Published in final edited form as:

Drug Discov Today Technol. 2016 March ; 19: 45–50. doi:10.1016/j.ddtec.2016.06.004.

Clinical trials for BET inhibitors run ahead of the science

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Abstract

Several cancer clinical trials for small molecule inhibitors of BET bromodomain proteins have been initiated. There is enthusiasm for the anti-proliferative effect of inhibiting BRD4, one of the targets of these inhibitors, which is thought to cooperate with MYC, a long-desired target for cancer therapeutics. However, no current inhibitor is selective for BRD4 among the three somatic BET proteins, which include BRD2 and BRD3; their respective functions are partially overlapping and none are functionally redundant with BRD4. Each BET protein controls distinct transcriptional pathways that are important for functions beyond cancer cell proliferation, including insulin production, cytokine gene transcription, T cell differentiation, adipogenesis and most seriously, active repression of dangerous latent viruses like HIV. BET inhibitors have been shown to reactivate HIV in human cells. Failure to appreciate that at concentrations used, no available BET inhibitor is member-selective, or to develop a sound biological basis to understand the diverse functions of BET proteins before undertaking for these clinical trials is reckless and likely to lead to adverse events. More mechanistic information from new basic science studies should enable proper focus on the most relevant cancers and define the expected side effect profiles.

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Author Contributions:
Concept and design: GVD
Acquisition of data: ACB
Analysis and interpretation of data: ACB
Composition and revision of the manuscript for important intellectual content: GA, ACB, GVD
Obtained funding: GVD

Conflict of Interest Disclosures: None

Keywords

bromodomain; cancer clinical trial; BET inhibitor; side effect profile; inflammation; macrophage; adipogenesis; pancreatic beta cell

Introduction

Ever since 1996, when we published the first report of BET (Bromodomain and Extra Terminal domain) protein function [1], there has been hope that this family of transcriptional co-regulators might be useful targets in cancer, if they could be ablated selectively and safely. This is a tall order, because BET proteins control diverse transcriptional networks as effectors of signal transduction, are ubiquitously expressed across human cell types, and have overlapping functions among the three principal somatic proteins BRD2 (originally named RING3), BRD3 (ORFX) and BRD4 (MCAP). Bromodomains, first identified in 1992 as a 110-amino acid motif present in certain transcription factors and chromatin-modifying factors [2] and solved structurally in 1999 [3], are 'readers' of *N*-ε-acetyl-lysine modifications in nucleosomal chromatin, long understood to bring chromatin modifying enzymes to targeted promoters [4]. Changes in acetyl-lysine and chromatin status are involved in the transcription of most induced genes, thus BET protein effects can be widespread in the genome. Genetic experiments to ablate BET proteins suggested that cell cycle and proliferation mechanisms might be useful processes to target for cancer therapeutics, but progress was slow until small molecule inhibitors became available.

The search for small molecule inhibitors of bromodomains has significantly expanded since the first reported inhibition of the PCAF bromodomain, to prevent association between HIV and Tat protein [5]. In 2010, there appeared two encouraging reports [6,7] of new small molecule inhibitors based on benzotriazolodiazepine (called I-BET762) and thienotriazolodiazepine (called JQ1) structures that competitively inhibit the binding of the amino terminal bromodomains to acetylated histones in chromatin, which are the anchor points for BET bromodomain protein binding to nucleosomal regions. These inhibitors showed anti-inflammatory and anti-cancer activity, respectively, but did not solve the problem of ubiquitous protein expression or structural similarity among the family members: the binding of all BET bromodomains to histones is effectively inhibited at low concentrations. Mixed lineage leukemia driven by *MLL* chromosomal translocation was shown to be susceptible to inhibition with a related compound I-BET151 [8], fulfilling our early prediction that BET proteins drive MLL because *fsh*, a *Drosophila* homolog of BET proteins is a known activator of *trithorax* [9], a *Drosophila* homolog of MLL [1]. The involvement of BET gene translocations in deadly and incurable cancers of the midline, called NUT midline carcinomas [10], provided a sound rationale for the first cancer clinical trial for a BET bromodomain inhibitor, I-BET762.

Cancer clinical trials

At present, seven BET bromodomain inhibitors are in active clinical trials for cancer. Although 'cancer patients want to live and oncologists want to help', in the current absence of rigorous biology, setbacks may be expected for cancer clinical trials of these inhibitors

[11]. Well intentioned but blunt attempts to cure an overly broad range of cancers now characterize this late, even decadent, stage of investigation of this class of epigenetically-directed, small molecule inhibitors. BET inhibitors are already in dose escalation clinical trials to investigate safety, tolerability, pharmacokinetics, pharmacodynamics, and clinical activity of the agent for a number of cancers, including OTX015 (NCT02259114) [12] and GSK525762 (NCT01587703) for NUT midline carcinoma, which has a clear justification. Other BET bromodomain inhibitors are in trials for treatment-refractory acute myeloid leukemia and myelodysplastic syndrome (NCT02308761), lymphoma (NCT01949883) and multiple myeloma (NCT02157636). Trials are also open for triple negative- and estrogen receptor- positive breast cancers, small cell and non-small cell lung cancers, castration resistant prostate cancer, pancreatic ductal adenocarcinoma, colorectal cancer, neuroblastoma and MYCN-driven solid tumors. Some trials are single agent, and others combinatorial. In these more recent trials, the rationale to use BET inhibitors is shaky because *BRD2, BRD3* or *BRD4* translocations are not involved in tumorigenesis. Sometimes the justification appears to rely on MYC inhibition [13,14]. However, mere anti-proliferative activity through ablation of MYC will likely have little advantage over traditional antimetabolite chemotherapeutic approaches, a fifty-year old technology. Furthermore, recent excitement has centered on the transcriptional co-activator activity of BET proteins, particularly for cell cycle and proliferation genes in cancer, but it has long been clear that BET proteins also function as co-repressors of transcription, depending on the signal transduction, cellular and gene context. Clinical trials are proceeding without much regard for numerous important genes that will obviously be transcriptionally activated upon inhibition of BET bromodomain proteins [15].

Complicating matters, no BET transgenic animal models are available for mechanistic studies, except our own BRD2-specific, B cell-restricted model [16] that produces a B cell tumor most similar to the ‘activated B cell’ form of diffuse large B cell lymphoma [17,18]. Animal models would be very helpful to anticipate phenotype and elucidate mechanism here. For example, an inducible BRD4 RNAi animal model [19] shows that BET protein ablation causes widespread toxicities, including stem cell depletion that should be expected from *trithorax* interactions and studies of hematopoiesis [15,20]. Problematically, high profile papers that employ BET inhibitors at irrelevant concentrations have undermined molecular mechanism studies. For example, although reported K_d values [6] for the first bromodomains of BRD2, -3 and -4 are about 128 nM, 60 nM and 50 nM, respectively, Shu *et al* [21] exposed breast cancer cell lines to JQ1 at 2 – 20 μM. These concentrations are extremely high, because potent biological effects are observed at 50 – 100 nM [20,22]. Reported reductions in cell numbers [21] are possibly attributable to non-specific poisoning of cellular energetics, and have nothing to do with BET protein functions, rendering any conclusions unreliable. This is only the most recent example of many poorly controlled experiments with BET inhibitors.

Safety concerns

Clinical trials have raced dangerously far ahead of settled biology. Notably, BRD4 interacts with P-TEFb, disrupting the interaction between the Human Immunodeficiency Virus (HIV) transactivator Tat and P-TEFb and suppressing *per se* HIV transcription in infected cells

[23]. Published warnings [15] show that JQ1 has demonstrated ability to disrupt the BRD4 — P-TEFb interaction and thereby reactivate HIV-1 transcription in latent infected human T cells, which we were the first to show in 2012 [24]. Nevertheless, recruitment criteria for cancer clinical trials of BET inhibitors still do not exclude patients with HIV-positive status, as they obviously should. Institutional review boards that oversee these studies should require monitoring for spikes in blood levels of HIV, human papilloma virus [25,26] or other BRD2- [27,28] or BRD4-regulated viruses [29] that might arise as adverse events in BET inhibitor trials. The troubling prospect of unanticipated, systemic viremias of uncertain origin should inspire greater caution, not unbridled enthusiasm.

Macrophages, Th17 cells and inflammation

We rigorously established the link between BET protein function and inflammation, and showed that bone marrow-derived macrophages from hypomorphic *BRD2*^{lo} mice are deficient in production of an array of pro-inflammatory cytokines in response to bacterial endotoxin challenge. This function could be recapitulated with BET member-selective shRNA against each of BRD2, BRD3 and BRD4, or JQ1 *ex vivo* [22]. Interestingly, inhibition effects are similar among shRNAs individually targeting BRD2, -3 and -4, which indicates that each is important for cytokine gene transcription, but when combined, the pooled shRNAs induce apoptosis, which is not seen with JQ1. This result suggests a hypothesis that the other domains including the extraterminal (ET) domain [26], but also conserved regions such as a N-terminal cluster of phosphorylation sites (NPS), a basic residue-enriched interaction domain (BID) or a phosphorylation-dependent interaction domain (PDID) [30], which are ablated with shRNA but not with JQ1, are responsible for additional functions that are required for viability. In fact, the BRD4 ET domain, a protein interaction domain with structure and properties that are independent of the amino-terminal bromodomains, interacts with NSD3, JMJD6, CHD4, GLTSCR1 and ATAD5 [26]. Furthermore, it has been reported that the BET conserved regions NPS, BID and PDID associate with transcription factors including p53 [30]. The functions of theses domains of BET proteins, independent of the bromodomains, should be tested in diverse cell types to fully unravel their biological properties. Experiments should use pooled shRNA experiments to ablate the whole proteins, unlike BET inhibitor experiments, which simply displace bromodomains from chromatin, leaving the polypeptide intact and available for other interactions with nuclear proteins. Such experiments will have value for mechanism.

There is also excitement about the possible utility of BET inhibitors in autoimmune diseases or other chronic inflammatory conditions that are mediated by Th17 cells [31], although the relative roles of BRD2, BRD3 and BRD4 in the transcriptional programs that control T cell differentiation are not yet elucidated. Our group has recently reported that a Th17 cytokine signature is characteristic of insulin-resistant obesity in humans [32], thus there may be therapeutic opportunities for BET inhibitors in the treatment of the inflammatory complications of type 2 diabetes.

BET proteins in transcriptional co-repression

Mass spectrometry analysis of BET protein complexes in nuclear extracts [33] has shown intriguing parallels with earlier biochemical analysis of the Mediator complex [34]. Several proteins of known importance for chromatin status (including SWI/SNF subunits like BRG1), histone modification enzymes, transcriptional co-regulators (including TATA Box Binding Protein-Associated Factors; TAFs) and DNA sequence-specific transcription factors are associated with BRD2. These data confirmed earlier hypotheses that BET protein complexes are dynamic, and include both transcriptional activation and repression activities, like SWI/SNF complexes, where the net activity depends on the promoter and signal transduction context of each cell type. The co-repression functions of BET proteins are well anticipated from *Drosophila* experiments, in which major functions of BET orthologs include repression of developmental proteins, and have been well discussed [15].

Pancreatic β-cells

One important mechanism of co-repression is observed in pancreatic β-cells. BRD2 plays a pleiotropic and profound role in energy metabolism, consistent with emerging evidence that epigenetic mechanisms and histone acetylation are important in obesity. In one provocative animal model of hypomorphic expression of BRD2, mice develop severe obesity on normal chow [35], yet with increased fatty acid oxidation, increased adipogenesis and increased insulin transcription and production, which together preserve metabolic functions. The transcriptional mechanisms can be recapitulated in cell culture models of pre-adipocytes and β-cells, respectively. Interestingly, insulin transcription in mouse embryonic stem cells is also de-repressed in BRD2-deficient stem cells [36]. Thus, BET proteins ordinarily oppose transcription of the insulin gene and PPARγ-controlled adipogenic genes. In data being prepared for publication, we show that different BET family members control different transcriptional programs in the pancreatic β-cell both in humans and mouse models, confirming that JQ1 and other non-selective pan-BET inhibitors likely create transcriptional confusion in these post-mitotic, differentiated cells, where BRD4 is likely playing a different role than in cancer.

A recent report showing that the small molecule BET inhibitor I-BET151 may have value for type-1 diabetes [37], has already prompted excitement for potentially expanded clinical implications [38]. The fact that different BET proteins are responsible for different functions in the β-cell and other cell types is not appreciated in the field; investigators frequently and incorrectly ascribe functions to BRD4 solely on the basis of small molecule BET inhibitors. Indeed, many journals continue to publish misleading reports that refer to JQ1, I-BET151 and related small molecules as ‘BRD4 inhibitors’. It is urgent for investigators to sort through BET family member-specific pathways in each cell type that is being targeted in order to understand side effect profiles of current or next-generation BET inhibitors in human trials. Studies of biological mechanism remain far behind clinical trial thinking; no currently available small molecule is selective for any individual BET protein. The consequences of inhibition of individual BET proteins in differentiated, somatic human cells are almost totally unknown, and the potential side effect profile is frightening to contemplate.

Adipocytes

We previously published a mass spectrometry investigation of the proteins that associate with BRD2, in which we identified proteins of the SWI/SNF complex, HDAC11, CAF1 and CBP/p300 [33]. It was clear that a number of the factors that interact with PPARγ also interact with BRD2; we later established that BRD2 and PPARγ interact directly to control transcription from PPAR-responsive elements in DNA [35]. We furthermore established that one of the functions of BRD2 is to oppose PPARγ-directed transcriptional activation, thus, low levels of BRD2 created either *in vivo* by gene disruption or *in vitro* by shRNA methods dramatically potentiate adipogenic transcription. In the hypomorphic *BRD2*^{lo} mouse model, this mechanism functions as a phenocopy of thiazolidinedione exposure and promotes whole body adiposity. In the 3T3-L1 model system, BRD2 knockdown greatly potentiates adipogenesis without interfering with adipogenic transcriptional programs, to enable remarkable formation of adipocytes in the absence of pioglitazone or other PPARγ ligand [35]. This analysis left open the question of whether the other interacting partners played a role in this transcriptional phenotype or whether ternary complexes of PPARγ, RXR and RARγ; were capable of mutual titration that had functional consequences.

BRD2 is expressed in the adipose tissues of mice in two forms, a long form that is found in omental, epididymal, inguinal, subcutaneous and intrascapular brown fat, and a short form that is restricted to brown fat [35]. Reduced expression of the long form in these tissues in BRD2 hypomorphic mice is associated with adipose depot expansion, increased adipogenesis and the development of a whole body form of ‘metabolically healthy obesity’ [15,35]. Knockdown of endogenous BRD2 mRNA by stable transfection of the 3T3-L1 pre-adipocyte dramatically promotes increased Oil Red O staining of cells upon induction of differentiation with insulin/isobutylmethylxanthine/dexamethasone, whereas forced expression of the long form of BRD2 through stable transfection dramatically reduces Oil Red O staining [35]. We established independently in co-immunoprecipitation experiments that BRD2 associates with PPARγ-containing multiprotein complexes [35]. Promoter reporter assays demonstrate that the long form of BRD2 functions to oppose the PPARγ-directed adipogenic transcriptional program in the 3T3-L1 pre-adipocyte [35]. Thus, reduced BRD2 expression shifts the PPARγ-directed transcriptional environment to one that functionally resembles exposure to a thiazolidinedione drug, *e.g.*, pioglitazone. Differential expression of spliced short and long forms of each BET protein may work as a transcriptional switch. The effects of BET protein-selective inhibition with small molecules in human pre-adipocytes or differentiated adipocytes have not been reported.

Observations that whole-body reduction of BRD2 in mice promotes obesity, and adipocyte-selective reduction of BRD2 promotes adipogenesis, prompted us to hypothesize that JQ1 treatment promotes obesity. When administering JQ1 to mice, we observed weight loss instead, which we attribute to possible stem cell depletion similar to hematopoietic models of BRD2 [19,20] and BRD3 [39], and to glucose uptake toxicity. There is reason to hope that next-generation, small molecule BET inhibitors that are selective for each BET family member will be able to resolve therapeutic benefits from toxicities.

Triple-negative breast cancer

Large-scale clinical studies and meta-analysis of prospective studies established that obesity (and high Body Mass Index *per se*) is a risk factor for multiple cancer types, including breast cancer [40]. Breast cancer is a heterogeneous malignancy that is clinically subdivided into several classifications, identified by differential expression of markers that predict grade, prognosis and therapeutic response. Triple-negative breast cancer, which represents the worst subgroup clinically, is very challenging and currently occupies an important place in the breast cancer research landscape. Triple-negative tumors that lack estrogen receptor, progesterone receptor and epidermal growth factor receptor 2 (ER^-, PR^-, $HER2^-$) are generally scored with high grade, are aggressive and disseminated, and present a poor prognosis for patients. Because of their restrictive phenotype and the lack of identified targets, therapeutic tools to treat triple-negative breast cancer are unacceptably weak [41]. It is increasingly urgent to identify precise and relevant targets that can efficiently broaden the therapeutic drug arsenal.

Six clinical trials are currently ongoing (5 of 6 are recruiting) to investigate the efficacy of BET bromodomain inhibitors in triple-negative breast cancer (and most of them actually monitor multiple types of advanced solid tumors). Only one trial was testing a compound for which results have been reported: OTX015 (OncoEthix, acquired by Merck in 2014) that has been described to target MYC transcription in B-cell lymphoma and acute leukemia [12,42].

Faced with such a huge misunderstanding of the molecular mechanisms of these compounds and their lack of selectivity for the different BET proteins, basic science research must react and undertake new mechanistic studies with next-generation BET bromodomain inhibitors'. JQ1 and other pan-BET bromodomain inhibitors' anti-cancer effects have been reported to rely mainly on down-regulation of MYC-dependent transcription programs [13,14]. Thus, pan-BET inhibition might pose a major problem with respect to the impact on numerous downstream transcriptional programs. However, other roles may be specifically attributable to each BET protein. BRD2, BRD3 and BRD4 are not fully functionally redundant. Specifically, neither $BRD2^{null}$ nor $BRD4^{null}$ mice are viable indicating that loss of one BET protein is not compensated for by continued function of the others [35,43]. One relevant example of a BRD4-only dependent function is its selective interaction with the transcription factor Twist, one of the master regulatory genes of epithelial-mesenchymal transition (EMT). BRD4 interacts with diacetylated Twist *via* its bromodomain 2, where other BET proteins do not. This interaction is crucial to regulate *WNT5A* expression, EMT transcription programs and tumorigenesis in basal-like breast cancer [44].

Because of their lack of selectivity, actual pan-BET bromodomain inhibitors constitute a major limiting issue for the elucidation of specific functions of BRD2, BRD3 or BRD4. Solving this problem by providing selective compounds of individual BET proteins is a crucial challenge to understand of their biological roles better, to unravel the molecular mechanisms of their signaling and to envisage relevant targeted therapeutic strategies. For example, it will be important to determine whether BET inhibitors intended for cancer therapy inadvertently cause temporary or permanent male sterility due to inhibition of the testis-specific BET member BRDT [45]. At the very least, the informed consent process

must explain this potential danger. Finally, a recent study suggested a mechanistic explanation for primary and acquired BET inhibition resistance in a model of acute myeloid leukemia [46]. The authors show that JQ1 resistance can be achieved through a remodeling of epigenetic marks and reactivation of the transcription of key BRD4 target genes. This study perfectly illustrates the urgent need for new compounds to confront the expected, acquired chemoresistance in cancer patients.

Of note, several studies are currently ongoing or have started to describe new compounds with a better selectivity regarding individual BET proteins. Recently, Raux *et al.* identified an acetylated-mimic xanthine derivate as the first low-micromolar, selective BRD4 bromodomain 1 inhibitor [47]. Interestingly, this compound exhibits a low but dose-responsive down-regulation of c-MYC, suggesting that the relationship between BET proteins and MYC is far more complex than previously appreciated.

Summary

Available data point to low-dose, ‘on-target’ effects for BET inhibition in tissues unrelated to cancer, including but not limited to: normal pro-inflammatory macrophages, T cells, pancreatic β-cells and adipocytes. The side effect profile of BET inhibitors can be guessed from animal models, which strongly suggest that, as far as molecular mechanism is concerned, cancer clinical trials have wandered deep into the forest without a map. The role of each BET protein in transcriptional control of secretory, differentiation and metabolic pathways in ‘post-mitotic’ cells is poorly understood, but urgent to investigate, in order to understand potential side effect profiles of BET inhibitors that are prematurely being evaluated in clinical trials not informed by mechanism. Overall, BET protein inhibition is very promising for the emergence of new therapeutic strategies, notably for several cancers that currently lack such efficient options like triple-negative breast cancer. However, the tendency to push compounds too fast in clinical trials without an appropriate biological knowledge of their properties represents a dangerous risk of failure that is unacceptable for both research and medical communities, and patients. Serious adverse events, such as accidental death at the Phase 1 stage as recently occurred in France [48], could abort any further development of new BET inhibitors that might have provided a real advantage in the clinic.

Acknowledgments

Funding: NCI U01 CA182898

Definitions

BET	bromodomain and extraterminal domain

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