3,5-Dimethylisoxazoles Act As Acetyl-lysine-mimetic Bromodomain Ligands†

David S. Hewings,†‡ Minghua Wang,‡ Martin Philpott,‡ Oleg Fedorov,‡ Sagar Uttarkar,‡ Panagis Filippakopoulos,† Sarah Picaud,‡ Chaitanya Vuppusetty,‡ Brian Marsden,‡ Stefan Knapp,‡ Stuart J. Conway,*‡ and Tom D. Heightman*‡

†Nuffield Department of Clinical Medicine, Structural Genomics Consortium, University of Oxford, Old Road Campus Research Building, Roosevelt Drive, Oxford OX3 7DQ, U.K.
‡Department of Chemistry, Chemistry Research Laboratory, University of Oxford, Mansfield Road, Oxford, OX1 3TA, U.K.

Supporting Information

Abstract: Histone—lysine acetylation is a vital chromatin post-translational modification involved in the epigenetic regulation of gene transcription. Bromodomains bind acetylated lysines, acting as readers of the histone-acetylation code. Competitive inhibitors of this interaction have antiproliferative and anti-inflammatory properties. With 57 distinct bromodomains known, the discovery of subtype-selective inhibitors of the histone—bromodomain interaction is of great importance. We have identified the 3,5-dimethylisoxazole moiety as a novel acetyl-lysine bioisostere, which displaces acetylated histone-mimicking peptides from bromodomains. Using X-ray crystallographic analysis, we have determined the interactions responsible for the activity and selectivity of 4-substituted 3,5-dimethylisoxazoles against a selection of phylogenetically diverse bromodomains. By exploiting these interactions, we have developed compound 4d, which has IC₅₀ values of <5 μM for the bromodomain-containing proteins BRD2(1) and BRD4(1). These compounds are promising leads for the further development of selective probes for the bromodomain and extra C-terminal domain (BET) family and CREBBP bromodomains.

Introduction

Genetic information is encoded in DNA by the specific linear sequence of its bases. The expression of this information is regulated by protein scaffolds that are capable of reading the complex code of post-translational modifications that occur on chromatin. Acetylation of histones lysine residues is one such essential component in the epigenetic regulation of gene expression.† Additionally, recent proteomics studies have demonstrated that lysine acetylation occurs in over 1750 cellular proteins involved in diverse roles such as cell cycle, splicing, nuclear transport, and actin nucleation.‡ Lysine acetylation on histones and other proteins is effected by the dynamic interplay of acetyltransferase (HAT) and deacetylase (HDAC) enzymes, which is analogous to the regulation of serine, threonine, and tyrosine phosphorylation by kinases and phosphatases.

Bromodomains are a family of conserved ~110 amino acid modules that bind selectively to acetylated lysines present in proteins, notably histones,‡ and are thereby thought to participate in deciphering the histone code.¶ Bromodomains have been classified into several distinct subgroups according to the function of their parent protein: (i) histone acetyltransferases (HATs), including CREBBP, GCN5, PCAF, and TAFII250; (ii) in components of ATP-dependent chromatin-remodeling complexes such as Sww2/SnF2; and (iii) the BET (bromodomain and extra C-terminal domain) family, a class of transcriptional regulators carrying tandem bromodomains and an extra terminal domain.¶

Although the biological roles of most bromodomains in the human genome remain elusive, those that have been characterized are fundamental. For example, the BET bromodomain-containing protein (BRD) 4 plays a key role in several cellular processes, including mitosis.¶ Expression levels of BRD4 correlate with breast cancer survival rates,‡ and in a subset of malignant squamous carcinomas, the N-terminal bromodomains of BRD4 are fused in frame to the NUT gene giving rise to extremely aggressive tumor growth.¶ Knockdown experiments have implicated BRD4 in the transcriptional regulation of viruses such as HIV and EBV,† as well as the degradation of HPV.‡ BRD4 was also shown to be required for transcriptional coactivation of NF-κB, regulating the transcription of P-TEFb-dependent pro-inflammatory target genes.¶ A second BET-family protein, the testis-specific BRDT, is essential for male germ cell differentiation in selective domain knockout mice that were viable but sterile.¶

The development of small molecule inhibitors of bromodomain binding to histones and other acetyl-lysine-containing proteins is in its infancy. Weakly potent small molecule ligands of the cAMP response element-binding protein (CREB) binding protein (CREBBP) bromodomain, discovered by NMR screening of acetyl-lysine (KAc) mimics, have been shown to modulate p53 expression in cancer cells.¶

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stability and function in response to DNA damage in cells. More recently, a 4-hydroxyphenylazobenzenesulfonic acid derivative was shown to bind to the CREBBP bromodomain, with \( K_D = 19 \mu M \), and consequently blocks p53-dependent apoptosis in cardiomyocytes under DNA-damage stress.

Ligands have also been developed for the BET family of bromodomains. A benzimidazole-derived BRD2(1) ligand with \( K_D = 28 \mu M \) reduced the transcription of BRD2-dependent genes. Anti-proliferative effects against a number of cell lines have been claimed in a patent for a series of thienotriazolodiazepine BRD2/3/4 blockers with nanomolar in vitro peptide displacement activity. The structurally related (+)-JQ1 (Figure 1A), recently characterized as a BET-family selective chemical probe, shows specific antiproliferative effects in BRD4-dependent cell lines and patient-derived xenograft models. In addition, a recently identified BET bromodomain blocker (I-BET, Figure 1B) suppressed lipopolysaccharide-inducible genes in macrophages and showed anti-inflammatory activity in mice. This work demonstrates the feasibility of inhibiting KAc binding to bromodomains with small molecules that occupy the well-defined KAc-binding pocket. It is possible that targeting this pocket might prove more tractable than developing inhibitors of catalytic activity in certain enzymes. In addition, small molecules that inhibit bromodomain–protein interactions might elicit subtly different pharmacological responses to the inhibition of the catalytic domain of the same protein.

The findings outlined above demonstrate clearly the need for systematic generation of high affinity subfamily selective bromodomain-binding small molecules that can be used as chemical probes to explore further the roles of individual bromodomains. These molecules will provide complementary data to studies using knockdown, peptide and antibody approaches, and will enable prediction of the likely phenotypes of eventual small molecule drugs. As part of our ongoing goal to generate cell-penetrant chemical probes for bromodomains, we have previously developed bromodomain–histone peptide binding assays to allow the identification of fragment and small molecule ligands. Herein, we describe the use of these assays, together with a structure-based design approach, to identify and develop the structure–activity relationships (SAR) of a novel series of 3,5-dimethylisoxazole-containing bromodomain–histone binding inhibitors.

We demonstrate that the 3,5-dimethylisoxazole moiety is an effective KAc mimic and show how differential substitution of the 3,5-dimethylisoxazole core gives derivatives that bind with some selectivity and useful affinity to either the first bromodomain of human BRD4 [BRD4(1)] or the bromodomain of human CREBBP.

**RESULTS AND DISCUSSION**

In the course of our work, we discovered that the solvent N-methyl pyrrolidinone (NMP) interacts with the KAc-binding pocket of a range of bromodomains, showing weak affinity but, because of its low molecular weight, high ligand efficiency (0.30–0.53, see Supporting Information, Table S1), with IC\(_{50}\) values ranging from 34 mM for the bromodomain adjacent to zinc finger domain 2B (BAZ2B) to 2.3 mM for CREBBP. During subsequent studies on methyl-bearing heterocycles, we purchased and tested the dihydroquinazolinone-containing 3,5-dimethylisoxazole derivative 1 (Figure 1C) and observed unexpected selectivity and useful affinity to either the first bromodomain of human BRD4 [BRD4(1)] or the bromodomain of human CREBBP.

The X-ray crystal structure of the BRD4(1) complex was solved at 1.98 Å (Figure 2A, PDB ID: 3SVF). It demonstrated that the 3,5-dimethylisoxazole was indeed acting as a KAc bioisostere, accepting a hydrogen bond from the conserved N-methyl pyrrolidinone (NMP) interacts with the KAc-binding pocket. We hypothesized that this product 2 might arise from oxidation at this position to form the corresponding iminium species, followed by the addition of ethylene glycol, avoiding the benzylic oxidation under laboratory conditions (Supporting Information, Figure S1), we hypothesized that this product 2 might arise from oxidation at this position to form the corresponding iminium species, followed by the addition of ethylene glycol,

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**Figure 1.** (A) Structure of the BET probe (+)-JQ1. (B) Structure of the BET probe I-BET. (C) Structure of dihydroquinazolinone-containing 3,5-dimethylisoxazole derivative 1. (D) The concept employed in the design of the 3,5-dimethylisoxazole-based bromodomain inhibitors.
which is present in the crystallization buffer (see Supporting Information, Figure S1 for mass spectrometry analysis). Interestingly, only one enantiomer was observed in the X-ray crystal structure; the ethylene glycol unit attached to the stereogenic center occupies a hydrophobic groove formed by W81 and P82 from the ZA loop and I146 and M149 from helix C. Occupation of this site by the 4-chlorophenyl substituent of (+)-JQ1 and I-BET (Figure 1A,B) is believed to contribute to the selectivity of these compounds for the BET subfamily over other bromodomains since a similar groove is not available in other bromodomains (Figure 2C).18 In addition, we observed that a sample of 1 displayed significant cytotoxicity in the HeLa human cell line, while (+)-JQ1 showed no cytotoxicity over the same concentration range (Supporting Information, Figure S2). Therefore, the development of analogues that do not possess a readily oxidized benzylic methylene unit, but that could form interactions with this groove, was pursued. The mono- and di-meta-substituted 3,5-dimethyl-4-phenylisoxazole scaffolds (3a–d and 4a–d) were chosen to allow extension from the aromatic core in two directions (Figure 1B). Initial docking studies suggested that a single meta-substituent would occupy a position similar to that of the ethylene glycol moiety of 2 (Supporting Information, Figure S3A). A second meta-substituent would be directed into a groove (referred to by Nicodeme et al.19a and Chung et al.19b as the ZA channel) defined by W81 on one side and L92 on the other (Supporting Information, Figure S3A). Consequently, the 3,5-dimethylisoxazole derivatives 3a–d and 4a–d were selected as target molecules that contain a range of substituents to enable exploration of the KAc-binding pocket of the bromodomains.

The syntheses of 3a–d and 4a–d are shown in Schemes 1–3. Initial work focused on the direct arylation of 3,5-dimethylisoxazole using Pd-catalyzed C–H bond activation.23 While this reaction proceeded well with bromobenzene 5, lower yields were obtained on reaction with 6 and 10 (Supporting Information, Scheme S1). Furthermore, the reaction has been reported to be less successful with electron-rich aryl bromides.23 Conversely, Molander et al. have demonstrated that potassium heteroaryltrifluoroborates react efficiently with both electron-rich and electron-deficient aryl halides, with low catalyst loadings and minimal protodeboronation.24 Therefore, we prepared potassium (3,5-dimethylisoxazol-4-yl)trifluoroborate 8 from (3,5-dimethylisoxazol-4-yl)boronic acid.24 Coupling of 8 to commercially available aryl bromides 5 and 6, and aryl bromide 7 (easily prepared from 3-bromophenol,25 Supporting Information, Scheme S2) afforded 3,5-dimethyl-4-phenylisoxazole 3a and the 3’-substituted 3,5-dimethyl-4-phenylisoxazoles 3b and 3c in excellent yields (Scheme 1). Although the coupling reactions did proceed when conducted with (3,5-dimethylisoxazol-4-yl)boronic acid (data not shown), the yields observed were lower than those in the route described above.

The 3’,5’-disubstituted 3,5-dimethyl-4-phenylisoxazoles 4a–c were prepared from 3-bromo-5-hydroxybenzoic acid 9 (Scheme 2). Alkylation of 9 afforded the ethyl ester 10, which was coupled to 8, yielding 4a. Subsequent LiOH-mediated hydrolysis furnished the carboxylic acid 4b. Alternatively, the ethyl ester of 10 was converted to the acetyl group via the Weinreb amide 12 in three steps; coupling, under the same conditions employed above, yielded the isoxazole 4c. The methyl ketones 3b and 4c were converted to the racemic secondary alcohols 3d and 4d by reduction with NaBH₄ (Scheme 3).
To enable accurate comparison with our new compounds, a sample of 1 was resynthesized (Supporting Information, Scheme S3).

Biological evaluation of 1 was conducted using freshly prepared DMSO solutions to minimize the possibility of oxidation (see Supporting Information for details).

To determine whether the compounds inhibited bromodomain/C0 histone binding, we utilized a peptide displacement assay based on AlphaScreen technology.21 For the first bromodomain of BRD2 (BRD2(1)) and BRD4(1), a histone H4 peptide acetylated at K5, 8, 12, and 16 was employed, while for CREBBP an H3 peptide acetylated at K56 was used. IC50 values for compounds 1, 3a–d, and 4a–d are shown in Table 1 (dose–response curves for 1, compounds 4b and 4d in BRD4(1), BRD2(1), and CREBBP are shown in Supporting Information, Figure S4). With the exception of 3a, all compounds inhibited BRD4(1) and BRD2(1) with IC50 ≤ 50 μM, the most potent compounds showing low micromolar activity. Introduction of an ethoxy substituent at R2 increases potency against BRD4(1) and BRD2(1). A variety of substituents at R1 are well tolerated, with the exception of R1 = CO2H in which the positioning of a negatively charged substituent in a hydrophobic site is likely to disfavor binding. IC50 values were generally higher against CREBBP, except for 4b (R1 = CO2H) where the IC50 for CREBBP was lower than that for BRD4(1) and similar to that for BRD2(1). Gratifyingly, 4b and 4d did not show any cytotoxicity in a human cell line (Supporting Information, Figure S2).

To investigate the selectivity of these compounds against other bromodomains, we employed a binding assay based on protein stability shift (Table 2).26 Of the compounds that produced substantial changes in melting temperature (ΔTm obs), most displayed selectivity for BET bromodomains. However, 4b also produced an appreciable ΔTm obs with CREBBP, in agreement with the IC50 values. Indeed, 4b showed selectivity, albeit modest, for CREBBP over the BET bromodomains.

In order to confirm that the dimethylisoxazole moiety was acting as a KAc mimic and to elucidate structure–activity profiles for the exception of R1 = CO2H in which the positioning of a negatively charged substituent in a hydrophobic site is likely to disfavor binding. IC50 values were generally higher against CREBBP, except for 4b (R1 = CO2H) where the IC50 for CREBBP was lower than that for BRD4(1) and similar to that for BRD2(1). Gratifyingly, 4b and 4d did not show any cytotoxicity in a human cell line (Supporting Information, Figure S2).

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these compounds, we obtained cocrystal structures of BRD4(1) with 4d and the CREBBP bromodomain with 4b (Figures 3 and 4).

The cocrystal structure of 4d bound to BRD4(1) was obtained at 1.68 Å resolution (PDB ID: 3SVG). Globally, the ligand-bound and apo structures (PDB ID: 2OSS) are very similar, with a rmsd of 0.17. This structure confirms that 4d binds with the dimethylisoxazole oriented into the KAc-binding pocket as anticipated by our docking studies (Supporting Information, Figure S3B). It is predicted that the isoxazole oxygen atom forms the expected hydrogen bond with the amine group of the conserved N140 (green, Figure 3A). The isoxazole nitrogen atom interacts with the phenol group of Y97 via the structured water molecule A (Figure 3B).

Although 4d was synthesized as a racemate, the electron density for this compound implies that the (R)-enantiomer crystallizes preferentially with BRD4(1). The secondary alcohol forms a network of hydrogen bonds with water molecules B–E, which interact with D144, D145, and the carbonyl oxygen atom of N140. The methyl group attached to the secondary alcohol binds within the hydrophobic pocket (pocket 2, Figure 3B) that is defined by I146, M149, and W81, the latter forming part of the WPF shelf (described by Nicodeme et al. as W81, P82, and F83). This is the same pocket that binds the ethylene glycol unit of 2 (Supporting Information, Figure S3C) and the chlorophenyl moieties of I-BET and (+)-JQ1 (Supporting Information, Figure S3D). The ethyl ether binds within the ZA channel (pocket 3, Figure 3B) that accommodates the thiophene moiety of (+)-JQ1 and the methylated phenol of I-BET. Therefore, analysis of the X-ray crystal structure of 4d in complex with BRD4(1) shows that this compound is forming interactions in many of the same regions as (+)-JQ1 and I-BET, perhaps explaining how a compound with relatively low molecular weight (261) still shows good affinity for BRD4(1) and reasonable selectivity for the BET subfamily of bromodomains. Indeed, 4d has ligand efficiencies of 0.39 for BRD4(1) and 0.43 for BRD2(1), which compare favorably with the value of 0.32 for (+)-JQ1 at BRD4(1) (see Supporting Information, Table S1 for details of ligand efficiency calculations). These ligand efficiencies suggest that compound 4d is a promising lead for the development of BET bromodomain

Table 2. ΔT\text{m}^{obs} against Selected Bromodomains

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<th>BRD3(1)</th>
<th>BRD3(2)</th>
<th>BRD3(3)</th>
<th>BRD4(1)</th>
<th>BRD4(2)</th>
<th>BRD4(3)</th>
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<th>CREBBP</th>
<th>LUC7L4</th>
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\textsuperscript{a} Compound concentration, 100 μM; protein concentration, 2 μM. Heat map shows relative ΔT\text{m}^{obs}; red indicates large ΔT\text{m}^{obs}, and green indicates small ΔT\text{m}^{obs}. Experiments were carried out in duplicate; average values are reported.

Figure 3. (A) X-ray crystal structure of 4d (carbon = yellow) bound to human BRD4(1) (PDB ID: 3SVG). The conserved N140 residue is highlighted in green. Black dashed lines represent hydrogen bonds between 4d, structured water molecules (red spheres), and interacting residues (labeled). Image generated using PyMOL. (B) A schematic representation of the key interactions formed between 4d and human BRD4(1).
ligands that display potencies similar to those of (+)-JQ1 and I-BET but with lower molecular mass than those of these compounds.

Identification of CREBBP bromodomain inhibitors has so far proved challenging, with only two studies identifying small-molecule ligands for this bromodomain.\textsuperscript{14,15} Therefore, given that proving challenging, with only two studies identifying small-molecule compounds. I-BET but with lower molecular mass than those of these ligands that display potencies similar to those of (+)-JQ1 and explain, at least in part, the preferential binding of this compound to CREBBP over the BET bromodomains. It should be noted that CREBBP crystallized as a dimer, with a second molecule of 4b bound at the dimer interface. This phenomenon is thought to result from the relatively high concentration of 4b used in obtaining the structure and is unlikely to be responsible for this compound’s ability to prevent the CREBBP–KAc interaction.

In summary, we have discovered an entirely new chemical class of bromodomain ligands comprising low-molecular weight 4-phenyl-3,5-dimethylisoxazole derivatives. Using structure-based approaches, we have demonstrated that the unstable dihydroquinazolinone moiety of lead 1 can be replaced with a single aromatic ring without significant loss of affinity. Most of the resultant 4-phenyl-3,5-dimethylisoxazoles are selective ligands for the BET family of bromodomains. However, compounds 4b–d produced a marked increase in T\textsubscript{m} (°C) for CREBBP, suggesting that 3,5-dimethylisoxazoles are also useful leads for selective inhibitors of CREBBP-KAc interactions, which have proved elusive to date. The costructures that we have obtained demonstrate that the 3,5-dimethylisoxazole moiety acts as a KAc bioisostere in two classes of bromodomains, corroborating the binding data and IC\textsubscript{50} values presented. As few chemical classes of small-molecule inhibitors of bromodomain–histone interactions have been described previously,\textsuperscript{15–20} this work represents a significant advance in the SAR of these important proteins.

In conclusion, we have exploited the serendipitous discovery of a KAc mimic to develop a novel class of compounds that inhibit bromodomains–histone interactions. Crystallographic analysis and rational design have provided insight into the structural requirements for potent, selective BRD4(1) and CREBBP bromodomain ligands. These compounds are invaluable leads in the continuing search for chemical probes of epigenetic targets.

### EXPERIMENTAL SECTION

**General Experimental.** \textsuperscript{1}H NMR spectra were recorded on Bruker DFX400 or DQX400 (400 MHz) or Bruker AVII 500 (500 MHz) using deuterochloroform (unless indicated otherwise) as a reference for the internal deuterium lock. The chemical shift data for each signal are given as δ\textsubscript{H} in units of parts per million (ppm) relative to tetramethylsilane.
experimentally determined hydrogen, carbon, and nitrogen composition was reported in Supporting Information. For all tested compounds, values are uncorrected.

Synthetic Procedures and Characterization for Compounds 3a–d, 4a–d, 3.5-Dimethyl-4-phenylisoxazol-3-yl)ethanone 3a.

**Method A.** To a dry 2–5 mL microwave vial were added 8 (108 mg, 532 μmol), Pd(OAc)2 (2 mg, 8 μmol), RuPhos (7 mg, 15 μmol), and anhydrous Na2CO3 (106 mg, 1.00 mmol). The vial was sealed and purged with argon (3 evacuations/fill). Compound 1 gave 3a as a pale yellow solid (49 μmol, 51%).

\[ 2(1H, m, 1H, 105 μmol), \text{and degassed } \text{EtOH} \text{ (3.8 mL) were added by syringe, and the mixture was heated at } 85 \text{ °C for } 15 \text{ min. The mixture was cooled to rt and filtered through a thin pad of silica (eluent } \text{CH}_2\text{Cl}_2 \text{ and concentrated in vacuo. Purification by flash column chromatography (gradient elution, gradient } 4 \rightarrow 30 \text{% EtOAc/petroleum ether }) \text{ gave } 3b \text{ as a pale yellow solid (55 mg, 51%).} \]

**Method B.** To a dry 2—5 mL microwave vial were added 108 mg, 532 μmol, Pd(OAc)2 (2 mg, 8 μmol), RuPhos (7 mg, 15 μmol), and anhydrous Na2CO3 (106 mg, 1.00 mmol). The vial was sealed and purged with argon (3 evacuations/fill). Compound 7 in degassed EtOH (178 μL, 2.8 mL, 499 μmol) was added by syringe, and the mixture was heated at 85 °C for 2 h. The mixture was cooled to rt and filtered through a thin pad of silica (eluent CH2Cl2 and concentrated in vacuo. Purification by flash column chromatography (gradient elution, gradient 4 → 30% EtOAc/petroleum ether) gave 3c as a pale yellow solid (49 μmol, 51%).
5.0 Hz, 3H), 4.07 (q, J = 7.0 Hz, 2H), 7.03 (dd, J = 7.0 Hz, 2H), 7.41 (dd, J = 1.5, 1.5 Hz, 1H), 7.46 (dd, J = 2.2, 1.5 Hz, 1H); HRMS m/z (ES') found [M + Na]+ 284.0886, C16H19NNaO4 requires M+ 284.0893; m/z (ES') 262 ([M + H]+, 32), 284 ([M + Na]+), 100, 545 ([2M + Na]+, 75).

To a 2–5 mL microwave vial were added 83 (88 mg, 433 μmol), 13 (100 mg, 411 μmol), Pd(OAc)2 (1 mg, 4 μmol), RuPhos (6 mg, 13 μmol), and anhydrous Na2CO3 (87 mg, 1 mL). The vial was sealed and purged with argon (3 × evacuation/fill). Degassed EtOH (2.3 mL) was added by syringe, and the mixture was heated at 85 °C for 64 h. The mixture was cooled to rt and filtered through a thin pad of silica (eluent CH2Cl2), and concentrated in vacuo. Purification by flash column chromatography (gradient elution, gradient 5 → 100% EtOAc/petroleum ether) gave 4a as a colorless solid (25 mg, 44%).

3-(3,5-Dimethylisoxazol-4-yl)-5-ethoxybenzoic acid 4b. To a solution of 4a (300 mg, 1.04 mmol) in THF (5 mL) were added water (2.5 mL) and LiOH (37 mg, 1.56 mmol), and the mixture was stirred for 16 h at rt. NaCl (1 M, 10 mL) was then added, and the mixture was washed with EtOAc (3 × 10 mL). The solution was acidified to pH 2 with HCl (1 M) and extracted with CH2Cl2 (3 × 30 mL). The combined organic layers were washed with brine (30 mL) and dried (MgSO4), and concentrated in vacuo.

Peptides. H4Ac4 peptide (BRD2 and BRD4 assays, H2N-SGRGK-(Ac)GGK(Ac)-GGK(GGK)GLGK(Ac)-GGAK(Ac)-RHRK(Biotin)-COOH) and H3K56Ac peptide (CREBBP assay, H2N-ALREIRRYQK(Ac)STELLI-RKLK(Biotin)-COOH) were synthesized by Tufts University Core Facility.

**Thermal Stability Shift Assay.** Thermal melting experiments were carried out using the Mx3005p real-time PCR machine (Agilent) and employing a protein concentration of 2 μM and an inhibitor concentration of 100 mM. Buffer conditions were 10 mM HEPES, pH 7.5, 500 mM NaCl and 1:1000 dilution of SyproOrange (Invitrogen, CA).

The assay and data evaluation were carried out as described previously.19 Experiments were carried out in duplicate; average values are reported.

**AlphaScreen Peptide Displacement Assay.** Bromodomain AlphaScreen assays were carried out as previously described.18,22 All experiments were carried out in duplicate on the same plate.

**Conformational Analysis of Lead Compounds.** Conformational analysis was performed for the two cocrystallized compounds (4b and 4d) using MacroModel (version 9.8) in Schrodinger Suite 2010 from Schrodinger LLC. The dihedral angle between the two ring systems was subjected to coordination scan with 1 degree of increment. The chemical structures from Ligprep were used as the starting structure. The default option was used, including a force field of OPLS2005, a solvent of water, and a PRCG minimization protocol with a convergence gradient of 0.05. The relative energy in kcal/mol of each minimized structure was then plotted versus the dihedral angle.

**Biological Evaluation of Compounds.** Protein Expression and Purification. Proteins were cloned, expressed, and purified as previously described.18

**Cytotoxicity Assay.** HeLa cells were subcultured in DMEM supplemented with 1-glutamine and 10% fetal calf serum. 20000 cells per well were seeded into a 96 well plate. After 24 h of incubation at 37 °C, the medium was changed, and compounds were applied at final concentrations of 10, 1, 0.1, and 0.01 μM. The cells were incubated for 24 or 48 h, after which the medium was changed, and MTI’ solution (5 mg/mL) was added to the plate. After 2 h of incubation at 37 °C, the medium was replaced with 200 μL of DMSO to solubilize the formazan crystals. After a further 15 min, absorbance was measured at 550 nm using a spectrophotometer. Experiments were performed in triplicate on the same plate.

**Accession Codes**

Atomic coordinates and structure factors for the reported crystal structures have been deposited with the Protein Data Bank under accession codes 3SVF (2), 3SVG (4d), and 3SVH (4b).

**Supporting Information.** Supporting figures, schemes, and tables, crystallographic information, further general experimental details, further characterization for compounds 3a–d, 4a–d, and synthesis and characterization of compounds 1, 7, 8, and 10–13. This material is available free of charge via the Internet at http://pubs.acs.org.

**ASSOCIATED CONTENT**

See Supporting Information.
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ABBREVIATION USED

BET, bromodomain and extra terminal domain; BRD, bromodomain-containing protein; BRD2(1)/(2), first/second bromodomain of BRD2; BRD4(1)/(2), first/second bromodomain of BRD4; BRDT, bromodomain, testis-specific; CREB, CAMP response element-binding protein; CREBBP, CREB binding protein; DMAc, N,N-dimethylacetamide; EBV, Epstein–Barr virus; GCN5, general control of amino acid synthesis, yeast, homologue-2; HAT, histone acetyltransferase; HBTU, O-(benzotriazol-1-yl)-N,N,N’,N”-tetramethyluronium hexafluorophosphate; HDAC, histone deacetylase; KAc, acetyl-lysine; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NUT, nuclear protein in testis; P-TEFb, positive transcription elongation factor b; PBL(5), fifth bromodomain of PB1 (BAF180), a unique subunit of the polybromo (PBAF) complex; PCAF, P300/CREBBP-associated factor; RuI’Phos, 2,6-dicyclohexylphosphino-2’,6’-diisopropoxyphenyl; TAFII250, TATA box binding protein-associate factor, 250 kDa

REFERENCES


