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REVIEW ARTICLE

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Inhibitors of bromodomain and extra-terminal proteins for treating multiple human diseases

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Abstract

Clinical development of bromodomain and extra-terminal (BET) protein inhibitors differs from the traditional course of drug development. These drugs are simultaneously being evaluated for treating a wide spectrum of human diseases due to their novel mechanism of action. BET proteins are epigenetic "readers," which play a primary role in transcription. Here, we briefly describe the BET family of proteins, of which BRD4 has been studied most extensively. We discuss BRD4 activity at latent enhancers as an example of BET protein function. We examine BRD4 redistribution and enhancer reprogramming in embryonic development, cancer, cardiovascular, autoimmune, and metabolic diseases, presenting hallmark studies that highlight BET proteins as attractive targets for therapeutic intervention. We review the currently available approaches to targeting BET proteins, methods of selectively targeting individual bromodomains, and review studies that compare the effects of selective BET inhibition to those of pan-BET inhibition. Lastly, we examine the current clinical landscape of BET inhibitor development.

KEYWORDS
BD-selective, BET, BETi, bromodomain (BD), enhancer

1 | INTRODUCTION

A new paradigm is emerging in drug development, centered on the regulation of epigenetic processes for treating a wide spectrum of human diseases. Epigenetic processes involve alterations to gene expression without altering the genetic code. Bromodomain and extra-terminal (BET) proteins are epigenetic "readers" that recognize and bind posttranslational modifications on histones and other transcriptional machinery to facilitate gene expression. BET proteins have received an increasing amount of attention in recent years,[1–49] and BET inhibitor(s) (BETi) that target the BET bromodomains (BD) are unique in drug development in that they are consecutively undergoing clinical investigation in vastly different disease areas. Evidence is mounting to support the use of BETi in treating cancer, metabolic, inflammatory, neurologic, cardiovascular, and musculoskeletal diseases.[1–3,14,19,20,29,31,44,50–62] Investigation of BETi potential as an antiviral has also recently garnered interest.[63–67]

Unlike other reviews in the field, we focus this review on explaining why targeting BET proteins shows potential benefit in seemingly unrelated disease states.[3,9,13,29,33,39,68–74] We begin with a brief description of BET protein structure and function. A significant amount of what is currently known about BET proteins was initially discovered in the fields of embryonic development and cancer. This study has led to the identification of BET proteins as key components of "superenhancers" (SE); chromatin structures comprised of clusters of enhancer regions designed to drive transcription.[75,76] An understanding of SEs leads into description of similar structures, termed "latent enhancers." These chromatin structures function in terminally differentiated cells to alter gene expression in disease. BET proteins, BRD4 being the best understood, play prominent roles in the development and progression of cardiovascular, metabolic, and inflammatory diseases. We present the landmark studies in these areas which demonstrate how BET proteins perpetuate disease at the transcriptional level, and provide the preliminary evidence for therapeutic potential of BETi in these areas. These studies support our explanation of why BETi benefit broad therapeutic categories, based on the intrinsic ability of cells from different lineages to respond to disease through evolutionarily conserved programs. We also describe various approaches to BET inhibition, what is known about the differential effects of pan- versus selective BD binding, and evidence supporting the use of BD-selective BETi, particularly in areas outside of cancer. BD-selective BET inhibition and its potential as a novel therapeutic approach is gaining ground within epigenetic drug development.

2 | BROMODOMAIN AND EXTRA-TERMINAL (BET) PROTEINS

BET proteins belong to a superfamily of bromodomain-containing proteins (46 members containing 61 BDs), within which they comprise a subfamily of 4 members; BRD2, BRD3, BRD4, and testes-specific BRDT.[77] The basic structure of BET proteins is comprised of two tandem ~110 amino acid bromodomains (BD1 and BD2) and an extra-terminal domain, with BRD4 and BRDT including a C-terminal motif (CTM) (Figure 1). As epigenetic "readers" BET proteins bind to their natural ligand, acetylated lysine, a posttranslational modification found on histone tails and transcription factors.[78] Exceptionally high concentrations of acetylated lysines are found on active chromatin; open regions of DNA containing genes that are accessible for transcription.[69,72,78–82] The binding of BET proteins to acetylated lysines on active chromatin plays an important role in gene transcription, as the BET proteins form docking scaffolds for transcription factors and other transcriptional machinery. In this way, BET proteins directly link epigenetic modifications to gene expression. Here, we describe some of the better-known functions of each BET protein. Little is known about BRD2, BRD3, and BRDT, while the most well-studied BET protein is BRD4.

BRD2 modulates gene transcription in connection with cell cycle activity of proliferating cells in embryonic development, neural development, and in cancers/tumors at later stages of development.[83,84] During embryogenesis, *Brd2*-deficient mice exhibit delayed fibroblast proliferation, neural tube closure defects, and overall delayed growth.[83,84] Though all BET proteins are highly structurally conserved and associate with numerous common proteins, BRD2's interactome displays the least overlap with other BET proteins.[30] For example, studies have shown that BRD2 primarily associates with E2F transcription factors.[22,85,86] In fibroblasts transfected with

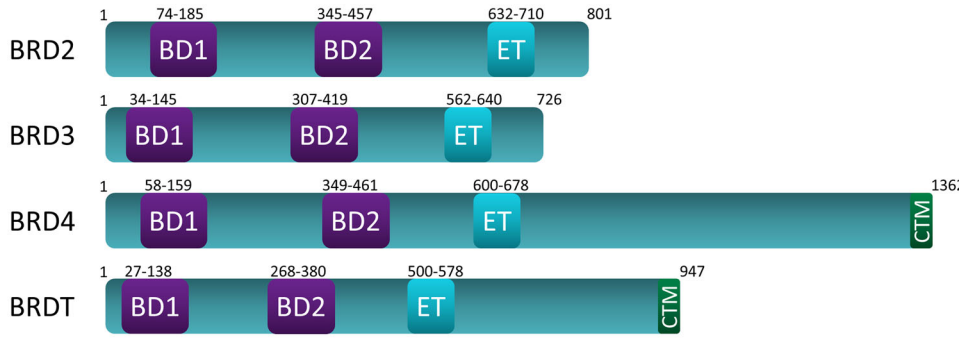

FIGURE 1 Schematic representation of basic domain organization of human BET (bromodomain and extra-terminal domain) family proteins; BRD2, BRD3, BRD4, and BRDT. BET proteins contain two ~110 amino acid bromodomains (BD1 and BD2) and an extra-terminal domain (ET). Only BRD4 and BRDT have a C-terminal motif (CTM)

BRD2, interruption of BRD2 interactions with E2F negatively regulated E2F-dependent cell differentiation and proliferation genes, such as cyclins A, D1, and E, which are required for the G1/S transition.[86,87] In addition to E2F, BRD2 interactions with RNA polymerase II (RNA Pol II), a complex that transcribes DNA into messenger, small nuclear and microRNA, are involved in transcription elongation in embryonic kidney cells.[87] BRD2's lack of a CTM indicates that its interactions with RNA Pol II are independent of positive transcription elongation factor b (PTEF-b), and thus differ from those of BRDT and BRD4 (described below). LeRoy et al. showed that BRD2 acts by chaperoning RNA Pol II through hyperacetylated nucleosomes that typically act as barriers to elongation, thus allowing for transcription.[71,87] In later stages of development, overexpression of BRD2 in adult mice has been shown to promote B-cell expansion and mitogenesis through interactions at the cyclin A promoter, leading to the development of B-cell lymphoma and leukemia.[88,89]

Known functions of BRD3, like BRD2, are related primarily to regulating the transcription of genes necessary for embryonic stem cell (ESC) development. BRD3 plays a role in erythroid cell differentiation through interactions with master erythroid transcription factor GATA-binding factor 1 (GATA1).[30,90,91] Disruption of BRD3-GATA1 binding in erythroid progenitor cells results in impaired GATA1-mediated cell maturation.[90] Additionally, a number of negative transcriptional regulators and chromatin remodelers preferentially associate with BRD3, such as the nucleosome remodeling and deacetylase (NuRD) complex.[30] BRD3 interactions with NuRD are closely linked to interactions with GATA-1, and thus are implicated in erythroid cell maturation.[30,92] BRD3 also interacts with RNA Pol II where it promotes transcription in a P-TEFb independent manner, similar to BRD2.[30] BRD3 in cancer cell lines may be antiproliferative, in contrast to commonly noted functions of other BET proteins in these cell types.[30] However, further research is needed to fully understand potential BRD3 antiproliferative effects in various cell types and stages of development.

BRDT expression is restricted to the testis. BRDT knockout or mutation in mice leads to lower sperm count and abnormal sperm morphology, indicating its role in spermatogenesis.[93–95] BRDT exhibits the least number of binding interactions with transcriptional regulators of all the BET proteins, associating primarily with the negative elongation factor (NELF) complex through its CTM binding with PTEF-b.[30] These findings are consistent with BRDT's organ specific localization, and its role in both facilitation of spermatogenic gene expression such as cyclin A1, Y-box protein 2, serine/threonine-protein kinase Plk1, aurora kinase C, and A-kinase anchor protein 4, and repression of genes that require silencing in this process, including nuclear RNA export factor 2 and testes-expressed gene 11.[93,94,96,97]

Among the BET protein family members, the BRD4 protein interactome and functionality have been most extensively studied. One of BRD4's many roles, which may be shared with BRD2,[98] lies in maintaining “mitotic memory” during cell cycle progression by remaining bound to chromatin at essential genes during mitosis. This allows transcriptional activity of these genes to be quickly reestablished as the cell cycle progresses to late- and

post-mitotic phases.[69,82,99,100] The critical role of BRD4 in fundamental cellular processes early in development is also apparent in homozygous BRD4 knockout mice, where BRD4 knockout is embryonically lethal before implantation.[101] BRD4, and likely other BET proteins, interact with histone acetyltransferases, deacetylases, and other chromatin remodelers.[102–104] Additionally, and possibly most importantly, BRD4 is involved in the recruitment of transcriptional machinery to actively transcribed genes through its interactions with RNA Pol II and PTEF-b (via its CTM, similar to BRDT), and numerous transcription factors and transcriptional coactivators.[30,105–108] BRD4 becomes the scaffold holding transcriptional complexes together at open chromatin, thereby facilitating transcription.[99] The central role of BRD4 in transcriptional regulation sets the stage for our discussion of its importance in driving disease-related transcriptional modifications.

Though we will focus the following discussion on BRD4, the BET protein for which the most is currently known, other BET proteins may play similar or opposing roles in these processes and are likely more important than currently understood. It is important to keep in mind that all four BET proteins exhibit overlap in interactors and in functionality in various cell types.[30]

2.1 | The role of BRD4 in transcription

Through its primary interactions with epigenetic coregulators, BRD4 plays a number of important roles in gene transcription. First, BRD4 is thought to assist chromatin de-compaction through histone acetyltransferase activity and association with other histone acetyltransferases, deacetylases, and chromatin remodelers.[82,102–104] For example, at enhancer regions in ESCs, BRD4 associates with the histone acetyltransferases P300 and CBP to enhance H3K27 acetylation.[102] Next, with access to the active chromatin, BRD4 binds to acetylated histones at enhancers, promoters, and transcriptional start sites. Binding to its natural ligand, acetylated lysine, on histones and transcription factors creates a scaffold for transcriptional machinery to come together. Mediator, a complex that transduces signals from transcription factors and activators at enhancers to promoters, is recruited by BRD4, which brings together all components of the active transcriptional complex; BRD4, Mediator, transcription factors, and RNA Pol II.[109–111] With the active transcriptional complex in place, BRD4's CTM binding with PTEF-b results in the phosphorylation of RNA Pol II (at serine 5) to initiate transcription[108,112–116] (Figure 2A). Following transcription initiation, BRD4 helps guide the complex along an active gene. Approximately 100 base pairs downstream of the transcription start site, where RNA Pol II pauses,[113,117] phosphorylation of RNA Pol II serine 2, permitted by maintained interaction between PTEF-b and BRD4, releases RNA Pol II pausing and advances elongation of the RNA transcript.[117,118] Therefore, BRD4's involvement in chromatin decompaction, recruitment of transcriptional complex components, as well as in initiation, pause release, and elongation stages of transcription makes it crucial in regulating gene expression.[61,72,99,119] The central role of BRD4 in these basic yet fundamental cellular processes underscores its importance in many aspects of cellular function.

2.2 | BRD4 at super-enhancers

Super-enhancers (SEs) are chromatin structures comprised of clusters of enhancers that are densely packed with BRD4, Mediator, transcription factors, acetylated histones, and coactivators that span regions of actively transcribed DNA.[75,111,120] While typical enhancers are approximately 100 base pairs in length, SEs can span as much as 50 kb pairs and contain significantly larger amounts of enhancer elements[111] (Figure 2B,C). SEs are located proximal to their target gene promoters, typically within 100 kb, and ensure that the transcriptional tool kit stays in close proximity to pre-selected gene promoter regions, priming them for gene responsiveness.[121] SEs were first described by Whyte et al.[111] in areas of the genome that are essential for lineage commitment in pluripotent ESCs. This developmental differentiation process involves a coordinated program requiring abundant expression of a well-defined, but limited panel of genes, including lineage-determining genes octamer-binding transcription factor

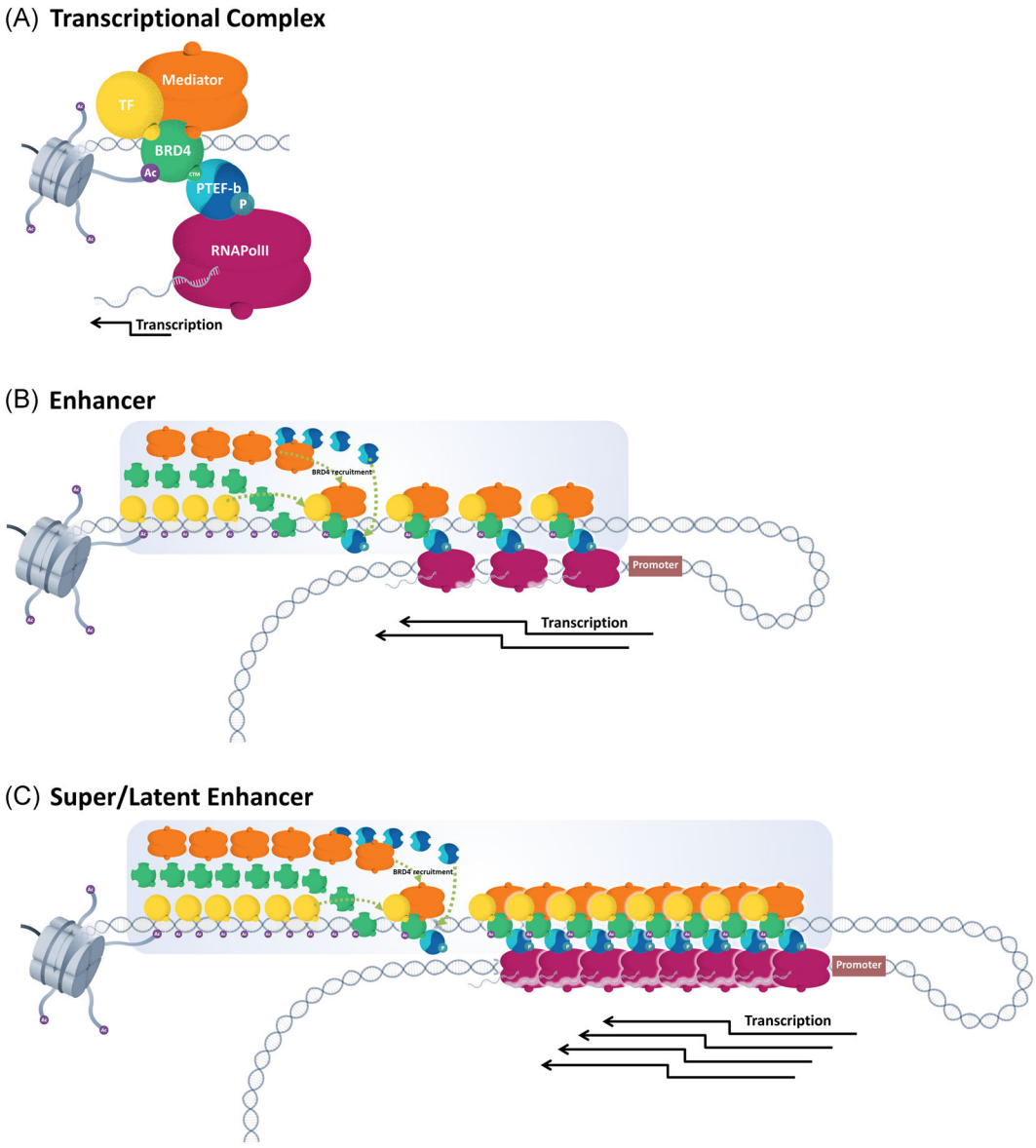

FIGURE 2 A, Transcriptional complex components. Ac, acetylated lysine; BRD4, bromodomain and extraterminal protein 4; P, phosphorylation; PTEFb, positive transcription elongation factor b; RNA Pol II, RNA polymerase II; TF, transcription factor. B, At typical enhancers, BRD4 binds its ligand, Ac, and becomes a scaffold for recruiting Mediator, transcription factors, and PTEFb to phosphorylate RNA Pol II at the target gene promoter, thereby initiating transcription. C, Latent and super-enhancers are densely packed with transcriptional complex components. The abundance of transcriptional machinery and BRD4 result in elevated levels of gene transcription

4 (*OCT4*), sex determining region Y-box transcription factor 2 (*SOX2*) and homeobox protein *NANOG*.[122] In ESCs, SEs boost the expression of these lineage-determining genes to levels needed to define cell identity.[75,111] BRD4 aids in the formation of ESC SE sites through its roles in chromatin decompaction, neutralizing positive charges on histones and other acetylated lysines, and recruiting the transcriptional machinery needed for rapid and abundant transcription.[75,109,123] Hnisz et al.[75] soon after expanded on Whyte's definition of SEs by examining 86 human cell

and tissue types, and determining that SEs are involved in cellular processes beyond development, namely those involved in cancer. For example, elevated levels of histone acetylation (particularly H3K27ac), BRD4, and Mediator are present at oncogenic SEs and are often used for SE identification. As at lineage determining SEs, BRD4 and Mediator aid in chromatin remodeling and the aggregation of transcriptional machinery, and BRD4's CTM binding with PTEF-b releases RNA Pol II pausing, advancing the elongation of oncogene transcripts.[76] These SEs form in proximity to well-known oncogenes like the MYC proto-oncogene (*MYC*), Immunoglobulin Lambda Like Polypeptide 5 (*IGLL5*), Interferon Regulatory Factor 4/Multiple Myeloma Oncogene 1 (*IRF4*), and X-Box Binding Protein 1 (*XBP1*) in multiple myeloma cells leading to their overexpression. In contrast to lineage determining SEs, oncogenic SEs facilitate maladaptive gene expression, resulting in tumor expansion and disease progression. With the loss of BRD4 function or availability comes the preferential loss of other key transcriptional coregulators from SEs, including Mediator and PTEF-b, repressing the transcriptional overexpression of oncogenes.[13,76,124] This finding further underscores the central role of BRD4 in SE-driven transcriptional upregulation, without it, overexpression does not occur.

2.3 | Latent enhancers

Both cellular differentiation and carcinogenesis rely on extensive transcriptional reprogramming that requires recruitment of massive amounts of transcriptional machinery to significantly alter expression of the specific genes which underlie these processes.[75,76,111] In metabolic, inflammatory, and cardiovascular diseases, transcriptional reprogramming also occurs through similar underlying processes.[4,14,53,56,75] When the cellular environment of terminally differentiated cells is disturbed by disease or other noxious stimuli, the cells must respond to the stressor. This response mechanism is the cells' intrinsic ability to alter chromatin structure and form new enhancers in proximity to response genes.[75,120,125] This “cellular plasticity” affords terminally differentiated cells the ability to facilitate their own transcriptional reprogramming in response to stimuli. Newly created enhancers form at genes that are then quickly expressed as the cell's response to the perturbation.

In response to a novel stimulus, the cell forms a new enhancer in proximity to the response gene promoter. With a single stimulus, enhancer elements at this genomic location may dissolve over time; however, a memory for that event is created speeding the response process for future insults. These primed enhancer regions are referred to as “latent” enhancers and are likely to play a key role in response to repeated cellular insults associated with disease.[120,126,127] Ostuni et al.[126] have outlined the characteristics of latent enhancers in terminally differentiated cells, distinguishing them from typical enhancers and SEs. Latent enhancers were defined in terminally differentiated cells as genomic regions that acquire the histone marks associated with typical enhancers or SEs in response to stimulation. In mouse bone marrow–derived macrophages, Ostuni et al. found that upon stimulation with lipopolysaccharide (LPS) and various other noxious stimuli, genomic regions that were unmarked in basal conditions acquired these marks, and thus were termed latent enhancers. In fact, identifying features of latent enhancers are similar to those for SEs, such as H3K27ac,[75,126] Mediator,[76,111,120] and BRD4.[75] The response to LPS was time-dependent in that the H3K27ac mark was present with 4 h LPS stimulation, but by 24 h stimulation H3K27ac had significantly reduced to near basal levels, suggesting that H3K27ac acts as an indicator of active enhancer regions. Additionally, H3K27ac appeared at both overlapping and unique genomic locations in response to various stimuli (LPS vs. TGFβ vs. IL-4, etc.). Ostuni et al. also noted that each stimulus induced a signature memory of histone methylation (H3K4me1) that persisted for days, and instilled a hyperresponsive state, in that subsequent stimulation resulted in faster and often greater histone acetylation than at previously unstimulated and unmarked genomic regions. Genes adjacent to the latent enhancer regions were also induced faster. Latent enhancers, in effect, afford the cell an opportunity to respond to a stimulus that it would be otherwise unable to respond to. Histone “marks” form a memory or “epigenomic signature” of the stimuli the cell has previously encountered, thus enabling it to respond quicker to the same and often similar stimuli later on.[126] Similar findings

in CD4+ T cells showed that BRD4 occupancy at genomic regions activated by stimulation correlated with several different histone acetylation marks (H4K5, H4K8, H3K9) including the enhancer identifier H3K27ac.[108] The pattern of BRD4 co-localization with various histone acetylation marks and other enhancer identifying factors such as Mediator, overlaps with regions identified as latent enhancers.

Evidence shows that BRD4 maintains its role in recruiting transcriptional machinery to these latent enhancers, perpetuating aberrant gene expression in response to disease stimuli.[82] Though the stimuli, transcription factors, and other coactivators may differ based on the disease conditions and cell type, BRD4 (or other BET proteins) are consistently found at latent enhancers across cell types. In terminally differentiated cells, latent enhancer formation can lead to maladaptive gene overexpression, particularly in disease states where there is recurring or sustained noxious stimuli within the cellular environment. This knowledge leads us to next focus on the studies that support the role of latent enhancers and BRD4's contribution to upregulating maladaptive gene expression at these regions in diseases outside of cancer. These studies are also some of the first to show the potential of BETi in diseases such as atherosclerosis, heart failure (HF), arthritis, and metabolic disorders through examination of disease-relevant cell types: endothelial cells, cardiac myocytes, immune cells, and adipose tissue.[4,14,29,53,56,128–132]

3 | BET INHIBITORS IN CARDIOVASCULAR, AUTOIMMUNE, AND METABOLIC DISEASES

In a hallmark study, Brown et al.[56] showed that BRD4 and latent enhancer formation play a significant role in atherosclerotic processes in vascular endothelial cells, a cell type that contributes tremendously to both the development and progression of the disease.[44,133,134] In these cells, inflammatory stimuli result in translocation of transcriptional regulator nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) from the cytoplasm to the nucleus. NF-κB engages in cross-talk with chromatin remodeling machinery, and its subunit v-rel avian reticuloendotheliosis viral oncogene homolog A (RELA or p65) binds to its consensus sequence near the transcription start site of proinflammatory genes. RELA binding, and subsequent acetylation, mark sites for latent enhancer formation. BRD4 is redistributed from genes associated with resting endothelial cell state, such as tyrosine kinase (TEK), to the latent enhancer regions. These latent enhancers are formed within 50 kb pairs of inflammation-responsive genes, such as the cytokine C-C motif chemokine ligand 2 (CCL2), as well as cell adhesion genes that drive atherosclerotic plaque formation such as E-selectin (SELE) and vascular cell adhesion molecule (VCAM1). These regions then become flooded with transcriptional machinery, increasing expression of these and other response genes.[56] Treatment of stimulated cells with BETi interrupts BRD4 redistribution to the CCL2 and other response gene promoter regions, which ultimately reduces their expression compared to that of untreated, stimulated cells. Brown et al.[56] demonstrate that disease-driven master transcription factors, such as NF-κB, induce rapid transcriptional responses through redistribution of BRD4 and other latent enhancer-binding factors to promote transcription of a new set of inflammatory response genes.[56] Their data also show that inhibition of BRD4 binding to the latent enhancer region of inflammatory and cell adhesion gene promoters in vitro is sufficient to interrupt aberrant transcription. In vivo, BETi also blocks the development of atherosclerosis in mouse models, showing the functional aspect of transcription regulation by BETi.[44,56] These groundbreaking discoveries are some of the first to show the prominence of BET proteins in inflammation-related vascular disease, and allude to BETi potential in therapeutic areas outside of cancer.

Other studies in the field of cardiovascular disease have focused on the role of BRD4 in HF. Though HF and atherosclerosis are both designated as cardiovascular diseases, the underlying pathophysiology is very different. In HF, prolonged stress causes pathological cardiac dysfunction and remodeling, which relies partially on alteration to cardiomyocyte cell state.[14,135–137] As has been shown in mouse hypertrophic cardiomyocytes and in agonist-induced hypertrophy in human-induced pluripotent stem cell–derived cardiomyocytes (iPSC-CMs), these cell state changes arise from altered gene expression; enhancer reprogramming, BRD4 redistribution, and overall global changes in histone acetylation and DNA methylation.[53,138] BRD4 has been shown to function as a critical

coactivator of pathologic gene transactivation in these models.[53] Mirroring what occurs in vascular endothelial cells in atherosclerosis, inflammatory signaling in cardiomyocytes is upregulated through activation of the master transcription factor NF-κB in HF.[56] Additionally, transforming growth factor beta (TGF-β) signaling is increased resulting in BRD4 and latent enhancer redistribution around profibrotic myocardial genes, such as connective tissue growth factor (*CTGF/CCN2*) and serpine1 (*Serpin E1/PAI-1*).[14,53,139] These alterations to the abundance of enhancer elements at new genomic locations (ie, latent enhancer formation) in cardiomyocytes ultimately lead to hypertrophy and contribute to HF. Using a number of currently available and structurally dissimilar BETi, the ability of BET inhibition to halt cardiomyocyte hypertrophy and HF was examined in various HF cell and animal models.[14,53] In these experiments, BETi consistently attenuated cardiac remodeling and pathologic hypertrophy, and suppressed key disease-related gene expression both in vivo and in vitro[14,53]. These studies further validate BET inhibition as a viable potential therapeutic option for cardiovascular diseases.

Importantly, however, the development of juvenile idiopathic arthritis, a debilitating disease of the joints affecting children under the age of 16, has also shown a dependency on latent enhancer formation and BRD4 redistribution. In this disease, defects in immune cell function result in a loss of immunological tolerance, which cannot be accounted for by genetic heritability in a large portion of affected individuals. It has now been shown that epigenetic alterations to chromatin and latent enhancer structures result in CD4+ T cell dysfunction. The gene C-X-C chemokine receptor type 4 (*CXCR4*), for example, was found proximal to latent enhancer regions in CD4+ T cells of juvenile idiopathic arthritis patients. The abundance of BRD4 at these latent enhancers and the disease-driven epigenetic changes in *CXCR4* expression were reversed by BETi.[29,129] Overall, BET inhibition was shown to preferentially alter an extensive list of juvenile idiopathic arthritis-specific genes. Though the functional consequences of these findings have yet to be explored, at the cellular level, juvenile idiopathic arthritis provides another example emphasizing the similarities in BRD4′s effect in terminally differentiated cell function across tissues, cell types, and disease states.

In metabolic disorders and obesity, latent enhancers enriched in BRD4 are involved in both adipocyte differentiation as well as chronic inflammation, both of which are key components of metabolic disease.[4] Adipocytes exhibit exceptional plasticity in response to environmental and metabolic changes, at least partially achieved by latent enhancer formation and BRD4 redistribution. Active enhancers placed near the promoter of the master adipogenic transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) result in BRD4 binding and upregulated PPARγ gene expression, which then activates a number of adipocyte-specific differentiation genes. This process has been shown in white, and browning (or beige) adipocytes, and inhibition of BRD4 binding with BETi interrupts PPARγ-mediated adipogenesis.[15,19,130,131,140] Though BRD4 activity at the PPARγ promoter is involved in adipocyte differentiation, it is not essential to maintain adipocyte identity.[140] However, BRD4 has been shown to play a role during fat storage and utilization, and in response to insulin resistance-inducing stimuli.[141,142] That is, obesity is characterized not only by accelerated adipocyte differentiation but also by increased accumulation of immune cells, primarily macrophages, within adipose tissue. Inflammatory cytokines produced by these macrophages trigger functional defects in differentiation, insulin resistance, lipolysis, and lipid storage, as well as a cascade of events leading to the activation of NF-κB.[25,40,130] As noted earlier in vascular endothelial cells and cardiomyocytes, NF-κB induces rapid redistribution of BRD4, transcription factors, and latent enhancer formation. NF-κB has a similar inflammation-inducing effect on both mouse and human adipocytes.[25,40,130,132] Remarkably, BETi suppreses the expression of inflammatory response genes associated with these latent enhancer regions in adipose tissue, as well as a number of genes involved in insulin sensitivity and lipolysis.[15,19,142] Not only do these studies show BRD4's involvement in both adipocyte-related differentiation and macrophage-driven inflammation, illustrating BRD4's breadth of effect in adipose tissue-related disorders, but they also show strong potential for the therapeutic use of BETi in metabolic disease and obesity.

Though latent enhancer formation is a beneficial response in acute attacks on the cellular environment, in disease states such as those described here, activation of this natural response becomes constant and maladaptive. In chronic diseases, latent enhancers contribute to sustained overexpression of response genes, which over time contributes to disease pathology and facilitates disease progression. Both the high levels of expression and the

chronic activation of these genes become harmful. The latent enhancer regions are flooded with the tools to maintain the overexpression of genes that are now detrimental to the cell and the organism. As key components of latent enhancers, and recruiters of other pertinent transcriptional machinery, BET proteins are pivotal in maintaining the disease-driven maladaptive activation of genes associated with chronic latent enhancer activation. As shown in the examples provided here, both in vitro and in vivo, in different cell types affected by different stimuli, the central role of BRD4 and likely other BET proteins as a component of latent enhancers puts these proteins at the cross-roads of controlling gene activity in different disease states. Depending on the situation, the specific HATs, acetylation marks, transcription factors, and coregulators may differ, but BRD4 holds its place in bringing together the transcriptional complex and exacerbating the expression of disease-driving genes. Inhibition of BET proteins, therefore, is an attractive target for broad therapeutic areas.

4 | TARGETING BET PROTEINS

Since BRD4 is a key mediator of transcription at latent enhancers and SEs, which can drive the expression of disease-causing genes, interruption of BRD4 activity has the potential to alter disease progression. In malignant cells, the cell assembles SEs surrounding oncogenes to then enable cancer cell immortality. In nonmalignant cells, overexpression of disease-specific genes perpetuates the disease phenotype. A truly remarkable feature of both malignant and nonmalignant disease states is that enhancer formation and location is chosen by cell signaling through evolutionarily conserved response programs. Since BRD4 is redistributed to latent enhancers to respond to a noxious cellular environment, it is thought that BETi treatment in these conditions will attenuate transcription primarily at the abnormally active genes. Controversy remains over the extent to which BET inhibition affects housekeeping genes and what these effects may mean functionally and phenotypically. However, it is generally thought, given current knowledge, that BETi are a means of targeting the altered transcription that occurs at latent enhancers called upon by the cell in responding to and perpetuating the disease state. The attractiveness of BETi is enhanced by the current availability and ongoing development of small molecules that compete for binding of BDs to the natural ligand, acetylated lysine.

4.1 | Approaches to targeting BET proteins

Various methods of targeting BET BDs, thus inhibiting their interaction with acetylated histones, have been developed. Proteolytic targeting chimera (PROTAC) compounds not only bind BET proteins, but they can induce BET degradation. These small-molecule BETi are linked to a ubiquitin ligase recognition module via a flexible linker. Thus, not only do the BETi displace BET proteins from acetylated lysines, they also target the BET protein for ubiquitination and degradation. ARV-825, ARV-771, dBETi, and MZ1 are examples of currently available PROTACs that have been tested in various cancer models[7,11,12,39,143–150] while others continue to be developed.[26] However, BET-targeting PROTAC research is currently in preclinical stages and thus the therapeutic potential is currently unknown.

Bivalent BETi, which bind both BDs of a BET protein simultaneously, are currently being developed.[151–153] Bivalent BD binding results in a stronger bond and greater potency than the monovalent binding of the more commonly known BETi, discussed below. The furthest bivalent BETi in development is AZD5153; an orally available BRD4 inhibitor.[153] Clinical development of AZD5153 is currently in phase 1, with ongoing studies focused on the treatment of lymphomas.[154,155]

Another class of BET inhibiting compounds currently in the early stages of development are covalent BETi. These molecules, such as those designed by Kharenko et al.,[28] form covalent bonds with residues within the BD binding pocket, creating a stronger interaction than noncovalent binding. Covalent BETi offer a longer lasting

effect and potential for pharmacological efficacy at lower concentrations compared to their noncovalent counterparts.[28,156] Currently, covalent BETi have yet to reach the clinical investigation stages.

The greatest number of currently available BETi belong to the noncovalent BETi class, which form monovalent interactions with individual BET BDs. Description of the first noncovalent BETi, JQ1[157] has lead the way for IBET-762, IBET-151, OTX015, ZEN-3694, and many others.[1,55] These BETi are commonly known for their ability to elicit antitumor activity in cancer cell lines as well as various murine cancer models.[57,58,158–160] For example, JQ1 has been proposed as a treatment for NUT-midline carcinoma because this disease stems from an oncoprotein arising from the fusion of BRD4 to NUT.[54,58,61,161–163] Unfortunately, JQ1 is not a clinical candidate due to unfavorable pharmacokinetic properties,[164] however, many other noncovalent BETi are being clinically evaluated for the treatment of cancer and cardiovascular disease.[52,59,165–200]

4.2 | Bromodomain-selective binding

BET bromodomains are comprised of a four-helix bundle: helices KZ, KA, KB, and KC. The long ZA loop between helices KZ and KA is connected to a BC loop between helices B and C. The binding pockets of both BD1 and BD2 are comprised of a WPF shelf, a ZA channel, and a peptide channel. Amino acid differences between the two BD binding pockets exist; BD1-specific residues of the binding pocket include Asp144, Ile146, Lys141, and Gln85. BD2-specific histidine (His433), Val435, Pro430, and Lys374 replace Asp144, Ile146, Lys141, and Gln85, respectively, leading to a narrower binding pocket as well as altered polarity and hydrophobicity compared to BD1[72,80,157,201] (Figure 3).

Most available BETi are pan-selective, in that they bind both BDs with near equal affinity. This is due to the highly conserved structure and shape of BDs. The chemical structure of pan-BETi JQ1, for example, allows for interactions with residues in the ZA- and BC-loops of both BD1 and BD2.[77,157] There are many pan-BETi currently available, such as JQ1, I-BET151, ZEN-3694, many of which are under clinical investigation for the treatment of cancer.[1,3,26,31,34,48,52,59,165–200]

There are, however, a limited number of BETi compounds that claim to exhibit BD selectivity. The most recognized example of a BD1 selective compound, Olinone, exhibits over 100-fold higher binding affinity to BD1 than BD2 in all BET proteins. The configuration of Olinone within the BD1-binding pocket, its specific contact with five key residue replacements between BD1 and BD2, and the number and mobility of hydrogen bonds in the ZA and BC loops drive its BD1 selectivity.[37,202] Though Olinone is the most well-known BD1 selective BETi, other scaffolds, configurations, and binding to unique amino acid residues within a given BD pocket can also be selective

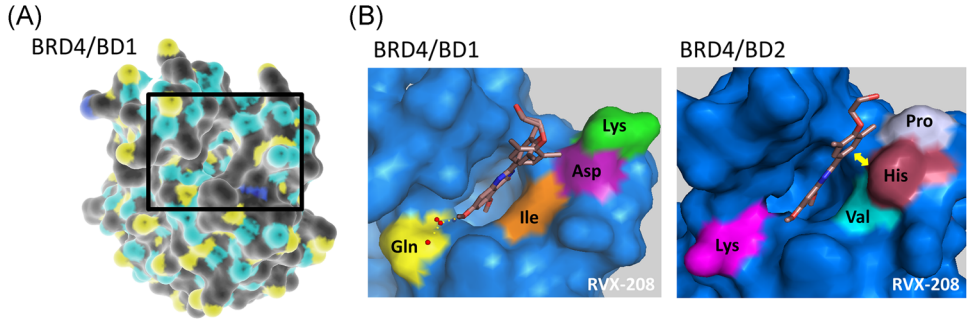

FIGURE 3 A, X-ray crystal structure of BRD4/BD1, outlined. B, X-ray crystal structure of BRD4/BD1 and BRD4/BD2 showing key residue replacements between bromodomains, with the example of apabetalone's (RVX-208) BD2-specific His433 binding (yellow arrows)

for BD1 over BD2.[156,203,204] A recent evaluation of GSK778, for example, shows >130-fold selectivity for BRD4 BD1 over BD2 through specific binding with the Asp144/His433 replacement in BD1 versus BD2, respectively.[205] Other examples of BD1 selective compounds, such as MS611 and MS436 and MS402, are derived from a unique chemical scaffold.[202,206–208] However, BD1 selective compounds have yet to be investigated in clinical trials.

Examples of BD2-selective compounds are increasing in number, and their unique therapeutic potential is gaining recognition. Available BD2-selective BETi in preclinical stages of development include BY27, which binds BD2 with 5–38-fold selectivity over BD1,[6] GSK620, and GSK046, each of which have been shown to have BD2-binding affinities >300-fold over BD1.[205] Two BD2-selective BETi currently in clinical development include ABBV-744, which has approximately 300-fold greater binding affinity to BD2 of BRD4 than BD1,[209] and apabetalone, which selectively binds BD2 of BET proteins with 20–30-fold greater affinity over BD1.[60,210] The first phase 1 clinical trial of ABBV-744 is in the recruiting stage, enrolling subjects with acute myeloid leukemia.[211] ABBV-744 was designed after the pan-BETi ABBV-075 to target the Asp144/His 437 and Ile146/Val439 sequence differences between BD1 and BD2.[5,212] Apabetalone, on the other hand, is the furthest BETi in clinical development, having recently been evaluated in a phase 3 trial in cardiovascular disease.[36,213,214] Apabetalone is unique in its interactions with BD2-specific His433, which causes the compound to maintain a single conformation in the narrow BD2-binding pocket[210] (Figure 3). This specific interaction prevents the multiple rotational positions that commonly occur in the BD1 pocket and highlights the specificity permissible by targeting unique BD features.[210] Another unique feature of apabetalone's binding in BD2 is the lack of interaction with the WPF shelf. Interestingly, initial findings from in vitro and early clinical investigations of both ABBV-744 and apabetalone suggest increased tolerability in humans compared to pan-selective agents,[212,215] suggesting that BD2-specificity may be linked to reduced toxicity.

4.3 | Pan-BETi versus selective BETi

There is now a considerable amount of data, generated by various groups, demonstrating differential outcomes of pan- versus selective BD inhibition.[16,38,45,157,210] In one investigational study, gene expression in hepatocellular carcinoma cell line (HepG2 cells) following treatment with the pan-BETi JQ1 was contrasted with that of BD2-selective apabetalone[210]; JQ1 exhibits equal binding affinity for both BDs, while apabetalone preferentially binds BD2.[157,210] JQ1 significantly affected the expression of at least 16 times more genes than apabetalone. More than 750 genes showed changes in expression of at least 1.5-fold with JQ1, while only 46 genes were altered by apabetalone to the same extent.[210] Genes modified by both apabetalone and JQ1 (42 genes) showed a much larger magnitude of change with JQ1, which ultimately resulted in limited overlap between the top genes affected by each compound. Tyler et al.[45] extended these findings with their discovery that binding BD2 with apabetalone, while subsequently adding JQ1, reduced the ability of JQ1 to engage BRD4 at chromatin in MV4-11 cells. These novel findings imply that dual BD binding is required for JQ1 to exhibit its full effect, and highlight the complex and dynamic interactions involved in pan- versus selective BET inhibition. Runcie et al.[38] also showed variation in the role of BD2 across BET proteins in human osteosarcoma U2OS cells. Compared to other BET proteins, BRD4-BD2 was essential for regulating gene expression, which suggests that BRD4-BD2 does, in fact, play a larger role in enhancer-driven transcriptional regulation than both BRD4-BD1 and other BET proteins in this cell type. These differential actions may prove to be of pivotal importance in therapeutic BETi use and development.

These and other studies confirm that pan-BETi lead to vast effects on gene expression and activity while BD2-selective binding often has less extensive effects.[45,157,210] They also suggest that the benefits of BD2-selective BETi may be more specific than pan-BETi in inhibiting those transcriptional changes associated with enhancer reprogramming and BRD4 redistribution to latent enhancer-driven response genes. That is, BD2-selective BETi may benefit disease states in which responses to disease-driving stimuli are less extensive, for example, in diseases

where inflammatory and profibrotic signaling are activated. On the other hand, disease states that result in more extensive alteration to cellular programming, such as cancer where fundamental cellular processes such as proliferation and cell survival signaling are triggered in combination with inflammatory signaling, may exhibit greater benefit from BD1 or pan-selective BET inhibition.[38,45,210] Preclinical support for the functional delineation of therapeutic BD-selectivity is indeed beginning to appear in the literature. The most promising example to date was provided by Gilan and colleagues, who have shown in K562 cells stimulated with interferon-gamma (IFNγ), THP-1 cells stimulated with phorbol 12-myristate 13-acetate, and CD4+ T cells stimulated with anti-CD3/CD28, that BD2-selective BET inhibition specifically targeted stimulus-induced gene expression, leaving basal gene expression largely unaffected.[205] This finding was corroborated by reduced recruitment of BET proteins to response genes associated with BD2 inhibition. While BD1-selective inhibition showed similar effects on proliferation, cell cycle arrest and apoptosis to that of pan-BETi in cancer models (both in vitro and in vivo), BD2-selective inhibition was effective in inflammation, metabolic disease, and profibrotic models.[205] Other studies have previously shown that BD2-selective compounds can directly alter BRD4 abundance specifically at latent enhancers.[18,44] In these studies, the effects of BD2-selective BETi on BRD4 redistribution and chromatin occupancy was examined in vascular smooth muscle cells subject to osteogenic (or calcifying) conditions,[18] and in endothelial cells stimulated with TNF-α.[44] In both studies, BD2-selective apabetalone reduced BRD4 occupancy at latent enhancers, transcription of genes proximal to latent enhancers, abundance, and activity of related proteins. These studies confirm that BD2-selective BETi can diminish BET protein redistribution and therefore latent enhancer formation and function in disease areas outside of cancer.

This preliminary evidence, combined with future research and clinical developments, will undoubtedly provide continued support for BD2-selective BETi in therapeutic research and development in diseases such as metabolic, inflammatory, and cardiovascular diseases.[4,14,19,53,56,129,205] By decreasing the negative effects of enhancer reprogramming and minimizing maladaptive transcription of disease-driving genes at latent enhancers, BETi with BD2-selectivity could be specifically beneficial in these areas.

4.4 | BD-selective BETi in the clinic: What human data do we have?

Though evidence of differential effects of pan- versus BD-selective BETi from the clinic is limited due to the early stage of BETi development, some preliminary patterns are beginning to emerge. Clinical trials of the BD2-selective BETi apabetalone provide the greatest repository of clinical data on BD2-selective BETi available, and allude to potential benefits and possible differential effects of BD2-selective BETi versus pan-selective BETi in humans. First, of all data collected to date from thousands of patients, some of the most important may be related to safety and tolerability. The safety and tolerability profiles of apabetalone far exceed those of pan-BETi in the clinic.[215] Whereas apabetalone-treated patients generally experience mild adverse events, pan-BETi–treated patients frequently experience more severe adverse events including increases in bilirubin, thrombocytopenia, and drug resistance.[3,32,35,52,215,216] Moreover, clinical data collected to date show that safety is not a limiting factor for chronic dosing of apabetalone, supporting the use of BD2-selective BETi in diseases outside of cancer.[32,214,215] Second, apabetalone trials have identified key pathways that benefit from BD2-selective BETi in cardiovascular and diabetes mellitus patients,[18,20,32,43,44,46] as well as chronic kidney disease patients.[47,217] This further underscores the role of BET proteins in driving the chronic maladaptive overexpression of genes and proteins which participate in networks and pathways that directly contribute to these disease states. Additional analysis of the recently completed phase 3 BETonMACE study will help to reveal more details of the unique biological effects of BD2-selectivity on BET protein function in diabetic, renal, and cardiovascular disease patients.[36,214] Overall, though the number of clinical trials is currently limited, existing evidence supports the use of BD2-selective BETi as a safe and efficacious therapy for the treatment of various chronic disease states.

4.5 | Current clinical landscape of BET inhibitors

The majority of current clinical advancement of BETi remains in the early stages. Generally, clinical trials of BETi focus on treating various forms of cancer. Trials of the pan-BETi CPI-0610 have included patients with multiple myeloma, lymphoma, myelofibrosis, myelocytic leukemia, myelodysplastic/myeloproliferative neoplasm, and myelodysplastic syndrome,[171–173,197] while the pan-BETi GSK525762 is being evaluated in ongoing clinical trials for solid tumors, brain tumors, and midline carcinoma.[176–178,218–225] ZEN-3694 is being tested in a clinical trial in castration-resistant prostate cancer and triple-negative breast cancer.[198–200] ABBV-744, a potential BD2-selective BETi,[16] is also in cancer trials, currently being evaluated in a phase 1 trial in acute myeloid leukemia.[211] Apabetalone, the furthest clinically advanced BETi, which is also BD2-selective, is to date the only BETi being evaluated in therapeutic areas outside of cancer. Apabetalone has been investigated in clinical trials in cardiovascular disease, cardiovascular disease combined with diabetes, chronic kidney disease, and planned clinical trials in pulmonary arterial hypertension are set to begin soon.[193,194,213,226–231] This brief list of examples (for a full list see www.clinicaltrials.gov) of clinical trials completed or currently underway shows that there is strong preclinical evidence that targeting one small protein family; BET proteins, can have the potential for therapeutic action on numerous cellular drivers of disease.[52,59,165–200,211,213,218–222,224–233] At the same time, the landscape of clinical trials suggests there is significant room for future development, particularly for BD-selective BETi.

5 | OUTLOOK

An understanding of the potential benefit of BETi in a wide spectrum of human diseases arises from their ability to target a single family of proteins which play a primary role in conserved cellular responses. BET proteins have a profound impact on transcription in both development and disease. We have shown that the breadth of BETi potential stems from the central role of BET proteins in super and latent enhancer formation and function. In many different diseases with different underlying pathologies, evolutionarily conserved cell signaling culminates in the activation of genes to respond to an insult. BET proteins and other transcriptional machinery are redistributed to enhancers to drive expression of these response genes. Evidence from studies focusing on BRD4 show that it is pivotal in the upregulation of disease-driving genes and contributes to their overexpression. Inhibition of BRD4 consistently counters the transcriptional upregulation associated with enhancer reprogramming. Thus, the central premise behind therapeutic BETi is based not on the ability of these compounds to target every BET occupied gene, but instead on the cell's response to a stimulus. In other words, the cell sets the stage for BETi sensitivity by activating genes required to respond to disease through redistributing BET proteins to these genetic locations. The genes, transcription factors, and other elements may differ across cell types and disease states, but the underlying processes of redistribution of these elements remains the same. BET proteins are a consistent requirement for the cell's response to be appropriately activated. In chronic disease conditions, such as cardiovascular disease or inflammatory disease, even mild alterations to gene expression can be compounded by sustained activation. That is, though the response to an insult may be protective initially, the constant activation of these pathways and the sustained overexpression of these genes become detrimental. The ability of BETi to target a key component of latent enhancers provides a potential for therapeutic benefit in a variety of diseases, all driven by these same processes.

The current state of BETi research suggests that BD2-selective BETi may be an effective way to safely target disease-driven transcriptional alterations in terminally differentiated cells in areas outside of cancer. There are still many questions remaining, but the future of BETi development shows significant promise, and the development of BD-selective BETi opens new possibilities. Focused development of BD-selective compounds is certain to alter and expand the clinical landscape for years to come.

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CONFLICT OF INTERESTS

Norman C. W. Wong, Brooke D. Rakai, and Ewelina Kulikowski are employees of Resverlogix Corp. and hold shares.

AUTHOR CONTRIBUTIONS

Norman C. W. Wong and Brooke D. Rakai researched and wrote the content; Brooke D. Rakai completed final editing for submission; Ewelina Kulikowski reviewed, edited, and approved content.

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