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Dual screening of BPTF and Brd4 using protein-observed fluorine NMR uncovers new bromodomain probe molecules

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Abstract

Bromodomain-containing protein dysregulation is linked to cancer, diabetes, and inflammation. Selective inhibition of bromodomain function is a newly proposed therapeutic strategy. We describe a ¹⁹F NMR dual screening method for small molecule discovery using fluorinated tryptophan resonances on two bromodomain-containing proteins. The chemical shift dispersion of ¹⁹F resonances within fluorine-labeled proteins enables the simultaneous analysis of two fluorinated bromodomains by NMR. A library of 229 small molecules was screened against the first bromodomain of Brd4 and the BPTF bromodomain. We report the first small molecule selective for BPTF over Brd4, termed AU1. The $K_d = 2.8 \mu\text{M}$ for AU1 which is active in a cell-based reporter assay. No binding is detected with Brd4. Three new Brd4 inhibitors with submicromolar affinity were also discovered. Brd4 hits were validated in a thermal stability assay and potency determined via fluorescence anisotropy. The speed, ease of interpretation, and low protein concentration needed for protein-observed ¹⁹F NMR experiments in a multi-protein format, offers a new method to discover and characterize selective ligands for bromodomain-containing proteins.

Graphical Abstract

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Supporting information: Additional experimental procedures, tables, figures and characterization data can be accessed free of charge via the internet at <http://www.pubs.acs.org>.

Introduction

Lysine acetylation is an important post-translational modification that is significant in the epigenetic regulation of both health and disease. Histone proteins that are acetylated are bound by bromodomain-containing proteins facilitating assembly of transcription complexes. Small molecules that enable characterizing the role of these epigenetic proteins will improve our understanding of signaling pathways and may ultimately lead to new therapeutics.¹ Clinical trials are underway evaluating inhibition of several members of the bromodomain and extra terminal family (BET) bromodomains (i.e, Brd2, 3, 4) in cancer, diabetes, and inflammation, supporting bromodomain modulation as a potential therapeutic approach.² The bromodomain PHD finger transcription factor, BPTF, is thought to play a significant role in melanoma,³ leukemia,⁴ colorectal,⁵ and bladder cancer⁶. Both the PHD finger and bromodomain are important for chromatin binding.⁷ No selective inhibitors for the BPTF bromodomain have been reported to test its role in regulating transcription or cancer, which motivates this research.

The lack of suitable ligands for competition-based experiments provides a challenge for developing reliable screens for bromodomain ligand development, specifically for BPTF.⁸ Direct binding experiments using NMR have become a valued method for screening, due to the ability to quantify small molecule protein interactions over a wide range of affinities, particularly weak ligands, and have been used for bromodomain ligand discovery.^{9–11} Protein-based methods using labelled amides (e.g., ¹H-¹⁵N HSQC) provide additional structural information for developing small molecules; however, the experiment can be material intensive and time consuming.¹² The fluorine nucleus is highly sensitive to changes in chemical environment. Using this environmental sensitivity, we and others reported on a protein-observed fluorine NMR method (PrOF NMR) for characterizing ligand binding at protein-protein interaction sites using fluorine-labelled side chains which showed a time enhancement of at least 2-fold over HSQC on 12 and 15 kDa proteins.^{13–17} ¹⁹F is 83% as sensitive as ¹H and 100% isotopically abundant (thus inexpensive), facilitating detection of ¹⁹F at low concentrations (μ M) for small and medium-sized proteins. We have previously applied PrOF NMR for fragment-based screening of over 500 small molecules and characterized the bromodomains Brd4, BrdT, and BPTF.^{13,14,18}

In this report, we demonstrate how the bromodomain for BPTF and the first bromodomain of Brd4 can be screened simultaneously due to the significant chemical shift dispersion and

simplified ^{19}F NMR spectra. This approach is similar to RAMPED-UP NMR developed by Zartler et al. who demonstrated the screening potential of three differentially labelled proteins via 2D-HSQC NMR experiments.¹⁹ These offer selectivity information up front and can lead to the discovery of new ligands for two to three biological targets in one screen. Selective targeting of bromodomains remains a significant challenge due to binding site similarity.¹⁸ We aimed to test if screening in the presence of another bromodomain could increase assay throughput, stringency, and binding information for finding selective inhibitors. Several structural classes of kinase inhibitors show preferential binding to BET bromodomains, including the PLK-1 kinase inhibitor BI2536.^{20,21} We reported its additional binding to a non-BET bromodomain BPTF using PrOF NMR.¹⁸ We have now screened 229 related compounds and disclose our findings for both Brd4 and BPTF selective compounds, including the first reported compound for BPTF. We validated our ligands using non-fluorinated proteins in protein stability, fluorescence anisotropy, and isothermal titration calorimetry (ITC) experiments (Figure 1). From these experiments we discovered submicromolar inhibitors for Brd4 and a selective BPTF ligand ($K_d = 2.8 \mu\text{M}$) that we term AU1, which is active in cell culture. Finally, we compare our screening hits to a prior screen using x-ray crystallography.²⁰ We anticipate new Brd4 and BPTF ligands will be valuable probes for studying the role of these bromodomains in various cancers.

Results and Discussion

Design of a dual Protein-Observed ^{19}F NMR screening assay with bromodomains Brd4 and BPTF

At least four aromatic amino acids for fluorine labeling are found in the histone binding site for more than half of all bromodomains.¹⁸ We chose to incorporate fluorinated tryptophan residues into both proteins (three for Brd4: W120, W81, and W75; one for BPTF: W2824). Tryptophan 81 of the first bromodomain of Brd4 is located in the “WPF shelf”, and is important for recognition of small molecule inhibitors including (+)-JQ1²² and BI2536²⁰. Tryptophan 2824 is the homologous residue in BPTF (Figure 2A). We can isolate our fluorinated proteins in high yield (84–88 mg/L with high fluorine labeling, >95%). We found fluorination to be only modestly perturbing to bromodomain structure and function.¹⁸ For Brd4, the binding affinity of (+)-JQ1 is 78 and 75 nM for the 5-fluorotryptophan (5FW)-labeled and unlabeled proteins respectively. Thermal stability is only affected by a two degree change of the thermal melting temperature (T_m) from 52 to 50 °C for 5FW-labeled Brd4. In the case of BPTF, we measure a negligible change in T_m for the single 5FW incorporation relative to the unlabeled protein ($T_m = 53$ °C in both cases) using variable temperature circular dichroism (Fig. S1, S2).²³

Fluorine NMR spectra can be obtained for both proteins at 50 μM in 5 minutes (which includes a short reference experiment) leading to four resolved fluorinated tryptophan resonances corresponding to fluorine signals from both proteins (Figure 2B bottom). The commonly used solvent dimethylsulfoxide (DMSO) for screening libraries can also inhibit bromodomains.²⁴ For NMR, addition of this solvent does not dramatically alter the chemical shift dispersion nor the binding ability of the dual Brd4/BPTF ligand BI2536 at 1% (v/v).

Therefore all screening experiments were carried out at 1% DMSO in the presence of 50 μ M Brd4 and BPTF.

The simultaneous screen of Brd4 and BPTF offers an efficient method to discover small molecule binders for both bromodomains at the same time. Due to the similarity of the acetylated-lysine binding pocket, it is common for molecules to bind with multiple bromodomains; therefore, selectivity information is necessary when designing bromodomain inhibitors. The published kinase inhibitor set (PKIS I and II) contains small molecules designed for kinase inhibitor campaigns and is made widely available by GlaxoSmithKline.²⁵ A portion of this library has already been screened by x-ray crystallography against Brd4 and allows a basis for comparison of our results.²⁰ As a initial test case, we studied two reported kinase inhibitors (TG-101348 and SB-202190) reported to be selective for Brd4 over BPTF.^{20,21} Our NMR experiment reproduced this selectivity (Fig. S3).²³ We then screened 229 compounds individually at 100 μ M. Chemical shift perturbations for all compounds were analyzed and the compounds were annotated as dual binders, selective BPTF or Brd4 binders, or non-binders (Table S2).²³

The ProF NMR binding information from the simultaneous screen was readily interpreted for most binders that induced significant resonance broadening and chemical shift changes (Fig. S4).²³ In some cases, the resonance was completely broadened into baseline (Figure 2B middle). NMR resonances exhibiting this broadening behavior from binding are dependent on both the residence time in the bound state and the chemical shift difference between the free and bound state.²⁶ In our experience, this is consistent with molecules with approximate K_d values from 0.1 to 100 μ M. Small molecules can be further titrated into the protein solution until the resonance grows out of baseline, and relative binding information can be estimated by the magnitude of change in chemical shift (Figure 2C). We and others have also described ProF NMR experiments yielding both slow and fast exchange binding kinetics, demonstrating the ability to detect molecules with dissociation constants in the low nanomolar up to millimolar range.^{13,14,16,17,27} Finally, binding site position can be estimated by which resonance is perturbed. In most cases, the W81 resonance of Brd4 located in the WPF shelf is the most dramatically perturbed by a binding interaction in the histone recognition pocket, while W120 on the other side of the protein remains unperturbed. The stability of W120 offers confidence that the protein is still well-folded and in solution. On BPTF, W2824, the only tryptophan present, is also located on the WPF shelf.

ProF NMR Screening Results for Brd4 Ligands

From our initial screen we further elucidated the 1,4,5- and 2,4,5-substituted-imidazole class that was previously disclosed as a ligand class for Brd4, and uncovered two new binding classes for both BPTF and Brd4, 1,2,5-oxadiazoles and arylureas containing 2,4-disubstituted pyrimidines. From the 1,2,5-oxadiazoles, 2/29 bound to BPTF (1 selective over Brd4). For the arylureas, 6/24 bound to BPTF (2 selective over Brd4, Figures 2,4). A hit is described as the resonance broadening into baseline, and selective hits are defined as perturbing the resonance of the other protein by less than one standard deviation based on the average from all NMR experiments in the screen for both chemical shift perturbation and change in resonance width at half height. Both of these classes also contain compounds that

resulted in moderate hits, where resonances were broadened but not significantly into baseline.

The ligandability from the PKIS set for the first bromodomain of Brd4 was found to be slightly higher than BPTF (six selective strong binders versus three selective strong binders), consistent with the druggability analysis of Vidler et al.²⁸ However, due to the similarity of many compounds this may also represent a limited amount of explored chemical space. Trisubstituted imidazole-containing compounds have been previously found to bind to Brd4, and we found additional compounds of this class that bind to this protein. Of the seven active trisubstituted imidazole-containing compounds (six of which were selective for Brd4), the most dramatic effects observed were for alkyl substitution on the phenylether at the 2-position on the pyrimidine. Ember et al. had previously reported a 2,3-dimethyl substitution pattern,²⁰ whereas in this study a 3,5-dimethyl, 2,5-dimethyl, and 4-ethyl were discovered (molecules **1**, **2**, and **4**, Figure 3). As NMR could only provide an estimate of the affinity, we turned our attention to complementary binding assays, using non-fluorinated bromodomains.

Thermal Stability Evaluation with Brd4 Ligands using Differential Scanning Fluorimetry

Differential scanning fluorimetry (DSF) has proven to be an effective screening tool for Brd4, demonstrating a linear correlation of ligand dissociation constants with thermal stability.^{20,22} To rank order our newly discovered Brd4 ligands using a non-fluorinated protein, we generated a correlation curve based on published affinity values using six known Brd4 binders and thermal melting temperatures measured by DSF under our experimental conditions (Figure 5, Table S3, Fig. S5).²³ We used this curve to estimate the affinity of newly discovered small molecules from our screen, as has previously been demonstrated by Ember et al.²⁰ Of the 31 discovered Brd4 ligands, 21 showed a stabilization > 1 °C. Eight non-binding compounds for Brd4 were also tested, five of which did not result in stabilization larger than 1 °C; none of the non-binders stabilized greater than 2.5 °C (Table S3).²³ The high confirmation rate supported the sensitivity of ProOF NMR for ligand detection, reducing concerns over potential false positive effects from fluorine incorporation. The trisubstituted imidazole SB-284851-BT (**1**) possessed one of the largest changes in thermal stability (T_m = 7.4 °C, Table 1, Figures 3, 5). Due to the only modest linear correlation in our calibration curve, the affinity of this compound and analogs were subsequently quantified in competitive inhibition assays.

Competitive Inhibition using Fluorescence Anisotropy for Brd4 Ligand Affinity Determination

To quantify the ligand affinity for Brd4, we designed a competitive inhibition assay using fluorescence anisotropy (FA) and a fluorescently labeled derivative of BI2536, which was chemically synthesized and reported previously as a fluorescent PLK-1 kinase probe.²⁹ Chung et al. previously published a similar assay using fluorescently labeled analog I-BET762 as a Brd4 ligand.³⁰ A competition experiment eliminates the need to fluorescently label all newly discovered small molecules which could alter binding and is higher throughput than ITC. We measured a K_d of 50 ± 11 nM for a BODIPY-conjugated BI2536 probe in direct binding experiments (Figure 6A), similar to reported values of 48 nM.²¹ Importantly, the probe could be self-competed with unlabeled BI2536 (IC_{50} = 130 nM for

Brd4). We also measured a K_d of $67 \pm 21 \mu\text{M}$ binding to BPTF, consistent with the moderate affinity detected in our NMR binding study and within 2-fold of our ITC measurements ($K_d = 37 \mu\text{M}$) (Fig. S6, S7).²³ We used this probe to determine dissociation constants from a competitive inhibition experiment (i.e., K_i) for our new Brd4 ligands.

We previously determined a K_d of $70 \pm 20 \mu\text{M}$ of a small molecule Dinaciclib binding to fluorinated Brd4,¹⁸ a known moderate affinity inhibitor.²⁰ Using our FA assay, when Dinaciclib was dissolved in ethylene glycol, a bromodomain compatible solvent, we obtained a partial inhibition curve with an estimated IC_{50} of $220 \mu\text{M}$ (Fig. S8, Table S3).²³ From this study, we conclude that inhibitors with weaker affinity than Dinaciclib will be difficult to characterize in this current FA format, and therefore we report only on a limited set.

The labs of Schönbrunn²⁰ and Knapp²¹ found that 1,4,5- and 2,4,5-trisubstituted imidazoles bound to Brd4. Trisubstituted imidazoles were also found to bind to BAZ2A/B.³¹ We have now discovered analogs in our PrOF NMR experiment with submicromolar affinity, the most potent of these scaffolds for Brd4 to date. For 1,4,5-trisubstituted imidazoles in the FA competitive inhibition experiment, we determined a K_i of 310 nM for SB-284851-BT (**1**) (Figure 6B, Table 1), our most promising candidate from DSF and PrOF NMR experiments, a modestly higher affinity than estimated from the calibration curve. The arylalkyl substitution pattern bonded to the pyrimidine is important for binding. Based on the results with SB-284851-BT (**1**) having a 3,5-dimethyl substitution pattern, we tested SB-284852-BT (**2**) (previously not screened by PrOF NMR) with a 2,5-dimethyl group substituted on the aromatic ring. SB-284852-BT (**2**) has a slightly higher K_i of 580 nM (Figure 6B, Table 1). A regioisomer with a 2,3-dimethyl substituted aromatic ring (SB-284847-BT (**3**)) was co-crystallized by Ember et al. with Brd4 but inhibited with a much weaker potency ($\text{IC}_{50} = 20 \mu\text{M}$).²⁰ A 4-ethyl group on SB-242719 (**4**) also results in 40-fold weaker affinity with a K_i of $13 \mu\text{M}$ in our assay and a $T_m = 2.1^\circ\text{C}$ by DSF (Figure 6B, Table 1).

Our newly discovered Brd4 inhibitors SB-284851-BT (**1**) and a 2,4,5-trisubstituted imidazole SB-590885-AAE (**5**) ($K_i = 400 \text{ nM}$, Table 1) were previously characterized as selective B-Raf kinase ($K_d = 0.3 \text{ nM}$)³² and p38 α kinase ($\text{IC}_{50} = 41 \text{ nM}$)³³ inhibitors. Although a variety of trisubstituted imidazoles have been reported to bind to Brd4, to date these two compounds are the most potent analogs, and may represent a starting point for ligand design or use in cell culture experiment to test the polypharmacology and potential synergy for p38 α or B-Raf and Brd4 inhibition in various cancer models.

PrOF NMR Screening Results and Structure Activity Relationships for BPTF Ligands

In addition to new Brd4 ligands, several selective compounds were also found to bind to BPTF. These results are encouraging due to an absence of reported ligands for the bromodomain of BPTF. The arylurea containing compounds were the tightest binders that we found for BPTF exhibiting greater than two standard deviation changes in chemical shift and resonance broadening into baseline. Two arylurea containing compounds completely selective for BPTF over Brd4 were obtained, GSK1379746A (**6**) and GSK1379725A. GSK1379725A was selected for follow-up studies, and so we now refer to it as AU1 (Figures 2,4, Fig. S9).²³ Negligible chemical shift changes were observed for Brd4 in the

presence of AU1 at up to 160 μM (Fig. S10).²³ In addition, a 1,2,5-oxadiazole (GSK561866B (**7**)) was highly selective for BPTF (Figure 4A). Non-selective arylurea containing compounds from the screen that have a trimethoxy-substituted aryl ring and bound in the intermediate exchange regime by ProOF NMR, are anticipated to bind with the trimethoxyphenyl ring in the acetyl-lysine binding pocket based on the binding mode of the trimethoxyphenyl ring of GW61228X bound to Brd4 reported by Ember et al.²⁰. Arylurea-containing compounds that lacked the trimethoxy-substituted aryl ring in some cases bound more weakly to Brd4.

Of the six high affinity BPTF binding compounds of the arylurea class, binding appears to be correlated to a benzene ring with a meta-substituted carbonyl present. Computational binding studies using the Phase^{34–36} module of Schrödinger indicate the placement of both the aromatic ring and hydrogen bond acceptor strongly correlate with binding (Table S1, Fig. S11, Fig. S12).²³ Docking studies indicate probable π – π interactions with the nearby phenylalanine 2887 and hydrogen bonds to the asparagine 2881 side chain in the binding site.^{37,38} Compound GSK1379724A (**8**) is similar to AU1 (Figure 4A), but contains a cyclic acetal in place of a carbonyl group on the phenyl ring. This compound is unable to bind to either BPTF or Brd4, and represents a useful negative control compound for future studies. Related molecules without a carbonyl group showed poor BPTF binding. Additional arylurea-containing compounds will be developed to determine a more extensive structure activity relationship prior to obtaining higher resolution structural information.

Dissociation Constant Determination for a Selective BPTF Ligand AU1

Due to the absence of ligands for the BPTF bromodomain, a correlation between DSF thermal stability values and K_d have yet to be reported. In fact, for BI2536 we measured a slight decrease in the T_m for BPTF of $-1.2\text{ }^\circ\text{C}$ consistent with other reports²¹, although we have determined a K_d of 37 μM and 67 μM via ITC and FA respectively (Fig. S7, S6).²³ The origins of the slight destabilization are yet unclear. From the NMR titration of AU1, the bound and unbound resonance were separated by 171 Hz (Figure 2C), providing an upper bound for the chemical exchange rate.²⁶ Assuming an association rate of $1 \times 10^8\text{ M}^{-1}\text{ s}^{-1}$ as a high end for a range of protein-small molecule interactions, (e.g., chymotrypsin:proflavin $k_1 = 1.2 \times 10^8\text{ M}^{-1}\text{ s}^{-1}$),³⁹ we estimate an upper K_d of 8 μM from this experiment. For a more accurate determination with a non-fluorinated protein, we used ITC as a complementary direct binding assay using unlabeled BPTF. We obtained a K_d of 2.8 μM (Figure 4B), consistent with our intermediate exchange resonance broadening by ProOF NMR. We are encouraged by this initial potency as a starting point for future cell-based studies, although new analogs are anticipated to be necessary, including the identification of the active stereoisomer and removal of the methyl ester hydrolysis susceptibility. A summary of our recorded data from the biochemical experiments described above is found in Table 1 along with a more extensive list in the supporting information.

Although AU1 has been demonstrated to be selective over Brd4, a full selectivity panel against other bromodomains will be needed. A database search using ChEMBL only showed AU1 to be active in five cellular assays with an EC_{50} of 500 nM carried out by Gamo et al.⁴⁰ However, the targets are unknown, which we are following up. Additionally, no kinase

activity has been reported for AU1 despite the growing screening use of the PKIS library. These results are encouraging regarding off target effects for this BPTF ligand.

AU1 transcriptional inhibition experiments in live cells

As an initial test for the effect of BPTF bromodomain inhibition on transcription, we first measured the cellular toxicity against healthy human cells. The prior study by Gamo et al. did not detect cellular toxicity in hepatocytes at 10 μ M compound,⁴⁰ which is more than 3-fold above the K_d of AU1. We also observe no cellular toxicity against HEK 293T cells after 24 hours at 3 and 10 μ M. Only after 48 hours of incubation at 10 μ M do cells have a reduced viability (76% viable) (Fig. S13).²³

Ruthenberg et al. demonstrated that both the PHD finger and bromodomain of BPTF were important for chromatin binding.⁷ Native chromatin precipitation experiments showed colocalization of BPTF at the MOBKL3 locus with histone H4, acetylated at lysine 16.⁷ To investigate the effect of AU1 treatment on BPTF-mediated gene regulation, we cloned a BPTF-regulatory region, localized upstream to the Mobkl3 promoter region,⁷ into a firefly luciferase reporter plasmid (pGL3_Mobkl3p). Transfection of the pGL3_Mobkl3p into Eph4, a normal mouse mammary gland cell line⁴¹ displayed robust luciferase activity (Figure 7), ~7-fold greater than those observed with a control firefly luciferase reporter plasmid (pGL3_empty) lacking a BPTF regulatory region (Figure 7). This data supports the association of factors to the Mobkl3 regulatory region, such as BPTF contributed to the increased luciferase activity.

To test if the pGL3_Mobkl3p luciferase levels were dependent on the bromodomain of BPTF, the Eph4 were again transfected with either the pGL3_Mobkl3p or the pGL3_empty vector and then treated with 5 μ M AU1 for 12 hours. A significant loss of luciferase activity (~4-fold) was observed in cells transfected with pGL3_Mobkl3p vector, suggesting that the Mobkl3 regulatory region requires BPTF activity to support the regulation of luciferase activity (Figure 7). Together our data suggests that binding of AU1 to the BPTF bromodomain interferes with its regulatory activity in cultured cells. This may represent a suitable system to study the net effects of the BPTF-bromodomain on gene regulation

Comparison of ProOF NMR to a prior X-ray Crystallography Screen

A combination of ProOF NMR, DSF, and FA have led to several new small molecules for both Brd4 and BPTF. In 2014, two parallel screens using kinase inhibitor libraries against Brd4 were reported providing a useful data set for assessing the application of ProOF NMR in a discovery mode.^{20,21} From the x-ray crystallography screen²⁰, 61 compounds overlapped with the molecules studied here. Of the 28 compounds where a high-resolution crystal structure was obtained (either apo or a co-crystal), ProOF NMR uncovered seven hits while x-ray crystallography uncovered two, with one compound identified by both screening techniques. Although our ProOF NMR results reveal more binders than this screen with the PKIS set, both methods discovered ligands with similar structural classes. In combination, ProOF NMR offers a complementary technique to x-ray crystallography, potentially guiding efforts for subsequent crystallization studies and improving on the structural biology efforts targeting bromodomain ligand discovery.

In conclusion, this study presents the first simultaneous screen of two proteins by PrOF NMR, enhancing the throughput and stringency for finding selective ligands for structurally related proteins. This study is reminiscent of RAMPED-UP NMR by Zartler et al. highlighting the screening potential of three differentially labeled proteins via 2D-HSQC NMR experiments.¹⁹ NMR screening has typically been employed in the area of fragment-based discovery. The hinge-binding kinase inhibitor classes have proven to be a privileged structural class for bromodomains allowing efficient exploration of chemical space and may be useful against other bromodomain family members outside the BET class. Several p38 α B-Raf inhibitors with submicromolar affinity for Brd4 will allow for the testing of synergistic effects of dual protein inhibition in cancer and inflammation. Our newest ligand for BPTF, AU1, represents a useful starting point for testing the unexplored role of its bromodomain in various cancer models. Biological studies are currently underway. Our method has been applied to BPTF and BET bromodomain Brd4. Discovery of highly isoform selective ligands for bromodomains within the BET bromodomain family has yet to be successful. In the future, PrOF NMR screening in the presence of multiple BET bromodomains may provide a method to achieve this goal.

Methods

Expression of 5FW-Brd4 (42–168), and 5FW-BPTF (2793–2911)

3FY and 5FW labeled Brd4(1) were expressed based on established methods¹³ using *E. coli* B121(DE3) + pRARE strains. To express the labeled protein, the secondary culture in LB media was grown until an O.D. at 600 nm of 0.6 was reached followed by harvesting. Cells were resuspended in defined media of Muchmore et al.⁴² containing 5-fluoroindole (60 mg/L) in place of tryptophan⁴³. The resuspended *E. coli* were incubated at 37 °C while shaking for 1.5 h followed by the cooling to 20 °C and media temperature equilibration for 30 min. Protein expression was induced with 1 mM IPTG overnight (14–16 h) at 20 °C. The cells were harvested and stored at –20 °C. 5FW-BPTF was expressed by the same protocol. Cell pellets were thawed at RT followed by the addition of lysis buffer (50 mM Phosphate pH 7.4, 300 mM NaCl) containing protease inhibitor PMSF (5 mM) as well as the Halt protease inhibitor and purified according to methods described in the Supporting Information using Ni-affinity chromatography. Yields following purification are 88 mg/L 5FW-Brd4 (>95% fluorine incorporation), 84 mg/L 5FW-BPTF (>95% fluorine incorporation). Purity of proteins was assessed by SDS-PAGE. Fluorinated amino acid incorporation efficiency in proteins was measured by mass spectrometry as described in the Supporting Information. Concentration was determined via absorbance at 280 nm.⁴⁴

Protein Observed Fluorine NMR

1D ¹⁹F NMR Parameters. ¹⁹F NMR spectra were acquired at 470 MHz on a Bruker 500 spectrometer with a 5 mm Prodigy TCI Cryoprobe without proton decoupling. Samples containing 40–50 μ M bromodomains were labeled in 50 mM TRIS, 100 mM NaCl, and 5% D₂O, pH 7.4 with 100 μ M ligand for binding assays unless otherwise stated. Spectra were referenced to trifluoroacetate (–76.55 ppm). Measurement parameters included a relaxation delay time of 0.7 s for 5FW-Brd4(1) and a 90° flip angle. An acquisition time of 0.05 s was used for all experiments. A sweepwidth of 10 ppm was used for 5FW-Brd4 spectra. All

screening experiments used 400 transients. Small molecules were titrated into the protein solution from concentrated stock solutions of DMSO (10 mM for all small molecules except acetaminophen). Final DMSO concentrations were kept at or below 1% DMSO.

Differential Scanning Fluorimetry (DSF)

The inhibitory activities of compounds against Brd4 were assessed by DSF using a CFX384 Real-Time PCR Detection System (BioRad). Purified Brd4 (4 μ M final concentration; 10 mM HEPES (pH 7.5), 100 mM NaCl, and 1 mM DTT) were assayed, in replicates of ten, in a 384-well plate. Inhibitors were added to a final concentration of 100 μ M and 1 % DMSO or ethylene glycol. SYPRO Orange Protein Gel Stain (1:5000; Sigma Aldrich) was used as the fluorescent probe. Protein stability was using 0.1 $^{\circ}$ C increments and 10 s incubations per increment from 25 to 75 $^{\circ}$ C. The inflection point of the transition curve/melting temperature (T_m) was calculated using the Boltzmann equation within the Protein Thermal Shift Software (v.1.1) (Applied Biosystems). (+)-JQ1 and dinaciclib were used as controls for strong and weak binders of Brd4, respectively. The T_m was calculated by using DMSO and ethylene glycol control wells as a reference.

Fluorescence Anisotropy Direct Binding and Competition Experiments

Fluorescence anisotropy was measured from an absorption wavelength of 485 nm and an emission wavelength of 535 nm on a Tecan Infinite 500 plate reader using 384-well plates (Corning 3676). All experiments were carried out in 50 mM Tris, 100 mM NaCl, 5 mM CHAPS at pH 7.4. The fluorescently labeled tracer, BODIPY-BI2536, was synthesized according to published methods.²⁹ For both direct binding and competition experiments, 25 μ M stocks of a BODIPY-BI2536 stock solution in DMSO were diluted to a final concentration of 25 nM. For direct binding experiments, Brd4 and BPTF were serially diluted from micromolar to subnanomolar concentrations. For competition experiments, Brd4 was kept at a constant concentration of 156 nM, equivalent to 80% bound tracer as determined in direct binding experiments, and the concentration of competing ligand was serially diluted from micromolar to subnanomolar concentrations. Because of the capacity of DMSO to perturb bromodomain binding, competitors were diluted from 10 mM ethylene glycol stocks. Data were collected within 30 minutes after plating to minimize Brd4 binding to the plate surface. Measurements taken after 30 minutes led to a significant decrease in the apparent dissociation constant at longer time points. All experiments were carried out in triplicate. Acquired data were fit using GraphPad Prism. K_d values were determined by fitting to equation 1, which accounts for ligand depletion. In this equation, b and c are the maximal and minimal anisotropy values, respectively, a is the concentration of fluorescently labeled tracer, x is the protein concentration, and y is the observed anisotropy value.

$$y=c+(b-c)\frac{(Kd+a+x)-\sqrt{(Kd+a+x)^2-4ax}}{2a} \quad (\text{Eq. 1})$$

IC₅₀ values were determined using GraphPad Prism's log(inhibitor) vs response function. K_i values were obtained using a variant of the Cheng-Prusoff equation from Huang et al.⁴⁵

Isothermal Titration Calorimetry

A Nano-ITC was used to perform isothermal titration calorimetry experiments. AU1 (46 μM) was diluted into the same buffer as BPTF (50 mM sodium phosphate pH 7.0) from a 10 mM DMSO stock solution. The resulting solution was sonicated immediately prior to use. Titrations were carried out by using a first injection of 0.5 μM followed by 19 injections of 2.5 μL volume of 635 μM BPTF with a releasing period of 8 seconds per injection with 300 second spacing time between injections. Heat liberated by each injection was integrated (ΔH) and plotted against molar ratio of ligand and protein. The obtained data was fit by using predefined two binding site model. The fitting of data was not sufficient with one binding site whereas a two binding site model produced an appropriate fit, indicating a potential low affinity binding ($K_d = 1.0 \text{ mM}$) site. Data was analyzed by NanoAnalyze software to calculate enthalpy of binding (ΔH) and dissociation constants (K_d). A two binding site binding model was used for data fitting. ITC data was obtained in a similar fashion for BI2536, using a concentration of 0.5 mM of BI2536 and 7 mM BPTF.

Viability Assays

Cell viability assay—Cell viability assay of HEK 293 cells were performed using resazurine dye based CellTiter-Blue® (Promega). HEK 293T cells were plated in 96 well plates and experiments conducted when cells were 80% confluent. Cells were treated with the 0, 1.0, 3.0 and 10.0 μM AU1 for 24 and 48 hours in 5% CO_2 ($n=5-6$, per condition). 20 μL of CellTiter-Blue® was added to the each well and incubated for 2.5 h at 37°C. As resazurin dye is reduced by viable cells to resorufin. Resorufin is fluorescent at 580 excitation and 590 emission. The data is normalized with the control (DMSO treated), (mean $\pm$ SE).

Luciferase reporter array

The BPTF-regulatory region localized upstream to the Mobkl3 promoter region (~380bp) was amplified by PCR, cloned into pCR2.1-TOPO vector (Invitrogen) and transferred to the firefly luciferase pGL3-Basic vector (Promega) using XhoI and HindIII restriction enzymes. pGL3_Mobkl3p and pGL3_empty vectors were propagated in TOP10 chemically competent bacteria (Invitrogen) and plasmid purification was performed with Qiagen kit (Qiagen). Purified plasmids were co-transfected into Eph4 cells, a normal mouse mammary gland cell line (gift from Dr. Celeste M. Nelson) with pRL Renilla luciferase control vector (Promega) using PEI (Sigma). Transfection was carried out for 8 hours, followed by media change and treatment with AU1 (12 hours). Cells were then washed once with 1xPBS and lysed with Passive Lysis Buffer (Promega) for 20 minutes with constant agitation. Luciferase activity was measured with the Dual-Glow luciferase reporter assay kit (Promega) on a Glomax 20/20 luminometer (Promega). Firefly luciferase levels were normalized to Renilla luciferase levels.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

A selection of compounds from the Public Kinase Inhibitor Set from GSK were screened against 5FW-BPTF and 5FW-Brd4. Hits for BPTF were then rank-ordered by titration of ligand, and then one final compound was analyzed by isothermal titration calorimetry (ITC). Hits for Brd4 were rank ordered by differential scanning fluorimetry (DSF), and then K_i information determined by fluorescence anisotropy (FA).

Figure 2.

A.) The tryptophan at the WPF shelf is in close proximity to the binding site in both Brd4 and BPTF. B.) A sample of NMR spectra from the screen with the second spectrum illustrating a selective Brd4 binder (only the W81 resonance is perturbed), and the third spectrum illustrating a selective BPTF binder (only the W2824 resonance is perturbed). C.) A titration of BPTF binder AU1 into a solution of 50 μ M 5FW-BPTF. Consistent with intermediate exchange kinetics, the BPTF resonance broadens into baseline and then begins to sharpen at saturating ligand concentrations that reach the solubility limit of AU1.

Figure 3.

Structures of trisubstituted imidazole-containing compounds that are selective for Brd4 over BPTF. Thermal stabilization and inhibitory potency is reported from differential scanning fluorimetry and fluorescence anisotropy experiments respectively. *SB-284847-BT reported by Ember et al.¹⁹ is shown for comparison.

Figure 4. Left.) Selected compounds of varying specificity for BPTF over Brd4, and non-binder GSK1379724A. Right.) ITC binding isotherm for AU1 (46 μM) titrated with bromodomain of BPTF (635 μM)

Figure 5. A DSF calibration curve of previously published Brd4 binders is shown in black, correlating change in thermal melting with reported binding affinity. Compounds from this screen are shown in red, plotted according to change in melting temperature for estimation of dissociation constants from the linear regression shown in hashed lines.

Figure 6.

Fluorescence anisotropy direct binding and competition experiments with Brd4. A) Direct binding between BODIPY-BI2536 and varying concentrations of Brd4 yields a K_d of 50 ± 11 nM B) Normalized fluorescence anisotropy signal is shown for Brd4 binders of various potencies, including SB242719 (red crosses), SB242851-BT (orange circles), SB242852-BT (black triangles), and unlabeled BI2536 (purple diamonds). Competition experiments were performed against bound BODIPY-BI2536.

Figure 7.

AU1 treatment interferes with BPTF activity in cells. Eph4 normal mouse mammary gland cells were transfected with firefly pGL3_empty (background control), firefly pGL3_Mobkl3p and pRL Renilla control vectors and luciferase activity was measured. Firefly luciferase activity was normalized to Renilla luciferase activity. N = 4 biological replicates. * pGL3_empty pGL3_Mobkl3p, $p=0.0002$. ** pGL3_Mobkl3p untreated x pGL3_Mobkl3p plus AU-1, $p=0.0006$.

Table 1

Comparative assay data from the Dual Bromodomain Screen

Structure	BPTF ¹⁹ F NMR ^a	Brd4 ¹⁹ F NMR ^a	Brd4 T _m (°C)	Brd4 FP K _i (μM)	BPTF ITC K _d (μM)
GSK1379725A (AU1)	+++	-	N/A	N/A	2.8
GSK1379746A (6)	+++	-	N/A	N/A	N/A
GSK1379767A*	+++	+++	3.1 ± 3.9	<i>b</i>	N/A
GSK260205A	+++	+++	4.2 ± 0.8	7 ± 12	N/A
GSK561866B (7)	++	-	N/A	N/A	N/A
SB-242719 (4)	+	+++	2.1 ± 0.5	13 ± 3	N/A

Structure	BPTF ¹⁹ F NMR ^a	Brd4 ¹⁹ F NMR ^a	Brd4 T _m (°C)	Brd4 FP K _d (μM)	BPTF ITC K _d (μM)
SB-284851-BT (1)	-	+++	7.4 ± 0.2	0.31 ± 0.04	N/A
SB-284852-BT (2)	N/A	N/A	N/A	0.58 ± 0.23	N/A
SB-590885-AAE (5)	-	+++	5.1 ± 0.4	0.40 ± 0.23	N/A

^a +++, a resonance from the protein in question has been completely broadened into baseline

++, a resonance from the protein in question has been perturbed by 2 standard deviations in either chemical shift or linewidth +, a resonance from the protein in question has been perturbed by 1 standard deviation in either chemical shift or linewidth

-, resonances are minimally to non-perturbed

^b Compound was not soluble in assay buffer