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Structural Features and Inhibitors of Bromodomains

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Abstract

Bromodomains are conserved structural modules responsible for recognizing acetylated-lysine residues on histone tails and other transcription-associated proteins, such as transcription factors and co-factors. Owing to their important functions in the regulation of ordered gene transcription in chromatin, bromodomains of the BET family proteins have recently been shown as druggable targets for a wide array of human diseases, including cancer and inflammation. Here we review the structural and functional features of the bromodomains and their small-molecule inhibitors. Additional new insights provided herein highlight the landscape of the ligand binding sites in the bromodomains that will hopefully facilitate further development of new inhibitors with optimal affinity and selectivity.

Introduction

The great diversity of the human proteome is due in part to the power of post-translational modifications (PTMs)[1], which are small chemical groups added to amino acid residues in proteins. These modifications have the ability to affect the functions of proteins, their cellular localization and the manner in which they interact with other proteins. Specifically, the post-translational modification of histones, the proteins around which DNA is wrapped to form nucleosomes, plays an essential role in the regulation of gene transcription in chromatin. Acetylation of lysine residues on histone tails is among the most abundant PTMs, and the protein domains responsible for recognizing these modifications are called bromodomains (BrDs). These functional domains are primarily responsible for reading these acetyl marks within the cell, but some have also recently shown to be capable of recognizing other PTMs, such as lysine butyrylation and lysine crotonylation[2]. As the reader of these marks, the BrDs plays a central role in the complex biological system of gene transcription in chromatin, as BrD-containing proteins are pivotal in recruiting chromatin remodeling proteins and different transcription factors to active transcription sites. Improper recognition of lysine PTMs in gene transcription has been linked to numerous human diseases including cancer, which brings BrD-containing proteins to the forefront as new therapeutic targets. This review highlights structural and functional features of BrDs, their chemical inhibitors as well as current drug candidates being evaluated in human clinical trials.

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Structural Coverage of the Bromodomains

A total of 42 genes encodes for at least 56 bromodomains in the human genome[3], and each of the BrD-containing protein contains between one and six BrDs. The first three-dimensional structure of a BrD (specifically, that of the histone acetyltransferase KAT2B also known as PCAF) was solved in 1999, using NMR spectroscopy[4]. This study also demonstrated for the first time the BrD function as a conserved acetyl-lysine (Kac) binding domain for histones. The structure was the first of many to exhibit the unique BrD fold as four alpha helices with a left-handed fold, denoted as α_Z , α_A , α_B , α_C (**Fig. 1A**). In addition, the unique BrD structure contains two loops one connecting the α_Z and α_A helices (called the ZA loop), and a second connecting the α_B and α_C helices (the BC loop). This NMR study used 2D ^1H - ^{15}N heteronuclear single quantum coherence (HSQC) spectra to establish that a lysine-acetylated peptide perturbed residues between the ZA and BC loops defined as the Kac binding site in BrDs (**Fig. 1B**), providing direct biochemical evidence that acetylated lysine binds at this location. One of the interesting features in the Kac binding site that was later confirmed is the presence of four conserved and difficult to displace water molecules, one of which is in direct contact with the acetylated lysine tail (**Fig. 1C**). It is important to note that the BrDs are often found to be associated with other epigenome reader domains within the context of a given BrD-containing protein[5] (e.g. plant homeodomains (PHDs), Pro-Trp-Trp-Pro (PWWP) domains) or other enzymatic domains (such as histone acetyltransferases (HATs)). These associations highlight the contribution of BrDs to the complex combinatorial recognition process in the regulation of gene transcription in chromatin[6,7].

BrDs can be classified into 8 subgroups[8] according to the similarity of their sequence (**Fig. 2A**). A total of 453 structures of 41 BrDs have been solved to date[9] (June, 30th 2016) with at least one crystal structure available for each subgroup. Due to massive interest in the biology and the development of chemical inhibitors for some BrDs, certain subgroups have been more investigated than others. Group II contains BET (bromodomain and extra-terminal family) BrDs (BRD2, BRD3, BRD4 and BRDT) and has the highest number of crystal structures available in the PDB with 206 structures to date. BRD4-BrD1 (the first BrD of BRD4) alone has 141 structures. This over-representation is due to the emergence of the members of this subgroup as new druggable targets, and the extensive development of chemical inhibitors that has followed. These structures have enabled a full three-dimensional analysis of the acetyl-lysine binding site. The Kac region includes a conserved asparagine amino-acid necessary for the recognition of acetyl-lysine histone peptides, the ZA and BC channels defined by the residues of the ZA and BC loops respectively, a hydrophobic area called the 'shelf' (named after the WPF shelf of the BET family), and the first residue of the α_C helix, called the 'gatekeeper residue' (which is usually hydrophobic) (**Fig. 3**)

Although BrDs are usually grouped and analyzed according their full BrD amino acid sequence, it is important to analyze the variation of the binding sites within a subgroup, as these differences can be subtle, yet quite important for ligand recognition. Thus, it is also important to consider a binding site classification (**Fig. 2B**) for inhibitor selectivity assessments, especially when the BrDs in question are *non-canonical* BrDs. Most BrD inhibitors developed to date were designed to target *canonical* BrDs, as these contain the

conserved asparagine residue that interacts with Kac-mimicking ligands. On the other hand, non-canonical BrDs contain a different amino acid at this position. Even though the overall sizes and shapes of the binding sites of these non-canonical BrDs will be comparable to those found in other members of their subgroup, these BrDs are distinct because of their inability to bind to the acetyl-lysine residues of histone tails. There are 12 non-canonical BrDs in the BrD family: ASH1L-BrD (Y instead of N), BRWD1-BrD2 (T), BRWD3-BrD2 (T), PB1-BrD1 (Y), PB1-BrD6 (D), PHIP-BrD2 (T), SP100-BrD (Y), SP110-BrD (Y), SP140-BrD (Y), SP140L-BrD (Y), TRIM28-BrD (T) and ZMYND8-BrD (Y). Binding site classification is useful for enhancing selectivity during the inhibitor design process, but attention must also be paid to the variations of the shelf residues and the gatekeeper residue because they also tend to lead to dramatic changes in inhibitor binding affinity. In terms of the highly-studied BET family, both overall BrD sequence-based and BrD binding site-based classification trees indicate that this family has high sequence and structural similarity, demonstrating the challenge of developing selective inhibitors within this family. The binding site classification tree (**Fig. 2B**) also reveals that Group IV is still orphan and that Group V has only three BrDs among 9 that were successfully inhibited by small molecules.

Small-Molecule Inhibitors of BrDs

(a) Non acetyl-lysine mimetic

Small-molecule inhibitors of canonical BrDs are typically designed to mimic an acetylated lysine residue, thus giving them the ability to disrupt the interaction between an acetylated histone and the Kac binding site within a BrD. An acetylated lysine from a histone peptide forms a hydrogen bond (H-bond) with the conserved asparagine of a canonical BrD, as well as a network of H-bonds with a group of conserved water molecules found at the base of the BrD binding site. BrD inhibitors that mimic acetyl-lysine are designed to preserve these interactions with the conserved asparagine and the binding site as a whole.

Interestingly, the first BrD inhibitor reported in 2005 by Zeng et al.[10] is not an acetyl-lysine mimetic. The authors identified a small molecule N-(3-aminopropyl)-4-methyl-2-nitroaniline (NP1, see **Fig. 4A**) that selectively inhibits the BrD of the histone acetyltransferase KAT2B (IC₅₀ of 1.6 μ M), and displaces its binding partner, K50-acetylated HIV trans-activator Tat, which is essential for the transcription and replication of the integrated HIV provirus. The NMR structure of NP1-KAT2B BrD shows that the compound makes an ionic interaction with the side chain of Glu750 of the ZA loop and possibly a H-bond with Tyr802 of the α_B helix. Soon after the discovery of this ligand, the tetrahydrocarbazolone ligand MS7972 was found to obstruct the association of the BrD of human transcriptional co-activator CREB-binding protein (alias CBP) and tumor suppressor p53 via the inhibition of the Kac binding site of CBP-BrD[11] (K_d of 19.8 μ M), thus modulating p53 action in response to DNA damage. Similarly to NP1, MS7972 mostly interacts with the ZA loop through van der Waals contacts (**Fig. 4B**). The more recently published BIC1 is another BrD inhibitor that does not serve as an acetyl-lysine mimetic. BIC1 blocks the transcriptional activity of BRD2-BrD1 (K_d of 28 μ M) in human cells by decreasing the activation of a BRD2-dependent luciferase gene[12]. Although BIC1 binds within the Kac and WPF shelf regions of BRD2-BrD1 binding site (**Fig. 4C**), it doesn't

interact with the conserved asparagine Asn156 via a H-bond, just as the other comparable compounds do not. Due to the lack of this interaction, *non acetyl-lysine mimetic* modulators typically show weaker BrD inhibition than their *acetyl-lysine mimetic* counterparts.

(b) Acetyl-lysine mimetic

In order to develop potent inhibitors for the BrD family, the most straightforward approach is to mimic histone peptide interactions. As previously described, the conserved asparagine in canonical BrDs is involved in the recognition process, as it forms a H-bond with the carbonyl of the acetylated lysine of acetylated histones. Strong inhibitors usually utilize this interaction. Several inhibitors of this variety have emerged in the past few years, with many of them targeting the BET family, and others targeting CBP-BrD and p300-BrD, and recently BRD7/BRD9 BrDs. Although the selectivity is usually contained within the BrD subgroup (**Fig. 2**), some chemical scaffolds are more promiscuous than others. As an example, the [1,2,4]Triazolo[4,3-*a*]phthalazines-based compounds led to the development of the Bromosporine (**Fig. 5A**) as pan-BrDs inhibitor[13]. Bromosporine is potent against the BET proteins, as well as the CBP, BRD9 and CECR2 BrDs[13].

BET inhibitors—The BrDs of the BET protein family are among the most investigated BrDs, largely due to the characteristics of the binding sites of the members of this family. The Kac binding site of the BET BrDs has the largest volume among the BrD family, making it more suitable for targeting with a small molecule. The first chemical probe[14] of the BET family BrDs to be published in the literature, JQ1, was disclosed in late 2010. This compound provided an important tool that led to the identification of BRD4 as an important target in the inhibition of cell proliferation and differentiation of NUT midline carcinoma cells[15]. In addition to JQ1, several thienodiazepines-based chemical probes have also been published, including MS417[16], CPI-203[17] and most recently OTX015 (MK-8628)[18]. It is important to note that these three compounds, while published recently, had been previously disclosed in a patent published by Mitsubishi Tanabe Pharma in 2000[19] even predating the publication of JQ1. Inhibiting BET BrDs with JQ1, also helped identify BRD4 as a target for the treatment of acute myeloid leukaemia (AML)[20], suppression of tumor growth in ovarian cancer[21] and attenuation of stem cell signaling of the malignant brain tumor medulloblastoma in patient overexpressing the MYC oncogene[22]. Similarly, MS417 helped identify the association of BET BrDs with NF- κ B-mediated inflammation and kidney injury in HIV-associated nephropathy (HIVAN)[16,23] and in colorectal cancer, as treatment with this compound slowed the growth and metastasis of diseased cells[24]. Finally, CPI-203 shed light on the role of BET BrDs as a potential therapeutic target for drug resistant myeloma[25], and inhibiting BET BrDs with OTX015 (MK-8628) both decreases c-MYC in acute leukemia cells[26,27] and shows anti-proliferative activity in a large panel of cell lines derived from lymphoid tumors[28]. The benzodiazepine I-BET762 is another BET inhibitor that has been studied in blood cancers, as it has shown anti-myeloma activity resulting in survival advantage in a systemic myeloma xenograft model[29]. Much like JQ1, I-BET762 induces cell cycle arrest that leads to a potent anti-proliferative effect against myeloma[29]. I-BET151 is a dimethyl-isoxazole BET inhibitor that has been shown to reduce the growth of medulloblastoma cells[30] and control tumor growth of glioblastoma

cells[31]. As a matter of fact, BET BrDs inhibition has shown great impact on the epigenetic regulation of various oncogenes[32].

On the whole, many molecular entities have been developed for the inhibition of BET BrDs and **Figure 6** depicts a sampling of the wide variety of compounds that have been developed. Inhibitors based on diazepine or triazepine chemical scaffolds have been optimized to utilize a methyl-triazole group to forge an interaction with the conserved asparagine residue, mimicking Kac histone binding. These compounds also make strong van der Waals contacts with the WPF shelf residues, the Ile (BrD1) / Val (BrD2) gatekeeper, a methionine of the α_C helix (Met149 in BRD4-BrD1) and two leucine residues from the ZA loop (Leu92 and Leu94 in BRD4-BrD1). Other inhibitors that comprise dimethyloxazole γ -carbolines, tetrahydroquinoline, acetyl-pyrrole, thiazolidinone, or indolizine derivatives chemical scaffolds have been also optimized to interact with the conserved asparagine, WPF shelf residues, and different residues of the ZA channel[15,18,33-36].

Depending on the scaffold, these studies have shown that there are two different ways by which the binding sites of the BET BrDs can be targeted. One is to form interactions with the residues of the ZA channel, and the other is to make contacts with important residues on the BC loop. BRD4-BrD1 selective ligands such as Olinone (K_d of 3.4 μ M for BRD4-BrD1 and $K_d > 300$ μ M for BRD4-BrD2) and MS436 ($K_i < 85$ nM for BRD4-BrD1 and K_i of 340 nM for BRD4-BrD2) have two distinct binding modes in the BrD binding site (**Fig. 6**), affirming that selectivity towards the first BrD domain of BET proteins can be achieved in more than one way. Certain variable residues at ligand binding sites between the BrD1 and BrD2 of BET proteins have been shown to be the keys to the development of selective inhibitors for both domains. Specifically, these include a residue in the ZA channel (Gln85 in BRD4-BrD1 vs. Lys374 in BRD4-BrD2), the gatekeeper residue (Ile146 in BRD4-BrD1 vs. Val435 in BRD4-BrD2), and a residue in the BC loop (Asp144 in BRD4-BrD1 vs. His433 in BRD4-BrD2). Raux et al.[37] recently reported a new inhibitor series of BET-BrD1 (BRD2 excluded) selective compounds based on a xanthine scaffold (**Fig. 7A**). One of these compounds, *Compound 1* exhibits a 10-fold selectivity toward BRD4-BrD1 (IC_{50} of 5 μ M) over BRD2-BrD1 and BRD3-BrD1 (both $IC_{50} > 50$ μ M). The crystal structure of *Compound 1* with BRD4-BrD1 sheds light on the ligand-induced flexibility of the ZA loop (**Fig. 7A**). The xanthine moiety of *Compound 1* in complex with BRD4-BrD1 interacts with Asn140 with two H-bonds, van der Waals contacts with Tyr139 from α_B helix and Leu94 from the ZA loop, while its triazolopyrimidinyl moiety makes van der Waals contacts with Trp81 and Pro82 from the WPF shelf, and with Gln85 of the ZA channel. In addition, it is involved in a H-bond interaction with the nitrogen of the backbone of Asp88. Although Asp88 is conserved among the BET-BrD1s, this residue seems to be less flexible in BRD4-BrD1 than in BRD2-BrD1 and BRD3-BrD1, thus providing evidence that the flexibility of the ZA loop appears to be important for developing selective inhibitors for each domain.

In contrast to the previously detailed BET BrD inhibitors, RVX-208 (Apabetalone) has recently been shown as a unique inhibitor selective for the second BrDs of the BET proteins[38,39]. RVX-208 mimics the Kac interaction through its pyridinone moiety binding to the conserved asparagine of the BrD. Surprisingly, RVX-208 forms no interactions with the WPF shelf, but it does make van der Waals contacts with Pro371, Leu381 and Leu383

from the ZA loop, the gatekeeper Val435 and His433 from the α_C helix. The methoxy-ethanol tail of RVX-208 is shown to be critical for selectivity for the BrD2s over the BrD1s, as its counterpart RVX-OH (which has been the same chemical structure, but not this tail) has the same affinity for both BrDs [Picaud, 2013 #70]. RVX-208's selectivity for the BrD2 domains can also be explained by a more favorable entropic effect in its BET-BrD2 binding, and additional van der Waals contacts with His433, resulting in better affinity, relative to its interaction with the aspartic acid residue at the corresponding location in BET-BrD1. Although RVX-208 is a relatively weak binder, with a K_d of 0.303 μ M for BRD4-BrD2 and a K_d of 8.93 μ M for BRD4-BrD1 [McLure, 2013 #71], this compound has been shown to be quite successful to date, as it is the first BET-BrD2 selective inhibitor to enter clinical trials. By taking advantage of certain structural features, diazepine- and triazepine-based inhibitors could also achieve selectivity towards BET-BrD2 [40]. Baud et al. [40] used a Bump-and-Hole approach to identify different chemical strategies to target potential residues that can introduce selectivity for each BET domain. One of these compounds, *Compound 28* exhibits 17-fold selectivity towards BRD2-BrD2 (K_d of 45 nM) over BRD2-BrD1 (K_d of 780 nM). The indole moiety of this inhibitor interacts with His433 of BRD2-BrD2 by an aromatic edge-to-face π - π stacking. This interaction is not possible in the first BrD because of the presence of Asp160 instead of His433 in BrD2 (**Fig. 7B**). Recently, RVX-297, a RVX-208 analog and BET-BrD2 selective (K_d of 1.44 μ M for BRD4-BrD1 and K_d of 0.18 μ M for BRD4-BrD2), has been reported [41] to exhibit both different pharmacodynamic profile and biological activity as demonstrated in mouse models for autoimmune disease of multiple sclerosis and arthritis [42,43].

Non-BET inhibitors—Generally, the Kac binding sites in non-BET BrDs are smaller than those in the BET BrDs, making it more challenging to develop small-molecule inhibitors of the former due to their limited buriedness and lipophilicity [44]. The BrDs of transcriptional co-activators CREBBP (CBP) and EP300 (alias p300), which are of great interest in biology, particularly in the p53 responses to DNA damage [11,45], are the second most investigated BrDs after those of the BET family. The first generation of CBP/p300 BrD inhibitors possess a micromolar binding affinity (**Fig. 5B, C, D, E**), but more selective inhibitors have since emerged. Some of the stronger BET inhibitors exhibits a weak affinity towards CBP/p300 BrDs such as isoxazoleazepines-based inhibitors. For example, *Compound 23* (CPI-3 analog, **Fig. 6**) has an IC_{50} of 2 μ M for CBP and IC_{50} of 0.032 μ M for BRD4-BrD1 [46], whereas triazolodiazepines-based inhibitors show potencies greater than 10 μ M for CBP-BrD [8,18].

The first highly potent and selective inhibitor of the CBP-BrD over the BET-BrDs to be published was SGC-CBP30, which displays 40-fold potency for the CBP-BrD [47] (**Fig. 8A, B**). This selectivity can be explained by the conformational change adopted by this inhibitor within those binding sites (**Fig. 8A, B**). Interestingly, there are structural differences between the BET-BrDs and CBP-BrD are located in residues of the ZA channel and α_C helix, and the WPF shelf of BET-BrD is replaced by a LPF shelf in the CBP-BrD. The conformation adopted by the inhibitor in the CBP-BrD binding site allows more favorable interactions, specifically with the LPF shelf and with Arg1173 (of the BC loop) through a cation- π interaction. Additionally, the methoxy group of the chlorobenzene moiety of the compound

forms a water mediated H-Bond with Arg1173 in the binding pocket (**Fig. 8A**). Another acetophenone-based ligand, named UL04 (**Fig. 8C**) was discovered by virtual screening and optimized using molecular dynamics[48,49]. The ligand is potent for CBP-BrD ($K_d = 0.77 \mu\text{M}$) and displays a 65-fold greater affinity for this BrD than BRD4-BrD1. The binding mode involves a H-bond interaction with the conserved Asn1168, as well as a water mediated H-bond with the backbone of Pro1110 and a polar interaction with Arg1173 of the BC loop (**Fig. 8C**). Indeed, Arg1173 is a critical residue for CBP-BrD ligand optimization, as specifically targeting it with modified ligands enables selectivity over BET-BrD proteins to be attained.

ATAD2 (ATPase family AAA domain-containing protein 2) is another emerging target for cancer therapies, as its up-regulation was observed in breast cancer and other human tumors[50,51]. Additionally, ATAD2 binds the MYC oncogene and stimulates its transcriptional activity[52]. Demont et al.[53] optimized a fragment hit based on a quinolinone scaffold that led to the first low micromolar inhibitors for the ATAD2-BrD. Subsequently, Bamborough et al.[54] used a structure-based approach to optimize a series of naphthyridones hits, yielding the high-affinity inhibitor named *Compound 38* (**Fig. 8D**), which shows a 100 fold selectivity for ATAD2-BrD (IC_{50} of 130 nM) over the BET-BrDs (IC_{50} of 16 μM for BRD4-BrD1). The authors utilized interactions with the BC loop and WPF shelf of the ATAD2-BrD to gain potency and selectivity over the BET BrDs. The selectivity for ATAD2 is due to the H-bond interactions the inhibitor forms with Asp1068 (water-mediated) from the BC loop, Asp1071 (a side chain residue of the α_c helix) and two H-bond interactions with Arg1077 (one water mediated).

Until recently, BRD9 was another under-studied BrD-containing protein, yet within the past few years, several inhibitors selective for BRD9 over the BET family have been found[55,56]. Among these inhibitors, I-BRD9 possesses 700-fold selectivity for BRD9-BrD over the BET BrDs and 200-fold selectivity over the highly structurally similar BRD7-BrD. The pyridinone moiety of I-BRD9 (**Fig. 8E**) interacts with the conserved Asn216 of BRD9, while other portions of the ligand interact with residues Phe160 and Phe161 of the GFF shelf via van der Waals contacts, with Tyr222 via π - π stacking, and with Ile169 and Arg217 via H-bonds. The ligand's selectivity for BRD9 over BRD7 can be explained from the residue difference in the BC and ZA loops (**Fig. 3**) that leads to a smaller binding site for BRD7 (pdb structure of apo BRD7: 2I7K).

BRPF1 (Peregrin or Bromodomain and PHD finger-containing protein 1) is another BrD-containing protein that is a subunit of the MOZ HAT (monocytic leukemic zinc-finger histone acetyltransferase) complex[57,58], a complex that has been associated with acute myeloid leukemia[57]. A first generation inhibitor based on a 1,3-dimethyl benzimidazolone scaffold was developed[59], as were dual BRPF1/TRIM24 BrD inhibitors[60]. *Compound 3* is a potent 1,3-dimethyl benzimidazolones inhibitor with a K_d of 10 nM for BRPF1-BrD. The key interactions between this compound and the BrD are van der Waals contacts with the NIF shelf, and Pro658, Glu661, and Val662 from the ZA loop, π - π stacking with the gatekeeper Phe714 and H-bonding with the conserved Asn708 (**Fig. 8F**). IACS-9571 is a dual BRPF1/TRIM24 BrDs inhibitor that expands the dimethyl benzimidazolones scaffold to enable more interactions with both the ZA channel and the BC loop, leading to a more

potent inhibitor for BRPF1-BrD. IACS-9571 binds to the BRPF-BrD with a K_d of 2.1 nM and to the TRIM24-BrD with K_d of 1.3 nM (**Fig. 8G**). The dimethylamino group added on this compound makes a polar interaction with Asp926 in TRIM24-BrD and with Glu655 in BRPF1-BrD at the same location. These two BrDs not only share a high structural similarity in the ZA channel but also a similar shelf, as TRIM24-BrD has a LAF shelf sequence and BRPF1-BrD has a NIF shelf sequence (**Fig. 8F, G**). Recently, Bamborough et al.[61] reported a new BRPF1-BrD chemical probe named GSK6853, which has K_d of 0.3 nM with 1600-fold selectivity for the BRPF1-BrD over all other bromodomains tested of the BROMOscan panel[61].

IACS-9571 also binds to the BAZ2A-BrD (K_d of 400 nM) and exhibits 7700-fold selectivity for this BrD over the BRD4-BrDs. BAZ2A and BAZ2B belong to the same binding site subgroup as TRIM24 (**Fig. 2B**). Although the overall binding site sequence similarity is high, the BAZ2A and BAZ2B BrDs possess a WPF shelf instead of the LAF shelf found in the TRIM24-BrD. While IACS-9571 shows affinity for these BrDs, GSK2801 was the first inhibitor designed specifically for the BAZ2A (K_d of 0.26 μ M) and BAZ2B (K_d of 0.14 μ M) BrDs. BAZ2B (**Fig. 8H**) has a smaller binding site relative to other BrDs due to its short BC loop. Additionally, possible van der Waals contacts between ligand and protein are obstructed by the fact that its gatekeeper residue, Ile2146, is close to the WPF shelf region. Therefore, most of the binding site of the BAZ2B-BrD is defined by its Kac region and ZA channel. The key interactions between GSK2801 and the BAZ2B-BrD are formed by van der Waals contacts with the gatekeeper Ile2146, as well as with Trp2083 and Pro2084 from the WPF shelf (**Fig. 8H**). Furthermore, the sulfonyl group of the ligand interacts with Asn2090 from the ZA loop through a water mediated H-bond interaction. GSK2801 has a selective profile for the BAZ2A and BAZ2B BrDs, and its *in vivo* characteristics make it a good chemical probe candidate.

The SWI/SNF (switch/sucrose nonfermentable) chromatin remodeling complex regulates gene transcription in an ATPase-dependent manner [62]. Its subunits transcription activator BRG1 (SMARCA4) and the global transcription activator SNF2L2 (SMARCA2) contain BrDs. The former has been linked to DNA damage response and validated as a target for improving cancer radiotherapy[63]. PFI-3 is a reported chemical probe for the SMARCA family BrDs with binding affinities of K_d of 86 nM and 54 nM towards SMARCA4-BrD and PB1-BrD5, respectively (PB1 is also a subunit of the SWI/SNF complex). The crystal structure of PFI-3 bound to the SMARCA4-BrD (**Fig. 8I**) shows that the carbonyl moiety of the compound interacts with the conserved Asn1540, the hydroxyl group is H-binding to Tyr1497 from the ZA loop. PFI-3 also has van der Waals contacts with the gatekeeper residues Ile1546 and Leu1494, Pro1489 from the ZA loop, which collectively makes PFI-3 bound deeply in the Kac binding site, displacing four water molecules that are present in the apo structure of the SMARCA4-BrD (pdb: 2GRC).

TAF1 (Transcription initiation factor TFIID subunit 1) is a member of the TFIID (Transcription factor II D) protein complex responsible of the placement of RNA Pol II (Polymerase II) to initiate transcription. TAF1-BrD2 has been shown to synergize with BRD4 in cancer cell proliferation and designated as a potential target for cancers driven by MYC oncogene[64]. A selective ligand, *Compound 5* for the TAF1-BrD2 over BRD4,

BRD9, CBP and CECR2 BrDs has recently been reported by Crawford et al.[65] and has IC₅₀ of 46 nM affinity for the TAF1-BrD2. The crystal structure of the complex highlights the rearrangement of the conserved water molecules that are observed in the TAF1-BrD2 apo structure. Specifically, *Compound 5* interacts with the conserved Asn1578 via its pyrrolopyridone moiety that is also involved in a π - π stacking with the gatekeeper residue Y1589; the butenyl substituent of the pyridine moiety of the ligand forms van der Waals contacts with Phe1528 side chain, and backbone atoms of Asp1549. The rearranged water molecules form a H-bond mediated interactions with the side-chain of Tyr1540 and Val1535 of the ZA loop. The phenylamide substituent on the 4-position of the pyrrolopyridone moiety interacts with an edge to face π - π stacking with Trp1526 of the WPF shelf and is H-bonding to the backbone nitrogen of Asn1533 via its carbonyl group. The butenyl substituent of *Compound 5* on its pyridine moiety appears to play a role in rearranging the two conserved water molecules in the BRD4-BrD1. However, a crotonyl substitution on that pyridone creates a narrow hydrophobic channel when bound to BRD9-BrD. This channel is located under the conserved water molecules between the conserved Asparagine and the GFF shelf[65], resulting in a selective inhibition of the BRD9-BrD (IC₅₀ of 0.16 nM). This study demonstrates that displacing or rearranging the bound water molecules in the Kac binding groove could yield ligand selectivity in different BrDs.

BrD Inhibitors in Human Clinical Trials

Due to the promise that they have shown in the laboratory setting, numerous pharmaceutical companies have entered their bromodomain inhibitors into clinical trials. As mentioned earlier in this review, the BET-BrD2 specific inhibitor RVX-208 (Apabetalone), developed by Resverlogix Corp., is currently in clinical trials. This compound has completed Phase II clinical trials for diabetes, coronary artery disease, atherosclerosis, dyslipidemia, and cardiovascular diseases[66]. At present, Resverlogix is recruiting participants for a Phase III trial that tests the ability of RVX-208 to reduce Major Adverse Cardiac Events (MACE) in high-risk cardiovascular and diabetes mellitus patients. The previously described benzodiazepine inhibitor, I-BET762, has been entered into two separate Phase I/II trials by GlaxoSmithKline – one for relapsed, refractory hematologic malignancies, and the other for NUT midline carcinoma and multiple other types of cancer. Merck, which acquired OncoEthix and its thienodiazepine OTX015 (MK-8628) in late 2014, is presiding over multiple different trials with this compound, including two designed to find the optimal dosage to treat certain solid tumors and certain hematologic malignancies. Other companies, namely Constellation Pharmaceuticals (CPI-0610), Tensha Therapeutics (TEN-010), Zenith Epigenetics (ZEN003694), AbbVie (ABBV-075), and Incyte (INCB054329 and INCB057643), have entered their compounds into clinical trials for different types of cancer, as well, and are currently recruiting participants for these trials. Additional information related to these trials can be found on <http://clinicaltrials.gov>.

Conclusion

In recent years, interest in targeting epigenome reader domains as new treatments for many human diseases has grown rapidly. At the forefront are bromodomains, the readers responsible for recognizing acetylated lysine residues on histones. Numerous campaigns to

develop tight-binding inhibitors of these domains have gone hand-in-hand with a great increase in the study of the biology of bromodomains. These multi-faceted advances have rapidly increased our knowledge of the roles played by bromodomains in numerous biological pathways, as well as in the onset and progression of certain diseases. This considerable progress has also generated important structural and molecular insights into target-ligand recognition for epigenetic protein-protein interactions, which promises to yield more advanced and selective next-generation inhibitors for similar epigenome reader domains. Such new inhibitors will serve both to answer additional questions that current inhibitors have not yet been answered about the functions of certain bromodomain proteins in cellular biology and physiology and to propel the development of novel treatments for challenging human diseases, particularly cancer and inflammatory disorders. Combined with the drug candidates currently being investigated in clinical trials, the potential of these additional advances is high and expected to have a tremendous impact on human health in the near future.

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Figure 1. Structure of the BrD fold and function

(A) The 3D structure of the conserved BrD fold. Highlighted are the four helices (α_Z , α_A , α_B , α_C), the ZA and BC loops that connect them, and the N and C termini of the bromodomain. (B) Superimposed region of the 2D ^{15}N -HSQC spectra of the BrD (0.5 mM) in its free form (red) and in complex with the Kac-containing H4 peptide (black) (molar ratio 1:6), adapted from Dhalluin et al.[4]. (C) The Kac binding site of a BrD, illustrated by a structure of BRD4-BrD1 in complex with H4K5acK8ac (pdb: 3UVW), with the four conserved water molecules, present in most of the BrDs, colored in red. Other water molecules in this specific binding pocket are colored in pink

Figure 2. Classification of the human BrD family, as based on protein sequence similarity

(A) Classification of the human BrD family based on full-length protein sequences of the BrDs. (B) Binding site sequence-based classification of the human BrD family. Binding site residues were manually selected for each BrD. Phylogenetic trees were generated using UPGMA clustering with the Geneious 8.1.7 software[67]. Non-canonical BrDs are color-coded in black. Symbols represent: [X] experimental apo structure available, [P] structure in complex with peptide available, [S] structure in complex with small-molecule available. The colored circles illustrate the range of affinities of ligands tested against a particular BrD. Affinities expressed as K_i , K_d , or IC_{50} were converted to pX_i , where $pX_i = -\log_{10}(X_i)$, with X_i expressed in M. The left side of the circle is the minimum value of pX_i and the right side the maximum value, which are color-coded according to the pX_i scale shown at the right lower corner. This figure was produced with data extracted from the ChEpiMod knowledgebase[9] as of December 2015.

	α_2	Shelf	ZA loop	α_A	α_B	N (BC loop)	G	α_C
BRD2-1	WKHQFAWPF	RQPVDAVKLG	LPDYHKI	IKQPMDMGT	IKRRLENN--YYWAASECMQDFNTMFTNCYIY	NKPTDD	IVLMAQTLEKI	IFLQKVASM
BRD3-1	WKHQFAWPF	YQPVDAIKLNL	PDYHKI	IKNPMDMGT	IKRRLENN--YYWSASECMQDFNTMFTNCYIY	NKPTDD	IVLMAQALEKI	IFLQKVAQM
BRD4-1	WKHQFAWPF	QQPVDAVKLNL	PDYHKI	IKTPMDMGT	IKRRLENN--YYWNAQECIQDFNTMFTNCYIY	NKPGDD	IVLMAEALEKL	IFLQKINEL
BRDT-1	WKHSFSWPF	RQPVDAVKLQL	PDYTI	IKNPMDLNT	IKRRLENK--YYAKASECIEDFNTMFSNCYLY	NKPGDD	IVLMAQALEKL	IFMQKLSQM
BRD2-2	KHAAYAWPF	YKPVDAASALGL	HDYHDI	IKHPMDLST	VKRKMENR--DYRDAQEFAADVRLMFNSCYKY	NPPDHD	VVAMARKLQDV	FEFRY---
BRD3-2	KHAAYAWPF	YKPVDAEALGL	HDYHDI	IKHPMDLST	VKRKMDGR--EYPDAGGFAADVRLMFNSCYKY	NPPDHE	VVAMARKLQDV	FEMRFAKM
BRD4-2	KHAAYAWPF	YKPVDEALGL	HDYCDI	IKHPMDMST	IKSKLEAR--EYRDAQEFGADVRLMFNSCYKY	NPPDHE	VVAMARKLQDV	FEMRFAKM
BRDT-2	KHFSYAWPF	YNPVDNALGLH	NYDVVN	NPMDLGT	IKKMDNQ--EYKDAYKFAADVRLMFNCYKY	NPPDHE	VTMARMQLQDV	FETHFSKI
CREBBP	RQDPESLPF	RQVPDPQLLGI	PDYFDIV	KNPMDLST	IKRKLDTG--QYQEPWQYVDDVWLMFNNAWLY	NKTSRV	YKFCSKLAEV	FEQEIDPV
EP300	RQDPESLPF	RQVPDPQLLGI	PDYFDIV	KNPMDLST	IKRKLDTG--QYQEPWQYVDDIWLMFNNAWLY	NKTSRV	YKFCSKLSEV	FEQEIDPV
ATAD2	AIDKRFRVET	KPVD--PDEV	PDYVTVIK	QPMDLSSV	ISKIDLH--KYLTVKDYLRDIDLICSNALEY	NPDRDP	GDRILIRH	RACALRDTAYAI
BRD7	QRKDPSAFF	SFPVT--DFI	APGYSMI	IKHPMDFST	MKEIKINN--DYQSIIEELKDNFKLMCTNAMIY	NKPETI	YYKAAKLLH	SGMKILSQE
BRD9	QRKDPHGF	FAFPVT--DAI	APGYSMI	IKHPMDFGT	MKDKIVAN--EYKSVTEFKADFKLMCDNAMTY	NRPDTV	YYKLAKKIL	HAGFKMMSKQ
TRIM24	-----	VPDYKIK	KNPMDLST	IKRRLQED	YSMSKPEDFVADFRLIFQNCAEF	NPDSEV	ANAGIKLE	NYFEELLKNL
BRPF1	QEKDTGNI	FSEPVP--LSE	VPDYLDHI	KKPMDFFT	TMQNLEAY--RYLNFDDFEEDFNLI	VSNCLKY	NKDTIFYRA	AVRLREQGGAVLRQA
BAZ2A	ESHDAWPF	LEPVN--PRL	VSGYRRI	IKNPMDFT	MRERLLRG--GYTSSEEFADALLVFDNCQTF	NEDDSE	VKGAGHIM	RFFESRWEFF
BAZ2B	ETHEDAWPF	LLPVN--LKL	VPGYKKV	IKKPMDFST	IREKLSSG--QYPNLETFALDVRLVFDNCETF	NEDDSI	GRAGHNMR	KYFEKKWTD
SMARCA4	SGRQLSEV	FIQLP	SR--KEL	PEYYELIR	KPVDFKKIKERIRNH--KYRSLNDLEKDVMLLCQNAQTF	NLEGLS	LIYEDSIV	LQSVFTSVRQKI
TAF1-2	MAVPDSWPF	HHPVNK--KFV	PDYKVI	VNPMDLET	IRKNISKH--KYQSRESFLDDVNLI	LANSVKY	NGPESQY	TKTAQEI
								VNVCYQTLTEY

Figure 3. Structure-based sequence alignment of 19 BrDs described in this review

Domain limits were extracted from Uniprot database. Sequence alignment was performed using profile-profile alignment using the PROMALS3D server[68]. The conserved asparagine is highlighted with the green letter N and the gatekeeper residue in the α_C helix in blue.

Figure 4. Structural insights of ligand recognition by BrDs

(A) NMR Structure of NP1 ligand binding to KAT2B-BrD (pdb: 1WUG). (B) NMR Structure of MS7972 ligand binding to CBP-BrD (pdb: 2D82). (C) Crystal structure of BIC1 ligand binding to BRD2-BrD1 (pdb: 3AQA).

Figure 5. Chemical structure of the Pan-BrD Bromosporine and CBP-BrD inhibitors

(A) Chemical structure of Bromosporine. (B) Chemical structure of MS7972 (K_d of 9.6 μM for CBP-BrD) (C) Chemical structure of Ischemin/MS120 (K_d of 19 μM for CBP-BrD). (D) Chemical structure of the Isoxazole inhibitor (IC_{50} of 0.771 for CBP-BrD). (E) Chemical structure of Xd14 (K_d of 1.6 μM for CBP-BrD).

Figure 6. Chemical scaffolds of major BET inhibitors, their structures and binding affinity

The central structure is H3K8acK12ac in complex with BRD4-BrD1 (pdb: 3UW9). This structure exhibits the major binding site features of BET BrD proteins. Three different regions are visible: the BC loop (green) containing the conserved asparagine, the gatekeeper residue on the α_c helix (light blue), the WPF shelf (gold) and the ZA loop (salmon). Conserved water molecules are represented with red balls. The circle is split into three regions covering Pan-BET inhibitors (green), Pan-BrD1 inhibitors (dark blue), and Pan-BrD2 inhibitors (orange). Within each region of the circle are the 2D chemical scaffolds commonly found in inhibitors of these domains. External to each region of the circle is a ligand containing the corresponding scaffold in complex with that BET BrD.

Figure 7. Structural comparison of ligand recognition by the BET BrDs

(A) Structural overlay of : *Compound 1* binding to BRD4-BrD1 (pdb: 5EGU) in gold color; structure of holo BRD4-BrD1 from MS417 complex (pdb: 4F3I) in light blue, and apo structure of BRD4-BrD1 (pdb: 4LYI) in white. This figure highlights the induced flexibility of the ZA loop of BRD4-BrD1 by the inhibitor *Compound 1*. (B) Structure of *Compound 28* binding to mutant W360F BRD2-BrD2 (pdb: 5DFD).

Figure 8. Structural insights of ligand recognition by selected non-BET BrDs

(A) Structure of the inhibitor (S)-SGC-CBP30 bound to CBP-BrD, pdb: 4NR7. (B) Structure of the inhibitor (S)-SGC-CBP30 bound to BRD4-BrD1, pdb: 5BT4. (C) Structure of the inhibitor UL04 bound to CBP-BrD, pdb: 4TQN. (D) Structure of the inhibitor *Compound 38* bound to ATAD2-BrD, pdb: 5A83. (E) Structure of the inhibitor I-BRD9 bound to BRD9-BrD, pdb: 4UIW. (F) Structure of the inhibitor *Compound 3* with BPRF1-BrD, pdb: 4UYE. (G) Structure of the inhibitor IACS-9571 bound to TRIM24-BrD, pdb: 4YC9. (H) Structure of the inhibitor GSK2801 bound to BAZ2B-BrD, pdb: 4RVR. (I) Structure of the inhibitor PFI-3 bound to SMARCA4-BrD, pdb: 5DKD. (J) Structure of the inhibitor *Compound 5* bound to TAF1-BrD2, pdb: 5I1Q.