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Bromodomains and Their Pharmacological Inhibitors

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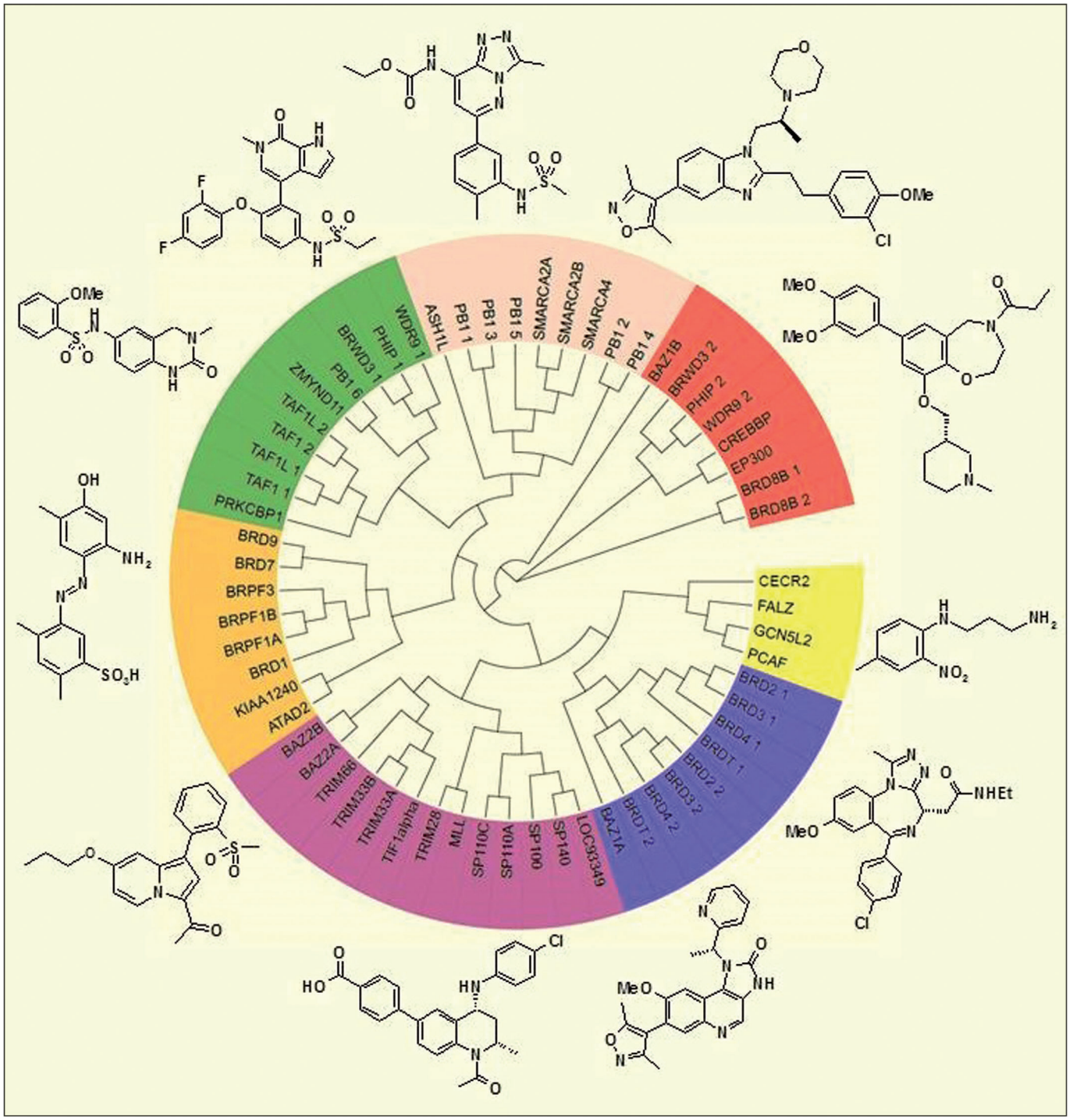

Over 60 bromodomains belonging to proteins with very different functions have been identified in humans. Several of them interact with acetylated lysine residues, leading to the recruitment and stabilization of protein complexes. The bromodomain and extra-terminal domain (BET) proteins contain tandem bromodomains which bind to acetylated histones and are thereby implicated in a number of DNA-centered processes, including the regulation of gene expression. The recent identification of inhibitors of BET and non-BET bromodomains is one of the few examples in which effective blockade of a protein–protein interaction can be achieved with a small molecule. This has led to major strides in the understanding of the function of bromodomain-containing proteins and their involvement in diseases such as cancer and inflammation. Indeed, BET bromodomain inhibitors are now being clinically evaluated for the treatment of hematological tumors and have also been tested in clinical trials for the relatively rare BRD-NUT midline carcinoma. This review gives an overview of the newest developments in the field, with a focus on the biology of selected bromodomain proteins on the one hand, and on reported pharmacological inhibitors on the other, including recent examples from the patent literature.

1. Introduction

Mammalian cells contain approximately two meters of linear DNA which need to be packed in the nucleus. This is achieved by association of the DNA with basic histone proteins to form highly condensed chromatin.[1] Nucleosomes represent the basic subunit of chromatin and are essentially composed of histone octamers formed of H2A, H2B, H3, and H4 dimers around which a DNA stretch of approximately 147 base pairs forming 1.7 left-handed turns is wrapped.[2] Nucleosomes are spaced by about 80 base pairs of linker DNA coated by histone H1.[3]

Numerous direct DNA–protein interactions take place in the nucleosome, with little specificity for the DNA sequence itself. Histones, however, may undergo multiple reversible, covalent post-translational modifications, mainly at their protruding N-terminal tails, which strongly impact their interactions with DNA and other histones.[4] The histone state affects all DNA-centered processes, including genome architecture and integrity, replication, repair, and gene transcription control.[5] Histone modifications are very complex and include acetylation and phosphorylation, which decrease the overall charge and thereby loosen the association with DNA. Additional modifications such as methylation, ubiquitylation, sumoylation, biotinylation, citrullination, ribosylation, and crotonylation have also been described.[6] Each can occur alone or in combination, and most correlate with the overall chromatin state. Not all combinations take place simultaneously, as some amino acids may undergo several modifications (e.g., lysine residues can be acetylated, methylated, or ubiquitylated) and some marks are present in different forms (lysine mono-, di-, and trimethylation; arginine symmetric and asymmetric dimethylation). Moreover, these modifications may be short-lived, occur sequentially or in a context-specific manner. Some of this information can be transmitted to daughter cells and is essential for epigenetic inheritance, for instance, for the maintenance of differentiated states.[7] The importance of the histone code is further documented by the fact that a number of pathologies, including cancer, are linked to a deregulation of this information storage system.[8]

The enzymes responsible for the addition of histone post-translational modifications are collectively known as writers, of which the most studied are the histone acetyltransferases (HAT), histone methyltransferases (HMT), and kinases. Enzymes that remove these marks are called erasers and include histone deacetylases (HDAC), demethylases, and phosphatases. Importantly, the histone deacetylase inhibitors vorinostat and romidepsin have been approved for the treatment of cutaneous T cell lymphoma, whereas valproic acid is used in neurology and psychiatry, underscoring the importance of histone acetylation status in various pathologies.[9] Histone methylation patterns have also been linked to disease, mainly cancer. Recently, the first histone methyltransferase inhibitors EPZ-6438 (targeting EZH2) and EPZ-5676 (targeting DOT1L) have shown efficacy in preclinical tumor models, which led to the initiation of clinical studies.[10] Altogether, these recent advances show that aberrations in histone post-translational modifications are linked to several pathologies and that several of the enzymes involved represent tractable drug targets.

Readers of histone marks often serve as platforms for the recruitment of a number of partners, thus forming large complexes with various cellular functions.[11] The interactions between reader proteins and histone marks are very specific and are further influenced by neighboring histone modifications, hence the concept of a histone code.[4a] Numerous studies indicate that individual local variations may have a dramatic effect on the binding of reader proteins, which in turn impacts the molecular outcome of the corresponding interactions.[12]

Blocking protein–protein interactions is generally challenging, and few examples of small molecules that can achieve this while maintaining appropriate pharmacokinetic properties have been reported. This is why the druggability of reader proteins has originally been considered low. However, the situation has dramatically changed following the discovery of small-molecule inhibitors of bromodomains, which are readers of histone acetylation marks.[13]

Acetylation of histones is an essential post-translational modification governing gene expression.[5] It affects a number of lysine residues in all histones and serves as a docking site for the evolutionarily highly conserved bromodomain module.[14] Following the recognition of discrete patterns of

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acetylation marks which, together with neighboring amino acids, mediate binding selectivity, bromodomain-containing proteins engage in a variety of biological functions.[15] They are highly conserved from humans to yeast, indicating a fundamental role in cellular processes. In line with this, deletion of bromodomain protein-encoding genes often results in early embryonic death or severe malformations. A total of 61 bromodomains found in 46 different proteins has been identified in the human proteome.[16] They form eight subfamilies with very different functions, and several of them have been implicated in human diseases, mainly cancer and inflammation.[17]

The understanding of the biology of bromodomain proteins has been facilitated by the recent identification of specific, small-molecule inhibitors. Bromodomains usually possess deep hydrophobic pockets with a small, tight binding site for the acetyllysine moiety. In addition, water molecules present at the bottom of the pocket have a significant impact on druggability. The interaction between bromodomains and acetylated proteins is also generally weak (low micromolar to millimolar K_D values), increasing the likelihood that potent inhibitors can be found.[17] Druggability assessment shows that the BET subgroup scores high within the bromodomain family,[18] which is confirmed by the identification of several potent small-molecule inhibitors with different scaffolds.[13] More recently, the first potent inhibitors of other bromodomain proteins such as cAMP response element binding protein-1 binding protein (CREBBP), E1A-associated protein p300 (EP300) and bromodomain adjacent to zinc finger domain (BAZ) have been reported. In this review, we outline new findings related to the biology of bromodomain proteins with a focus on those that may play an important role in cancer. We also review the pharmacological inhibitors that have recently been described for several of them and discuss their potential impact on future disease treatment.

2. Biology

2.1. BET subfamily

BET proteins are related to the *Drosophila fsh* gene, which plays a major role in fly development. They derive their name from the presence of two related tandem bromodomains named BD1 and BD2, and of a unique extra-terminal region in the C-terminal moiety. BET proteins are found in the cell nucleus, where they bind via their two bromodomains to acetylated proteins, including histones H3 and H4. Detailed investigations revealed important differences in the binding preferences of individual BET proteins to acetylated histone motifs.[16] BET proteins also possess regions weakly reminiscent of kinase motifs, hence their additional classification as atypical kinases.[19] They are ubiquitously expressed (except the BRDT form, see below) and play essential roles in epigenetic memory and in maintaining chromatin architecture. They regulate cell-cycle progression by controlling gene transcription. Interactions with various viral proteins have furthermore been reported. Four BET members are found in humans: BRD4, which acts as a mitotic bookmark and transcription facilitator; BRD3, which binds

to acetylated GATA1 and regulates erythroid target genes; BRD2, a regulator of body energy balance; and BRDT, which is expressed selectively in the testis and is essential for spermatogenic gene expression. The unique function of each BET protein is furthermore evidenced by the very different phenotypes of knock-out animals. BRD4-deficient mice die shortly after implantation, whereas heterozygous animals exhibit severe growth defects due to decreased cell proliferation.[20] BRD2-deficient mice die early during embryogenesis.[21] Surprisingly, hypomorphic animals with a mutated BRD2 promoter develop severe obesity, however with low blood glucose levels and elevated glucose tolerance.[22] BRDT knock-out mice are viable, but have major defects in spermatogenesis and are sub-fertile. Data on BRD3-deficient mice have not been published yet. The recent identification of potent inhibitors (see below) has helped tremendously in the deeper understanding of the BET protein family.

2.1.1. BRD4

BRD4 is the best-studied member of the BET family. There are three isoforms expressed in humans: one long isoform of 1362 residues, and two shorter isoforms (722 and 796 residues) that differ by a unique 75-residue coding exon at the C terminus.[23] The corresponding gene maps to chromosomal region 19p13.1. BRD4 is involved in epigenetic memory as a bookmark that remains attached to chromosomes during cell division and allows rapid reactivation of target genes immediately after mitosis completion.[24] The 722-residue short isoform of BRD4 interacts directly with the acetylated cyclin T1 subunit of the positive transcription elongation factor b (P-TEFb). However, predominantly via its P-TEFb interacting domain (PID), the BRD4 long isoform binds to the P-TEFb complex, releasing HEXIM1 and 7SK snRNA from the complex, thus leading to the formation of an active form.[25] The active complex stimulates transcription elongation by phosphorylation of the C-terminal end of RNA polymerase II by cyclin-dependent kinase 9 (CDK9). BRD4 furthermore interacts with the mediator complex, the replication factor C, and signal-induced proliferation-associated protein 1,[26] as well as with a number of viral proteins.[27] Genome-wide studies indicate that BRD4 binding correlates with gene expression and that beside promoter regions, intergenic and intragenic regions are also recognized.[28] BRD4 binding sites are also concentrated in large super-enhancer regions characteristic of oncogenic drivers.[29] Recent studies with the *Drosophila* homologue indicate that the short isoform is responsible for transcriptional regulation, whereas the long isoform co-localizes with insulator proteins and is important for chromatin architecture.[30]

BRD4 interacts with different transcription factors to regulate downstream gene expression. It binds to acetylated RelA, thus leading to stabilization of nuclear NF-κB and expression regulation of genes involved in inflammatory processes.[31] Association with the N-terminal region of the retinoid acid receptor α for regulation of a discrete set of genes,[32] and with p53 leading to regulation of p21 expression, have also been observed.[33] In addition, BRD4 interacts with several chromatin modifiers, including the histone methyltransferase NSD3 and the hydroxylase JMJD6.[34] BRD4 is an important regulator of the expression of genes involved in S-phase progression. BRD4 target genes such as c-Myc, c-Fos, aurora B, cyclin D1, and cyclin D2 are all involved in cell-cycle control.[29,33,35] It was shown very recently that BRD4 has an essential role in regulating LMO2, which is involved in erythroid differentiation.[36] Interestingly, blocking BRD4 function often results in only a transient down-regulation of target genes, and normal levels are observed at 24 h or later. Recent data show that the short BRD4 isoform also participates in DNA damage signaling.[23] It interacts with the condensin complex and attenuates H2AX phosphorylation, thus modulating the response to DNA damage. Finally, BRD4 is also implicated in the regulation of the apolipoprotein A1 (ApoA1) gene,[37] thereby modulating the levels of high-density lipoproteins, which are involved in the pathology of arteriosclerosis.[38]

Different regions essential for BRD4 function have been described (Figure 1). Both bromodomains are required for recognition of acetylated histones and other proteins. Concerning the interaction with the P-TEFb complex, it was found that the BD2 region binds to tri-acetylated cyclin T and that the extreme C-terminal region containing the essential PID makes contact with CDK9 and cyclin T1.[39] Importantly, the PID is absent in the short BRD4 isoforms.[25b] Interaction with replication factor C and signal-induced proliferation-associated protein 1 necessitates BRD4 BD2.[26b] A 12-residue-long region acting as nuclear localization signal (NLS) is located between both bromodomains.[40] It is part of the previously defined motif A.[41] Motif B contains a dimerization signal essential for the function of BRD2 and is conserved in BRD4.[42] It may mediate homodimerization as well as heterodimerization between BET proteins. The extra-terminal region is recognized by sever-

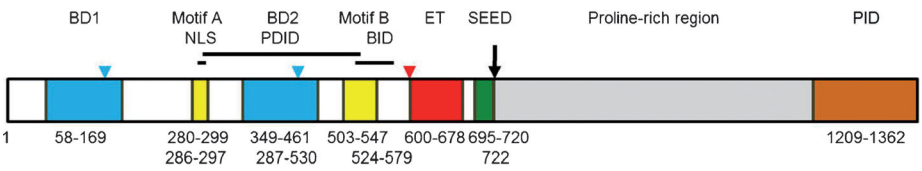


Figure 1. Schematic representation of human BRD4 protein. The domains discussed in the text are shown as colored boxes with their respective locations indicated below. The nuclear localization signal (NLS), the acidic phosphorylation-dependent interaction domain (PDID) and basic residue-enriched interaction domain (BID) are overlined, and their limits indicated in the lower line. The black arrow shows the C-terminal end of the short isoform with the exact location given below. The blue arrowheads show the location of the essential asparagine residues involved in hydrogen bonding in the bromodomains, and the red arrowhead points to a putative ATP binding site.

al proteins involved in epigenetic modifications, including NSD3 and JMJD6.[34] Studies with the yeast orthologues show that this region interacts with the general transcription factor TFIID.[43] The serine-rich SEED motif is found next to the extra-terminal region and is needed for binding of BRD2 to transcription factors of the E2F family.[44] The large proline-rich region is located in the C-terminal moiety and plays a role in mesenchymal transition.[45] Recently, two regions involved in the interaction with p53 were identified: the basic residue-enriched interaction domain BID and the acidic phosphorylation-dependent interaction domain PDID, which contains several putative phosphorylation sites.[33] Point mutations introduced into each bromodomain allowed the identification of individual amino acids implicated in BRD4 function. Biochemical and cellular assays show that N140 and N433 in the first and second bromodomain, respectively, which form hydrogen bonds with acetyllysine, as well as Y139 and Y432, are essential for chromatin interaction.[46] A putative ATP binding site has been described, in line with the potential kinase activity of BET proteins.[19]

The understanding of the function of BRD4 was much improved by the identification of potent BET-selective inhibitors such as (+)-JQ1 ((+)-**1**) and I-BET762 (**2**) (Figure 2).[47] Crystal structures show these inhibitors to mimic the acetyllysine group and to engage in similar interactions with individual amino acids.[47a,48] The studies also demonstrate that BRD4 is implicated in a number of diseases. Chromosomal translocation leading to the expression of a fusion between BRD4 (or BRD3) and the nuclear protein in testis (NUT) causes a rare form of cancer known as NUT midline carcinoma (NMC).[49] Patient-derived NMC tumors are inhibited by BET-selective compounds in in vivo xenograft models.[47a,50] BRD4 also plays a crucial role in a number of hematological tumors, including acute myeloid lymphoma,[51] acute lymphoblastic leukemia,[52] lymphoma,[35e] and multiple myeloma.[35b] Furthermore, a role in solid tumors such as neuroblastoma,[53] glioblastoma,[54] lung cancer,[55] and melanoma[21] has been reported. The oncogenic role of BRD4 is due, at least in part, to its role in regulating the expression of a number of genes involved in the cell cycle, including c-Myc and cyclin D1.[35d,e,56] Apoptosis is furthermore observed in cells treated with an inhibitor; however, higher doses are required.[51b,56]

Clinical studies have recently been initiated with different BET inhibitors to evaluate their effect on NMC and hematological tumors. Another pathology in which BRD4 is implicated is inflammation, as evidenced by the protective role of the BET inhibitor I-BET762 (**2**) against endotoxic shock and sepsis.[47b] Another BET inhibitor named MS417, which is structurally almost identical to (+)-**1** (having a methyl ester instead of a *tert*-butyl ester moiety), decreased the expression of pro-inflammatory genes in tumor necrosis factor α (TNFα)-treated kidney cells.[57] Recently, a role of BRD4 in cardiac hypertrophy has also been proposed.[58] Finally, hijacking of BRD4 activity is essential for the life cycle of a number of viruses, including herpes, papilloma, and the murine leukemia viruses.[59] These viruses take advantage of the retention of BRD4 to the host mitotic chromosomes for their propagation during cell division.

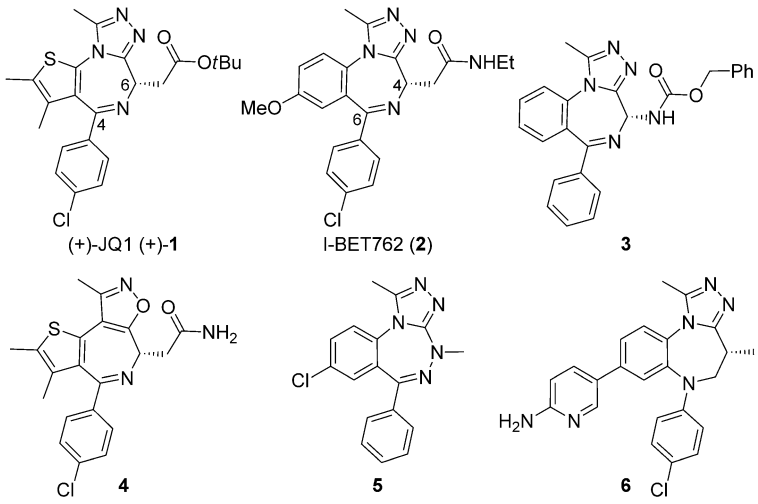


Figure 2. Methyltriazolodiazepine BET bromodomain inhibitors and structurally related compounds reported to date.

2.1.2. BRD3

BRD3 is a 726-residue protein encoded by a gene located at chromosome position 9q34. It was originally reported to bind to genome regions that are hyperacetylated.[60] It interacts with GATA1, a transcription factor with an essential role in hematopoiesis.[61] Binding of BRD3 to GATA1 is observed both at active and inactive gene regions, and independent of the acetylation status of histones. The interaction requires acetylation of GATA1 and can be abrogated by a point mutation in the first bromodomain of BRD3, demonstrating the high selectivity of this protein–protein interaction. Like other BET proteins, BRD3 interacts with a number of viral proteins.[62]

2.1.3. BRD2

BRD2 was originally identified under the name RING3 as a mitogen-activated nuclear kinase.[63] There are three main isoforms of 836, 801, and 754 residues, and the corresponding gene maps near the major histocompatibility complex genes at 6p21.3.[64] It is present in several multiprotein transcriptional complexes that associate with hyperacetylated chromatin regions and are involved in cell-cycle progression.[60] In line with this, transgenic mice that overexpress BRD2 in the lymphoid compartment develop lymphoma and leukemia.[65] Preferential binding of BRD2 to acetylated H4 and H2A.Z histones has been reported.[66] BRD2 interacts with E2F transcription factors through a serine-rich region.[44] BRD2-containing complexes regulate a number of genes involved in cell proliferation including cyclin A and cy-

clin D1,[60,67] and also androgen receptor (AR) target genes.[66] An implication in the inflammatory response of macrophages via direct binding to regulatory regions of cytokine genes has furthermore been evidenced.[68] BRD2 is also involved in neuronal differentiation via the E2F1 pathway.[69] A role in epilepsy has been proposed, based on linkage studies and mice models.[70] However, another study did not show a link between BRD2 and photosensitive epilepsy.[71] As mentioned above, BRD2 hypomorphic mouse models are severely obese, but are protected from glucose intolerance and insulin resistance.[22,72] This may be linked to epigenetically deregulated expression of genes involved in energy metabolism. An interaction between BRD2 and proteins from different viruses including the mouse herpes and human immunodeficiency viruses has also been reported.[62,73]

2.1.4. BRDT

The gene encoding the 947-residue-long BRDT protein maps to chromosome region 1p22.1.[74] It is specifically expressed in testis and plays an essential role in compacting the sperm genome.[75] It binds to hyperacetylated H4 via its tandem bromodomains and is involved in the chromatin remodeling that takes place during spermiogenesis.[76] It controls meiotic divisions and post-meiotic repackaging of the genome.[77] In addition, BRDT regulates spermatogenic gene expression by activating or repressing a number of essential genes. Ablation of the BRDT BD1 region leads to disrupted spermiogenesis and sterility in mice.[78] Interestingly, a number of RNA-splicing genes are deregulated in the round spermatids of these animals. Together with the observed interaction between BRDT and proteins involved in transcript maturation, this indicates that beside the regulation of transcription, BRDT plays an additional role in mRNA alternative splicing.[78,79] The crystal structure of BRDT BD1 reveals that it cooperatively recognizes two histone acetylation marks.[80] This may also be true for other bromodomains, suggesting that combinatorial readout of histone marks may be a general feature of acetylation readers.

2.2. Non-BET bromodomain proteins

2.2.1. ATAD2

ATAD2 (also known as ANCCA) is a highly conserved protein that belongs to the structurally highly diverse "ATPase associated with diverse cellular activities" family.[81] The ATPase region is necessary for protein multimerization,[82] but little more is known about its function. The C-terminally located bromodomain binds to several acetylated lysines on histones, including H3K14ac[83] and H4K5ac,[82] allowing interaction with chromatin and transcriptional regulation of target genes.

ATAD2 regulates the function of several transcription factors. It is a co-activator of the AR, thus stimulating the expression of a number of androgen target genes in prostate cancer cells.[84] ATAD2 expression is itself stimulated by androgens,[85] and this is mediated by an AR binding site located in the distal enhancer region and by an E2F1 binding site in the promoter region of the ATAD2 gene, leading to cooperative activation by androgens.[84a] In addition, ATAD2 regulates the expression of the H3K27 methyltransferase EZH2, which is one of the most strongly up-regulated genes in prostate cancer.[85] Similar to androgens, estrogens also stimulate ATAD2 expression, and ATAD2 is a co-activator of the estrogen receptor (ER), which is essential for the recruitment of further co-activators and for histone hyperacetylation at estrogen target genes.[86] ATAD2 also binds to the c-Myc protein to stimulate its activity.[87] Co-amplification of the ATAD2 and c-Myc genes which co-localize to the 8q24.12 locus and concomitant overexpression have been reported in several tumor types. This leads to the stimulation of downstream genes related to cell proliferation and resistance to apoptotic response,[87,88] thereby implying an important function in tumor growth.

A role of ATAD2 in cancer is furthermore substantiated by knock-down experiments. ATAD2 silencing in vitro is followed by cell proliferation arrest,[84b] elevated sensitivity to cell-damaging agents, and increased apoptosis.[82] Conversely, ATAD2 overexpression leads to uncontrolled cell proliferation and loss of a normal apoptotic cell response. In line with this, ATAD2 is highly expressed in different tumors, including lung, breast, and endometrial cancer.[81,82,88,89] In smokers, a driver function for ATAD2 in lung cancer has been postulated.[88a] The related ATAD2B form is also overexpressed in tumors,[90] but little is known about its precise function.

Altogether, these results point to an important role of ATAD2 in reprogramming the genome toward a malignant phenotype.[81] It alters chromatin dynamics, gene transcription programs and the apoptotic response, thereby contributing to multiple oncogenic processes. Selective inhibitors would further help in the understanding of the biological function of ATAD2, but have not yet been described.

2.2.2. PCAF

The p300/CBP-associated factor PCAF (also named KAT2B) possesses a lysine acetyltransferase function and contains a bromodomain, thereby combining a writer and reader function in chromatin modification. It is part of a larger HAT complex with an unusual catalytic mechanism.[91] It modifies histone H3, mainly at position K9, and histone H4,[92] thus regulating the expression of numerous genes, including p21[93] and insulin.[94] A role in controlling the expression of ribosomal genes and miRNAs has also been reported.[95] The PCAF bromodomain binds to the same acetyllysine position that is targeted by the HAT region for acetylation.[96] By this, PCAF is poised to spread the acetyl mark to neighboring histones, thereby reinforcing transcriptional activation by a feed-forward mechanism.[97]

Beside histones, PCAF acetylates a number of transcription factors including c-Myc,[98] β-catenin,[99] FOXO1,[100] Fli1,[101] and p53,[102] with different outcomes. It also acetylates p27, thus affecting the stability of this cell-cycle inhibitor.[103]

PCAF possesses E3 ubiquitin ligase activity, enabling it to ubiquitylate Hdm2[104] and Gli1.[105] Hdm2 participates in the regulation of p53 levels, giving PCAF an additional, albeit indirect, involvement in p53-mediated cell-cycle regulation.

PCAF overexpression is observed in central nervous system tumors,[106] but conversely, decrease expression is observed in esophageal[107] and gastrointestinal[108] cancers. A role of PCAF in cell proliferation and invasion, and in drug resistance has been reported in urothelial cancer cells.[109] Also, the overexpression of PCAF increases drug resistance of cisplatin-resistant cells, via stimulation of E2F1 expression.[110] Inhibition of PCAF acetyltransferase activity using high concentrations of isothiazolone ultimately leads to a decrease in proliferation of colon and liver cancer cells.[111] Blocking bromodomain-mediated interaction with chromatin and transcription factors has the potential to block PCAF function as well. This has been successfully shown with arylpropane-diamine compounds for preventing interaction with the acetylated HIV Tat protein.[112]

2.2.3. CREBBP and EP300

CREBBP (KAT3A) and EP300 (also named p300 and KAT3B) are general transcriptional co-activators with lysine acetyltransferase activity. They are highly homologous, share a similar domain organization, and possess partly overlapping functions. Both proteins are characterized by several zinc finger domains, a bromodomain followed by a plant homology domain (PHD) and a HAT domain, and a CREB binding domain.[113] They bind to multiple partner proteins, mainly transcription factors and other co-activators (reviewed by Wang et al.[113b]) and also to several acetyllysines in histones.[96,114] Binding preferences for acetylated histones are different between CREBBP and EP300, as observed in peptide binding assays.[16] CREBBP and EP300 play essential roles in histone acetylation, mainly at histone H3 positions K18 and K27.[114] Genome-wide studies show that CREBBP and EP300 bind to active gene regions with high histone acetylation but also to repressed regions, suggesting that these proteins may mediate the switch between both chromatin states.[115] A model for EP300 auto-acetylation and substrate acetylation, based on the crystal structure of the catalytic core, has recently been proposed.[116] CREBBP and EP300 are important in DNA replication and repair, and also for regulation of cell growth and genomic stability.[117] They are involved in the control of genes from a number of signaling pathways.[118]

Depending on the context, CREBBP and EP300 may act as oncogenes or tumor suppressors.[113b,119] Their deregulation has been associated with tumor growth. For instance, chromosomal translocations giving rise to gene fusions between MOZ or MLL and CREBBP or EP300 and including their respective bromodomains are reported in leukemias and lymphomas.[113b,120] In vitro experiments show that CREBP/EP300 is involved in cell-cycle regulation[121] and in senescence.[122] As co-activators of transcription factors such as c-Myc and AR, they may be implicated in various tumors including prostate and colon cancer.[123] Recently, an activating role of EP300 on T regulatory cell function linked to tumor immunity has been evidenced.[124] Finally, an implication in drug resistance has been reported.[125] A role as tumor suppressor is suggested by several observations.[114] Patients with the Rubinstein–Taybi syndrome have germline mutations of CREBBP or EP300 and an increased incidence of hematological tumors.[126] Truncating EP300 mutations were found in primary samples or cell lines from breast, colon, and pancreatic cancer.[127] It was also reported that heterologous CREBBP-deficient mice develop hematologic malignancies.[128]

Given that the acetyltransferase activity is critical for the function of CREBBP and EP300, first inhibitors were designed to disrupt their catalytic function (for reviews, see Wang et al.,[113b] Dekker and Haisma,[119b] and Heery and Fischer[129]). Suppression of histone acetylation and of cell proliferation was observed after treatment with various HAT inhibitors and in vivo efficacy reported in breast cancer xenografts after treatment with one compound.[130] Since then, and with the knowledge that chromatin binding domains can be successfully targeted for small-molecule inhibition, inhibitors of the bromodomains of CREBBP and EP300 have been generated (see below).

2.2.4. BAZ family

Four members form the BAZ protein family: BAZ1A, BAZ1B, BAZ2A, and BAZ2B.[131] They contain a PHD finger located near a bromodomain in their C-terminal moiety, as well as a WAKZ motif and several additional conserved regions. The presence of a WAC motif in BAZ1A and BAZ1B, and of a ZB2 motif in BAZ2A and BAZ2B led to a further sub-classification. The distribution of BAZ transcripts is ubiquitous, however, with considerable differences in expression levels.

BAZ1 proteins are found in chromatin-remodeling complexes. BAZ1A (hACF1) is a component of the ACF nucleosome-remodeling complex which is involved in the chromatin assembly process and in the regulation of nucleosome spacing.[132] The ACF complex furthermore interacts with KU70 and is needed for DNA double-strand break repair and for G_2M damage checkpoint.[133] BAZ1B (WSTF) was originally identified due to its implication in the development disorder pathology named Williams syndrome.[134] Indeed, some of the defects reported in Williams syndrome are found in BAZ1B-deficient mice.[135] BAZ1B belongs to a chromatin-remodeling complex that mediates the activity of the vitamin D receptor.[135] BAZ1B also has tyrosine kinase activity toward H2A.X and is involved in the DNA damage response.[136] Additional functions of BAZ1B in chromatin assembly, RNA polymerase I and III gene regulation or DNA repair, depending on the protein complex to which it belongs, have been described.[137]

BAZ2 proteins may be involved in deregulated miRNA pathways in cancers of the prostate and ovary,[138] as well as in chronic lymphocytic leukemia.[139] BAZ2A (also called TIP5 and TTF5) is a component of the nucleolar remodeling complex NoRC.[140] The ATPase SNF2h and BAZ2A are the main components of this complex, which is essential for the silencing of rRNA expression. Interaction of NoRC with the DNA regions coding for rRNA modifies the local histone marks and increases DNA methylation, thus leading to transcriptional shutdown. Binding of the BAZ2A bromodomain to acetylated nucleosomes and cooperation with the neighboring PHD finger which binds to other chromatin modifiers including DNMTs, HDAC1 and SNF2h, are necessary for NoRC function.[141] Different BAZ2A regions play a role in nuclear matrix association, nu-

cleolar targeting, and DNA binding.[142] In addition, the NoRC complex is implicated in the upkeep of high-order chromatin structure at telomere and centromere regions of chromosomes.[140a] In line with this, silencing of BAZ2A expression is followed by increased recombination between telomere repeats and chromosomal translocations.[143] Very little is known about BAZ2B, although the histone binding preferences of the bromodomain have recently been described.[144] A recent genome-wide association study showed the BAZ2B locus is associated with sudden cardiac death.[145]

2.2.5. TRIM24, TRIM28, and TRIM33

TRIM24 (TIF-1α), TRIM28 (KAP1, TIF-1β), and TRIM33 (TIF-1γ) belong to the TIF1 family which is characterized by an N-terminal tripartite motif, a nuclear receptor interaction box, and a C-terminal PHD-bromodomain region.[146] The tripartite motif comprises a RING finger domain also found in other E3 ligases, followed by one or two B-boxes and a coiled-coil region. PHD is a common motif in chromatin-associated proteins, and is often a site of histone binding or other protein–protein interactions. TRIM24, TRIM28, and TRIM33 mainly act as transcriptional repressors, a function which requires the PHD-bromodomain as well as the E3 ligase functions. A tumor-suppressor role in liver cancer has been reported, based on a knock-out mouse model.[147] Homodimers as well as heterodimers and heterotrimers of various TRIM proteins have been purified.[147,148]

TRIM24 contains a bromodomain with the characteristic acetyllysine binding function that specifically contacts H3K23ac.[149] The neighboring PHD region interacts with unmodified H3K4me0 within the same nucleosome, making the two domains a single histone H3 binding module. TRIM24 overexpression contributes to faulty cell-cycle regulation and uncontrolled cell proliferation. It binds to the ER and the AR, leading to transcription stimulation of estrogen- or androgen-dependent genes.[149,150] TRIM24 is believed to recruit the ER following an interaction with acetylated, unmethylated chromatin. Subsequently, estrogen-dependent genes are up-regulated, enhancing cell proliferation and tumor progression. Elevated levels of TRIM24 are associated with poor prognosis in breast cancer.[149,151] High TRIM24 expression is furthermore observed in head and neck squamous cell carcinoma,[152] acute myeloid leukemia,[152] non-small-cell lung cancer,[153] and sporadic colorectal cancers. In addition, TRIM24 negatively regulates p53 stability through ubiquitylation.[154] Knock-down of TRIM24 expression leads to apoptosis, cell-cycle arrest, and/or decreased cell proliferation likely by these and perhaps other mechanisms in several tumor types.[152–154] On the other hand, studies performed with knock-out mice suggest that TRIM24 acts as a tumor suppressor in the liver in the context of retinoic acid signaling.[147,155] Indeed, a ligand-dependent interaction of TRIM24 with the retinoic acid receptor has been shown.[156] Interestingly, sexual dimorphic expression of the TRIM24 gene was observed in mouse liver,[157] possibly linked to its regulation by the female-specific liver transcription factor CUX2.[158]

TRIM28 is found in a number of chromatin-remodeling complexes, but does not directly bind to DNA.[159] Its atypical bromodomain shows no interaction with acetylated histones. It is known to interact with KRAB-ZNF proteins, a very large family of zinc finger proteins that regulate numerous cellular processes. Interestingly, TRIM28 binds to the 3′ ends of KRAB-ZNF genes without affecting their expression levels.[160] Several studies indicate that TRIM28 acts as a transcription regulator, mostly as a repressor. Following binding of TRIM28 to promoter regions, RNA polymerase II is removed, and active chromatin marks are erased while a repressive H3K9me3 mark is written. This necessitates sumoylation at several lysine residues of the bromodomain mediated by the neighboring PHD region.[161] TRIM28 negatively regulates IRF7 by sumoylation.[162] In addition, TRIM28 has important functions not linked to transcriptional regulation. It regulates apoptosis by promoting p53 ubiquitylation, is involved in DNA repair following its phosphorylation by ATM, and may suppress DNA recombination. Overexpression of TRIM28 in gastric cancer has been reported.[163] Conversely, a tumor suppressor role in early lung cancer has been postulated.[164]

TRIM33 binds to chromatin preferentially at unmodified H3K4, methylated H3K9, and acetylated H3K18 and H3K23.[165] After sumoylation at specific lysine residues, it ubiquitylates SMAD4 to repress its activity.[166] TRIM33 is reported to have tumor suppressor functions and has roles in DNA repair.[167] It is also involved in hematopoiesis by controlling the expression of erythroid genes.[168]

3. Bromodomain inhibitors

3.1. BET inhibitors

3.1.1. Introduction

Since 2010, most of the work on developing small-molecule bromodomain inhibitors has focused on the BET family of bromodomains. In addition to the diseases discussed, potential use of (+)-JQ1 ((+)-**1**) as a male contraceptive has been postulated.[169]

I-BET762[170] (**2**, Figure 2) from GlaxoSmithKline (for the treatment of NMC) as well as OTX015[171] (for the treatment of hematological malignancies; structure not yet disclosed) from OncoEthix (in-licensed from Mitsubishi Pharmaceuticals) and CPI-0610[172] from Constellation Pharmaceuticals (for the treatment of lymphomas; structure not yet disclosed) have entered clinical trials, underscoring the therapeutic potential of this class of inhibitors. The first BET bromodomain inhibitors were published almost simultaneously by Filippakopoulos et al.[47a] ((+)-JQ1, (+)-**1**) and Nicodeme et al.[47b] (I-BET762, **2**). Since then, a number of different chemotypes have been described as BET-selective bromodomain inhibitors, and are discussed in greater detail in the following sections along with recent developments from the patent literature. A representative selection of reported inhibitors can be classified into the following structurally diverse main classes: triazolodiazepines[47a,b,48b] **1**–**3** and structurally related compounds (triazolotriazepines[46b] **5**, isoxazoloazepines[173] **4**, and triazolodihydrodiazepines[174] **6**;

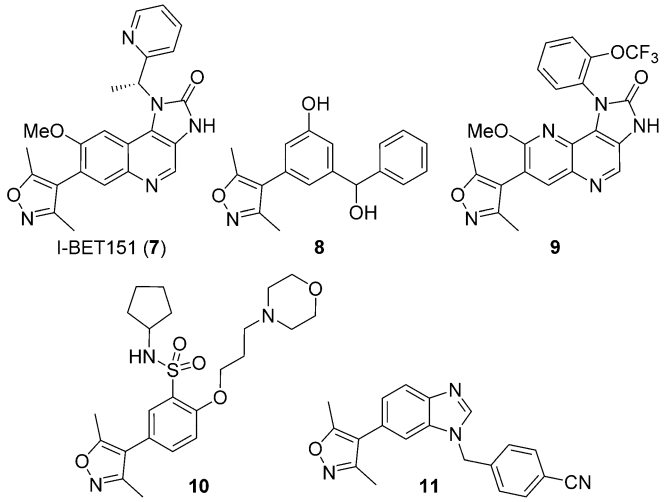


Figure 3. 3,5-Dimethylisoxazole BET bromodomain inhibitors reported to date.

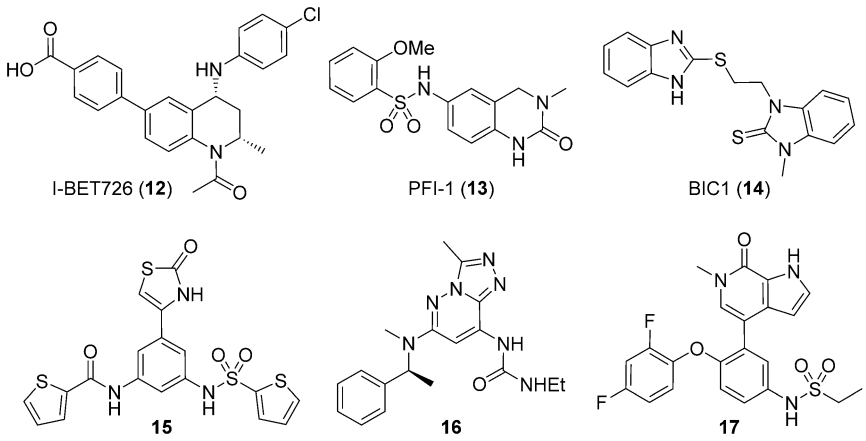


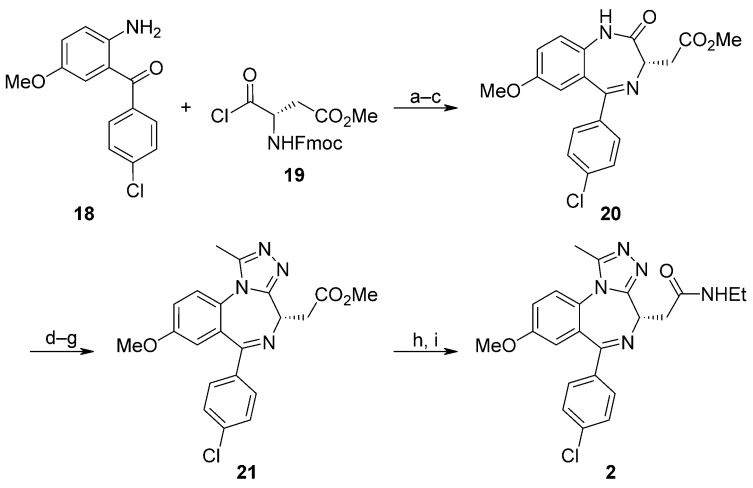
Figure 4. Other chemotypes reported as BET bromodomain inhibitors including 1-acyltetrahydroquinolines[176] (**12**), 3-methyldihydroquinazolinones[56,177] (**13**), benzimidazoles[178] (**14**), 2-thiazolidinones[179] (**15**), triazolopyridazines[180] (**16**), and pyrrolopyridinones[181] (**17**).

Figure 2), 3,5-dimethylisoxazoles[37,51a,175] **7**–**11** (Figure 3), 1-acyltetrahydroquinolines[176] **12**, 3-methyldihydroquinazolinones[56,177] **13**, benzimidazoles[178] **14**, 2-thiazolidinones[179] **15**, pyrazolopyridazines[180] **16**, and pyrrolopyridinones[181] **17** (Figure 4).

3.1.2. Methyltriazolodiazepines and structurally related chemotypes

Methyltriazolodiazepine **3** (Figure 2) was one of the first selective BET bromodomain ligands and was discovered by using a phenotypic screen to identify stimulators of ApoA1 expression, the up-regulation of which is associated with protection from atherosclerosis progression and anti-inflammatory effects.[47b,48b] Compound **3** was identified in a luciferase reporter-based screen and showed an EC_{50} value of 440 nM for the induction of the ApoA1 reporter gene. The potency and pharmacokinetic properties required for in vivo efficacy were further optimized by structure–activity relationship (SAR) investigations, leading to compound **2** (Figure 2) with an improved EC_{50} value of 700 nM in the reporter gene assay.[48b] SAR investigations revealed the importance of the benzodiazepine core and of the correct stereochemistry at the benzodiazepine 4-position for a high activity. All active analogues contained an aryl group at the benzodiazepine 6-position, and a variety of groups were tolerated at the 4-position, making this a useful moiety for the modification of physicochemical and pharmacokinetic properties. However, the molecular target was still unknown and could not be identified by a screen against a panel of known drug targets such as kinases, ion channels, nuclear receptors, or G-protein-coupled receptors. Following a chemoproteomic approach, the cellular targets of compounds **2** and **3** were identified.[48b] Lysates from HepG2 human liver carcinoma cells were passed over an affinity matrix of the active analogue **2** as well as a control matrix with an inactive analogue. After extensive washing, a defined pattern of proteins was identified by affinity chromatography. These were retained on the active matrix, but were absent on the control matrix. LC–MS/MS analysis revealed the BET bromodomain proteins BRD2, BRD3, and BRD4 as molecular targets of **2**, and the potency of **2** was confirmed in a fluorescence resonance energy transfer (FRET) assay (IC_{50} = 36 nM, BRD4(BD1)) and by isothermal titration calorimetry (ITC) (K_D = 55 nM, BRD4(BD1)).[48b] No affinity for other bromodomains was observed for compound **2** by ITC.[48b] It was shown that **2** suppressed the expression of key pro-inflammatory genes in an in vivo mouse model.[48b] In addition, it was demonstrated that compound **2** inhibited cell growth in a panel of tumor cell lines.[48b] As described very recently, I-BET762 (**2**) shows a good preclinical profile, including high in vitro permeability (167 nm s^{-1}), high solubility in physiologically relevant media (> 3 mg mL^{-1}), low potential to inhibit the major human cytochrome P450 isoforms in vitro (IC_{50} > 33 μM), and low in vitro clearance in liver microsomes and hepatocytes (CL_i < 1.7 mL min^{-1} g^{-1} liver; all species including humans).[47c,182] The pharmacokinetic profiles observed in mouse, dog, and primate, and predicted in humans, led to the selection of this compound for further characterization.[47c,182] In vivo studies demonstrated the efficacy of **2** in several tumor and immune-inflammatory models so that I-BET762 (**2**) recently progressed into the clinical development phase for oncology indications.[47c,170,182]

An enantioselective synthesis of **2** is shown in Scheme 1. The benzodiazepine core was formed by condensation of ben-



Scheme 1. Enantioselective synthesis of **2**.[47c,182] *Reagents and conditions*: a) $CHCl_3$, 60 °C; b) Et_3N, CH_2Cl_2, reflux; c) AcOH, 1,2-dichloroethane, 60 °C, 30%; d) P_4S_{10}, Na_2CO_3, 1,2-dichloroethane, 65 °C, 67%; e) H_2NNH_2, THF, 5–15 °C; f) AcCl, THF, 0 °C; g) AcOH, THF, RT, 86%; h) $NaOH_{(aq)}$, THF, RT, 89%; i) $EtNH_2$, HBTU, iPr_2NEt, THF, RT, 47%.

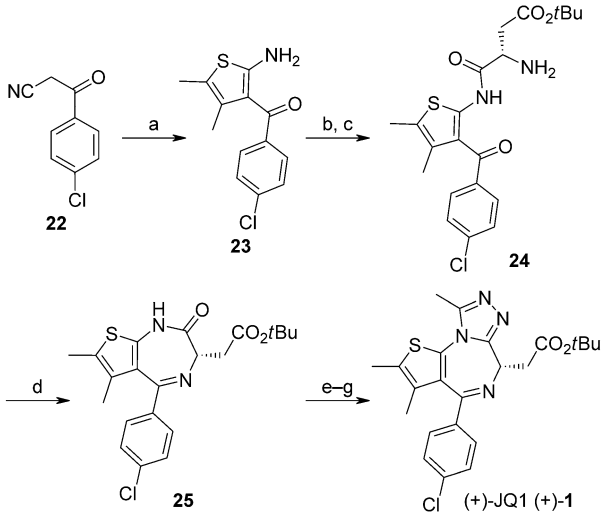
zophenone **18** with an acid chloride derivative of aspartate **19** followed by a four-step procedure to build up the methyltriazole ring. The thioamide of **20**, formed by the reaction of amide **20** with phosphorus pentasulfide, was reacted with hydrazine followed by acetyl chloride to form an intermediate acetylhydrazone, which was finally cyclized to triazole **21** in acetic acid. Saponification and amide formation under standard conditions afforded enantiopure **2**.

Another triazolodiazepine (+)-**1** was reported as a BET bromodomain-selective ligand.[47a] It is structurally related to compounds from a patent application filed by Mitsubishi Pharmaceuticals in which the inhibition of binding between an acetylated histone and bromodomain-containing protein BRD4 is described.[183] By applying molecular modeling studies based on the apo-crystal structure of the first bromodomain of BRD4, Filippakopoulos et al. designed the thienotriazolodiazepine (+)-**1** as a prototype ligand.[47a] The bulky *tert*-butyl ester was incorporated to avoid strong interactions with other known benzodiazepine binding proteins. Similar to **2**, only the *S* enantiomer (+)-**1** was active, as confirmed by ITC ($K_D = 49$ nM, BRD4(BD1)) and an AlphaScreen assay ($IC_{50} = 77$ nM, BRD4(BD1)). The selectivity of compound (+)-**1** for the BET bromodomain family was confirmed by differential scanning fluorimetry. Compound (+)-**1** showed antiproliferative effects in various tumor cell lines, and in vivo activity was demonstrated in mice bearing xenograft models of NMC. Further studies describe the efficacy of (+)-**1** in mouse models of metabolic disease[184] and of acute myeloid leukemia.[185] A potential application for male contraception was also discussed.[169a] Very recently, Shimamura et al. provided experimental evidence that (+)-JQ1 ((+)-**1**) exerts antitumor efficacy in a subset of non-small-cell lung cancer cells harboring the KRAS mutation.[186]

The synthesis of enantiomerically enriched (+)-**1** is shown in Scheme 2. In the first step, formation of aminothiophene **23** was achieved by condensation of 2-butanone and 4-chlorobenzylacetonitrile **22** in the presence of elemental sulfur. Amide formation with an L-aspartate derivative and subsequent deprotection of the Fmoc group under standard conditions yielded amine **24**, which was cyclized under mild conditions to form the diazepine core **25**. Conditions for the formation of the triazole ring were optimized to decrease racemization at this late stage of the synthesis. Compound **25** was transformed into a phosphorylimidate at low temperature to activate the amide for nucleophilic attack by acetic hydrazide. The initially obtained enantiomeric purity (90% *ee*) was increased to >99% by preparative chiral HPLC.

X-ray crystal structures of (+)-**1** and **2** in complex with BRD4(BD1) revealed nearly identical interactions, with the methyltriazole moiety binding to the acetyllysine (KAc) binding site of the protein (see Figure 5 for a simplified representation).[47a,b,187] This binding site comprises three distinct pockets, each forming key interactions with the endogenous ligands. The 3-methyl-1,2,4-triazole of (+)-**1** and **2** acts as an acetyllysine mimic, binding to the KAc binding pocket, which is conserved in the majority of the bromodomains.[48b] The methyl group of the triazole ring binds to a small hydrophobic pocket that is responsible for recognition of the acetyl methyl group of the native acetyllysine ligand. The two adjacent nitrogen atoms of the 1,2,4-triazole ring mimic the carboxyl group of acetyllysine by one hydrogen bond interaction with the side chain NH_2 group of a conserved asparagine residue (N140 in BRD4(BD1)) and another water-mediated hydrogen bond interaction with the hydroxy group of a conserved tyrosine residue (Y97 in BRD4(BD1); Figure 5).

Compound **2** forms an additional hydrogen bond between its amide NH and the carbonyl oxygen atom of N140 (Figure 5b).[48b] The fused aromatic ring of the triazolodiazepine



Scheme 2. Synthesis of enantiomerically enriched (+)-**1**.[47a] *Reagents and conditions*: a) 2-butanone, sulfur, morpholine, EtOH, 70 °C, 70%; b) Fmoc-Asp(O*t*Bu)-OH, PyBOP, iPr_2NEt, DMF, RT, 72%; c) piperidine, DMF, RT, 90%; d) SiO_2, toluene, 90 °C, 95%; e) KO*t*Bu, THF, −78 °C→RT; f) $POCl(OEt)_2$, THF, −78 °C→−10 °C; g) $AcNHNH_2$, *n*BuOH, 90 °C, 92% (90% *ee*).

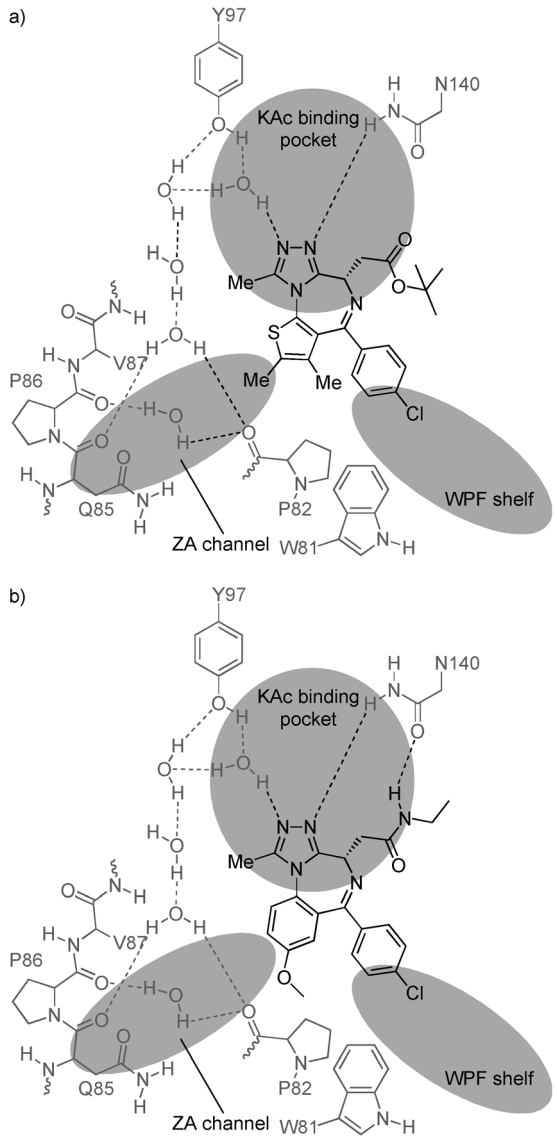


Figure 5. Simplified representation of the critical binding interactions of compounds a) (+)-**1** and b) **2** with BRD4(BD1) including the KAc binding pocket, the ZA channel, and the WPF shelf (in analogy to the graphical representation reported by Hewings et al.[187]).

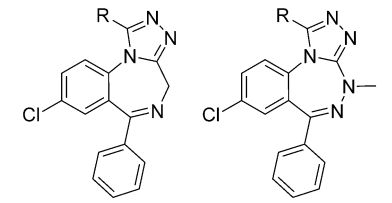


Figure 6. Structurally closely related marketed drugs alprazolam (**26**) and estazolam (**27**) as well as triazolobenzotriazepines **5**, **28**, and **29** reported by Filippakopoulos et al. as BET bromodomain inhibitors.[46b]

(dimethylthiophene for (+)-**1** and methoxybenzene for **2**) is directed into a lipophilic pocket called the ZA channel, which is not occupied by the native histone peptide. The 4-chlorophenyl group of (+)-**1** or **2** interacts with another hydrophobic region called the WPF shelf. All members of the BET family share this conserved motif, and it is believed that occupying this region is critical for good binding affinities as well as for gaining selectivity over non-BET bromodomains.[48b]

In a study by Filippakopoulos et al., several clinically approved benzodiazepine-containing drugs (e.g., **26** and **27**) as well as novel benzotriazepine compounds (e.g., **5**) were described as BET bromodomain inhibitors (Figure 6).[46b] In addition, the GABA receptor positive allosteric modulator etizolam (structure not shown) has been described very recently to show inhibitory activity against several BET bromodomains.[47c,182] The anxiolytic drug alprazolam **26** and benzotriazepine **5** bind to BRD4(BD1) with respective K_D values of 2.5 and 0.64 μM in ITC assays. Further SAR investigations underscored the importance of the 3-methyl substituent of the triazole for binding affinity, as its replacement with an ethyl or NH_2 group (compounds **28** and **29**) results in a loss of BET bromodomain binding activity.

Recently, a group from Constellation Pharmaceuticals reported a selective isoxazoloazepine BET bromodomain inhibitor (compound **4**) with good potency in biochemical (IC_{50} = 26 nM; BRD4(BD1) AlphaScreen) and cellular (IC_{50} = 140 nM; c-Myc expression) assays.[173] They identified aminoisoxazole **30** (Figure 7) by a fragment screen, as it showed potency in the

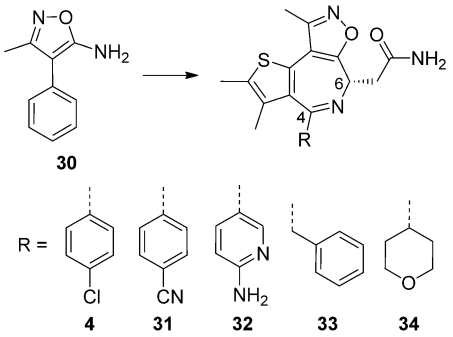


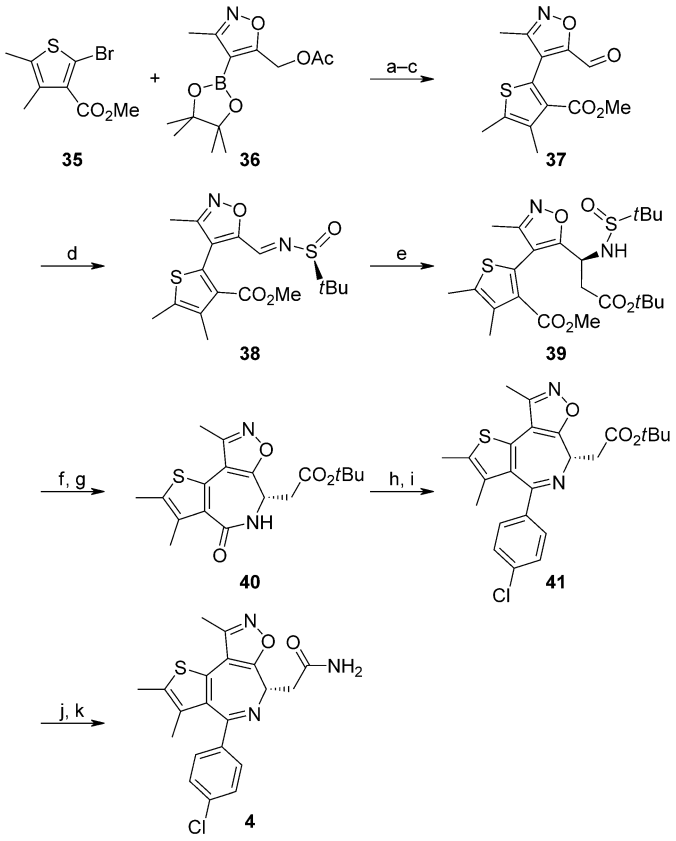
Figure 7. Fragment hit **30** and isoxazoloazepines **4** and **31**–**34** reported by Gehling et al. as BET bromodomain inhibitors.[173]

low micromolar range and successfully co-crystallized it with BRD4(BD1). Aminoisoxazole **30** made similar hydrogen bonding contacts within the KAc binding pocket, as already described for the methyl-1,2,4-triazole moiety of (+)-**1** and **2**. A similar 3,5-dimethylisoxazole motif was reported earlier as a KAc mimic, and is discussed in greater detail in the following section.[51a,175c,e] Replacement of the methyltriazole in (+)-**1** with the newly identified methylisoxazole led to compounds with biochemical and cellular activities similar to that of (+)-**1** (Figure 7, compounds **4** and **31**–**34**).

Several variations of the azepine side chain at the 6-position were tolerated. The 4-chlorophenyl substituent in the azepine 4-position could be replaced with other (hetero)aromatic residues while maintaining high activity (compounds **31**–**32**). However, a benzylic (**33**) or polar, saturated (**34**) residue was

not tolerated ($IC_{50} > 5$ μM, BRD4(BD1) AlphaScreen assay). Compound **4** showed an acceptable in vivo pharmacokinetic profile and inhibited c-Myc mRNA expression in an in vivo mouse model after oral application. As observed in the crystal structure of **4** in complex with BRD4(BD1), the isoxazole moiety forms a hydrogen bond with N140 and a water-mediated hydrogen bond with Y97, effectively mimicking the acetyllysine head group.

The synthesis of isoxazoloazepine **4** is shown in Scheme 3. Aldehyde **37** was synthesized in three steps starting with a Suzuki–Miyaura coupling of bromothiophene **35** with isoxazoloboronic ester **36** followed by deprotection of the acetate and oxidation of the resultant primary alcohol under Swern conditions to yield aldehyde **37**. Imine formation with (*S*)-*tert*-butylsulfinamide gave sulfinyl imine **38**, which was allowed to react with the Reformatsky enolate of *tert*-butylacetate. Sulfinamine **39** was obtained with modest diastereoselectivity (d.r. 7:1 to 5:1) in favor of the desired diastereomer. After deprotection of compound **39** under acidic conditions, the resulting amine was cyclized to the appropriate lactam **40**. Chlorination of the amide functionality to an imidoyl chloride and subsequent Suzuki–Miyaura cross-coupling with 4-chlorophenylboronic acid yielded the isoxazoloazepine **41**. Cleavage of the *tert*-butyl ester and amide formation under standard conditions afforded the desired compound **4**.



Scheme 3. Synthesis of **4** reported by Gehling et al.[173a] *Reagents and conditions*: a) $Pd_2(dba)_3$, SPhos, K_3PO_4, *n*BuOH, 100 °C; b) NaOMe (cat.), MeOH, 88%; c) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C→RT; d) $Ti(OEt)_4$, (*S*)-*tert*-butyl sulfinylamide, 91%; e) 2-(*tert*-butoxy-2-oxoethyl)zinc(II) chloride, NMP, −10 °C, 89% (d.r. 7:1 to 5:1); f) HCl, MeOH; g) *i*PrMgBr, THF, 80%; h) PCl_5, CH_2Cl_2, 65%; i) $Pd(PPh_3)_4$, K_2CO_3, 4-chlorophenylboronic acid, toluene/H_2O (10:1), 95 °C, 82%; j) TFA, $CHCl_3$, 36 °C; k) HATU, NH_4Cl, Et_3N, CH_2Cl_2, 80%.

In published patent applications a number of BET bromodomain inhibitors were reported which share the central triazolodiazepine core of (+)-**1** and **2** or are structurally related to them. A group from Constellation Pharmaceuticals reported compounds with a spiro-cyclopropane or -cyclobutane substituent at the 6-position of the triazolodiazepine (compounds **42**–**48**, Figure 8) with IC_{50} values < 500 nM (BRD4(BD1), AlphaScreen assay).[188]

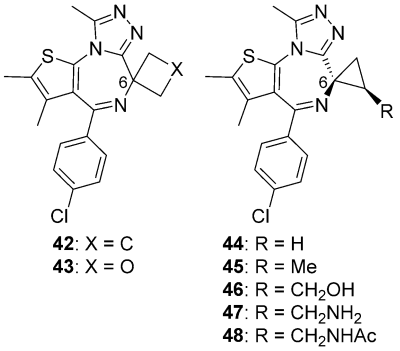


Figure 8. 6-Spiro-substituted triazolodiazepines **42**–**48** reported as BET bromodomain inhibitors in a patent application from Constellation Pharmaceuticals.[188]

Another patent application from Constellation Pharmaceuticals claims compounds with a dihydrobenzodiazepine core (Figure 9) as potent BET bromodomain inhibitors, which showed high activity in cell-based assays (c-Myc mRNA quantification assay and cell-based interleukin 6 (IL-6) quantification assay).[174] A selection of the most potent compounds (IC_{50} < 100 nM, BRD4(BD1) AlphaScreen assay) bears a methyl sub-

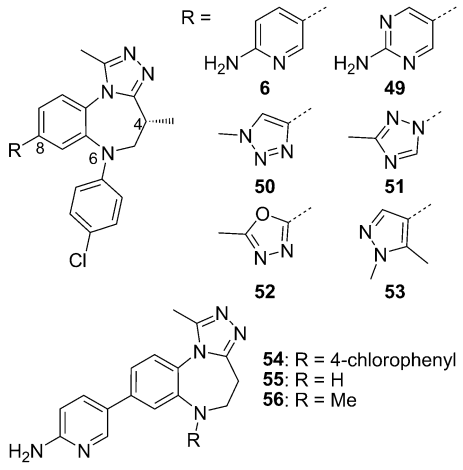
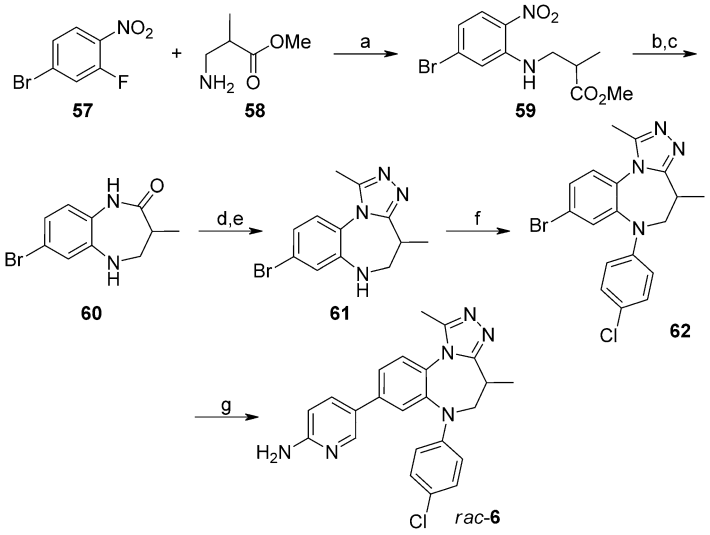


Figure 9. Dihydrobenzodiazepines **6** and **49**–**56** reported as BET bromodomain inhibitors in a patent application from Constellation Pharmaceuticals.[174]

stituent at the dihydrobenzodiazepine 4-position, and a variety of heteroaromatic moieties were tolerated at the 8-position (compounds **6** and **49**–**53**, Figure 9). Compounds without any substitution at the dihydrobenzodiazepine 4-position showed high activities ($IC_{50} < 100$ nM, BRD4(BD1) AlphaScreen assay) as well (compound **54**, Figure 9), whereas a 4-chlorophenyl group or other aromatic residues at the 6-position proved to be beneficial for high BRD4 affinity (compounds **55**–**56** with $IC_{50} > 1$ μM, BRD4(BD1), Figure 9).

A racemic synthesis of one representative compound is shown in Scheme 4. A nucleophilic aromatic substitution between amine **58** and aryl fluoride **57** afforded compound **59**. Reduction of the aromatic nitro group and subsequent cyclization resulted in the formation of tetrahydrobenzodiazepinone **60**. Methyltriazole **61** was built up by the reaction of the intermediate thioamide with acetic hydrazide, and the 4-chlorophenyl substituent was introduced by a Buchwald coupling reaction to yield **62**. A Suzuki–Miyaura cross-coupling reaction with the appropriate pyridyl boronic ester yielded the desired compound *rac*-**6**. The two enantiomers were finally separated by chiral HPLC.



Scheme 4. Racemic synthesis of dihydrobenzodiazepine *rac*-**6** reported in a patent application from Constellation Pharmaceuticals.[174] *Reagents and conditions*: a) K_2CO_3, THF, reflux, 59%; b) Fe, NH_4Cl, EtOH/H_2O, 60 °C, 83%; c) NaOMe, MeOH, 0 °C→RT, 34%; d) Lawesson's reagent, THF, 80 °C, 79%; e) $AcNHNH_2$, *n*BuOH, 130 °C, 62%; f) 1-chloro-4-iodobenzene, $Pd_2(dba)_3$, SPhos, Cs_2CO_3, toluene, 110 °C, 20%; g) 5-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)pyridin-2-amine, $Pd(PPh_3)_4$, K_2CO_3, THF/H_2O, 100 °C, microwave irradiation, 66%.

3.1.3. Identification of structural motifs acting as acetyllysine (KAc) mimics

Since the discovery of triazolodiazepines (+)-**1** and **2**, much effort has been invested in identifying new chemotypes as selective BET bromodomain inhibitors. One frequently applied strategy was the identification of an alternative acetyllysine-mimicking moiety binding to the conserved KAc binding pocket of the bromodomain by a fragment-based drug discovery approach and a subsequent structure-based optimization program. In this way, several fragments were identified as acetyllysine mimics, shown in Figure 10.[187]

Similar to the known 3-methyl-1,2,4-triazole (Figure 10a), the 3,5-dimethylisoxazole[51a,175c,e] (Figure 10b), the 3-methyl-3,4-dihydroquinazolinone[176a,177a] (Figure 10c), the *N*-acetyl-2-methyltetrahydroquinoline[176] (Figure 10d), and the 2-thiazolidinone (Figure 10e) moieties share the same essential features required for binding to the KAc binding pocket: a hydrogen-bonding functionality to interact with the conserved asparagine and tyrosine (water-mediated) residues and a small alkyl substituent binding to a small hydrophobic pocket that is responsible for recognition of the KAc acetyl methyl group (except for the 2-thiazolidinone fragment, Figure 10e). In the case of the 3-methyl-3,4-dihydroquinazolinone moiety (Figure 10c) an additional hydrogen bond interaction is formed between the urea NH proton and the carbonyl function of the asparagine amide. In the following section, the fragment-based discovery of several KAc mimics and their optimization to improve potency and pharmacokinetic properties is reviewed. At this point, most work has been done on compounds bearing a 3,5-dimethylisoxazole moiety.

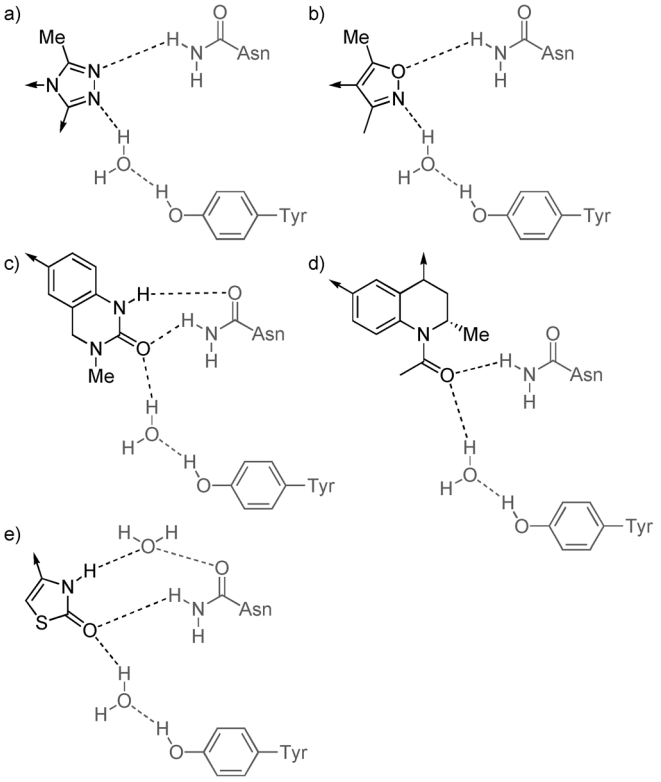


Figure 10. Schematic representations of the binding interactions of reported KAc mimics with the bromodomain KAc binding pocket. a) 1-methyl-1,2,4-triazole, b) 3,5-dimethylisoxazole, c) 3-methyl-3,4-dihydroquinazolinone, d) *N*-acetyl-2-methyltetrahydroquinoline, and e) 2-thiazolidinone (in analogy to the graphical representation reported by Hewings et al.[187]).

3.1.4. 3,5-Dimethylisoxazoles

The 3,5-dimethylisoxazole moiety has been independently identified as KAc mimic by Bamborough et al.,[175e] Hewings et al.,[175c] and Dawson et al.[51a] Hewings et al. found 3,5-dimethylisoxazoles as a KAc bioisostere from an X-ray crystal structure of compound **63** (Figure 11) bound to BRD4(BD1).[175c]

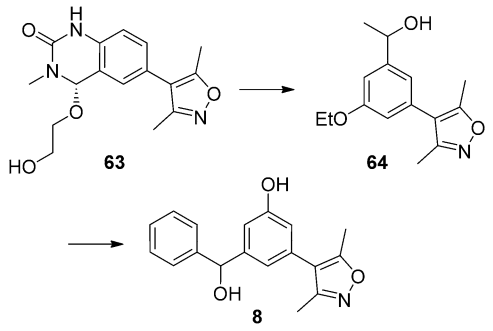


Figure 11. Starting point **63** and optimized 3,5-dimethylisoxazolo compounds **64** and **8** reported by Hewings et al. as BET bromodomain inhibitors.[175c,d]

They identified the 3,5-dimethylisoxazole moiety as a KAc mimic with the isoxazole oxygen atom forming a hydrogen bond with the amide NH_2 of asparagine residue N140 and the isoxazole nitrogen atom forming a water-mediated hydrogen bond with tyrosine residue Y97 (Figure 10a). The ethylene glycol substituent of **63** was observed to be directed into the hydrophobic WPF shelf, which is occupied by a 4-chlorophenyl substituent in the case of (+)-**1** and **2** (see Figure 5). With the help of molecular modeling calculations, a di-*meta*-substituted 3,5-dimethyl-4-phenylisoxazole scaffold was designed. Compound **64** showed activity in the low-micromolar range (IC_{50} = 4.8 μM, BRD4(BD1) AlphaScreen) as well as selectivity for BET bromodomains (determined by ITC). A crystal structure of **64** bound to BRD4(BD1) was solved and confirmed the binding mode of the dimethylisoxazole as a KAc mimic to be identical to that of **63** (Figure 12).

However, in a successive study the ethoxy substituent (directed into the ZA channel; Figure 12) and the methyl substituent of **64** (directed into the hydrophobic WPF shelf; Figure 12) were chosen for SAR investigations to enhance the ligand–protein interactions.[175d] Compound **8** was identified as having higher binding affinities for BRD4(BD1) (IC_{50} = 382 nM for (*S*)-**8** and 386 nM for (*R*)-**8**, AlphaScreen assay) than that of **64** and anti-proliferative effects in an acute myeloid leukemia cell line (sub-micromolar IC_{50} values). Compound **8** showed moderate selectivity over the CREBBP bromodomain and excellent selectivity over other phylogenetically diverse bromodomain classes. The crystal structure of (*S*)-**8** bound to human BRD4(BD1) revealed that the phenyl ring occupies the WPF shelf more effectively than the smaller methyl substituent of **64**, and that the phenol hydroxy group forms an additional hydrogen bond to a conserved water molecule located in the ZA channel. The combination of these two interactions pushes the

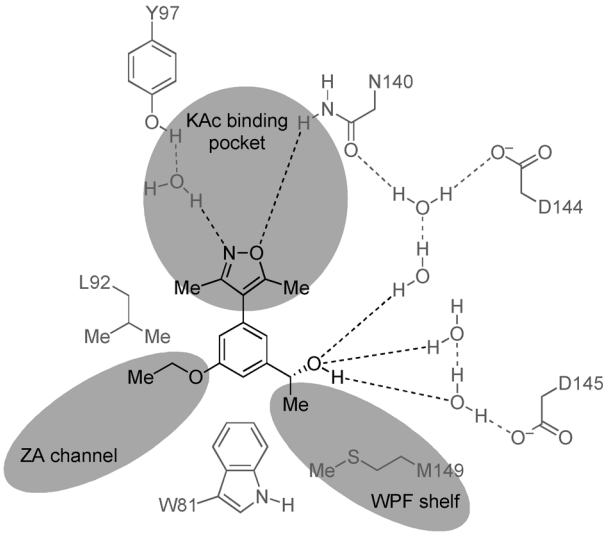


Figure 12. Schematic representation of the key ligand–protein interactions formed between **64** and human BRD4(BD1) as reported by Hewings et al.[175c]

3,5-dimethylisoxazole moiety further into the KAc binding pocket than **64**.

Bamborough et al. identified 3,5-dimethyl-4-phenylisoxazole (structure not shown) from a rationally selected screening set as a KAc mimic by a fluorescence anisotropy assay and confirmed the binding mode to be exactly the same as reported by Hewings et al.[175e]

Compounds with a sulfonamide substituent on the phenyl ring *meta* to the isoxazole were chosen from a 3D pharmacophore model as a strategy to improve binding affinity. This led to compounds **65**–**66** (Figure 13) with IC_{50} values in the low-

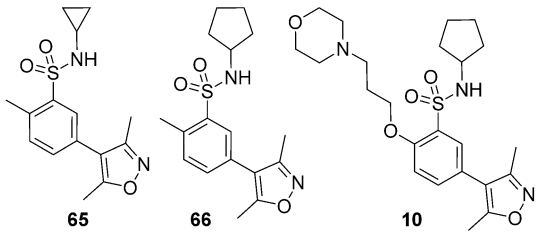


Figure 13. 3,5-Dimethylisoxazoles **10**, **65**, and **66** reported as BET bromodomain inhibitors by Bamborough et al.[175e]

micromolar range (fluorescence and FRET assays). As apparent from the X-ray crystal structures, the sulfonamide in the *meta* position introduces a bend in the shape of the molecule and directs the cycloalkyl residues into the hydrophobic WPF shelf (similarly to compounds **64** and **8** reported by Hewings et al.[175c,d]). The sulfonamide itself is involved in a water-mediated hydrogen bonding network. To improve the solubility of the compound series, polar and solubilizing groups were attached to the phenyl *para* position, as this position points toward the solvent, and variations have little effect on potency. Compound **10** retained the affinity of the simpler analogues

65–**66**, but showed a 1000-fold increased solubility (1125 μg mL^{-1} for **10** relative to <1 μg mL^{-1} for **66**, pH 5.0). Compounds **10** and **65** showed high selectivity for the BET bromodomains in a thermal shift assay as well as activity in a cell-based IL-6 assay, with IC_{50} values in the low-micromolar range.

Referring to the findings of Hewings et al.,[175c] Bamborough et al.,[175e] and Dawson et al.,[51a] 3,5-dimethylisoxazole-substituted benzimidazoles have recently been identified as potent and selective inhibitors of BRD4(BD1) by Hay et al.[175f] They extended the core of 4-aryl-3,5-dimethylisoxazoles (e.g., compound **64**, Figure 14) reported by Hewings et al.[175c] by fusing a five-membered ring to the aryl moiety. They found indanol **67** to have an IC_{50} value of ~1.3 μM (BRD4(BD1) AlphaScreen assay), and an X-ray crystal structure of compound **67** bound to BRD4(BD1) revealed the binding mode to be essentially the same as that of other known 3,5-dimethylisoxazoles (e.g., **8**, **10**, **64**) with the 3,5-dimethylisoxazole moiety occupying the KAc binding pocket. The phenyl ring of **67** was directed straight into the hydrophobic WPF shelf. After some optimization, benzimidazoles **11** and **68**–**69** (Figure 14) were identified as preferred core structures with benzyl substituents being superior in their biological activity to phenyl substituents at the benzimidazole 1-position.

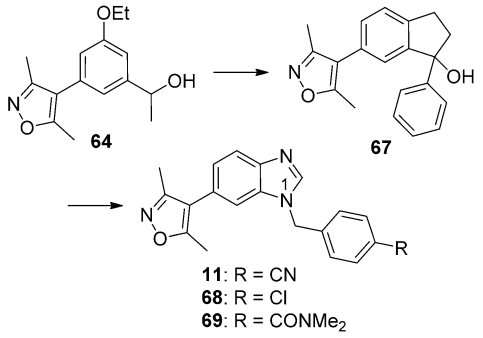
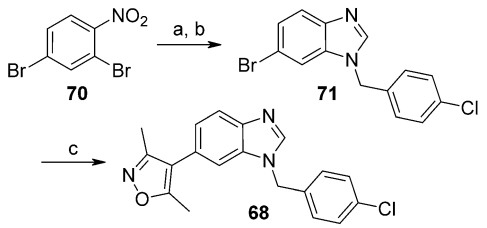


Figure 14. Discovery of 3,5-dimethylisoxazole-substituted benzimidazoles as BET bromodomain inhibitors as reported by Hay et al.[175f]

Compound **11** showed an IC_{50} value of 180 nM and a 100-fold selectivity over the CREBBP bromodomain (confirmed by an AlphaScreen assay). As noted by the authors, the easy three-step synthesis of the scaffold shown in Scheme 5 makes it an attractive template for bromodomain lead discovery projects.[175f] Nucleophilic substitution of 2,4-dibromonitrobenzene **70** with 4-chlorobenzylamine was followed by reduction of the nitro group under standard conditions and cyclization to the benzimidazole core with formic acid. A final Suzuki–Miyaura cross-coupling of aryl bromide **71** with 3,5-dimethylisoxazolylboronic acid afforded the final compound **68**.

Researchers from GlaxoSmithKline were the third to describe the 3,5-dimethylisoxazole moiety as a KAc mimic.[37,51a,175a] Similar to the discovery of I-BET762 (**2**), the 3,5-dimethylisoxazole-substituted quinoline **72** (Figure 15) was identified as a robust hit from a phenotypic HTS campaign in the search for up-regulators of ApoA1 ($EC_{170}=500$ nM compared with $EC_{170}=200$ nM for **2**).



Scheme 5. Representative synthesis of 3,5-dimethylisoxazole-substituted benzimidazole **68** reported by Hay et al.[175f] *Reagents and conditions*: a) 4-chlorobenzylamine, Na_2CO_3, DMSO, 76 °C, 23%; b) Fe, HCO_2H, 85 °C, 52%; c) 3,5-dimethylisoxazole-4-ylboronic acid, $Pd(PPh_3)_4$, K_2CO_3, dioxane/H_2O, 120 °C, 65%.

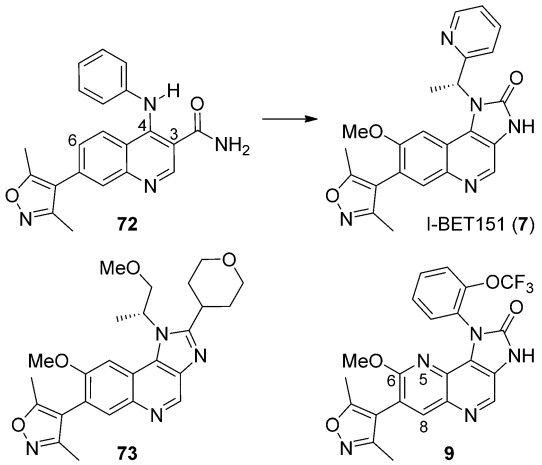
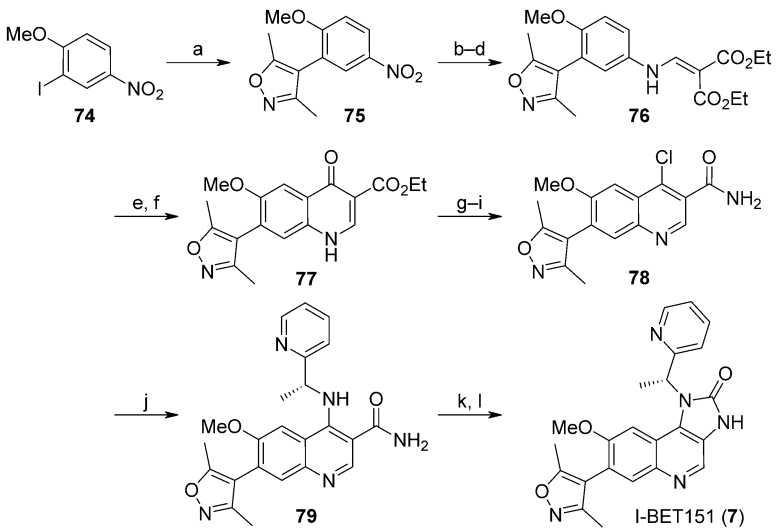


Figure 15. Initial HTS hit **72** and optimized compounds **7**, **9**, and **73** reported by researchers from GlaxoSmithKline.[37,51a,175b,189]

SAR studies revealed that the carboxamide at the 3-position is ideal for potency on ApoA1 up-regulation and that a variety of aromatic, benzylic, or aliphatic substituents at the 4-position are well tolerated. To freeze the likely active conformation of **72**, resulting from a postulated intramolecular hydrogen bond between the C3 carboxamide and the C4 NH group, and to eliminate the free carboxamide moiety, cyclized analogues with an imidazolone ring were synthesized. The careful choice of the amine substituent and the incorporation of a methoxy group on the quinoline 6-position resulted in the discovery of compound **7** (I-BET151), which displayed high potency on ApoA1 up-regulation ($EC_{170}=90$ nM) and abolished the cytochrome P450 liabilities observed in both quinoline series (with or without imidazolone). Compound **7** showed activities similar to that of **2** in biochemical and cellular assays, but an improved in vivo pharmacokinetic profile relative to (+)-**1** or **2**. Its efficacy for the treatment of inflammation was demonstrated in a murine model of endotoxic shock in prophylactic and therapeutic dosing regimens. In addition, **7** was shown to be effective in two distinct mouse models of murine and human leukemia. The X-ray crystal structure of **7** bound to human BRD4(BD1) was solved and showed the 3,5-dimethylisoxazole bound to the KAc binding pocket to form similar interactions



Scheme 6. Synthesis of compound **7** reported by Dawson et al.[51a] *Reagents and conditions*: a) 3,5-dimethylisoxazole-4-ylboronic acid, Cs_2CO_3, PEPPSI, DME/H_2O, 90 °C, 83%; b) 5% $Na_2SO_{3(aq)}$, EtOAc, RT; c) H_2, Pd/C, EtOH, RT, 100%; d) diethyl 2-(ethoxymethylene)malonate, 150 °C, 100%; e) Ph_2O, 255 °C; f) EtOAc, reflux, 64%; g) $NaOH_{(aq)}$, EtOH, reflux, 85%; h) $POCl_3$, 120 °C; i) NH_4OH, THF, 0 °C, 89%; j) [(1*R*)-1-(2-pyridinyl)ethyl]amine, iPr_2NEt, NMP, 120 °C, 77%; k) KOH, MeOH; l) $PhI(OAc)_2$, 0 °C, 77%.

within the pocket as reported for **8** or **66**, mimicking the lysine acetyl head group. The pyridyl group bound to the WPF shelf and the quinoline nitrogen atom accepted a hydrogen bond from a water molecule conserved in the ZA channel (similar to the phenolic hydroxy group of **8**). A representative synthesis of **7** is shown in Scheme 6.[51a]

In the first step, the isoxazole was introduced via a Suzuki–Miyaura cross-coupling of aryl iodide **74** with 3,5-dimethylisoxazole boronic acid. The nitro group was reduced to an aniline intermediate, which was transformed into quinolinone **77** by condensation of the aniline amino group with diethyl 2-(ethoxymethylene)malonate and subsequent thermal cyclization of the resultant enamine **76**. Saponification of the ethyl ester was followed by a $POCl_3$-mediated chlorination to give the aryl chloride as well as the appropriate acid chloride of the carboxylic acid function, which was converted into carboxamide **78** by treatment with ammonia. Nucleophilic substitution of the aryl chlorine atom with the appropriate amine led to **79**, which was transformed into the desired compound **7** by a Hofmann rearrangement of the carboxamide functionality and an intramolecular attack of the adjacent secondary amine to the intermediate isocyanate.

Recently, compound **73** (Figure 15) was reported in a patent application from GlaxoSmithKline.[189] Relative to **7**, the imidazolone and the pyridine-2-ylmethyl substituents were replaced by a substituted imidazole group and a 1-methoxypropan-2-yl residue, respectively. The activity of compound **73** was confirmed in different cell-based assays in which anti-inflammatory and anti-proliferation effects were measured as well as in an in vivo efficacy mouse model of acute inflammation.

Referring to their previous findings on compound **7**, Mirguet et al. very recently published the discovery of 1,5-naphthyridine core analogues of **7** as novel BET family bromodomain inhibitors.[175b] They discovered that the introduction of an additional nitrogen atom is ideal at the 5-position instead of the 6- or 8-positions, as a nitrogen atom adjacent to the inter-ring bond (linkage between naphthyridine core and isoxazole) was found to disfavor the relatively non-planar conformation between the naphthyridine core and the isoxazole moiety that the compounds are forced to adopt by the binding site. Compound **9** showed BET binding (pIC_{50} = 6.2, BRD4(BD1)) and cellular activity (pIC_{50} = 6.5, IL-6 assay), and favorable in vivo pharmacokinetics in rat, associated with good solubility. Its efficacy was demonstrated in a mouse model of inflammation. The X-ray crystal structure of **9** bound to BRD4(BD1) was solved and revealed the binding mode to be similar to that of **7** with the 3,5-dimethylisoxazole bound to the KAc binding pocket. Steric bulk at the 2-position of the phenyl ring was shown to be beneficial for high potency, as this substituent was directed into the lipophilic WPF shelf.

3.1.5. 3-Methyldihydroquinazolinones

Chung et al.[176a] and Fish et al.[177a] independently discovered the 3,4-dihydro-3-methyl-2(1*H*)-quinazolinone fragment **80** and its 6-brominated equivalent **81** as a KAc mimic showing activity in a peptide displacement biochemical assay (AlphaScreen) in the range of 30–90 μM against BRD4 and CREBBP (Figure 16).

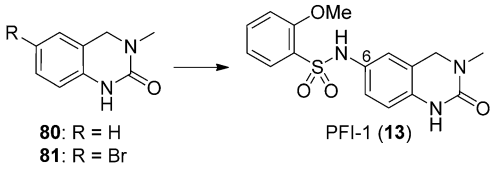


Figure 16. Initial fragment hits **80** and **81** and optimized compound **13** reported as BET bromodomain inhibitor by Fish et al.[177a]

The binding mode of fragment **80** was confirmed by X-ray crystal structure determination and showed the cyclic urea buried in the KAc binding pocket of the protein mimicking the acetyl NH group of the natural acetyl lysine ligand. The 6-position was selected for attachment of an additional side chain to the fragment hit **81**. Guided by the X-ray crystal structure of **81** in complex with BRD4(BD1), sulfonamides were chosen as substituents, as the sulfonamide moiety was expected to

induce a kink in the 6-substituent directing a side chain into the hydrophobic WPF shelf (similar to the findings of Bamborough et al.[175e]). Sulfonamide **13** was identified as one of the most potent ligands, with an IC_{50} value of 220 nM for BRD4 (AlphaScreen) and high selectivity for the BET bromodomains, as confirmed by a temperature shift assay. The X-ray crystal structure of **13** bound to BRD4(BD1) showed the key interactions of the urea carbonyl with N140 and Y97 (water-mediated) as well as an additional hydrogen bond between the urea NH proton and the amide carbonyl oxygen of the asparagine residue N140 in the KAc binding pocket (see Figure 10c).[56] The *ortho*-methoxy-substituted phenyl ring was directed into the hydrophobic WPF shelf, and the sulfonamide NH function was further shown to be involved in a water-mediated hydrogen bond to a backbone carbonyl group of the ZA loop. The cellular activity of **13** was confirmed in an IL-6 assay as well as the antiproliferative effects on leukemia cell lines.[56,177] Moreover, **13** showed an acceptable in vivo pharmacokinetic profile for oral administration.[56,177]

3.1.6. N*-acetyl-2-methyltetrahydroquinolines*

Chung et al. discovered the *N*-acetyl-2-methyltetrahydroquinoline (THQ) fragment **82** by screening a focused fragment set of putative KAc mimics which was assembled using the X-ray crystal structural knowledge of BET bromodomains (Figure 17).[176a] The binding mode of fragment **82** was confirmed by its X-ray crystal structure in complex with BRD2(BD1) and showed the THQ *N*-acetyl group of **82** to mimic the acetyllysine side chain of the natural ligand and to form interactions with N156 and the bridging water molecule described earlier (see Figure 10d). Its methyl group bound in the same pocket as the KAc methyl group. The *S* configuration of the THQ C2 atom placed the methyl group into another small lipophilic pocket, which is not occupied by the acetyllysine side chain. Compounds based on the THQ moiety were reported in patent applications from GlaxoSmithKline, and a representative selection is shown in Figure 17.[176b,190] The majority of these compounds share the common *N*-acetyl-2-methyltetrahydroquinoline core, although some exceptions are reported with an *N*-propanoyl residue or an ethyl substituent at the 2-position. In the 4-position of the THQ core an amino group derivatized as a carbamate (isopropyl carbamate in particular), aniline, or an amino heterocycle is present in most of the exemplified compounds. On the (hetero)aryl ring attached at the THQ 6-position numerous heteroaromatic, basic, neutral, and acidic functionalities were reported. A number of the claimed compounds were found to be potent in a fluorescence anisotropy binding assay as well as in whole-blood assays measuring levels of inflammatory mediators (e.g., TNFα and IL-6).[176b] The efficacy of compound **12** was further confirmed in an in vivo mouse endotoxemia model, and its activity as a cell growth inhibitor in several tumor cell lines was demonstrated.[176b] Wyce et al. very recently identified I-BET726 (**12**) as an effective compound for the treatment of neuroblastoma tumors in vivo in mouse xenograft models after oral administration.[191] They also confirmed the binding mode of compound **12** in complex with BRD4(BD1) by X-ray crystal structure determination with the THQ *N*-acetyl group binding to the KAc binding pocket. The selectivity of I-BET726 (**12**) for the BET family of bromodomains was demonstrated by a fluorescent thermal shift assay.[191]

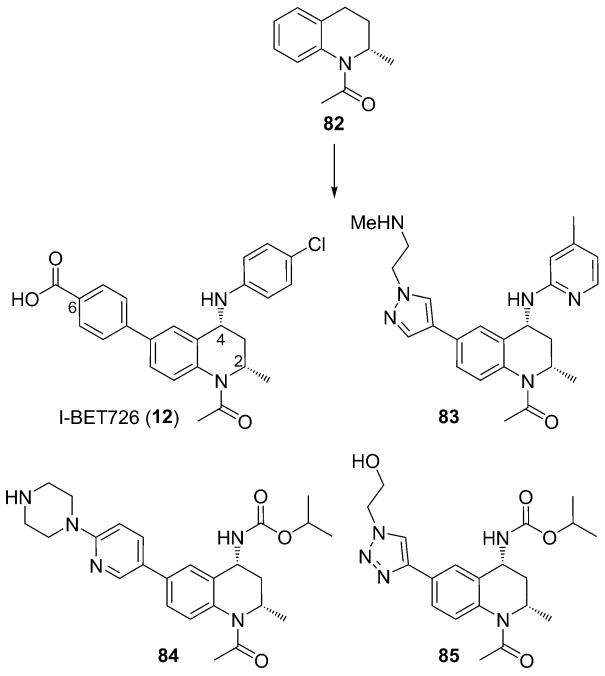
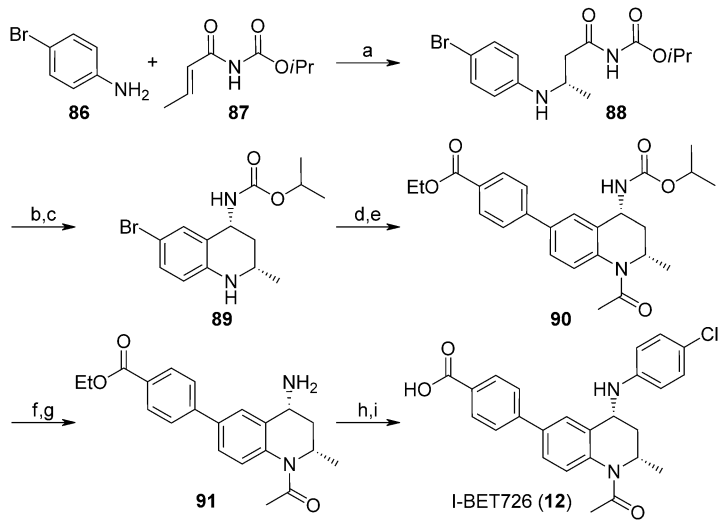


Figure 17. THQ fragment **82**[176a] and acyltetrahydroquinolines **12** and **83**–**85** reported in patent applications from GlaxoSmithKline.[176b,190]

The synthesis of representative compound **12** is shown in Scheme 7. An enantioselective aza-Michael addition of aniline **86** with α,β-unsaturated carbamate **87** in the presence of a chiral Pd^{II} catalyst gave carbamate **88** in good yield and high enantiomeric purity. The tetrahydroquinoline core was formed by Lewis-acid-mediated reduction of the amide carbonyl function and subsequent cyclization to tetrahydroquinoline **89**. The C2 stereochemistry directed the cyclization to give a single diastereomer of **89**. Acetylation of the tetrahydroquinoline nitrogen atom under standard conditions was followed by a Suzuki–Miyaura cross-coupling reaction with (4-ethoxycarbonylphenyl)boronic acid to yield compound **90**. The carbamate moiety was cleaved with aluminum trichloride, and a subsequent Buchwald coupling of amine **91** with 1-bromo-4-chlorobenzene followed by saponification of the ethyl ester gave the final compound **12**.

3.1.7. 2-Thiazolidinones

More recently, the 2-thiazolidinone moiety was identified as an additional chemotype acting as acetyllysine mimic by Zhao et al.[179] Fragment **92** (Figure 18) was selected on the basis of a computational docking study and was subsequently crystallized in complex with BRD4(BD1). Similar to the isoxazole moiety described earlier, the 2-thiazolidinone ring bound to the KAc binding pocket by a direct hydrogen bond of its carbonyl group with the amide NH_2 of N140 and a water-mediated hydrogen bond with Y97. In addition, the nitrogen atom of



Scheme 7. Representative synthesis of compound **12** reported in a patent application from GlaxoSmithKline.[176b] *Reagents and conditions*: a) [(*R*)-BINAP(MeCN)$_2$]Pd(OTf)$_2$, toluene, RT, 67% (92% *ee*); b) $NaBH_4$, $MgCl_2{\cdot}6H_2O$, EtOH/H_2O, −10 °C→RT; c) HCl, citric acid, EtOH/CH_2Cl_2, 83%; d) AcCl, pyridine, CH_2Cl_2, 78%; e) (4-ethoxycarbonylphenyl)boronic acid, Na_2CO_3, $Pd(PPh_3)_4$, DME/H_2O, 105 °C, 89%; f) $AlCl_3$, CH_2Cl_2, 0 °C; g) Et_3N, MeOH, 0 °C, 84%; h) 1-bromo-4-chlorobenzene, NaO*t*Bu, $Pd_2(dba)_2$, DavePhos, toluene, 70 °C, 32%; i) NaOH, H_2O/MeOH, 87%.

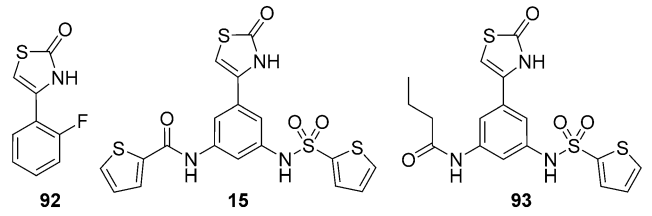


Figure 18. Fragment **92** and optimized 2-thiazolidinones **15** and **93** reported by Zhao et al.[179]

the 2-thiazolidinone core formed another water-mediated hydrogen bond with the amide CO group of N140 (see Figure 10e). Similar to the findings of Bamborough et al.[175e] and Fish et al.,[177a] a sulfonamide functionality was attached at the *meta* position of the phenyl ring to direct a side chain into the hydrophobic WPF shelf (compounds **15** and **93**, Figure 18). Moreover, the incorporation of an amide side chain at the second phenyl *meta* position was found to further improve binding affinities.

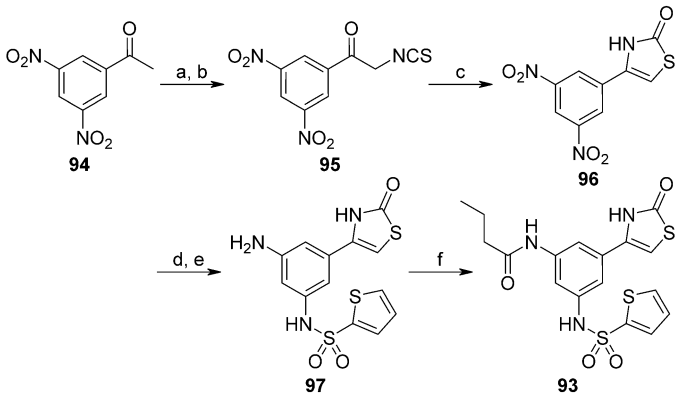
An X-ray crystal structure reveals that the amide functionality of compound **93** extends into the ZA channel and makes extensive hydrogen bonding interactions with residues lining this channel. One of the most potent compounds (**15**, IC_{50} = 230 nM, BRD4(BD1) fluorescence anisotropy assay) showed good in vitro metabolic stability.

A representative synthesis of compound **93** is shown in Scheme 8. Treatment of acetophenone **94** with copper(II) bromide gave the desired 2-bromoacetophenone, which was transformed into thiocyanate **95**. Cyclization under acidic conditions yielded thiazolidinone **96**. Reduction of both nitro groups and consecutive acylation of the aniline amino groups to install the amide as well as the sulfonamide side chain gave the final compound **93**.

3.1.8. Other chemotypes as BET bromodomain inhibitors

A benzimidazole-based BET bromodomain inhibitor (BIC1 **14**, Figure 4) was reported by Ito et al.[178] Compound **14** was identified by surface plasmon resonance measurements, and its K_D value was determined to be 28 μM for BRD2(BD1). Interestingly, the X-ray crystal structure of **14** in complex with BRD2(BD1) revealed that the benzimidazole benzene ring occupies the KAc binding pocket. In contrast to the structures described in the previous section, no hydrogen bonds to the conserved asparagine and tyrosine residues of the KAc binding pocket were observed. The benzimidazole-2-thione moiety was directed toward the WPF shelf.

Further examples for BET bromodomain ligands have been published in the patent literature. Pyrazolopyridazines (**16**, **98**–**103**) were reported in a patent application from Constellation Pharmaceuticals (Figure 19).[180] The most potent compounds (**16**, **98**–**101**) showed IC_{50} values $<$500 nM in biochemical



Scheme 8. Representative synthesis of compound **93** reported by Zhao et al.[179] *Reagents and conditions*: a) $CuBr_2$, EtOAc, 80 °C; b) KSCN, acetone, RT; c) H_2SO_4, HOAc, 100 °C; d) Fe, NH_4Cl, EtOH, 80 °C; e) thiophene-2-sulfonyl chloride, CH_2Cl_2, pyridine, RT; f) butanoyl chloride, pyridine, CH_2Cl_2, RT; (no yields given).

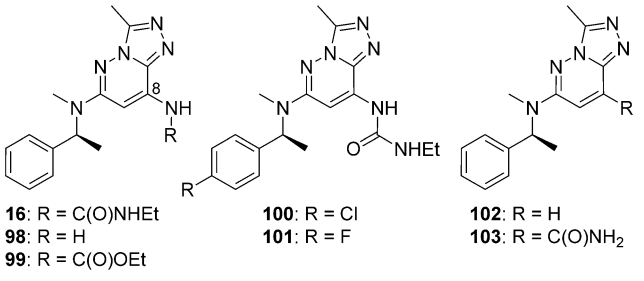
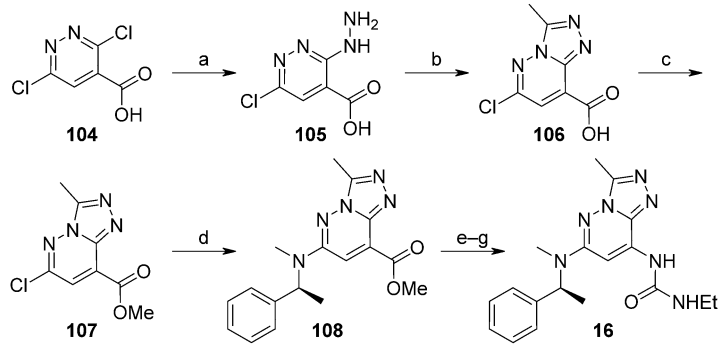


Figure 19. Triazolopyridazines **16** and **98**–**103** reported as BET bromodomain ligands in a patent application from Constellation Pharmaceuticals.[180]



Scheme 9. Synthesis of representative compound **16** reported in a patent application from Constellation Pharmaceuticals.[180] *Reagents and conditions*: a) H_2NNH_2, Et_3N, EtOH, 85 °C; b) AcOH, 100 °C, 73%; c) $SOCl_2$, MeOH, 80 °C, 72%; d) (1*S*)-*N*-methyl-1-phenylethanamine, DMSO, 130 °C, 15%; e) LiOH, MeOH/H_2O, RT, 85%; f) DPPA, Et_3N, dioxane, 115 °C; g) $EtNH_2$, 0→60 °C, 4%.

(BRD4(BD1) AlphaScreen assay) as well as cell-based (c-Myc mRNA quantification and IL-6 quantification) assays. An amine, urea, or carbamate functionality at the pyrazolopyridazine 8-position proved to be essential for a high activity, as compounds **102** and **103** showed IC_{50} values >1 μM (BRD4(BD1) AlphaScreen assay). Synthesis of the representative compound **16** (Scheme 9) started with the formation of hydrazine **105** from 3,6-dichloropyridazine-4-carboxylic acid **104**. The cyclization to pyrazolopyridazine **106** was accomplished by thermal condensation of **105** in acetic acid. The carboxylic acid was converted into its methyl ester **107** and subsequent substitution of the aryl chlorine atom with (1*S*)-*N*-methyl-1-phenylethanamine afforded compound **108**. Saponification of the ester followed by a Curtius rearrangement and reaction of the intermediate isocyanate with ethyl amine gave the final compound **16**.

In addition to GlaxoSmithKline, Constellation Pharmaceuticals, Pfizer, and the Dana-Farber Cancer Institute, Abbott Laboratories/Abbvie have filed a patent application on pyrrolopyridinones and pyrrolopyridazinones as potent BET bromodomain inhibitors.[181] Exemplary compounds **17** and **109**–**114** (Figure 20) showed IC_{50} values <20 nM (FRET assay, BRD4(BD1)) as well as EC_{50} values <100 nM (except compound **115**) in a cancer cell proliferation assay (MX-1 cell line). A sulfone or sulfonamide moiety at the 1-position of the phenyl ring attached to the pyrrolopyridinone core was essential for high activity, and a polar substituent at the pyrrolopyridinone 3-position was not tolerated, as compounds **115** and **116** showed IC_{50} values >1 μM (FRET assay, BRD4(BD1)). The efficacy of compounds **17** and **109**–**114** was demonstrated in vivo in different cancer xenograft mouse models as well as the efficacy of compound **17** in an in vivo rat arthritis model.

A representative synthesis of compound **17** is shown in Scheme 10. Enamine **118** was synthesized from **117** by treatment with the formylating reagent *N*,*N*-dimethylformamide dimethyl acetal under basic conditions. Reduction of the nitro group with Raney nickel engendered cyclization and loss of dimethylamine to give pyrrolopyridine **119**. The pyrrolo nitrogen was tosylated, and after cleavage of the methoxy ether the resulting intermediate amide was methylated to yield pyrrolopyridinone **120**. The aryl bromide was transformed into boronic acid pinacol ester **121**, which was reacted with 3-bromo-4-(2,4-difluorophenoxy)aniline in a Suzuki–Miyaura cross-coupling reaction to give aniline **122**. The aniline nitrogen was sulfonylated and after cleavage of the pyrrolo *N*-tosylate, the final compound **17** was obtained.

Martin et al. reported the ability of the CDK inhibitor dinaciclib (**123**), currently in Phase III clinical trials for the treatment of leukemia,[192] to interact with the acetyllysine binding site of bromodomains.[193] They solved a crystal structure of compound **123** bound to BRDT and observed that the pyridine-*N*-oxide interacts with residue N109 of the KAc binding pocket (albeit to a much lesser extent than (+)-**1** or **7**),

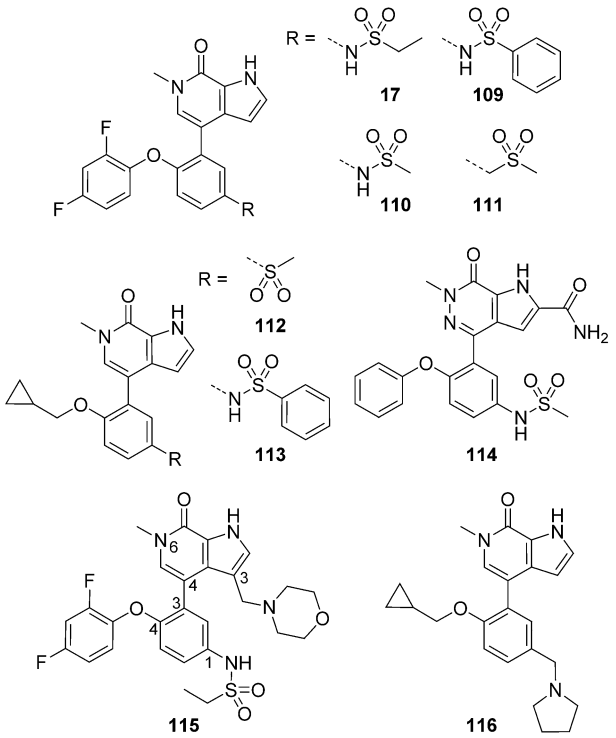
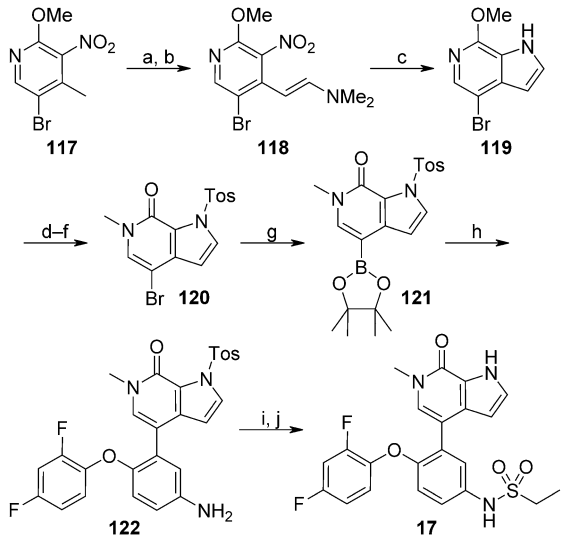


Figure 20. Representative pyrrolopyridinones and pyrrolopyridazinones reported as BET bromodomain inhibitors in patent applications from Abbott Laboratories/Abbvie.[181]

which is the target residue of the triazole ring of (+)-JQ1 ((+)-**1**) and the 3,5-dimethylisoxazole ring of I-BET151 (**7**) (Figure 21). In contrast to (+)-**1**, dinaciclib (**123**) interacts with two coordinated water molecules located at the ZA channel via its structural hinge binding motif (depicted as "pseudo-hinge of BRDT" by the authors[194]), similar to the quinolone nitrogen of I-BET151 (**7**).[51a] Profiling against a panel of 24 bromodomains revealed that dinaciclib (**123**) predominantly bound to members of the BET family. As noted by the authors, it is unlikely that compound **123** affects bromodomains at clin-



Scheme 10. Synthesis of representative compound **17** reported in a patent application from Abbott Laboratories.[181] *Reagents and conditions*: a) LiOMe, DMF, 100 °C; b) *N*,*N*-dimethylformamide dimethyl acetal, 95 °C, 76%; c) Raney-Ni, EtOAc/H_2O, RT, 72%; d) *p*TsCl, NaH, DMF, RT, 100%; e) HCl, dioxane, 40 °C, 94%; f) MeI, NaH, DMF, RT, 96%; g) bis(pinacolato)diboron, KOAc, $Pd_2(dba)_3$, XPhos, dioxane, 80 °C, 73%; h) 3-bromo-4-(2,4-difluorophenoxy)aniline, 1,3,5,7-tetramethyl-6-phenyl-2,4,8-trioxa-6-phosphaadamantane, $Pd_2(dba)_3$, K_3PO_4, dioxane/H_2O, 60 °C, 72%; i) ethanesulfonyl chloride, CH_2Cl_2, 0 °C→RT, 91%; j) KOH, hexadecyltrimethylammonium bromide, THF/H_2O, 90 °C, 82%.

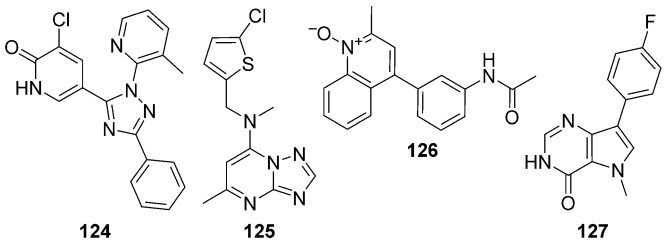


Figure 22. Structurally new KAc mimics **124**–**127** described as BRD4 inhibitors by Vidler et al.[194]

In conclusion, tremendous efforts have been invested over the past three years in the identification of selective small-molecule inhibitors of the BET family of bromodomains both in academia and in the pharmaceutical industry. This led to the discovery of chemically diverse ligands, the majority of which share a similar binding mode to the highly conserved KAc binding site of the bromodomain pocket. Critical ligand–protein interactions responsible for high affinity and selectivity have been revealed by extensive X-ray crystal structure determinations, thus helping in the optimization of binding properties. The first BET bromodomain inhibitors have already entered clinical trials, and it will soon be seen whether their expected therapeutic use can be confirmed.

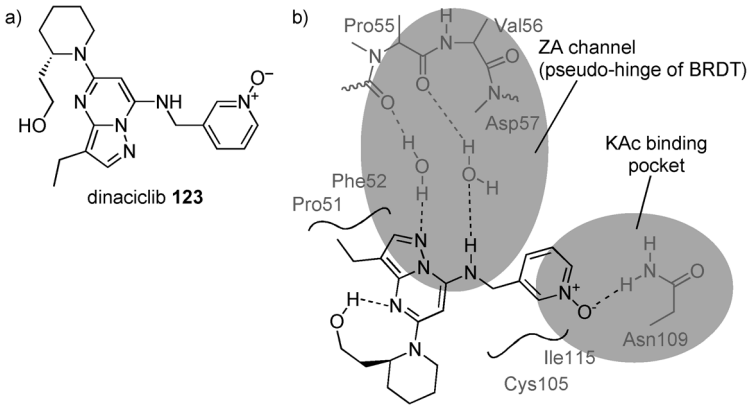


Figure 21. a) CDK inhibitor dinaciclib (**123**), currently in phase III clinical trials at Merck; b) simplified representation of the critical binding interactions of **123** with BRDT.

ically relevant doses, as it binds to CDK2 with 30000-fold higher affinity.[193]

Very recently, Vidler et al. described the discovery of four new unprecedented KAc mimics (Figure 22).[194] They identified compounds **124**–**127** by a structure-based virtual screening approach and a subsequent improvement of the biological activity of the initial screening hits through the use of co-crystal structures and structure-based design. Compounds **124**–**127** showed activity in the low-micromolar range against BRD4(BD1) (IC_{50} = 4.7 μM for **124**, 24 μM for **125**, 42 μM for **126**, and 16 μM for **127**; AlphaScreen assay).[194]

3.2. Non-BET bromodomain inhibitors

3.2.1. PCAF bromodomain inhibitors

Zhou and co-workers performed pioneering experiments in the development of small-molecule bromodomain ligands. They obtained the first solution structure of the PCAF bromodomain and showed that this protein binds specifically to acetylated lysine 50 of the human immunodeficiency virus Tat protein.[195] The Tat-KAc50–PCAF interaction is important for viral transcription and could potentially be targeted for therapy.[196] Nuclear magnetic resonance screening was used to discover small molecules that bind to the PCAF bromodomain. Compound **128** (Figure 23) was identified as inhibiting the interaction of biotinylated Tat-KAc50 peptide with an IC_{50} value of 1.6 μM. Despite further efforts to optimize compound **128**, no significant increase in potency was reported.[112]

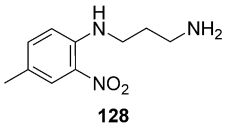


Figure 23. Structure of PCAF inhibitor **128**.

3.2.2. Bromosporine

In comparison with the field of kinase inhibitors, the variety of chemotypes of bromodomain inhibitors is still relatively limited. In analogy to staurosporine, a kinase inhibitor with broad coverage of a diverse set of kinases, Knapp and colleagues from the Structural Genomics Consortium (SGC) and the Uni-

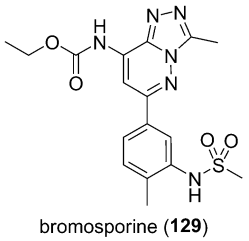


Figure 24. Structure of bromosporine (**129**).

versity of Oxford designed and prepared bromosporine **129** (Figure 24) as a tool compound with biological activity on a variety of different bromodomains.[197] Bromosporine **129** is structurally related to compounds **16** and **98**–**101** reported in a patent application from Constellation Pharmaceuticals.[180] So far, the synthesis and pharmacological properties of **129** have been reported on the SGC homepage exclusively.[197] It should be mentioned at this point that the SGC is aiming to map the entire epigenetic class of proteins and that their activities are not limited to bromodomains only.[197] Bromosporine **129** is a broad-spectrum inhibitor for bromodomains, and such a chemical tool might be very useful in further elucidating the biological role of various reader domains as well as for validating functional assays. In differential scanning fluorimetry experiments the compound showed melting shifts at 10 μM with the following proteins: BRD2, BRD3, BRD4, BRDT, PB1, CECR2, TAF1, BAZ2A, TIF1α, BRD9, CREBBP, and ATAD2. Bromosporine is moderately cytotoxic in HeLa cells at 18 μM.

Starting from commercially available 3,4,6-trichloropyridazine **130**, bromosporine **129** is accessible in nine chemical steps (Scheme 11). The methyl triazole moiety is formed in three steps, giving triazolopyridazine **133**. After Boc protection of the free amino function, the aryl chloride functionality of **134** is coupled with the appropriate boronic acid in a Suzuki Miyaura cross-coupling reaction to yield compound **135**. Boc deprotection and subsequent formation of the ethyl carbamate is followed by reduction of the nitro group of intermediate **137** to give aniline **138**. Formation of the sulfonamide under standard conditions yields the final compound bromosporine **129**.

3.2.3. CREBBP bromodomain inhibitors

CREBBP is an emerging bromodomain-containing target for small-molecule drug discovery.[198] The first small-molecule ligands *rac*-**82** and **139** (Figure 25) for the CREBBP bromodomain were identified by Zhou et al. by screening a focused library of 200 compounds with a special focus on KAc mimics to interact with the KAc binding pocket. As the entrance to the CREBBP bromodomain pocket is positively charged, electron-rich functional groups were preferred in compound collection. The library was screened by using $^{1}H–^{15}N$ HSQC spectra measured in the presence and absence of a mixture of eight fragments. Fourteen compounds binding to the CREBBP bromodomain were identified in this screen. The p53 KAc382–CREBBP bromodomain interaction was blocked by compounds *rac*-**82** (MS 2126) at 100 μM and **139** at 50 μM.[199] Furthermore, the effect of compounds *rac*-**82** and **139** on p53 translational activation was studied in human bone osteosarcoma epithelial cells. In another screen of a library of 3000 compounds using the $^{1}H–^{15}N$ HSQC-based assay, the same group identified the 4-hydroxyphenylazobenzenesulfonic acid (MS 456).[200] Further optimization resulted in the more potent compound ischemin **140** (Figure 25), with a K_D value of 19 μM and an IC_{50} value of 5 μM in the cell-based p21 luciferase assay. An NMR study showed that **140** occupies the peptide binding groove of the CREBBP bromodomain. However, the compound does not protrude far into the KAc binding pocket.[96] The selectivity of ischemin **140** for the bromodomain of CREBBP over other bromodomains was investigated using a fluorescence assay. Com-

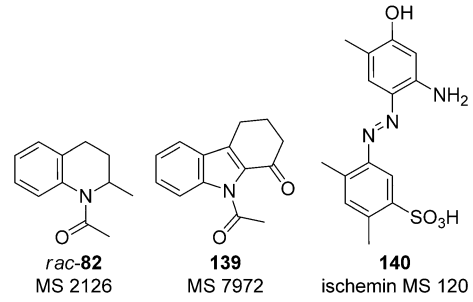
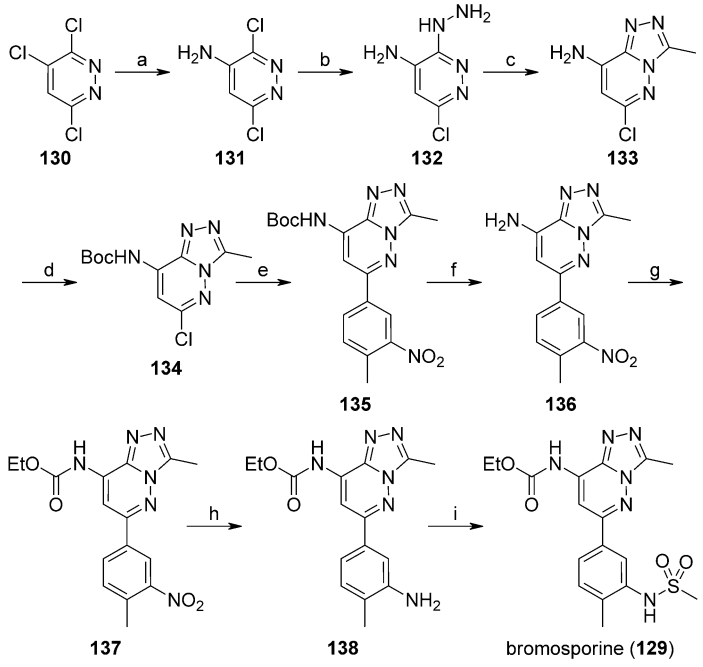


Figure 25. Reported CREBBP inhibitors MS 2126 (*rac*-**82**), MS 7972 (**139**), and ischemin MS 120 (**140**).



Scheme 11. Synthesis of bromosporine **129**. *Reagents and conditions*: a) NH_3, EtOH; b) H_2NNH_2, H_2O; c) AcOH, d) Boc_2O, DMAP, THF; e) (4-methyl-3-nitrophenyl)boronic acid, $Pd(PPh_3)_4$, K_2CO_3, dioxane/H_2O, reflux; f) TFA, CH_2Cl_2; g) ethyl chloroformate, Et_3N, THF; h) $SnCl_2$, H_2O, EtOH; i) MsCl, pyridine; (yields not given).[197]

pound **140** was up to fivefold selective for CREBBP bromodomain over PCAF, BRD4(BD1), and BAZ2B.

Conway, Heightman, and co-workers reported several 3,5-dimethylisoxazole derivatives which bind to the CREBBP bromodomain in addition to BET BD1 pockets.[175c] The 3,5-dimethylisoxazole acts as a KAc mimic that binds in the KAc binding pocket in a similar orientation as the 3,5-dimethylisoxazole-based BET bromodomain ligands. Compound **141** (Figure 26) has an IC_{50} value of 32 μM against the CREBBP bromodomain and shows modest selectivity for this bromodomain over BRD4(BD1). However, it has an IC_{50} value of 28 μM against BRD2(BD1). Despite this modest potency, compound **141** might be considered as a prototype for the development of more potent and selective 3,5-dimethylisoxazole-based CREBBP bromodomain ligands.

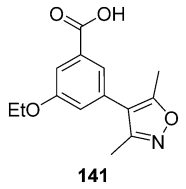


Figure 26. Structure of CREBBP inhibitor **141.**

Further optimization of 3,5-dimethylisoxazole-based CREBBP bromodomain ligands has been reported by Brennan.[201] BDOIA298 (**142**, Figure 27) is a potent CREBBP inhibitor with

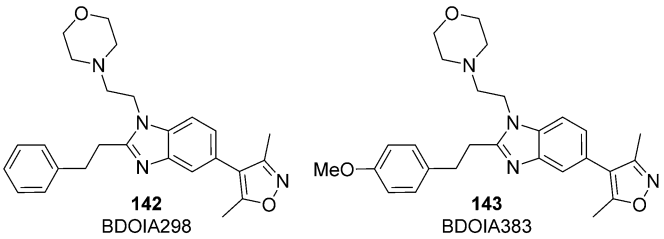


Figure 27. Optimized CREBBP inhibitors BDOIA298 (**142**) and BDOIA383 (**143**).

an AlphaScreen IC_{50} value of 170 nM. Furthermore, it was described to be 11-fold selective against BRD4 (IC_{50} 1.8 μM) and 92-fold selective against CECR2 (10.3 μM). BDOIA383 (**143**, Figure 27) was claimed to be even more selective, with a CREBBP K_D value of 50 nM.

SGC-CBP30 (**144**, Figure 28), which is a CREBBP/EP300-selective chemical probe recently released by the SGC, seems to be a further optimized derivative of this 3,5-dimethylisoxazole-based series with a CREBBP (AlphaScreen assay) IC_{50} value of 69 nM.[202] The compound has moderate cytotoxicity in U2OS and HeLa cells.

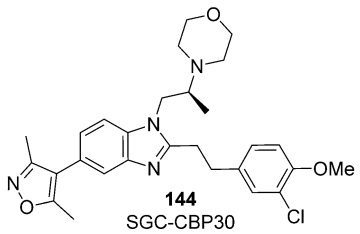


Figure 28. Optimized CREBBP inhibitor SGC-CBP30 (**144**).

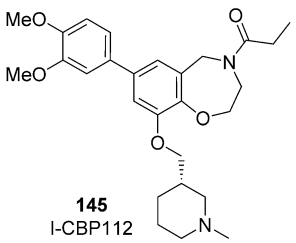


Figure 29. Structure of CREBBP inhibitor I-CBP112 (**145**).

Another CREBBP/EP300-selective chemical probe from a completely different structural class which was also developed by the SGC is I-CBP112 (**145**, Figure 29).[203] I-CBP112 (**145**) has an IC_{50} value of 170 nM in the CREBBP AlphaScreen assay, and is selective against the bromodomain proteins ATAD2, BAZ2B, BRD2(BD2), BRD4 (BD1), PB1(BD5), PCAF, PHIP(BD2), TRIM24/TIF-1α. In U2OS cells no significant cytotoxicity up to 50 μM was found. Similar to bromosporine **129**, data related to SGC-CBP30 (**144**) and I-CBP112 (**145**) are reported on the SGC homepage exclusively.[197]

3.2.4. BAZ bromodomain inhibitors

The BAZ proteins belong to a family of ubiquitously expressed bromodomain-containing proteins. GSK2801 (**146**, Figure 30) was recently reported by the SGC to be a selective chemical probe for BAZ2A and BAZ2B bromodomains[204] with an IC_{50} value in the BAZ2B AlphaScreen of 9–350 nM. No interaction of **146** was detected for the bromodomains of BRD4(BD1), CREBBP, TRIM24/TIF-1α, PB1(BD5), PCAF, or ATAD2. As mentioned in the previous sections, data related to GSK2801 (**146**) have also been reported on the SGC homepage exclusively thus far.

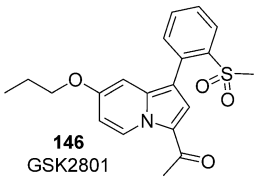


Figure 30. Structure of BAZ2A/B inhibitor GSK2801 (**146**).

4. Outlook

Bromodomains are an exciting class of novel targets for therapeutic intervention. Highly potent and selective small-molecule modulators with drug-like properties have been identified recently for several bromodomains. As these compounds bind to the KAc binding pocket of bromodomains, they act as effective inhibitors of protein–protein interactions that are usually difficult to tackle by small molecules. Especially for the BET bromodomain family, various chemotypes have been found independently by academic and industrial research groups, and the first BET bromodomain inhibitors have now reached clinical testing for the treatment of cancer patients. The first selective and potent chemical probes for other bromodomains have been published, and these novel tools will be an important prerequisite for a deeper understanding of the pharmacological relevance of non-BET proteins. Although a main focus of

current research is the treatment of cancer patients, there might be additional opportunities for selective bromodomain inhibitors in more indications, in light of the implication of bromodomain proteins in several other pathologies. Success of such approaches will depend on whether the therapeutic window of bromodomain inhibitors will be broad enough for safe long-term treatment. Given the current high interest and intensive ongoing efforts in this field, the development of further chemical probes and novel drug candidates, for non-BET bromodomains as well, can be expected in the near future. This will enable deeper insight into the biology of this fascinating class of proteins and has the potential to offer new treatment options for patients in various diseases.

Abbreviations

AcCl, acetyl chloride; AcOH, acetic acid; ApoA1, apolipoprotein A1; AR, androgen receptor; BAZ, bromodomain adjacent to zinc finger domain; BET, bromodomain and extra-terminal domain; BID, basic residue-enriched interaction domain; BINAP, 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl; Boc, *tert*-butyloxycarbonyl; *n*BuOH, *n*-butanol; CDK, cyclin-dependent kinase; CREBBP, cAMP response element binding protein-1 binding protein; DavePhos, 2-dicyclohexylphosphino-2′-(*N*,*N*-dimethylamino)biphenyl; dba, dibenzylidene-acetone; DMAP, 4-dimethylaminopyridine; DME, dimethoxyethane; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulfoxide; DPPA, diphenylphosphoryl azide; EP300, E1A-associated protein p300; ER, estrogen receptor; EtOAc, ethyl acetate; EtOH, ethanol; Fmoc, fluorenylmethoxycarbonyl; FRET, fluorescence resonance energy transfer; GABA, γ-aminobutyric acid; HAT, histone acetyltransferase; HATU, (dimethylamino)-*N*,*N*-dimethyl(3*H*-[1,2,3]triazolo[4,5-b]pyridin-3-yloxy)methaniminium hexafluorophosphate; HBTU, *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HDAC, histone deacetylase; HMT, histone methylfransferase; HPLC, high-performance liquid chromatography; IL-6, interleukin-6; ITC, isothermal calorimetry; MeCN, acetonitrile; MeOH, methanol; MsCl, methanesulfonyl chloride; NMC, NUT midline carcinoma; NMP, *N*-methyl-2-pyrrolidone; NUT, nuclear protein in testis; OTf, trifluoromethanesulfonate; P-TEFb, positive transcription elongation factor b; PCAF, p300/CBP-associated factor; PDID, phosphorylation-dependent interaction domain; PEPPSI, pyridine-enhanced precatalyst preparation stabilization and initiation; PHD, plant homology domain; PID, P-TEFb-interacting domain; PyBOP, (benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate; SAR, structure–activity relationship; SPhos, 2-dicyclohexylphosphino-2′,6′-dimethoxybiphenyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran; *p*TsCl, 4-toluenesulfonyl chloride; XPhos, 2-dicyclohexylphosphino-2′,4′,6′-triisopropylbiphenyl.

Keywords: antitumor agents · bromodomains · cancer · drug design · inflammation · targeted therapy

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