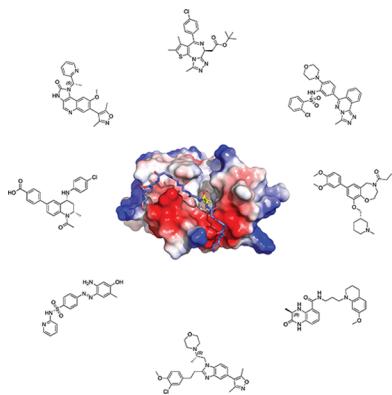


Discovery of Chemical Inhibitors of Human Bromodomains

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1. INTRODUCTION

1.1. Lysine Acetylation

Acetylation of lysines on nucleosomal histones has long been perceived as being responsible for weakening DNA–histone interactions, thereby influencing gene transcriptional activation.¹ It was not until the late 1970s, however, that evidence supporting the direct contribution of histone tail lysine acetylation to the opening of chromatin and activation of transcription was presented.² Lysine acetylation is a reversible process, occurring at the ε-amino group of site-specific lysine residues in histones. The combined effects of acetylation, namely, charge neutralization and steric hindrance, relax chromatin structure and enhance the accessibility of DNA for recognition by transcription factors³ (Figure 1). Additionally, the discovery of HAT (histone acetyltransferase) activity of transcriptional cofactors directly linked histone acetylation to gene transcriptional activation in chromatin.^{4–6} Indeed, a series of transcriptional cofactors has been shown to possess intrinsic histone HAT activities, including GCN5 (general control of amino acid synthesis-5),⁴ CBP (p300/CREB-binding protein),^{7,8} P/CAF (p300/CBP-associated factor),⁹ and TAF (TBP (TATA-binding protein)-associated factor).¹⁰ The role of lysine acetylation in gene transcriptional activation was further highlighted by the identification of the bromodomain—a conserved structural motif found in many eukaryotic transcription cofactors^{11,12} whose primary function is to recognize acetylated lysine in histones and transcription proteins.¹³ Most nuclear HATs (type A) in the GNAT and p300/CBP families contain a bromodomain¹⁴ that can serve as

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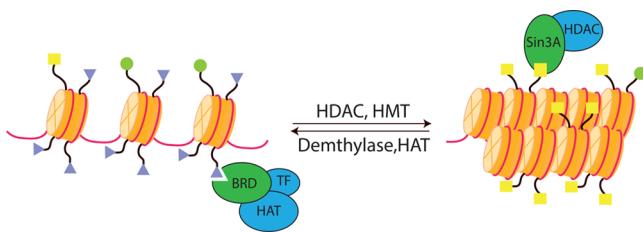


Figure 1. Histone acetylation and methylation are two post-translational modifications that play a direct role in the activation and repression of gene transcription. The relaxed/open form of chromatin is called euchromatin and is associated with transcriptional activation. In this chromatin state, HATs have acetylated numerous lysine residues on histone tails, dislodging DNA from the core histones due to an increase in steric hindrance and a decrease in positive charge. As such, DNA is more accessible to transcription factors and other cofactors, allowing for active transcription. The condensed form of chromatin is called heterochromatin, and transcription is repressed in this state. Nucleosomes are very tightly packed, leaving the DNA and histone tails with minimal exposure to transcription factors and other cofactors. HDACs have removed acetylation marks from the histone tail lysines, and they have been replaced by methylation marks added by HMTs, which are a hallmark of transcriptionally repressed chromatin. Yellow squares, blue triangles, and green circles represent methylation, acetylation, and phosphorylation marks, respectively.

an anchor to histones, allowing such HATs to exert their catalytic activity by acetylating other nearby lysine residues and drive active transcription. Clearly, the acetyl-lysine-reading ability of the bromodomain places it at the heart of gene transcriptional activation in chromatin.^{5,13,15}

Notably, lysine acetylation occurs not only in the nucleus but also in the mitochondria and the cytoplasm. Indeed, many lysine acetyltransferases (KATs) are present exclusively in the cytoplasm, and a significant number of KATs exist in either the nucleus or the cytoplasm.¹⁶ Unlike type A HATs, type B HATs (including HAT1,^{17–19} HatB3.1,²⁰ and HAT4²¹) primarily act on newly synthesized histones in the cytoplasm⁵ before they translocate to the nucleus and incorporate into chromatin. As they lack bromodomains, type B HATs recognize newly synthesized core histones that are typically nonacetylated. In this review, the HATs referred to are type A HATs, as they are directly involved in regulation of gene transcription in chromatin through the functions of their HAT catalytic domains and bromodomains.⁴

Interestingly, ϵ -N-lysine acetylation does not occur randomly; different HATs have distinct preferences for site-specific lysines on histones, thus producing unique modification patterns. For example, the preferred acetylation sites for yeast GCN5 are histone H3 at lysine 14 (H3K14), H4K8, and H4K16.²² As would be expected, site-specific histone acetylation marks are associated with different biological functions. H4K16 acetylation (H4K16ac) and H3K14ac were found to affect chromatin structure²³ and play a role in DNA damage repair.^{24–26} The global reduction of H4K16ac has been linked to cancer²⁷ and chromatin decondensation,²³ while H4K12ac has been shown to be relevant to early embryonic development,²⁸ cognitive decline, and age-related memory loss.²⁹ H4K8ac is generally recognized as a transcriptional activator.^{30,31} Along with other post-translational modifications (e.g., methylation, phosphorylation, etc.), these well-defined modifications work in concert with one another to influence various biological processes. These distinctive patterns create a complex regulatory system that is capable of controlling gene

activation and silencing in chromatin in an orderly fashion.³² Outside of the realm of chromatin modifications, HATs have also been shown to acetylate a number of nonhistone substrates.^{33,34}

The level of acetyl-lysine is also regulated by histone deacetylases (HDACs), which counter the activity of HATs, removing acetyl groups from histone and nonhistone proteins. The dynamic interplay between HATs and HDACs maintains histone acetylation at an appropriate level for proper transcriptional regulation. To date, 18 mammalian HDACs with diverse biological functions have been identified and grouped into four major categories, namely, classes I, II, III, and IV.³⁵ Class III HDACs are distinctive from the other classes as they are NAD dependent.³⁶ Classical HDAC inhibitors such as TSA (trichostatin A), SAHA (vorinostat), and romidepsin are mostly pan-HDAC (class I, II, IV) inhibitors, lacking selectivity for a specific class, which limits the scope of their applications in cancer treatment to cutaneous T cell lymphoma (CTCL). However, next-generation HDAC inhibitors with improved selectivity profiles have entered clinical trials as anticancer agents.³⁷ It is noteworthy to point out that HDACs also modify other nonhistone proteins such as p53, MEF2 (myocyte enhancer factor 2), Rb (retinoblastoma protein), Runx (Runt domain transcription factors), NF- κ B (nuclear factor kappa-light-chain-enhancer of activated B cells), and HSP90 (heat shock protein 90).³⁸

In summary, lysine acetylation is one of the most widespread post-translational modifications associated with the regulation of gene transcription in chromatin. To date, over 27 650 sites for protein acetylation have been identified (PhosphoSite-Plus).³⁹ These modifications influence a wide array of biological processes, ranging from chromatin organization and gene transcription to apoptosis, DNA repair, and differentiation.

1.2. Discovery of the Bromodomain

Bromodomains began to draw serious attention just as researchers were fervently investigating HATs and HDACs as transcriptional activators and repressors. In 1992, a common motif was first found in *Drosophila brahma* (*brm*) and female-sterile homeotic (*fsh*) proteins and named the “bromodomain”.¹¹ Despite its functional importance and sequence consensus among different proteins, the precise secondary structure of the bromodomain remained elusive and could only be predicted via computational methods at the time.¹² In 1999, however, the three-dimensional solution structure of PCAF bromodomain was solved for the first time using nuclear magnetic resonance (NMR) spectroscopy, unveiling a unique left-handed four-helix bundle structural fold.¹³ Important structural features of the bromodomain include the interhelical ZA and BC loops, both located on the one end of the helix bundle, flanking a well-conserved hydrophobic pocket responsible for acetyl-lysine recognition.^{13,40}

Bromodomains are found in a wide variety of nuclear proteins. These bromodomain-containing proteins play many roles within the cell, but in each, the unique ability of the bromodomain to bind to acetyl-lysine is required for the function of the protein in some capacity. Protein families that contain bromodomains include HATs (CBP, EP300 (E1A binding protein p300)), histone methyltransferases (ASH1L (absent small and homeotic disks protein 1 homologue), MLL (mixed lineage leukemia)), transcriptional regulators (BET (bromodomain and extraterminal), SP100 (nuclear antigen Sp100), ATAD2 (ATPase family AAA domain-containing

Timeline of Bromodomain Milestones

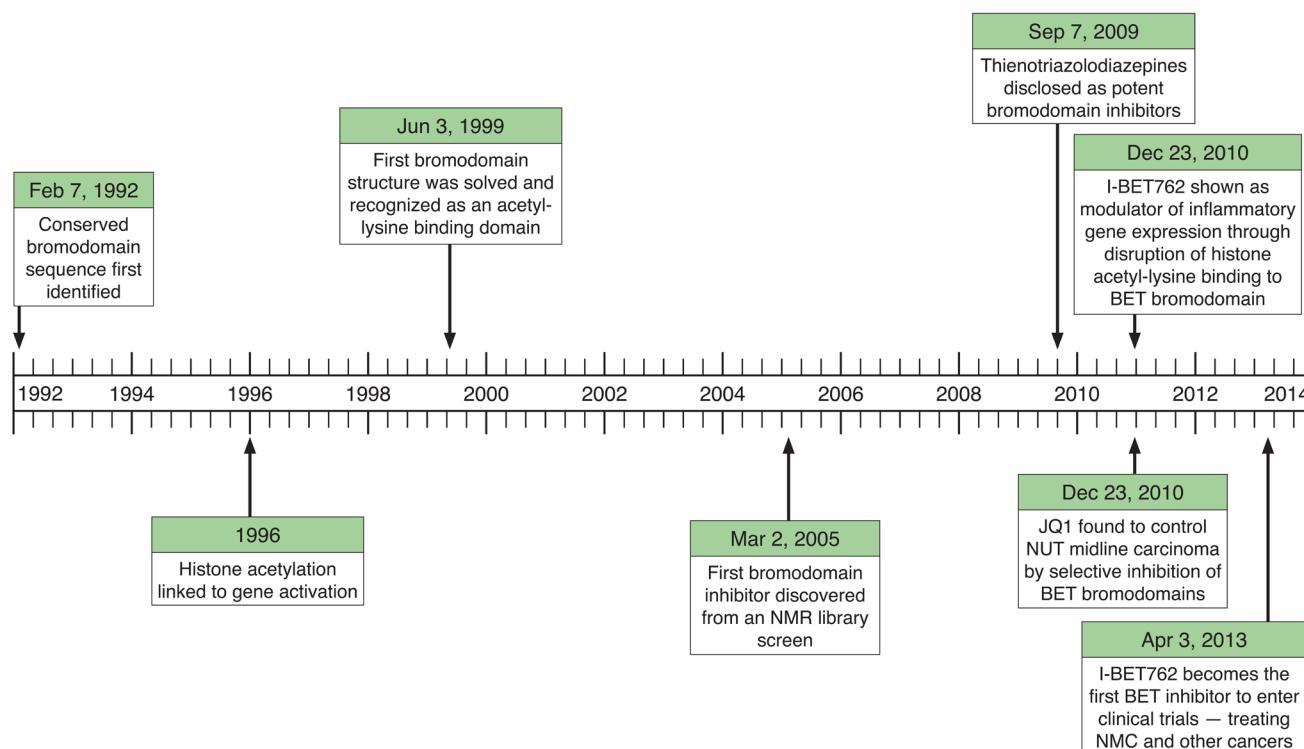


Figure 2. Timeline of notable milestones in the development of bromodomain inhibitors.

protein)), nuclear-scaffolding proteins (PB1 (polybromo 1)), chromatin-remodeling factors (SMARCA2 (SWI/SNF-related matrix-associated actin-dependent regulator of chromatin a2), SMARCA4), ATP-dependent chromatin-remodeling complexes (BAZ1B (BRD adjacent to zinc finger domain protein)), and transcriptional coactivators (TRIM28 (tripartite motif-containing 28), TAFs). The ubiquitous presence of bromodomains in nuclear proteins of different functions suggests their essential role in various transcription regulation processes.

The identification of the bromodomain as the dedicated acetyl-lysine binding module also built a solid foundation upon which bromodomains became considered viable drug targets. As such, large compound library screens were initiated in an effort to spur the development of small-molecule regulators of bromodomains. The first bromodomain inhibitor, a small-molecule modulator of the PCAF bromodomain, was reported in 2005. This inhibitor blocked the pivotal interaction between the PCAF bromodomain and acetylated lysine 50 of HIV-1 Tat (trans-activator of transcription), preventing the recruitment and activation of Tat for HIV transcription.^{41,42} This study showed that bromodomains were indeed druggable targets, opening the door for the development of other small molecules that could displace an acetylated peptide from the conserved binding pocket of a given bromodomain.

A major breakthrough occurred when a series of highly potent thienotriazolodiazepines that could inhibit the binding of acetylated histones to the BET bromodomain BRD4 were disclosed in a patent in 2009.⁴³ These compounds demonstrated extraordinary inhibitory activities against a panel of cancer cells,⁴⁴ showing their potential in the treatment of

refractory cancers such as acute myeloid leukemia, acute lymphoblastic leukemia, and multiple myeloma. Thienotriazolodiazepines are analogs of benzodiazepines, which have a long-standing history in the clinic as treatments for anxiety, insomnia, and seizure. Their medical use suggests these scaffolds bear favorable pharmacological and toxicological profiles. In 2010, two independent studies identified thienotriazolodiazepines and benzotriazolodiazepines as potent inhibitors of a specific subset of human bromodomains—BET bromodomains.^{45,46} In one study, investigators demonstrated that a compound known as JQ1 successfully inhibited cell proliferation and differentiation in a NUT (nuclear protein in testis) midline carcinoma model.⁴⁵ In the other study, researchers showed that I-BET (also referred to as I-BET762), an inhibitor of BET bromodomains, suppressed inflammatory gene expression in a LPS-induced macrophage model.⁴⁶ In 2011, a separate study targeted MLL-fusion leukemia by disrupting the association between the MLL-fusion protein and the BET family of proteins in the super elongation complex.⁴⁷ In 2014, researchers developed a novel approach to treat castration-resistant prostate cancer by interfering with the association between BET bromodomain proteins and androgen receptors.⁴⁸ These results, as well as many others, collectively validate BET bromodomains as a drug target.⁴⁹

Due to their promise shown in these studies, numerous bromodomain inhibitors have entered the clinic in the recent years. RVX-208, a compound that utilizes BET bromodomain inhibition to stimulate apolipoprotein (ApoA1) gene expression, entered a clinical trial for coronary artery disease in 2010.^{50,51} In 2012, I-BET762 entered a Phase I trial for midline

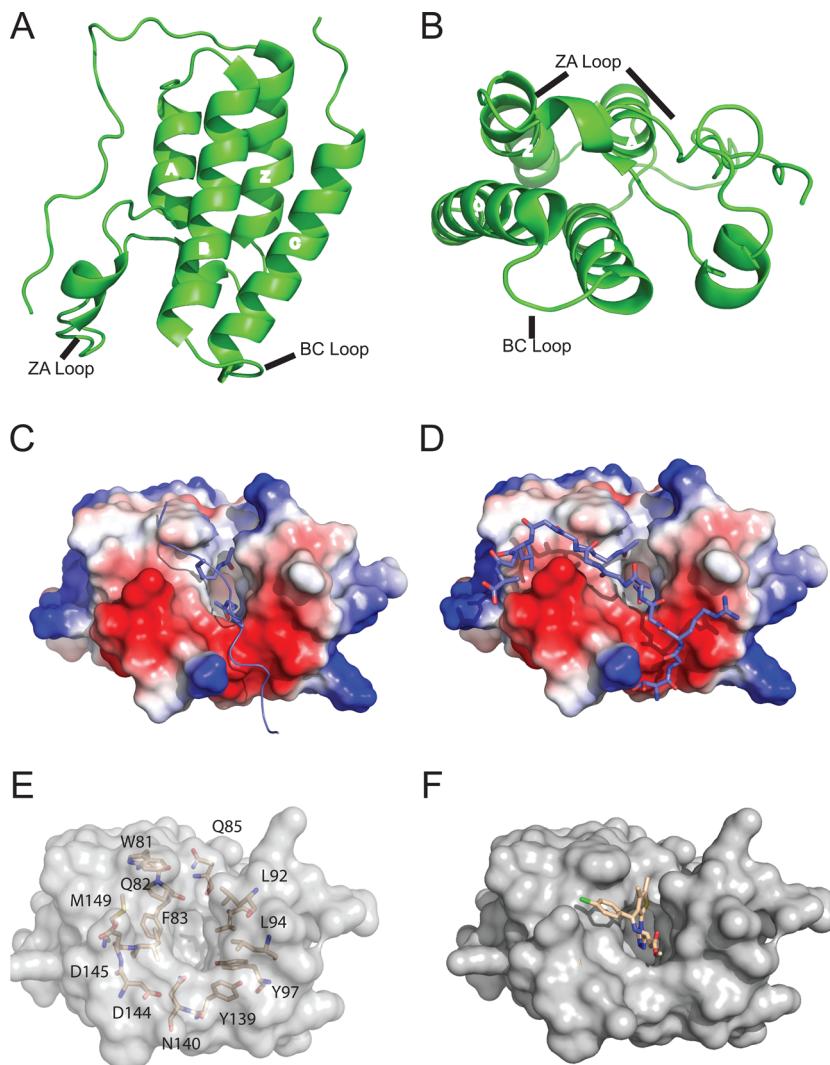


Figure 3. Crystal structures of bromodomains. (A) Cartoon representation of a typical bromodomain-containing protein, BRD4-BrD1 (PDB ID 4LYI), viewed from the side. The structure of the bromodomain is an evolutionarily conserved left-handed four-helix bundle packed in an antiparallel fashion, with helices named αZ , αA , αB , and αC . These helices are connected by two loops—a long ZA loop (connecting helices αZ and αA) and a short BC loop (connecting helices αB and αC). (B) Top view of the cartoon representation of BRD4-BrD1. The acetyl-lysine binding pocket is largely defined by the interhelical loops. (C) Electrostatic surface representation of BRD4-BrD1 (PDB ID 3UVW), in which red, blue, and white colors represent negatively charged, positively charged, and noncharged hydrophobic regions, respectively. BRD4 has a net negative charge overall, and the acetyl-lysine binding pocket is hydrophobic in nature. (D) BRD4 in complex with the diacetylated histone peptide H4K5acK8ac. The long peptide fits nicely in the valley on the BRD4 surface near the acetyl-lysine binding pocket. K5ac is anchored in the pocket by the conserved residue Asn140. (E) Stick representations of the key residues that are involved in ligand binding of BRD4-BrD1 (PDB ID 4F3I). (F) BRD4-BrD1 in complex with a thienotriazolodiazepine ligand MS417, which has a perfect shape complementarity to the acetyl-lysine binding pocket. Ligand atoms are colored as follows: oxygen, red; nitrogen, blue; chlorine, green; carbon, salmon.

carcinoma, and another inhibitor, OTX015, entered clinical trials for acute leukemia and other hematological malignancies in the same year. In 2013, TEN-010 and CPI610 entered clinical trials for solid tumors and lymphoma, respectively. A timeline of the development of bromodomains as therapeutic targets is outlined in Figure 2.

1.3. Bromodomain Structures

In spite of variations in length and sequence, bromodomains share a distinct left-handed α -helix bundle comprised of four α helices, referred to as αZ , αA , αB , and αC . These helices are packed tightly against each other in an antiparallel fashion and connected together by two long loops, termed the ZA and BC loops, which have diverse amino acid compositions. The ZA and BC loops form a hydrophobic pocket located on the one

end of the helix bundle, which is the site at which acetylated lysine binds (Figure 3A and 3B).⁵² Numerous bromodomain structures have been determined to date, supporting the conserved nature of this structural module.

At the base of the hydrophobic pocket is a key asparagine residue that interacts directly with the acetyl group of acetyl-lysine and is the main determinant for bromodomain/acetyl-lysine recognition.^{13,53–55} Additionally, water molecules located at the base of the binding pockets of almost all bromodomains play an indispensable role in the stabilization of the bromodomain–ligand complex structure.⁵⁶ Interestingly, researchers have also discovered that the bromodomains of the BET proteins of the bromodomain protein family have the ability to bind two acetylated lysines simultaneously.⁵⁷ This finding is demonstrated in the NMR solution structure of the

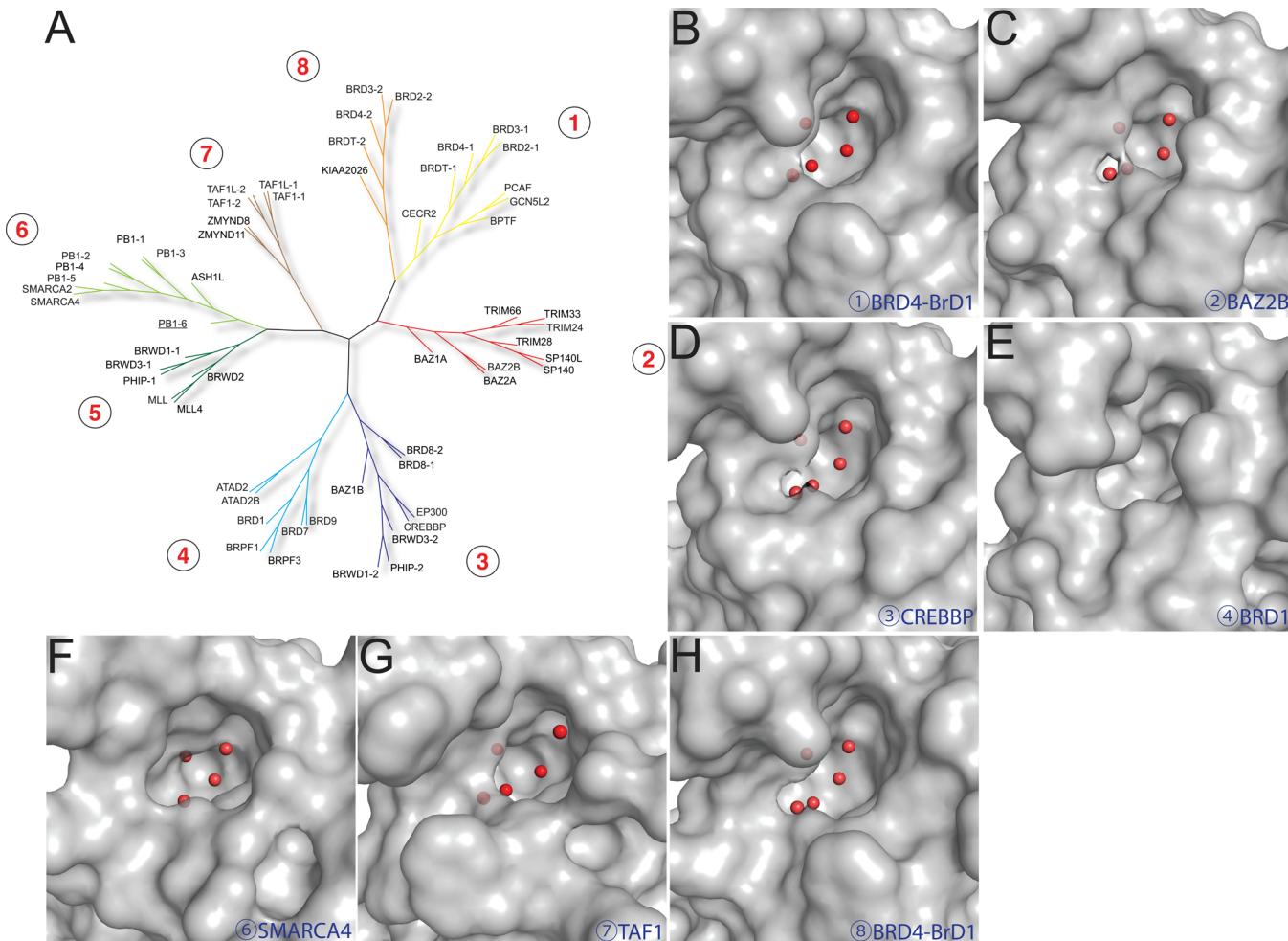


Figure 4. Classification of the bromodomain family. (A) Phylogenetic tree of the bromodomain family based on sequence similarity. To date, a total of 61 human bromodomains have been discovered. On the basis of their similarities in sequence and structure, they have been categorized into eight subfamilies. Eight bromodomain-containing proteins are selected and displayed further, serving as a representative for each subfamily. The binding pockets of these proteins vary in size and enclosure. (B) Group 1 BRD4-BrD1 (PDB ID 3MXF). (C) Group 2 BAZ2B (PDB ID 3G0L). (D) Group 3 CREBBP (PDB ID 3P1C). (E) Group 4 BRD1 (PDB ID 3RCW). (F) Group 6 SMARCA4 (PDB ID 2GRC). (G) Group 7 TAF1 (PDB ID 3UV4). (H) Group 8 BRD2-BrD2 (PDB ID 2YEM). It is worth noting that several water molecules are always conserved in the acetyl-lysine binding pocket. These water molecules, represented by red spheres, are integral parts of the proteins, contributing to their stabilization.

diacetylated transcription factor TWIST in complex with the second bromodomain of BRD4. One acetyl-lysine (K73ac) binds within the canonical acetyl-lysine binding pocket by forming a hydrogen bond with the conserved asparagine (Asn433), while the second acetyl-lysine (K76ac) is bound to a small hydrophobic cavity in the vicinity of the first pocket (Figure 3C). The first bromodomain of BRD4 can also bind diacetylated histone H4, as illustrated in a recently published high-resolution crystal structure.⁵⁸ While one histone mark (H4K5ac) binds within the classical acetyl-lysine binding pocket, the other (H4K8ac) is held in place due to the stabilization energy contributed by a network of water-associated hydrogen bonds (Figure 3D). Numerous structural and functional characteristics of bromodomains, from their ability to bind acetyl-lysine within a hydrophobic binding pocket to the evidence that links them to the recruitment of transcription factors and cofactors in gene activation, make these domains ideal targets in epigenetic drug discovery.⁵⁹

1.4. Classification and Druggability Assessment of the Bromodomain Protein Family

Despite the overall conservation of the four-helix bundle fold across the 61 bromodomains that have been identified in the human genome, the disparity in sequence and length of the interhelical loops within the bromodomain family can be quite significant. Any sequence variation in the ZA and BC loops of a given bromodomain would lead to a change in the size, accessibility, and charge of the binding pocket formed by the loops. The subtle variations in these loops seen across the entire family serve as indicators of the differing structures, functions, and binding capabilities of different bromodomains.^{52,58}

In the recent years, there has been an exponential increase in the number of available bromodomain structures. In an effort to curate this wealth of data and learn about similarities and differences in the family as a whole, comprehensive structural analyses have recently been conducted on the human bromodomains.⁶⁰ On the basis of structure-based sequence alignment, the human bromodomain family was subdivided into eight groups (Figure 4A). The binding pockets for a

representative of each group are shown in Figure 4B–I. Additionally, druggabilities of bromodomains were predicted and ranked using the Dscore calculated from SiteMap—BET bromodomains, such as BRD4, are deemed “druggable”; CREBBP and PB1 are considered “intermediate”; EP300 and SMARCA4 are termed “difficult”.⁶⁰ The range and distribution of the druggability of bromodomains with available structures is shown in Figure 5. While these results certainly provide a

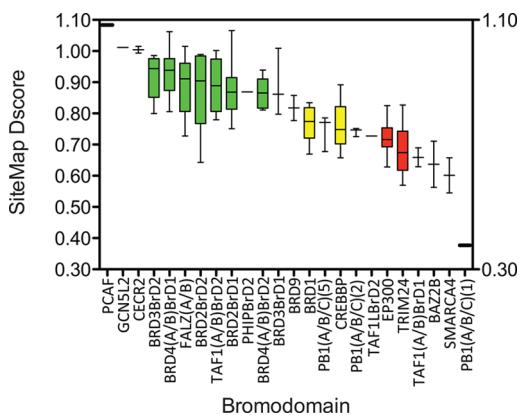


Figure 5. Druggability of selected bromodomains. Using the protocol developed by Vidler et al.,⁶⁰ selected bromodomains were ranked based on available crystal structures and their corresponding DScores: green, druggable; yellow, intermediate; red, difficult. This box-and-whisker plot was generated using the GraphPad Prism 5.0 software.

guideline for future drug discovery endeavors, they are limited by a number of factors, including the scoring function used, number of available structures, filter selection, and the overall impact of water molecules. Even as these types of studies remain helpful, one example that shows the difficulty of computationally determining the druggability of a given bromodomain is the development of PFI-3, a highly potent and selective chemical probe for SMARCA4. Despite the fact that this bromodomain was predicted to be difficult to target, PFI-3 proved to be a high-affinity ligand toward SMARCA4 as well as SMARCA2 and PB1.⁶¹

1.5. BET Family

Among the eight subfamilies of bromodomains (Figure 4A), the BET bromodomain subfamily is unique in that it has a well-defined and enclosed hydrophobic acetyl-lysine binding pocket. The size of the pocket is amenable to acetyl-lysine binding, and as such, BET bromodomains have generally been determined to have good druggabilities.⁶⁰ The BET family includes BRD2, BRD3, BRD4, and testis-specific BRDT in humans, each of which contains a pair of tandem bromodomains, namely, the first bromodomain (BrD1) and the second bromodomain (BrD2), followed by the ET domain, hence the name of the BET proteins.^{52,58} According to structure-based sequence alignment, these bromodomains have a high degree of sequence conservation (Figure 6A). This is especially the case among the BrD1s and BrD2s of the BET family, as the key residues that are responsible for acetyl-lysine binding are largely retained. Another important feature of BET bromodomains is the conserved water molecules that are bound deep inside the acetyl-lysine binding pocket.^{56,60} These waters are an integral part of the protein and greatly contribute to the stabilization of the structure by coordinating with the pocket residues (Figure 4B–I). Researchers have made attempts to displace these

waters with small molecules,⁶² but so far these efforts have been unsuccessful.

Aside from the two N-terminal bromodomains (BrD1 and BrD2), each BET protein contains a conserved N-terminal motif (A), an N-terminal phosphorylation site (NPS), a basic residue-enriched domain (BID), an extra-terminal (ET) domain, and a divergent C-terminal motif (CTM) (Figure 6B).^{63–66} Motif A is a conserved module within the long linker region between BrD1 and BrD2 with an unknown function. NPS is located downstream of BrD2, regulating it in a phosphorylation-based fashion and ultimately impacting its binding to acetylated histones. NPS can also contact BID downstream. The ET domain reportedly confers transcriptional activation by engaging in protein/protein interactions with NSD3, JMJD6, CHD4, GLTSCR1, and ATADS.^{66,67} Finally, the CTM of BRD4 (but not of other members of the BET family) mediates the recruitment of positive transcription elongation factor b (p-TEFb).⁶⁸ More recently, BRD4 has been reported to function as an atypical kinase that phosphorylates Ser2 at the C-terminus of RNA PolII.⁶⁹

2. DISCOVERY OF BROMODOMAIN INHIBITORS

In recent years, bromodomains have become popular drug targets.⁷⁰ A summary of the biological functions and disease indications of the members of the human bromodomain family is presented in Table 1.^{46,47,71–116} Also presented in Table 1 is a look at the progress that has been made in the area of bromodomain inhibitor development; while the most extensive development programs in recent years have focused on inhibitors of the BET bromodomains, many bromodomain-containing proteins are the focus of active investigation.. There are two major applications for these useful small molecules: first, these molecules can become therapeutic agents to treat various human diseases such as cancer, inflammatory diseases, and autoimmune diseases; second, these molecules can serve as chemical probes to investigate the biological function of bromodomain modules in chromatin-signaling pathways and in transcriptional activation and silencing. In this review, we will summarize the different chemical scaffolds that have been used by medicinal chemists to develop bromodomain inhibitors, allowing us to examine the past, present, and future of these drug discovery efforts.

2.1. N-Arylpropanediamine

As mentioned earlier in this review, the earliest development of bromodomain inhibitors dates back to 2005, when an NMR-based screen yielded the PCAF bromodomain inhibitor NP1, an N¹-aryl-propane-diamine (Figure 7A). This compound blocks the interaction between HIV/Tat and PCAF by selective inhibition of the PCAF bromodomain, thereby stopping HIV transcription.^{41,42} In terms of its affinity and selectivity, NP1 has an IC₅₀ (half-maximal inhibitory concentration) value of 1.6 μM in competition with HIV/Tat peptide and did not bind the structurally similar CBP or TIF1β (transcription intermediary factor 1β) bromodomains. These interactions were evaluated by 2D ¹⁵N-HSQC (two-dimensional heteronuclear single-quantum coherence) NMR spectra.^{41,42} The basic principle of this technique involves the observation of the changes in chemical shifts of protein signals upon ligand binding in a 2D ¹⁵N-HSQC spectrum. The residues with perturbed chemical shifts are most likely to reside at the binding interface between ligand and protein. The advantages of this NMR-based method are its high sensitivity (it is able to detect weak interactions at

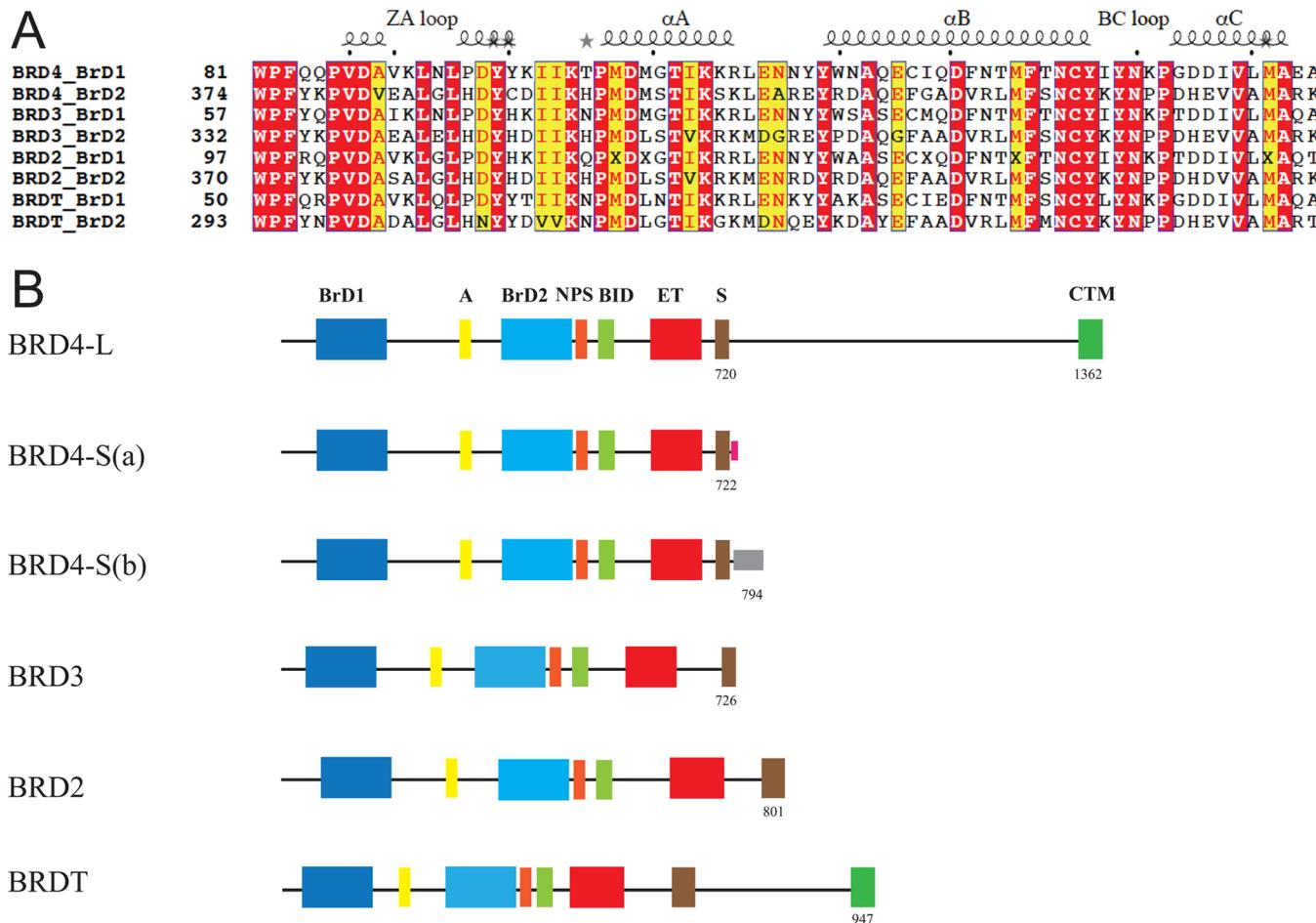


Figure 6. Sequence alignment and domain organization of BET bromodomains. (A) Sequence alignment of all eight bromodomains in the BET family. Residues blocked in red are completely conserved; residues blocked in yellow are largely conserved. This sequence alignment was created using ClustalW2. (B) Domain organization of BET bromodomains. A is a conserved motif that was discovered based on sequence alignment. BrD1 and BrD2 are the tandem bromodomains of each BET family member; NPS stands for N-terminal phosphorylation site; BID stands for a basic residue-enriched domain; ET stands for extra terminal domain; CTM stands for C-terminal motif. Although the domain organizations are highly similar, the specific length and sequence of each motif is different.

millimolar concentration) and ability to detail the precise binding locations of ligands. In addition, it is also possible to assess the impact of ligand binding on the global structure of target proteins. An NMR-based method is particularly helpful in cases in which X-ray crystal structures of bromodomain/ligand complexes are hard to obtain. Although affinities of hits discovered from 2D ¹⁵N-HSQC spectrum-based screening can be moderate and additional chemical modifications are typically required to improve the potency of the screen hits, this method provides a useful starting point for drug development for interesting bromodomain targets. Finally, the *in vitro* activity of NP1 was further assessed by competing biotinylated Tat-K50ac peptides immobilized on streptavidin in a Western blot.^{41,42}

The three-dimensional structure of this compound in complex with the PCAF bromodomain delineated that the nitro group of this compound is in range to hydrogen bond with Tyr802 and Tyr809, and the aromatic ring of NP1 is sandwiched between a group of hydrophobic residues (Figure 8A and 8B). However, NP1 lies external to the acetyl-lysine binding pocket and does not gain entry deep into the pocket. It has no interaction with the conserved Asn798 and therefore is not considered an acetyl-lysine mimetic. This is in direct contrast to the Tat peptide, whose K50ac residue buries deep within the binding pocket. The fact that NP1 does not bind

deeply within the acetyl-lysine pocket provides an explanation for its modest affinity for its target. Recently, researchers have improved the activity of NP1 by making 2-(3-aminopropylamin)pyridine 1-oxide derivatives.¹¹⁷ The best compound, 20 (Figure 7A), demonstrated an IC₅₀ value of 0.93 μM in a fluorescence polarization (FP) assay. This compound was tested against human T lymphocyte cell line C8166 and HIV-1IIIB and showed a half-maximal effective concentration (EC₅₀) of 11.5 μM.

2.2. Tetrahydrocarbazolone

In 2006, a CREBBP bromodomain inhibitor named MS7972 was identified through use of a similar NMR-based approach. This compound disrupted the association between human tumor suppressor p53/K382ac with coactivator CREBBP, modulating the function of p53 in response to DNA damage.¹¹⁸ It demonstrated a binding constant value (K_d) of 19.6 μM in a tryptophan fluorescence quenching assay. More recently, structure-guided design produced a selective compound named Olinone (tetrahydro-γ-carbolinone, Figure 7B), which is a synthetic analog of tetrahydrocarbazolone. Olinone displayed a dissociation constant value of 3.4 μM, as measured by isothermal titration calorimetry (ITC), toward the first bromodomain (BrD1) of BRD4, as opposed to a dissociation

Table 1. Bromodomains in Biology and Their Implications in Human Diseases

protein	name	also known as	protein functions	disease indications	compound ^a
ASH1L	Ash1 (absent, small, or homeotic) like	ASH1, KMT2H	histone methyltransferase transcriptional regulator	outated in lung cancer, ⁷¹ linked to facioscapulohumeral muscular dystrophy ⁷²	
ATAD2	ATPase family, AAA domain containing 2	ANCCA, CT137	transcriptional regulator	overexpressed in several types of tumors ⁷³	hits; see section 2.13.4 and Figure 29
ATAD2B	ATPase family, AAA domain containing 2B	KIAA1240, ACFL, WCRE180, WALP1	histone-binding factor chromatin remodeling factor	expressed in multiple neural tumor types ⁷⁴	
BAZ1A	bromodomain adjacent to zinc finger domain, 1A	WSTF, WBSCR9, WBSCR10	tyrosine kinase, chromatin remodeling factor, transcriptional regulator	has a role in spermatogenesis and possibly male infertility ⁷⁵	
BAZ1B	bromodomain adjacent to zinc finger domain, 1B	TIP5, WALP3	transcriptional repressor	overexpressed in colorectal tumors, ⁷⁶ deleted in Williams–Beuren syndrome ⁷⁷	lead; see section 2.13.1 and Figure 26
BAZ2A	bromodomain adjacent to zinc finger domain, 2A	WALP4	unknown	overexpressed in prostate cancer ⁷⁸	probe; see section 2.13.1 and Figure 26
BAZ2B	bromodomain adjacent to zinc finger domain, 2B	FAC1, FALZ	transcriptional regulator	associated with sudden cardiac death ⁷⁹	
BPTF	bromodomain PHD finger transcription factor	BRL, BRPF2	transcriptional regulator	plays a role in melanoma progression ⁸⁰ and neurodegenerative diseases ⁸¹	
BRD1	bromodomain containing 1	FSH, RING3	transcriptional regulator	plays a role in acute lymphoblastic leukemia ⁸³	
BRD2	bromodomain containing 2	ORFX, RING3L	transcriptional regulator	altered in B-cell lymphomagenesis ⁸²	
BRD3	bromodomain containing 3	CAP, MCAP, HUNK1	transcriptional regulator	implicated in NUT midline carcinoma ⁸⁴ and numerous aggressive leukemias ⁴⁷	
BRD4	bromodomain containing 4	BP75, NAG4, CELTIX1	transcriptional regulator, tumor suppressor	implicated in NUT midline carcinoma, ⁸⁵ numerous aggressive leukemias, ⁴⁷ and many other cancers and diseases of the immune system and inflammatory pathways ⁴⁶	
H	BRD7	SMAP, SMAP2	transcriptional regulator	associated with numerous types of cancer ^{86,87}	
	BRD8	BRD6	transcriptional regulator	linked to colorectal cancer ⁸⁸	N/A
BRD9	bromodomain containing 9	BRD6	chromatin remodeling factor	possible roles in immune function and multiple types of cancer ^{87,89}	
BRDT	bromodomain, testis-specific	BR140, Pergrin	transcriptional activator	expressed in lung cancer ⁹⁰	
BRPF1	bromodomain and PHD finger containing 1				
BRPF3	bromodomain and PHD finger containing 3	BRODL	component of MOZ/MORF H3 acetyltransferase complex		
BRWD3	bromodomain and WD repeat domain containing 3		chromatin-modifying factor	aberrantly expressed in B-cell chronic lymphocytic leukemia ⁹¹	
CECR2	cat eye syndrome chromosome region, candidate 2	CBP, KAT3A	chromatin-remodeling factor		
CREBBP	CREB binding protein	p300, KAT3B, RSTS2	histone acetyltransferase	implicated in acute myeloid leukemia, ⁹² acute lymphoblastic leukemia, ⁹³ B-cell lymphoma, ⁹⁴ and mutated in Rubinstein–Taybi syndrome ⁹⁵	probe; see section 2.13.2 and Figures 9, 12, and 14
EP300	E1A binding protein p300	GCN5, GCN5L2	histone acetyltransferase transcriptional activator	implicated in acute myeloid leukemia ⁹⁶ and other types of cancer ⁹⁶	probe; see section 2.13.2 and Figures 9, 12, and 14
KAT2A	lysine acetyltransferase 2A	KMT2A, HRX, TRX	histone acetyltransferase histone methyltransferase	implicated in nonsmall cell lung cancer ⁹⁷	
MLL	mixed-lineage leukemia	PBL, BAF180	chromatin remodeling factor	translocations of the gene implicated in the development of multiple leukemias ⁹⁸	
PBRM1	Polybromo 1	KAT2B	histone acetyltransferase	mutated in renal cell carcinoma ⁹⁹	
PCAF	P300/CBP-associated factor			involved in cancer cell proliferation pathways ¹⁰⁰	hit; see section 2.1 and Figure 7A

Table 1. continued

protein	name	also known as	protein functions	disease indications	compound ^a
PHP	pleckstrin homology domain interacting protein	WDR11, BRWD2, DCAF14	insulin signaling	expressed in myeloma and epidermoid carcinoma cells ¹⁰¹	
PRKCBP1	protein kinase C-binding protein 1	ZMYND8, RACK7	transcriptional regulator	overexpressed in cervical cancer ¹⁰²	
SMARCA2	SWI/SNF related, matrix associated, actin-dependent regulator of chromatin, subfamily a, member 2	BRM, SNF2L2	chromatin remodeling factor	associated with head and neck squamous cell carcinoma, ¹⁰³ altered in hepatocellular carcinoma ¹⁰⁴	
SMARCA4	SWI/SNF related, matrix-associated, actin-dependent regulator of chromatin, subfamily a, member 4	BRGL, SNF2L4	chromatin remodeling factor	altered in hepatocellular carcinoma, ¹⁰⁴ also mutated in many other types of cancer, including lung, breast, and pancreatic ^{105–107}	
SP100	SP100 nuclear antigen	IPRI, VODI	transcriptional regulator	downregulated in laryngeal cancer ¹⁰⁸	
SP110	SP110 nuclear body protein	LYSP100	transcriptional regulator	mutations associated with immunodeficiency and hepatic veno-occlusive disease, ¹⁰⁹ copy number changes in desmoplastic melanoma and malignant peripheral nerve sheath tumor ¹¹⁰	
SP140	SP140 nuclear body protein	TAF2A, TAF (II)250	transcription initiation	linked to chronic lymphocytic leukemia ¹¹¹	
SP140L	SP140 nuclear body protein-like	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 250 kDa	TAF2A2, TAF(II)210	transcription initiation	
TAF1	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 210 kDa like	TIF1 α , PTC6, RNF82	transcription regulator	associated with prostate cancer ¹¹²	
TAF1L	TAF1 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 210 kDa like	TIF1 β , KAP1, RNF96	transcription regulator	altered in numerous types of cancer, including papillary thyroid cancer and breast cancer ¹¹³	
TRIM24	Tripartite motif containing 24	TIF1 γ , ECTO, PTC7, RFG7	transcription regulator	associated with gastric cancer ¹¹⁴	
TRIM28	Tripartite motif containing 28	TIF1 δ	transcriptional repressor		
TRIM33	Tripartite motif containing 33	BRWD1	chromatin remodeling factor	linked to chronic myelomonocytic leukemia ¹¹⁵	
TRIM66	Tripartite motif containing 66	BS69, BRAM1, MRD30	transcriptional repressor	associated with repression of breast cancer cell growth ¹¹⁶	
WDR9	WD repeat domain 9				
ZMYND11	zinc finger, MYND-type containing 11				

^aFor the compounds, “probe” refers to a compound with an affinity of 250 nM or better, “lead” refers to a compound with an affinity between 250 nM and 1 μ M, and “hit” refers to a compound with an affinity above 1 μ M.

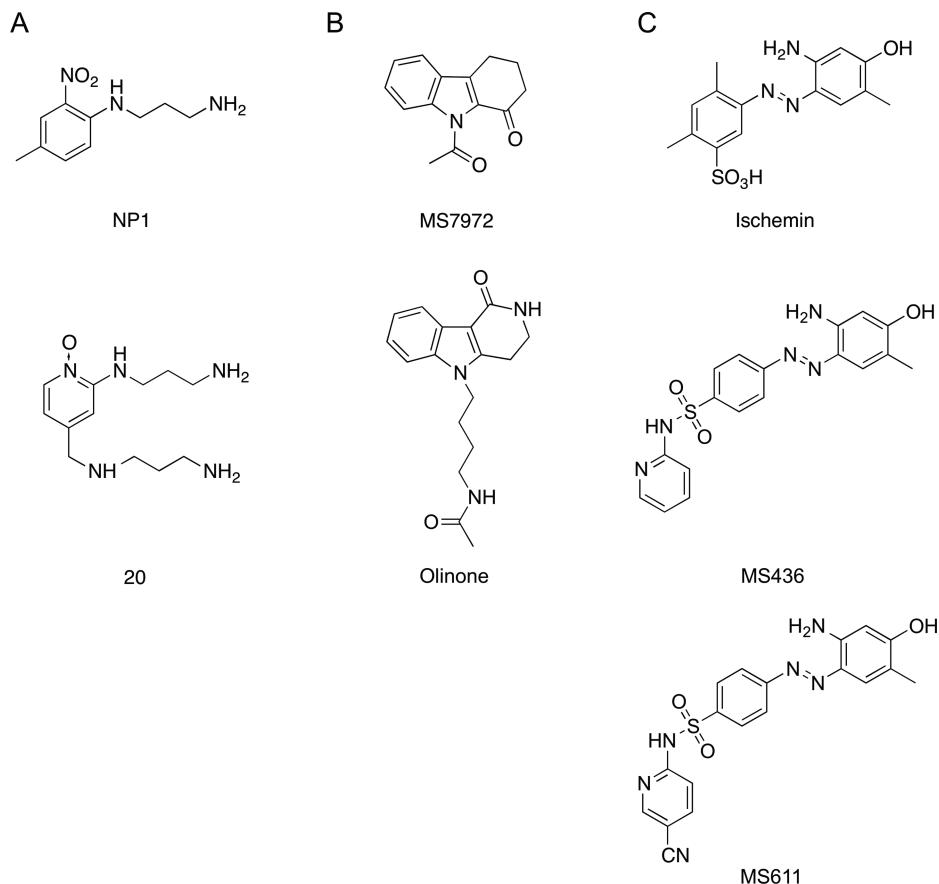


Figure 7. Early bromodomain inhibitor hits discovered via NMR-based screening. These molecules have been further optimized and repurposed using medicinal chemistry and structure-based drug design. (A) NP1 was the first reported bromodomain inhibitor for PCAF. Further optimization led to 2-(3-aminopropylamino) pyridine 1-oxide derivatives. (B) MS7972 was initially screened as a CREBBP inhibitor, and optimization by medicinal chemistry turned it into a highly specific inhibitor for BRD4-BrD1. (C) Ischemin was identified early in the bromodomain drug discovery process as a CREBBP inhibitor. It was later repurposed and modified, resulting in MS436, a highly potent BET bromodomain inhibitor with a modest selectivity between BrD1 and BrD2 of BRD4.

constant of over 300 μM toward the second bromodomain (BrD2) of BRD4. This preference for BrD1 was also seen in the other BET bromodomains (i.e., BRD3 and BRD2).¹¹⁹ A high-resolution X-ray crystal structure of the BRD4-BrD1/Olinone complex revealed the tricyclic moiety of Olinone interacts with Asp144, which is one of the few differing residues within the acetyl-lysine binding pocket that distinguishes BrD1 from BrD2. According to structure-based alignment, the corresponding residue in BrD2 is His437, which is bulkier and could possibly clash with the tricycle, preventing binding. The acetyl group at the tail of Olinone is delivered to the hydrophobic pocket by an alkyl chain and forms a hydrogen bond with the highly conserved Asn140 in an identical fashion as histone peptides H4K5ac/K8ac (Figure 8C and 8D). Two-dimensional $^1\text{H}-^{15}\text{N}$ HSQC showed Olinone had moderate activity toward CREBBP and PHIP (Pleckstrin homology domain-interacting protein) with K_d values of 33 and 103.4 μM , respectively. It is almost completely inactive toward other subgroups of the bromodomain family, as represented by BRD7, PCAF, ASH1L, TAF1L, SMARCA4, BAZ1A, BAZ1B, ATAD2, BPTF (Bromodomain PHD Finger Transcription Factor), and TRIM28. Olinone displayed a unique biological function, as it was found to enhance the differentiation of primary oligodendrocyte progenitors in mice, while a pan-BET bromodomain inhibitor hampered differentiation.¹¹⁹ This study paved the way for

developing future treatments of demyelinating diseases by selectively targeting BET bromodomains.

2.3. Diazobenzene

In 2011, a compound with a diazobenzene scaffold was identified as a CREBBP bromodomain inhibitor after 2D $^1\text{H}-^{15}\text{N}$ -HSQC NMR spectra showed that backbone amide protons of ^{15}N -labeled CREBBP were perturbed upon the addition of the compound. A drug design effort based on structure–activity relationship (SAR) data yielded the optimized compound named Ischemin, which was found to have a K_d value of 19 μM toward CREBBP BRD in an in vitro fluorescence binding assay.¹²⁰ Its moderate affinity notwithstanding, Ischemin interfered with the recruitment of CREBBP by p53 and the target gene expression of p53, protecting ischemic cardiomyocytes from apoptosis. Ischemin was determined to have an IC_{50} of 5 μM toward the CREBBP bromodomain through use of a p53-induced p21 luciferase assay.¹²⁰ Furthermore, Ischemin displayed modest selectivity for the CREBBP bromodomain over other human bromodomains such as PCAF, BAZ1A, BAZ1B, and BRD4 in vitro. The phenoxy group of Ischemin forms a hydrogen bond with the amide nitrogen of Asn1168 in CREBBP, mimicking the interaction of the acetyl-lysine of H4K20ac with CREBBP (Figure 8E). Compared with H4K20ac, Ischemin was not buried as deep as the histone peptide, an observation that

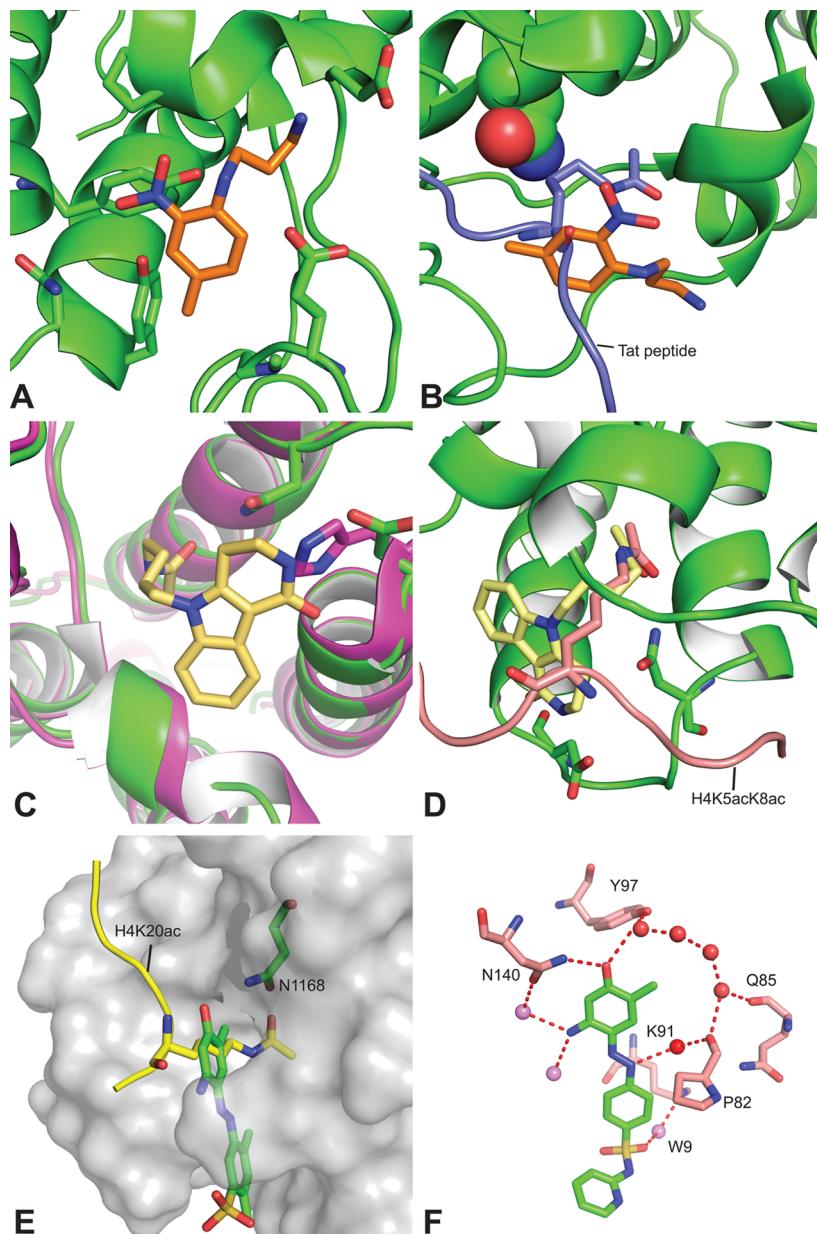
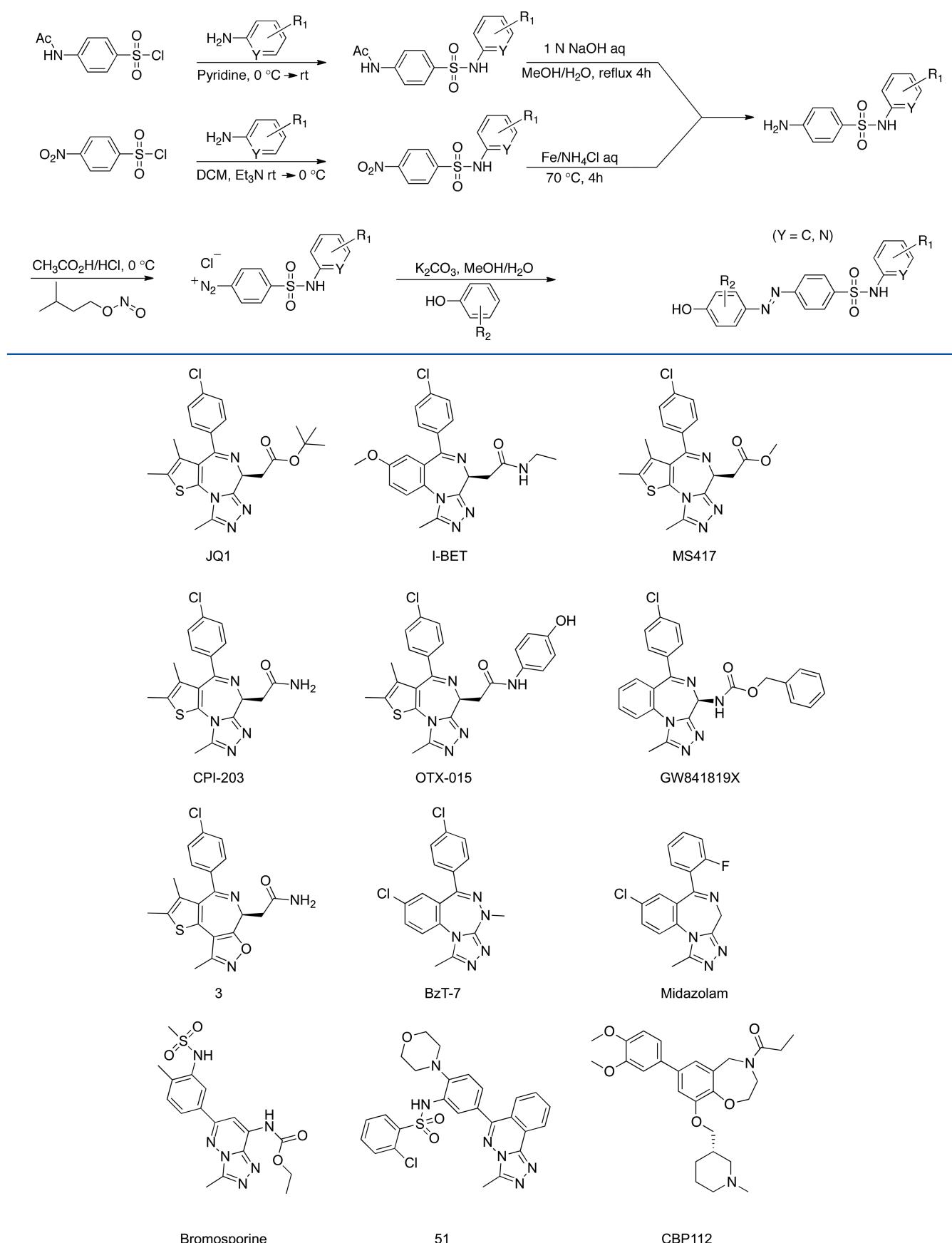


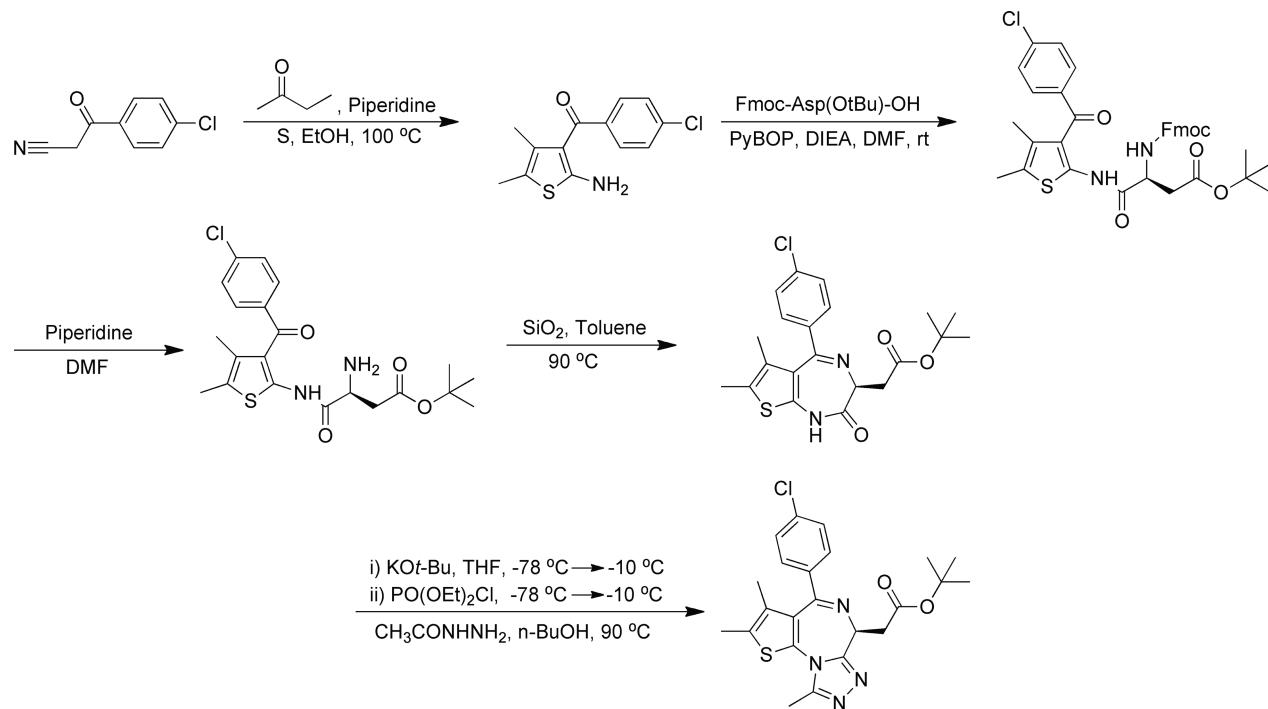
Figure 8. Structures of bromodomain-containing proteins in complex with early hits. (A) Solution structure of the PCAF/NP1 complex (1WUG). (B) Solution structure of PCAF in complex with the Tat peptide (1JM4), with NP1 superimposed on the structure. Atoms of the conserved asparagine residue are represented by spheres. NP1 is unable to reach deep into the pocket and therefore does not act as a traditional acetyl-lysine mimic. (C) Superimposition of high-resolution X-ray crystal structures of the BRD4-BrD1/Olinone complex (4QBS, green) and BRD4-BrD2 (2YEM, purple). Olinone interacts with Asp144 in the BrD1, while the corresponding residue at this location in the BrD2 is His437. Due to the significant difference in size between the residues, Olinone will most likely clash with the BrD2, explaining why Olinone is a BrD1-specific inhibitor of BRD4. (D) BRD4-BrD1/Olinone complex (4QBS, green) in alignment with H4K5acK8ac (3UVW, salmon). This comparison reveals that the tail of Olinone binds deep inside the acetyl-lysine pocket, almost in an identical manner as the acetylated histone H4 peptide. (E) Solution structure of CREBBP with MS120 (2L84) in alignment with histone peptide H4K20ac (4N3W). This result suggests that ischemin binds at the entrance of the acetyl-lysine binding pocket, providing some insight into potential further ligand modifications. (F) Key molecular interactions between BRD4-BrD1 and MS436 (4NUD). MS436 interacts with the conserved Asn140 through its phenoxy group. MS436 is stabilized by its interaction with the five water molecules at the base of the binding pocket, which partially influences its potency and selectivity. The conserved water molecules in the acetyl-lysine pocket are colored red, and the additional interacting water molecules are colored purple.

provides a future direction for potential chemical modifications of this scaffold.¹²¹ In addition, Ischemin was used as an effective tool to investigate the impact of double modifications of CREB on CREBBP recruitment at the promoter region.¹²²

In 2013, Ischemin was further optimized by a comprehensive medicinal chemistry campaign. The resulting compound, a much-improved diazobenzene named MS436, is a specific

inhibitor of BET bromodomains. MS436 has an estimated inhibition constant (K_i) value of 30–50 nM for BrD1 of BRD4, as determined by a fluorescence polarization assay.^{123,124} High-resolution X-ray crystal structures of the top compounds synthesized show that the extended pyridine ring (as compared to Ischemin) forms a $\pi-\pi$ interaction with Trp81 of BRD4-BrD1. In addition to improved potency, there is also a modest

Scheme 1. Synthesis of Diazebenzene MS436 and Its Derivatives¹²³**Figure 9.** Chemical structures of thienotriazolodiazepine and triazolobenzodiazepine inhibitors and their analogs.

Scheme 2. Stereospecific Synthesis of (+)-JQ1⁴⁵

10-fold selectivity for BrD1 over BrD2 of BRD4 by MS436. This selectivity originates from a unique water-associated hydrogen bond between W9 and Lys91 in BrD1. The corresponding residue in BrD2 is Ala384, which is not in the proper position to interact with the ligand or water molecule. A number of additional bound water molecules are also responsible for the enhanced binding affinity of MS436, as they engage in an extensive hydrogen-bonding network (Figure 8F). MS436 exhibited effective inhibitory activity of NF- κ B-regulated production of nitric oxide as well as pro-inflammatory cytokine interleukin-6 (IL-6) in murine macrophages. Inhibition of BRD4 by MS436 altered human and mouse embryonic stem cell colony integrity.¹²⁵ This class of molecules was further modified, yielding the optimized compound MS611 (Figure 7C), which displayed 40-fold selectivity for BrD1 over BrD2 in a FP assay.¹¹⁹ Like Olinone, MS611 facilitates the progression of primary oligodendrocyte progenitors toward differentiation.¹¹⁹ The chemical synthesis of MS436 and its analogs is outlined in Scheme 1.

2.4. Thienotriazolodiazepine and Its Analogs

2.4.1. Thienotriazolodiazepine and Benzotriazolodiazepine. The diazepine ring has a long history of clinical applications and is widely accepted as a privileged chemical scaffold in drug discovery.¹²⁶ Many compounds have scaffolds that build upon the simple diazepine ring, such as thienotriazolodiazepines (TTDs), which have an extensive research history that dates back to 1974. Historically, TTDs such as brotizolam are known to be tranquilizers, anesthetics, hypnotics, anxiolytics, and immunosuppressants.^{127–129} TTDs have also shown indications as platelet activating factor (PAF) antagonists, implying that they may have potential as therapeutics in cardiovascular diseases, including coronary heart disease.^{130–132} TTDs are analogs of benzodiazepines (BZDs), and they bind to the benzodiazepine binding site on the GABA_A receptor and act as allosteric agonists.¹³³ TTDs were linked to increased levels of Apolipoprotein A1

(ApoA1),^{134,135} which is the major component of plasma high-density lipoprotein (HDL). Low levels of plasma HDL are associated with higher incidence of coronary artery disease. Furthermore, TTDs were shown to be effective in treating inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease.¹³⁶ In 2006, TTDs showed effectiveness in CD-28-dependent T-cell suppression, which can be particularly useful in minimizing the risk of organ transplant rejection and in treating autoimmune diseases.⁴³

In a 2009 patent, scientists in Japan disclosed that TTDs acted as antitumor agents via the inhibition of BET bromodomains. These TTDs displayed low nanomolar IC₅₀ values against BRD4 in a time-resolved fluorescence resonance energy transfer (TR-FRET) assay. These TTDs were also screened against a panel of cancer cell lines and showed significant inhibitory activity toward cell proliferation. TTDs demonstrated broad potential indications in acute promyelocytic leukemia, acute myelocytic leukemia (AML), myeloma, acute lymphoblastic leukemia (ALL), liver cancer, ovarian cancer, prostate cancer, lung squamous cell carcinoma, osteosarcoma, and colon carcinoma in cell-based studies.⁴⁴ This disclosure was a hallmark event in the field of bromodomain drug discovery (Figure 2), as it represented the first highly potent and clinically relevant chemotype that was an acetyl-lysine mimic. This work laid a solid foundation for later investigations and provided great insights into potential clinical implications of bromodomain targeting. BZDs and TTDs enhance the activity of the neurotransmitter GABA, and their pharmacological properties have been investigated extensively. Their affinity for BET bromodomains could help elucidate certain cancer biology pathways from an epigenetic perspective once their sedative, hypnotic, and anxiolytic effects are removed by chemical modifications that prevent them from binding to the GABA_A receptor.

In 2010, two separate groups independently reported the small molecules JQ1 and I-BET (Figure 9) as highly selective

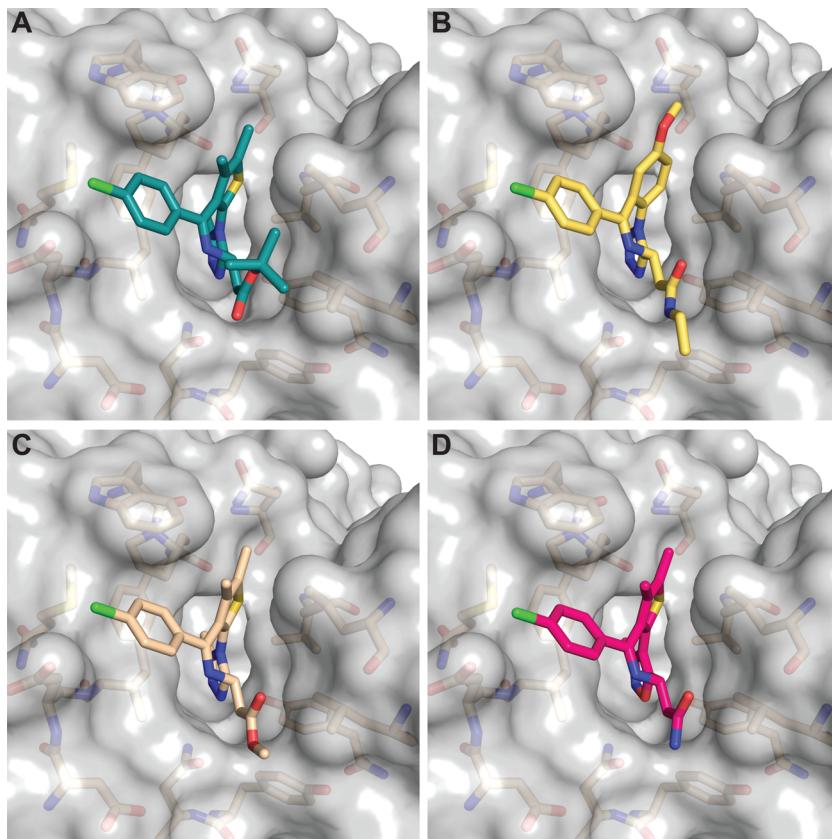
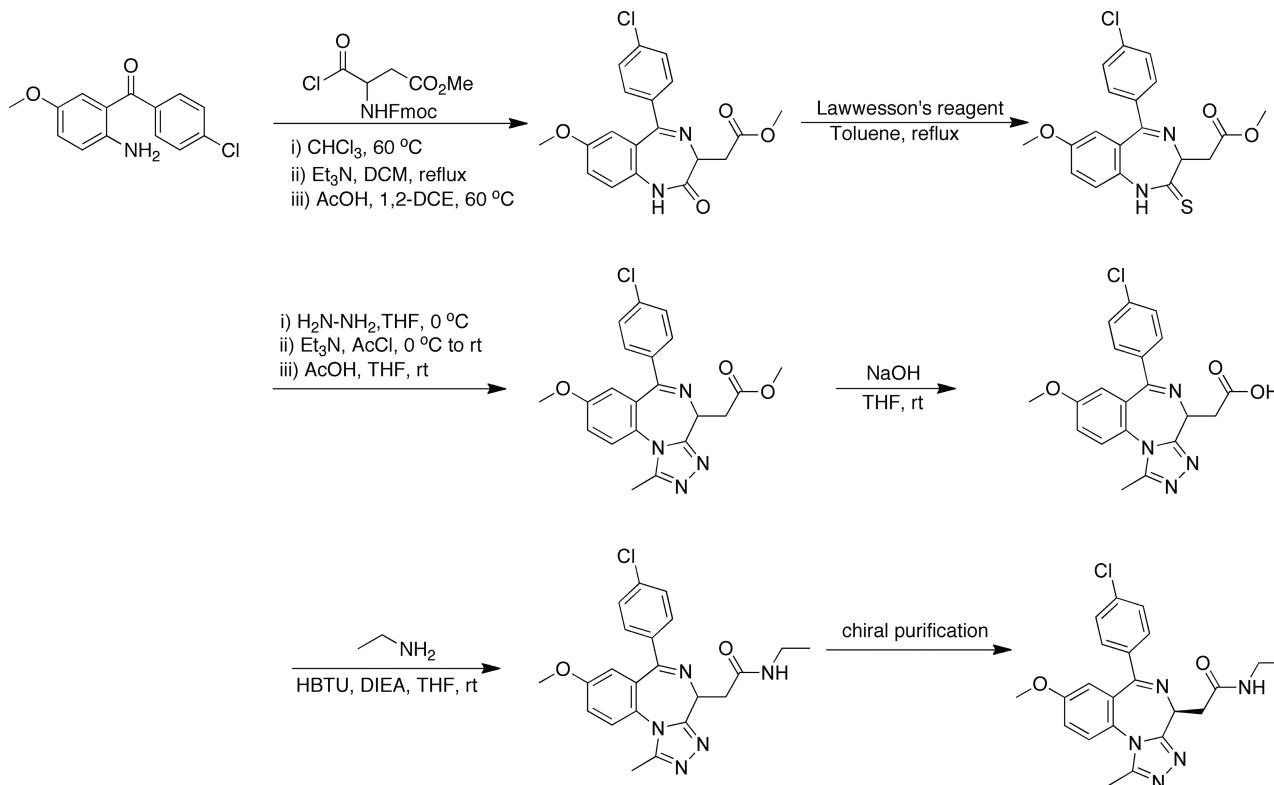


Figure 10. X-ray crystal structures of BRD4-BrD1 in complex with thienotriazolodiazepine analogs: (A) JQ1 (3MXF), (B) I-BET (3PSO), (C) MS417 (4F3I), and (D) benzoioxazoloazepine (4LRG). The binding modes of TTDs and TBZDs are very similar, the lone exception being the orientation of the *tert*-butyl ester on the pendant side chain of JQ1. The standard binding mode is exemplified by the methyl ester moiety of MS417, which makes additional hydrophobic interactions with BRD4-BrD1 within a small hydrophobic cavity, which is made up of Leu95, Tyr97, and Tyr139. On the other hand, the bulky *tert*-butyl group of JQ1 cannot be accommodated within this pocket and flips outward, explaining the compound's lower affinity for its target relative to MS417.

inhibitors of the BET bromodomains. (+)-JQ1, a close derivative of the TTDs disclosed in the Japanese patent, binds BRD4 in its acetyl-lysine binding pocket. (+)-JQ1 had a K_d value of 49 and 90 nM toward the first and second bromodomain of BRD4, respectively, according to ITC measurement.⁴⁵ In an amplified luminescence proximity homogeneous assay (AlphaScreen), (+)-JQ1 competed with acetylated histone peptide and had IC_{50} values of 77 and 33 nM, respectively, for the first and second bromodomains of BRD4. A thermal shift assay shows a temperature shift of over 9 and 7–9 °C upon (+)-JQ1 binding to BRD4-BrD1 and BRD4-BrD2, respectively.⁴⁵ Research into this compound also showed that (+)-JQ1, the *S*-enantiomer (*R/S* nomenclature system), is over 100-fold more potent than (−)-JQ1, providing a stereospecific consideration to the development of potent bromodomain inhibitors. JQ1 can be readily accessed via a stereospecific synthesis (Scheme 2). In terms of its selectivity for the BET family, JQ1 displayed very weak binding to bromodomains outside of the BET family, as suggested by the data from the researchers' protein stability shift assay. High-resolution X-ray crystal structures of BRD4-BrD1/JQ1 and BRD2-BrD2/JQ1 complexes detail the interactions between JQ1 and the acetyl-lysine binding pocket. The triazole ring serves as an acetyl-lysine mimic, forming a hydrogen bond with the conserved asparagine residue (Asn 140 in BRD4-BrD1 and Asn429 in BRD2-BrD2). JQ1 also forms extensive hydrophobic interactions with hydrophobic residues (such as Val87, Leu92,

Leu94, Tyr97, Tyr139, Cys136, Met149, Pro82, and Trp81 in BRD4-BrD1) in the ZA and BC loops (Figure 3E and 3F). Computational modeling suggests (−)-JQ1 (*R*-enantiomer) clashes with Leu92 and Leu94, rationalizing its markedly reduced activity. The pendant side chain on the chiral carbon of JQ1 is critical because it prevents the compound from binding to GABA receptors, lowering the potential for off-target effects. Additionally, JQ1 demonstrated its ability to competitively bind to BRD4 in osteosarcoma cells in a fluorescence recovery after photobleaching (FRAP) assay, and JQ1 induced apoptosis by targeting the BRD4-NUT fusion protein in human carcinoma cells and attenuated death in a NUT midline carcinoma (NMC) xenograft model.⁴⁵

An independent group reported that a different BET bromodomain-specific inhibitor, I-BET, suppressed inflammation by disrupting the formation of chromatin complexes that are critical in inflammatory gene activation.⁴⁶ I-BET is a benzodiazepine (BZD), a subcategory of the diazepine family that also has a long-standing history of medicinal use. BZDs are useful in treating medical conditions such as anxiety,¹³⁷ insomnia,¹³⁸ and seizure¹³⁹ like TTDs but are more widely used than TTDs in the clinic. As measured by ITC, I-BET has K_d values of 50.5–61.3 nM toward the members of the BET bromodomain family. I-BET binds to tandem BET bromodomains BRD2, BRD3, and BRD4 competitively and was found to displace acetylated histone H4 peptide with IC_{50} values of 32.5–42.5 nM in a fluorescence resonance energy transfer

Scheme 3. Synthesis of I-BET762⁴⁶

(FRET) assay. As is the case with JQ1, the *S*-enantiomer of I-BET has no activity toward HATs or other families of proteins (e.g., GPCRs, ion channels, kinases, and transporters).⁴⁶ In bone marrow-derived macrophages, I-BET suppressed the expression of key lipopolysaccharide (LPS)-induced chemokines and cytokines selectively. In LPS-induced endotoxic shock and bacteria-induced sepsis models, I-BET promoted prolonged survival of mice. Crystallographic data confirms that I-BET has the anticipated binding mode in that its triazole ring sits deep within the Kac binding pocket and hydrogen bonds with Asn140 and a network of water molecules in BRD4-BrD1. Its side chain amide is also in range of hydrogen bonding with Asn140. The selectivity of I-BET is potentially determined by its interaction with the hydrophobic ZA channel and WPF shelf, with the conserved Ile147 acting as a gatekeeper residue.⁴⁶ These observations are consistent with the binding mode of JQ1 (Figure 10A and 10B). The organic synthesis of this class of benzotriazolodiazepine (BZTD) is outlined in Scheme 3.^{46,140} In comparison with TTDs, BZTDs are more stable and capable of handling harsher synthetic conditions.

Another example of the TTD class of compounds is MS417 (Figure 9). Sharing a high structural similarity to both JQ1 and I-BET, MS417 had IC₅₀ values of 30 and 46 nM, respectively, toward BrD1 and BrD2 of BRD4 in a FP assay.¹⁴¹ This is approximately a 10-fold improvement over JQ1, as per the data described in a comparative study.⁵⁷ High-resolution X-ray crystallography depicted a different orientation of the ester moiety of MS417, in line with the comparable moiety in I-BET. Embedded in a small hydrophobic cavity determined by Leu95, Tyr97, and Tyr139, the methyl ester moiety of MS417 has the ability to make additional hydrophobic interactions with BRD4-BrD1, while the more bulky *tert*-butyl group found on JQ1 could not be accommodated in this pocket. Instead of making

the contacts made by the methyl ester, the *tert*-butyl group rotates 180° and projects outward from the protein surface (Figure 10A and 10C). Biologically, MS417 was found to impede master transcription factor NF-κB from binding to BRD4 and disable the assembly of transcriptional complex with p-TEFb and RNA polymerase II. Moreover, MS417 conferred protection for kidney injury in HIV-1 transgenic mice by suppressing NF-κB-mediated inflammation,¹⁴¹ showed anti-proliferative activity in melanoma cells and impaired melanoma tumor growth *in vivo*,¹⁴² and blocked the interaction between BRD4 and Twist, suppressing tumor progression in basal-like breast cancer.⁵⁷

CPI-203 (Figure 9), another closely related TTD analog, displayed an IC₅₀ value of 37 nM in an AlphaScreen assay and showed comparable *in vitro* and cellular activities to JQ1. In two separate cellular assays, CPI-203 had IC₅₀ values of 99 and 30 nM against MYC and IL-6, respectively.⁶⁹ Recently, CPI-203 was reported to be highly efficacious as a monotherapy as well as a combination therapy *in vivo* in a refractory form of mantle cell lymphoma (MCL) in combination with lenalidomide.¹⁴³ CPI-203 also demonstrated its antitumor activities both as a single agent and in combination with rapamycin in inhibiting cell proliferation in pancreatic neuroendocrine tumors.¹⁴⁴ OTX015 (Figure 9) is also a potent BET bromodomain inhibitor. OTX015 competed with acetylated H4 peptide and had IC₅₀ values of 92–112 nM and EC₅₀ values of 10–19 nM toward BET bromodomains in a TR-FRET assay. OTX015 also displayed antiproliferative activity against the growth of a panel of human cancer cell lines, especially hematological malignancies (GI₅₀ values 60–200 nM).¹⁴⁵ OTX015 was also effective in inhibiting the growth of a variety of solid tumor cell lines and was reported to induce apoptosis and senescence.¹⁴⁶ Evidence has emerged that OTX015 targets NF-κB, toll-like

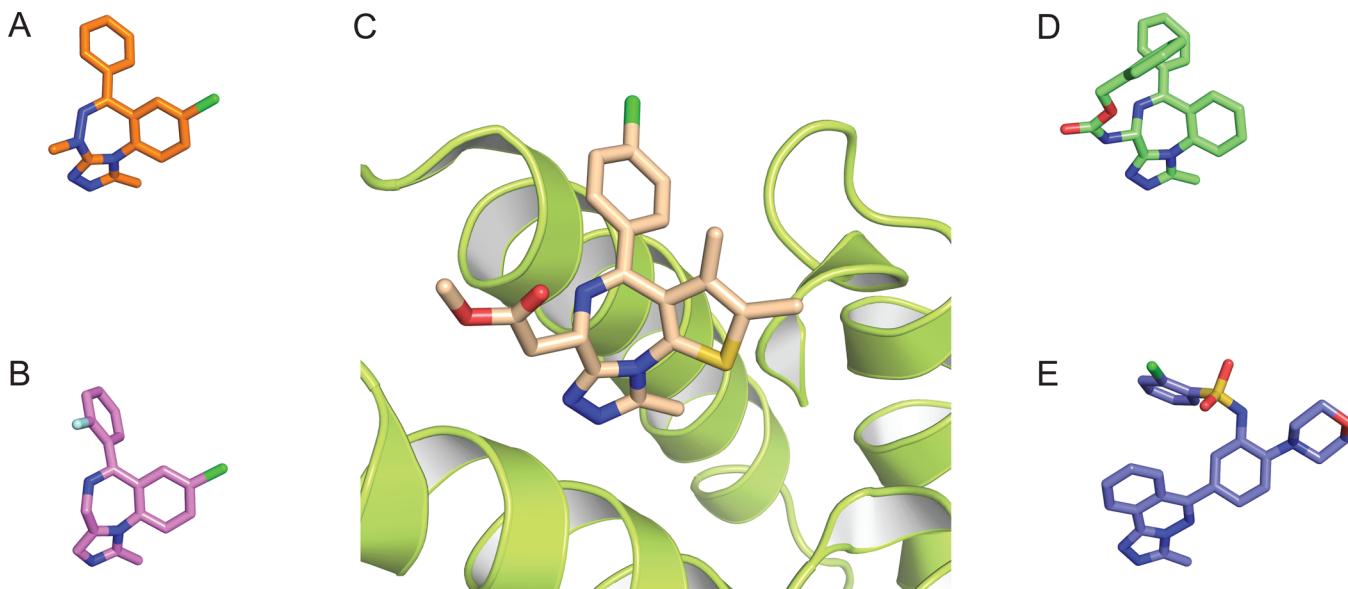
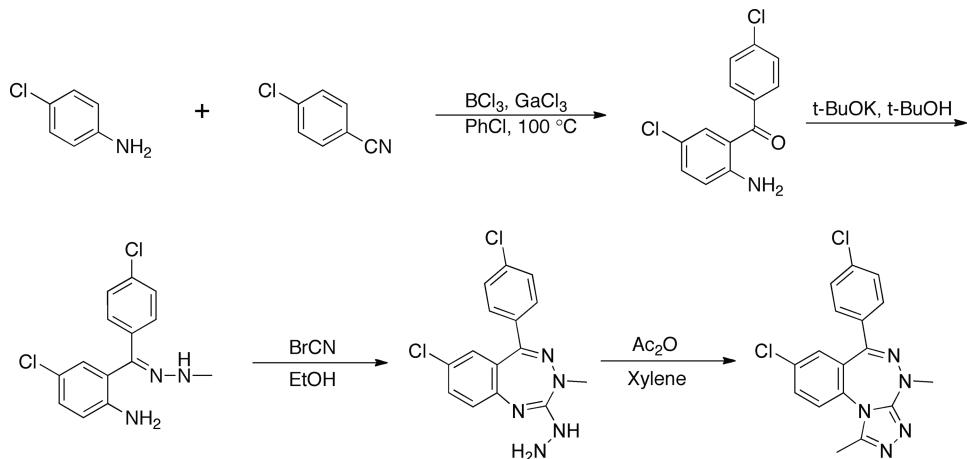


Figure 11. Comparison of binding modes to BRD4-BrD1 between thienotriazolodiazepine analogs. Binding poses were extracted from their corresponding complex structures. (A) BzT-7 (3U5L) and (B) midazolam (3U5K) bind BRD4-BrD1 in almost an identical fashion as (C) MS417 (4F3I). The differences in the side chains of the compounds explain the order of affinity toward BRD4-BrD1. Midazolam has no pendant side chain and thus makes no hydrophobic interactions with the ZA channel. BzT-7 retained some hydrophobic interaction because of its methyl group but could not reach far enough into the pocket to maintain the potency found with MS417. (D) GW841819X (2YEL) maintains the conformation of CM417, albeit with a far bulkier side chain. Its benzyl group points toward the solvent-accessible surface, making this interaction closer to the manner in which JQ1 interacts with BRD4-BrD1. Because (E) triazolophthalazine 51 (4NQM) relinquishes the traditional diazepine framework and adopts a rather planar aromatic system, only the position of its triazole ring remains constant when compared to other inhibitors. The rest of the molecule binds BRD4-BrD1 in a completely different manner, which alters the specificity of the molecule. For visual clarity, the ZA loop is hidden.

Scheme 4. Synthesis of Triazolobenzotriazepine BzT-7^{151,152}



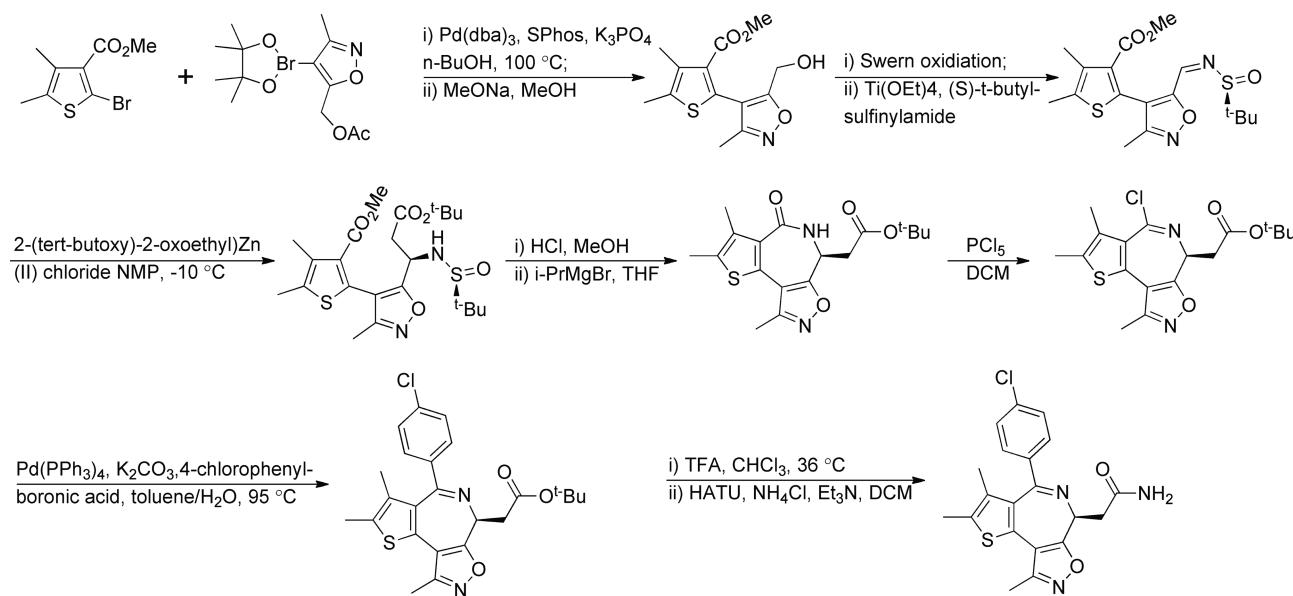
receptors (TLR), and the JAK/STAT pathways and appears promising in treating mature B-cell tumors, both as a single agent and in combination with a targeted anticancer agent.^{147–149}

Researchers also investigated the activity of clinically approved anxiolytic BZTDs toward BET bromodomains. An X-ray crystal structure revealed alprazolam (Figure 9) bound BRD4-BrD1 in an almost identical mode as I-BET and the other diazepine-based inhibitors and maintained hydrogen bonding with Asn140 (Figure 11). However, alprazolam lacks the entire side chain found on the diazepine bromodomain inhibitors, resulting in a loss of hydrophobic interaction and as such a loss of affinity. Alprozolam had a K_d of $2.5 \mu\text{M}$ toward the first bromodomain of BRD4 in an ITC assay, which is 50 times less potent than I-BET, making it unlikely to impose off-

target effects through inhibition of the BET bromodomains.^{62,150}

In summary, the efficacy of thienotriazolodiazepines can be traced to the unique geometry of the 1,4-diazepine, which fits tightly into the binding pockets of the BET bromodomains and presents numerous functional groups that can interact with the protein to increase affinity. Medicinal chemists are certain to explore many more analogs of this exceptional scaffold in an effort to develop potent and selective ligands.

2.4.2. Benzotriazepine. Benzotriazepines are analogs of benzodiazepines, differing only by an additional nitrogen atom at the location occupied by a chiral carbon in the latter (Figure 9). Benzotriazepines have a comparable binding mode, adopting the same geometry within the binding pocket as the BZDs (Figure 11). The triazepine ring can be readily

Scheme 5. Synthesis of Thienoisoxazolodiazepine¹⁵⁶

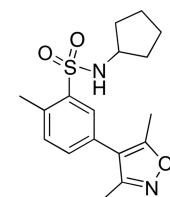
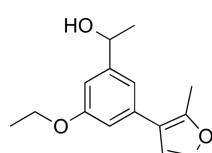
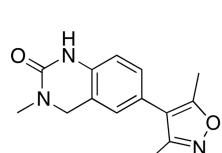
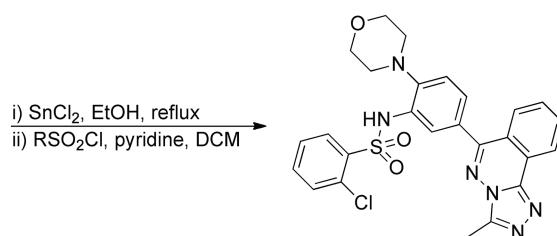
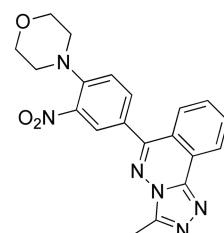
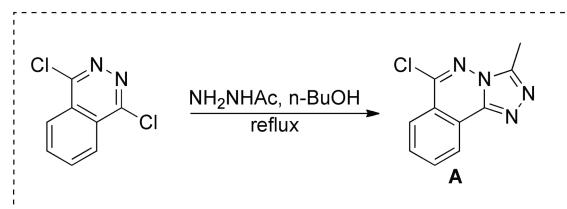
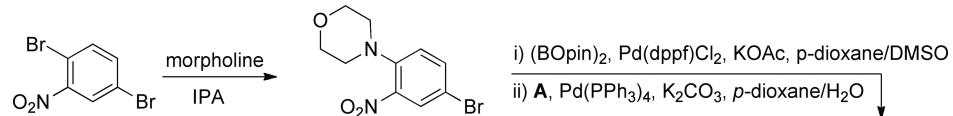
constructed as shown in **Scheme 4**.^{151,152} However, the substitution of carbon with a heteroatom abrogated the chirality and synthetic flexibility of tested inhibitors. One compound, BzT-7, had a K_d value of 0.64 μM toward BRD4-BrD1 in an ITC assay.⁶²

2.4.3. 3-Amino-triazolobenzodiazepine. While conducting the small-molecule screen that would eventually yield the compound I-BET, researchers came across a 3-amino-triazolobenzodiazepine analog GW841819X (**Figure 9**) that is a potent inducer of ApoA1 reporter genes. GW841819X showed an EC₅₀ of 440 nM.^{46,153} This compound displays K_d values of ~50 nM toward various constructs of BRD2 and K_i values between 15 and 40 nM toward tandem BET bromodomains (except BRDT) in a FRET assay. Molecular interaction analysis based on the X-ray crystal structure of the BRD4/GW841819X complex shows that the 1,4-diazepine moiety is perfectly superimposable with the comparable moiety in both I-BET and MS417. The difference lies in the orientation of the side chain of GW841819X, as it possesses a phenyl ring that flips up and points to the solvent-accessible surface, comparable to the way JQ1 binds (**Figure 11**). The carbamate moiety of this compound makes fewer interactions with the pocket, possibly contributing to the compound's overall weaker binding affinity toward BET bromodomains. Further chemical modifications of this compound eliminated its GABA activity. Additionally, GW841819X possesses great anti-inflammatory properties and inhibited the production of IL-6 with an IC₅₀ value of 160 nM in LPS-induced peripheral blood mononuclear cells (PBMC).^{46,153} This compound blocks the interactions between BRD3 and acetylated GATA1.¹⁵⁴ GW841819X and its derivatives underwent quick degradation at low pH, undermining its clinical potential due to poor bioavailability.

2.4.4. Thienoisoxazoloazepine. The design of the thienoisoxazoloazepine compound 3 (**Figure 9**) originated from a fragment uncovered from a screen. In spite of the low affinity of the isoxazole fragment, an X-ray crystal structure in complex with BRD4-BrD1 suggested it could serve as an isostere of the triazole, making it a perfect alternative to endogenous acetyl-lysine. Thienoisoxazoloazepines replace the

triazole ring with an isoxazole ring and they, as their name indicates, can be viewed as an amalgam of thienoazepine and isoxazole (see **section 2.5** below) rings. Thienoisoxazoloazepines can be synthesized via a complicated multistep route (**Scheme 5**). Crystallographic data from the complex of BRD4-BrD1 and compound 3, the most active compound with this scaffold, shows the retention of the hydrogen bond between Asn140 and the isoxazole moiety. Of note, both the oxygen and the nitrogen atoms are involved in this hydrogen bond, which could possibly increase the bond strength. The water-associated hydrogen-bond network seen in other bromodomain–ligand complexes is also preserved (**Figure 10D**). Compound 3 had an IC₅₀ value of 26 nM against BRD4-BrD1 in an AlphaScreen assay. It suppressed MYC expression in Raji cells in a MOA (Mechanism of Action) cell assay with an IC₅₀ value of 140 nM. More importantly, compound 3 showed an excellent pharmacokinetic (PK) profile in a canine model. At the dose of 100 mg/kg, compound 3 suppressed MYC expression for 8 h.¹⁵⁵ Researchers further extended the scope of their study by making a large number of benzoisoxazoloazepines (BZIAs, analogs of thienoisoxazoloazepines), which had GI₅₀ values of 5–300 nM toward a panel of cancer cell lines. Additionally, these BZIAs were effective in controlling tumor growth in an MV4-11 xenograft mouse model.¹⁵⁶

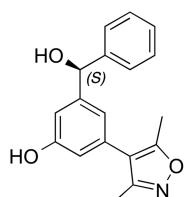
2.4.5. Triazolophthalazin. Inspired by the molecules disclosed in recent patents,^{157,158} researchers gained interest in using triazolophthalazines as replacements for TTDs. In a departure from the previously detailed TTD analogs, triazolophthalazines do not utilize a seven-membered ring as their core. The notion was this alteration in geometry could make the inhibitors more selective toward non-BET bromodomains because their acetyl-lysine binding pockets are smaller (**Figure 4B–E**). A collection of commercially available triazolophthalazines was screened against 17 bromodomains from different branches of the phylogenetic tree by a differential scanning fluorimetry (DSF) assay. Indeed, the results suggested these compounds had higher potencies against BRD4-BrD1, BRD9, CECR2, CREBBP, and TAF1L, with the highest against BRD9 and CREBBP.¹⁵⁹ Endeavors were made to improve the selectivity of these compounds for specific bromodomains, but

Scheme 6. Synthesis of Triazolophthalazine 51¹⁵⁹

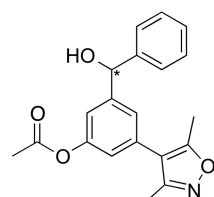
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4d

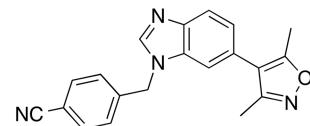
6a



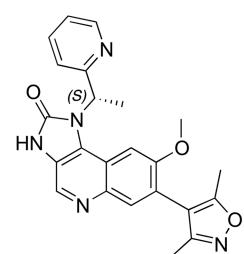
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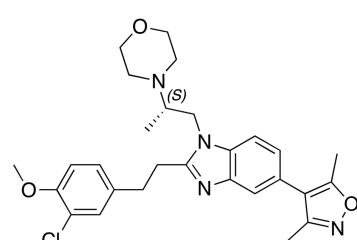
12



28a



I-BET151



59 (CBP30)

Figure 12. Chemical structures of bromodomain inhibitors based on an isoxazole scaffold.

the resulting compounds either lost affinity or remained promiscuous. The best compound in this series (compound 51, Figure 9) had IC₅₀ values of 200 nM for BRD4-BrD1, BRD9, and CREBBP. There is no evidence that such polypharmacology would be beneficial in any disease model investigated to date. X-ray crystal structures of compound 51 in complex with BRD4-BrD1 indicate the retention of the

hydrogen bonds between the triazole ring and the conserved asparagine. The phthalazine ring is stabilized by hydrophobic interactions with Leu94 and Ile146 in the binding pocket. The sulfonamide nitrogen, possibly deprotonated, is in range to hydrogen bond with Trp81, an interaction not demonstrated in prior BET/ligand complexes. There is no evidence that the morpholine ring engaged in binding, as it was pointing toward

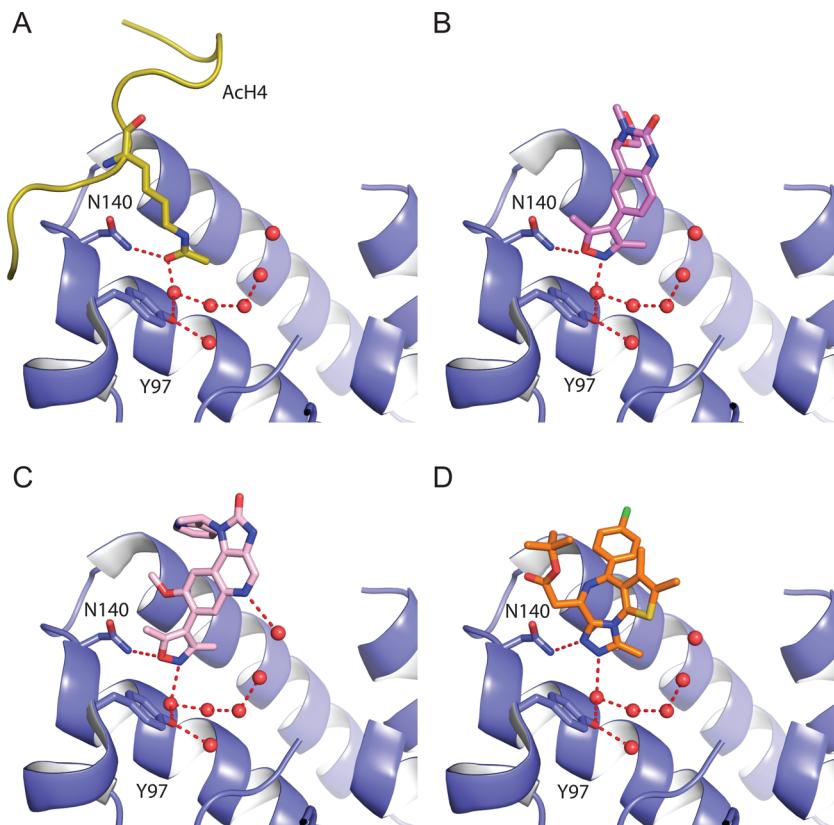


Figure 13. Binding mode of I-BET151 in comparison with other known inhibitors. (A) Acetylated H4 peptide bound to the acetyl-lysine binding pocket of BRD4-BrD1 (3UVW). (B) Compound 2 contains a dimethylisoxazole moiety, which serves as an acetyl-lysine mimetic. It maintains the same hydrogen bonds as the peptide: one with Asn140 and another with a conserved water molecule. The chiral carbon on the dihydroquinazolinone ring directs the ethylene glycol moiety to the hydrophobic pocket occupied by a chlorophenyl moiety in JQ1 (3SVF). (C) I-BET151 binds to BRD4 in a similar pose as compound 2. However, the different alignment of the rings in the scaffold allows the nitrogen atom on the quinoline ring to engage in an additional hydrogen bond with another conserved water molecule. The chiral carbon on the imidazole ring delivers the pyridine ring to the hydrophobic pocket (3ZYU). By comparison, the binding mode of I-BET151 is markedly different from that of (D) JQ1 (3MXF). For visual clarity, the ZA loops are hidden from these graphical representations.

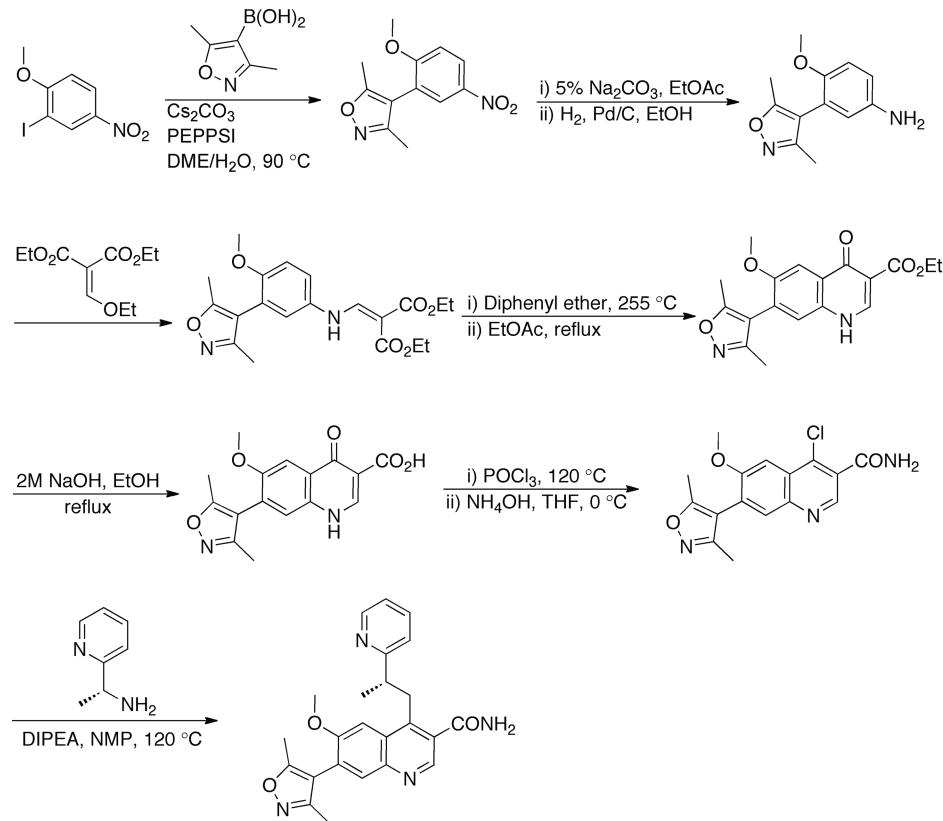
the solvent-accessible surface (Figure 11). In a FRAP assay, triazolophthalazines displaced CREBBP in U2OS cells.¹⁵⁹ Bromosporine (Figure 9) was reported to be highly potent toward CECR2 and had a better selectivity profile than compound S1 in a thermal shift assay.^{160,161} The modular synthesis of compound S1 is outlined in Scheme 6.

2.4.6. Benzooxazepine. A compound with a novel chemotype, the benzooxazepine CBP112, was recently disclosed as a specific inhibitor of CREBBP and the related protein EP300.¹⁶² The introduction of an oxygen atom into the 7-membered ring made it incapable of further functionalization at the 4 position, most likely altering the conformation of the molecule. The X-ray crystal structure of CBP112 in complex with CREBBP has yet to be reported. CBP112 (Figure 9) had K_d values of 0.151 and 0.625 μM toward CREBBP and EP300, respectively. When tested across the entire human bromodomain panel, CBP112 showed weak activity toward BET bromodomains. This compound showed no significant cytotoxicity at up to 50 μM in U2OS cells.¹⁶² CBP112 disrupts the acetylation of H3K56ac by CREBBP.¹⁶³

2.5. Isoxazole

The isoxazole is another major chemotype among bromodomain-targeting small molecules. More specifically, the 4-phenyl-3,5-dimethylisoxazole was discovered as one of the hits from a large compound library screen and found to be an effective

inhibitor.^{164–167} One of these early hits, a dihydroquinazolinone-containing 3,5-dimethylisoxazole derivative, compound 1 (Figure 12), had IC₅₀ values of 4.8 and 3.4 μM toward BRD4-BrD1 and CREBBP, respectively, in an AlphaScreen peptide displacement assay.^{164,165} A similar compound, compound 4c, has shown potency toward BRD2-BrD1. The X-ray crystal structure of compound 2 (Figure 13B), a derivative of compound 1, shows compound 2 binds BRD4-BrD1 in a very similar mode as JQ1, confirming this compound as an effective mimic of acetyl-lysine. The dihydroquinazolinone moiety replaces the thiophene ring in JQ1, and the pendant alkyl chain binds in the same area as the chlorophenyl moiety in JQ1. Subsequently, a sulfonamide derivative of isoxazole (6a, Figure 12) was developed and shown to have an IC₅₀ value of 500 nM toward BET bromodomains in a FP assay and a TR-FRAP assay.¹⁶⁵ Further lead optimization yielded the phenol and acetate derivatives, 8 and 12 (Figure 12). The S-enantiomer of compound 8 had IC₅₀ values of 382 nM toward BRD4-BrD1 and 771 nM toward CREBBP. Compound 12 was more selective toward BRD4-BrD1, as evidenced by its IC₅₀ value of 371 nM in an AlphaScreen assay. Overlay of the crystal structure of the compound 8/BrD4-BrD1 complex and JQ1 shows that the phenyl ring of compound 8 interacts with the WPF shelf of the bromodomain in an almost identical manner as the chlorophenyl moiety of JQ1. Compound 8 was tested against a variety of hematological malignancies and particularly

Scheme 7. Synthesis of Isoxazole I-BET151⁴⁷

effective against AML cell line MV4-11.¹⁶⁶ A structure-guided design approach was then used to produce an even more potent isoxazole, compound 28a. Compound 28a (Figure 12) has an IC₅₀ value of 180 nM toward BRD4-BrD1 and is at least 100-fold selective for this domain over CREBBP.¹⁶⁷ The isoxazole scaffold was further derivatized into naphthyridine isomers, with the 1,5-isomer bearing favorable inhibitory activities against BET proteins.¹⁶⁸ Among the many isoxazole-based compounds that have been tested, however, I-BET151 (Figure 12) is the most effective toward BET bromodomains.

Researchers developed a novel small-molecule inhibitor named I-BET151 to inhibit the BET bromodomains as a strategy to disrupt the formation of the super elongation complex (SEC) and polymerase associated factor complex (PAFc).^{47,169} The organic synthesis of I-BET151 has been outlined in Scheme 7. As was touched upon earlier in this review, BET bromodomains play a critical role in the regulation of transcriptional elongation. Additionally, the control of the SEC is of particular importance, making the targeting of the BET bromodomains involved in its formation of a plausible strategy to control the progression of MLL-fusion-driven cancer. I-BET151 abrogated the binding of BRD3/4, PAFc, and key components of the SEC from chromatin, thereby stopping the transcription of key genes such as *BCL-2*, *C-MYC*, and *CDK6*. I-BET151 is a modified dimethylisoxazole with excellent selectivity and target potency—in a fluorescent thermal shift assay, only BET bromodomains showed significant temperature shifts ($T_m > 7$ °C), and a proteomic profiling study showed similar results.⁴⁷ I-BET151 displayed IC₅₀ values from 250 to 790 nM in a FP ligand displacement assay and K_d values of 20–100 nM in surface plasmon resonance (SPR) measurement against BRD2/BRD3/BRD4.

In a mesoscale discovery of human IL-6 assay, I-BET151 had IC₅₀ values of 160 and 1260 nM in human PBMC cells and whole blood, respectively, suppressing the production of LPS-induced cytokine IL-6. These results demonstrate that I-BET151 is a more potent therapeutic agent toward BET bromodomains than I-BET. In addition, I-BET151 displays much better pharmacokinetic properties, such as bioavailability and half-life, than both I-BET and JQ1.⁴⁷

A high-resolution X-ray crystal structure of BRD4-BrD1/I-BET151⁴⁷ shows that the isoxazole moiety is the acetyl-lysine mimicking portion of the ligand, binding deep within the pocket in a similar fashion as the natural ligand, the acetylated H4 peptide. The oxygen of the isoxazole forms a hydrogen bond with the conserved Asn140, while the nitrogen hydrogen bonds with a conserved water molecule (Figure 13A and 13C). However, other aspects of the binding mode of I-BET151 are vastly different than that of JQ1 (Figure 13D) or MS417. The core of I-BET151 sits slightly differently in the binding pocket than the diazepine core of the other two compounds. Unlike the triazolodiazepines, there is a rotatable bond on the quinoline that determines the orientation of the isoxazole moiety, potentially leading to a thermodynamic penalty and an alteration in affinity. I-BET151 also interacts with the WPF shelf through a pyridine ring in its slightly different orientation, which is dictated by the stereochemistry on the neighboring carbon. I-BET151 was tested against a spectrum of leukemia cell lines and showed IC₅₀ values of 15–192 nM for the cell lines driven by MLL-fusion.⁴⁷ As a result of its activity, I-BET151 induced apoptosis and G0/G1 cell-cycle arrest in cell lines that harbored MLL-fusion. I-BET151 selectively abrogated the recruitment of BRD3/4 and ultimately resulted in inadequate recruitment of Pol II. I-BET151 was efficacious in

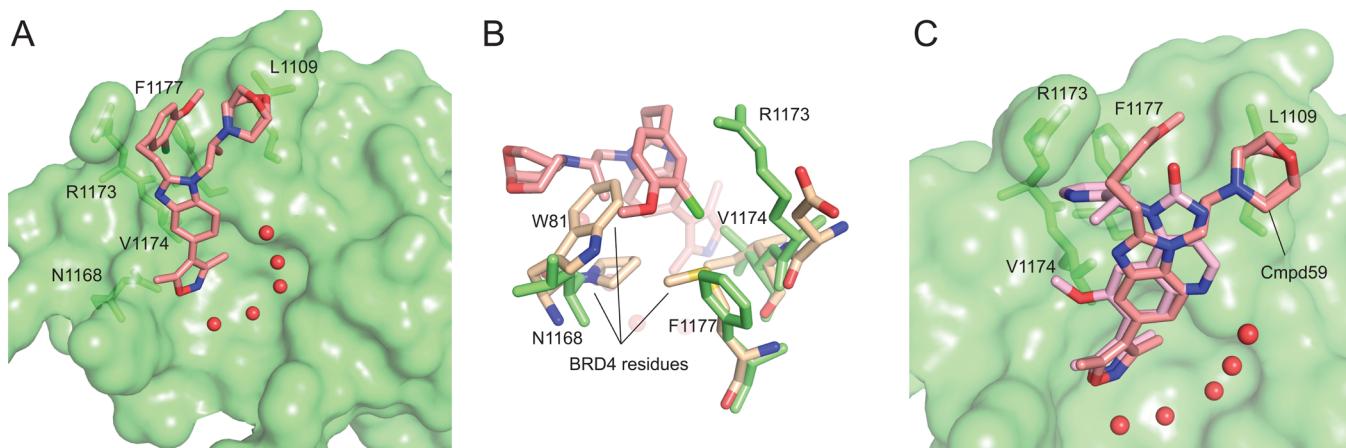
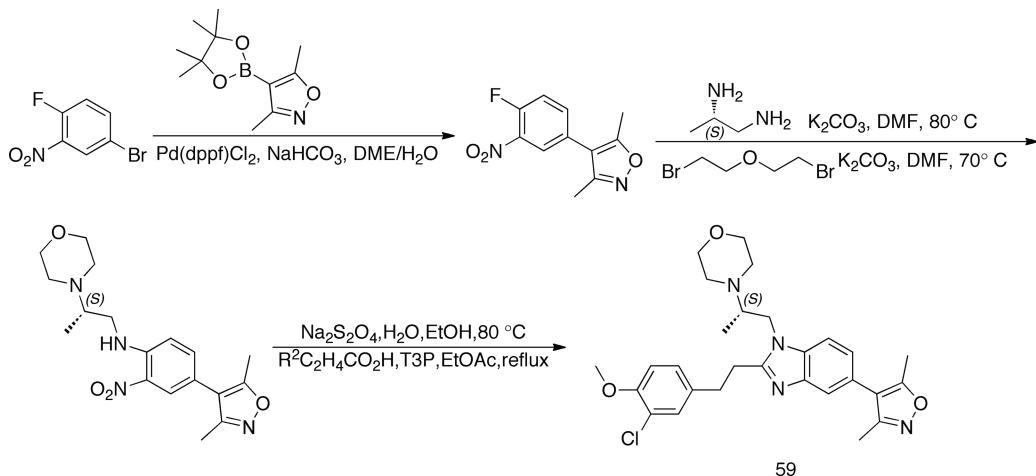
Scheme 8. Synthesis of CBP-Specific Compound 59 (CBP30)¹⁷⁰

Figure 14. Structural basis for the selective binding of isoxazole compound 59 (also known as CBP30) to CREBBP. (A) Compound 59 binds tightly to the CREBBP BrD (4NR7). Key residues are presented as sticks inside of the surface representation. Red spheres represent the conserved water molecules at the base of the binding pocket. For visual clarity, residues from the ZA loop are hidden. (B) Structure-based alignment of the CREBBP BrD and BRD4-BrD1 (3ZYU) reveals the structural basis for the selectivity of compound 59. Arg1173 forms a cation–π interaction with the substituted phenyl ring; the corresponding residue in BRD4-BrD1 is Asp145, too small to make effective contact with the ligand. Leu1109, together with Val1174 and Phe1177, form two small pockets for the Cl and the methoxy group on the phenyl ring of 59; the residue in BRD4-BrD1 that corresponds to Leu1109 is Trp81, which is far too bulky to accommodate the phenyl ring. (C) Overlay of compound 59 and I-BET151. For clarity, only the surface of CREBBP is shown. The pyridine ring of I-BET151 is in partial contact with Arg1173. One distinct feature of compound 59 is its extension on N-1 through an alkylated morpholine. The limited resolution of the structure has prevented the exact conformation of morpholine group from being determined.

controlling the progression of MLL-infused leukemia in two murine models.

Interestingly, a few variants have been developed from this isoxazole template for different targets. Researchers recently engineered this usually BET-specific scaffold to reverse its selectivity and develop ligands that were highly potent toward CREBBP.¹⁷⁰ Guided by a series of X-ray crystal structures of N-1- and C-2-modified 5-isoxazolyl-benzimidazoles in complex with CREBBP, researchers were able to develop a stereospecific and highly potent ligand through a comprehensive SAR campaign. The synthesis of lead compound 59 (also known as CBP30) is outlined in Scheme 8. It has K_d values of 21 nM toward CREBBP, 850 nM toward BRD4-BrD1, and 5200 nM toward BRD4-BrD2, displaying a 40–250-fold of selectivity between CREBBP and BRD4 bromodomains. These results were consistent in ITC, AlphaScreen, and DSF assays. Compound 59 was also potent toward the structurally related p300 bromodomain.

The selectivity for the CREBBP bromodomain over the two bromodomains of BRD4 can be explained by the X-ray crystal structure of the CREBBP/compound 59 complex. The pendant substituted aryl ring forms an apparent cation–π interaction with Arg1173. An induced pocket formed by the movement of the side chain of Arg1173 accommodates the aromatic ring. The corresponding residue in BRD4-BrD1 is Asp145, which is much smaller in size, preventing an interaction from taking place. The chlorine atom on the aryl ring of compound 59 takes part in hydrophobic interactions with Val1174 and Phe1177 (Figure 14A). The chiral methyl group sits beneath the aromatic ring, possibly fixing the ring into position. This induced fit mechanism could be the key for selective CREBBP inhibition. In the case of BRD4-BrD1, the methyl group is most likely to clash with Trp81, as this residue is far bulkier than its counterpart Leu1109 in CREBBP. The interaction with this specific residue also explains the difference in selectivity between compound 59 and its enantiomer 58 (Figure 14B).

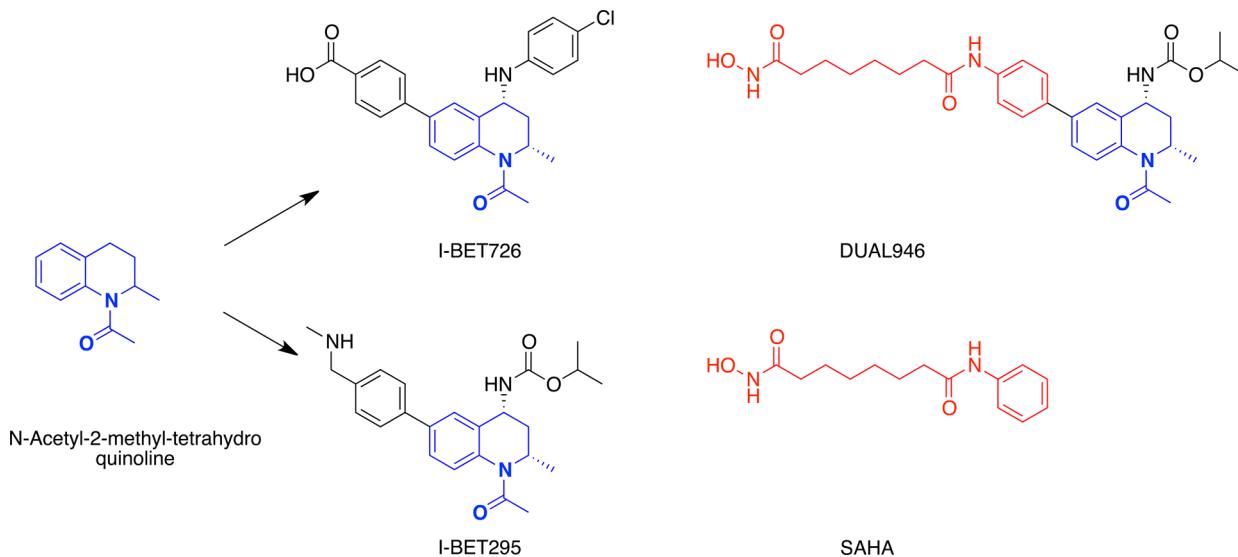


Figure 15. Chemical structures of tetrahydroquinoline-based bromodomain inhibitors.

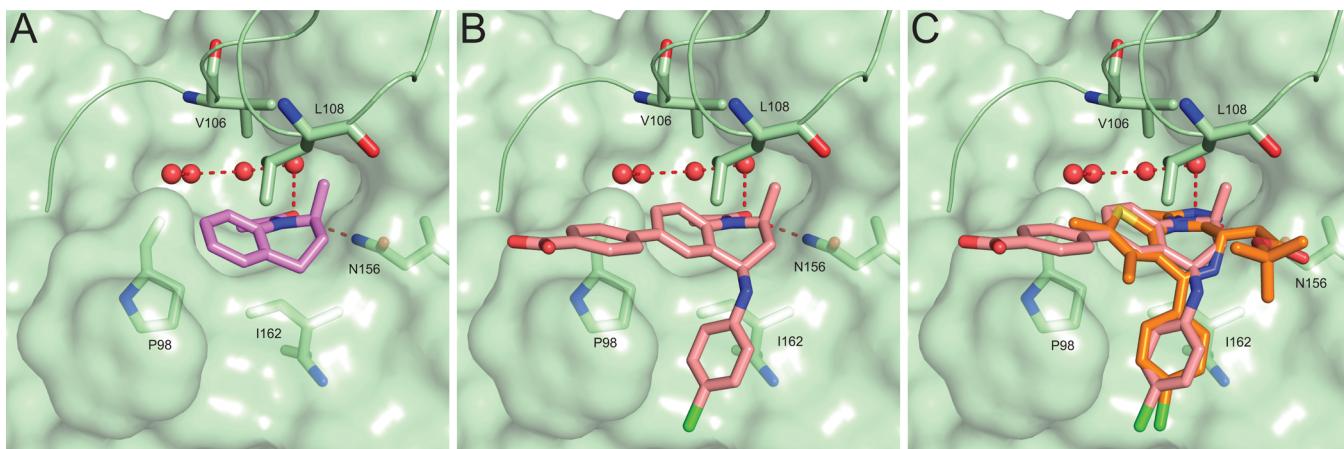


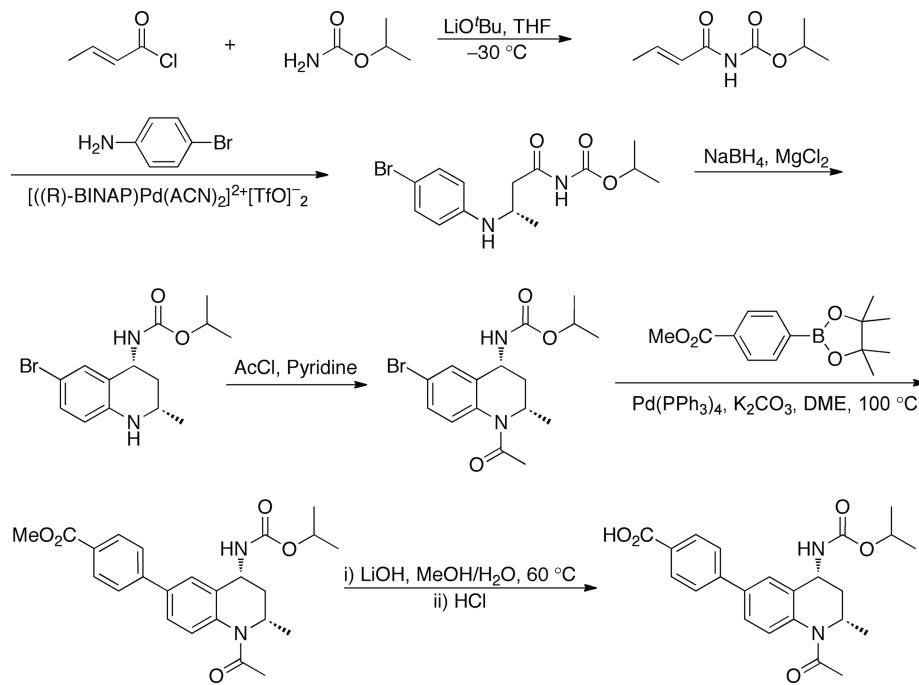
Figure 16. Crystal structures of tetrahydroquinolines (THQs) in complex with bromodomains. (A) The first THQ fragment was identified from chemical screening (4A9H), shown in complex with human BRD2-BrD1. The *N*-acetyl group mimics acetyl-lysine and fits snugly in the pocket defined by hydrophobic residues such as Pro98, Val106, Leu108, and Ile108. The carbonyl of the acetyl-lysine mimic forms hydrogen bonds with the conserved Asn156 and a bridged water molecule. (B) I-BET726 adopts the same envelope confirmation as in the THQ fragment (4BJX), in complex with human BRD4-BrD1. Moreover, this ligand extends into the WPF shelf due to chemical modifications designed to make the ligand resemble. (C) JQ1 (in orange). For simplicity, the ZA loop is shown as a ribbon (instead of as a part of the surface) in each panel.

Superimposition of compound 59 and I-BET151 allows the binding modes of the two compounds to be compared and contrasted. First, the phenyl ring on C-2 of compound 59 inserts into the same pocket as the pyridine ring on the chiral carbon of I-BET151, though the cation–π interaction of compound 59 is more efficient. These compounds are similar enough that one would expect I-BET151 to have decent activity toward CREBBP bromodomain; however, this conflicts with the thermal shift assay result ($\Delta T_m < 2$ °C) published previously.⁴⁷ Second, the morpholine ring on N-1 of compound 59 protrudes into an area I-BET151 never reaches, colliding with Trp81 of BRD4-BrD1, explaining its low affinity toward BRD4-BrD1 (Figure 14C). Promisingly, compound 59 inhibited p53 activity with an IC_{50} value of 1.5 μ M, and it displaced acetylated chromatin in HeLa cells in a FRAP assay. However, compound 59 displayed a minor cytotoxicity ($CC_{50} = 80$ μ M) related to BRD4 and a fast clearance, creating a potential hurdle for in vivo testing. Furthermore, when screened against other classical biological targets, compound

59 demonstrated activities toward adrenergic receptors (α 2C (110 nM), α 2A (570 nM)), phosphodiesterase-5 (150 nM), and PAF (540 nM).¹⁷⁰

2.6. Tetrahydroquinoline

Tetrahydroquinoline (THQ) was identified as one of the few lead fragments that preferably targeted BRD2-BrD1 in biochemical screening (Figure 15).^{164,171} X-ray crystallography was then used to characterize the binding mode of selective fragment sets. The molecular mimicry of acetyl-lysine by THQ is illustrated by the crystal structure of BRD2-BrD1/THQ complex. The amide mimics acetyl-lysine, interacting with the conserved Asn156 and bridging water molecules in a network of hydrogen bonds. The ring system sits well in the hydrophobic portion of the bromodomain defined by Pro98, Val106, Leu108, and Ile162 (Figure 16A). The methyl group on the 2 position of the ring is located in a small hydrophobic pocket. It is noteworthy to point out that once again there is a stereochemical consideration for this bromodomain inhibitor,

Scheme 9. Synthesis of I-BET726¹⁷²

as only the *S*-enantiomer was active, and the cocrystal structure, suggesting that the *R*-enantiomer would clash with the protein. On the basis of its promising inhibitory activity and favorable ligand efficiency, the THQ fragment was selected as a candidate for extensive further chemical modifications.^{172–174} A compound developed from these modifications, I-BET726 (Figure 15), was recently reported to be highly potent and selective toward BET bromodomains. I-BET726 had IC₅₀ values of 22–41 nM against BET bromodomains in a TR-FRET assay, showing at least 1000-fold more potency against these bromodomains than its interaction with the bromodomain of CREBBP. In a MYC-driven hematological cancer model, I-BET726 exhibited a GI₅₀ value of 75 nM against a panel of neuroblastoma cell lines, an activity five times more potent than that of I-BET151.¹⁷⁵ Additionally, X-ray crystallography showed that the binding mode of I-BET726 was very similar to that of JQ1. The six-membered ring adopts a fascinating envelope conformation, perfectly superimposable with the previously described TTD framework. The benzoic acid functionality extends further into the WPF shelf, making this compound distinct from JQ1 (Figure 16 B and 16C). I-BET726 directly suppressed *N*-Myc and *BCL2* and regulated the gene expressions in apoptosis and Myc signaling.¹⁷⁵ I-BET726 was effective in treating neuroblastoma,¹⁷⁵ tumor growth, and myeloma¹⁷⁶ in xenograft models, setting the stage for its use as an antineuroblastoma and antimyeloma agent. I-BET726 can be accessed following the route described in Scheme 9.¹⁷²

More recently, researchers developed DUAL946 (Figure 15), a dual HDAC/BET inhibitor that incorporated elements of both THQ and SAHA.¹⁷⁷ The drive to develop such a ligand came from the observation that HDAC inhibitors and BET inhibitors have common phenotypes such as apoptosis and cell-cycle arrest, leading to the hypothesis that such a dual inhibitor may be effective.^{45,178,179} The organic synthesis of DUAL946 is outlined in Scheme 5. DUAL946 had an IC₅₀ value of 50 nM against BRD4-BrD1 in a TR-FRET assay. Apparent dissociation constants (K_d^{app}) were determined in a chemo-proteomic

competitive binding assay. DUAL946 had K_d^{app} values of 32–112 nM toward BET and 207–955 nM toward HDACs. However, the antiproliferative activities of DUAL946 in NMC and AML cells were not any better than its parent compound I-BET295 (Figure 15). When combining JQ1 and panobinostat, no significant synergistic effects were observed.

2.7. Dihydroquinazolinone

Dihydroquinazolinone (DHQ) was another fragment identified in the initial biological screening for inhibitors of BRD2-BrD1 (Figure 17).¹⁷¹ Unlike other fragment hits, this scaffold bound

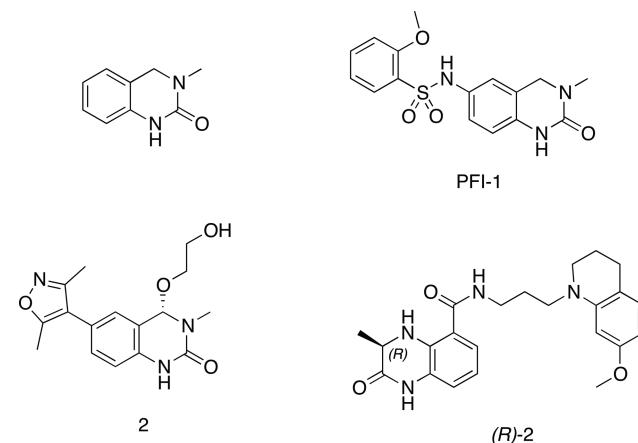


Figure 17. Chemical structures of bromodomain inhibitors based on a dihydroquinazolinone scaffold.

in the acetyl-lysine binding pocket without presenting an acetyl group or acetyl-group mimic. An X-ray crystal structure showed that the carbonyl of the DHQ group was the hydrogen-bond acceptor that paired with the conserved Asn156. In terms of the interactions of the specific atoms of DHQ, N-1 of the ring system is also a donor and in range for hydrogen bonding with the side chain of Asn156. The introduction of an extra N (as

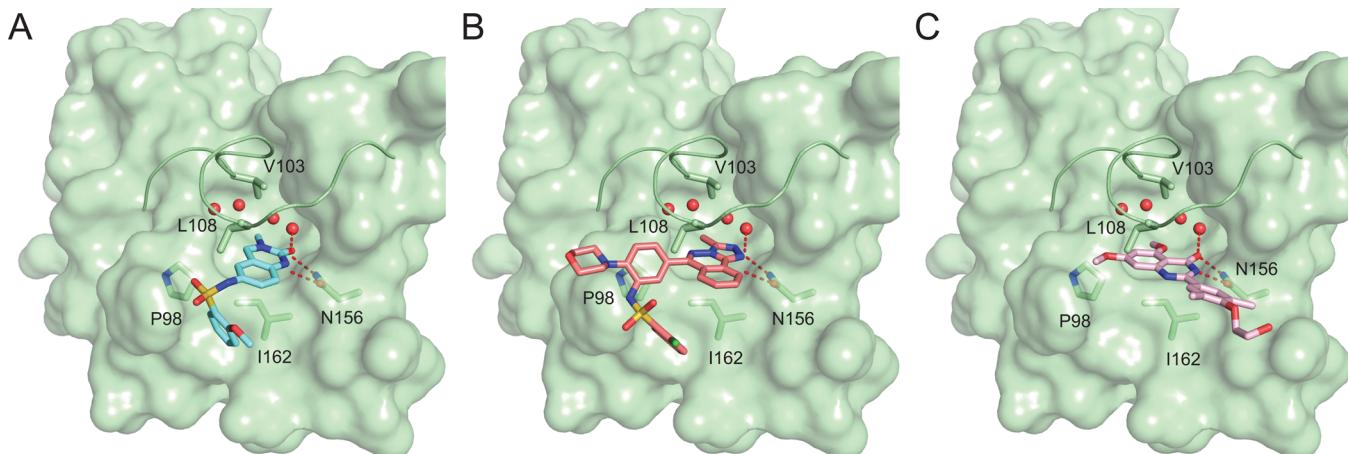
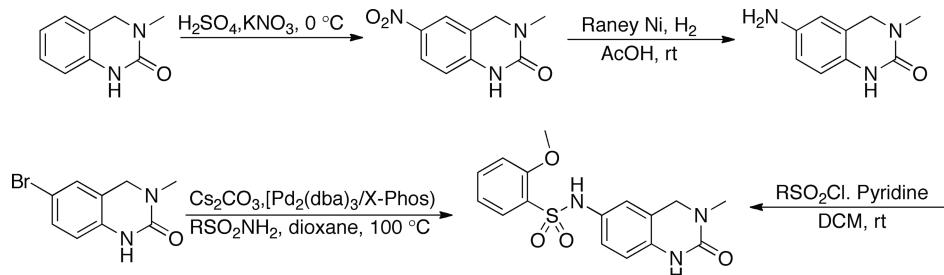


Figure 18. Crystal structure of PFI-1 in complex with BRD4-BrD1 (4E96). (A) The dihydroquinazolinone moiety of PFI-1 mimics acetyl-lysine. One unique feature of this chemotype is that the carbonyl and the neighboring --NH form a canonical hydrogen-bond pair with the conserved Asn156 residue. A sulfonamide linker was employed to further position an aromatic ring in the hydrophobic pocket. The binding mode of PFI-1 is very close to that of (B) triazolophthalazine 51 (4NQM). For clarity, BRD2 is represented as a protein surface. (C) The quinazolinone RVX-208 (4MR6), as shown when bound to BRD2-BrD2, leaves the ZA channel unfilled. Its phenyl ring does, however, extend into the BC channel.

Scheme 10. Synthesis of PFI-1 and Its Analogs¹⁸⁰



compared to the THQ group) provides additional binding opportunities with other parts of the binding pocket, and substitutions at the C-6 position led to hydrophobic interaction with WPF shelf.^{164,180,181}

Structure-based design efforts led to PFI-1, an improved inhibitor based on the DHQ scaffold. Aside from the ring structure and the cyclic urea moiety contained in the DHQ fragment, a sulfonamide linker was employed on the other end of the molecule to introduce an aromatic ring that would be presented deep in the hydrophobic pocket^{180,182} (Figure 18A). The planar core structure and sulfonamide linker feature of PFI-1 are shared with triazolophthalazine 51, which bound BET bromodomains in a similar pose (Figure 18B). The improved PFI-1 displayed an IC_{50} value of 180 nM toward BRD4-BrD1 in an AlphaScreen assay.¹⁸⁰ Its weak binding to CREBBP ($K_d = 49 \mu\text{M}$ in an SPR measurement) confirms that it is a BET-specific inhibitor. Beyond its inhibitory data, PFI-1 inhibited the production of IL-6 in PBMCs with an EC_{50} value of $1.9 \mu\text{M}$ in a cell-based assay and displays a 1 h half-life when administered intravenously and a 2 h half-life when administered subcutaneously. Furthermore, PFI-1 downregulates Aurora B kinase significantly, offering an alternative approach to inhibit this well-known oncological target.¹⁸² The organic synthesis of PFI-1 is outlined in Scheme 10.

2.8. Quinazolinone

Quinazolinone and its derivatives first garnered attention due to their potential usage against cardiovascular diseases.¹⁸³ RVX-208 (Figure 19) increases the level of ApoA1 and high-density lipoprotein (HDL)¹⁸⁴ in cells and is considered a promising

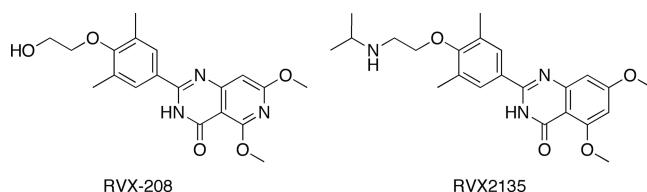


Figure 19. Chemical structure of the quinazolinones RVX-208 and RVX2135.

agent in treating coronary heart disease. It was also shown that RVX-208 reduces the atherosclerosis symptoms in hyperlipidemic ApoE-deficient mice.¹⁸⁵ Recently, RVX-208 was reported to be a BET bromodomain inhibitor, with selectivity toward the second bromodomains of the members of the family.^{51,186} RVX-208 has K_d values of 4.1 and $0.2 \mu\text{M}$ toward BrD1 and BrD2 of BRD3, respectively, determined by ITC measurement. This selectivity (approximately 20-fold) is most pronounced in BRD3 of all the BET family members. Removal of the ethylene glycol moiety from the benzene ring led to a complete loss of selectivity between BrD1 and BrD2 of BRD3, indicated by ITC measurement. The cocrystal structure of RVX-208 and BRD4-BrD1 confirmed that the amide moiety of the quinazolinone serves as the compound's acetyl-lysine mimetic, as it hydrogen bonds with the conserved Asn140 and takes part in water-mediated hydrogen bonding with both Tyr97 and Gln85 (Figure 20A). The ethylene glycol moiety protrudes from the acetyl-lysine pocket, weakly interacting with a few surface residues. In contrast to the interactions found in

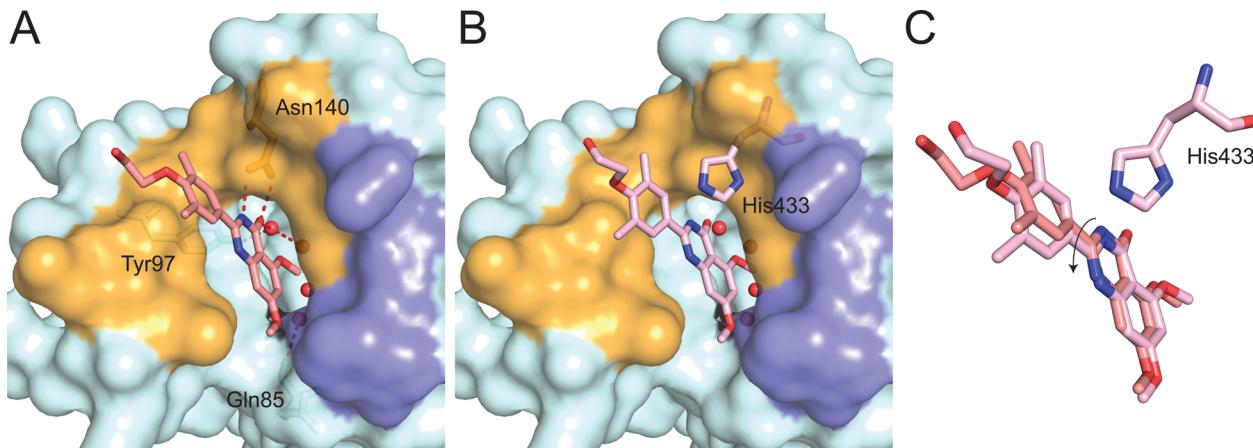
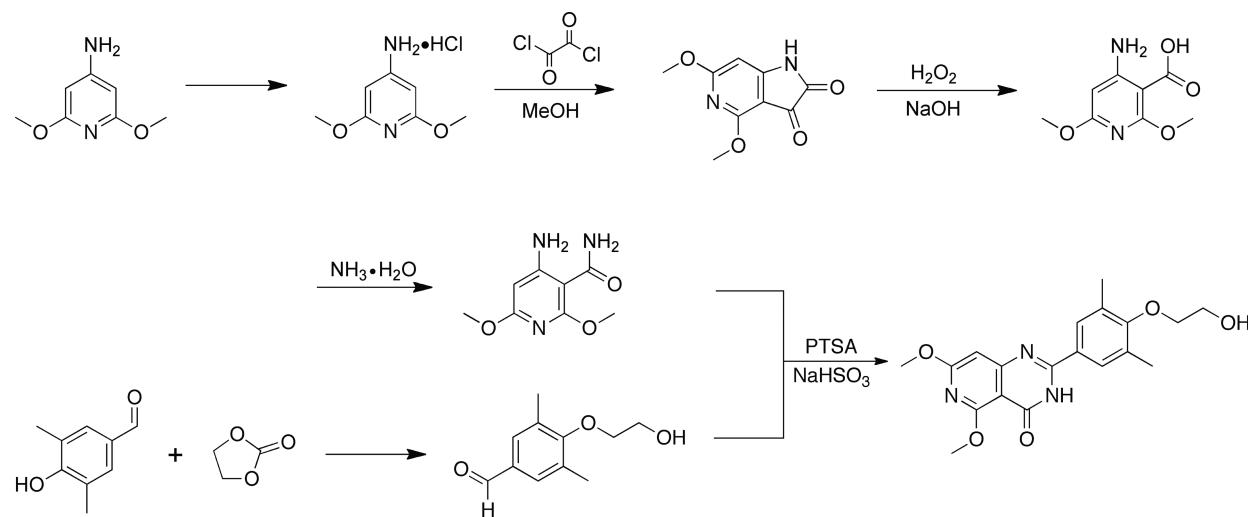


Figure 20. Crystal structures of RVX-208 in complex with BET bromodomains. (A) RVX-208 forms hydrogen bonds with the conserved Asn140 and a water molecule in BRD4-BrD1 (4MR4), but it does not interact with the WPF shelf, decreasing its binding affinity for this specific bromodomain. (B) In the case of the interaction between RVX-208 and BRD2-BrD2 (4MR6), His433 flips outward and forms a π - π interaction with the phenyl ring on the quinazolinone. In the stick representation of the two binding modes in C, it can be seen that the phenyl ring rotates approximately 25° to optimize the alignment of the two aromatic systems. In A and B, the WPF shelf is colored blue and the ZA loop is colored orange.

Scheme 11. Synthesis of RVX-208¹⁸³



many other bromodomain inhibitors, the WPF shelf is not occupied by RVX-208. Although RVX-208 largely retains its binding mode in BrD2 of BRD4, His433—a unique residue for BrD2—flips into the acetyl-lysine pocket, engaging in an additional interaction with the phenyl ring. The phenyl ring rotates approximately 25° in order to establish an efficient π - π interaction, providing an explanation for the preference for BrD2 over BrD1 (Figure 20B and 20C). RVX-208 was found to have difficulty displacing BRD3 from acetylated chromatin (likely due to its low affinity for BrD1) in a FRAP assay in U2OS osteosarcoma cells.^{51,187} Investigation of transcriptional regulation of gene expression in liver carcinoma cells indicated that RVX-208 only played an insignificant role, reaffirming that BrD1 is required in this gene regulation process.^{57,119,141} Because its metabolism is well understood,¹⁸⁸ RVX-208 shows the potential for an expansion in its overall therapeutic impact. The synthesis of RVX-208 is depicted in Scheme 11. RVX2135 (Figure 19), an analog of RVX-208, inhibits proliferation and induces apoptosis in Myc-induced murine lymphoma.¹⁷⁹

2.9. Dihydroquinoxalinone

The dihydroquinoxalinone scaffold is derived from 3,4-dihydro-3-methyl-2(1H)-quinazolinone (DHQ, Figure 17),¹⁷¹ which carries moderate binding activity toward CREBBP. In an effort to improve its undesirable stability, researchers developed the dihydroquinoxalinone (R)-2 (Figure 17) as a CREBBP inhibitor. Interestingly, the molecule is stabilized by an intramolecular hydrogen bond, occurring between an amine hydrogen on the dihydroquinoxalinone and the carbonyl of the amide bond external to the ring. Compound (R)-2 had a K_d value of 0.39 μM toward CREBBP in an ITC experiment, showing a modest selectivity over BRD4-BrD1 ($K_d = 1.4 \mu\text{M}$). X-ray crystallography of (R)-2 bound to CREBBP led to the discovery of a key induced-fit pocket, stabilized by cation- π interaction between the tetrahydroquinoline moiety and Arg1173. In this case, Arg1173 flipped up (relative to its position in the Kac-bound crystal structure), making room for tetrahydroquinoline. The dihydroquinoxalinone establishes two hydrogen bonds with Asn1168 and a water-mediated hydrogen bond with Tyr1125, while its methyl group sits on the base of

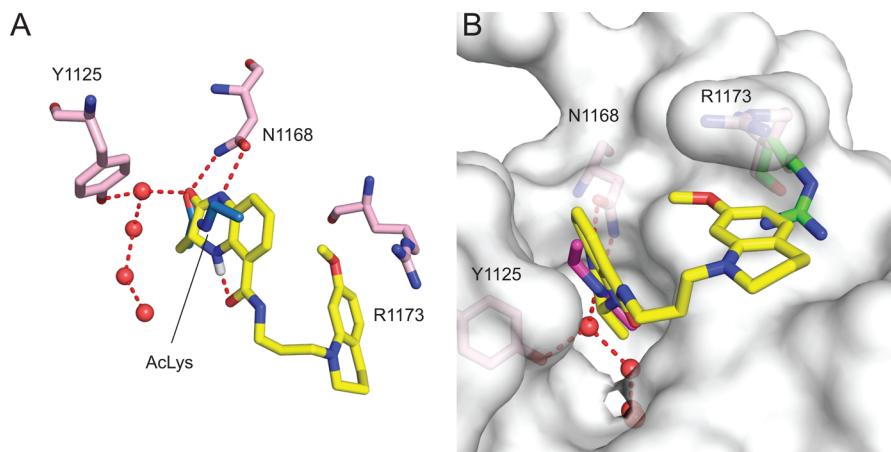


Figure 21. Comparison of the binding modes of acetylated lysine and (R)-2 to CREBBP BrD. (A) (R)-2 binds CREBBP BrD (4NYX) in a similar way as acetyllysine (3P1C) via its dihydroquinoxaline moiety, which forms hydrogen bonds with the conserved Asn1168 and a conserved water. (R)-2 also forms a cation–π interaction with Arg1173 via its tetrahydroquinoline moiety. (B) Surface representation shows that Arg1173 flipped up when (R)-2 bound to CREBBP BrD. This alteration made room to accommodate the tetrahydroquinoline moiety by fitting in an induced-fit pocket. Acetylated lysines and the corresponding Arg1173 are colored in slate.

the acetyl-lysine binding pocket. The intramolecular hydrogen bond and the interaction between the carbonyl oxygen and conserved water molecules dictate the position of the tetrahydroquinoline moiety, positioning it to fit into the induced pocket with the aid of a nearby methoxy group (Figure 21). In terms of its biological effect, (R)-2 displaced CREBBP from chromatin in U2OS cell in a FRAP assay.¹⁸⁹

2.10. Thiazolidinone

The discovery of the thiazolidinone scaffold as a bromodomain inhibitor stemmed from a combined approach of molecular docking and crystallography. A collection of fragments was screened against BRD4-BrD1 in silico using the program Glide,^{190,191} after which the top hits were then screened and validated by X-ray crystallography. 2-Thiazolidinone was chosen as a prominent fragment that could be pursued further in SAR studies. Structure-guided design led to an optimized compound 40a (Figure 22), which had an IC₅₀ value of 230 nM toward BRD4-BrD1 in a FP assay. The X-ray crystal structure of 40d (a derivative of 40a, Figure 22) in complex with BRD4-BrD1 indicated that the carbonyl in thiazolidinone forms a direct hydrogen bond with the conserved Asn140, while the

nitrogen atom binds to Asn140 with the aid of a bridged water molecule. The sulfonamide linker delivers the thiophene ring to the WPF shelf, while the amide linker positions the molecule's lipophilic substituent in the ZA channel outside of the acetyl-lysine pocket. 40d does not occupy the BC channel (Figure 23A). 40a was stable as shown in mice liver microsomes.¹⁹² Unfortunately, 40a showed limited activity (a GI₅₀ value of >50 μM) in an HT-29 cell line. More recently, this scaffold was further improved by structure-based optimization, yielding the lead compound 33b (Figure 22), which had an IC₅₀ value of 60 nM in a FP assay, consistent with the result from a thermal shift assay. 33b also showed a GI₅₀ of 141 nM and an antiproliferative effect against MV4-11 cancer cells. In terms of its specificity, this compound is highly specific toward BET bromodomains and showed little activity against EP300, BRG1, and ATAD2.¹⁹³

2.11. Acylpyrrole

Following a similar method that was used for the development of thiazolidinone, 4-acylpyrrole was identified via high-throughput virtual screening of a library containing approximately 7 million compounds. Instead of fragments, researchers screened drug-like molecules taken from the ChEMBL^{194,195} and ZINC¹⁹⁶ databases. This search yielded a 4-acylpyrrole derivative named XD-14 (Figure 22), which stood out as a promising hit toward BRD4-BrD1.¹⁹⁷ Indeed, XD14 was validated as an inhibitor of BRD4-BrD1, as an ITC measurement showed it has a K_d of 237 nM for the domain. Overlay of XD14 and acetylated H4 peptide in complex with BRD4-BrD1 revealed that the 4-acyl and 5-methyl groups of this compound mimicked acetyl-lysine. The pyrrole ring buries deep inside of the pocket, with N1 forming a hydrogen bond with the carbonyl of Pro82. This positioning places the 2-carbonyl in range for hydrogen bonding with a conserved water molecule. The sulfonamide moiety enters the ZA canal, in close range to Trp81, forming a CH–π interaction. A unique feature of the complex structure is the orthogonal interaction between Trp81 and the phenyl ring of XD14, which also interacts with Leu92 (Figure 23B). According to results from a BROMOscan assay, XD14 is a potent inhibitor of BRD2, BRD3, and BRD4 but not BRDT. Unfortunately, XD14 lacks specificity for the BET bromodomains, as it also has high affinities for the CREBBP

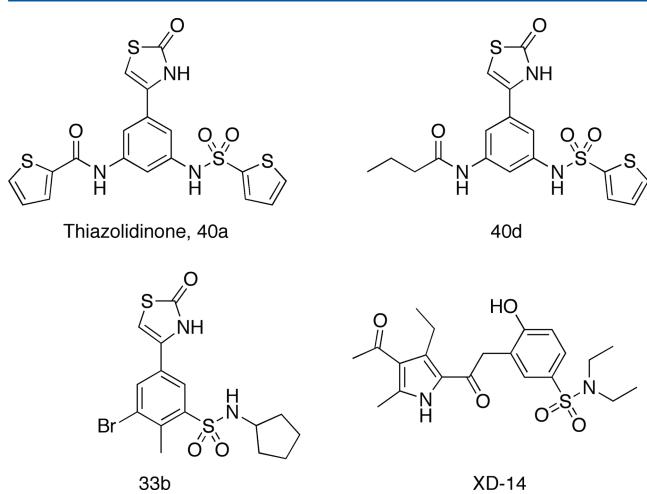


Figure 22. Chemical structures of thiazolidinone and acylpyrroles.

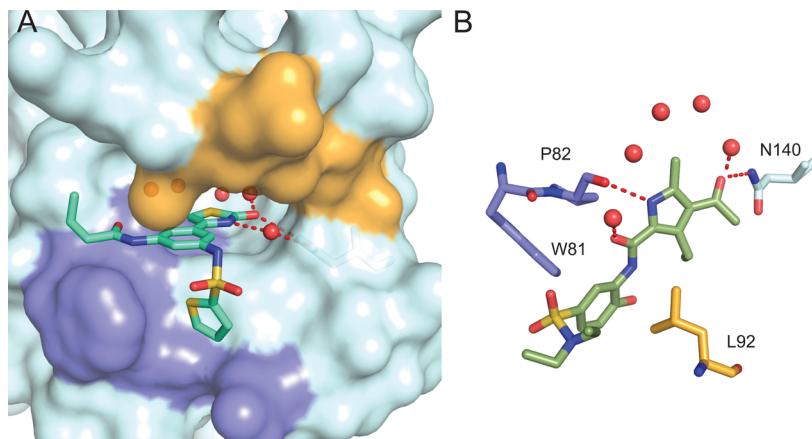


Figure 23. Key molecular interactions between thiazolidinone/acylpyprole and BRD4-BrD1. (A) Thiazolidinone 40d forms two hydrogen bonds with Asn140: one directly via its carbonyl moiety and the other indirectly through a bridged water molecule. The sulfonamide linker directs the thiophene ring to the WPF shelf, and the amide linker positions an aliphatic chain in the ZA channel, leaving the BC channel unoccupied (4HXM). (B) The key interactions between XD14 and BRD4-BrD1 include hydrogen bonding of the acetyl group with Asn140 and the pyrrole NH with the Pro82 backbone. The crystal structure reveals an unusual CH–π interaction between Trp81 and the phenyl ring, which are orthogonally oriented. In this figure, residues of the WPF shelf are colored blue, the ZA surface is colored orange, and the rest of the protein surface is colored cyan.

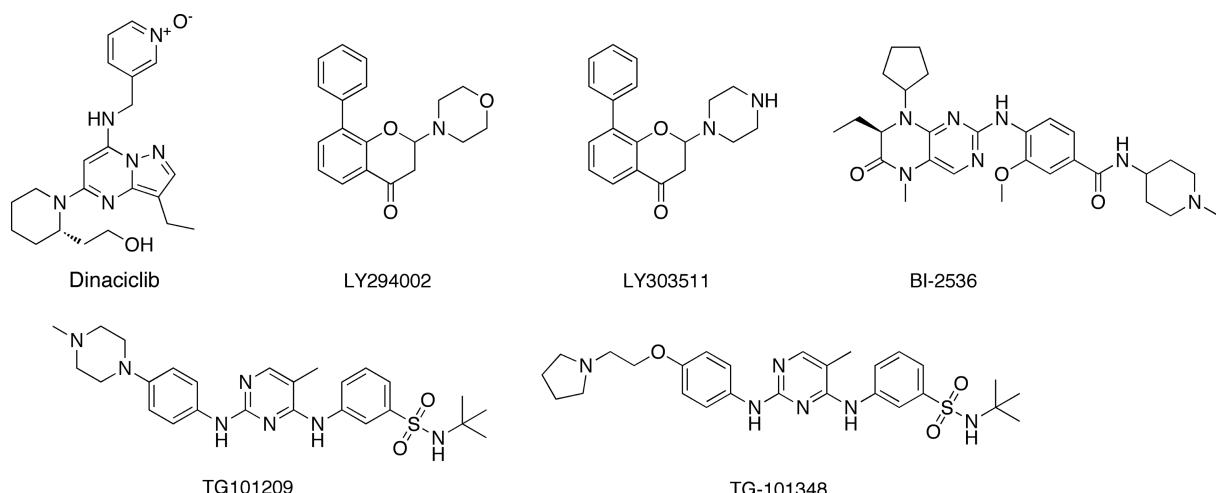


Figure 24. Chemical structures of representative kinase-BET dual inhibitors.

and EP300 bromodomains. A GI_{50} value of 20 μM was measured for XD14 in HL60 cells, but the compound produced no detectable antiproliferative activity in HeLa cells, even in concentrations as high as 50 μM .¹⁹⁷ XD14 was also applied against NCI60 human tumor cell lines and demonstrated particular activity against leukemia cells such as HL60 and SR. Finally, XD14 carries favorable chemicophysical properties that give it the potential to become an even more useful bromodomain inhibitor should development of similar compounds be pursued.

2.12. Dual Kinase-Bromodomain Inhibitors

As the development of bromodomain inhibitors progressed, researchers discovered that BRD4 is an atypical kinase⁶⁹ and comparisons between the binding pockets of bromodomains and kinases began.^{56,198} A series of reports revealed that selected kinase inhibitors (Figure 24) also interacted with bromodomains with high potency.^{199–202} The first reported dual kinase-bromodomain inhibitor was the cyclin-dependent kinase (CDK) inhibitor dinaciclib. Dinaciclib is a selective inhibitor for CDK1, CDK2, CDK5, and CDK9 and has antiproliferative activities toward cancer cell lines at low

nanomolar concentrations. Structural analysis showed that dinaciclib has an excellent shape complementary with the ATP-binding pocket of CDK (Figure 25A), explaining its potent activity. Dinaciclib's high affinity can also be attributed to an intricate network of molecular interactions. In particular, the pyrazolopyrimidine moiety of the compound formed a pair of hydrogen bonds with the hinge region of the kinase (residues 81–83), the hydroxyethyl group interacted with the conserved Lys33, which was within close range to Asp145 in the DFG motif, and the nitroxy group interacted with the ϵ -amino group of Lys89. In addition, dinaciclib made extensive hydrophobic interactions with the ATP-binding pocket, including the gatekeeper residue Phe80 (Figure 25B). Intriguingly, dinaciclib bound BRDT in a very different way. When binding to this bromodomain, pyridine-N-oxide acts as acetyl-lysine mimetic and anchors the molecule into the binding pocket by interacting with Asn109, but the longer distance relative to other bromodomain inhibitors' interactions with the conserved asparagine (3.5 Å) indicated a weaker interaction than JQ1 or I-BET151 has with this residue (Figure 25C–F). The pyrazolopyrimidine moiety was situated near the WPF shelf,

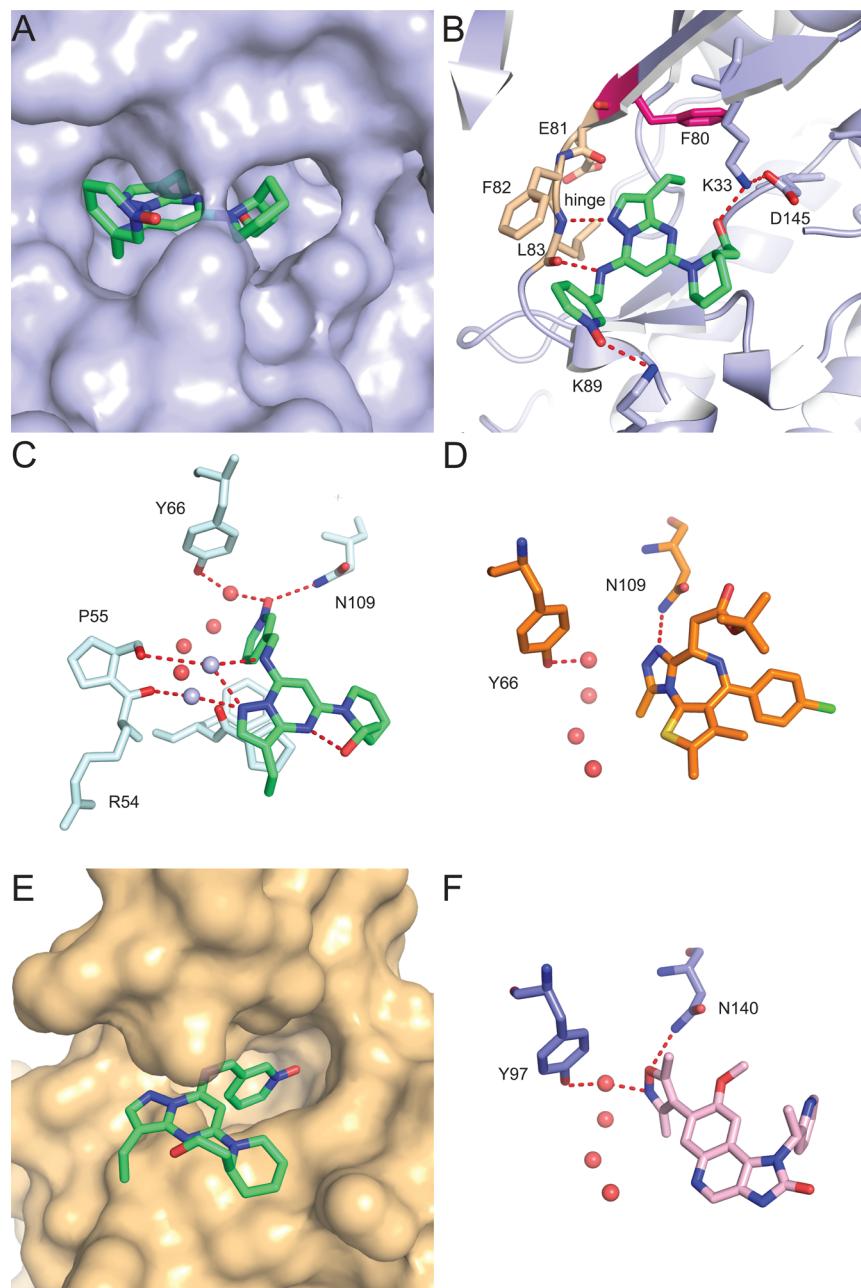


Figure 25. Crystal structures of dinacliclib in complex with CDK2 and BRDT. (A) Surface representation shows dinacliclib fits snugly in the enclosed ATP-binding pocket of CDK2 (4KD1). (B) The dinacliclib/CDK2 complex structure is stabilized by hydrogen bonding between pyrazoloprimidine and the hinge region of the protein (backbones of Leu83 and Phe82). Nitroxy and ethoxy groups interact with Lys89 and Lys33, respectively. Dinacliclib engages in extensive hydrophobic interactions with surface residues, including the gatekeeper residue Phe80. The residues of the hinge region are colored wheat. The gatekeeper residue Phe80 is colored pink. The rest of the protein is colored light blue. For clarity, part of the β -sheet is hidden. (C) JQ1 binds to BRDT-BrD1 (4FLP) in a vastly different mode than does dinacliclib. There is no hydrogen bond between JQ1 and the conserved water molecules. (D) Detailed analyses of the molecular interactions between dinacliclib and BRDT-BrD1. In addition to the highly conserved four water molecules (in red) within the acetyl-lysine pocket, two additional waters (in light blue) outside the pocket are also involved. Dinacliclib interacts with Pro55 and Arg54 via these two water molecules. These residues serve as a pseudo hinge, comparable to the hinge area of CDK2 (shown in B). (E) Surface representation shows that dinacliclib also bound to BRDT (4KCX). However, it is clear that the surface of BRDT is less enclosed and the molecule does not bind deeply into the acetyl-lysine pocket. (F) I-BET151 interacts with a conserved water molecule through its isoxazole moiety when bound to BRD4-BrD1 (3ZYU).

possibly stabilized by van der Waals forces. Interestingly, dinacliclib establishes water-associated hydrogen bonds with the backbone atoms of Pro55 and Val56, interactions not seen in the complexes between the BET bromodomains and other established inhibitors (e.g., JQ1 and I-BET151). The two water molecules involved in this interaction are outside of the acetyl-

lysine pocket and serve as a pseudohinge motif. In terms of affinity, dinacliclib has a K_d value of 37 μM in a dose-dependent qPCR-based assay against BRDT. Aside from its activity toward BET bromodomains, dinacliclib showed appreciable activity toward the TAF1 bromodomain.¹⁹⁹ In a separate study, an inhibitor of phosphoinositide 3-kinase (PI3K), LY294002, and

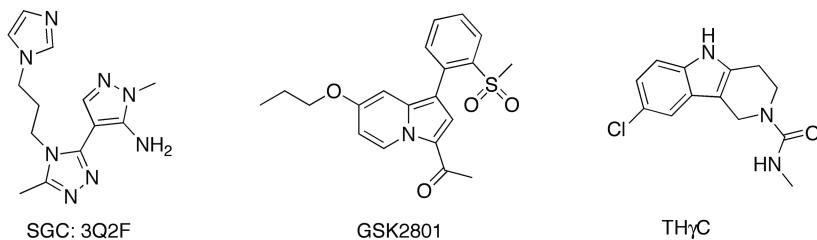


Figure 26. Chemical structures of known BAZ2B inhibitors.

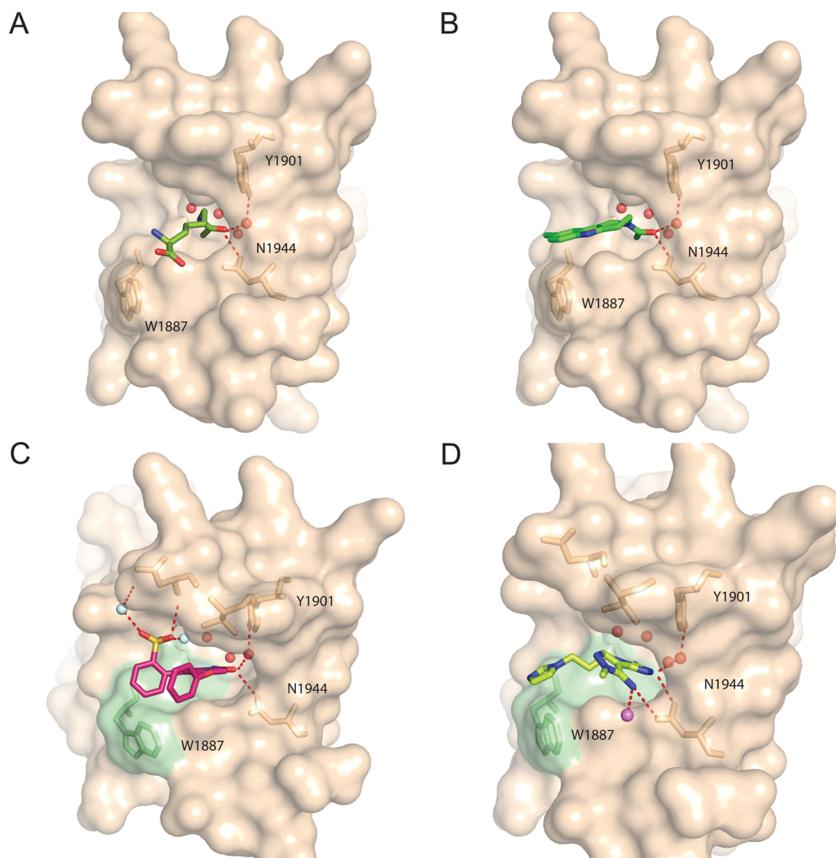


Figure 27. Crystal structures of BAZ2B in complex with various ligands. (A) Acetylated lysine bound to BAZ2B (4NR9). (B) THyC fragment bound BAZ2B in an acetyl-lysine competitive fashion (4NRA). The saturated ring adopts a conformation that enables an edge-to-face $\pi-\pi$ interaction with Trp1887. The N-acetyl group hydrogen bonds with the conserved Asn1944 and also interacts with Tyr1901 via a bridging conserved water molecule. (C) A GSK2801 analog binds BAZ2B (4IR6) differently than does THyC. Its sulfonyl group forms contacts with two water molecules (colored in cyan) and the main chain of Asn1894, significantly enhancing its binding activity. In addition to the interactions with WPF shelf (colored in light green), the indolizine core of this compound engages in hydrophobic interactions with Val1898 and possibly with Leu1897 through its extended aliphatic tail (substituted by an aromatic ring in this analog). (D) IPTP forms a complex with BAZ2B (3Q2F). The triazole moiety of this compound serves as an acetyl-lysine mimetic. This complex is stabilized by a series of hydrogen bonds. Specifically, the amino group on the pyrazole ring forms a hydrogen bond with a surface water molecule (colored in pink).

its analog, LY303511, demonstrated IC_{50} values of 7–12 μM against BET bromodomains in a pull-down assay. Mutagenesis studies suggested these two compounds are BrD1 specific, but no binding affinities were measured against BrD2. These two PI3K inhibitors exhibited an immunosuppressive effect in MV4-11 cells but had significant off-target effects.²⁰⁰

Although dinaciclib and LY294002 are active toward BET bromodomains, their inhibitory activities are insignificant compared with their activities against their corresponding target kinases. On the other hand, the discovery that the polo-like kinase (PLK1) inhibitor BI2536 and the janus kinase 2 (JAK2) inhibitor TG101209 have strong BET bromodomain inhibitory properties and have since changed the landscape of

the dual inhibitor field. BI2536 displayed an IC_{50} value of 25 nM toward BRD4-BrD1 in an AlphaScreen assay²⁰¹ and a K_d value of 37 nM in an ITC experiment.²⁰² BI2536 was also moderately active toward TAF1-2, TAF1L-2, and BRD7. Structural analyses confirmed that BI2536 bound BRD4-BrD1 in a hinge-binding manner via water molecules, much in the same way that dinaciclib binds. Biologically, BI2536 suppressed the expression of c-Myc in the multiple myeloma cell line MM.1S, possibly exerting its therapeutic effect by targeting BET and JAK family proteins simultaneously. Nevertheless, there are no immediately apparent medical benefits of these dual inhibitors *in vivo* when compared with a single BET or kinase inhibitor. More detailed comparative studies are needed

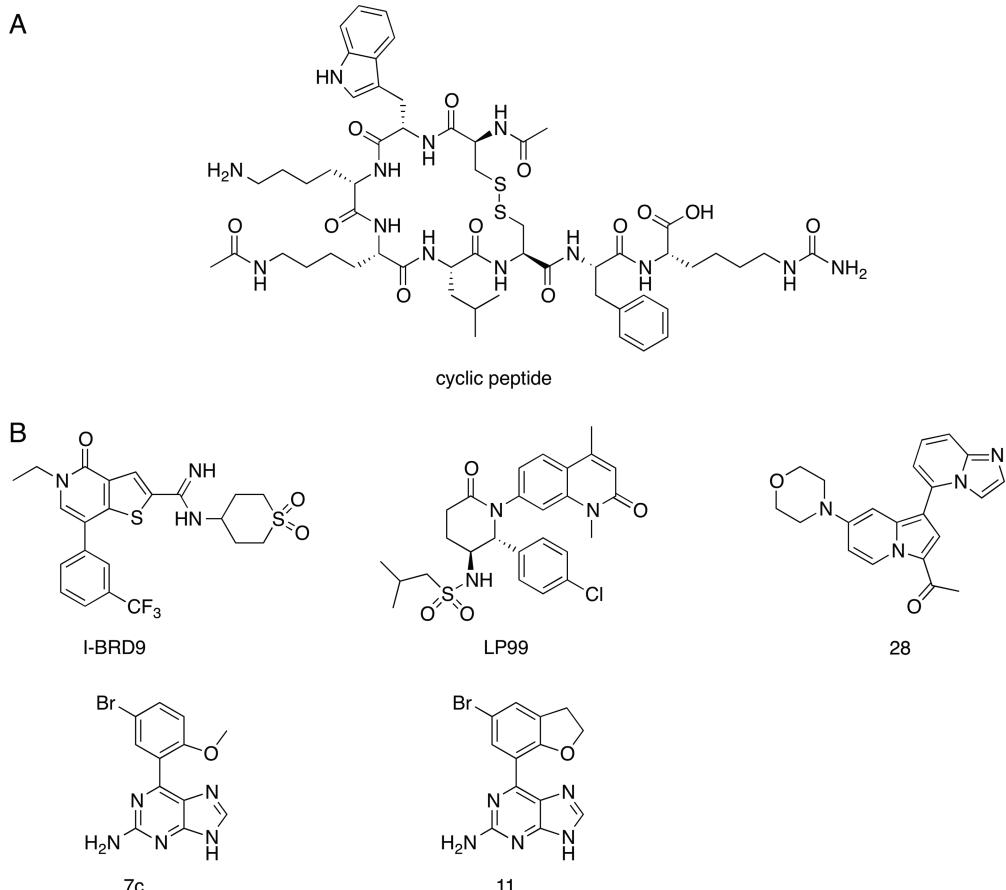


Figure 28. Chemical structures of non-BET bromodomain inhibitors. (A) Cyclic peptide as a CREBBP BrD inhibitor. (B) Inhibitors of BRD9.

in vivo to investigate whether kinase-bromodomain dual inhibition is indeed pharmacologically advantageous.

2.13. Inhibitors of Other Bromodomains

2.13.1. BAZ2B. BAZ2B is characterized as a C-terminal bromodomain adjacent to a zinc finger and has no known biological functions. Unlike the BET bromodomains, BAZ2B has a significantly smaller pocket (Figure 4E) and is projected to have a very low druggability (Figure 5).⁶⁰ Recently, researchers discovered a compound with a tetrahydro- γ -carboline (TH γ C) scaffold that served as a BAZ2B bromodomain inhibitor (Figure 26) using a fragment-based screening approach. The best compound in this class had a K_d value of 9 μM against BAZ2B in an ITC assay.²⁰³ An X-ray crystal structure of the BAZ2B/TH γ C complex revealed the unsaturated ring distorts the molecule in such a way that the indole ring forms an edge-to-face $\pi-\pi$ interaction with Trp1887 of the bromodomain. The N-acetyl group is in range for hydrogen bonding with the conserved Asn1944 and water molecule, comparable to the conventional binding mode of acetylated lysine (Figure 27A and 27B).

Another compound, GSK2801 (Figure 26), was recently disclosed and shown to have a high affinity and selectivity toward BAZ2B/A.^{161,204,205} GSK2801 has displayed K_d values of 136 and 257 nM against BAZ2B and BAZ2A, respectively. In terms of its selectivity, the compound did not inhibit BRD4-BrD1, CREBBP, and PCAF at a concentration of 1 μM . The X-ray crystal structure of a close analog of GSK2801 in complex with BAZ2B indicated that the sulfonyl group of the compound interacts with two water molecules and the backbone of

Asn1894, significantly enhancing its binding activity. Aside from its interaction with the WPF shelf, the indolizine core of GSK2801 engages in hydrophobic interaction with Val1898 and possibly with Leu1897 via its extended aliphatic tail (Figure 27C). Another interesting molecule, 4-(4-(3-(1H-imidazol-1-yl)propyl)-5-methyl-4H-1,2,4-triazol-3-yl)-1-methyl-1H-pyrazol-5-amine (IPTP),²⁰⁶ which was built on a triazole scaffold, also showed promise as a useful probe for BAZ2B. One unique feature of IPTP is an amino group on the pyrazole ring, which can form hydrogen bonds with both the amide side chain of Asn1944 and a water molecule on the BAZ2B protein surface (Figure 27D).

2.13.2. CREBBP. The CREBBP bromodomain has a medium druggability score,⁶⁰ but multiple inhibitors developed for this bromodomain have found some degree of success (Figure 5). As mentioned above, small molecules such as ischemin (Figure 7),^{120,207} MS7972 (Figure 7),^{41,208} isoxazole compound 8 (Figure 12),¹⁶⁵ (*R*)-2,¹⁸⁹ isoxazole compound 59 (also known as CBP30) (Figures 12 and 14),²⁰⁹ and I-CBP112 (Figure 9)¹⁶² are all CREBBP-specific inhibitors. Researchers have also developed cyclic peptides²¹⁰ as inhibitors for CREBBP (Figure 28A). These peptides were cyclized via a disulfide bond, and the top-performing one exhibited a K_d value of 8 μM in a FP binding assay.

2.13.3. BRD9. Prior to recent investigation into the function of BRD9, not much was known about its biological function outside of its presence as a subunit in the chromatin remodeling SWI/SNF complex.²¹¹ A previously reported pan-bromodomain inhibitor was shown to have activity against BRD9,¹⁵⁹ but only recently have more selective compounds for

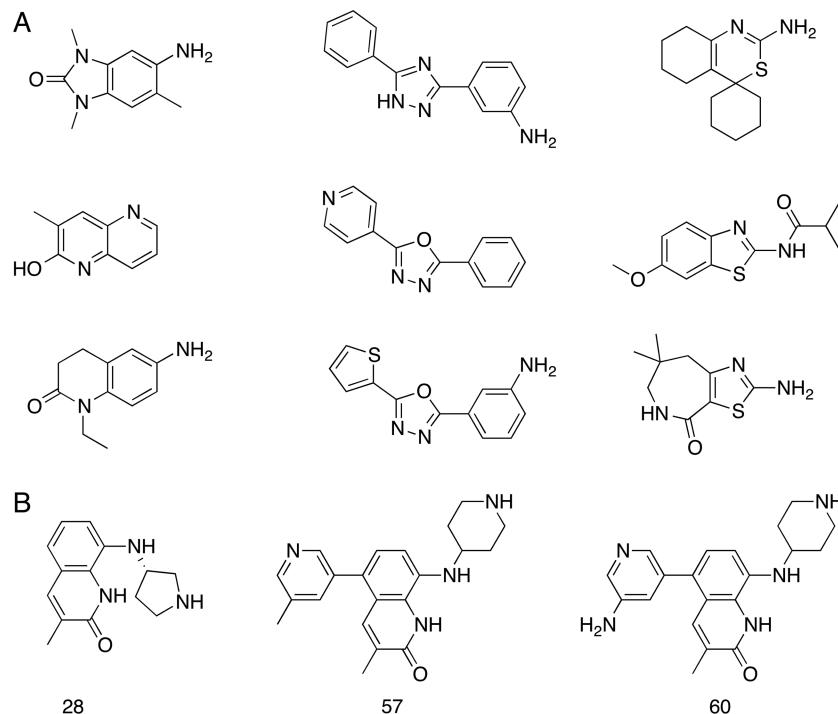


Figure 29. Chemical structures of ATAD2 screening hits and inhibitors. (A) Selective screening hits.²²⁶ (B) Inhibitors.²²⁷

this bromodomain been developed. Two of these compounds, the quinolone-fused lactam LP99²¹² and the imidazopyridine compound 28²¹³ (Figure 28B), have recently been published as inhibitors of both the BRD9 bromodomain and the structurally similar BRD7 bromodomain. Specifically, LP99 has a K_d value of 99 nM against BRD9, shows slightly less activity against BRD7, and shows little to no appreciable activity against any of the other bromodomains it was tested against.²¹² Compound 28 showed a similar affinity and selectivity profile, with a K_d of 68 nM against BRD9 and a K_d of 368 nM against BRD7.²¹³ Both compounds proved to be potent in cellular assays as well. An additional series of compounds with 9H-purine scaffolds is also in development as BRD9 inhibitors and has been tested for their selectivity for BRD9 over BRD4-BrD1. The two highlighted compounds from this study are compound 7d, which displayed a K_d of 397 nM toward BRD9 and a K_d of 4.65 μM toward BRD4-BrD1 in an ITC assay, and compound 11 (Figure 28B), which displayed a K_d of 278 nM toward BRD9 and a K_d of 1.37 μM toward BRD4-BrD1 in an ITC assay.²¹⁴

A compound with further selectivity for BRD9 over all other bromodomains, the thienopyridine I-BRD9 (Figure 28B), was also recently disclosed.²¹⁵ I-BRD9 shows low nanomolar potency against BRD9 as well as >700-fold selectivity over the BET family and, in contrast to the BRD7/9 inhibitors detailed previously, >200-fold selectivity over BRD7. Because of its overwhelming selectivity, even over BRD7 and other closely related bromodomains, I-BRD9's use in a cell-based assay enabled the discovery of genes specifically regulated by BRD9 in cancer and immunological pathways.²¹⁵

2.13.4. ATAD2. ATAD2 contains both a bromodomain and an ATPase domain. It has been shown that ATAD2 is an important cofactor for transcription factors such as E2F,²¹⁶ MYC,²¹⁷ estrogen receptors,^{218,219} and androgen receptors²²⁰ and contributes to the progression of aggressive cancers such as triple negative breast cancer,²²⁰ prostate cancer,²²¹ lung

cancer,²²² cervical cancer,²²³ endometrial carcinoma,²²⁴ and hepatocellular carcinoma.²²⁵

The discovery and development of ATAD2 inhibitors is still in its early stages. Researchers have identified approximately 65 fragments (selected hits are shown in Figure 29) from a NMR-based biomolecular screening as compounds to build upon as ATAD2 inhibitors. Although these fragments only bind ATAD2 with K_d values in the hundreds of micromolar range in a SOFAST-HMQC experiment,²²⁶ they provide a foundation for further improvement in affinity. More recently, a new study took advantage of an earlier hit for BRD4-BrD1 and made variants based on the quinolinone and naphthyridone scaffolds (Figure 30).²²⁷ These are the first reported ATAD2 inhibitors

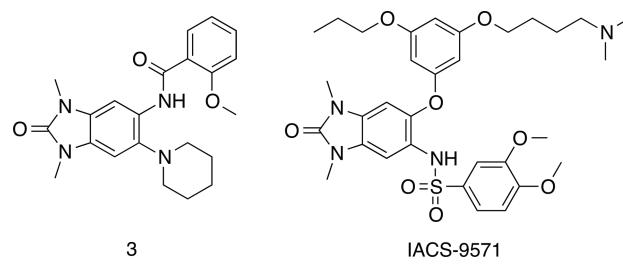


Figure 30. Chemical structures of BRPF1 inhibitors.

with low micromolar potency (measured in a TR-FRET assay). The disadvantage of these compounds is they are also highly active ($\text{IC}_{50} \approx$ low micromolar) toward BET bromodomains. Future efforts to develop ATAD2 inhibitors will look to improve the selectivity profile of these compounds.

2.13.5. BRPF1. BRPF1 (Human bromodomain-PHD finger protein 1) is composed of multiple reader domains, including a double PHD and zinc finger assembly, a bromodomain, and a PWWP domain. These effector domains have known functions in chromatin binding and gene transcription.²²⁸ Only recently has more been uncovered about the biological function of

BRPF1, as researchers were able to determine that it is essential in regulating the growth and proliferation of the embryo.²²⁹ New findings reveal that BRPF1 interacts with multiple acetyl-lysine residues on the N-terminus of histones, specifically H2AK5Ac, H4K12Ac, and H3K14Ac.²²⁸ Moreover, BRPF1 forms a quaternary complex with the monocytic leukemic zinc finger (MOZ), inhibitor of growth 5 (ING5), and the human Esa1-associated factor 6 ortholog (hEaf6) and further enhances the HAT function of MOZ.²³⁰

To avoid the diverse phenotypes that have been reported when BET bromodomains are inhibited, it is absolutely crucial for the inhibitors of BRPF1 to be highly selective. It was not until late 2014 that a research group developed a series of compounds based on a benzimidazolone scaffold to meet this challenge. Compound 3 (Figure 30), the best compound in this class, demonstrated good selectivity among BRPFs (pIC_{50} BRPF1/2/3 7.1/5.1/ < 4.0 in a TR-FRET assay). This compound also has great selectivity over BET bromodomains, as shown in TR-FRET and BROMOScan assays.²³¹ Interestingly, a separate research group discovered a dual BRPF1 and TRIM24 inhibitor IACS-9571 (Figure 30) based on the same scaffold with exceptional in vitro binding activity (BRFP1, K_d = 14 nM; TRIM, K_d = 31 nM in ITC).²³² This compound also has excellent cellular activity and favorable pharmacokinetic properties (EC_{50} = 50 nM). Although IACS-9571 has good selectivity over BAZ2B, BRD4 (BrD1 and BrD2), and TAF (BrD2), it displays little selectivity among the BRPF1/2/3 bromodomains.²³²

2.14. Evaluation of Bromodomain Inhibitors

Although quite a few biophysical techniques have been developed for the evaluation of bromodomain inhibitors in vitro, the results have to be interpreted with caution. Fluorescence/luminescence-based assays such as AlphaScreen (and its variants, such as a peptide displacement assay²³³), DSF, and FP have been widely adopted as high-throughput screening methods due to their high sensitivity and ease of implementation in microplate formats. However, these assays are prone to false positives,²³⁴ and the assay sensitivities tend to fluctuate, as exemplified by some studies mentioned above.^{45,51,170,187} Therefore, they are excellent tools for initial screens, but a more accurate and reliable secondary method, such as ITC or SPR, should be used as a complementary method for validation of binding. However, ITC and SPR usually require compounds to have good solubility, adding another degree of difficulty to the drug design and testing process. Even though these secondary assays are low throughput, they are important for determining when true inhibition is taking place. These assays have already replaced NMR as the mainstays of precise determination of binding affinities, yet NMR is still important to many screening efforts.²²⁶

The affinities of these molecules could vary (in some cases, dramatically) due to different protein constructs used when assaying them. For example, binding of BET inhibitor GW841819X to His6-tagged BRD2-BrD1 can be highly temperature dependent: at 26 °C, K_d is higher than 1 μM; at 16 °C, K_d is 46 nM, as determined by ITC.²³⁵ For a longer tandem domain construct, his-tagged BRD2-BrD12 (1-473), binding was found to be in the tens of nanomolar range for both BrD1 and BrD2 using the same technique. These observations tell us the behavior of different constructs can be different, and as such, so can the binding affinities of their

binding partners. It is advisable to use caution when interpreting in vitro binding data.

2.15. Summary of Bromodomain Inhibitors

With the advancement of intense research into bromodomain biology, many chemotypes have emerged as bromodomain inhibitors. These compounds are important not only as potential therapeutics but also as invaluable probes for target validation and functional studies. These diverse inhibitors all bind bromodomains by occupying the acetyl-lysine binding pocket and forming various interactions with the ZA channel, BC channel, WPF shelf, and/or ZA surfaces of a target bromodomain (Figure 1). The orientations of these inhibitors are determined by the conformation of the core scaffold, intramolecular hydrogen bonds, and stereochemistry. Some distinct scaffolds have begun to address questions about bromodomains seen as being on the lower end of the druggability scale, such as CREBBP, PCAF, and BAZ2B/A. High-performing compounds that have passed the initial testing stages are currently making their way through clinical trials and have the potential to find widespread use combatting cancer, inflammatory diseases, and viral infections in humans.

Thus far, the vast majority of the ligands developed are BET specific. BET family proteins are unique bromodomain-containing proteins that remain attached to chromosomes during mitosis.²³⁶ BET proteins represent only one of eight subgroups of the bromodomain protein family—other bromodomain proteins such as CREBBP also exist in the nucleus, so it is essential that bromodomain inhibitors have great selectivity profiles. Further, bromodomains in some proteins (e.g., ASH1L) do not bind acetyl-lysine, and inhibitor discovery efforts related to these bromodomain proteins will most likely require different chemotypes than the ones that are currently being explored.

3. BROMODOMAIN INHIBITION AND HUMAN DISEASES

3.1. Bromodomain Inhibitors as Anticancer Agents

The roles played by bromodomains in cancer are complex—there is not a single specific way in which bromodomains are associated with a cancer-causing pathway. One such cancer-causing mechanism that involves the bromodomain is the formation of oncogenic fusion proteins. Such a fusion protein can be found in NUT midline carcinoma (NMC), which is a very aggressive form of squamous cell carcinoma.^{84,237,238} The fusion protein in NMC is formed by a chromosomal rearrangement involving *NUT*, a gene located on chromosome 15. In most NMC patients, a fused *BRD4-NUT* gene is created by inserting the coding region of *NUT* into the 3' end of *BRD4* on chromosome 19. In rare cases, a *BRD3-NUT* fusion gene has been observed.²³⁸ *BRD3-NUT* and *BRD4-NUT* fusion proteins are stringently nuclear, suggesting *BRD* retains *NUT* in the nucleus by interacting with acetylated chromatin at a discrete locus. Knockdown of *BRD4-NUT* and *BRD3-NUT* in NMC cells leads to squamous differentiation and cell-cycle arrest.⁸⁴

Collectively, *BRD*-*NUT*s are oncoproteins that intervene in the transcriptional program that controls differentiation. The pan-BET inhibitor JQ1 treatment resulted in cell differentiation and attenuated cell proliferation in cultured NMC squamous cells.⁴⁵ The expression levels of three typical squamous tissue genes were considerably reduced as measured by RT-PCR. JQ1 was also shown to promote tumor regression and a prolonged

survival rate in a murine NMC model.⁶² However, the mechanism of how BET inhibitors reverse the effect of the BRD-NUT fusion protein remains unclear. Recently, it was reported the BRD-NUT chimera included not only two bromodomains and an ET domain but also a p300-binding domain.^{84,239} This would make blocking BRD-NUT fusion proteins much more difficult, as p300 is very important in the transcriptional regulation of processes such as proliferation and differentiation.^{96,240,241} One possibility is that BRD-NUT proteins block cell differentiation by sequestering p300, thereby suppressing the expression of genes that are in charge of squamous differentiation, but further investigation is needed to address this precise mechanism.

Chromosomal translocations involving *MLL* gene fusions are responsible for the onset of an aggressive subset of leukemia. Similar to the NUT fusion proteins, MLL fusions have also been linked to the presence and activity of BET bromodomains. Leukemia cell lines MOLM13 and MV4-11 are driven by MLL-AF9 and MLL-AF4, respectively. I-BET151 displayed exceptional therapeutic values in MLL-fusion cell lines and efficacies in murine and human MLL fusion leukemia models.⁴⁷ Other bromodomain proteins are also observed as fusion partners of MLL. *MLL-p300*²⁴² and *MLL-CBP*^{243–245} fusion genes are caused by translocations t(1;22;11)(q44;q13;q23) and t(11;16)(q23;q13) in AML. Homologous *p300/CBP* encodes the HAT *p300/CBP*, which contains a bromodomain. In a number of solid tumors and hematological malignancies, mutations of *p300/CBP* are detected, suggesting the association of these genes with human cancer. *p300/CBP* function as typical tumor suppressor genes in tumor formation in a transgenic mouse model.⁹⁶ This finding helped establish the important role of these two transcription cofactors in tumorigenic pathways such as p53 and transforming growth factor β (TGF- β). *MOZ* can also be translocated to other loci to generate fusions with *p300*²⁴⁶/*CBP*.²⁴⁷ A *MOZ-p300/CBP* (*MOZ*, monocytic leukemia zinc finger protein) fusion protein was generated by a reciprocal chromosomal rearrangement t(8;16)(p11;p13) in AML. The translocation disrupted *MYST3/MOZ* fusion on chromosome region 8p11 to *p300/CBP* on 16p13. *MOZ-CBP* blocked cell differentiation through the inhibition of *RUNX1* regulated transcription.²⁴⁸ A *MOZ*-related gene, *MORF*, also fused with *CBP* due to a t(10;16)(q22;p13) translocation in AML.²⁴⁹ *p300/CBP* are also transcriptional cofactors for other nuclear oncogenetic proteins such as *MYB*, *JUN*, and *FOS*.⁹⁶ Although a detailed mechanism of how bromodomain-related fusion proteins work has yet to be fully elucidated, these discoveries open a promising avenue into this pathway and future cancer therapies. Additionally, work is needed to confirm the mechanisms driving bromodomain fusion protein formation in other bromodomain-dependent diseases.

Bromodomains can also interact with oncogenic drivers in advanced cancer. One such cancer is metastatic castration-resistant prostate cancer (CRPC), which is dominantly regulated by androgen receptor (AR) pathways.²⁵⁰ BRD4-BrD1 interacts directly with the N-terminal domain of AR, and inhibition of BRD4 impedes the recruitment of AR to its target gene, preventing the expression of AR-mediated genes. In fact, the role of BRD4 in this process was made even clearer when it was shown that JQ1 was more effective than the AR antagonists abiraterone and MDV3100 as a potential treatment for CRPC. JQ1 was potent toward AR-signaling positive prostate cancer cell lines and proved effective in CRPC xenograft models,

providing a feasible alternative treatment for refractory prostate cancer.⁴⁸ AR is also associated with other bromodomain containing proteins, such as CBP, which serves as a coactivator in AR-mediated signaling²⁵¹ and enhances AR activities after stimulation with an AR antagonist. CBP is highly expressed in advanced prostate cancer and in direct contact with AR in vitro²⁵² and in vivo.²⁵¹ CBP is an integrator between AR and various nuclear signaling pathways (i.e., NF- κ B), and regulation of CBP is of critical importance pertaining to transcriptional responses to AR. It is yet to be determined whether CBP is also a target in prostate cancer as regulated by AR pathways.

3.2. Bromodomain Inhibitors as Anti-Inflammatory Agents

Excessive immune response is a main feature of many inflammatory diseases. Inflammatory responses hinge both on the activation state of signaling proteins that control the regulation of inflammatory genes and on the induced assembly of nuclear chromatin complexes that initiate mRNA expression.^{253,254} Proper recognition of post-translational histone modifications by nuclear proteins is crucial for the initiation and elongation of mRNA expression and in the context of inflammation could lead to the transcription of inflammatory genes.^{255–257} Researchers established a system to monitor pro-inflammatory cytokine production in bone marrow-derived macrophages (BMDMs) challenged by bacterial endotoxin.⁴⁶ I-BET suppressed the expression of important pro-inflammatory cytokines and chemokines such as IL-1 β , IL-6, IL12, CXCL9, and CCL12 in BMDMs. The binding of BRD2, BRD3, and BRD4 at the promoter genes *IL-6* and *TNF* was significantly lower when treated with I-BET in a LPS-induced macrophage model. IV injection of I-BET rescued mice with sepsis as induced by a cecal ligation and puncture procedure. The therapeutic benefit of I-BET was also demonstrated through the expansion of the lifespan of mice in an LPS- and heat-killed *Salmonella typhimurium*-induced endotoxin shock model.⁴⁶ These results collectively substantiate that targeting BET bromodomains is a promising approach for intervention in inflammatory diseases.

Of the many proteins upregulated by I-BET, hexamethylene bis-acetamide inducible protein 1 (HEXIM1)⁴⁶ is of particular interest. HEXIM1 inhibits p-TEFb as well as NF- κ B-dependent target genes, thereby inhibiting the production of pro-inflammatory cytokines.²⁵⁸ Therefore, this represents a new approach to target NF- κ B as a measure to treat NF- κ B-directed inflammation. Indeed, MS417 interrupted the transcriptional activity of NF- κ B by preventing cofactor BRD4 from interacting with the transcriptional complex containing p-TEFb and RNA Pol II. Additionally, MS417 attenuated inflammation and kidney injury in HIV-1 transgenic mice.⁵⁵

Furthermore, outside of their potential usages as anticancer agents and anti-inflammatory agents, bromodomain inhibitors also exhibit potential in combatting viral infections such as HIV,^{259,260} largely due to the important role of bromodomain proteins in HIV infection.²⁶¹

3.3. Bromodomain Inhibitors in Clinical Trials

Researchers have begun to translate the knowledge acquired in the laboratory about the potential effects of bromodomain inhibitors on various diseases into medicines that can have a true impact in the clinic on disease prevention and treatment. Of the many chemotypes summarized above, triazolodiazepines (TTDs and BZTDs) and their close analogs have been broadly studied and possess favorable DMPK properties, making them excellent candidates to be examined in humans.^{45,140,155}

Table 2. Bromodomain Inhibitors in Human Clinical Testing

compound	company	stage	trial ID	implications	status
OTX015	OncoEthix	Phase I	NCT01713582	acute leukemia and other hematological malignancies	completed
		Phase I	NCT02259114	advanced solid tumors	recruiting
		Phase I	NCT02303782	newly diagnosed AML	not recruiting yet
CPI-0610	Constellation Pharmaceuticals	Phase II	NCT02296476	recurrent glioblastoma multiforme	recruiting
		Phase I	NCT01949883	progressive lymphoma	recruiting
		Phase I	NCT02157636	multiple myeloma	recruiting
TEN-010	Tensha Therapeutics	Phase I	NCT01987362	acute leukemia, myelodysplastic syndrome, myelodysplastic/myeloproliferative, neoplasms	recruiting
		Phase I	NCT02308761	solid tumors	recruiting
		Phase I	NCT01587703	acute myeloid leukemia and myelodysplastic syndrome	recruiting
GSK525762	GlaxoSmithKline	Phase I	NCT01943851	midline carcinoma and other cancers	recruiting
		Phase I	NCT01728467	hematological malignancies	recruiting
		Phase II	NCT01058018	diabetes	completed
RVX-208	Resverlogix Corp	Phase II	NCT01423188	atherosclerosis, coronary artery disease	completed
		Phase II	NCT01067820	dyslipidemia, coronary artery disease	completed
		Phase II	NCT00768274	coronary artery disease	completed
		Phase II	NCT01863225	dyslipidemia, atherosclerosis, acute coronary syndrome, cardiovascular disease	completed
ZEN-3365	Zenith Epigenetics	Phase II	NCT02238522	dyslipidemia, coronary artery disease	terminated
		Phase I	NCT02238522	lymphoproliferative malignancies	withdrawn
					acute myeloid leukemia

On the basis of the studies of the *BRD4-NUT* and *BRD3-NUT* gene fusions, Tensha Therapeutics' BET bromodomain inhibitor TEN-010 entered a Phase I clinical trial for the treatment of solid tumors in late 2013 (Figure 2 and Table 2). As NMC is a very rare form of cancer, a genetic marker is needed to predict a given patient's response to epigenetic inhibitors such as TEN-010. In 2014, another Phase I clinical trial was initiated to study TEN-010 in patients with AML and Myelodysplastic Syndrome (Table 2). Researchers have also screened a collection of cancer cell lines and found that four neuroblastoma cell lines with amplified *NMYC* were sensitive to JQ1.²⁶² Additionally, GSK had previously initiated two Phase I clinical trials of I-BET762 (GSK525762) in 2012 for testing in various cancers, such as NMC, multiple myeloma, small cell lung cancer, colorectal cancer, neuroblastoma, *NMYC*-amplified solid tumors, and other hematological malignancies.²⁶³

OTX015 (also known as Y-803) is another drug candidate targeting BET bromodomains. A private company named OncoEthix in-licensed OTX015 from Mitsubishi Tanabe Pharmaceutical for clinical development. OTX015 entered a clinical trial in 2012 for specific indications in acute myeloid leukemia, B-cell lymphoma, multiple myeloma, and other hematological malignancies and has now successfully completed an oral administration Phase I trial (Table 2). Interim results showed OTX015 had preferable pharmacokinetics and excellent tolerance at a once-daily 80 mg/kg dose. Complete responses were seen in patients with refractory and aggressive leukemia when the drug was administered as a single agent. This study was complete by 2014. Two more Phase I studies include single-agent OTX015 in pediatric and adult solid tumors and a combination therapy in selected hematological malignancies are ongoing. More recently, OncoEthix started a Phase II trial for OTX015 in patients with recurrent glioblastoma multiforme (Table 2). At the end of 2014, OncoEthix was acquired by Merck, who will presumably continue exploring this portfolio of drugs. OTX-015 is now

renamed MK-8628 and has shown some therapeutic benefits in advanced nonsmall cell lung cancer and small cell lung cancer²⁶⁴ as well as triple negative breast cancer²⁶⁵ in clinical trials.

Constellation Pharmaceuticals initiated a Phase I trial (Table 2) in 2013 for their compound, CPI-0610, in patients with progressive lymphoma. A second Phase I trial initiated by Constellation looks to test the potential of CPI0610 as an antimyeloma therapy. Special emphasis has been placed upon recruiting patients with relapsed or refractory multiple myeloma in an effort to test the compound against these specific disease states. Another trial is also underway for patients with acute leukemia and myelodysplastic syndrome, based upon preclinical data that blood-related cancers are potentially treated by the inhibition of BET bromodomains.

The previously described RVX-208²⁶⁶ (also known as RVX-000222), a compound originally developed by Resverlogix Corp. to induce the production of ApoA1 and HDL, has completed Phase II clinical trials for diabetes, coronary artery disease, atherosclerosis, dyslipidemia, and cardiovascular diseases (Table 2). Recently, RVX-208 was identified as a BET bromodomain inhibitor with selectivity toward the second bromodomains of BET family members.⁵¹ Preliminary findings from a recently completed Phase II trial (Table 2) suggested RVX-208 reduced major adverse cardiovascular events (MACE) in patients with diabetes mellitus (DM).²⁶⁷ These results reinforced the therapeutic benefits of RVX-208 in lowering the risk of MACE and provided insight into other metabolic parameters for high-risk patients with DM and low HDL levels. In a Phase IIb study of Quantitative Serial Trends in Lipids With Apo protein A1 Stimulation (SUSTAIN, NCT01423188, Table 2), RVX-208 successfully increased the levels of ApoA1 and large HDL particles—critical factors in reverse cholesterol transport.²⁶⁸ However, in an ApoA1 Synthesis Stimulation and Intravascular Ultrasound for Coronary Atheroma Regression Evaluation (ASSURE) trial (Table 2), RVX-208 was unable to increase HDL levels and

coronary plaque build-up.²⁶⁹ At present, a Phase III clinical trial of RVX-208 for acute coronary syndrome is being planned. In August 2014, Resverlogix Corp. spun out all research and development activities related to its epigenetics platform, excluding the clinical program related to RVX-208, to Zenith Epigenetics. Zenith Epigenetics announced their proprietary compound ZEN-3365 would enter Phase I clinical trials for AML, lympho-proliferative disorder (LPD), and solid tumors.²⁷⁰ However, this trial was recently withdrawn. Zenith Epigenetics also has compounds in the pipeline to address autoimmune diseases.

4. TOOLS TO AID THE INVESTIGATION OF EPIGENETIC THERAPY

With the quick expansion of the field of epigenetic therapies, effective tools have been developed to assist the collective endeavors of researchers worldwide. Statistics on acetylation and other protein modification sites are available on phosphosite (www.phosphosite.org). For structure-based design, large volumes of crystal structures of bromodomain proteins in complex with small-molecule ligands or peptides are available in the RCSB protein databank (<http://www.rcsb.org/pdb/home/home.do>). Millions of drug-like molecules are assembled into integrated virtual libraries (e.g., ChEMBL^{194,195} (<https://www.ebi.ac.uk/chembl/>), ZINC¹⁹⁶ (<http://zinc.docking.org>)) for in silico screening. ChEpiMod (<http://chepimod.org/db/>) is a knowledge-based database of ligands for the epigenome reader domains (i.e., bromodomains, chromodomains, and PHD, etc.) with activity data and information on molecular contacts.²⁷¹ Furthermore, a collection of highly useful chemical probes for various bromodomains is available at Structural Genomic Consortium (SGC, <http://www.thescg.org/chemical-probes/epigenetics>). Detailed information on the status of pharmaceutical trials can be found in databases developed by either NIH (<https://ClinicalTrials.gov>) or private companies (<http://sigma.larvol.com>).

5. CHALLENGES AND FUTURE DIRECTIONS

As has been described extensively in this review, bromodomains have become attractive targets for the development of epigenetic therapies for a spectrum of human diseases, ranging from cancer and inflammation to viral infection. In recent years, researchers have achieved great successes in developing potent inhibitors for the BET bromodomain family, members of which play an indispensable role in the assembly of transcriptional complexes. These compounds not only will serve as useful chemical probes that can enhance our understanding of biology but also can be developed into therapeutic agents in the treatment of various human diseases. However, it is important to emphasize that the role of the BET bromodomains is highly context specific, and BET proteins are involved in several important nuclear signaling pathways involving key transcription factors such as MYC, ETS, and AR. Therefore, it is essential to understand other genes that are also transcriptionally regulated within these contexts throughout the drug discovery and target validation process.²⁷²

The issue of specificity remains front and center in epigenetic drug development. In the case of bromodomain-containing proteins, their structural features are largely the same, but it is the subtle differences in the bromodomain structures and functions that make them extremely important to the biological pathways of which they are a part. For example, the BET

bromodomains (BRD2, BRD3, BRD4, and BRDT) share high sequence identities,⁵² and they are all involved in the transcriptional activation complex; nonetheless, they have distinctive roles based on different nuclear signaling context. For example, BRD4 (but not BRD2) knockdown leads to significant reduction in the release of IL-6 and CXCL8, suggesting BRD4 plays an important role in inducing inflammation.²⁷³ BRD4 is also essential in NF-κB-mediated induction of IL-6 and IL-8.²⁷⁴ In one instance, individual silencing of BRD2 and BRD3 did not affect ESC colony formation, but when BRD4 was depleted, ESC colony formation was perturbed.^{49,125} On the other hand, BRD2 (but not BRD3/BRD4) is known to reduce STAT5 expression.²⁷⁵ Knockdown of BRD4 but not BRD2 and BRD3 led to an increased level of ApoA1 expression.¹⁸⁶ In addition to the differences of the four BET family proteins, growing evidence indicates that the individual bromodomains of the BET proteins, namely, BrD1 and BrD2, seem to have distinct functions in regulation of gene transcription. For instance, the BrD1 of BRD4 is dedicated for binding to histone H4 at K5ac and K8ac, whereas the BrD2 can interact with various lysine-acetylated transcription factors as well as cyclin T1 of the p-TEFb complex that is important for transcription factor recruitment and activation of transcription elongation required for productive gene transcription. Therefore, there is growing demand for new bromodomain inhibitors that are selective for each BET protein as well as for individual BET bromodomains.

It is important to note that the BET family bromodomains are highly druggable, as such most of the developed bromodomain inhibitors target the BET bromodomains. Bromodomains such as BAZ2B, SMARCA4, and PB1 have very low druggability due to their limited pocket volume, enclosure, and degree of hydrophobicity.⁶⁰ Strides have been made in the development of inhibitors for these bromodomains, but continued efforts with structure-guided drug design is required to improve these lead inhibitors for these more difficult targets.

Researchers must also overcome recently discovered long-term side effects of BET bromodomain inhibition, as they are an important challenge that needs to be addressed in the development of new therapeutic agents targeting bromodomain proteins. Side effects in the epithelial cells of the small intestine and skin cells of a mouse model upon inhibition of the BET bromodomains with small-molecule inhibitors or RNAi have recently been reported.²⁷⁶ These toxicities can manifest as reversible epidermal hyperplasia, alopecia, and dramatic decline in stem cell count in small intestine. Possibility also exists for additional adverse synergistic effects, as BRD4 suppression sensitizes the intestinal response to internal and external stimuli. These results from a transgenic mouse model predict the possible consequences of potent and sustained inhibition of BET protein. This research echoes the prior work¹⁴¹ that suggested that inhibition of BET bromodomains should be temporal rather than permanent. This study also provided some guidelines for potential combination therapy to offset the detrimental effects of BET bromodomain inhibitors to normal tissues.

In summary, the structure-guided drug design strategy has played an important role in the recent discovery and development of bromodomain inhibitors that has yielded a multitude of chemotypes as described in this review. Going forward, we expect that this structure-based approach will

continue to guide medicinal chemists in lead optimization of bromodomain inhibitors and facilitate the efforts to develop new inhibitors for less druggable bromodomains. Collectively, these emerging new chemical inhibitors will empower our ongoing investigation to better understand the roles of bromodomain proteins in control of gene transcription in human biology and disease pathology. These small-molecule inhibitors can also be developed into new epigenetic therapies for new effective treatments of various human diseases including cancer and inflammatory disorders.

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