



## Discovery of alkoxy benzamide derivatives as novel BPTF bromodomain inhibitors via structure-based virtual screening

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### ABSTRACT

Bromodomain PHD finger transcription factor (BPTF), a bromodomain-containing protein, plays a crucial role in the regulation of downstream gene expression through the specific recognition of lysine acetylation on bulk histones. The dysfunction of BPTF is closely involved with the development and progression of many human diseases, especially cancer. Therefore, BPTF bromodomain has become a promising drug target for epigenetic cancer therapy. However, unlike BET family inhibitors, few BPTF bromodomain inhibitors have been reported. In this study, by integrating docking-based virtual screening with biochemical analysis, we identified a novel selective BPTF bromodomain inhibitor **DCB29** with the  $IC_{50}$  value of  $13.2 \pm 1.6 \mu\text{M}$  by homogenous time-resolved fluorescence resonance energy transfer (HTRF) assays. The binding between **DCB29** and BPTF was confirmed by NMR and SPR. Molecular docking disclosed that **DCB29** occupied the pocket of acetylated H4 peptide substrate and provided detailed SAR explanations for its derivatives. Collectively, **DCB29** presented great potential as a powerful tool for BPTF-related biological research and further medicinal chemistry optimization.

### 1. Introduction

Lysine acetylation on bulk histones regulates the chromatin architecture and access to DNA, which has emerged as the best-characterized post-translational modification (PTM) in epigenetic research. The acetylated lysine in histones could be specifically recognized by

bromodomain (BRD) family. So far 61 BRDs have been identified and could be categorized into eight distinct families according to their relative functions [1]: HAT component (GCN5, PCAF, etc.), ATP-dependent chromatin remodeling complex component (BAZ1B, etc.), helicase component (SMARCA), methyltransferase component (MLL, ASH1L, etc.), transcriptional co-activation factor components (TRIM, TAF, etc.),

**Abbreviations:** BPTF, bromodomain PHD finger transcription factor; HTRF, homogenous time-resolved fluorescence resonance energy transfer; PTM, post-translational modification; BCPs, bromodomain-containing proteins; SPR, surface plasmon resonance assays; PAIS, pan assay interference compounds; GST, glutathione S-transferase; SP, standard precision; BRD, bromodomain; CPMG, Carr-Purcell-Meiboom-Gill; ALPHA, amplified luminescent proximity homogeneous assay; NMR, nuclear magnetic resonance

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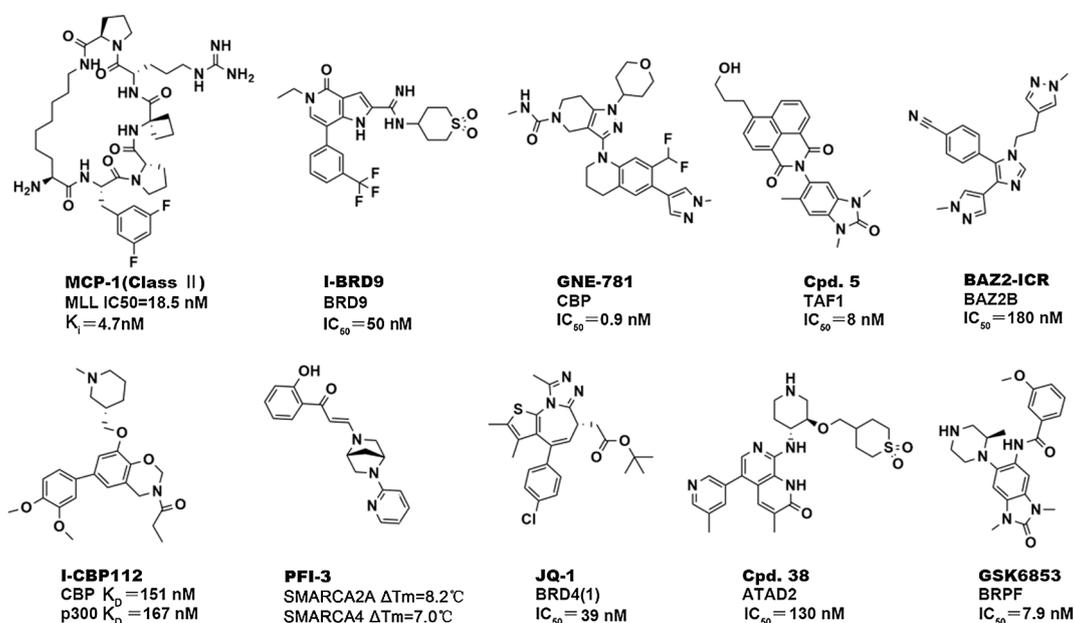


Fig. 1. Representatives of small-molecule inhibitors targeting BCPs.

transcriptional mediator component (TAF1), nuclear skeleton protein component (PB1), and BET family component (BRD2/3/4/T) [2].

The earliest BRD structure was determined by NMR-imaging in 1990 by Haynes and co-workers [3,4]. The BRD family was found to have a conserved hydrophobic pocket surrounded by four reverse  $\alpha$ -helices bundles ( $\alpha$ Z,  $\alpha$ A,  $\alpha$ B,  $\alpha$ C) [5]. The bromodomain-containing proteins (BCPs) can thus act as “readers” of acetylated histones recruiting related chromatin remodeling factors and transcription factors to specific gene transcription sites, which is essential for downstream gene expression [6,7].

Aberrant expression and activation of BCPs have been implicated in the development and progression of many diseases, such as obesity [8], HIV [9], malignant cancers and inflammation [10–12]. Hitherto, a large number of small-molecule inhibitors targeting BCPs have been developed for the treatment of BCPs-related diseases and could be used as functional chemical probes to explore the biological function of BCPs (Fig. 1).

Among BCPs, bromodomain and extra-terminal domain (BET) family was most well-studied in both academia and industry due to its unique role in the maintenance of higher-order architecture of chromatin and the regulation of oncogenes transcriptional activation including *MYC*. BET family inhibitors OTX-015 and I-BET762 have successfully entered into clinical trials for the treatment of leukemia and solid tumors [13,14]. Instead, compared to impressive progress in the drug discovery and development for BET family, the development of potent and selective inhibitors for non-BET BCPs lags behind. Bromodomain PHD finger transcription factor (BPTF), which contains a non-BET bromodomain motif, plays a pivotal role in chromatin remodeling [15,16], transcriptional regulation [17], cell differentiation [18], embryogenesis [19], and so on. Dysfunction of BPTF has been reported to be associated with the development and progression of malignant cancers including bladder cancer [20], colorectal cancer [21], melanoma [22,23], leukemia [24] and so on. Knockdown of BPTF enhances anti-tumor immunity mediated by CD8<sup>+</sup> T cell or NK cell [25]. Collectively, BPTF has emerged as the novel anti-cancer drug target for chemical intervention in the era of target therapy.

Nonetheless, little progress has been achieved in the drug discovery against the BPTF bromodomain. Mishra and co-workers reported that BI-2536 binds to 5FW-BPTF by a <sup>19</sup>F NMR method using fluorine-labeled aromatic amino acids in 2014 [26]. In 2016, Sarah et al. discovered bromosporine as the BPTF inhibitor with the inhibitory activity

at micromolar levels. However, it's more potent against other BRDs including BRD2, BRD4, BRD9, and CECR2 [27]. Therefore, there is urgent need to develop potent and selective BPTF bromodomain inhibitors with novel scaffolds for BPTF-related mechanism studies.

In this work, through docking-based virtual screening, we identified a novel, selective BPTF bromodomain inhibitor **DCB29** with the IC<sub>50</sub> value of 13.2 ± 1.6  $\mu$ M measured by HTRF. **DCB29** displayed high selectivity over other BCPs and epi-targets. Further biophysical assays like NMR and surface plasmon resonance assays (SPR) also confirmed the direct binding between **DCB29** and BPTF bromodomain. Further molecular docking results demonstrated that **DCB29** occupied the pocket of H4 peptide substrate, which disclosed the molecular mechanism of its inhibitory activity.

## 2. Results and discussion

### 2.1. Structure-based virtual screening

Nowadays, with the rapid development of computational methodologies, virtual screening has been widely applied for the discovery of lead compounds in both academia and industry. It has become a powerful and efficient tool and made great achievement in epigenetic-related research in recent decades [28–31]. Herein, docking-based virtual screening was performed to identify novel BPTF inhibitors from the in-house compound library, which has been optimized by Lipinski's rule of five [32] and “pan-assay interference compounds” rules [33–35] using Pipeline Pilot, version 7.5 (Pipeline Pilot; Accelrys Software Inc., San Diego, CA) based on SPECS chemical database (Fig. 2). An indexed multi-conformer 3D database containing 20,000 compounds derived from preprocessed compound library with all stereoisomers and different protonation states was generated in Accelrys Discovery Studio 3.0 as previously described by Meng et al [28,36]. Based on human crystal structure of BPTF bromodomain (PDB: 3QZS), residues located within 10 Å of N148 were defined as the binding pocket. Then molecular docking was performed using Glide SP mode integrated in Maestro [37]. The top-ranked 1000 candidates were selected for further cluster analysis and visual inspection to rule out the compounds with poor shape and repeated chemical scaffold. Finally, 105 compounds were purchased and tested by the homogenous time-resolved fluorescence resonance energy transfer (HTRF) assay to validate the biochemical activities.

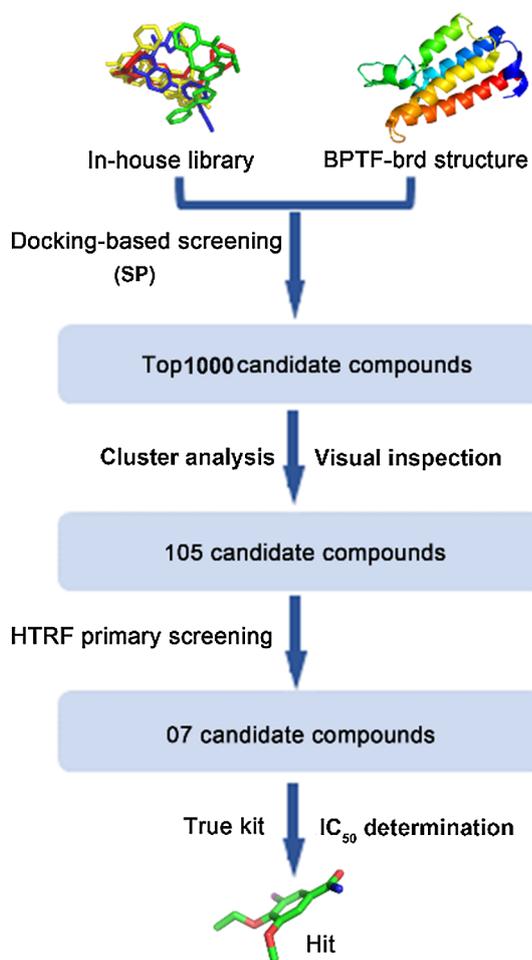


Fig. 2. The flow chart of the hit discovery by docking-based virtual screening.

## 2.2. HTRF biochemical assay

The 105 candidate compounds mentioned above were measured for further validation of inhibitory activity against BPTF by HTRF assay (Fig. 3A). Firstly, different pH conditions (5.8 to 8.7) were explored to test the pH effect on protein stability using thermal shift assays. Among them, BPTF in Bis-Tris buffer at pH 7.0 displayed higher  $T_m$  values (Fig. 3B). Therefore, Bis-Tris buffer at pH 7.0 was chosen for the development of HTRF assays. Based on titration assays, 8 nM BPTF bromodomain protein and 200 nM biotinylated H4 peptide were chosen to provide reliable HTRF signals and used to detection assay (Fig. 3C). Thus, a robust biochemical HTRF assay platform was established for preliminary hit evaluation. Among 105 candidates, seven compounds displayed promising inhibition against BPTF with inhibitory activity of BPTF > 50% at 100  $\mu$ M (Fig. 3D, 4A).

In order to eliminate false positives possibly in HTRF assays, GST-biotin without the presence of BPTF protein was used in HTRF. Among candidate compounds, DCB45 and DCB101 showed inhibitory activity > 50% at 100  $\mu$ M, which was excluded in future validation assays (Fig. 4B). Then, the  $IC_{50}$  values of remaining five compounds against BPTF were determined (Fig. 4C). Among them, DCB14, DCB29, DCB77 and DCB89 presented inhibitory activity with the  $IC_{50}$  value of  $38.0 \pm 2.0 \mu$ M,  $13.2 \pm 1.6 \mu$ M,  $65.3 \pm 4.8 \mu$ M and  $101.4 \pm 2.0 \mu$ M, respectively. Thus, the most potent compound DCB29 was kept for follow-up biophysical validation (Fig. 4C).

## 2.3. Biophysical binding assays

To further investigate the binding between DCB29 and BPTF

bromodomain protein, the NMR study was performed. As shown in Fig. 4D, the Carr-Purcell-Meiboom-Gill (CPMG) NMR experiment revealed a significant decrease signal of DCB29 in the presence of 4  $\mu$ M and 6  $\mu$ M BPTF bromodomain protein, indicating that DCB29 binds directly to the BPTF bromodomain. The binding between DCB29 and BPTF bromodomain was also validated by surface plasmon resonance (SPR) assays. The equilibrium dissociation constant ( $K_D$ ) value of DCB29 was 17.9  $\mu$ M in consistent with the detected inhibitory activity in HTRF assays. Collectively, these biophysical evidence proved that DCB29 binds to BPTF bromodomain *in vitro*.

## 2.4. Selectivity profiling

Considering the fact that the structure of bromodomains in all families of BCPs is highly conserved, we further performed selectivity profiling to evaluate the selectivity. DCB29 displayed significant selectivity over other BCPs including SMARCA2-BRD, BRD4-BD1, CBP-BRD and p300-BRD and some other epi-targets at 100  $\mu$ M. These results demonstrated that DCB29 was a selective inhibitor against BPTF bromodomain, which is a promising starting point for further structure decoration.

## 2.5. 2D similarity search and structure activity relationship (SAR) exploration

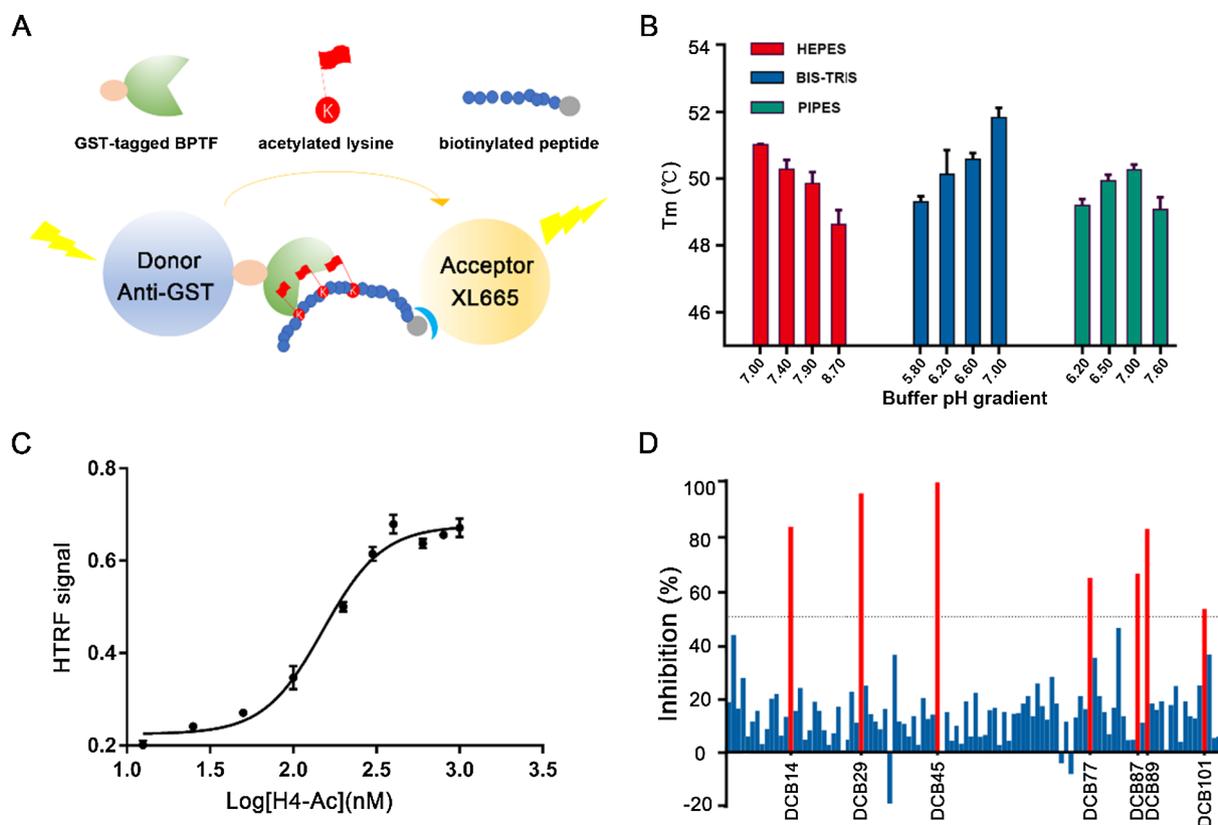
To further explore the SAR of DCB29, 2D similarity search was applied and five analogues with alkoxy benzamide scaffold were purchased from SPECS (<https://www.specs.net/>) and tested by HTRF. As is shown in Table 1, compared with DCB29\_1 (Fig. S1), the addition of methoxy group in DCB29 significantly increased the inhibitory activity. Replacement of methoxy group and ethoxy group with larger groups like benzyl or phenethyl group led to DCB29\_4 and DCB29\_3, which displayed little inhibitory activity against BPTF bromodomain protein at 50  $\mu$ M while the minor change of methoxy group appeared little influence. The  $IC_{50}$  values of DCB29\_2 and DCB29\_5 against BPTF bromodomain were  $24.6 \pm 2.1 \mu$ M and  $20.1 \pm 2.9 \mu$ M, respectively (Fig. S1).

To clarify the mechanism of action (MOA) of DCB29, we carried out molecular docking studies to explore the potential binding mode between BPTF bromodomain and DCB29. The results indicated that DCB29 occupied the acetylated H4 peptide substrate pocket, which accounts for its *in vitro* activity (Fig. 5A-B). The methoxy group of DCB29 formed a hydrogen bond to the amide of N148 which clearly explained the differences in the inhibitory activity between DCB29 and DCB29\_1. The lack of methoxy group in DCB29\_1 significantly diminished potency (Table 1). Additionally, the other hydrogen bond was observed between the amide group of DCB29 and the carbonyl group of D101. Besides polar interactions, DCB29 also formed hydrophobic interactions with P92, F194, and Y145, Y105, V97, respectively (Fig. 5C-D). The replacement of methoxy or ethoxy with larger groups display significant steric clashes, which provided the structural explanation for impaired activity of DCB29\_3 and DCB29\_4. The hydrophobic cavity cannot make the enough structural accommodations for larger groups to provide potent binding, which is in accordance with biochemical activities *in vitro*.

## 3. Materials and methods

### 3.1. Molecular docking-based virtual screening and analogue searching

The protein crystal structure of BPTF bromodomain was retrieved from the Protein Data Bank (PDB ID: 3QZS). The monomer in the asymmetric unit was extracted and the substrate peptide was deleted. Water molecules were deleted except the conserved structural water molecular wat54, wat175, wat176. The Protein Preparation Wizard module in the Maestro Program was applied for the optimization of protein status with the pH value of  $7.0 \pm 2.0$ . Other parameters were



**Fig. 3.** *In vitro* BPTF bromodomain HTRF assays for compounds evaluation. (A) The schematic diagram of HTRF principles. (B) The thermostability of BPTF bromodomain in buffers with different pH gradients. (C) HTRF saturation binding curves with increasing concentrations of H4 peptide in optimized buffer. (D) Inhibitory activities of the 105 compounds at 100  $\mu$ M.

set default. The in-house compound database was optimized based on Lipinski's rule of five [32]. The pan-assay interference compounds (PAINS) were removed using pipeline Pilot, version 7.5 (Accelrys Software Inc., San Diego, CA, USA) [33,34,38,39]. All stereoisomers and different protonation states were prepared in Ligprep module in the Maestro program. Finally, an indexed multiconformer 3D database containing 20,000 compounds was generated in Accelrys Discovery Studio 3.0. GLIDE was applied for virtual screening with standard precision (SP) mode [40]. The grid box was set to  $25 \text{ \AA} \times 25 \text{ \AA} \times 25 \text{ \AA}$  centered at the N. The top-ranked 1,000 molecules were kept for structure cluster analysis followed by visual inspection. And 105 compounds were selected and purchased for biological testing. The similarity-based analogue searching was performed using Pipeline Pilot, version 7.5 (Pipeline Pilot; Accelrys Software Inc., San Diego, CA).

### 3.2. Protein expression and purification

The human BPTF bromodomain (residues 2914–3037) was cloned into pGEX6p-1 vector with an N-terminal glutathione S-transferase (GST) tag. The protein was overexpressed in *Escherichia coli* BL21-Codonplus (DE3)-RIPL cells (stratagene). The cells were grown in the LB medium for 4–6 h until  $OD_{600}$  reached 0.6–0.8 at 37 °C and then induced with 0.4 mM isopropyl  $\beta$ -D-thiogalactoside at 16 °C for 14–16 h. Cell pellets were harvested by centrifugation at 5,000 rpm for 8 mins and resuspended in the pre-cooled lysis buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP (Sigma C4706)). After cell lysis and centrifugation, the GST-tagged fusion protein was purified by GST affinity column with wash buffer (50 mM HEPES pH 7.4, 500 mM NaCl, 5% glycerol, 0.5 mM TCEP, 20 mM L-glutathione (Sigma G4251)). The GST tag was cleaved by PreScission protease overnight. The protein was further purified with size-exclusion chromatography on Superdex 75. The purified protein was concentrated and stored at  $-80 \text{ }^\circ\text{C}$  in final

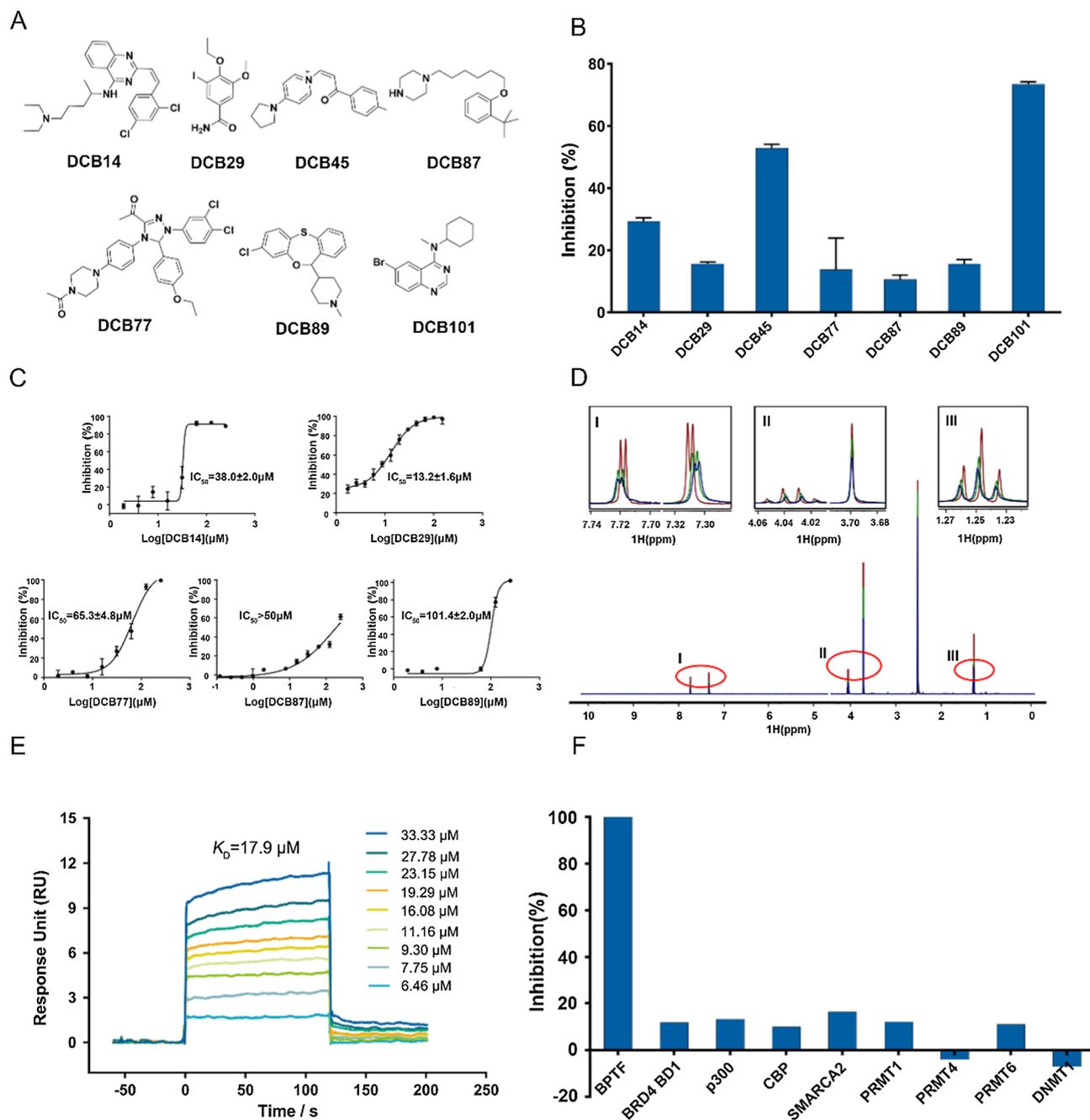
buffer containing 10 mM HEPES pH 7.4, 50 mM NaCl, 5% glycol, and 1 mM DTT (No. CSN10878, CSNpharm, Chicago, USA).

### 3.3. Protein thermal shift assays

QuantStudio™ 6 Flex Real-time PCR system was used to carry out protein thermal shift assays in order to test the BPTF bromodomain protein stability. Different assay buffers with various pH gradients were tested, including HEPES (pH = 7.0, 7.4, 7.9, 8.7), Bis-tris (pH = 5.8, 6.2, 6.6, 7.0), PIPES (pH = 6.2, 6.5, 7.0, 7.6).  $5 \times$  SYPRO orange (Invitrogen) and 5  $\mu$ M BPTF protein were mixed and added into 96-well plates (DN Biotech, Cat. No.5371112). Fluorescence signal was monitored and collected from 25 to 95 °C within half an hour. Protein Thermal Shift™ Software version 1.2 was applied for the determination of the  $T_m$  values of BPTF bromodomain.

### 3.4. BPTF bromodomain inhibition assay based on HTRF

The biotinylated peptide KGGK(Ac)GLGK(Ac)GGAK(Ac)RHRK(Ac)VLRDN-Biotin was synthesized by Shanghai China Peptides Corporation and purified to 96.4% as previously described [41]. The compounds dissolved in DMSO were diluted by dilution buffer (20 mM Bis-Tris pH 7.0, 150 mM NaCl) in the HTRF screen assay in the presence of 2% DMSO. The protein, peptide and beads were dissolved in the assay buffer (20 mM Bis-Tris pH 7.0, 150 mM NaCl, 0.01% v/v Triton X-100, 0.1% w/v bovine serum albumin, and 1 mM DTT). Firstly, 2.5  $\mu$ L compounds and 2.5  $\mu$ L proteins were added into 384-well plates (OptiPlate-384, PerkinElmer). After incubating at room temperature for 30 min, 5  $\mu$ L peptides were transferred into plates. Followed by incubation for another 30 min, 10  $\mu$ L beads (5  $\mu$ L streptavidin-XL665 and 5  $\mu$ L Anti GST-Eu cryptate) were added into each well and incubated at room temperature for 1 h. The signals were read in HTRF mode



**Fig. 4.** Biophysical and biochemical validation of identified BPTF BRD inhibitors. (A) Chemical structures of seven candidate compounds after preliminary biological evaluation. (B) Counter screen validation to exclude possible assay interference compounds. (C) The IC<sub>50</sub> values of remaining five compounds excluding false positives. (D) <sup>1</sup>H NMR spectra of DCB29 in presence of 4 μM (green), 6 μM (blue) or absence (red) of BPTF bromodomain protein. (E) SPR curves for BPTF bromodomain binding with DCB29.

(excitation at 620 nm and emission at 665 nm) with an EnVision Multilabel Plate Reader.

### 3.5. Selectivity profiling

The selectivity against BRD-family protein including BRD4-BD1, p300, CBP and SMARCA2 was determined by Alpha Screen assay. 12.5 nM BRD4-BD1 and 25 nM acetylated H4, 40 nM p300-BRD and 100 nM acetylated H4, 20 nM CBP-BRD and 200 nM acetylated H4, were mixed and incubated respectively in 384-well plated for 1 h before signal collection. The selectivity against other epi-targets including PRMT1, PRMT4, PRMT6 and DNMT1 was assessed using the radioisotope assays. The signal was acquired after reacting for 1 h at room temperature.

### 3.6. NMR spectroscopy

To investigate interactions between DCB29 and BPTF bromodomain, Carr-Purcell-Meiboom-Gill NMR experiments were carried out as previously described. Bruker Avance III-600 MHz (proton frequency) spectrometer equipped with a cryogenically cooled probe (Bruker biospin, Germany) was applied for all NMR spectra collection at 25 °C. The protein sample BPTF bromodomain was prepared in phosphate buffer (20 mM NaH<sub>2</sub>PO<sub>4</sub>, 20 mM Na<sub>2</sub>HPO<sub>4</sub>, 100 mM NaCl, pH 7.4) at 200 μM and diluted into 4 μM and 6 μM with D<sub>2</sub>O for NMR data acquisition. Compounds were dissolved in 5% DMSO-*d*<sub>6</sub> to concentration of 200 μM.

By the [RD-90°-(τ-180°-τ)*n*-ACQ] pulse sequence, the solvent-suppressed <sup>1</sup>D-<sup>1</sup>H CPMG was acquired. A 54.78 dB pulse during the

**Table 1**  
Inhibitory activity of alkoxy benzamide derivatives.

ID	R <sub>1</sub>	R <sub>2</sub>	R <sub>3</sub>	Inh at 50 μM	IC <sub>50</sub> (μM)
DCB29	I		—CH <sub>3</sub>	93%	13.2±1.6
DCB29_1	Br		—	51%	48.0±10.7
DCB29_2	I			58%	24.6±2.1
DCB29_3	I		—CH <sub>3</sub>	−2%	N.D.
DCB29_4	I			13%	N.D.
DCB29_5	I	—CH <sub>3</sub>	—CH <sub>3</sub>	85%	20.1±2.9

N.D. Denotes for not determined.

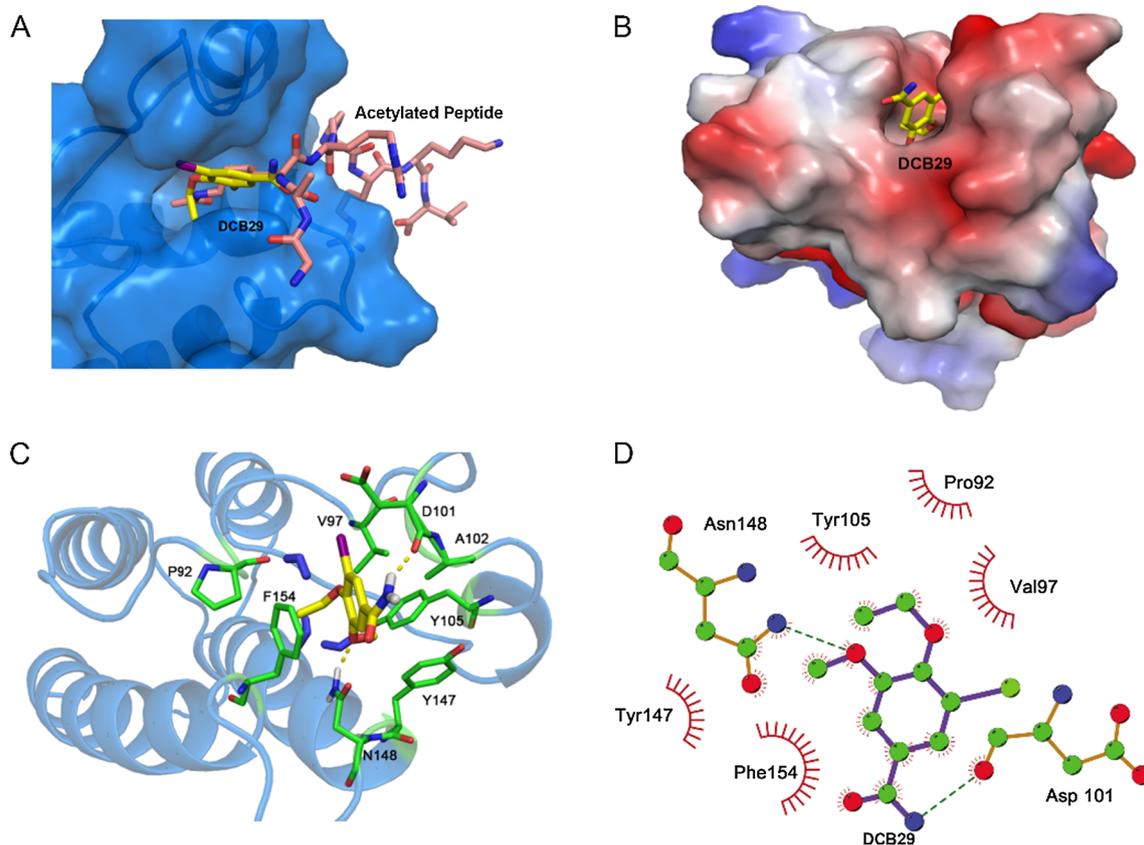
recycle delay of 4 s was applied to eliminate the influence of water resonance. We adjusted the 90° pulse length to around 11.82 μs and collected 4 dummy scans and 64 free induction decays into 64 K acquisition point with a spectral width of 12 kHz (20 ppm) and an acquisition time of 2.73 s.

### 3.7. Surface plasmon resonance based binding assays

Biacore T200 instrument (GE Healthcare) was used to conduct the SPR binding assays and performed at 25 °C. BPTF bromodomain protein was covalently immobilized on a CM5 chip using a standard amine-coupling procedure in 10 mM sodium acetate pH 5.0. Firstly, the chip was equilibrated with HBS-EP buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 0.05% v/v P20 for 3 h. For kinetic measurement, DCB29 was diluted in HBS buffer (50 mM Hepes pH 7.4, 150 mM NaCl, 0.05% v/v P20 and 0.2% v/v DMSO) at concentrations ranging from 6 to 33 μM. DCB29 was injected to bind with the protein for 120 s and dissociate for another 120 s. The *K<sub>D</sub>* value of DCB29 against BPTF bromodomain was determined by Biacore T200 evaluation software (GE Healthcare).

### 4. Conclusion

The acetylated lysine residues on bulk histones were specifically recognized by BRDs. BRD family proteins play the critical role in chromatin remodeling, transcriptional regulation, cell differentiation and embryogenesis serving as one of the most significant anticancer targets in both academia and industry. However, compared with BET family, fewer inhibitors targeting non-BET family especially BPTF have been reported so far. In this work, docking-based virtual screening was conducted to identify the hit compounds targeting BPTF bromodomain. In combination with virtual screening and biochemical evaluation, seven candidate compounds showed promising inhibitory activity at 100 μM detected by HTRF. Among them, **DCB29** was characterized as one of the most potent hits with the IC<sub>50</sub> value of 13.2 ± 1.6 μM. The direct binding between **DCB29** and BPTF bromodomain protein was



**Fig. 5.** The putative binding mode between **DCB29** and BPTF. (A) The alignment of acetylated H4 peptide binding site of BPTF (PDB ID: 3QZS) and predicted binding conformation of **DCB29**; acetylated H4 peptide is depicted as pink sticks, while **DCB29** is depicted as yellow sticks. BPTF is shown in marine surface. (B) The binding pocket of BPTF for **DCB29**. **DCB29** is depicted as yellow sticks. BPTF is shown in electrostatic potential surface. (C) Close-up view of the key interactions stabilizing **DCB29** in H4 peptide binding pocket. **DCB29** is depicted as yellow sticks, the surrounding key residues are shown in green sticks and labelled. Hydrogen bonds are shown as yellow dot lines. BPTF is shown in marine surface. (D) Schematic diagram showing detailed interactions between BPTF and **DCB29**. Residues involved in the hydrophobic interactions are depicted as red starbursts, and hydrogen-bonding interactions are shown as green lines.

validated by NMR and SPR assays. Further 2D similarity-based analogue searching and molecular docking studies provided comprehensive structural explanations for SAR study. Collectively, DCB29 could serve as a qualified starting point to develop more potent and selective BPTF inhibitors, which shed light on the treatment of BPTF-related diseases.

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### Competing interests

The authors declare no competing financial and non-financial interests in the submission of the manuscript.

### Appendix A. Supplementary material

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bioorg.2019.01.035>.

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