151–153), suggesting that BETi may suppress metastatic progression, at least in part, by modulating AP4 activity.

In another study by Andrieu and colleagues, JQ1 suppressed migration and invasion in MDA-MB-231 and SUM149T cells (55). In this case, *JAG1* was identified as a key target of BRD4. This gene encodes Jagged, a Notch receptor family ligand involved in EMT, metastasis, proliferation, survival, and resistance to therapy (154). The clinical relevance of this finding is demonstrated by Kaplan-Meier analysis of 664 breast cancer patients (155) revealing that high co-expression of *BRD4* and *JAG1* is associated with shorter distant metastasis-free survival (55). BRD4 binding to the promoter of *JAG1* is enhanced by the pro-inflammatory cytokine interleukin 6 (IL6), which is linked to EMT, migration, invasion, and metastasis and is secreted by the tumor microenvironment (156–158) and leads to increased Jagged1 protein levels. Increased Jagged1 in turn activates Notch1 to promote migration and invasion. Consistently, treatment with JQ1 prevents the recruitment of BRD4 to the *JAG1* promoter, reducing Notch1 activation. BETi have previously been shown to have anti-inflammatory activity (62, 64), and these findings are further enhanced by the discovery that BETi can combat the pro-metastatic effects of IL6 from the tumor microenvironment by modulating Jagged1/Notch signaling. These data further support the notion that BETi can inhibit the secretion of pro-metastatic factors into the tumor microenvironment and may improve patient outcomes. However, the specific impact of BETi on metastatic spread was not assessed.

Another mechanism by which BRD4 can directly modulate motility and invasion of breast cancer cells involves its interaction with Twist, a transcription factor that plays an essential role in the activation of EMT (159). When Twist is di-acetylated, it interacts with the second BRD of BRD4 (90). This Twist-BRD4 complex drives expression of a set of EMT genes and thus maintains mesenchymal characteristics of TNBC cells. One of the gene targets of this complex is *WNT5A*. As mentioned above, WNT5A is a secreted factor that regulates various aspects of cancer cell properties, including proliferation, self-renewal, migration, and invasion (160). The *WNT5A* gene has a putative super-enhancer, and binding of Twist to this locus is important for the recruitment of BRD4, P-TEFb, and RNAPII to the *WNT5A* enhancer and promoter (90). BETi disrupts the interaction between BRD4 and Twist, leading to the suppression of invasiveness in TNBC cells. Both JQ1 treatment and gene silencing of BRD4 in five basal-like breast cancer cell lines suppressed the expression of *WNT5A* as well as invasion and tumorsphere formation. These data suggested that BRD4 may modulate metastatic outgrowth. However, similar to the analyses by Andrieu and colleagues, this was not directly tested. Rather, the authors reported that two BETi, JQ1 and MS417, inhibited growth of primary SUM1315 tumors partially via the suppression of *WNT5A* expression.

Only two studies have directly assessed the ability of BETi to impact the breast cancer metastatic cascade *in vivo*. We found that JQ1 treatment reduces the number of liver macrometastases in mice with tumors derived from the metastatic TNBC cell line, MDA-MB-231 (109). However, a second study using two highly metastatic cell lines (Mvt-1 and 6DT1) revealed that while I-BET151 lowered primary tumor weight, it did not suppress the incidence of pulmonary metastasis (23). Several differences between the models used may explain this discrepancy. MDA-MB-231 cells are a claudin-low human breast cancer cell

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line that was examined in immune-compromised mice whereas Mvt-1 and 6DT1 murine mammary cancer cells were studied as allografts in immune-competent mice. In addition, the molecular classification of Mvt-1 and 6DT1 cell lines is mixed with both having elements of luminal and claudin-low gene expression signatures (161). The use of cell lines that have their own unique transcriptomes could account for the apparent differences in the impact of BETi on metastasis in these two studies. Lastly, these studies interrogated metastatic potential to different organs. The impact of I-BET151 on metastasis of Mvt-1 or 6DT1 to the liver or organs other than the lungs was not assessed, and it is possible that BETi modulate microenvironmental sensing in one tissue context but not another. Additional analysis of a broader spectrum of tumor models will be necessary to elucidate the molecular drivers that define the impact of BETi on metastasis.

4.7 BET inhibitors and metabolism

Deregulated cellular metabolism is a hallmark of cancer, with cancer cells having different energy needs than normal cells due to increased cell division and proliferation as well as altered access to nutrients (122). Very little is known regarding the impact of BETi on cellular metabolism in breast cancer. One study found the BETi, XD14, significantly altered the expression of 67 metabolites in the ER+ MCF7 cell line (162). These metabolites included amino acids, fatty acids, lipids, and phospholipids and could be grouped into 12 pathways including those that regulate amino acid levels. Eight amino acids as well as glucose were elevated following XD14 treatment, suggesting that XD14-treated MFC7 cells consumed less energy than vehicle-treated cells. Similarly, we found that “metabolism” was one of the top Reactome pathways altered in HCC70 cells after treatment with JQ1 (44). To complement these descriptive reports, mechanistic and functional studies are necessary to clarify the effect of BETi on metabolism and how this impacts breast cancer pathogenesis.

4.8 Mechanism(s) of action of BET inhibitors in breast cancer

BET proteins act as co-activators or co-repressors depending on their binding partners and cellular context. Considering their critical roles in modulating transcription, it is not surprising that gene expression analyses have revealed that BETi alter expression of hundreds of genes in breast cancer cells (28, 44, 54, 57, 91, 101, 103, 104, 163). The genes impacted by BETi vary depending on breast cancer subtype and the cell lines used. However, one of the most consistent findings among many of these reports is that BET inhibition induces cell cycle arrest. The majority of studies that performed cell cycle analysis on BETi-treated breast cancer cells found that multiple ER+ and TNBC cell lines arrest in the G1 phase as early as 24 hours after drug addition (53–59). This occurred following the suppression of cell cycle genes (44, 104). Nonetheless, the mechanism by which cell cycle arrest occurs seems to be subtype, and even cell line, dependent.

A major regulator of proliferation in varied cell types is MYC. In numerous non-breast cancer models, BETi dramatically suppress expression of c-MYC, and overexpression of c-MYC can reduce sensitivity to BETi (164, 165). Thus, BETi have been touted as “Myc inhibitors.” However, multiple studies have found that neither *MYC* amplification nor BETi-mediated suppression of c-Myc were involved in the response of TNBC to BETi, because BETi had an inconsistent impact on c-Myc expression in various cell lines representing this

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subtype of breast cancer (53, 54, 58, 109, 110). Furthermore, overexpression of *MYC* in SUM149, HCC38, and EVSAT cells did not induce JQ1 resistance (92), indicating that modulation of MYC likely contributes only modestly to the BETi responsiveness of TNBC cells. In contrast, c-Myc suppression may play a more substantial role in the BETi response of ER+ and HER2+ breast cancers. Several studies reported downregulation of c-Myc in cell lines representing both of these subtypes, including those with acquired drug resistance (58, 91, 101–104). Furthermore, MYC-overexpressing BT474 cells were less sensitive to JQ1 treatment (101). It is possible these results could be partially driven by the time point selected, as at least one study showed c-Myc expression initially decreased but then rebounded in BETi-treated MCF7 cells (104). Another caveat of these studies is that they utilized a limited number of cell lines which may not fully represent the response of the luminal and HER2+ subtypes as a whole. Indeed, qRT-PCR analysis of 24 breast cancer cell lines representing the luminal, HER2+, and TNBC subtypes treated with JQ1 revealed variable regulation of *MYC* mRNA, and the suppression or upregulation of *MYC* did not correlate with growth sensitivity to JQ1 (92). Interestingly, GSEA of MDA-MB-231 and MDA-MB-468 cells treated with vehicle or OTX015 revealed that MYC target genes were downregulated following OTX015 treatment even though *MYC* itself was not suppressed (54). This could indicate that BETi can regulate MYC-responsive genes through a mechanism other than by the direct transcriptional repression of the *MYC* gene itself.

In contrast to MYC, we found that BETi profoundly disrupted mitosis in TNBC, partially due to the downregulation of Aurora kinases A and B (*AURKA*/*B*) (109). AURKA and AURKB play critical roles in multiple steps of mitosis, and altered expression of these genes induces polyploidy (166–169). Indeed, treatment with JQ1 suppresses expression of AURKA and AURKB, leading to polyploidy in MDA-MB-231 cells and multi-nucleation in several TNBC cell lines. The impact of BETi on *AURKA* and *AURKB* gene expression is direct. BRD4 binds to the promoter regions of both genes and JQ1 reduces BRD4 recruitment to these loci. In addition, treatment of TNBC cells with an AURKA-selective (MLN8237) or an AURKB-selective (AZD1152) inhibitor phenocopies JQ1; both induced multi-nucleation followed by either senescence or apoptosis. These data suggest Aurora kinases could potentially be used as biomarkers to predict response to BETi therapy.

We further reported that mitotic dysfunction induced by BETi results in mitotic catastrophe, as indicated by the suppressed expression of mitosis/cytokinesis-associated genes, increased mitotic timing, and acquisition of multi-nucleation or induction of apoptosis in or immediately following mitotic exit (44). The global downregulation of genes involved in mitosis/cytokinesis was mediated, at least in part, by the suppression of the critical mitosis regulator *LIN9* by JQ1. Silencing *LIN9* alone suppressed similar cell cycle genes as JQ1 and led to multi-nucleation, indicating that *LIN9* is an important regulator of mitotic progression in TNBC that is also a key target of BETi. In addition, genes that were highly correlated with LIN9 in breast cancer or had a LIN9 binding site in HeLa cells were more likely to be downregulated by JQ1 than those that were not correlated. Interrogation of publically available datasets revealed that *LIN9* is amplified and/or overexpressed in two-thirds of basal breast cancers, and its expression is linked to poor prognosis, underscoring its clinical impact on this disease and its potential utility as a biomarker for predicting BETi responsiveness of TNBCs.

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As in other cancers, SEs have been detected in several oncogenes in breast cancer cell lines (44, 53, 170–172). Shu, *et al.* identified 219 SEs in SUM159 cells and 159 SEs in SUM149 cells, and SEs were found at known gene drivers of TNBC, including *MYC* and *HIF1A* (53). Treatment of SUM159 and SUM149 cells with JQ1 led to a rapid (within 12 hours) suppression of transcription of SE-associated genes, and the number of SEs increased when cells developed BETi resistance. These studies suggested that SE modulation may play a key role in mediating the effects of BETi in breast cancers. However, we found that the impact of BETi on mitosis in TNBC was not related to the suppression of SE-associated genes, as *LIN9* and four other JQ1-regulated master mitosis transcription factors were not associated with SEs (44). Cancer cells are particularly sensitive not only to SE disruption but also to mitotic catastrophe (173). We thus hypothesize that the selectivity of BETi for cancer cells, at least in TNBC, is due to a combination of loss of SEs and mitotic dysregulation via the induction of mitotic catastrophe.

The observed downregulation of critical genes in breast cancer cells following BETi treatment can also result from the prevention of transcriptional elongation rather than disruption of SEs. In a study using ER+ breast cancer cells, Sengupta, *et al.* found that while BETi did not impact ERα expression or recruitment to promoters and EREs, it does alter transcription of estrogen-target genes (56). JQ1 only slightly suppressed the recruitment of RNAPII to the promoters of the estrogen-target genes *TFF1*, *GREB1*, and *XBP1*. In contrast, there was a large reduction in RNAPII binding to the bodies of these gene following JQ1 treatment. This indicates that the transcription complex was able to form and initiate transcription in the presence of BETi but transcription elongation was inhibited. BRD4 recruits P-TEFb to the chromatin which releases RNAPII from its paused state at promoters to stimulate elongation (39). It is likely that the impact of BETi on elongation is due to the loss of P-TEFb presence at gene promoters, thus restraining RNAPII at the promoter and preventing its progression to the gene body.

4.9 Resistance to BET inhibitors

Thus far, two mechanisms of resistance to BETi have been identified in breast cancer. One of these is activation of the PI3K pathway (Figure 2A). JQ1 did not inhibit proliferation of cell lines derived from mouse mammary tumors with amplified *Myc* in conjunction with either an activating mutation in *PI3K* (MCCL-278) or deletion of *Pten* (MCCL-357), despite suppressing MYC protein expression at higher (≥2 μM) doses (174). A second BETi, MS417, did suppress growth in both cell lines by at least 50% but only at high (≥4 μM) doses. Similar observations were made using the TNBC cell line SUM159 which has an activating mutation in *PIK3CA* and amplified *MYC* (175). These results indicate that tumors with activated PI3K pathway and high MYC expression could be intrinsically resistant to BETi. Supporting this conclusion, analysis of shRNA dropout screens in 82 breast cancer and mammary epithelial cell lines using the algorithm siMEM also identified *PIK3CA* mutation as a potential BETi resistance mechanism (92). Cell lines that did not die in response to BETi were more likely to have a mutated form of *PIK3CA*, and SKBR3 cells overexpressing wild type or mutated *PIK3CA* were less susceptible to JQ1-induced growth inhibition. Although tumors with altered PIK3CA are less sensitive than their wild type counterparts, they can still respond to BETi to some degree. Indeed, the BETi, MS417,

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suppressed growth of both MCCL-278 and MCCL-357 tumors by about 50% (174), and a separate study found growth of SUM159 xenografts was significantly reduced in response to JQ1 treatment (53). The partial responsiveness of PIK3CA mutant cancers to BETi suggests that combining AKT pathway inhibitors with BETi should provide an effective therapeutic approach for these cancers. This is supported by preclinical *in vivo* data that will be discussed below in the section focused on combination therapies.

A second mode of BETi resistance was discovered by Shu, *et al.* (53). In this case, the authors developed BETi-resistant SUM159 (SUM159R) and SUM149 (SUM149R) cells via long-term culture with gradually increasing doses of JQ1. BRD2, BRD3, and BRD4 still localized to the chromatin despite the presence of JQ1, indicating that a factor that controls BET protein binding to other chromatin-associated proteins may be altered. SUM149R and SUM159R cells had higher levels of phosphorylated BRD4 (pBRD4), and there was enhanced binding of pBRD4 to MED1 compared to unphosphorylated BRD4, allowing pBRD4 to localize to the chromatin even in the presence of BETi (Figure 2B). PP2A was identified as a BRD4 phosphatase, and PP2A activity was diminished in BETi-resistant cells. These data indicate phosphorylation of BRD4 can confer BRD-independent functions and maintain activity of BRD4, thus providing a mechanism to overcome the effects of inhibitors targeted to the BET BRDs. They further suggest that activators of PP2A may prevent BETi resistance. Lastly, these studies support the development of alternative approaches for inhibiting BET protein function that do not rely on the BRDs. Inroads in this last effort will be discussed later in this review.

4.10 BET inhibitor adverse effects

BETi are generally non-toxic in mice. Mice treated with BETi did not lose weight (57, 88, 103, 104, 109, 176), and the only adverse effects observed were reversible male infertility and reduced long-term memory formation (76, 177). BETi can also decrease uterine size and weight in female mice due to the suppression of estrogen-target genes (28), but the potential impact of BETi on fertility in female patients is unknown. In the normal adult mammary gland, BETi did not alter rates of proliferation or apoptosis and had no impact on ductal branching following one week of treatment, indicating BETi, at least in the short-term, do not negatively impact the virgin adult gland (109). However, it is unclear if and how long-term BETi treatment will affect the normal breast architecture or function in women, especially those who are pre-menopausal. Estrogen signaling is critical for terminal end bud formation during puberty in mice, and estrogen is involved in the formation of milk ducts in women during the menstrual cycle (178). Because BETi suppress expression of estrogen-target genes (56), it will be important to determine if BETi alter the expression of such genes in the normal adult breast in order to predict the potential impact of BETi on this tissue, especially in pre-menopausal women.

One study has also utilized BRD4 silencing to predict untoward effects of BETi. In mice, loss of BRD4 reduced the T lymphoid and hematopoetic stem cell populations, indicating BETi may prevent normal hematopoiesis (179). Furthermore, after five weeks of silencing, the mice experienced alopecia and skin hyperplasia. These animals also had reduced cellular diversity in the intestine, including a loss of several cell types that compose the secretory

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lineage as well as stem cells in the crypts. While the intestines of shBRD4 mice seemed to function normally, they displayed impaired regenerative capability following insult by radiation or chemotherapy agents that are known to damage the intestine. This suggests that combining BETi with chemotherapies may result in intolerable gastrointestinal and skin effects in patients.

Following these preclinical studies, BETi are currently being assessed in clinical trials in a wide variety of hematologic and solid tumors, and results from three phase 1 studies assessing OTX015 in acute leukemia (41 patients), NUT midline carcinoma (NMC; 4 patients), lymphoma (33 patients), and multiple myeloma (12 patients) have been published (180–182). Across these three studies, the most frequent grade 3–4 adverse event was thrombocytopenia, with other common side effects including diarrhea, anemia, neutropenia, fatigue, hyperglycemia, and nausea. The NMC study is the only trial thus far to assess any clinical activity of BETi in solid tumors. NMC is a rare but highly aggressive disease with a median survival of less than one year. It is driven by the translocation of *BRD3* or *BRD4* which results in the fusion of *BRD3/BRD4* and *NUT* genes (183, 184). The encoded BRD3/4-NUT fusion proteins have oncogenic activity and prevent epithelial differentiation, thereby contributing to the poorly differentiated phenotype of cells in NMC tumors (184). Of the four NMC patients treated with OTX015 in the published phase 1 trial, one patient achieved disease stabilization while two experienced tumor regression (180). Although these three patients initially rapidly responded to OTX015, they all relapsed, highlighting the importance of developing effective combination therapies with BETi. However, two patients survived significantly longer than the median 6.7 month survival time for NMC (18 and 19 months from initial diagnosis). Given the phase I nature of this study and the small cohort size, it is not possible to make broad conclusions regarding BETi efficacy in this disease. Nevertheless, it is promising that BETi more than doubled the survival time for two of four patients.

5. ALTERNATIVE BET PROTEIN TARGETING MECHANISMS

As described above, small molecule competitive inhibitors of BET proteins have profound anti-tumor effects in breast cancer models via suppression of multiple oncogenic pathways. However, the utility of BETi in the clinic could be hampered by toxicity associated with long-term treatment and/or the development of drug resistance. Additional therapeutic approaches targeting BET proteins are currently being developed, including combining BETi with other therapies (Figure 3), degrading BET proteins, and using dual kinase/BET protein inhibitors. These strategies and their performance in breast cancer models are reviewed below. We also discuss CDK7 inhibitors which may function in a similar manner to BETi and thus may provide similar therapeutic benefits to breast cancer patients.

5.1 Combination therapies

BETi synergize with anti-mitotic (docetaxel and vinorelbine) and DNA-damaging (cisplatin and carboplatin) agents (57, 88). Treatment of the TNBC cell lines MDA-MB-231 and HS578T with any of these drugs in combination with BETi induces greater apoptosis and growth inhibition than the single agents. It has also recently been reported that BETi

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synergize with platinum-containing agents in high-grade serous ovarian carcinoma (185), which has a high degree of genetic and transcriptomic similarity with TNBC (2). Therapies that are effective in TNBC or ovarian cancer may also be efficacious in the other (2), further supporting the continued study of BETi and chemotherapy combinations in TNBC, with a particular need to assess its synergy with platinum-containing agents.

Another strategy that could be effective in TNBC is combining BETi with PARP inhibitors (PARPi). Three separate BETi (JQ1, I-BET762, and OTX015) have been shown to synergize with two PARPi (olaparib and veliparib) in MDA-MB-231 cells, with the combination of BETi and PARPi also being effective in ovarian and prostate cancers (88). In mice, combined BET protein and PARP inhibition significantly reduced growth of orthotopic MDA-MB-231 tumors compared to either agent alone, and this treatment strategy did not induce obvious toxicity. In addition to increasing growth inhibition in anchorage-dependent and anchorage-independent conditions, adding BETi to PARPi treatment enhanced DNA damage. JQ1 decreased homologous recombination in response to ionizing radiation and instead increased repair by non-homologous end joining, supporting a mechanism for the synergy between BETi and PARPi. This resulted, at least in part, from the JQ1-mediated suppression of *BRCA1* and *RAD51*, genes that encode proteins that repair DNA damage via homologous recombination. Reduced binding of BRD2/3/4 at the promoter regions of these genes was observed in response to BETi.

Interestingly, cancer cell lines with *BRD4* amplification were less sensitive to PARPi, suggesting that adding BETi to PARPi treatment could increase PARPi sensitivity of tumors with amplification of *BRD4*. PARPi are particularly effective in tumors that are unable to perform repair of double strand breaks by homologous recombination, such as those with *BRCA1/2* mutations (186, 187). However, while *BRCA1/2* mutations are frequent in TNBC, a large number of breast cancers maintain the ability to perform homologous recombination (188). Data from this study indicate that combining BETi with PARPi can increase the efficacy of PARPi in tumors that lack *BRCA1/2* mutations and those that acquire homologous repair proficiency.

Histone deacetylase inhibitors (HDACi) have anti-tumor effects, are being evaluated in clinical trials, and several have been approved for use in specific types of cancer (189). Because BETi inhibit the recognition of acetylated histones and HDACi block histone acetylation, it was logical to expect that these drug classes would demonstrate synergism. Indeed, BETi synergize with HDACi in AML, pancreatic ductal carcinoma, melanoma, murine lymphoma, and T-cell acute lymphoblastic leukemia (T-ALL) (190–194). A few studies have found that this drug combination could also be a plausible treatment strategy in breast cancer. Comparing drug signatures of four HDACi (trichostatin A, vorinostat, scriptaid, and CP-690334-01) from the Connectivity Map database (CMAP) (195) with GSEA results from two TNBC cell lines (MDA-MB-231 and MDA-MB-468) treated with OTX015 revealed that HDACi and OTX015 regulated similar genes, further supporting the potential for synergy between these two drug classes in TNBC (54). Indeed, combination treatment of HDACi with BETi suppressed the growth of MCF7, MDA-MB-231, and BT549 cells *in vitro* and inhibited growth of two PDX TNBC tumor models *in vivo* to a greater extent than either the HDACi or BETi alone (91, 176). To identify mechanisms of synergy,

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Borbely and colleagues performed gene expression profiling in MDA-MB-231 cells. This revealed that several members of the USP17 family of deubiquitinating enzymes were upregulated following co-treatment with HDACi and BETi (91). The upregulation of *USP17* and *USP17L5* was confirmed in MCF7, T47D, MDA-MB-231, and BT549 cells, indicating activity in both luminal and TNBC cells. USP17 suppresses proliferation, and part of its anti-proliferative effect stems from its ability to inhibit Ras, thereby decreasing the activity of the Ras/MAPK pathway (196, 197). Combining BETi and HDACi repressed Ras at the protein level as well as phosphorylation of MEK and ERK1/2, indicating that changes in Ras function may underlie the synergy between the two agents. Recently, a dual HDAC/BRD4 inhibitor has been developed that inhibits growth of AML and chronic myelogenous leukemia (CML), *in vitro* (198–200). These studies suggest that either combining HDACi and BETi or use of a dual HDAC/BRD4 inhibitor may have efficacy in at least two subtypes of breast cancer.

BETi have also been shown to combat resistance to standard therapies in various breast cancer models. Tamoxifen resistance is a major clinical hurdle in the treatment of ER+ breast cancers. Roughly half of ER+ cancers do not respond to tamoxifen and as many as 40% of patients whose tumors initially respond relapse and succumb to their disease (201). This highlights the need to develop new treatment options for women with *de novo* or acquired tamoxifen resistance. Of the patients whose tumors acquire resistance to tamoxifen, 20% respond to second-line treatment with aromatase inhibitors or the ERα degrader fulvestrant. Fulvestrant is also approved as a first-line treatment for postmenopausal ER+ breast cancer (202). In tamoxifen-resistant (Tam-R) MCF-7 xenografts, BETi can restore fulvestrant sensitivity (104). Analysis of these tumors revealed reduced ERα protein expression and decreased Ki67 and histone H3 p-Ser10 staining in tumors treated with the combination therapy compared to either agent alone. These results suggest that combining BETi with fulvestrant could provide a novel therapeutic option for women with Tam-R breast cancer. Indeed, the BETi GS-5892 is currently being investigated in a phase 1 trial in combination with fulvestrant or the aromatase inhibitor exemestane in post-menopausal women with advanced ER+ breast cancer (NCT02392611).

A second study found that estrogen regulates the binding of BRD4 to both the transcription start site of estrogen-dependent genes as well as distal ERα-regulated enhancers (36). The presence of H4K12ac was highly correlated with BRD4 binding at these loci. When comparing global H4K12ac in MCF7 (ER+ breast cancer) to MCF10A (ER− non-transformed mammary epithelium) cells, MCF7 cells had higher levels of this histone mark. In addition, treatment of MCF7 cells with fulvestrant globally reduced H4K12ac levels, indicating that BET proteins work with ERα to position these marks. These results support the finding by Feng, *et al.* that combining BETi with fulvestrant or other anti-estrogens could be a therapeutic strategy in Tam-R ER+ breast cancers (104). These findings also suggest the utilization of agents that target HATs and HDACs (described above) that modulate H4K12 acetylation could prevent BRD4 localization to the chromatin and thus have anti-tumor effects in ER+ breast cancers.

Numerous kinase inhibitors have been approved for use in patients with breast cancer. These agents target receptor tyrosine kinases (RTKs) as well as various signaling intermediates.

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However, acquired resistance to kinase inhibitors is common and can occur via multiple mechanisms, including mutation of the targeted protein(s) or the activation of alternative pathways or pathway members, such as RTKs, that bypass the suppression of the targeted kinase and maintain activity of an oncogenic pathway (203, 204). It is critical to develop new therapeutic options that will prevent or overcome resistance to kinase inhibitors to extend their clinical utility and prevent disease recurrence. In this regard, BETi have been shown to synergize with a wide variety of kinase inhibitors. Nieto-Jiménez, *et al.* performed gene expression analysis of a publically available dataset (205, 206) comparing normal epithelial cells and basal-like breast tumors to identify differentially expressed kinases (59). Several druggable mitotic kinases, including AURKA/B and polo-like kinase 1 (PLK1), were expressed at higher levels in breast tumors compared to the normal epithelium. The IC_{50}s for drugs targeting these kinases were determined in four basal-like cell lines (MDA-MB-231, HS578T, BT549, and HCC3153) with volasertib, a PLK1 inhibitor, having the lowest IC_{50} in all four cell lines. Moreover, this drug synergized with JQ1 to repress growth and stimulate apoptosis. This is particularly notable given our studies that have demonstrated that BETi induce profound mitotic dysfunction in TNBC cells and underscores the potential utility of targeting mitotic vulnerabilities through varied avenues (44).

Using the connectivity maps (CMAP) described above to compare drug signatures to the results of gene expression profiling of two TNBC cell lines treated with OTX015, Vázquez, *et al.* found that OTX015-target genes overlapped with those that are modulated by a number of agents, including the PI3K inhibitor LY294002 (54). This highlights the potential utility of combining BETi with agents targeting the PI3K/AKT/mTOR pathway. Treatment of two TNBC cell lines (MDA-MB-231 and HCC1937) with a combination of OTX015 and the mTOR inhibitor everolimus for 72 hours had an additive effect, suppressing growth in both lines. In contrast, the combination was antagonistic in MDA-MB-468 cells. It is unclear what determines the outcome of combining BETi and inhibitors of the PI3K/AKT/mTOR pathway, and this warrants future study. The *in vivo* efficacy of combining OTX015 and everolimus was also assessed. Mice with xenografted tumors derived from MDA-MB-231 cells were treated with OTX015, everolimus, or the combination for four weeks. As previously reported (207), the MDA-MB-231 model was resistant to mTOR inhibition. In contrast, OTX015-treated tumors were significantly smaller than vehicle-treated tumors. More importantly, the combination of these two drugs significantly reduced tumor size and was more effective than either single agent. In addition, tumors treated with the combination remained smaller than tumors from the other treatment groups 20 days after the end of treatment, indicating a durable response to BETi/everolimus in this model. Notably, the combination of OTX015 and everolimus was also more effective than treatment with paclitaxel, the current standard of care for TNBC (208).

In contrast to TNBC, mTOR inhibitors are much more efficacious in luminal breast cancer models, and everolimus is currently FDA-approved for treating patients with ER+ breast cancers when used in combination with exemestane (209). However, everolimus resistance is common and understanding the mechanisms underlying resistance may reveal new therapeutic approaches. Relevant to BETi, enhanced BRD4 binding at the *MYC* locus can confer resistance to everolimus in parental ER+ breast cancer cells and in long-term estrogen deprived (LTED) ER+ breast cancer cells that mimic tumors with acquired resistance to

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tamoxifen or aromatase inhibitors (102, 210). Treatment of everolimus-resistant (Eve-R) parental and Eve-R LTED MCF7 cells with JQ1 reduced BRD4 binding to the *MYC* gene and suppressed MYC expression (102). JQ1 also resensitized Eve-R parental and LTED cells to everolimus, as the combination treatment had a greater growth suppressive effect and induced cell death. To assess the *in vivo* efficacy of combining BETi and everolimus, MCF7 xenografts were treated with vehicle, JQ1, everolimus, or JQ1+everolimus for three weeks. Everolimus suppressed tumor growth by approximately 50%, while JQ1 alone had no impact. However, combining JQ1 and everolimus synergized to significantly reduce tumor size compared to either single agent. In addition, when treatment was removed and tumors were allowed to regrow for 25 days, tumors that had been treated with the combination therapy were significantly smaller than everolimus-treated tumors. A second study confirmed these findings. In this case, JQ1 synergized with the mTOR inhibitors rapamycin or Torin to suppress growth of MCF7 and T47D cells (92). MCF7 xenografts began to regress within 15 days of JQ1 and everolimus co-treatment, while JQ1 alone did not impact tumor growth and everolimus alone suppressed tumor growth but did not induce regression. These results further affirm the potential addition of BETi to everolimus to provide greater therapeutic benefit to patients with ER+ breast cancer and potentially overcome resistance to mTOR inhibitors. It will be necessary in the future to assess if these findings are applicable to other mTOR-targeting agents.

Lapatinib, a reversible competitive inhibitor of EGFR and HER2, is FDA-approved for use in patients with HER2+ breast cancer who have already been treated with an anthracycline, a taxane, or trastuzumab. While tumors initially respond to this small molecule, resistance is common (211). During the development of resistance, cancer cells undergo kinome reprogramming wherein a variety of protein kinases are upregulated to overcome loss of HER2-mediated signaling. Stuhlmiller, *et al.* found that an entire network of kinases are altered within 48 hours of lapatinib treatment *in vitro*, and the kinases responsible for lapatinib resistance were cell-line dependent (101). The authors reasoned that utilizing a single kinase inhibitor would be unable to address the heterogeneity of response to lapatinib due to the variety of kinases that drive lapatinib resistance. Instead, they focused on preventing the adaptive kinome response by targeting epigenetic modulating enzymes and found BETi could not only resensitize resistant HER2+ cells to lapatinib but also prevent the onset of lapatinib resistance. Combination treatment of lapatinib with JQ1 increased the extent of apoptosis and was more effective than combinations of lapatinib with dasatinib (Bcr-Abl and Src family kinase inhibitor) or other kinase inhibitors targeting IGF1R/INSR (BMS754), FAK (PF228), or FGFR (BGJ298) in parental and lapatinib resistant (Lap-R) cells. In the presence of lapatinib, BETi inhibited expression of the kinases involved in lapatinib resistance in both parental and Lap-R HER2+ cells. BETi prevented BRD4 localization to the promoters and enhancers of lapatinib-responsive genes and decreased the accumulation of p-Ser2 RNAPII at their promoters and gene bodies. Addition of BETi to lapatinib further inhibited the recruitment of BRD4 and RNAPII to the chromatin. This suggests that the mechanism by which BETi prevent lapatinib-induced kinome reprogramming is by inhibiting the recruitment and formation of the P-TEFb complex with BRD4 and RNAPII, thus leaving RNAPII paused at the promoters of lapatinib-responsive genes. This mechanism is consistent with the model proposed by Sengupta, *et al.* for ER+

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breast cancer where BETi prevent transcription elongation at estrogen-target genes (56) and suggests that a common path by which BETi prevents therapeutic resistance is through blocking the reprogramming of the active genome in response to various growth-inhibitory drugs.

The MEK inhibitor trametinib is approved for use in patients with melanoma and non-small cell lung cancer with activated BRAF and is currently being evaluated for efficacy in TNBC. While tumors initially respond, resistance occurs due to the upregulation and activation of other kinase pathways that can overcome dependence on the MEK-ERK signaling pathway (212). In TNBC cell culture, mouse xenografts, and biopsied TNBC patient tumors, trametinib treatment quickly induced the upregulation of several tyrosine kinases with dramatic restructuring of the enhancer landscape and acquisition of over 1,000 enhancers, including numerous SEs (170). This is very similar to the kinome reprogramming observed in lapatinib-treated breast cancer cells discussed above, and, consistent with that study, BETi can collaborate with trametinib in SUM159PT and MDA-MB-231 cells to inhibit proliferation better than either agent alone. Adding a BETi also prevents the *de novo* formation of typical enhancer and SE regions observed with trametinib treatment alone. As anticipated, the combination treatment also blocked the upregulation of several genes involved in the adaptive response. While the authors did not show evidence of apoptosis, the combination also increased the expression of BIM, suggesting an increase in BAX/BAK priming. Lastly, BETi were able to overcome acquired resistance to trametinib in SUM159 cells, similar to what was observed in lapatinib-resistant HER2+ cells (101). *In vivo,* the combination of trametinib and I-BET151 significantly inhibited tumor growth compared to either agent alone in SUM159PT xenografts as well as in two TNBC orthotopic syngeneic mouse tumor transplant models. This study provides foundational support for clinically assessing the utility of adding BETi to kinase inhibitors to prevent acquired resistance that occurs via adaptive bypass signaling.

In addition to using BETi to overcoming resistance to various therapies, approaches for blocking resistance to BETi themselves have also been identified. As mentioned above, Shu, *et al.* discovered that hyperphosphorylated BRD4 can confer resistance to BETi in TNBC (53). Either targeting CK2, the kinase responsible for phosphorylating BRD4, using the CK2 inhibitor CX-4945 or activating the phosphatase PP2A using perphenazine suppressed growth of both parental and BETi-resistant TNBC cell lines when used in concert with JQ1. In addition to excessive phosphorylation of BRD4 in BETi resistant cells, these cells acquire an SE at the gene encoding Bcl-xL. ABT737, a pan-Bcl-2 family inhibitor, synergized with JQ1 in both parental and resistant cell lines.

As previously mentioned, human breast cancer and mouse mammary epithelial cell lines with either mutated PI3K alone or mutated PI3K plus amplified MYC are resistant to BETi (92, 174). Co-inhibition of BET proteins and PI3K effectively suppressed growth and induced apoptosis of breast cancer cells. T47D and MCF7 cells treated with JQ1 and A66 (PIK3CA-selective inhibitor) underwent greater growth inhibition compared to JQ1 alone or JQ1 plus TGX221, a PIK3CB-selective inhibitor (92). The combination of JQ1 and PIK3CAi was also effective in the basal cell line SUM159 which has an activating *PIK3CA* mutation. In another study, the combination of BETi and GDC-0941, a PI3K inhibitor,

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suppressed growth and induced cell death more than either single agent *in vitro* and *in vivo* (174). Treatment with GDC-0941 alone induced rebound activation of AKT as well as enhanced expression of a number of RTKs, both of which indicate reactivation of the PI3K signaling pathway, an established mechanism of resistance. However, BETi prevented these compensatory changes by reducing BRD4 binding to GDC-0941-responsive genes. Adding BETi to GDC-0941 blocked the reactivation of the PI3K pathway in cell lines representing numerous cancer types, including seven breast cancer cell lines, six of which had activating mutations in the PI3K pathway but lacked *MYC* amplification. Hence, dual inhibition of BET proteins and PI3K could impact a broad spectrum of cancers by preventing feedback reactivation of the PI3K pathway. These data also suggest combining BETi with inhibitors that target the PI3K signaling pathway will be efficacious in patients with *PIK3CA* mutations and may combat PIK3CA-mediated BETi resistance, underscoring the need for clinical trials that assess the efficacy of combining BETi with PI3K/AKT/mTOR-targeting agents.

5.2 BET protein degraders

Generally speaking, small-molecule inhibitors are ideal agents for cancer therapy and have provided immense clinical benefit. However, they do have notable disadvantages (213), including the typical requirement for high concentrations to provide efficacy, increasing the risk for off-target effects. Small molecules that act by competitive inhibition such as ATP analogs also leave the targeted pathogenic protein(s) intact, providing an opportunity for resistance to develop. Small-molecule-based proteolysis-targeting chimeras (PROTACs) present an improved small-molecule approach that reduces the expression of target proteins by marking them for proteasomal degradation. A PROTAC is a heterobifunctional compound that combines two moieties: one that targets the protein of interest while the other binds a specific E3 ligase, effectively recruiting the target protein to the E3 ligase for ubiquitination and subsequent degradation by the proteasome (213). Several BET PROTACS have been developed, including dBET1, ARV-825, and ARV-771, all of which degrade BET proteins. These inhibitors link JQ1 and thalidomide (engaging cereblon E3 ligase), OTX-015 and pomalidomide (also engaging cereblon E3 ligase), or OTX-015 and a ligand that targets the von Hippel-Landau E3 ligase, and have shown greater efficacy against AML, Burkitt's lymphoma, mantle cell lymphoma, and castration resistant prostate cancer cell lines and PDX models compared to standard BETi treatment (214–217).

Thus far, only one report has examined the utility of PROTAC technology in breast cancer. Bai, *et al.* developed a PROTAC molecule (BETd-246) that tethers the potent BETi, BETi-211, to thalidomide (163). Treatment of several TNBC cell lines with BETd-246 effectively and selectively degraded the majority of BRD2, BRD3, and BRD4 within three hours. BETd-246 suppressed growth of 13 TNBC cell lines and was more potent than the parent BETi-211 compound. In the majority of TNBC cell lines tested, BETd-246 rapidly induced apoptosis, activating multiple apoptotic pathways within as little as five hours in some cell lines. Interestingly, RNA-seq analysis revealed BETd-246 evoked a unique transcriptional response compared to BETi-211 in TNBC cells, suggesting that the mechanism(s) of action of BETi may not be the same as BET PROTACs. BETd-246 also synergized with two dual Bcl-2/Bcl-xL inhibitors and a selective Bcl-xL inhibitor in six

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TNBC cell lines to produce strong anti-proliferative and pro-apoptotic responses. When examined *in vivo*, no toxicity was observed in several models representing immunocompetent or immunodeficient mice. Treatment of the Washington Human in Mouse (WHIM) PDX model and MDA-MB-453 xenografts with BETd-246 suppressed tumor growth and, at higher doses (10 mg/kg vs. 3 mg/kg), induced partial tumor regression. In these tumors, BET protein levels were significantly reduced within one hour after the first dose but rebounded once BETd-246 was cleared from the tissue (around 12 hours). In contrast to these results, BETd-246 was not effective in MDA-MB-231 or MDA-MB-468 xenografts due to poor delivery of the drug to the tumor tissue. This led to the optimization of BETd-246 to generate BETd-260. This derivative had anti-proliferative activity in TNBC cells, *in vitro*, but more importantly, suppressed growth of MDA-MB-231 and MDA-MB-468 xenografts without inducing observable toxic effects. Overall, these data suggest that PROTAC approaches may be more efficacious for breast cancer than BET inhibition and may prevent some of the acquired resistance mechanisms identified for BETi treatment due to the ability of PROTACs to selectively degrade their protein targets.

5.3 Dual protein kinase/BET protein inhibitors

One of the hallmarks of cancer is sustained proliferative signaling via the dysregulation of signaling pathways and aberrant regulation of signaling kinases (122). While oncogenic kinases are attractive therapeutic targets, compensatory pathways that cause acquired resistance have limited their long-term therapeutic impact (218, 219). Combination therapies have been proposed to subvert these issues. However, this approach has its own complications, including toxicities, drug-drug interactions, and increased costs. Ciceri, *et al.* proposed an alternative approach to combination therapy that could provide similar benefits while avoiding the same clinical development challenges by targeting multiple proteins with a single small molecule (220). They screened 628 kinase inhibitors for evidence of binding to BRD4 and found that nine directly interacted with BRD4 and prevented its binding to acetylated H4. These kinase inhibitors docked into the first BRD of BRD4 and included the PLK inhibitors BI-2536 and volasertib, the JAK inhibitor TG-101348, and the PI3K-mTOR inhibitors GSK2636771 and PP-242. Using BI-2536 and TG-101348 as exemplars, they further confirmed that these kinase inhibitors prevented the recruitment of BRD4 to chromatin and suppressed expression of c-Myc in the multiple myeloma cell line MM.1S. In addition, TG-101348 inhibited growth of MM.1S cells. Growth inhibition was restricted to these two inhibitors, as other selective PLK and JAK inhibitors did not interact with BRD4, repress c-Myc, or inhibit growth in MM.1S cells. Following this preliminary study, additional dual kinase/BET inhibitors have since been identified, and their efficacy has been assessed in a limited number of cancer models (221–227). While these preliminary studies are promising, more work is necessary to fully characterize the efficacy and mechanism(s) of action of dual kinase/BET inhibitors in different cancer types, including breast cancer.

5.4 CDK7 inhibitors

One of the mechanisms of action of BETi is the dismantling of SEs. Thus, agents that disrupt SE architecture through different mechanisms should also be useful either as single agents or in combination with BETi. In this regard, CDK7 inhibitors (CDK7i) have recently been shown to alter the composition of SEs in many cell types. Similar to BRD4, inhibition

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of CDK7 results in the preferential downregulation of SE-associated genes, including *RUNX1* in T-ALL, *MYCN* and *PHOX2B* in neuroblastoma, *SOX2* and *SOX4* in small cell lung cancer, and *PAK4* and *YAP1* in esophageal squamous cell carcinoma (228–231). CDK7 is responsible for phosphorylating, and thus activating, cell cycle CDKs as well as RNAPII at transcriptional start sites (232). CDK7, cyclin H, and MAT1 form a subcomplex of transcription factor II human (TFIIH) which plays roles in both transcription and DNA nucleotide excision repair. Before transcription can begin, TFIIH is recruited to the pre-initiation complex and unwinds a small portion of the DNA, giving RNAPII access to the transcriptional start site of the target gene. CDK7 phosphorylates RNAPII at Ser5, the first of two phosphorylation events required before transcription elongation can occur.

CDK7, cyclin H, and MAT1 expression levels are elevated in breast cancers, particularly in ER+ tumors, compared to normal tissue and are associated with better clinical outcome (233). However, analyses of publically available datasets and tissue microarrays have revealed high CDK7 protein expression is associated with poor outcome of TNBC patients (234), suggesting the potential utility of inhibiting CDK7 in TNBC. Several small molecules have been developed that target CDK7, and they have shown preclinical efficacy in breast cancer. CDK7i prevent phosphorylation of RNAPII, thus inhibiting transcription (235–237). This has a profound effect on short-lived proteins, including the depletion of c-Myc and the anti-apoptotic proteins Mcl-1 and X-linked inhibitor of apoptosis protein (XIAP) (237, 238). In ER+ breast cancer cells, CDK7i (BS-181, roscovitine, ICEC-0782, and SNS-032) suppressed proliferation and induced cell cycle arrest and apoptosis (235–239). These drugs also inhibited growth of MCF7 and MDA-MB-453 xenografts without inducing overt toxicity or impacting the normal mammary gland (235, 237, 238). In TNBC, shRNA-mediated knockdown of CDK7 reduced proliferation and migration and increased sensitivity to doxorubicin supporting the potential utility of CDK7i in TNBC (234). Indeed, the CDK7i THZ1 and BS-181 suppressed proliferation, induced apoptosis, reduced phosphorylation of CDK1 and RNAPII, and lowered expression of c-Myc and Mcl-1 in TNBC models. THZ1 additionally synergized with the BH3 mimetic ABT-263 to suppress growth and induce apoptosis.

While BETi and CDK7i produce similar outcomes in cancer cells, BRD4 and CDK7 control different sets of genes. For example, in diffuse intrinsic pontine glioma (DIPG), JQ1 altered expression of genes involved in nervous system development while THZ1 disrupted transcription-associated genes (240). Inhibition of CDK7 may thus provide an alternative to BETi, and it will be important in the future to determine which inhibitor class is more effective. It may also be possible to use co-inhibition of BET proteins and CDK7 as a therapeutic strategy, as JQ1 and THZ1 synergized in DIPG (240).

6. FUTURE PERSPECTIVES AND CONCLUSIONS

BET proteins are epigenetic readers that bind acetylated lysines in histones and transcription factors and tether transcriptional regulators to chromatin, thus impacting gene expression. The functions of BET proteins are dictated by their binding partners, which are context dependent. Several diseases, including cancer, have been linked to excessive BET activity, highlighting the clinical significance of developing drugs that target this protein family. In

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breast cancer, BETi impact the expression of genes that drive multiple hallmarks of cancer (122) such as inducing angiogenesis, resisting programmed cell death, activating invasion and metastasis, and deregulating cellular energetics (Figure 4). Furthermore, multiple mechanisms of action of BETi have been identified in breast cancer, including the suppression of *LIN9* expression and the disruption of SEs. It is unlikely that the efficacy of BETi is due to the suppression of a single gene or signaling pathway. Rather, BETi likely alter several key oncogenic pathways that work in concert to suppress cancer-associated pathologies.

Breast cancer is a highly heterogeneous disease comprised of several distinct subtypes, each of which is driven by a unique transcriptome. Despite the diversity within this collection of diseases, BETi elicit anti-tumor responses in several subtypes of breast cancer. However, to date there are no studies evaluating the response of inflammatory breast cancer (IBC) to BET-targeting agents. IBC is a rare but very aggressive subtype of breast cancer that is associated with poor prognosis. The role of BET proteins in inflammation and the observation that suppression of BET proteins reduces the expression of pro-inflammatory cytokines (53, 55, 60–62, 100) suggests BETi may provide a new treatment avenue for this poorly understood disease, and it will be important to test this possibility.

While BETi have a profound effect on hematologic cancers, many studies of BETi in solid tumors, including breast cancer, have concluded that BETi are more likely to induce tumor stasis rather than regression. Understanding the mechanism(s) of action of BETi in the individual subtypes of breast cancer should aid in the selection of appropriate patients for BETi therapy. For example, we discovered that LIN9 is a critical mediator of the BETi response in TNBC (44). This suggests tumors with high LIN9 expression may be particularly responsive to treatment with BETi, and LIN9 could potentially be used as a predictor of response to BETi in the clinic. In addition, single-agent therapies are risky due to the likelihood of acquired resistance. Therefore, it is important to identify alternative strategies to single-agent BETi treatment to improve their clinical utility. Numerous preclinical studies have revealed that BETi can be combined with agents targeting various oncogenic pathways to induce more durable, improved responses over single-agent treatment and to overcome acquired resistance. Many of the combination strategies involved agents that are already approved for use in various cancers. In addition, several FDA-approved kinase inhibitors also target BET proteins. Thus, alternatives to single-agent BETi treatment should be rapidly assessed in clinical trials following the conclusion of phase I studies examining the safety of these drugs. Overall, the impact of BETi in preclinical models of breast cancer is promising and further studies into their mechanisms of action should facilitate identification of the most effective combinations to accelerate their use in this heterogeneous disease.

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Abbreviations

BETi	Bromodomain and Extraterminal protein inhibitor
ER	estrogen receptor
PR	progesterone receptor
HER2	human epidermal growth factor receptor 2
TNBC	triple-negative breast cancer
EMT	epithelial-to-mesenchymal transition
BRD	bromodomain
HAT	histone acetyltransferase
RNAPII	RNA polymerase II
ERE	estrogen response element
H4K12ac	histone H4 acetylated at lysine 12
SE	super-enhancer
DCIS	ductal carcinoma in situ
siMEM	the siRNA/shRNA mixed effect model
Tam-R	tamoxifen-resistant
PDX	patient-derived xenograft
GSEA	gene set enrichment analysis
HIF	hypoxia-inducible factor
CA9	carbonic anhydrase 9
CSC	cancer stem cell
AML	acute myeloid leukemia
ECM	extracellular matrix
NMC	NUT midline carcinoma
PARPi	PARP inhibitor
HDACi	histone deacetylase inhibitor
CMAP	Connectivity Map database
RTK	receptor tyrosine kinase
LTED	long-term estrogen deprived

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Eve-R	everolimus-resistant
Lap-R	lapatinib resistant
PROTAC	proteolysis-targeting chimeras
CDK7i	CDK7 inhibitor
IBC	inflammatory breast cancer

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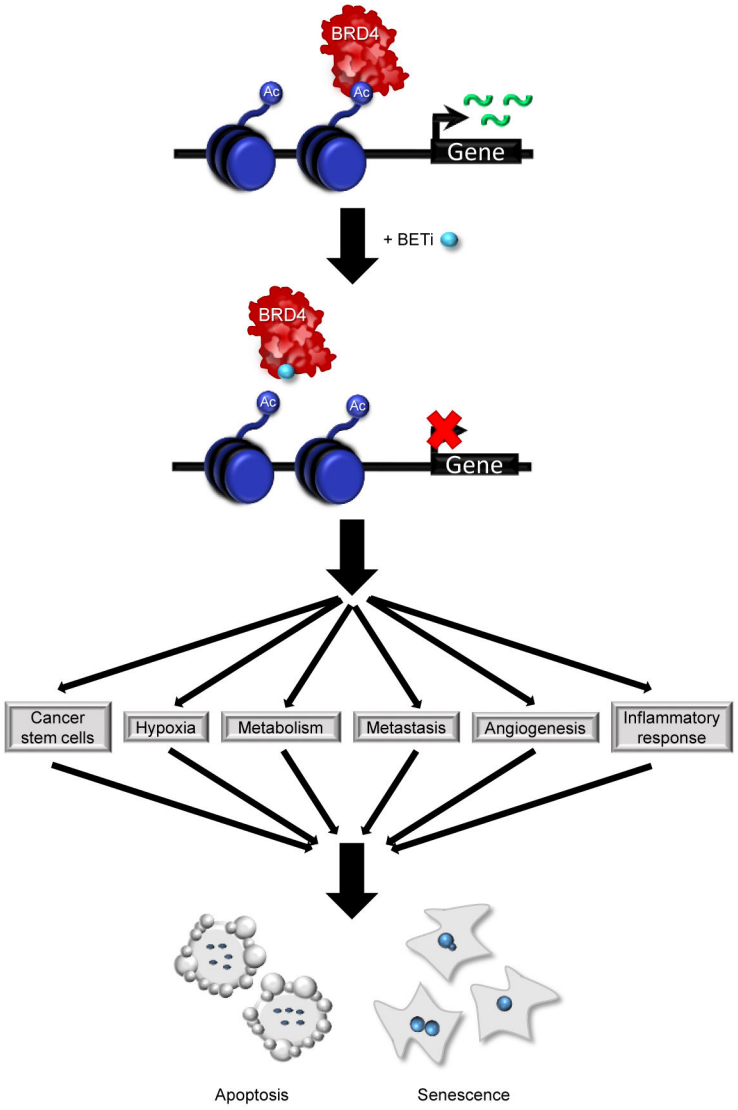

Figure 1. BET inhibitors suppress several oncogenic pathways in breast cancer

Binding of BETi to the BRD of BET proteins, such as BRD4, prevent BET proteins from binding to acetylated lysines in histone tails. This blocks BET protein localization to the promoter and enhancer regions of target genes, thus inhibiting their transcription. In breast cancer, outcomes of BETi include suppression of self-renewal properties in cancer stem cells, inhibition of hypoxia-induced pathways, altered metabolism, decreased metastatic potential, inhibition of angiogenic factors, and reduction in the pro-inflammatory response. In response to the disruption of several of these oncogenic pathways, breast cancer cells either undergo apoptosis or senescence.

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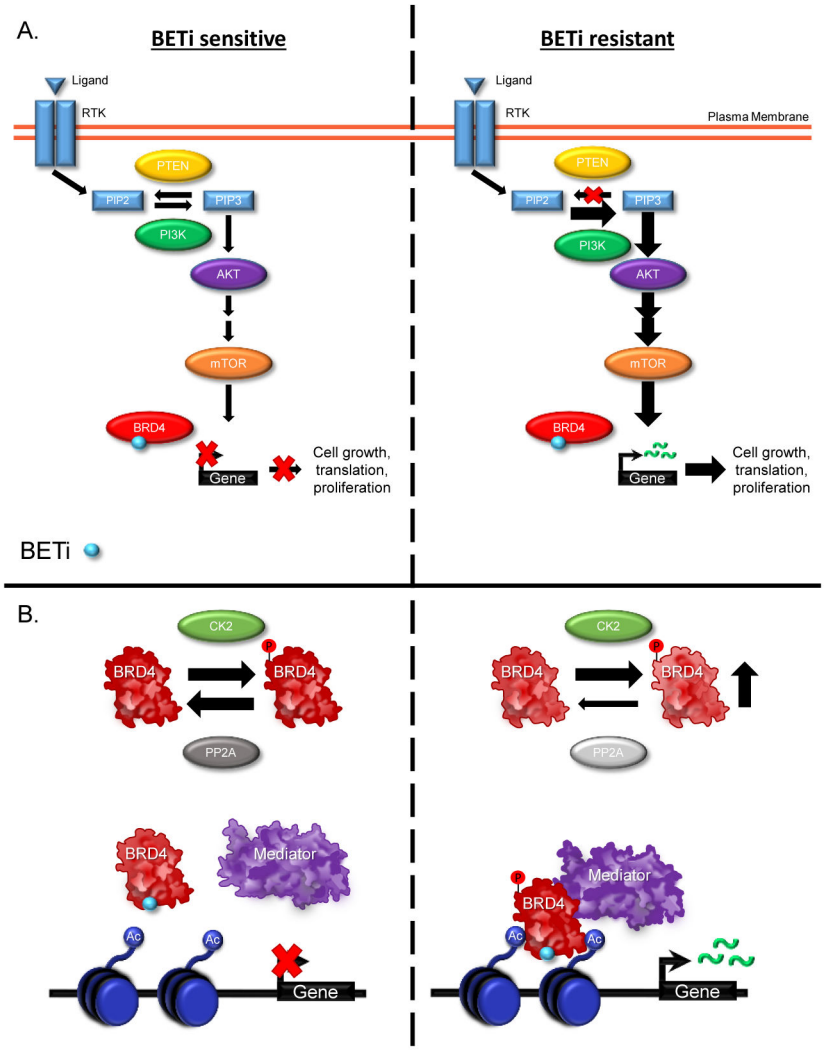

Figure 2. Mechanisms of BET inhibitor resistance in breast cancer

A. Increased activity of the PI3K/AKT/mTOR pathway. *Left:* BET inhibition and normal PI3K/AKT/mTOR signaling. The binding of ligands, such as growth factors, hormones, or cytokines, to receptor tyrosine kinases (RTKs) activates PI3K. PI3K coverts PIP2 to PIP3, and PTEN counteracts PI3K by dephosphorylating PIP3. PIP3 mediates the phosphorylation, and thus activation, of AKT which leads to the activation of mTOR. mTOR signaling normally drives critical cellular process including cell growth, translation, and proliferation, but BETi suppress expression of the genes responsible for these processes. *Right:* The PI3K/AKT/mTOR pathway can remain active if PTEN is mutated, preventing it from converting PIP3 to PIP2, or if PI3K has an activating mutation. Both mutations increase phosphorylation of PIP3, maintaining activity of AKT and mTOR and overcoming BETi-mediated suppression of gene expression. **B.** Increased phosphorylation of BRD4. *Left*: BRD4 is phosphorylated by CK2 and dephosphorylated by PP2A. When BETi (blue circle) binds to BRD4, it prevents localization of BRD4 to the chromatin. BRD4 can thus no longer recruit Mediator to the DNA, silencing target gene expression. *Right:* Reduced PP2A activity increases the abundance of phosphorylated BRD4 (pBRD4). Despite the binding of

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BETi to its BRD regions, pBRD4 can still localize to the chromatin and interact with Mediator, maintaining transcription of target genes.

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Figure 3. Combination treatments with BET inhibitors in breast cancer

Drugs that have been combined with BETi and resulted in greater efficacy are listed and color-coded according to the outcome of the combination treatment. Gray circles: drug synergized with BETi. Green circle: drug synergized with BETi, and the combination overcame tamoxifen resistance. Orange circle: drug synergized with BETi, and the combination overcame everolimus resistance. Purple circle: drug synergized with BETi, the combination overcame BETi resistance, and the combination prevented reactivation of the PI3K pathway. Yellow circle: the combination overcame and prevented lapatinib resistance. Red circle: drug synergized with BETi, and the combination overcame and prevented MEK inhibitor resistance.

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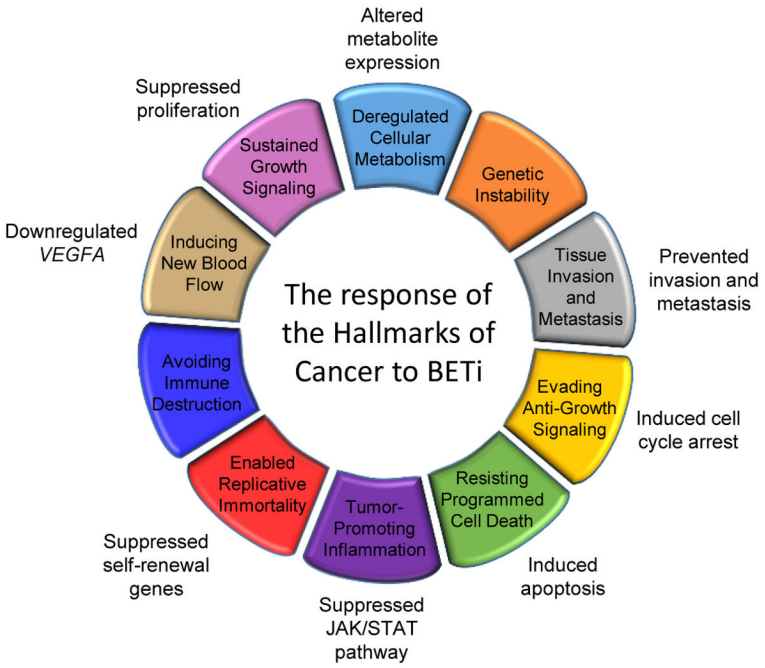

Figure 4. The response of the hallmarks of cancer to BET inhibitor treatment in breast cancer

BETi impact most of the hallmarks of cancer described by Hanahan and Weinberg. An example of a BETi-induced effect is listed next to the hallmarks that were altered by BETi in breast cancer cells.

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Table 1

Breast cell lines used to study BET proteins and their inhibitors.

Human Cell Lines		
Cell Line	**Origin**	**Comments**
MCF10A	Fibrocystic disease	ER− non-transformed mammary epithelium
MCF7	Invasive ductal breast carcinoma; derived from pleural effusion	ER+; luminal A
T47D	Invasive ductal breast carcinoma; derived from pleural effusion	ER+; luminal A
BT474	Invasive ductal breast carcinoma	ER+; luminal B
ZR-75-1	Invasive ductal breast carcinoma; derived from ascites	ER+; luminal B
MDA-MB-453	Breast adenocarcinoma; derived from pericardial effusion	HER2+; also subtypes as TNBC
HCC70	Ductal breast carcinoma	TNBC; basal-like
HCC1806	Primary breast acantholytic squamous cell carcinoma	TNBC; basal-like
HCC1937	Primary ductal breast carcinoma	TNBC; basal-like; *BRCA1* mutation
HCC3153	Ductal breast carcinoma	TNBC; basal-like; *BRCA1* mutation
MDA-MB-468	Breast adenocarcinoma; derived from pleural effusion	TNBC; basal-like
SUM149/SUM149PT	Invasive ductal breast carcinoma	TNBC; basal-like; inflammatory breast cancer; *BRCA1* mutation
BT549	Invasive ductal breast carcinoma	TNBC; claudin-low
HCC38	Primary ductal breast carcinoma	TNBC; claudin-low
HS578T	Invasive ductal breast carcinoma	TNBC; claudin-low
MDA-MB-231	Breast adenocarcinoma; derived from pleural effusion	TNBC; claudin-low; highly metastatic
MDA-MB-436	Invasive ductal breast carcinoma; derived from pleural effusion	TNBC; claudin-low *BRCA1* mutation
SUM159/SUM159PT	Anaplastic breast carcinoma	TNBC; claudin-low; activating *PiK3CA* mutation; amplified *MYC*
SUM1315	Invasive ductal breast carcinoma; derived from skin	TNBC; claudin-low; *BRCA1* mutation
EVSAT	Invasive ductal breast carcinoma; derived from ascites	ER−/HER2−; unknown subtype
Mouse Cell Lines		
6DT1	Mammary tumor from MMTV-Myc mouse	Highly metastatic
MCCL-278	Mammary tumor from MMTV-*Myc; Pik3ca*$^{H1047R+}$; *Wap*^{Cre} mouse	Activating *PI3K* mutation; amplified *Myc*
MCCL-357	Mammary tumor from MMTV-*Myc; Pten*$^{flox/flox}$; *Wap*^{Cre} mouse	Deletion of *Pten*; amplified *Myc*
Mvt-1	Mammary tumor from MMTV-Myc-VEGF mouse	Highly metastatic