



Targeting Cancer Cells with BET Bromodomain Inhibitors

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Cancer cells are often hypersensitive to the targeting of transcriptional regulators, which may reflect the deregulated gene expression programs that underlie malignant transformation. One of the most prominent transcriptional vulnerabilities in human cancer to emerge in recent years is the bromodomain and extraterminal (BET) family of proteins, which are coactivators that link acetylated transcription factors and histones to the activation of RNA polymerase II. Despite unclear mechanisms underlying the gene specificity of BET protein function, small molecules targeting these regulators preferentially suppress the transcription of cancer-promoting genes. As a consequence, BET inhibitors elicit anticancer activity in numerous malignant contexts at doses that can be tolerated by normal tissues, a finding supported by animal studies and by phase I clinical trials in human cancer patients. In this review, we will discuss the remarkable, and often perplexing, therapeutic effects of BET bromodomain inhibition in cancer.

In eukaryotic cells, sequence-specific DNA-binding transcription factors (TFs) activate their target genes by recruiting multisubunit coactivator complexes, which use diverse biochemical mechanisms to activate RNA polymerase II (Pol II). One important class of coactivators possess lysine acetyltransferase (KAT) activity, which transfers acetyl groups from acetyl-coenzyme A to the epsilon amino group of lysine residues of various substrate proteins. Many KAT enzymes (e.g., p300/CBP) have permissive substrate specificity and will acetylate unstructured, lysine-rich peptides found on TFs, histones, and various other components of the transcription apparatus (Dancy and Cole 2015). The pervasiveness of TF–KAT interactions in transcriptional regulation leads to a global partitioning of eukaryotic genomes

into hyperacetylated and hypoacetylated domains, which strongly correlate with active and inactive *cis*-regulatory regulatory elements, respectively (Wang et al. 2008).

One mechanism by which acetylation influences transcription is by neutralizing the positive charge of lysine side chains to disrupt electrostatic interactions (e.g., between histones and DNA), which can lead to chromatin decompaction (Roth et al. 2001). In an alternative mechanism, lysine side-chain acetylation of many transcriptional regulators will create docking sites for proteins possessing acetyllysine binding/reader domains. In this setting, acetyllysine serves a vital function in the assembly of the transcriptional apparatus at enhancer and promoter elements. The most well established acetyllysine reader is the bromodomain,

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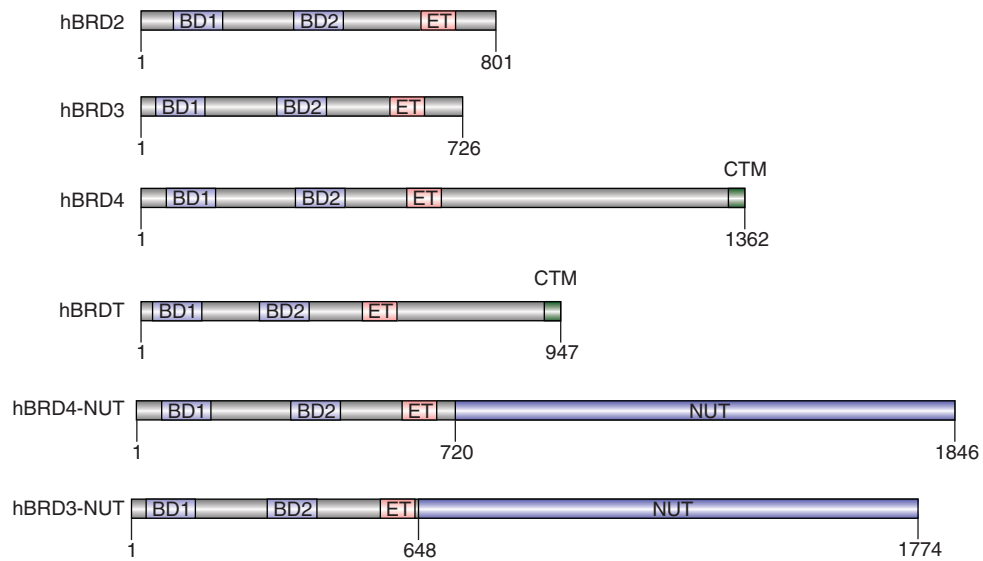


Figure 1. Domain structure of the bromodomain and extraterminal (BET) protein family and BET–NUT fusion proteins. Each BET protein contains two bromodomains (BD1 and BD2) and an extraterminal (ET) domain. BRD4 and BRDT have an additional carboxy-terminal motif (CTM). BRD3/BRD4–NUT fusion proteins found in NUT midline carcinoma (NMC) patients fuse the amino terminus of BRD4 (or BRD3) with almost the entire NUT protein.

which is present on 46 different proteins encoded in the human genome (Dhalluin et al. 1999; Filippakopoulos et al. 2012). A bromodomain is composed of a left-handed bundle of four α helices, with interhelical loops forming a hydrophobic binding pocket that engages in acetyllysine recognition (Dhalluin et al. 1999). Studies spanning nearly two decades have implicated bromodomain-containing coactivator proteins as integral components of TF-mediated gene regulation by linking lysine acetylation to downstream effects on chromatin structure and transcription (Sanchez and Zhou 2009). Moreover, the functional diversity and emerging “drugability” of bromodomain modules with small molecules has motivated a widespread interest in this class of proteins as therapeutic targets (Filippakopoulos and Knapp 2014).

BET PROTEIN FAMILY OF TRANSCRIPTIONAL COACTIVATORS

The mammalian BET (bromodomain and extraterminal domain-containing) protein family consists of four members, including the ubiqu-

itously expressed BRD2, BRD3, BRD4, and the germ-cell-specific BRDT (Fig. 1) (Wu and Chiang 2007; Shi and Vakoc 2014). All four BET proteins have two conserved bromodomains that preferentially bind to multiacetylated peptides (Fig. 1) (Dey et al. 2003; Moriniere et al. 2009; Gamsjaeger et al. 2011; Filippakopoulos et al. 2012). The preferred ligand of the first bromodomain (BD1) is $K^{\text{ac}}\text{XXX}^{\text{ac}}$, with the intervening X amino acids having small side chains (e.g., glycine or alanine), whereas the second bromodomain (BD2) is more permissive to binding multiacetylated peptides in diverse sequence contexts (Dey et al. 2003; Moriniere et al. 2009; Gamsjaeger et al. 2011; Filippakopoulos et al. 2012). The acetylated tails of core histones H3 and H4 and acetylated regions of TFs are the most well-validated binding partners of BET bromodomains, which are generated as consequence of TF-mediated KAT recruitment (Dey et al. 2003; Lamonica et al. 2011; Shi et al. 2014; Roe et al. 2015). ChIP-seq studies in several cell types have shown that BRD4 localizes preferentially to the nucleosome-free site occupied by TFs at enhancers and promoters, which is

consistent with acetylated TFs being important recruiters of BET proteins (Roe et al. 2015; Stonestrom et al. 2015). It is likely that a multitude of acetylated peptides contribute to BET protein recruitment to a particular DNA element, with different acetylated peptides being relevant at different *cis* elements. However, it is challenging to pinpoint the complete repertoire of acetylated peptides responsible for recruiting BET proteins to chromatin.

In addition to two bromodomains, all four BET proteins possess a conserved extraterminal (ET) domain that performs an effector role in transcriptional activation and in chromatin remodeling. The ET domain interacts with several different cofactors, including the demethylase protein JMJD6, the methyltransferase/adaptor protein NSD3, and the chromatin remodeling ATPases CHD4 and BRG1 (Rahman et al. 2011; Shen et al. 2015). The ET domain also interacts with the virally encoded proteins, such as murine leukemia virus (MLV) integrase and latency-associated nuclear antigen (LANA) peptide of Kaposi's sarcoma-associated herpesvirus (KSHV) (Hellert et al. 2013; Crowe et al. 2016). All of these interactions are mediated by a hydrophobic groove on the ET domain that recognizes a consensus motif of alternating lysine and hydrophobic residues found on several of the above-mentioned cofactors (e.g., an IKLKI motif on NSD3 and a LKIKL motif on CHD4) (Hellert et al. 2013; Shen et al. 2015; Crowe et al. 2016; Zhang et al. 2016). The available evidence indicates that BRD4 relies on a unique subset of these ET-interacting proteins for transcriptional activation in particular cell types. In HEK293T cells, BRD4 uses JMJD6 as its ET-domain effector, which will demethylate histones and noncoding RNA to promote enhancer-mediated gene activation (Liu et al. 2013). In acute myeloid leukemia (AML) cells, the ET domain of BRD4 activates transcription by interacting with NSD3, which functions as a scaffold to recruit the chromatin-remodeling enzyme CHD8 (Shen et al. 2015). This apparent context-specificity of ET domain function is not well understood at present.

BRD4 and BRDT possess a unique carboxy-terminal motif (CTM), which binds to the

serine/threonine kinase P-TEFb as an additional mechanism of gene activation (Bisgrove et al. 2007; Krueger et al. 2010). P-TEFb is a heterodimer of the kinase Cdk9 and a K, T1, or T2-type cyclin, which together can phosphorylate the serine 2 position of the Pol II carboxy-terminal domain (CTD), as well as serine and threonine residues on the pausing factors DSIF and NELF (Krueger et al. 2010; Jonkers and Lis 2015). Thus, BRD4-mediated P-TEFb recruitment will drive a variety of local phosphorylation events to bypass the paused state of Pol II and promote transcription elongation. At the biochemical level, BRD4 uses multiple mechanisms to regulate P-TEFb activity. Using purified proteins, the interaction with BRD4 is sufficient to stimulate P-TEFb kinase activity (Itzen et al. 2014). In cells, this BRD4 interaction is competitive with the interaction of P-TEFb with HEXIM1/7SK RNA, which are inhibitors of its kinase activity (Jang et al. 2005; Yang et al. 2005). BRD4 also contributes to the localization of P-TEFb to hyperacetylated enhancers and promoters across the genome, thus guiding P-TEFb to its relevant substrates near TF-bound sites (Jang et al. 2005; Yang et al. 2005). Another key player in the functional linkage between BRD4 and P-TEFb is the Mediator complex, which is a 30 subunit coactivator complex that physically associates with BRD4 and with P-TEFb (Jiang et al. 1998; Jang et al. 2005; Donner et al. 2010; Allen and Taatjes 2015). Although the precise interaction surface between BRD4 and Mediator has yet to be mapped, the MED23 subunit has been implicated in this interaction (Wang et al. 2013). BRD4 and Mediator stabilize each other's occupancy at specific sites across the genome, and these two machineries cooperate in recruiting P-TEFb (Jang et al. 2005; Donner et al. 2010; Bhagwat et al. 2016). It should be noted that the CTM region is found on BRD4 (and BRDT), but not on BRD2 and BRD3, which may explain why BRD4 performs a broader nonredundant role in transcriptional activation than the other BET proteins. For example, genetic inactivation of BRD4 will lead to slow growth phenotypes in essentially all mammalian cell lines, whereas BRD2 and BRD3 lead

to only subtle phenotypes when targeted (Vakoc CR, unpubl.). Only a few cellular contexts have been identified in which redundancy exists among the BET proteins, as was recently shown for BRD2 and BRD3 in hematopoietic cells (Stonestrom et al. 2015).

Recent studies have found that BRD4 protein possesses intrinsic kinase and KAT activity in in vitro assays (Devaiah et al. 2012, 2016). Similar to P-TEFb, BRD4 can directly phosphorylate the CTD of Pol II at the serine 2 position and can acetylate multiple residues on histone H3 and H4, including H3K112 found on the globular region of the nucleosome (Devaiah et al. 2012, 2016). Earlier work had also identified an intrinsic kinase activity in purified BRD2 and in FSH, the *Drosophila melanogaster* ortholog of BRD4 (Denis and Green 1996; Chang et al. 2007). The presence of these activities in biochemical assays is difficult to reconcile with the lack of an obvious kinase or KAT domain in the BRD4 polypeptide, and hence these activities should be considered provisional at present and await further validation to confirm their importance in vivo.

SMALL-MOLECULE INHIBITORS OF BET BROMODOMAINS

The above description of BET proteins suggests a general role of these regulators in transcriptional control, particularly because acetylated TFs and histones are found at all active promoters and enhancers in the genomes. It is only recently that the attention of the field has turned toward identifying biological processes that are disproportionately BET protein-dependent. This avenue of research was invigorated by two studies published in 2010 describing the first selective small-molecule inhibitors of BET bromodomains (Filippakopoulos et al. 2010; Nicodeme et al. 2010). The potency, specificity, and in vivo activity of these molecules in modulating BET proteins has allowed numerous studies in a myriad of animal models of disease. This work has exposed a remarkable gene specificity of transcriptional effects of BET inhibition that underpins a broad interest in BET proteins as therapeutic targets.

The first class of BET bromodomain inhibitors, which are a series of thienotriazolodiazepines, were originally filed as patents by the Mitsubishi Tanabe Pharmaceutical Corporation (Adachi et al. 2006; Miyoshi et al. 2010, 2013). The compounds belong to the diazepine family and are analogs of benzodiazepine, which has been used extensively in the clinic as psychoactive drug (Smith et al. 2014). Thereafter, the Bradner laboratory and researchers at GlaxoSmithKline independently published the highly potent and selective BET bromodomain inhibitors JQ1 (a thienotriazolodiazepine) and iBET (a benzodiazepine), respectively (Filippakopoulos et al. 2010; Nicodeme et al. 2010). Of note, both JQ1 and iBET are pan-BET bromodomain inhibitors, which do not discriminate between the two bromodomains within the same BET protein, nor among the four BET family members (Filippakopoulos et al. 2010; Nicodeme et al. 2010). Because the tandem bromodomains within the same BET protein have distinct functions and binding affinities toward acetylated peptides, the lack of specificity of JQ1 and iBET limits the potential use of these chemical probes to study the roles of individual BET proteins. Nonetheless, the paninhibitory activity of these compounds may contribute to their high potency in modulating biological processes in vivo. Because BRD4 tends to be the dominant transcriptional regulator within the BET protein family in somatic cell types, most studies have linked the transcriptional effects of BET inhibitors to BRD4 inhibition, with BRD2 and BRD3 contributing to a lesser degree.

Chemists have continued to optimize these compounds with the aim to improve the selectivity among the BET protein family and enhance drug potency and in vivo pharmacodynamics, in an effort to make these compounds suitable for clinical investigation. This includes multiple pan inhibitors, some of which have entered clinical trials (see below), BD1 selective inhibitors, such as MS-436, Olinone, and BI-2536, as well as the BD2 selective inhibitors RVX-208 and RVX-297 (Steggmaier et al. 2007; Park et al. 2013; Picaud et al. 2013; Zhang et al. 2013; McLure et al. 2014; Kharenko et al. 2016). As expected, inhibition of individual BET bro-



mododomains will lead to different transcriptional and phenotypic outcomes. For example, the BD1-specific inhibitor Olineone induces differentiation of mouse primary oligodendrocytes, whereas pan BET bromodomain inhibitors have the opposite effect (Gacias et al. 2014). The BD2-selective inhibitor RVX-208 was originally identified in a cell-based chemical screen to enhance the production of apoA-1, and only recently was discovered to target BET proteins after completing phase III clinical trials for treatment of atherosclerosis (Bailey et al. 2010; Nicholls et al. 2011, 2012; Picaud et al. 2013; McLure et al. 2014). Interestingly, RVX-208 causes a milder effect on the transcriptome of cells when compared with inhibitors that bind to both BET bromodomains (Picaud et al. 2013).

All of the compounds described above bind to BET bromodomains in a competitive manner with acetyllysine to displace BET-containing protein complexes from chromatin. A more recent innovation in BET inhibitor design has been to conjugate JQ1 with chemical moieties that promote recruitment of E3 ubiquitin ligases, which leads to polyubiquitylation of BET proteins and proteasome-dependent degradation, a strategy known as proteolysis targeting chimera (PROTAC) (Lu et al. 2015; Winter et al. 2015; Zengerle et al. 2015). This new generation of inhibitors (known as dBET1, ARV-825, or MZ1) leads to more potent suppression of BET proteins in cells, and may provide an additional strategy for therapeutic targeting.

Recent studies have shown that many clinical-stage kinase inhibitors, including the CDK inhibitor dinaciclib, the JAK2 inhibitor TG101209, and the PLK1 inhibitor BI-2536, potentially inhibit BET bromodomains as an unintended off-target effect (Martin et al. 2013; Ciceri et al. 2014; Dittmann et al. 2014; Ember et al. 2014). These findings raise the possibility that the off-target effect on BET proteins might contribute to the therapeutic effect of these kinase inhibitors. Moreover, these findings reveal an opportunity for the rational design of drugs that simultaneously target specific kinases and BET proteins to augment anticancer activity or prevent drug resistance (Ciceri et al. 2014).

TARGETING THE BRD4–NUT FUSION ONCOPROTEIN IN NUT MIDLINE CARCINOMA

The first malignant context in which BET proteins were proposed as therapeutic targets is in a rare cancer called NUT midline carcinoma (NMC), which is an aggressive subtype of squamous cell carcinoma with a median survival of only 6.7 months (French et al. 2003; French 2010). Most cases of NMC possess a chromosomal translocation that generates a fusion of the amino terminus of BRD4 (or less commonly BRD3 or NSD3) to the carboxyl terminus of NUT, which is normally only expressed in testes (French et al. 2007, 2014; French 2010). The resulting BRD4–NUT oncoprotein retains the two bromodomains and the ET domain fused to a region of NUT that binds to p300, a protein with KAT activity (French et al. 2003, 2007; Reynoird et al. 2010; Wang and You 2015). It is interesting to note that BRD4–NUT (and presumably BRD3–NUT) requires an interaction with NSD3 via its ET domain for its oncogenic function, whereas the NSD3–NUT fusion requires its BRD4-binding motif for its oncogenic function (French et al. 2014). This highlights a remarkable convergence of molecular functions among BRD3–, BRD4–, and NSD3–NUT fusion proteins, and implies that each of these proteins function through similar multisubunit complexes to regulate transcription.

The tumorigenic properties of BRD4–NUT stems from the coupling of its bromodomains with the p300 binding site on NUT, which leads to a positive feedback loop that generates hyperacetylation-driven nuclear foci (Fig. 2) (French et al. 2007, 2014; Yan et al. 2011; Grayson et al. 2014; Alekseyenko et al. 2015; Wang and You 2015). At a genomic level, this positive feedback loop of acetylation and bromodomain-mediated binding generates large contiguous “megadomains” of active chromatin, which are enriched for BRD4–NUT, p300, and histone hyperacetylation, which span >1 Mb of the genome (Alekseyenko et al. 2015). The BRD4–NUT-induced active chromatin domain seems to propagate unfettered until it

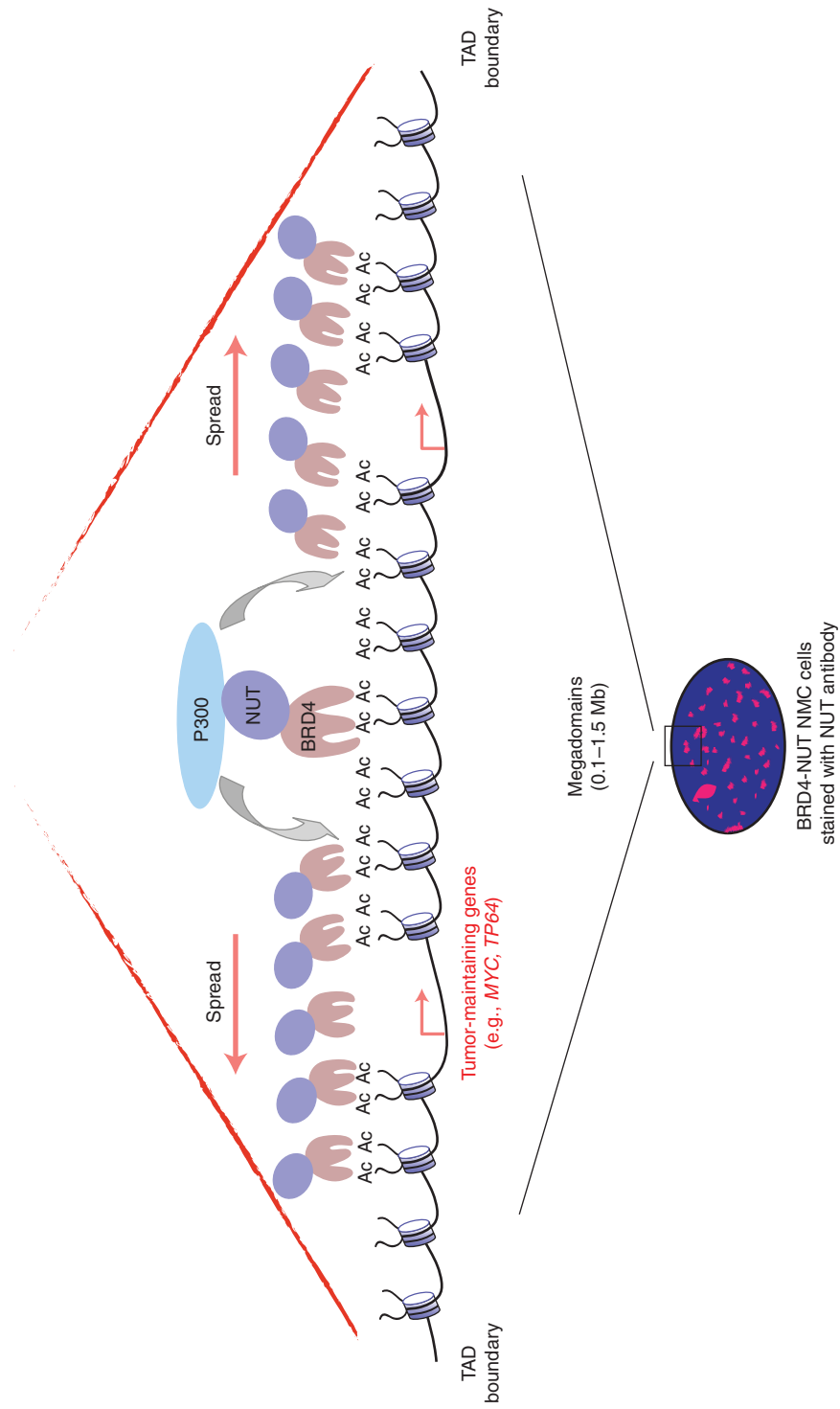


Figure 2. Oncogenic mechanism of the BRD4-NUT fusion protein. In NUT midline carcinoma (NMC) cells, BRD4-NUT forms 80 to 100 large nuclear foci, due to the aggregation of BRD4-NUT on the chromatin. On a molecular level, BRD4-NUT occupies a stretch of highly acetylated chromatin, spanning 0.1–1.5 Mb, termed “mega-domains.” The NUT portion of BRD4-NUT recruits p300, which is a histone acetyltransferase that drives further increases in local histone acetylation, thus forming a feed forward loop. The size and spreading of megadomains tends to be restricted to specific topologically associated domains (TADs). Megadomains can occur over genes that are important for the survival of NMC cells, like *MYC* and *TP63*.

encounters the edge of a topological domain defined by CTCF/cohesion (Alekseyenko et al. 2015). Active chromatin domains of this size are not observed in normal mammalian chromatin, highlighting a unique chromatin-based mechanism of transformation used by BRD4–NUT. Despite the enormity of these megadomains, only a specific program of genes becomes aberrantly expressed by BRD4–NUT function, which includes *MYC* and the epithelial fate determinant *TP63* (Alekseyenko et al. 2015).

Because the bromodomains of BRD4–NUT are essential to its oncogenic function, NMC provides a clear rationale for evaluating the therapeutic activity of BET bromodomain inhibitors. Exposing cultured NMC cell lines to JQ1 leads to a rapid eviction of BRD4–NUT from chromatin, followed by a rapid suppression of its direct target genes, such as *MYC*, and the induction of terminal squamous cell differentiation (Filippakopoulos et al. 2010; Yan et al. 2011; Grayson et al. 2014; Alekseyenko et al. 2015). Moreover, JQ1 treatment of mice bearing subcutaneous NMC patient-derived xenograft leads to a pronounced inhibition of tumor growth in vivo, with minimal toxicity to normal tissues (Filippakopoulos et al. 2010). This remarkable study provided the first evidence for BET inhibition having a therapeutic index in treating cancer, and provided rationale for subsequent clinical studies of BET inhibition in NMC patients (see below).

A WIDESPREAD SENSITIVITY OF CANCER CELLS TO BET BROMODOMAIN INHIBITION

Given the rarity of NMC, a key question arises as to whether malignancies that lack *BRD4* rearrangements would also be sensitive to BET inhibitors. Because the original description of NMC sensitivity to BET inhibition, numerous studies have shown that BRD4 is a non-oncogene dependency in several forms of cancer. Two studies published in 2011 implicated BRD4 as a vulnerability in the MLL-rearranged subtype of AML (Dawson et al. 2011; Zuber et al. 2011). One used shRNA screening to reveal that AML cells were hypersensitive to genetic

knockdown of BRD4, whereas the other study used a proteomic approach to link BRD4 with MLL–fusion cofactors (Dawson et al. 2011; Zuber et al. 2011). These studies, as well as others using multiple myeloma and lymphoma models, showed that BET inhibitors show therapeutic effects in diverse genetic contexts of hematological malignancy, with effects comparable to observations in NMC models (Dawson et al. 2011; Delmore et al. 2011; Mertz et al. 2011; Zuber et al. 2011). Although BET inhibitors show broad efficacy in the blood malignancies, the precise pattern of gene expression changes incurred by BET inhibitor treatment is remarkably heterogeneous among different cancer cell lines. In addition, some cancer cell types will terminally differentiate in response to JQ1/iBET exposure, whereas others undergo apoptosis (Dawson et al. 2011; Delmore et al. 2011; Mertz et al. 2011; Zuber et al. 2011). Although there is generally a lack of consistent gene-expression alterations following BET inhibitor treatment in these malignancies, the well-established oncogenes *MYC*, *BCL2*, and *CDK6* are often suppressed by these drugs, whereas housekeeping genes tend to be unaffected (Shi and Vakoc 2014).

Over the past 5 years, the efficacy of BET inhibitors has been shown in numerous preclinical solid tumor models, including tumors of the prostate, breast, colon, intestine, pancreas, liver, and brain (Sahai et al. 2016). Large-scale profiling studies in human cancer cell lines have suggested that a specific subset of these different tumors harbor exceptional sensitivity to BET inhibitors (Rathert et al. 2015). However, it has been challenging to identify biomarkers that predict hypersensitivity to BET inhibition that might guide patient enrollment into clinical trials. Nonetheless, we can now appreciate that sensitivity to BET inhibition is pervasive across different malignancies.

Although the broad anticancer activity of BET inhibitors is remarkable, it should be emphasized that normal cell types are also affected in unique ways by these agents. This leads to pleiotropic phenotypes in the normal tissues of mice, and presumably in humans. Some of these effects may present therapeutic opportu-

nities in areas outside of oncology. For example, iBET will attenuate cytokine production in innate immune cells, which can allow iBET-treated mice to survive septic shock (Nicodeme et al. 2010). BET inhibitors will also suppress the pathological remodeling of cardiomyocytes in response to pressure overload (Anand et al. 2013). It has also been proposed that BET inhibitors might be used as a male contraceptive, owing to the reversible impairment in spermatogenesis consequent to BRDT inhibition in the testes (Matzuk et al. 2012; Berkovits and Wolgemuth 2013). BET inhibition will reactivate latent HIV infection, which might prove useful to eliminate viral reservoirs in infected patients (Banerjee et al. 2012; Zhang et al. 2012; Zhu et al. 2012; Li et al. 2013). However, it remains to be determined whether these indications for BET inhibition will be translated into human clinical investigation.

There are also more concerning effects of BET inhibition, such as an impairment in memory formation and an autism-like syndrome in the central nervous system and a worsening of viral/bacterial infections consequent to immunosuppression (Marazzi et al. 2012; Korb et al. 2015; Sullivan et al. 2015). Transgenic *Brd4* shRNA mice, in which BRD4 levels are reduced conditionally in adult tissues, show stem cell depletion in the small intestine and hyperplasia of epidermal tissues (Bolden et al. 2014). The latter phenotype may reflect the emerging role for BRD4 in tumor suppression, and hence BET inhibition might be expected to worsen certain malignancies (Alsarraj and Hunter 2012; Fernandez et al. 2014; Tasdemir et al. 2016). Notably, the side effects of BRD4 knockdown in vivo are known to be reversible after restoring BET protein function (Matzuk et al. 2012; Bolden et al. 2014; Nagarajan et al. 2014).

PHASE I CLINICAL STUDIES OF BET INHIBITORS IN HUMAN CANCER PATIENTS

The efficacy of BET inhibitors in preclinical cancer models provided the rationale for a multitude of ongoing human clinical trials, which includes patients with hematological malignancies, BRD4–NUT-expressing NMC, and various

solid tumors. A summary of these ongoing trials can be found in Table 1. Although we cannot determine at the present time the ultimate impact BET inhibitors will have in oncology, there are a few key observations that have been made from the initial administration of these agents to human patients thus far. Importantly, the toxicities in humans have been determined for three clinical BET inhibitors (OTX015, TEN-010, and CPI-0610) (Abramson et al. 2015; Shapiro et al. 2015; Amorim et al. 2016; Berthon et al. 2016; Stathis et al. 2016). In one set of phase I trials in hematological cancers, OTX015 was administered orally once or twice a day for 21-day cycles. At the higher doses (120–160 mg/d), OTX015 resulted in a substantial, yet reversible thrombocytopenia (drop in platelet counts), severe gastrointestinal events, and fatigue (Amorim et al. 2016; Berthon et al. 2016). The recommended dose for leukemia and lymphoma patients was identified in this study as 80 mg/d given in repetitive cycles of 14 days on followed by 7 days off (Amorim et al. 2016; Berthon et al. 2016). At different doses in these trials, evidence was reported of disease reduction in five out of 37 acute leukemia patients and five out of 17 diffuse large B cell lymphoma patients, whereas no responses were observed in any of the 12 multiple myeloma patients treated (Amorim et al. 2016; Berthon et al. 2016). Consistent with observations in mouse models, OTX015 was found to cause the terminal differentiation of myeloid leukemia cells, as indicated by an increase in peripheral neutrophil counts during treatment (Dawson et al. 2011; Zuber et al. 2011; Berthon et al. 2016). Unfortunately, a specific genetic mutation in leukemia patients has yet to be identified that correlates with responses to OTX015. This highlights the formidable challenge of identifying a predictive biomarker to guide patient enrollment in future studies. In NMC patients, rapid responses to OTX015 have also been identified, in association with tumor regression (Shapiro et al. 2015; Stathis et al. 2016). Although these findings are encouraging and will motivate further phase II evaluation, many of the patients that initially responded later relapsed several months after initiating

Table 1. Clinical trials of BET bromodomain inhibitors

Compound	Sponsor	NCT identifier	Conditions	Clinical phase
ABBV-075	AbbVie	NCT02391480	Advanced cancer; breast cancer; non-small-cell lung cancer (NSCLC); acute myeloid leukemia (AML); multiple myeloma	Phase I (recruiting)
BAY 1238097	Bayer	NCT02369029	Neoplasms	Phase I (terminated)
BI 894999	Boehringer Ingelheim	NCT02516553	Neoplasms	Phase I (recruiting)
BMS-986158	Bristol-Myers Squibb	NCT02419417	Multiple indications cancer	Phase I/IIa (recruiting)
		NCT01949883	Lymphoma	Phase I (recruiting)
CPI-0610	Constellation Pharmaceuticals	NCT02157636	Multiple myeloma	Phase I (recruiting)
		NCT02158858	Leukemia, myelocytic, acute; myelodysplastic syndrome (MDS); myelodysplastic/myeloproliferative neoplasm, unclassifiable; myelofibrosis	Phase I (recruiting)
FT-1101	Forma Therapeutics	NCT02543879	AML; acute myelogenous leukemia; myelodysplastic syndrome	Phase I (recruiting)
GS-5829	Gilead Sciences	NCT02607228	Metastatic castration-resistant prostate cancer (CRPC) (as a single agent or in combination with enzalutamide)	Phase I (recruiting)
GSK2820151	GlaxoSmithKline	NCT02630251	Cancer	Phase I (not yet open for recruiting)
GSK525762/I-BET762	GlaxoSmithKline	NCT01587703	Carcinoma, midline	Phase I (recruiting)
		NCT01943851	Cancer	Phase I (recruiting)
INCB054329	Incyte Corporation	NCT02431260	Advanced cancer	Phase I/II (recruiting)
		NCT02698189	AML including AML de novo and AML secondary to MDSs; diffuse large B-cell lymphoma (DLBCL)	Phase I (recruiting)
MK-8628	Merck Sharp & Dohme Corp.	NCT02698176	NUT-midline carcinoma (NMC); triple-negative breast cancer (TNBC); NSCLC; CRPC	Phase I (recruiting)
N-methyl-2-pyrrolidone	Peter MacCallum Cancer Centre, Australia	NCT02468687	Multiple myeloma	Phase I (recruiting)
		NCT01713582	AML; DLBCL; acute lymphoblastic leukemia; multiple myeloma	Phase I (active, not recruiting)
OTX015/MK-8628	OncoEthix GmbH/Merck	NCT02259114	NMC; TNBC; NSCLC with rearranged ALK gene/fusion protein or KRAS mutation; CRPC; pancreatic ductal adenocarcinoma	Phase I (active, not recruiting)

Continued

Table 1. *Continued*

Compound	Sponsor	NCT identifier	Conditions	Clinical phase
		NCT02296476	Glioblastoma multiforme	Phase I (terminated)
RVX-208/ RVX 000222	Resverlogix Corp.	NCT02586155	Diabetes mellitus, type 2; coronary artery disease; cardiovascular diseases	Phase III (recruiting)
TEN 010	Tensha Therapeutics/ Roche	NCT01987362	Solid tumors; advanced solid tumors	Phase I (recruiting)
		NCT02308761	MDSs; AML	Phase I (recruiting)
ZEN003694	Zenith Epigenetics	NCT02711956	Metastatic CRPC (in combination with enzalutamide)	Phase I (not yet open for recruiting)
		NCT02705469	Metastatic CRPC	Phase I (recruiting)

See clinicaltrials.gov.

treatment (Stathis et al. 2016). This indicates the importance of studying mechanisms of resistance and the potential of combining BET inhibitors with other agents to provide more durable responses. Taken together, these initial studies have generated sufficient enthusiasm within the pharmaceutical industry to justify a continuing of phase II clinical investigation.

WHY ARE CANCER GENES HYPERSENSITIVE TO BET INHIBITION?

When considering the basic molecular function of BRD4 described above, it is difficult to understand why chemical inhibition of BET proteins would lead to preferential impairment to cancer cells versus nontransformed cell types. Transcriptome-level studies have revealed that BET inhibitors suppress hundreds of genes in each cell type (Anand et al. 2013; Chapuy et al. 2013; Lovén et al. 2013; Asangani et al. 2014). The identity of BET-dependent genes varies dramatically from cell type to cell type, which poses a major challenge in proposing a unified mechanism to explain the anticancer effects of BET inhibitors. At present, our understanding of these effects is limited to correlative observations that these compounds will preferentially suppress expression of cancer-promoting genes versus that of housekeeping genes (Chapuy et al.

2013; Lovén et al. 2013; Nagarajan et al. 2014; Roe et al. 2015; Bhagwat et al. 2016; Henssen et al. 2016). An alternative summation of the available evidence is that JQ1 preferentially suppresses “highly regulated” genes, that is, genes that are dynamically expressed in response to exogenous stimuli or those genes that are expressed in a lineage-specific manner, and hence are influenced by numerous *trans*- and *cis*-acting regulators. Indeed, many growth/cancer-promoting genes (e.g., *MYC* and *BCL2*) fall into this broad category (Lovén et al. 2013; Asangani et al. 2014; Roe et al. 2015; Shu et al. 2016). Such a model would also explain why JQ1 suppresses cytokine genes in immune cells and the immediate-early genes in cardiomyocytes and neurons (Nicodeme et al. 2010; Anand et al. 2013; Brown et al. 2014; Korb et al. 2015; Toniolo et al. 2015). It is important to note that the effects of JQ1 on transcription have been shown to be reversible, that is, withdrawing JQ1 leads to a rapid restoration of the preexisting transcription level (Mertz et al. 2011). This is likely to account for why normal tissues are able to recover in the setting of 14-day-on, 7-day-off BET inhibition treatment-cycles in humans (Amorim et al. 2016; Berthon et al. 2016). Cancer cells are perhaps less able to recover following BET inhibitor treatment, owing to their “addiction” to oncogenes like *MYC*

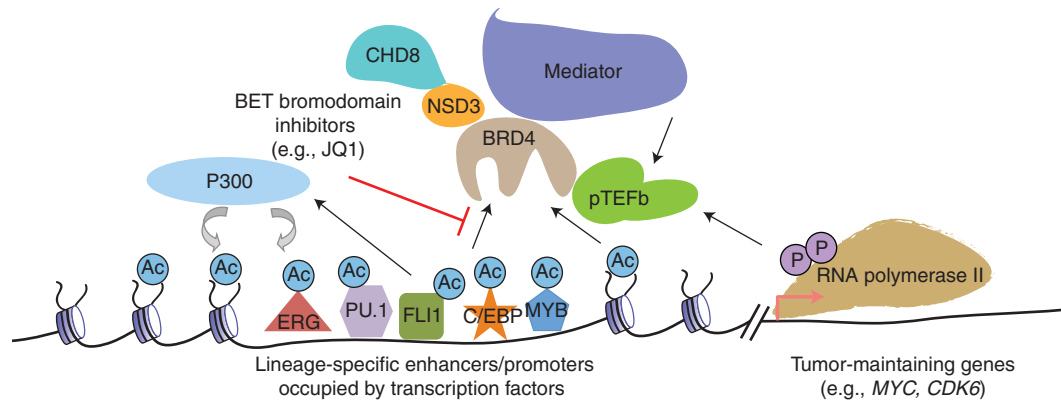


Figure 3. The BRD4 pathway in acute myeloid leukemia (AML) cells. In AML cells, BRD4 is recruited to lineage-specific enhancers and promoters by acetylated histones and transcription factors (TFs), which are acetylated by p300. BRD4 will then recruit several proteins to regions through direct physical interaction, including NSD3/CHD8, the Mediator complex, and p-TEFb, which promotes transcriptional activation. BET bromodomain inhibitors will release BRD4 from chromatin along with its cofactors to suppress transcription. Because many BRD4-occupied enhancers are located at distal upstream or downstream sites, several of these activities are occurring at a distance from the target gene promoter.

(Arvanitis and Felsher 2006). Nevertheless, the specific pattern of genes that are suppressed by BET inhibition in each cell type is clearly of central importance to the therapeutic efficacy of these agents. Below, we describe studies that have defined molecular mechanisms that underlie the gene-specific transcriptional effects of BET bromodomain inhibition.

One explanation for the context-specific effects of BET inhibition is that each cell type expresses a different complement of acetylated TFs that bind to BET bromodomains. In AML cells, for example, it has been found that BRD4 binding across the genome is highly correlated with a set of hematopoietic lineage TFs (ERG, FLI1, PU.1, MYB, C/EBP α , and C/EBP β), which can each physically associate BRD4 in either a bromodomain-dependent or independent manner (Roe et al. 2015). In JQ1-treated leukemia cells, the downstream target genes of this set of TFs are rapidly suppressed, whereas TF occupancy on DNA remains unaffected (Fig. 3) (Roe et al. 2015). Moreover, ectopic expression of these hematopoietic TFs in fibroblasts can recapitulate the JQ1 transcriptional response seen in leukemia cells, indicated that these TFs are sufficient to specify the effects of BET inhibition (Roe et al. 2015). An expanding

body of literature is continuing to link the anticancer effects of BET inhibition to the functional suppression key TFs—the androgen receptor in prostate cancer (Asangani et al. 2014), the estrogen receptor and TWIST in breast cancer (Nagarajan et al. 2014; Shi et al. 2014), NF- κ B in lymphoma and lung cancers (Asangani et al. 2014; Nagarajan et al. 2014; Zou et al. 2014; Gao et al. 2015). The broad suppression of TF function in normal cell types is also likely to be related to the on-target toxicities of BET inhibitors seen in human patients. As an example, the demonstrated interaction between the diacetylated TF GATA-1 and BET proteins provides a potential explanation for the thrombocytopenia observed in BET inhibitor-treated patients (Gamsjaeger et al. 2011; Lamonica et al. 2011; Stonestrom et al. 2015).

It has been observed that many of the genes that are sensitive to BET inhibition have an unusually large repertoire of enhancer elements in their vicinity, which might be interpreted as another indicator of genes that are “highly regulated.” Indeed, genes with a large number of enhancers tend to encode lineage-specific and growth-regulatory factors (Hnisz et al. 2013; Whyte et al. 2013). Such enhancers have been given numerous labels (superenhancers,

locus control regions, stretch enhancers) and often exist in clusters and harbor high levels of BRD4 enrichment (Li et al. 2002; Chapuy et al. 2013; Lovén et al. 2013; Parker et al. 2013). Using genomic approaches applied to several different cell types, it has been observed that genes with “superenhancers” nearby tend to be more suppressed by JQ1 than randomly chosen expressed genes (Chapuy et al. 2013; Lovén et al. 2013; Peeters et al. 2015). More recent evidence indicates that only a minority of superenhancers are in fact targeted by JQ1, as indicated by measurements of Mediator eviction following BET inhibitor exposure (Bhagwat et al. 2016).

From these studies, it is clear that specific *cis*-elements in the genome are more suppressed by BET inhibition than others, and this contributes to the biased effects of JQ1 on certain genes. The mechanistic basis underlying these heterogeneous effects is still not understood. One possibility is that enhancer-binding proteins like BRD4 have variable on–off rates at each *cis*-regulatory element, and that perhaps BET inhibitors will preferentially evict BRD4-containing proteins complexes from chromatin at sites that are more dynamic. Taken together, the context-specific consequences of BET inhibition can be attributed, at least in part, to the specific complement of TF-bound *cis*-elements (enhancers and promoters) that are suppressed by these small molecules.

MECHANISMS OF RESISTANCE TO BET INHIBITION

All cancer monotherapies are limited by the emergence of drug-resistant cell populations, with the ongoing clinical trials indicating that BET inhibitors are not an exception. Hence, an important area of ongoing investigation has been to define mechanisms of resistance to BET bromodomain inhibition. Several recent studies have shown that acquired resistance to BET inhibition is associated with nongenetic mechanisms, in association with a global alteration of gene expression that compensates for the effects of BET inhibition (Tang et al. 2014; Fong et al. 2015; Rathert et al. 2015; Shu et al. 2016). In MLL-fusion AML, this compensatory

change in gene expression is linked to activation of the WNT signaling pathway, which can restore *MYC* expression despite chemical blockade of BRD4 (Fong et al. 2015; Rathert et al. 2015). In breast cancer cells, JQ1 resistance is associated with an elevated level of BRD4 phosphorylation, which in turn will bind more tightly to the Mediator complex to achieve bromodomain-independent recruitment to chromatin (Shu et al. 2016). Across a panel of heterogeneous cell lines, it has been found that the sensitivity to BET inhibitors can be correlated with the preexisting expression level of genes that encode apoptosis regulators (Conery et al. 2016). For example, leukemia cell lines with high *BCL2* expression and low expression of *BCL2L1* (also called *BCL-xl*) or *BAD* are generally correlated with higher sensitivity to BET inhibition (Conery et al. 2016). Consistent with this observation, acquired resistance to BET inhibition can be linked to an increase in the expression of *BCL2L1* by gaining super enhancers upstream of the *BCL2L1* gene (Shu et al. 2016). What is notable across these studies is that resistance to BET inhibition is not associated with BRD2/BRD3/BRD4 mutations, but instead is associated with selection for a rare (presumably preexisting) cell population harboring a pattern of gene expression that bypasses BET inhibition (Fong et al. 2015; Rathert et al. 2015; Conery et al. 2016).

A clear objective for future clinical investigation is to identify drugs that synergize with BET inhibitors in causing anticancer effects, but without having a synergistic increase in toxicity. One promising area of drug combinations is to use BET inhibition as a means to eliminate drug resistance to other targeted agents. In T-cell leukemia driven by activating NOTCH1 mutations, drug-tolerant cells are able to survive NOTCH pathway inhibition (Knoechel et al. 2014). These resistant T-ALL cells are more sensitive to BET bromodomain inhibition than the parental population, thus providing a rationale to combine NOTCH and BET-targeting agents in this disease (Knoechel et al. 2014). A similar scenario is found in breast cancer driven by PI3 kinase mutations, in which sensitivity to PI3K inhibitors is attenuated by feedback pathways



that bypass PI3K through the activation of tyrosine kinase pathways (Stratikopoulos et al. 2015). Remarkably, these bypass pathways can be suppressed transcriptionally via BET bromodomain inhibition. Hence, combinations of BET and PI3K inhibitors are a promising therapeutic approach in breast cancer. Resistance to estrogen receptor modulation, Sonic hedgehog, and androgen receptor blockade can also be overcome by BET bromodomain inhibition, thus providing numerous opportunities to explore drug combinations in the clinic (Asangani et al. 2014; Nagarajan et al. 2014; Tang et al. 2014; Shu et al. 2016).

CONCLUDING REMARKS

There is a long history of treating cancer patients with agents that disrupt fundamental cellular processes (e.g., antimetabolites and DNA alkylating agents), which can cause more severe cell death responses in cancer cells than in normal tissues. A similar description could be applied to BET bromodomain inhibitors, which target a set of transcriptional coactivators to disrupt an important hub for numerous TF pathways. As we have outlined in this review, there is clearly specificity in the transcriptional effects of BET inhibition, but only to a degree. Hence, these agents can cause detrimental effects to cancer cells in association with tolerable pleiotropic biological effects in normal tissues. The basic and preclinical research performed in this field has provided a roadmap for the implementation of BET inhibition in the clinic. In our view, the success of BET inhibition in clinical studies will rest squarely on our ability to find predictive biomarkers of therapeutic response and the most effective drug combinations for achieving durable disease remissions.

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