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Protein degradation for drug discovery

Small-molecule PROTAC degraders of the Bromodomain and Extra Terminal (BET) proteins — A review

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The PROteolysis TArgeting Chimeric (PROTAC) concept has provided an opportunity for the discovery and development of a completely new type of therapy involving induction of protein degradation. The BET proteins, comprised of BRD2, BRD3, BRD4 and the testis-specific BRDT protein, are epigenetic readers and master transcription coactivators. Extremely potent and efficacious small-molecule PROTAC degraders of the BET proteins, based on available, potent and selective BET inhibitors, have been reported. BET degraders differ from BET inhibitors in their cellular potency, phenotypic effects, pharmacokinetic properties and toxicity profiles. Herein, we provide a review of BET degraders and the differential outcome observed in the cellular and animal models for BET degraders in comparison to BET inhibitors.

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Introduction

The PROteolysis TArgeting Chimeric (PROTAC) concept, first described more than 15 years ago [1,2], calls for the design of a bifunctional molecule capable of inducing targeted protein degradation. A typical PROTAC degrader consists of a linker group and two moieties, one binding to the protein of interest (POI) and the other to an E3 ligase degradation complex to recruit the POI to the E3 ligase complex for ubiquitination and subsequent degradation by the proteasome.

Several E3 ligases have been employed for the design of PROTAC degraders. The multi-protein Cullin-Ring E3 ubiquitin ligase (CRL) machinery is a platform that has frequently been employed in the design of PROTAC molecules. Small molecule degraders engaging directly a single RING E3 ligase such as MDM2 [3] and cIAP1, as in SNIPERs (Specific and Nongenetic IAP-dependent Protein Erasers) [4,5] have also been reported. The discovery of the immunomodulatory drugs (IMiDs) [6] as ligands for cereblon (CRBN), a substrate receptor in the CRL4 E3 ubiquitin ligase [7–9] has provided a new opportunity for the design of PROTAC molecules [10,11]. The expanding strategies of chemical induction of protein degradation provide powerful chemical probes with which to examine potential drug targets [12,13] and their subsequent advancement as drug candidates for clinical development. Currently, one PROTAC drug candidate inducing

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degradation of the androgen receptor (AR) from Arvinas is in Phase I clinical trial and more candidates are expected to advance into clinical development in the near future. A number of excellent reviews covering this rapidly evolving field have appeared [2,14–16]. In this review, we will discuss recent progress made in the discovery of PROTAC BET (Bromodomain and Extra Terminal) proteins degraders with a focus on some of the key differences between degraders and the corresponding inhibitors.

In the following sections, we first briefly review the BET proteins, and then discuss the design of BET degraders and the major differences between BET degraders and BET inhibitors in terms of their biological consequences and pharmacological outcomes.

The BET proteins

Acetylation of lysine in histone tails has been associated with the epigenetic switching of a gene from repressive to active states [17]. A key modular domain recognizing the acetyl-lysines in a histone is the bromodomain (BRD) [18]. To date, 41 BRD-containing proteins have been identified [19] and 30 of these have been shown to bind to the acetyl-lysine coded in different segments of the histone tails [18]. BRD-containing proteins are known to play a role in cancer and a number of other human diseases [20–24]. For example, NUT midline carcinoma (NMC) [25], a rare form of cancer, contains a fusion gene of BRD4 with the nuclear protein in testis gene (NUT). In other cases, BRDs either acquire mutations in diseases [26,27] or participate directly in the aberrant epigenetic regulation by collaborating with other dysfunctional chromatin modifying enzymes and transcription factors [28].

The BET proteins are one class of BRD-containing proteins and have four family members including BRD2, BRD3, BRD4 and testis-specific BRDT [29]. Although all BET proteins bind to acetylated chromatin, BRD4 functions as a transcription coactivator that stimulates the transcription elongation activity of the RNA Polymerase (Pol) II transcription complex by recruiting P-TEFb to the promoter [30] or directly phosphorylating Pol II [31]. The discovery of (+)-JQ-1 and other classes of small-molecule inhibitors of the BET proteins provided evidence that BET proteins can be therapeutically targeted [32–34]. Currently, 13 small-molecule BET inhibitors are in clinical trials for the treatment of human cancers and other diseases [35,36]. Early phase clinical trials results however, show that BET inhibitors achieve only modest clinical activity as single agents in patients with advanced cancer [35,36]. Because BET proteins contain multiple functional domains including an extra terminal domain that interacts with transcription factors responsible for ribosomal RNA production [37], small-molecule inhibitors such as JQ-1 may only block their chromatin binding functions. Consequently, new therapeutic approaches, such as targeted protein degradation, could be more much effective for the treatment of human diseases in which BET proteins play a key role.

BET proteins contain several conserved domains, namely bromodomain 1 (BD1) and bromodomain 2 (BD2), an extra-terminal domain (ET), and the Ser/Glu/Asp (SEED)-rich domain region (Fig. 1). Three isoforms have been reported for BRD4. BRDT and the longest isoform of BRD4 contains the C-terminal p-TEFb-interacting domain (PID) that recruits RNA

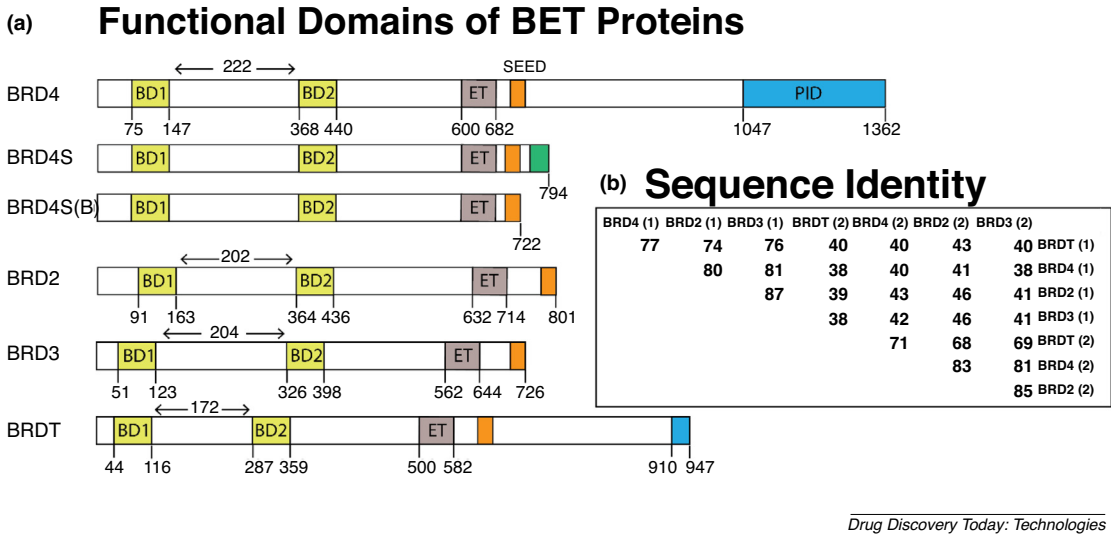

Fig. 1. Functional domains of BET proteins and the sequence identity between BET BD domains. (A) Functional domains of BET proteins in the UNIPROT database are shown and the amino acid numbers corresponding to each domain are labelled. The number of amino acids between BD1 and BD2 for each BET proteins is also noted. (B) Sequence identity between BET BD domains. *Abbreviations*: BD1/2, bromodomain 1/2; ET, extra terminal; SEED, Ser/Glu/Asp rich domain; PID, p-TEFb-interacting domain.

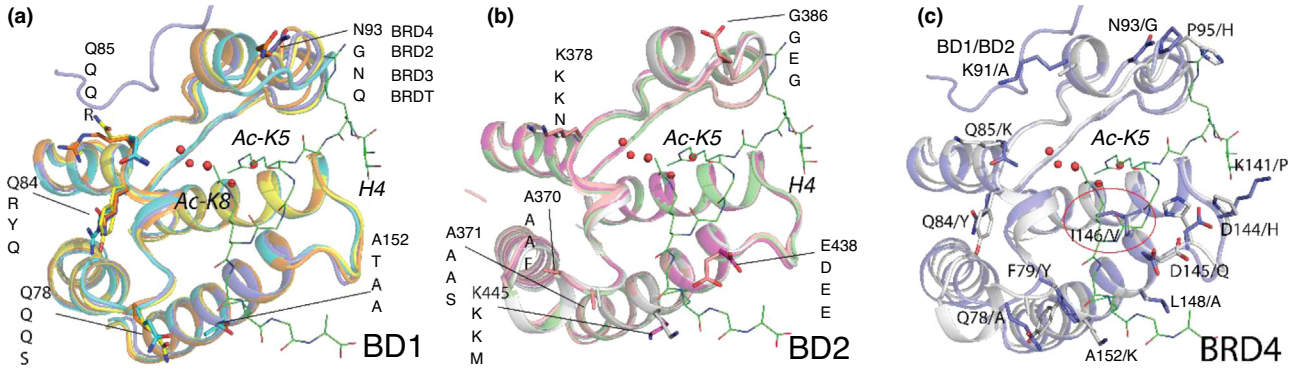

Fig. 2. Amino acid differences in the acetyl-lysine binding sites of BET BDs. Structures of BET proteins (A) BD1 (PDB: 3ZYU, 4ALG, 3S91, 4FLP) and (B) BD2 (PDB: 2YEM, 3ONI, 3S92, 2WP1). The proteins are aligned and amino acids that are different in different BET proteins are depicted and labelled. The acetylated H4 peptide bound to BRD2-BD1 (PDB: 4ALG) is showed in green for reference. (C) The differences in the amino acids at the binding sites between BRD4-BD1 and BRD4-BD2 are highlighted.

polymerase II. The PID is replaced by different amino acid sequences in BRD4S which are absent in BRD4S(B) [38]. There is a high sequence identity between different BD1 or BD2 domains in BRD2-4 versus BRDT but a relatively low sequence identity between BD1 and BD2 in the same BET protein (Fig. 2A–B). For BD1 or BD2 in different BET proteins, residues that interact directly with acetyl-lysine are well conserved. However, there are a number of residue differences between BD1 and BD2 in each BET protein. In BRD4, 13 amino acids in BD1 are different from those in BD2 including one located at the binding site that interacts with acetyl-lysine (L146 in BD1 versus V439 in BD2 in Fig. 2C). The differences between BD1 and BD2 within the same BET protein provide a structural basis for the design of selective inhibitors of BD1 or BD2, and, as an example, a compound (ABBV-744) with a 300-fold selectivity for BD2 over BD1 was recently reported [39]. However, the high degree of amino acid conservation for the same BD1 or BD2 domains between different BET members makes it difficult to design highly selective inhibitors that target a single BET member.

Essential components in the PROTAC degrader design

A PROTAC degrader requires a ligand to bind to the POI. Since the discovery of the first BET inhibitor, (+)-JQ1 [32], a number of classes of BET inhibitors have been reported [35,36] and these provide ample choices for BET degrader development. In early proof-of-concept studies, (+)-JQ1 (Fig. 3) was used for the design of both pan-BET degraders (dBET1 [10], ARV-825 [11]) and selective BRD4 degraders (MZ1 [40], dBET23 [41]). Our laboratory has developed CF-53 [42,43] and QCA276 [44] (Fig. 3) as two classes of BET inhibitors with nanomolar binding affinities to BET proteins, which have been employed for the design of BET degraders [44–46].

Another consideration in the design of PROTAC degraders is the selection of the E3 ligase systems. Both Cullin-4- and Cullin-2-Ring E3 ubiquitin ligase (CRL4, CRL2) platforms have been shown to be effective, inducing POI ubiquitination and subsequent degradation in recent examples. The challenge of selecting other CRLs is the dearth of small molecules that have been shown to recruit E3 ligases [47]. Engagement of a single RING E3 ligase using MDM2 [3] and cIAP1 [4,5] has been reported, but the degradation of POIs via the RING E3 ligase is less efficient in the examples reported [48,49]. Subcellular localization of the ubiquitination machinery can be a factor to the success of the PROTAC concept.

Other critical considerations in the design of PROTAC degraders include the linkage positions on the inhibitor and the length and chemical composition of the linker. Available crystal structures can guide the choices of the pivotal points in the molecules to avoid steric clashes between the linker segment and POI. The linker length can control the proximity to the POI and influence the ubiquitination efficiency. For example, a 16-atom chain was found to be optimal in the degradation of ER using a HIF-1α motif in PROTAC molecules [50]. Notwithstanding the fact that the linker length can be subject to optimization, a long and flexible linker is typically used in the initial design of PROTAC molecules. For this purpose, a multi-unit polyethylene glycol [51] and an extended carbon chain [52] are frequently used as linkers in the design of initial PROTAC degraders.

Design of BET degraders

In the first BET degrader reported by Winter et al. (dBET1, Figs. 3 and 4A) [10], an eight-atom linker, *N*-butyl-2-hydroxyacetamide was used to connect (+)-JQ1 with thalidomide — the molecule that binds to CRBN in the $CRL4^{CRBN}$ complex. When a human AML cell line (MV4:11) was treated with 100 nM concentration

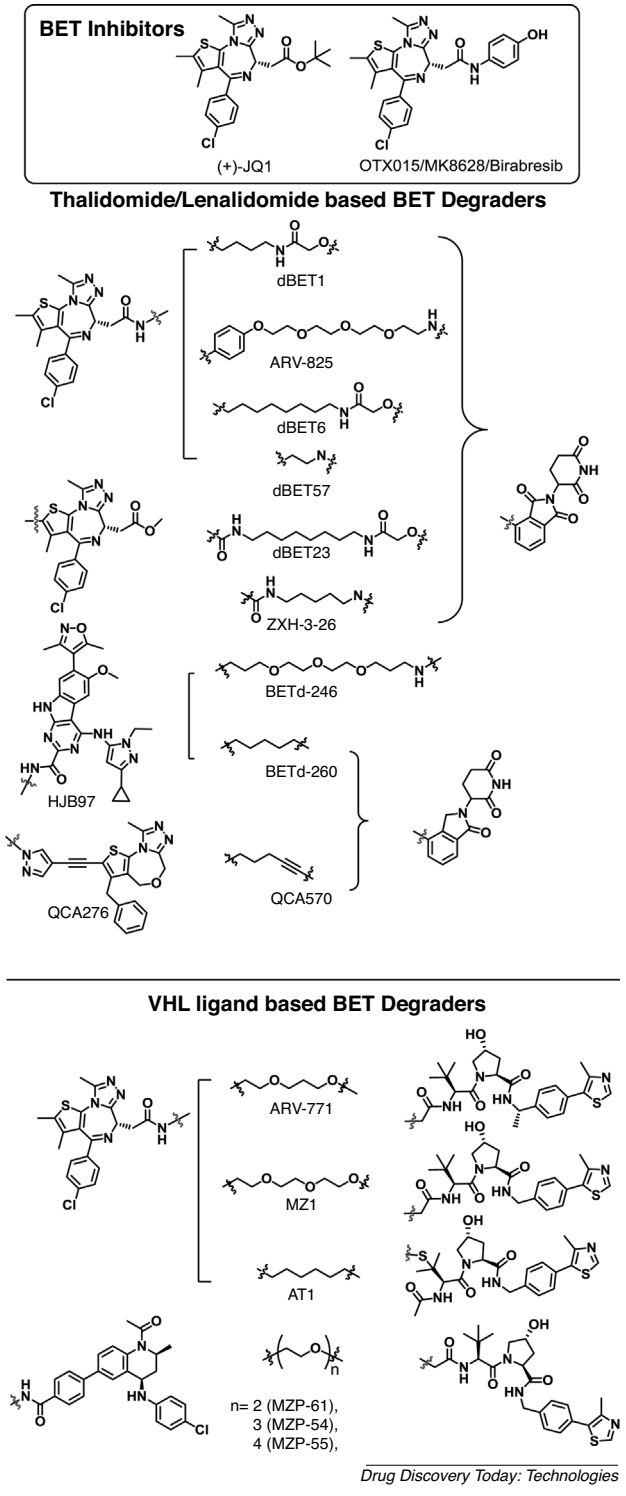

Fig. 3. Reported BET degrader design strategies utilizing thalidomide, lenalidomide and VHL ligands as the motifs with which to recruit CRL molecules.

of dBET1, depletion of BRD4 started at 1 h but partial recovery of BRD4 occurred at 24 h. Another BET degrader ARV825 (Fig. 3), developed by Lu et al. at Arvinas [11] uses a different linker and OTX015 to bind with BRD4. Using the Burkett's lymphoma (BL) cell lines, the DC_{50} (the drug concentration that results in 50% protein degradation) of the BRD4 degradation for ARV825 was estimated to be below 1 nM. Besides thalidomide/lenalidomide, an optimized VHL ligand that recruits VHL in the $CRL2^{VHL}$ system has been used extensively for the design of BET degraders (Fig. 3). A proof-of-concept BET degrader using a VHL ligand is ARV-771 (Fig. 3) that was shown to induce rapid BRD2/3/4 degradation with $DC_{50} < 5$ nM in castration resistant prostate cancer (CRPC) cell lines and tumor regression in the 22Rv1 CRPC xenograft mouse model [53].

Our laboratory has reported two different classes of BET degraders, based on our potent BET inhibitors and ligands for CRBN and represented by BETd-246/BETd-260 [45] and QCA570 [40]. In designing BETd-246/BETd-260, the co-crystal structure of CF53 complexed with BRD4-BD1 protein (PDB: 6C7R) suggested the replacement of the solvent-exposed methyl group (Fig. 4B) in the tricyclic system with an amide group (HJB97 in Fig. 3, $K_i < 1$ nM to BET proteins) to build the linker group. In the case of QCA570 [40], modeling showed that the triple bond in QCA276 goes across the channel formed by W81, P82, Q85, L92 in BRD4-BD1 and the solvent exposed 1-methyl-1H-pyrazole group (Fig. 4C) contains a suitable site at which to anchor a chemical linker. Both BETd-260 and QCA570 exhibit picomolar cellular potencies in several cancer cell lines and QCA570 is arguably the most potent BET degrader reported to date. At concentrations of 10–30 pM, both BETd-260 and QCA570 significantly reduce the levels of BRD4 protein in leukemia cells. BETd-260 achieves IC_{50} values of 51 pM and 2.3 nM in cell growth inhibition in the RS4;11 and MOLM13 cell lines, respectively and is thus 10-500 times more potent than its parent inhibitor HJB97 [44]. QCA570 inhibits cell growth in MV4;11, MOLM-13, and RS4;11 cell lines with IC_{50} values of 8.3, 62, and 32 pM, respectively. In direct comparison, QCA570 is at least 3000-times more potent in the cell growth inhibition than its corresponding BET inhibitor QCA276 [40].

By harnessing an appropriate linker length with a pan BET inhibitor, Zengerle et al. was the first to report the discovery of MZ1 as a selective BRD4 degrader [40]. This selective degradation of BRD4 over BRD2 and BRD3 proteins is rationalized by the ternary crystal structure of VHL:ElonginC: ElonginB/BRD4-BD2/MZ1 (PDB: 5T35, Fig. 4E) that shows acquired protein-protein and protein-ligand interactions in three-body binding [54]. The degree of favorable (or unfavorable) three-body interaction can be quantified by a cooperativity index [54]. The ternary structure guides the design of AT1 that degrades BRD4 more selectively [54]. Recently, co-crystal structures of CRBN/BRD4-BD1/BET degraders (PDBs: 6BN7, 6BOY, Fig. 4D) were reported [41]. Despite the linkage at two different sites in (+)-JQ1, dBET6 and dBET23 (binding preferentially to BRD4-BD1) were shown to induce the formation of a similar ternary structure pertaining to preferred BRD4-BD1/CRBN binding (Fig. 4D). This finding led to a computational protein-protein docking calculation to identify low-energy binding complexes between CRBN and BRD4-

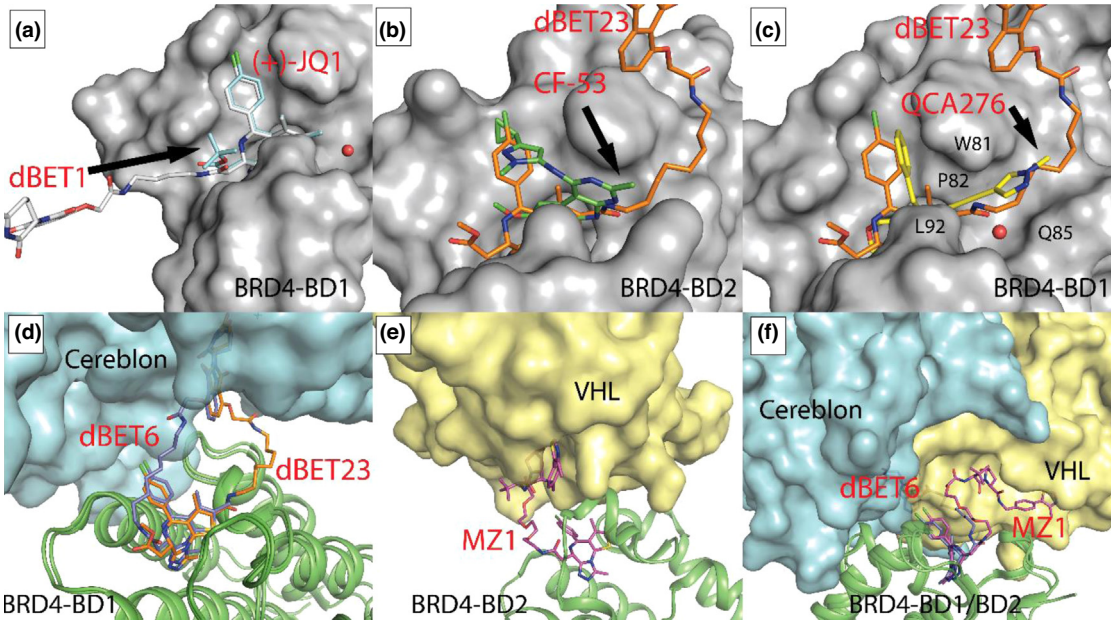

Fig. 4. Linkage positions of BET inhibitors in the development of BET degraders. Alignment of crystal structures of (A) BRD4-BD1/(+)-JQ1 (PDB: 3MXF) and BRD4-BD1/dBET1 (PDB: 4ZC9), (B) BRD4-BD2/CF-53 (PDB: 6C7R) and BRD4-BD1/dBET23 (PDB: 6BN7), (C) BRD4-BD1/dBET23 and BRD4-BD1/QCA276 (model), (D) The same ternary structure is formed by the same BET inhibitor linked at two positions. CRBN(cyan)/BRD4-BD1(green)/dBET23(orange) (PDB: 6BN7) and CRBN (not shown)/BRD4-BD1(green)/dBET6(blue) (PDB: 6BOY), (E) MZ1 stabilizes a different interaction interface between BRD4-BD2 and VHL compared with (D). VHL(yellow)/BRD4-BD2(green)/MZ1(purple) (PDB:5T35), (F) BRD4-BD1 and BRD4-BD2 are aligned using the ternary complexes of CRBN (cyan)/BRD4-BD1(green)/dBET6(blue) and VHL(yellow)/BRD4-BD2(green)/MZ1(purple) to highlight the different protein-protein interaction interfaces between BRD4 and CRBN and VHL. The arrows indicate the linkage positions used in the design of BET degraders.

BD1 that were used to develop a BRD4 selective BET degrader, ZXH-3-26, giving DC_{50} ~5 nM at 5 h [41]. Comparison of the protein complexes mediated by dBET23 and MZ1 indicates that BRD4 engages different surface areas to bind with CRBN and VHL (Fig. 4F). These ternary crystal structures also suggest that the interaction gained between the POI and the receptor protein (determined by the cooperativity index) may deliver degradation selectivity in POIs that possess high sequence similarity at the binding sites.

Recently, a series of compounds such as MZP-61, based on a different BET inhibitor (I-BET726), were used to study the impact of the target warhead and linkage direction on BET protein degradation [55]. The study suggested that potent inhibitors are not sufficient to generate potent PROTAC molecules [55–57]. However, use of potent inhibitors for PROTAC design can minimize unintended recruitment of promiscuous POIs to the ubiquitination machinery.

We have explored how the chemical composition and rigidity of the linker affect the effectiveness of the degrader [44,46]. In our study [44], it was found that five-atom linkers between QCA570 and either thalidomide or lenalidomide were optimal to induce degradation of BET proteins. Furthermore, a linker with a rigid triple bond in QCA570 analogs [44] performed better than an all-carbon linker or a linker containing an NH group. In general, optimization of the linker, including reduction of the length and number of rotatable bonds, offers opportunities to improve drug-like properties of protein degraders [58–61].

Biological consequences

BET degraders cause widespread downregulation of gene transcription in tumor cells

BET proteins have both bromodomain (BD)-dependent and BD-independent functions [37,62,63]. Therefore, depletion of BET proteins by a degrader is expected to have a more profound effect on BET-mediated transcriptional complexes than a BET inhibitor which displaces BET proteins from their binding to acetyl-lysines [10,53,64,65]. Compared to occupancy-centric small-molecule inhibitors, much lower concentrations of degraders can achieve profound therapeutic effects by efficiently eliminating targeted proteins [11,66]. For example, BET degraders such as dBET1/dBET6, ARV-825/ARV-771, BETd-246/BETd-260 and QCA-570 suppress expression of genes (e.g., c-MYC) regulated by BET proteins much more effectively than the corresponding BET inhibitors. This has resulted in marked inhibition of cell growth and induction of apoptosis in preclinical models of solid tumors and hematologic malignancies [10,11]. Glioblastoma cells treated with dBET6 exhibit significant depleted chromatin occupancy of BET proteins, reduced RNA-pol2 activity and impaired transcription program regulated by E2F1 [67]. In AML cells, ARV-825 was found to impose a greater perturbation to the

mRNA levels than the inhibitor, OTX015 [65]. Interestingly, both BET inhibitor and degrader treatment induced comparable numbers of up- and down-regulated genes in AML cells [65]. In our study in triple negative breast cancer models [45], BETd-246 treatment was found to cause a global downregulation of gene transcription, consistent with the role of BET proteins as master regulators of global transcription elongation [10]. Analysis of gene set enrichment and gene ontology enrichment reveals that multiple biological processes such as regulation of transcription and survival signaling pathways are significantly affected by BETd-246 [45]. For example, the gene signature for IL6-STAT3 signaling is significantly downregulated by BETd-246 in TNBC. Our profiling of transcriptomes also reveals pronounced downregulation of the antiapoptotic MCL1 [45], which may contribute greatly to the strong pro-apoptotic activity of BETd-246, BETd-260, QCA570 and other BET degraders.

Oncogene c-MYC has long been regarded as ''undruggable'' [68,69]. Downregulation of c-MYC expression upon BET inhibition is commonly found in both solid tumors and hematologic malignancies in preclinical studies. Downregulation of c-MYC correlates with BET inhibitor sensitivity in multiple preclinical models including multiple myeloma [70], acute myeloid leukemia [71–73], Burkitt lymphoma [72], acute lymphoblastic leukemia [74], diffuse large B cell lymphoma [75,76], glioblastoma [77], prostate cancer [78], MYC-amplified medulloblastoma [79], K-ras-mutant non-small cell lung cancer [80] and Merkel cell carcinoma [81,82] and it has been suggested that it may be accountable for the antitumor activities of BET inhibitors. We found that treatment with BET-d246 results in transcriptional downregulation of c-MYC in TNBC and AML cell lines [44–46]. This effect however is largely transient in TNBC cell lines whose mRNA and protein levels recover rapidly, suggesting that c-MYC downregulation is unlikely to be the main cause for the antitumor activity of BET degradation in breast cancer, in agreement with other studies employing BET inhibitors [37,63]. Instead, we suggest that the broad suppression of gene transcription by BET degraders collectively may contribute to their profound antitumor activities in breast cancer cells.

BET degraders exert antitumor activity superior to that of their parent BET inhibitors in preclinical tumor models

Extensive preclinical studies have shown that the effects of BET inhibitors are largely cytostatic, especially in solid tumor models, and induction of apoptosis is seen in only a few blood cancer models [70,75,83]. Conery et al. [84] recently reported that in melanoma and leukemia models, the preclinical antitumor efficacy of BET inhibitors is determined by the magnitude of the apoptotic, rather than the cytostatic response. This is consistent with earlier studies which show that induction of apoptosis by BET inhibitors is often associated with their preclinical efficacy in leukemia, multiple myeloma, peripheral nerve sheath tumors and neuroblastoma [34,70,72,74,85,86]. We found that the BET inhibitor BETi-211 provokes minimal or very modest apoptosis in TNBC and in contrast, the BET degraders BETd-246 and BETd-260 elicit strong apoptotic response in the majority of TNBC cell lines [45]. Treatment with the BET degraders BETd-246 or BETd-260 inhibits the growth of TNBC tumors much more effectively than the corresponding BET inhibitor [45]. Notably, BETd-260 or QCA-570 both completely eliminate the tumors of RS4;11 in mouse xenograft models [44,46]. The superiority of the antitumor activity of BET degraders to that of the corresponding inhibitors is in agreement with the extent of their effects on the transcriptome.

BET degraders are well tolerated in mice

Previous studies showed that BRD4-depleted mice display reversible epidermal hyperplasia, alopecia, and decreased cellular diversity and Lgr5+ stem cell depletion in the small intestine [87]. In our preclinical studies, BET degraders BETd-246/260 and QCA-570 are well tolerated in mice with no overt toxicity [44,45]. However, in view of their pleiotropic effects on global gene expression, the toxicity of BET degraders should be carefully assessed, in rodent and non-rodent species, before such compounds can be advanced into human clinical trials.

Conclusion

The discovery of (+)-JQ1 has provoked intense interest in the development of BET inhibitors and more than 10 BET inhibitors have advanced to early stage clinical trials for patients with different types of cancer. However, BET inhibitors demonstrate very limited antitumor activity in patients, suggesting a new therapeutic approach is needed to target BET proteins. Employing the PROTAC strategy, highly potent and efficacious BET degraders have now been developed. Both pan BET and selective BRD4 degraders have been discovered. Preclinical data have shown that BET inhibition and degradation produce very different cellular fates and biological outcomes. PROTAC BET degraders may offer a new therapeutic opportunity to target BET proteins and to significantly improve the limited clinical efficacy of BET inhibitors.

Despite the impressive preclinical antitumor activity demonstrated by pan-BET degraders in vitro and in vivo, no BET degrader has been advanced into clinical development, suggesting that either a selective BRD4 degrader or a tissue-specific BET degrader may be needed for clinical development. Nevertheless, the knowledge we have gained from the successful design of extremely potent and efficacious BET degraders is very valuable for the design of degraders for different protein targets. PROTAC degraders are not only powerful biological and pharmacological agents for research but also may have the great potential to be developed as a

completely new type of therapies for the treatment of human cancers and other diseases.

Notes

The University of Michigan has filed a number of patent applications on the BET inhibitors and degraders reviewed in this work. The patents have been licensed to Oncopia Therapeutics Inc. S. Wang, Q. Chong, C.-Y. Yang and L. Bai are co-inventors on one or more of these patents. S. Wang is a co-founder and paid consultant of Oncopia Therapeutics, Inc, and owns stock in Oncopia. The University of Michigan also owns stock in Oncopia.

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