

[1,2,4]Triazolo[4,3-*a*]phthalazines: Inhibitors of Diverse Bromodomains

Oleg Fedorov,^{†,‡} Hannah Lingard,[†] Chris Wells,[†] Octovia P. Monteiro,^{†,‡} Sarah Picaud,^{†,§} Tracy Keates,[†] Clarence Yapp,[†] Martin Philpott,[†] Sarah J. Martin,[†] Ildiko Felletar,[†] Brian D. Marsden,^{†,||} Panagis Filippakopoulos,^{†,§} Susanne Müller,[†] Stefan Knapp,^{†,‡} and Paul E. Brennan^{*,†,‡}

[†]Structural Genomics Consortium, Nuffield Department of Medicine, University of Oxford, Old Road Campus Research Building, Roosevelt Drive, Oxford OX3 7DQ, U.K.

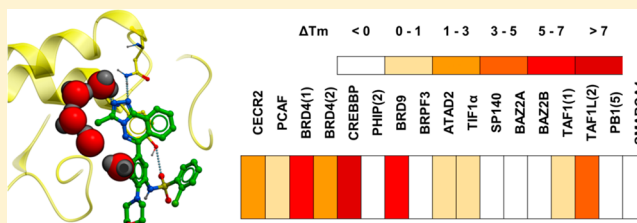
[‡]Target Discovery Institute, Nuffield Department of Medicine, University of Oxford, Roosevelt Drive, Oxford OX3 7FZ, U.K.

[§]Ludwig Institute for Cancer Research, Nuffield Department of Medicine, University of Oxford, Old Road Campus Research Building, Roosevelt Drive, Oxford OX3 7DQ, U.K.

^{||}Kennedy Institute of Rheumatology, University of Oxford, Roosevelt Drive, Oxford OX3 7FY, U.K.

S Supporting Information

ABSTRACT: Bromodomains are gaining increasing interest as drug targets. Commercially sourced and de novo synthesized substituted [1,2,4]triazolo[4,3-*a*]phthalazines are potent inhibitors of both the BET bromodomains such as BRD4 as well as bromodomains outside the BET family such as BRD9, CECR2, and CREBBP. This new series of compounds is the first example of submicromolar inhibitors of bromodomains outside the BET subfamily. Representative compounds are active in cells exhibiting potent cellular inhibition activity in a FRAP model of CREBBP and chromatin association. The compounds described are valuable starting points for discovery of selective bromodomain inhibitors and inhibitors with mixed bromodomain pharmacology.



INTRODUCTION

The rapidly expanding field of epigenetics can be broadly divided into two levels of processes: DNA methylation and histone modification. Various post-translational modifications of histone proteins contribute to the epigenetic code including methylation, acetylation, phosphorylation, ubiquitinylation, and citrullination.¹ Acetylation of lysine residues plays an important role in the regulation of chromatin structure and ultimately transcription due to the charge neutralization that occurs, leading to changes in protein conformation and protein–protein interactions. It is similar to phosphorylation in its prevalence and has been particularly studied on unstructured histone tails. Aberrant lysine acetylation frequently leads to alterations in gene expression, causing activation of pro-survival and proliferation-promoting pathways and inactivation of tumor suppressor functions. Insight into the regulation of *ε*-N-acetyl-lysine (Kac) marks is therefore desirable in the understanding of and development of novel drugs for cancer treatment. Consequently, enzymes that write (acetyltransferases, HATs) and erase (histone deacetylases, HDACs) these marks have emerged as interesting targets.^{2,3}

The bromodomain family of protein interaction modules specifically recognizes the acetyl-lysine mark, and these acetyl-lysine reader domains have likewise gained interest of late as novel targets for pharmacological intervention.^{4,5} Bromodomain-containing proteins are key components in diverse

biological processes and are involved in mediating the assembly of various nuclear protein complexes, including the recruitment of chromatin modifying enzymes and transcriptional regulators to acetylated chromatin. Chromosomal rearrangements, aberrant expression of bromodomain-containing proteins, and protein dysfunctions have been tightly linked to tumorigenesis,⁶ and new avenues for the development of antineoplastic drugs have recently been highlighted by the potent antitumor activity exhibited by inhibitors which selectively target bromodomains.⁷ Known bromodomain inhibitors mainly target the BET family of bromodomains, including BRD3 and BRD4, but many other bromodomain-containing proteins such as CREBBP, TIF1 α , ATAD2, and SMARCA4 have been implicated in a variety of diseases.⁴

Potent BET inhibitors generally fall into three structural classes: isoxazoles,⁸ amides/ureas,⁹ and 1,2,4-triazoles.⁵ Most BET inhibitors described to date have a methyl group adjacent to a hydrogen bond acceptor which mimics the acetyl group of acetyl lysine. The structurally related thieno- and benzodiazepine triazoles (+)-JQ1 and I-BET762 (Figure 1A) were shown to be potent inhibitors of the BET bromodomains and have potential for use in inflammatory disease,¹⁰ atherosclerosis,¹¹ NUT-midline carcinoma,⁷ acute leukemia,¹² lympho-

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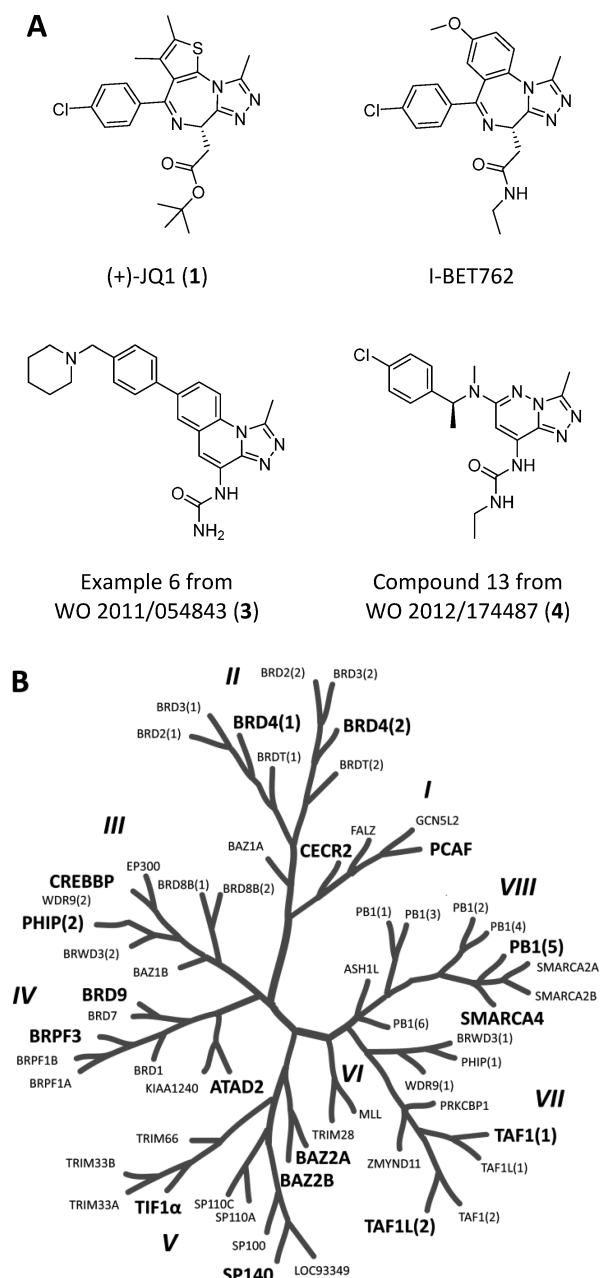


Figure 1. (A) Triazole-containing BET inhibitors. (B) The bromodomain family is made of eight subfamilies (large italic).¹⁷ Family members screened in this work are shown in larger typeface.

ma,¹³ and HIV infection.¹⁴ Two structurally related fused triazoles (compounds 3 and 4) have been exemplified in patents from GSK¹⁵ and Constellation¹⁶ as potent BET inhibitors.

Given the success of the chemical probes (+)-JQ1 and I-BET762 in deciphering the role of the BET subfamily of bromodomains in disease, it is clear that there is an urgent need for inhibitors for the remaining subfamilies of bromodomain-containing proteins in order to investigate their biological function and therapeutic potential.

RESULTS AND DISCUSSION

The selectivity of (+)-JQ1 and I-BET762 for the BETs has been attributed to the 4-chlorophenyl moiety which forms hydrophobic interactions with residues on the edge of the

acetyl-lysine binding pocket including W81. It was hoped that by keeping the 3-methyl-[1,2,4]-triazolo motif but varying the fused ring and pendant substituents, novel compounds could be found that would maintain bromodomain potency with altered selectivity for non-BET proteins. To find new starting points for bromodomains outside the BET family, a number of triazole-containing commercial compounds were purchased and profiled against 17 BRDs in the bromodomain family tree by differential scanning fluorimetry (DSF) (Figure 1B).^{7,18} The [1,2,4]triazolo[4,3-*a*]phthalazines shown in Figure 2 were thought to be attractive potential BRD inhibitors due to the presence of the 5-methyl group adjacent to the triazole H-bond acceptor.¹⁹ It has been shown by Chung et al. that potent BRD inhibitors can be discovered by focusing on privileged substructures.²⁰ By testing the potential inhibitors against bromodomains from the entire protein family by the operationally simple DSF assay in a platform discovery approach, a rapid assessment of BRD potency and selectivity was established.

When tested in a panel of bromodomains, these initial compounds showed hits for BRD4(1), BRD9, CECR2, CREBBP, and TAF1L(2), with the greatest potencies against BRD9 (compounds 7 and 15) and CREBBP (compounds 9, 14, and 17). Very little activity was observed against BAZ2A, BAZ2B, PB1(5), and TIF1 α . All of the commercially available triazolophthalazines shared an amide or sulfonamide substituent at the *meta*-position (R₁) and a methyl group at the *para*-position (R₂) of the pendant phenyl group. Sulfonamides were observed to have greater potency than amides (e.g., compounds 7 and 15 vs compound 5). *N*- and *S*-linked sulfonamides were found to have similar potencies (compound 7 vs 15). A variety of substituents were tolerated on the sulfonamide, both aryl and alkyl. To examine the effect of the omnipresent *para*-methyl group in the commercial compounds, compound 13 was synthesized (Scheme 1). This methyl group was shown to positively influence activity as the *des*-methyl analogue 13 was less active in the DSF assay against all bromodomains tested.

As these compounds showed binding to multiple bromodomains, compound 17 was chosen as a representative for docking into the bromodomain of CREBBP. Although there were other compounds as potent as compound 17, it was chosen for docking studies as it had a good combination of high potency and rigidity which provided a small number of calculated poses. All but the five water molecules previously shown to be important in CREBBP^{8c} were removed from the published complex of CREBBP with a fragment ligand (PDB ID 3SVH), and compound 17 was docked into the protein using the ligedit functionality of ICM-Pro.²¹ As seen in Figure 3, compound 17 has an excellent fit to the bromodomain. The triazole forms two hydrogen bonds via adjacent nitrogen atoms to a structural water molecule and the conserved asparagine residue (N1168) found in most bromodomains. The triazole's methyl group fits well into the cavity formed by the remaining water molecules. The *meta*-sulfonamide formed two hydrogen bonds to arginine (R1173) in CREBBP.

To confirm the potencies initially established by DSF, AlphaScreen competition assays were used to test whether representative compounds 6–8 and 15 could displace labeled peptides from seven representative bromodomains (Table 1).²² These compounds were chosen as representatives as they all had similar structures but differed in their bromodomain inhibition profiles. With one exception (compound 8 with

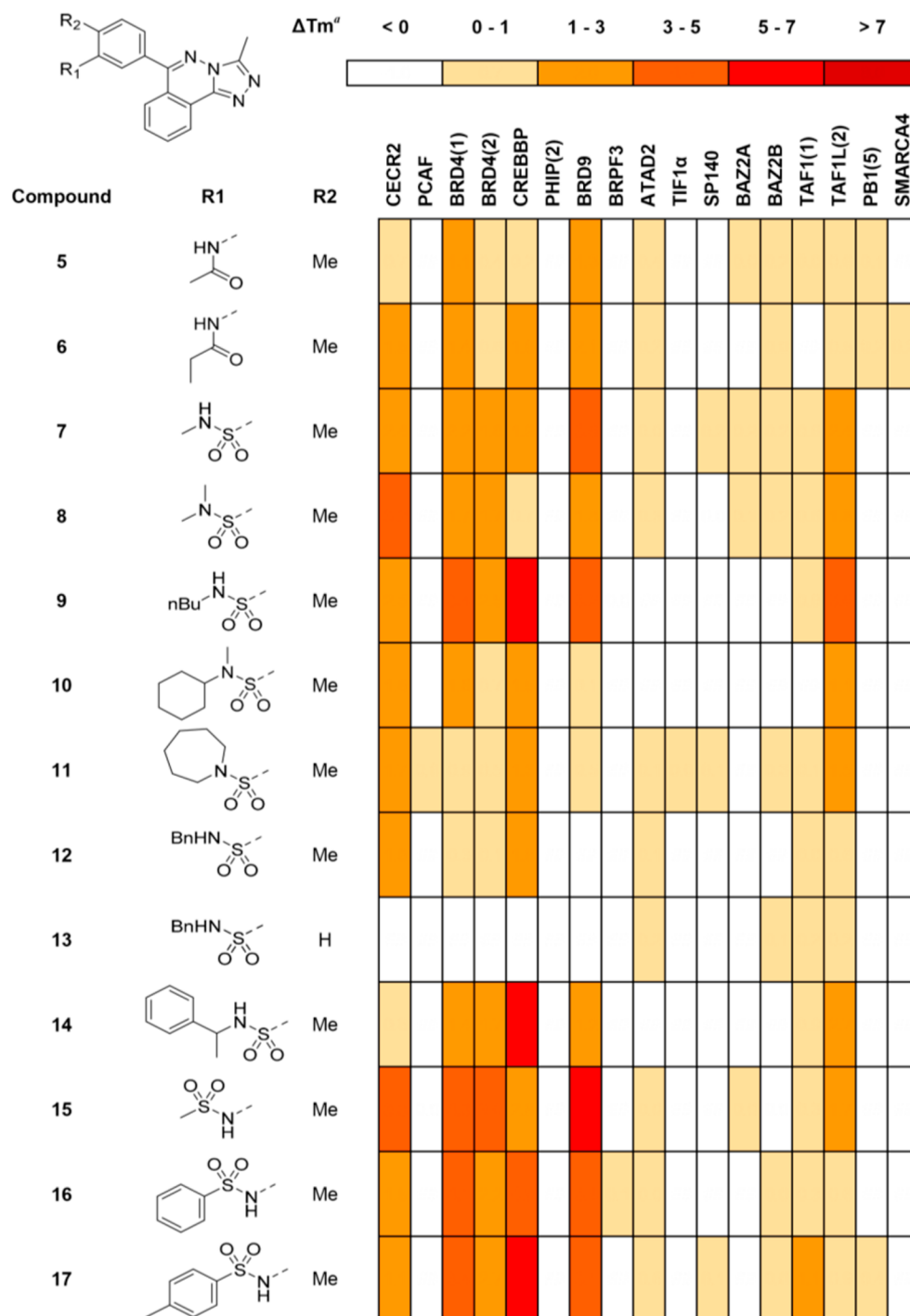
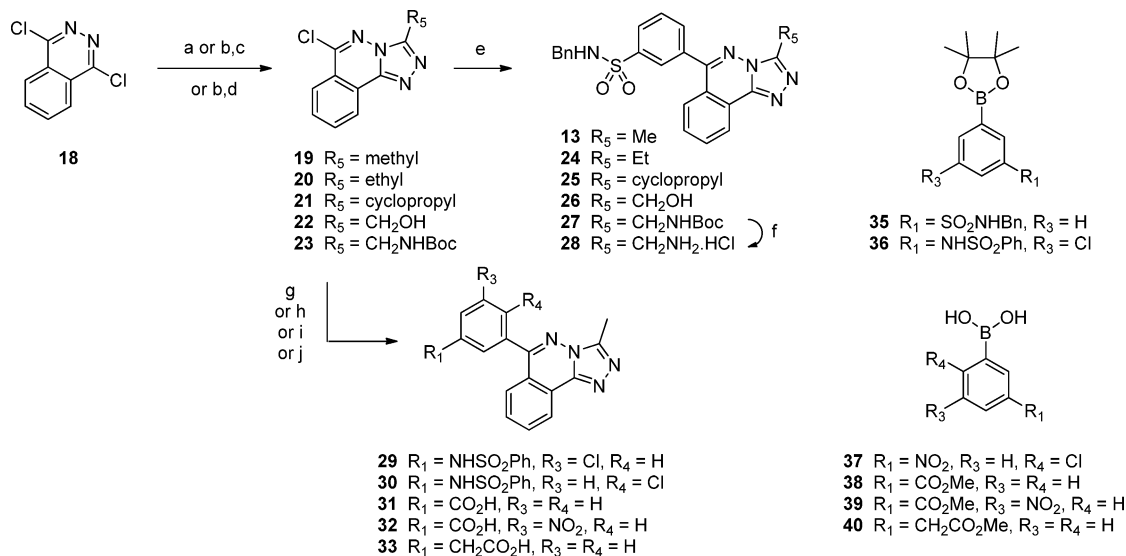


Figure 2. Commercial [1,2,4]triazolo[4,3-a]phthalazines are potent inhibitors of multiple bromodomains by DSF screening.

CREBBP), the pIC_{50} values measured for the representative compounds were consistent with DSF T_m shifts (ΔT_m). The coefficient of determination (R^2) between the two assay formats was high enough that the general and operationally simple DSF assay was felt to be a useful surrogate for the more complex AlphaScreen in further efforts to increase the potency of the compounds.

With an interesting series of inhibitors discovered through compound purchasing, additional analogues were designed and synthesized as shown in Scheme 1. Initial analogues focused on optimizing compounds 12 and 17 for CECR2 and CREBBP potency. Compound 12, although not the most potent

analogue, had a preference for CECR2 and CREBBP by DSF T_m shift. Compound 17 was an attractive CREBBP lead due to its potency and synthetic accessibility. The fused methyl triazole ring was formed by substitution of 1,4-dichlorophthalazine 18 with acetyl hydrazide and in situ condensation. Suzuki coupling of the resulting aryl chloride with boronic ester 35 gave compound 13. Stepwise chloride displacement of compound 18 with hydrazine and subsequent condensation with carboxylic acids gave more elaborate substitution at the 2-position of the triazole in compounds 24–28 following Suzuki reaction. It was hoped that triazole substituents larger than methyl might compensate for the loss of activity on CECR2

Scheme 1. The Synthesis of Compounds 13 and 24–33^a

^aReagents and conditions: (a) NH₂NHAc, *n*BuOH, reflux (41%); (b) NH₂NH₂, THF; (c) R₅CO₂H, *p*-dioxane, reflux (32–42%); (d) Boc-glycine, THF, reflux (39%); (e) **35**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O (29–80%); (f) HCl, EtOAc (100%); (g) **36**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O (25%); (h) (i) **37**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O, (ii) SnCl₄, EtOH, reflux, (iii) PhSO₂Cl, pyridine, THF (16% over 3 steps); (i) **39**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O (35%); (j) (i) **38** or **40**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O, (ii) KOH, MeOH (39–79% over 2 steps).

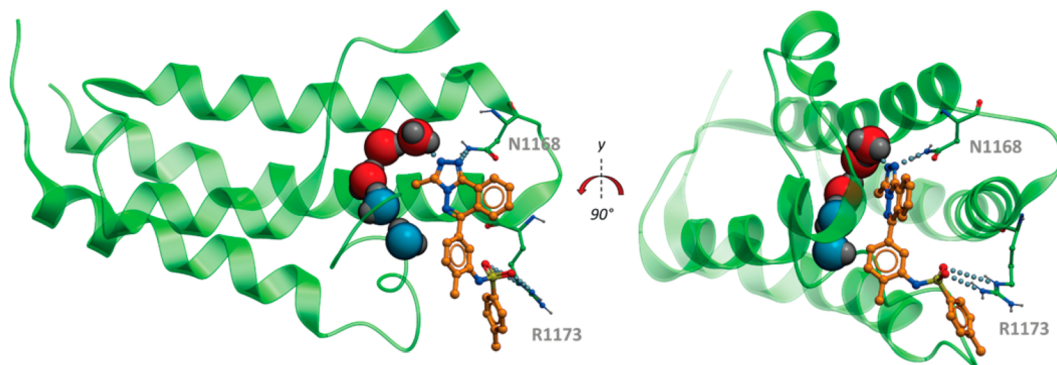


Figure 3. Docking of compound **17** (orange ball and stick) in bromodomain of CREBBP (PDB ID, 3SVH; protein, green ribbon and sticks; water molecules, red and blue spheres; top loop removed for clarity in first image). The triazole moiety forms two H-bonds (dashed line) to a conserved water (red sphere) and asparagine 1168 (N1168). The sulfonamide accepts two H-bonds from arginine 1173 (R1173).

and CREBBP seen between compounds **12** and **13** by displacing conserved waters in the bromodomain pocket (blue sphere in Figure 3). The hydrophobic ethyl and cyclopropyl groups of compounds **24** and **25**, and hydrogen bonding groups of compounds **26** and **28** provided no additional binding beyond the methyl group of compound **13** and could not rescue the loss of the *para*-methyl group from compound **12** (Figure 4).¹⁷

The next group of analogues explored alternative additional substituents to the aryl sulfonamide. Compound **29** was made from the commercially available sulfonamide-containing boronic ester **36**. To examine the effect of a chlorine in the 6-position of the phenyl ring, 2-chloro-5-nitrophenylboronic acid **37** was coupled to intermediate **19**. Reduction and sulfonylation gave compound **30**. Although compound **29** showed only weak binding to all bromodomains, compound **30** had modest binding to BRD4(1) and CREBBP.

As highlighted in Figure 3, CREBBP has an arginine (R1173) at the mouth of the acetylated peptide binding pocket and it was thought that the arylsulfonamide (calculated pK_a 8.1)²³ of

compound **15** might be interacting with this charged arginine. Carboxylic acid-containing compounds were synthesized to try to exploit a potential salt bridge to R1173 and create CREBBP selective inhibitors. Compounds **31**–**33** were synthesized from compound **19** via Suzuki reaction with boronic acids **38**–**40** and hydrolysis of the methyl esters if required. Of the three, compound **33** with its extended acid was the most active against CREBBP but did not show selectivity over BRD4(1). Comparing compound **33** to compounds **12** and **13** showed that the acetic acid group could compensate to some extent for the loss of activity going from **12** to **13** and turn a CECR2 and CREBBP favoring inhibitor into a BRD4(1) and CREBBP inhibitor. Removing all substituents from the 6-phenyl group gave the simplest molecule described so far, compound **34**, which, although it is only a modest inhibitor, has an intriguing selectivity for BRD9.²⁴

The structure–activity relationship (SAR) established thus far had highlighted the importance of a group at the 4-position of the 6-phenyl substituent and the utility of a sulfonamide at the 3-position. The potent and lipophilic analogues in Figure 1,

Table 1. pIC₅₀ of Representative Compounds As Measured by AlphaScreen and ΔT_m As Measured by DSF

target	6		7		8		15	
	ΔT _m °C ^{a,b}	pIC ₅₀	ΔT _m °C ^{a,b}	pIC ₅₀	ΔT _m °C ^{a,b}	pIC ₅₀	ΔT _m °C ^{a,b}	pIC ₅₀
BRD4(1)	1.4 ± 0.40 (3)	4.7 (4.6–4.8) ^c	2.2 ± 0.15 (3)	5.7 (5.6–5.8) ^c	1.7 ± 0.08 (3)	5.9 (5.9–5.9) ^c	4.3 ± 0.16 (3)	5.3 ± 0.54 (2) ^a
BRD9	2.1 ± 0.56 (3)	ND	3.6 ± 0.31 (3)	5.9 (5.7–6.1) ^c	1.8 ± 0.22 (3)	5.8 (5.6–6) ^c	5.6 ± 0.30 (3)	6.3 ± 0.12 (2) ^a
CECR2	1.5 ± 0.54 (3)	5.3 (5.1–5.5) ^c	2.4 ± 0.7 (3)	5.5 (4.9–6.0) ^c	3.2 ± 0.87 (3)	6.6 (6.5–6.7) ^c	3.3 ± 0.14 (3)	6.3 ± 0.17 (2) ^a
CREBBP	1.6 ± 0.10 (3)	5.2 ± 0.48 (2) ^a	1.9 ± 0.12 (3)	5.8 (5.5–6.1) ^c	0.7 ± 0.1 (3)	5.9 (5.7–6.2) ^c	2.8 ± 0.13 (3)	4.8 ± 0.061 (3) ^a
BAZ2B	0.03 ± 0.03 (3)	<4 ^d	0.23 ± 0.09 (3)	<4 ^d	0.20 ± 0.11 (3)	<4 ^d	−0.013 ± 0.18 (3)	<4 ^d
PB1(S)	0.16 ± 0.03 (3)	<4 ^d	−0.18 ± 0.13 (3)	<4 ^d	−0.33 ± 0.05 (3)	<4 ^d	−0.070 ± 0.087 (3)	<4 ^d
TIF1α	−0.14 ± 0.04 (3)	<4 ^d	−0.27 ± 0.05 (3)	<4 ^d	−0.19 ± 0.06 (3)	<4 ^d	−0.12 ± 0.082 (3)	<4 ^d
R ^{2c}	0.90	0.90	0.90	0.80	0.82			

^aMean ± standard error of the mean (number of determinations). ^bCompound concentration 10 μM. ^cpIC₅₀ (95% CI based on duplicate pIC₅₀ measurements). ^d<25% inhibition at 100 μM. ^eCoefficient of determination based on a linear correlation between DSF T_m shift (abscissa) and AlphaScreen pIC₅₀ (ordinate).

such as compounds **14** and **17**, suffered from poor solubility. To identify more potent sulfonamides, without further loss in solubility, a polar replacement for the critical 4-methyl group was sought. As the docking of compound **17** in CREBBP (Figure 3) did not show a direct binding role of the 4-methyl group, its influence was hypothesized to be due to a positive conformational effect on the sulfonamide. *N*-Morpholino- and 4-methylpiperazinyl- groups were chosen as polar methyl substitutes, and analogues incorporating each were synthesized as shown in Scheme 2.

In a route designed to allow synthesis of sulfonamide variants from a common intermediate, 2,5-dibromonitrobenzene **41** was selectively reacted with morpholine or 4-methylpiperazine to give compounds **42** and **43**. After bromide to boronate substitution, Suzuki coupling with compound **19** gave compounds **45** and **47**. Reduction with tin(II) chloride gave the anilines **46** and **48**, which were capped with sulfonyl chlorides to give the inhibitors **49** – **55**.

The resulting compounds **47**–**55** were tested by DSF in the panel of 17 bromodomains, and the results are summarized in Figure 5. The strategy to increase potency by replacing the *para*-methyl group with a cyclic amine was very successful; compound **50** was as potent as compound **15**. However, increasing the size of the sulfonamide from methyl to *para*-tolyl to give compound **49** was detrimental to solubility and accurate T_m shift measurements were difficult to obtain. The chloro-substituted analogues of compound **49** were soluble, and compounds **51**, **52**, and **53** were potent and showed an apparent preference for CREBBP.

Compound **53** is an interesting BRD4-, BRD9-, and CREBBP-favoring inhibitor targeting three different BRDs implicated in leukemia.^{12,25} More polar replacements for the morpholine and chlorophenyl substituents were incorporated in compound **55** in the form of 4-methylpiperazine and 4-methoxyphenylsulfonyl groups, respectively, which gave a preference for BRD9 over other bromodomains.

In a final attempt to displace two of the conserved waters in the bromodomain acetyl-lysine binding pocket (blue sphere in Figure 3), two analogues of the potent inhibitor **53** with triazole methyl group extensions, compounds **56** and **57**, were synthesized from compound **42**. But as in compounds **24**–**28**, they both lost potency compared to the methyl analogue **53**.

Four representative compounds (**50**, **51**, **53**, and **55**) were chosen for confirmation screening and IC₅₀ determination in AlphaScreen peptide displacement assays against five bromodomains (Table 2). In general, all compounds were inactive against TIF1α but the AlphaScreen assays showed less discrimination than the DSF assays for the remaining four bromodomains. This may be due to assay differences; DSF T_m shift is a measure of protein stability increased by ligand interaction, whereas AlphaScreen competition IC₅₀ is also a function of the competing peptide's affinity for the BRD. In addition, the AlphaScreen IC₅₀ is consistently measured at 20–25 °C, whereas the DSF T_m shift is measured at the melting temperature of the protein which varies between proteins. Any compounds that bind with a large entropic contribution would be expected to show differences in potencies between assays run at different temperatures. Compound **50** was a promiscuous inhibitor and showed submicromolar IC₅₀ values against BRD4(1), BRD9, CECR2, and CREBBP, whereas compound **51** was at least 100-fold selective for BRD4(1), BRD9, and CREBBP over CECR2. Compound **53** was similar

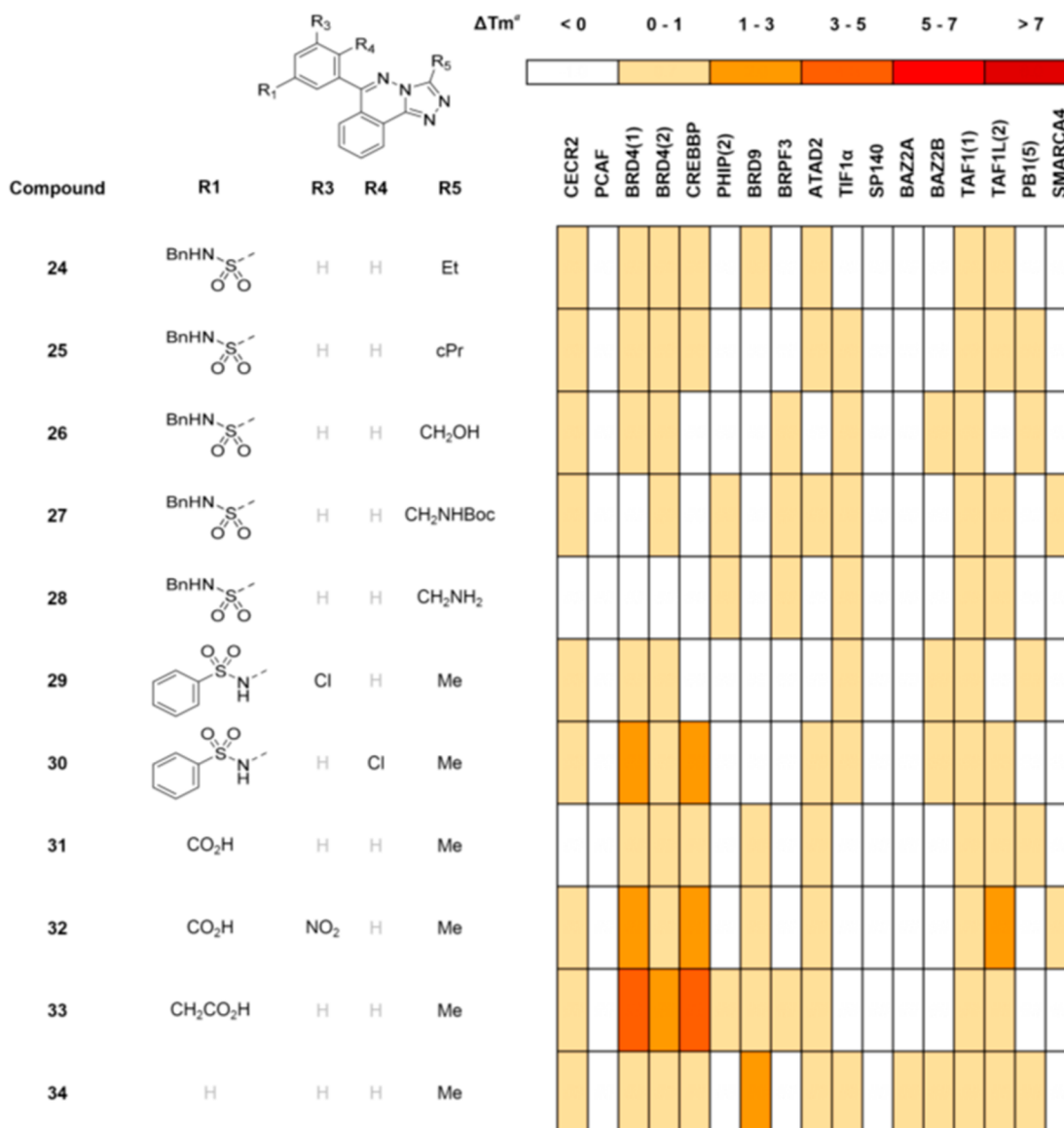


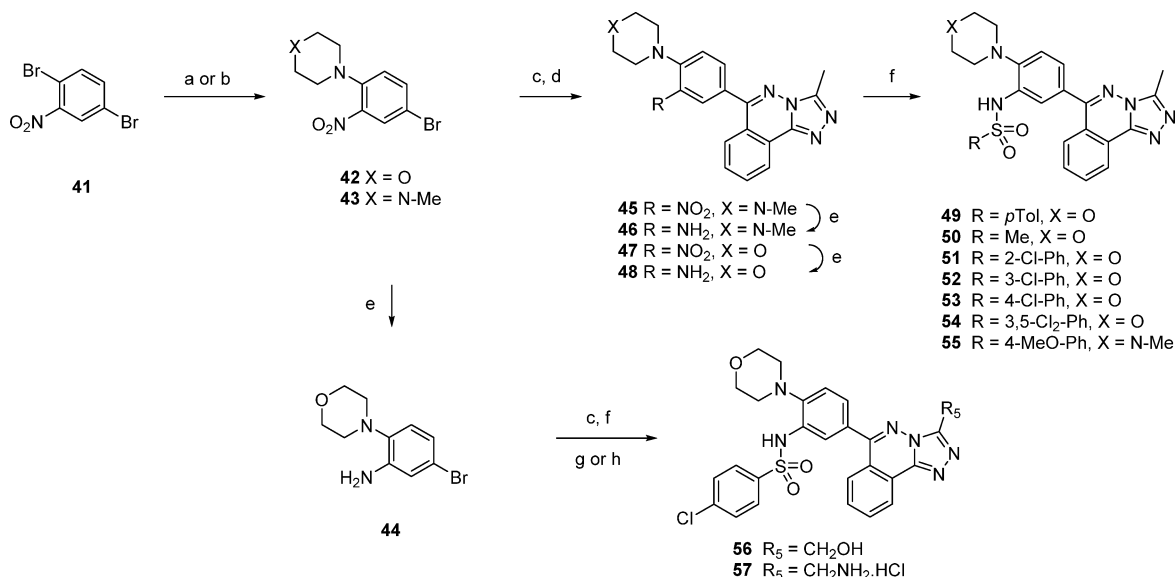
Figure 4. Synthetic inhibitors of multiple bromodomains.

in profile to compound 51, with less discrimination over CECR2. The only piperazine derivative, compound 55, had a slight preference for CECR2 over BRD4(1), but despite inclusion of polar substituents, poor solubility of this compound showed variable IC₅₀ determinations for BRD9 and CREBBP as shown by the high standard errors.

It is noteworthy that the compounds disclosed in this work are potent inhibitors of the CECR2, BRD4(1)/(2), CREBBP, BRD9, and TAF1L(2) bromodomains to varying degrees. The inhibited bromodomains do not cluster as expected from the sequence-based phylogenetic tree (Figure 1b). From the phylogenetic tree it would be expected that closely related BRDs such as PCAF (subfamily I shared with CECR2), PHIP(2) (subfamily III shared with CREBBP), BRPF3 and ATAD2 (subfamily IV shared with BRD9), and TAF1(1) (subfamily VII shared with TAF1L(2)) would also be affected by the inhibitors. It could be argued that the phylogenetic tree in Figure 1b described by Filippakopoulos et al.¹⁷ is based on a sequence alignment of the entire bromodomain, whereas only the residues in the ligand binding region are relevant to selectivity. A more ligand-focused phylogenetic tree has been

described by Vidler et al. which clusters BRDs based on binding site residues.²⁶ This refined analysis goes part of the way to explaining compound selectivity as it clusters PHIP(2) with PB1(5) (both not inhibited) and moves ATAD2 to its own branch, but it does not explain the remaining inconsistencies as PCAF still clusters with CECR2, BRPF3 with BRD9, and TAF1(1) with TAF1L(2). Calculated druggability is also insufficient to explain inhibitor preference as both PCAF and TAF1(1) have similar SiteMap D-scores to CECR2 and TAF1L(2).²⁶

Compound 51 was chosen as a representative of this series, and co-crystallization was attempted with multiple bromodomains. High resolution structures were obtained with BRD4(1) and BRD9 (Figure 6). The overall pose of the compound with the two bromodomains was exactly as expected from the initial docking studies of the predecessor 17 in CREBBP (Figure 3). The triazole moiety formed hydrogen bonds to the conserved asparagine N140 in BRD4(1) and N100 in BRD9 and to a conserved pocket water molecule in both proteins. The phthalazine ring system was sandwiched securely between L94 and I146 in BRD4 and I53 and Y106 in BRD9. The

Scheme 2. The Synthesis of Compounds 49–57^a

^aReagents and conditions: (a) morpholine, *i*PrOH (100%); (b) 4-methylpiperazine, Et₃N, *i*PrOH (100%); (c) (BOpin)₂, Pd(dppf)Cl₂, KOAc, *p*-dioxane/DMSO (47–72%); (d) **19**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O (32–50%); (e) SnCl₂, EtOH, reflux (26–100%); (f) RSO₂Cl, Et₃N, *p*-dioxane or RSO₂Cl, pyridine, DCM (47–81%); (g) **22**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O (62%); (h) (i) **23**, Pd(PPh₃)₄, K₂CO₃, *p*-dioxane/H₂O, (ii) HCl, EtOAc (60%).

pendant aryl ring allowed further interactions of the sulfonamide. In BRD4(1), the nitrogen of the sulfonamide appears to be deprotonated and acting as an H-bond acceptor to tryptophan-81 with a heavy atom distance of 2.1 Å. This unusual interaction has not been described for previous BET inhibitors, which rely on a hydrophobic interaction with W81 for potency. In BRD9, the sulfonamide is rotated to allow a hydrophobic interaction between the 2-chlorophenyl group and I53. This leaves the sulfonamide in a position to accept an H-bond from the phenol of Y106. The morpholine substituent does not make any direct interactions with the protein but keeps the sulfonamide conformation favorable for protein interactions. Although compound **51** was not crystallized in CECR2, it was hypothesized that the difference in selectivity could be rationalized by the large 2-chlorophenylsulfonamide being harder to accommodate in the smaller pocket of CECR2 than the smaller methylsulfonamide of the more promiscuous compound **50**.

The ability of inhibitors to displace the bromodomain of CREBBP from chromatin was assessed using fluorescence recovery after photobleaching (FRAP). A construct consisting of the multimerised bromodomain of CREBBP as well as a similar construct in which the conserved asparagine responsible for binding of acetylated lysine has been mutated to a phenylalanine was transfected into U2OS cells. Cells were treated with the histone deacetylase (HDAC) inhibitor SAHA in order to globally increase lysine acetylation, resulting in a better assay window (Figure 7A). Treatment of the cells with compounds **50**, **51**, **53**, and **55** significantly decreased FRAP recovery times (Figures 7B), indicative of displacement of the BRD construct from hyper-acetylated chromatin. The piperazine derivative, compound **55**, showed a slightly decreased recovery time, indicating a stronger binding to the CREBBP bromodomain.

CONCLUSIONS

A series of potent BRD inhibitor compounds has been developed. Initial SAR in this series shows the potential to develop selective inhibitors for individual bromodomains, with compounds showing some preference for the BRDs of CECR2, BRD4(1), CREBBP, BRD9, and TAF1L(2) over the likes of PCAF, PHIP(2), BRPF3, ATAD2, TIF1 α , SP140, BAZ2A/B, TAF1(1), PB1(5), and SMARCA4. It is not clear from sequence- and structure-based clustering why these novel inhibitors have preference for some BRDs over others. Inhibitors with *in vitro* IC₅₀ < 1 μ M have been identified for the previously untargeted BRDs of BRD9 and CECR2. A modular synthetic route allows diversification of multiple positions of the core and will be used to further explore this scaffold to find more selective molecules to probe the biological function of the less well studied members of this epigenetic reader family. Using a FRAP assay, selected compounds have been shown to be cell active. By adopting a chemical probe approach²⁷ rather than a preselected target approach and characterizing compounds across the entire BRD family, compounds with intriguing polypharmacology have also been uncovered such as compound **53**, which selectively inhibits three bromodomain-containing proteins implicated in leukemia (BRD4, CREBBP, and BRD9).

EXPERIMENTAL SECTION

General Experimental. Commercial reagents were used as received without further purification. Commercial anhydrous solvents were used in reactions, and HPLC grade solvents were employed for workup and chromatography. NMR spectra were recorded using a Varian Mercury 300 or 400 MHz for ¹H and 75 or 101 MHz for ¹³C. The solvent was used as internal deuterium lock. Coupling constants (*J*) are quoted in Hz and are recorded to the nearest 0.5 Hz. Identical proton coupling constants are averaged in each spectrum and reported to the nearest 0.1 Hz. When peak multiplicities are reported, the following abbreviations are used: s = singlet, d = doublet, t = triplet, m = multiplet, br = broadened, dd = doublet of doublets, dt = doublet of

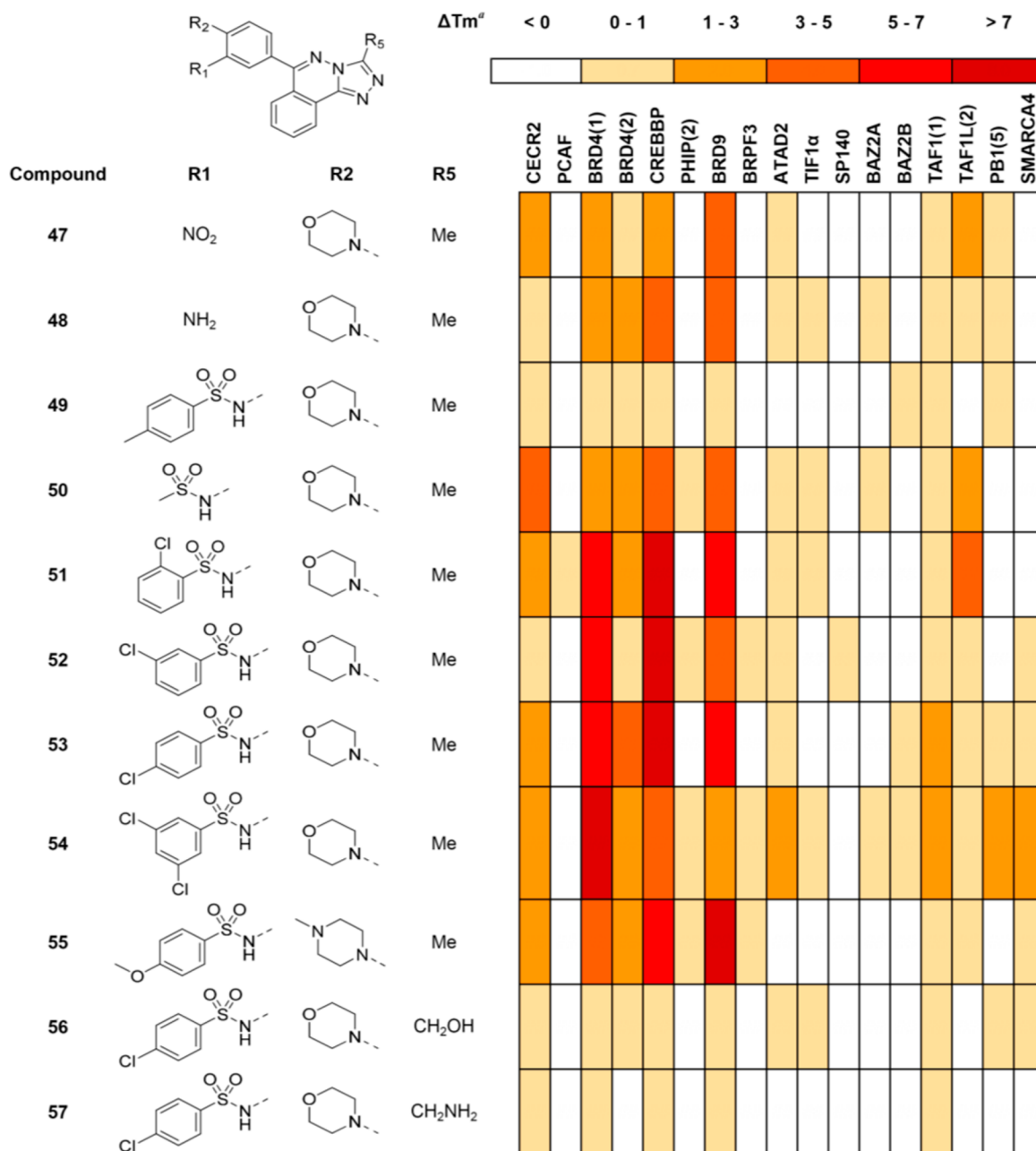


Figure 5. Triazolophthalazines with *para*-aminophenyl substituents are potent bromodomain inhibitors.

triplets. LRMS employed an electrospray ionization source acquiring in positive and negative ionization mode. m/z values are reported in Daltons. Analytical HPLC was carried out on an Agilent 1100 equipped with photodiode array detector (DAD), quaternary gradient pump, and micro plate sampler (Agilent 220). Separation of the analytes was performed upon CenturySil C18-AQ + 5 μ m, 50 mm \times 4.6 mm (Johnson). The flow rate of the mobile phase was kept at 3.5 mL/min. Mobile phases B and C were acetonitrile with 0.35% CF₃CO₂H and water with 0.35% CF₃CO₂H, respectively. The gradient conditions were as follows: 0–0.5 min 1% B and 99% C, 3.7 min 90% B and 10% C, 5 min 99% B and 1% C. The injection volume was 10 μ L. All compounds tested in biological assays were \geq 95% pure by HPLC at 254 nm and by evaporative light scattering detection (ELSD).

Synthetic Procedure and Characterization of Compounds 13, 24–34, 49–57. 6-Chloro-3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazine (19). 1,4-Dichlorophthalazine 18 (5 g, 25.1 mmol) was mixed with *n*-butanol (100 mL) under argon, and acetic hydrazide (3.7 g, 50.2 mmol) was added. The reaction was stirred at reflux overnight.

The mixture was cooled to room temperature, followed by filtration. The solid was washed with EtOAc and MeOH. The solid residue was purified by flash column chromatography (EtOAc:petroleum ether 1:3) to obtain title compound 19 (2.26 g, 41%). MS (ES⁺): m/z calcd for (C₁₀H₇ClN₄ + H)⁺ 219.0, found 219.0. Purity (ELSD) >95%.

N-Benzyl-3-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-benzenesulfonamide (13). A mixture of compound 19 (45 mg, 0.21 mmol), boronate 35 (50 mg, 0.17 mmol), Pd(PPh₃)₄ (20 mg), and K₂CO₃ (58 mg, 0.43 mmol) in dioxane and water was stirred under argon at 120 °C. The reaction was monitored by TLC. Upon completion, water was added and the aqueous layer was extracted with DCM. The organic layers were combined, washed with brine, and dried (Na₂SO₄). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 30:1) to give the title compound 13 (30 mg, 33%). MS (ESI): m/z calcd for (C₂₃H₁₉N₅O₂S + H)⁺ 430.1, found 429.9. ¹H NMR (DMSO-*d*₆) δ 8.60 (1H, d, *J* = 7.8), 8.11–8.02 (3H, m), 7.96–7.79 (3H, m), 7.66 (1H, d, *J* = 8.1), 7.24–7.11 (5H, m), 4.10 (2H, s), 2.71 (3H, s).

Table 2. pIC₅₀ of Representative Compounds As Measured by AlphaScreen and ΔT_m As Measured by DSF

target	50		51		53		55	
	ΔT _m °C ^{a,b,c}	pIC ₅₀ ^{a,d}	ΔT _m °C ^{a,b,c}	pIC ₅₀ ^{a,d}	ΔT _m °C ^{a,b,c}	pIC ₅₀ ^{a,d}	ΔT _m °C ^{a,b,c}	pIC ₅₀ ^{a,d}
BRD4(1)	2.6 ± 0.099 (6)	6.6 ± 0.49 (2)	4.4 ± 0.81 (11)	6.8 ± 0.12 (2)	4.4 ± 0.38 (9)	6.0 ± 0.16 (2)	2.5 ± 0.38 (8)	5.3 ± 0.014 (2)
BRD9	4.9 ± 0.24 (4)	6.5 ± 0.092 (3)	6.6 ± 0.82 (6)	6.7 ± 0.11 (3)	6.2 ± 0.35 (5)	6.0 ± 0.11 (2)	7.3 ± 0.44 (3)	6.2 ± 0.99 (2)
CECR2	3.7 ± 0.16 (3)	6.8 ± 0.17 (2)	1.62 ± 0.48 (4)	4.6 ± 0.24 (2)	2.0 ± 0.16 (3)	4.7 ± 0.10 (2)	2.0 ± 0.16 (3)	6.0 ± 0.14 (2)
CREBBP	3.9 ± 0.33 (4)	6.2 ± 0.62 (2)	7.6 ± 0.94 (7)	6.7 ± 0.074 (2)	7.9 ± 0.33 (6)	6.2 ± 0.57 (2)	6.7 ± 0.35 (6)	6.1 ± 0.61 (2)

^aMean ± standard error of the mean (number of determinations). ^bCompound concentration 10 μM. ^cAll compounds tested showed ΔT_m < 0.2 °C for TIF1α. ^dAll compounds tested showed <30% inhibition of TIF1α at 50 μM.

N-Benzyl-3-(3-ethyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-benzenesulfonamide (**24**). 1-Chloro-4-hydrazinylphthalazine²⁸ (300 mg, 1.54 mmol) was dissolved in propanoic acid (3 mL), and the solution was heated to reflux. The reaction was monitored by TLC. Upon completion, the solvent was removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 20:1) to provide the intermediate 6-chloro-3-ethyl-[1,2,4]triazolo[3,4-*a*]phthalazine (**20**) (149 mg, 42%). A mixture of compound **20** (45 mg, 0.21 mmol), boronate **35** (50 mg, 0.17 mmol), Pd(PPh₃)₄ (20 mg), and K₂CO₃ (58 mg, 0.43 mmol) in dioxane and water was heated and stirred under argon at 120 °C. The reaction was monitored by TLC. Upon completion, water was added and the mixture was extracted with DCM. The organic layers were combined, washed with brine, and dried (Na₂SO₄). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 30:1) to give the title compound **24** (30 mg, 33%). MS (ESI): *m/z* calcd for (C₂₄H₂₁N₃O₂S + H)⁺ 444.1, found 443.9. ¹H NMR (DMSO-*d*₆) δ 8.62 (1H, d, *J* = 7.8), 8.12–7.80 (6H, m), 7.69 (1H, d, *J* = 8.4), 7.25–7.18 (5H, m), 4.12 (2H, s), 3.14 (2H, q, *J* = 7.5), 1.42 (3H, t, *J* = 7.8). HPLC retention time 3.088 min.

N-Benzyl-3-(3-cyclopropyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-benzenesulfonamide (**25**). Following the same procedure as for **24**, the title compound was obtained in 80% yield via compound **21**. MS (ESI): *m/z* calcd for (C₂₅H₂₁N₃O₂S + H)⁺ 456.1, found 455.9. ¹H NMR (DMSO-*d*₆) δ 8.59 (1H, d, *J* = 7.2), 8.10–8.04 (3H, m), 7.95 (1H, d, *J* = 7.8), 7.91–7.80 (2H, m), 7.66 (1H, d, *J* = 7.8), 7.25–7.17 (5H, m), 4.11 (2H, s), 2.45 (1H, m), 1.21–1.15 (4H, m).

N-Benzyl-3-(3-(hydroxymethyl)-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)benzenesulfonamide (**26**). Following the same procedure as for **24**, title compound **26** was obtained in 37% yield via compound **22**. MS (ESI): *m/z* calcd for (C₂₃H₁₉N₃O₃S + H)⁺ 446.1, found 446.1. ¹H NMR (DMSO-*d*₆) δ 8.65 (1H, d, *J* = 8.1), 8.14–8.04 (3H, m), 7.99–7.90 (2H, m), 7.83 (1H, t, *J* = 7.8), 7.69 (1H, d, *J* = 7.8), 7.25–7.18 (5H, m), 4.96 (2H, s), 4.11 (2H, s).

tert-Butyl ((6-(3-(*N*-Benzylsulfamoyl)phenyl)-[1,2,4]triazolo[3,4-*a*]phthalazin-3-yl)-methyl)carbamate (**27**). 1-Chloro-4-hydrazinylphthalazine²⁸ (1.6 g, 8.24 mmol) and Boc-glycine (7.2 g, 40 mmol) were dissolved in THF (150 mL), and the mixture was stirred at reflux. The reaction was monitored by TLC. Upon completion, the mixture was concentrated in vacuo, followed by dilution with H₂O (50 mL) and extraction with DCM (3 × 20 mL). The combined organic layers were dried (Na₂SO₄), concentrated in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 30:1) to provide the intermediate **23** (237 mg, 39%).

A mixture of tricyclic triazole **23** (169 mg, 0.51 mmol), boronate **35** (162 mg, 0.56 mmol), Pd(PPh₃)₄ (58 mg, 0.1 equiv), and K₂CO₃ (175 mg, 1.27 mmol) in dioxane (5 mL) and water (0.5 mL) was stirred and heated under argon at 120 °C. The reaction was monitored by TLC. Upon completion, water (20 mL) was added and the mixture was extracted with DCM (3 × 20 mL). The combined organic layers were dried (Na₂SO₄), concentrated in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 40:1) to give the title compound **27** (80 mg, 29%). MS (ESI): *m/z* calcd for (C₂₈H₂₈N₆O₄S + H)⁺ 545.2, found 545.1. ¹H NMR (CDCl₃): δ 8.72 (1H, d, *J* = 7.8), 8.17 (1H, m), 8.09 (1H, m), 7.95 (1H, m), 7.87 (1H, m), 7.76–7.72 (3H, m), 7.26–7.22 (5H, m), 5.47 (2H, br), 4.92 (2H, d, *J* = 5.7), 4.27 (2H, d, *J* = 6.3), 1.42 (9H, s).

3-(3-(Aminomethyl)-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-*N*-benzylbenzenesulfonamide (**28**). Compound **27** (42 mg, 0.08 mmol) was dissolved in a solution of HCl in EtOAc. The mixture was stirred at room temperature, and the reaction was monitored by TLC. Upon completion, the precipitate was filtered to afford the title compound (40 mg, 100%) as the HCl salt. MS (ESI): *m/z* calcd for (C₂₃H₂₀N₆O₂S + H)⁺ 445.1, found 445.1. ¹H NMR (DMSO-*d*₆): δ 8.70 (1H, d, *J* = 7.8), 8.17 (1H, m), 8.10–8.07 (2H, m), 8.01–7.98 (2H, m), 7.85 (1H, m), 7.75 (1H, d, *J* = 7.8), 7.27–7.22 (5H, m), 4.65 (2H, s), 4.12 (2H, s).

N-(3-Chloro-5-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-phenyl)benzenesulfonamide (**29**). Benzenesulfonyl chloride (0.26 mL, 2 mmol) was added to a mixture of 3-bromo-5-chloroaniline (350

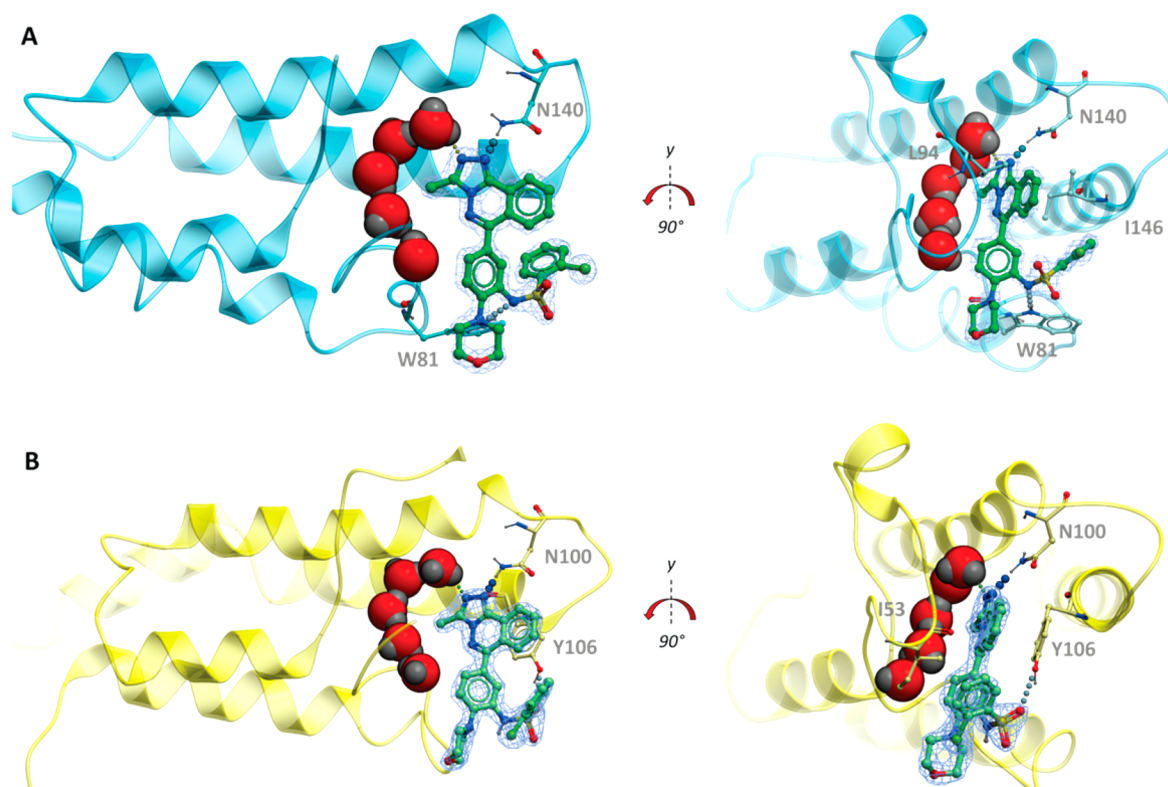


Figure 6. (A) Compound **51** (green stick) in complex with BRD4(1) (PDB ID: 4NQM, blue ribbon and stick, top loop removed for clarity in first image) shows H-bonds (dashed lines) between the triazole moiety, the conserved asparagine (N140), and a pocket water (red sphere). The anionic sulfonamide forms an additional hydrogen bond to tryptophan (W81). (B) In complex with BRD9 (PDB ID: 4NQN, yellow ribbon and stick (the ZA loop was removed for clarity in first image)), compound **51** forms H-bonds to N100 and water but acts as an H-bond acceptor via a sulfonamide oxygen to Y106. The electron density map from the X-ray refinement is shown as a dark-blue mesh around the ligand.

mg, 1.7 mmol) and pyridine (0.16 mL, 2 mmol) in THF (8 mL), and the resulting mixture was stirred at room temperature for 6 h. Water was added, and the aqueous layer was extracted with DCM. The organic layers were combined, washed with brine, and dried (Na_2SO_4). The solvent was removed in vacuo, and the residue was purified by flash column chromatography (EtOAc:petroleum ether 8:1) to give the intermediate *N*-(3-bromo-5-chlorophenyl)-benzene sulfonamide (380 mg).

A mixture of the sulfonamide from the preceding reaction (180 mg, 0.52 mmol), bis(pinacolato)diboron (145 mg, 0.57 mmol), KOAc (101 mg, 1.04 mmol), and $\text{Pd}(\text{dppf})\text{Cl}_2$ (11 mg, 0.016 mmol) in *p*-dioxane (3 mL) and DMSO (0.1 mL) was degassed with argon and heated at 85 °C for 26 h. The reaction was monitored by TLC. Upon completion, the solvents were removed in vacuo, aqueous NaOH (2 M, 20 mL) was added to the residue, and the mixture was stirred for 10 min at room temperature. The mixture was extracted with EtOAc, 6 M HCl was added to the aqueous layer to adjust the pH to 3–4, and it was then extracted with EtOAc. The organic layer was washed with brine, dried (Na_2SO_4), concentrated in vacuo, and purified by flash column chromatography to provide the compound **36** (126 mg).

A mixture of tricyclic triazole **19** (40 mg, 0.19 mmol), boronate **36** (70 mg, 0.18 mmol), $\text{Pd}(\text{PPh}_3)_4$ (20 mg), and K_2CO_3 (6 mg, 0.43 mmol) in dioxane and water was stirred and heated under argon at 120 °C. The reaction was monitored by TLC. Upon completion, water was added and the aqueous layer was extracted with DCM. The organic layers were combined, washed with brine, and dried (Na_2SO_4). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 30:1) to give title compound **29** (20 mg, 25%). MS (ESI): m/z calcd for $(\text{C}_{22}\text{H}_{16}\text{ClN}_5\text{O}_2\text{S} + \text{H})^+$ 450.1 (^{35}Cl) and 452.1 (^{37}Cl), found 449.8 (^{35}Cl) and 451.9 (^{37}Cl). ^1H NMR (DMSO- d_6) δ 8.57 (1H, d, $J = 7.2$), 8.06 (1H, t, $J = 7.2$), 7.81–7.77 (3H, m), 7.64–7.51 (5H, m), 7.34–7.27 (2H, m), 2.69 (3H, s).

N-(4-Chloro-3-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-phenyl)benzenesulfonamide (**30**). **Step 1:** 6-(2-Chloro-5-nitrophenyl)-3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazine. A mixture of tricyclic triazole **19** (500 mg, 2.28 mmol), 2-chloro-5-nitrophenylboronic acid (690 mg, 3.43 mmol), $\text{Pd}(\text{PPh}_3)_4$ (264 mg, 10%), and K_2CO_3 (789 mg, 5.72 mmol) in dioxane and water was stirred and heated under argon at 120 °C. The reaction was monitored by TLC. Upon completion, water was added and the aqueous layer was extracted with DCM. The organic layers were combined, washed with brine, and dried (Na_2SO_4). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (EtOAc:petroleum ether 10:1) to give the title compound (307 mg, 39%). MS (ESI): m/z calcd for $(\text{C}_{16}\text{H}_{10}\text{ClN}_5\text{O}_2 + \text{H})^+$ 340.0 (^{35}Cl) and 342.0 (^{37}Cl), found 339.9 (^{35}Cl) and 341.9 (^{37}Cl). ^1H NMR (CDCl_3) δ 8.79 (1H, d, $J = 7.8$), 8.46–8.42 (2H, m), 7.99 (1H, m), 7.82 (1H, d, $J = 9.6$), 7.74 (1H, m), 7.43 (1H, d, $J = 8.1$), 2.84 (3H, s).

Step 2: 4-Chloro-3-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)aniline. A mixture of tricyclic triazole from step 1 (115 mg, 0.34 mmol) and SnCl_2 (381 mg, 1.69 mmol) in ethanol was stirred under reflux for 5 h. Water was added, the pH was adjusted to 7–8 using saturated aqueous NaHCO_3 , and the aqueous layers were extracted with DCM. The organic layers were combined, washed with brine, and dried (Na_2SO_4). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 20:1) to give the title compound (100 mg, 95%). MS (ESI): m/z calcd for $(\text{C}_{16}\text{H}_{12}\text{ClN}_5 + \text{H})^+$ 310.0 (^{35}Cl) and 312.0 (^{37}Cl), found 309.9 (^{35}Cl) and 311.9 (^{37}Cl). ^1H NMR (CDCl_3) δ 8.71 (1H, m), 7.91 (1H, m), 7.70 (1H, m), 7.56 (1H, m), 7.33 (1H, d, $J = 8.7$), 6.86 (1H, dd, $J = 8.4, 2.7$), 6.79 (1H, d, $J = 2.7$), 3.63 (2H, br s), 2.83 (3H, s).

Step 3. Benzene sulfonyl chloride (0.03 mL, 0.24 mmol) was added to a solution of aniline from step 2 (50 mg, 0.16 mmol) in anhydrous THF (3 mL), followed by addition of pyridine (0.026 mL, 0.32 mmol). The resultant mixture was stirred at room temperature, and

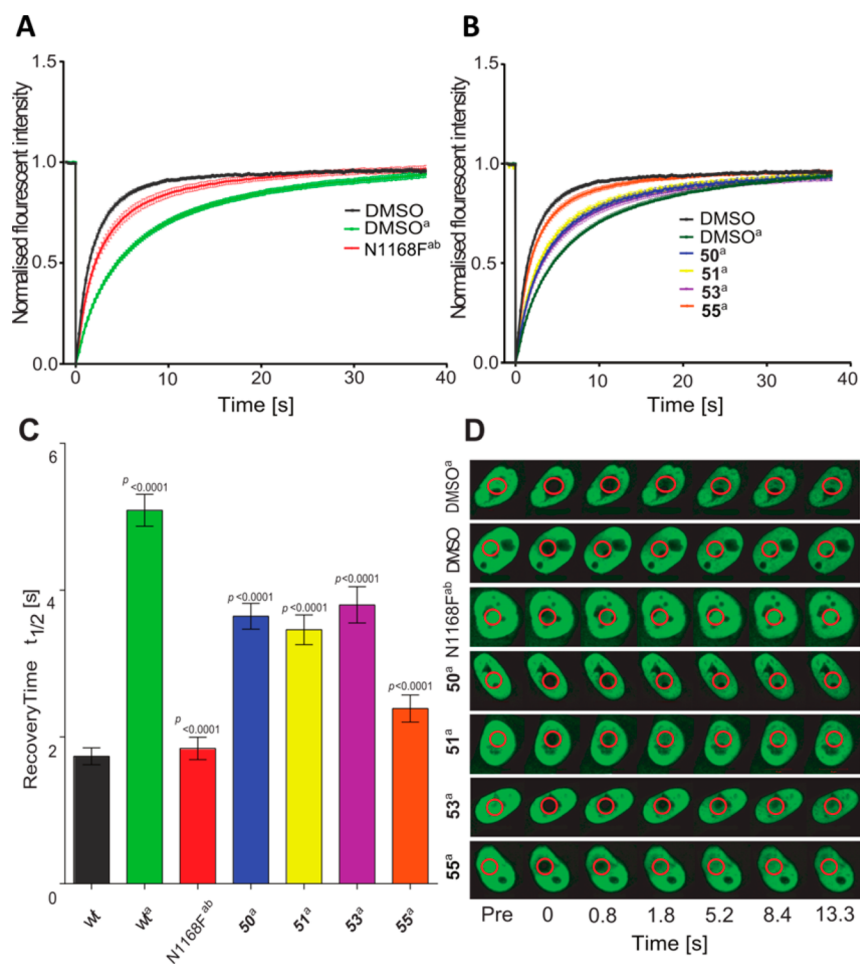


Figure 7. (A) Cells transfected with a trimerized CREBBP-BRD-GFP construct show rapid recovery of fluorescent intensity after photobleaching (FRAP) (black). Recovery time is increased by pretreating cells with 2.5 μ M SAHA* (green) and restored by transfecting with incompetent mutant protein (N1168F, red). (B) Cells treated with SAHA^a (2.5 μ M) and compounds 50 (blue), 51 (yellow), 53 (purple), and 55 (red) (1 μ M) show increased recovery rates. (C) Recovery half-lives of transfected (black), SAHA treated (green), and SAHA plus compound treated cells. (D) Fluorescent images of cells show rapid recovery of photobleached area (red circle) after compound treatment. *SAHA treated cells; ^bN1168F, mutation of N1168 to Phe.

the reaction was monitored by TLC. Upon completion, water was added and the aqueous layer was extracted with DCM. The organic layers were combined, washed with brine, and dried (Na_2SO_4). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 30:1) to give title compound 30 (30 mg, 42%). MS (ESI): m/z calcd for ($\text{C}_{22}\text{H}_{16}\text{ClN}_5\text{O}_2\text{S} + \text{H}$)⁺ 450.1 (^{35}Cl) and 452.1 (^{37}Cl), found 449.9 (^{35}Cl) and 451.9 (^{37}Cl). ^1H NMR (DMSO- d_6) δ 8.58 (1H, d, $J = 7.8$), 8.07 (1H, t, $J = 7.8$), 7.85–7.79 (3H, m), 7.71–7.57 (4H, m), 7.39 (1H, dd, $J = 8.7, 2.7$), 7.32 (1H, d, $J = 2.7$), 7.19 (1H, d, $J = 8.1$), 2.69 (3H, s).

3-(3-Methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)benzoic Acid (31). A mixture of tricyclic triazole 19 (24 mg, 0.11 mmol), boronic acid 38 (21 mg, 0.12 mmol), $\text{Pd}(\text{PPh}_3)_4$ (13 mg, 0.1 equiv), and K_2CO_3 (37 mg, 0.27 mmol) in dioxane and water was stirred and heated under argon at 120 $^\circ\text{C}$. The reaction was monitored by TLC. Upon completion, water was added and the aqueous layers were extracted with DCM. The organic layers were combined and dried (Na_2SO_4). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 30:1) to give the Suzuki adduct methyl 3-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)benzoate (29 mg, 83%). The intermediate ester (29 mg, 0.09 mmol) was dissolved in methanol (14 mL), and KOH (99 mg, 1.76 mmol) was added. The reaction was monitored by TLC, and upon completion water (21 mL) was added. The mixture was adjusted to pH 3–4 with 6 M HCl and extracted with DCM. The combined organic layers were dried (Na_2SO_4), and the solvent was removed in

vacuo to give the title compound 31 (26 mg, 95%). MS (ESI): m/z calcd for ($\text{C}_{17}\text{H}_{12}\text{N}_4\text{O}_2 + \text{H}$)⁺ 305.1, found 305.1. ^1H NMR (DMSO- d_6) δ 8.60 (1H, d, $J = 7.8$), 8.25–8.19 (2H, m), 8.08 (1H, m), 7.98 (1H, m), 7.88 (1H, m), 7.80–7.76 (2H, m), 2.72 (3H, s).

3-(3-Methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-5-nitrobenzoic Acid (32). Following the same procedure as for compound 31 step 1, the methyl ester hydrolyzed under the reaction conditions to yield title compound 32 directly (35%). MS (ESI): m/z calcd for ($\text{C}_{17}\text{H}_{11}\text{N}_5\text{O}_4 + \text{H}$)⁺ 350.1, found 350.0. ^1H NMR (DMSO- d_6) δ 8.84–8.79 (2H, m), 8.64–8.60 (2H, m), 8.09 (1H, m), 7.90–7.80 (2H, m), 2.73 (3H, s).

2-(3-(3-Methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)phenyl)acetic Acid (33). Tricyclic triazole 19 and boronic acid 40 were coupled and hydrolyzed using the same procedures as for compound 31 give the desired compound (Suzuki, 53%; hydrolysis, 73%). MS (ESI): m/z calcd for ($\text{C}_{18}\text{H}_{14}\text{N}_4\text{O}_2 + \text{H}$)⁺ 319.1, found 319.1. ^1H NMR (DMSO- d_6) δ 8.58 (1H, d, $J = 7.8$), 8.21 (1H, m), 7.94–7.76 (2H, m), 7.69–7.46 (4H, m), 3.75 (2H, s), 2.72 (3H, s).

3-Methyl-6-phenyl-[1,2,4]triazolo[3,4-*a*]phthalazine (34). 1-Hydrazino-4-phenyl-phthalazine (300 mg, 1.27 mmol) was dissolved in acetic acid (3 mL) and refluxed for 2 h. The reaction mixture was concentrated in vacuo and the residue purified by flash column chromatography (EtOAc:petroleum ether 1:3) to give title compound 34 (269 mg, 81% yield). MS (ESI): m/z calcd for ($\text{C}_{16}\text{H}_{12}\text{N}_4 + \text{H}$)⁺ 261.1, found 261.0. ^1H NMR (CDCl_3) δ 8.67 (1H, d, $J = 6.0$), 7.89–7.82 (2H, m), 7.64–7.49 (6H, m), 2.77 (3H, s).

4-(4-(3-Methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-nitrophenyl)morpholine (**47**). 4-(4-Bromo-2-nitrophenyl)morpholine²⁹ (1.03 g) was treated with bis(pinacolato)diboron (1 g, 3.94 mmol), KOAc (703 mg, 7.16 mmol), and Pd(dppf)Cl₂ (89 mg) in *p*-dioxane (20 mL) and DMSO (0.5 mL) and heated to reflux under argon overnight. After removal of the solvents in vacuo, aqueous NaOH (2 M, 10 mL) was added to the residue and the mixture was stirred for 30 min at room temperature. The mixture was extracted with EtOAc, and 6 M HCl was added to the aqueous layer to adjust the pH to 3–4. The precipitate formed was collected by filtration and dried then purified by flash column chromatography (DCM:MeOH 50:1) to provide the corresponding boronate (860 mg, 72%) which was used directly.

A mixture of aryl chloride **19** (59 mg, 0.27 mmol), boronate from the previous reaction (100 mg, 0.30 mmol), K₂CO₃ (94 mg, 0.68 mmol), and Pd(PPh₃)₄ (31 mg) in *p*-dioxane (5 mL) and water (0.5 mL) was heated to reflux under argon. The reaction was monitored by TLC. Upon completion, the mixture was filtered, and the filtrate was evaporated to dryness. The residue was partitioned between EtOAc and water. The organic layer was washed with brine, dried (Na₂SO₄), concentrated in vacuo, and the crude residue was purified by flash column chromatography (petroleum ether:EtOAc 1:10) to give the title compound **47** (50 mg, 50%). MS (ESI): *m/z* calcd for (C₂₀H₁₈N₆O₃ + H)⁺ 391.1, found 390.9. ¹H NMR (CDCl₃) δ 8.81 (1H, d, *J* = 8.1), 8.19 (1H, d, *J* = 2.1), 8.02–7.77 (4H, m), 7.34 (1H, d, *J* = 8.7), 3.92 (4H, t, *J* = 4.5), 3.23 (4H, t, *J* = 4.5), 2.86 (3H, s).

5-(3-Methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholinoniline (**48**). A mixture of nitrophenyl **45** (50 mg, 0.13 mmol) and SnCl₂ (144 mg, 0.64 mmol) in ethanol was stirred under reflux for 5 h. Water was added, and the pH was adjusted to 7–8 by adding saturated aqueous NaHCO₃. The mixture was filtered to remove the precipitate, and the filtrate was extracted with DCM. The organic layers were combined, washed with brine, and dried (Na₂SO₄). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 60:1) to give title compound **48** (12 mg, 26%). MS (ESI): *m/z* calcd for (C₂₀H₂₀N₆O + H)⁺ 361.1, found 361.1. ¹H NMR (CDCl₃) δ 8.72 (1H, d, *J* = 7.8), 7.99 (1H, d, *J* = 8.1), 7.91 (1H, t, *J* = 7.5), 7.71 (1H, t, *J* = 7.8), 7.17 (1H, m), 7.05–7.04 (2H, m), 3.92 (4H, t, *J* = 4.2), 3.04 (4H, t, *J* = 4.2), 2.83 (3H, s).

4-Methyl-N-(5-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholinophenyl)benzenesulfonamide (**49**). Et₃N (36 μL, 0.249 mmol) was added to a solution of **48** (30 mg, 0.083 mmol) in *p*-dioxane (5 mL), followed by addition of *p*-toluenesulfonyl chloride (36 mg, 0.19 mmol). The reaction mixture was stirred at room temperature and monitored by TLC. Upon completion, water was added and the aqueous layers were extracted with DCM. The organic layers were combined, washed with brine, and dried (Na₂SO₄). The solvents were removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 60:1) to give title compound **49** (27 mg, 63%). MS (ESI): *m/z* calcd for (C₂₇H₂₆N₆O₃S + H)⁺ 515.1, found 514.9. ¹H NMR (CDCl₃) δ 8.78 (1H, d, *J* = 7.8), 8.01–7.70 (7H, m), 7.39–7.25 (3H, m), 3.86 (4H, t, *J* = 4.2), 2.84 (3H, s), 2.74 (4H, t, *J* = 4.2), 2.40 (3H, s).

N-(5-(3-Methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholinophenyl)methane-sulfonamide (**50**). Methanesulfonyl chloride (14 mg, 0.12 mmol) was added to a solution of **48** (22 mg, 0.06 mmol) in DCM (1.6 mL), followed by addition of pyridine (15 μL, 0.03 mmol). The resulting mixture was stirred at room temperature, and the reaction was monitored by TLC. Upon completion, water was added and the aqueous layers were extracted with DCM. The organic layers were combined and dried (Na₂SO₄). The solvent was removed in vacuo, and the residue was purified by flash column chromatography (DCM:MeOH 30:1) to give compound **50** (16 mg, 60%). MS (ESI): *m/z* calcd for (C₂₁H₂₂N₆O₃S + H)⁺ 439.1, found 439.1. ¹H NMR (CDCl₃) δ 8.79 (1H, d, *J* = 7.8), 8.00–7.94 (2H, m), 7.90–7.85 (2H, m), 7.78 (1H, m), 7.48 (2H, m), 3.98–3.95 (4H, m), 3.20 (3H, s), 3.04–3.01 (4H, m), 2.86 (3H, s).

2-Chloro-N-(5-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholinophenyl)benzenesulfonamide (**51**). Following the same procedure as for compound **50** gave compound **51** (29 mg, 81%). MS

(ESI): *m/z* calcd for (C₂₆H₂₃ClN₆O₃S + H)⁺ 535.1 for ³⁵Cl and 537.1 for ³⁷Cl, found 535.0 and 537.0. ¹H NMR (CDCl₃) δ 8.80 (2H, m), 8.13 (1H, d, *J* = 7.4), 7.96 (1H, m), 7.79–7.64 (3H, m), 7.56–7.45 (2H, m), 7.41–7.30 (3H, m), 3.98–3.87 (4H, m), 3.05–2.90 (4H, m), 2.80 (3H, s).

3-Chloro-N-[5-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholinophenyl]benzenesulfonamide (**52**). Following the same procedure as for compound **50** gave compound **52** (36 mg, 78%). MS (ESI): *m/z* calcd for (C₂₆H₂₃ClN₆O₃S + H)⁺ 535.1 for ³⁵Cl and 537.1 for ³⁷Cl, found 535.0 and 537.0. ¹H NMR (CDCl₃) δ 8.77 (1H, d, *J* = 8.0), 8.09 (1H, s), 7.96 (1H, m), 7.88–7.81 (2H, m), 7.81–7.70 (3H, m), 7.55 (1H, m), 7.40 (3H, m), 3.95–3.79 (4H, m), 2.82 (3H, s), 2.78–2.66 (4H, m).

4-Chloro-N-[5-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholinophenyl]benzenesulfonamide (**53**). Following the same procedure as for compound **50** gave compound **53** (37 mg, 64%). MS (ESI): *m/z* calcd for (C₂₆H₂₃ClN₆O₃S + H)⁺ 535.1 for ³⁵Cl and 537.1 for ³⁷Cl, found 535.1 and 537.0. ¹H NMR (CDCl₃) δ 8.83 (1H, d, *J* = 7.8), 8.06 (1H, s), 7.98 (1H, m), 7.85–7.69 (5H, m), 7.46–7.34 (4H, m), 3.93–3.81 (4H, m), 2.85 (3H, s), 2.81–2.70 (4H, m).

3,5-Dichloro-N-[5-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholinophenyl]benzenesulfonamide (**54**). Following the same procedure as for compound **50** gave compound **54** (33 mg, 64%). MS (ESI): *m/z* calcd for (C₂₆H₂₂Cl₂N₆O₃S + H)⁺ 569.1 for ³⁵Cl₂ and 571.1 for ³⁵Cl³⁷Cl, found 569.0 and 571.0. ¹H NMR (CDCl₃) δ 8.76 (1H, d, *J* = 7.9), 8.17 (1H, br s), 7.95 (1H, m), 7.86–7.61 (5H, m), 7.54 (1H, m), 7.48–7.32 (2H, m), 3.90 (4H, m), 2.83 (3H, s), 2.77 (4H, m).

4-Methoxy-N-[5-(3-methyl-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-(4-methylpiperazin-1-yl)phenyl]benzenesulfonamide (**55**). Starting from compound **41** and 4-methylpiperazine, compound **55** (16 mg) was synthesized in a manner analogous to compound **49**. MS (ESI): *m/z* calcd for (C₂₈H₂₉N₇O₃S + H)⁺ 544.2, found 544.3. ¹H NMR (300 MHz, CDCl₃): δ 8.75 (1H, d, *J* = 7.2), 8.03 (1H, br s), 7.93 (1H, m), 7.86–7.80 (4H, m), 7.73 (1H, m), 7.36–7.35 (2H, m), 6.92 (2H, d, *J* = 9.0), 3.85 (3H, s), 2.84 (7H, m), 2.69 (4H, br s), 2.46 (3H, s).

4-Chloro-N-[5-(3-(hydroxymethyl)-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl)-2-morpholino-phenyl]benzenesulfonamide (**56**). 5-Bromo-2-morpholinoniline²⁹ **44** (640 mg, 2.49 mmol), bis(pinacolato)diboron (945 mg, 3.73 mmol), Pd(dppf)Cl₂ (91 mg), and KOAc (610 mg, 6.23 mmol) were dissolved in dioxane (30 mL) and DMSO (1 mL) and heated to 85 °C under argon. The reaction was stirred and monitored by TLC; upon completion, the solvent was removed in vacuo and the residue was dissolved in 2 N NaOH (20 mL) and stirred for 10 min. The mixture was extracted with diethyl ether, and the combined organic layers were dried (Na₂SO₄). After removal of the solvent in vacuo, the residue was purified by flash column chromatography (petroleum ether:EtOAc 4:1) to give the intermediate boronate (960 mg, 100%).

4-Chlorobenzene-1-sulfonyl chloride (435 mg, 2.06 mmol) was added to a solution of the boronate from the previous reaction (314 mg, 1.03 mmol) in DCM (5 mL), followed by addition of pyridine (0.25 mL, 3.09 mmol). The mixture was stirred at room temperature, and the reaction was monitored by TLC. Upon completion, water was added and the aqueous layer was extracted with DCM. The combined organic layers were dried (Na₂SO₄), and the solvent was removed in vacuo. The residue was purified by flash column chromatography (petroleum ether:EtOAc 6:1) to give the sulfonamide (232 mg, 47%), which was coupled to the heteroaryl chloride **22** as in the preparation of compound **26** to give the title compound **56** (23 mg, 62%). MS (ESI): *m/z* calcd for (C₂₆H₂₃ClN₆O₄S + H)⁺ 551.1 for ³⁵Cl and 553.1 for ³⁷Cl, found 551.1 and 553.1. ¹H NMR (300 MHz, CDCl₃) δ 8.87 (1H, d, *J* = 7.8), 8.05 (2H, m), 7.89–7.81 (5H, m), 7.49 (2H, d, *J* = 8.7), 7.44–7.37 (2H, m), 5.33 (2H, s), 3.91 (4H, m), 2.82 (4H, m).

N-[5-[3-(Aminomethyl)-[1,2,4]triazolo[3,4-*a*]phthalazin-6-yl]-2-morpholinophenyl]-4-chlorobenzenesulfonamide (**57**). Following analogous procedures as for the preparation of compound **56** gave compound **57** from compound **44** (16 mg, 60%). MS (ESI): *m/z* calcd for (C₂₆H₂₄ClN₇O₃S + H)⁺ 550.1 for ³⁵Cl and 552.1 for ³⁷Cl, found

550.1 and 552.0. ^1H NMR (300 MHz, $\text{CDCl}_3/\text{CD}_3\text{OD}$) δ 8.65 (1H, d, $J = 8.1$), 8.02 (1H, m), 7.84 (2H, d, $J = 4.2$), 7.64 (3H, m), 7.43 (1H, d, $J = 8.1$), 7.36–7.32 (3H, m), 4.64 (2H, s), 3.75 (4H, br s), 2.73 (4H, br s).

Biological Evaluation. Protein Expression and Purification. Proteins were cloned, expressed, and purified as previously described.⁷

Peptides. H4Ac4 peptide (BRD4 and CECR2 assays, H_2N -YSGRGK(Ac)GGK(Ac)GLGK(Ac)-GGAK(Ac)RHRK-(Biotin)- CO_2H), H3K56(Ac) peptide (CREBBP assay, H_2N -ALREIRRYQK(Ac)-STELLIRLKLK(Biotin)- CO_2H), H2K9(Ac)K13(Ac)K15(Ac) peptide (BRD9 assay, H_2N -YSGRGKQGGK(Ac)ARAK(Ac)AK(Ac)-TRSSRA-biotin), H3K14(Ac) peptide (BAZ2B, PB1(S) and TIF1 α assays, H_2N -YQTARKSTGGK(Ac)APRKQLATKA-K(biotin)- CO_2H) were synthesized by Tufts University Core Facility, Pepceuticals, or Alta Biosciences.

DSF T_m Shift Assay. Bromodomain DSF T_m shift assays were carried out as previously described.^{8a}

AlphaScreen Peptide Displacement Assay. Bromodomain AlphaScreen assays were carried out as previously described.^{8a} All experiments were carried out in duplicate on the same plate.

CREBBP Fluorescence Recovery After Photobleaching (FRAP) Assay. FRAP studies were performed using a protocol modified from previous studies.^{7,30,31} In brief, U2OS cells were transfected (Lipofectamine 2000, Life Technologies) with mammalian overexpression constructs encoding a GFP chimera with three tandem repeats of the CREBBP bromodomain (corresponding to amino acids 869–1341, with or without N1168F mutagenesis, of RefSeq CREBBP (NM_004380)) in pcDNA6.2/N-EmGFP-DEST (Life Technologies). SAHA was added 4 h post transfection and compounds as indicated 16 h post transfection. The FRAP and imaging system consisted of a Zeiss LSM 710 scanhead (Zeiss GmbH, Jena, Germany) coupled to an inverted Zeiss Axio Observer.Z1 microscope equipped with a high-numerical-aperture (NA 1.3) 40 \times oil immersion objective (Zeiss GmbH, Jena, Germany) equipped with a heated chamber set at 37 $^\circ\text{C}$. FRAP and GFP fluorescence imaging were carried out with an argon-ion laser (488 nm) and with a piezomultiplier tube (PMT) detector set to detect fluorescence between 500 and 550 nm. A 27.5 μm^2 region of a GFP-positive nucleus was selected, and after 5 prescans, the region was bleached. A time-lapse series was then taken to record GFP recovery using 1% of the power used for bleaching with an interval time of ~ 0.25 s. The image data sets and fluorescence recovery data were exported from ZEN 2009, the microscope control software, into Microsoft Excel. The average intensity at each imaging time point was measured for three regions of interest: the bleached region (I_t), the total cell nucleus (T_t), and a random region outside of the cell for background subtraction (BG). The relative fluorescence signal in the bleached region was calculated for each time point t , with the following equation:³²

$$\frac{(T_{\text{average pre-bleach}} - \text{BG})(I_t - \text{BG})}{(I_{\text{average pre-bleach}} - \text{BG})(T_t - \text{BG})}$$

The baseline was normalized to zero and the prebleach to 1. Normalized data was imported into GraphPad Prism 6.0, and half times of recovery were calculated from individual single exponential curve fittings and presented as the mean. P Values were calculated using the unpaired t test.

X-ray Crystallography. Crystallization. Aliquots of the purified proteins were set up for crystallization using a mosquito crystallization robot (TTP Labtech, Royston UK). Coarse screens were typically set up onto Greiner 3-well plates using three different drop ratios of precipitant to protein per condition (100 + 50 nL, 75 + 75 nL, and 50 + 100 nL). Initial hits were optimized further by scaling up the drop sizes. All crystallizations were carried out using the sitting drop vapor diffusion method at 4 $^\circ\text{C}$. Crystals of BRD4(1) with compound 51 were grown by mixing 200 nL of the protein (9.2 mg/mL and 5 mM final ligand concentration) with 100 nL of reservoir solution containing 0.1 M SPG pH 8.0 and 60% MPD. BRD9 crystals with compound 51 were grown by mixing 100 nL of protein (14.9 mg/mL and 5 mM final ligand concentration) with 200 nL of reservoir

solution containing 0.2 M KSCN and 20% PEG3350. In both cases, diffraction quality crystals grew within a few days.

Data Collection and Structure Solution. BRD9 crystals were cryoprotected using the well solution supplemented with additional ethylene glycol and were flash-frozen in liquid nitrogen. BRD4 crystals were frozen without any additional cryoprotection. Data were collected in-house on a Rigaku FRET rotating anode system equipped with a RAXIS-IV detector at 1.52 \AA . Indexing and integration was carried out using MOSFLM,³³ and scaling was performed with SCALA.³⁴ Initial phases were calculated by molecular replacement with PHASER³⁵ using the known models of BRD4(1) (PDB ID 2OSS) and BRD9 (PDB ID 3HME). Initial models were built by ARP/wARP,³⁶ followed by manual building in COOT.³⁷ Refinement was carried out in REFMAC5.³⁸ In all cases, thermal motions were analyzed using TLSMD³⁹ and hydrogen atoms were included in late refinement cycles. Data collection and refinement statistics can be found in Table 3. The models and structure factors have been deposited with PDB accession codes: 4NQM (BRD4(1)/compound 51), 4NQN (BRD9/compound 51).

Table 3. Data Collection and Refinement Statistics for BRD4(1) and BRD9 Complexes

Data Collection		
PDB ID	4NQM	4NQN
protein	BRD4(1)	BRD9
ligand	compd 51	compd 51
space group	$P2_12_12_1$	$P2_12_12_1$
cell dimensions		
a, b, c (\AA)	45.51, 46.57, 62.38	47.12, 48.42, 69.32
α, β, γ (deg)	90.00, 90.00, 90.00	90.00, 90.00, 90.00
resolution* (\AA)	1.58 (1.66–1.58)	1.73 (1.82–1.73)
unique observations*	18694 (2514)	17147 (2438)
completeness* (%)	98.7 (93.5)	99.9 (99.8)
redundancy*	3.9 (2.8)	4.4 (4.1)
R_{merge} *	0.049 (0.439)	0.060 (0.523)
$I/\sigma I$ *	15.7 (2.0)	12.6 (2.0)
Refinement		
resolution (\AA)	1.58	1.73
$R_{\text{work}}/R_{\text{free}}$ (%)	16.9/20.3	19.0/24.5
no. of atoms (protein/other/water)	1082/45/166	946/37/158
B -factors (\AA^2) (protein/other/water)	17.04/18.34/30.66	30.71/22.55/37.40
rmsd bonds (\AA)	0.015	0.016
rmsd angles (deg)	1.666	1.587
Ramachadran favored (%)	99.17	98.11
allowed (%)	0.83	1.89
disallowed (%)	0.00	0.00

*Values in parentheses correspond to the highest resolution shell.

■ ASSOCIATED CONTENT

Supporting Information

DSF T_m shifts with replicates and errors (XLSX). This material is available free of charge via the Internet at <http://pubs.acs.org>.

■ AUTHOR INFORMATION

Corresponding Author

*Phone: +44 (0)1865 612 932. E-mail: paul.brennan@sgc.ox.ac.uk.

Author Contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

HAT, histone acetyl transferase; HDAC, histone deacetylase; BET, bromodomain and extra terminal domain; BRD, bromodomain-containing protein or bromodomain; DSF, differential scanning fluorimetry; BRD4(1)/(2), first/second bromodomain of BRD4; BRDT, bromodomain, testis-specific; CREBBP, CREB (cyclic-AMP response element binding) binding protein; BRD9, bromodomain-containing protein 9; CECR2, cat eye syndrome chromosome region, candidate 2; TAF1, TBP-associated factor RNA polymerase 1; TAF1L, TAF1-like; TIF1 α , transcription intermediary factor 1-alpha; ATAD2, ATPase family, AAA domain containing protein 2; SMARCA4, SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 4; P300 or EP300, E1A binding protein p300; BAZ2A, bromodomain adjacent to zinc finger domain, 2A; BAZ2B, bromodomain adjacent to zinc finger domain, 2B; PB1, polybromo-1; PCAF, P300/CBP-associated factor; BRPF3, bromodomain and PHD finger containing protein 3; PHIP, pleckstrin homology domain interacting protein; FRAP, fluorescence recovery after photo-bleaching

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