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Article

Identification of small molecule inhibitors targeting the SMARCA2 bromodomain from a high-throughput screening assay

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Abstract

SMARCA2 is a critical catalytic subunit of the switch/sucrose non-fermenting (SWI/SNF) chromatin remodeling complexes. Dysregulation of SMARCA2 is associated with several diseases, including some cancers. SMARCA2 is multi-domain protein containing a bromodomain (BRD) that specifically recognizes acetylated lysine residues in histone tails, thus playing an important role in chromatin remodeling. Many potent and specific inhibitors targeting other BRDs have recently been discovered and have been widely used for cancer treatments and biological research. However, hit discovery targeting SMARCA2-BRD is particularly lacking. To date, there is a paucity of reported high-throughput screening (HTS) assays targeting the SMARCA2-BRD interface. In this study, we developed an AlphaScreen HTS system for the discovery of SMARCA2-BRD inhibitors and optimized the physicochemical conditions including pH, salt concentrations and detergent levels. Through an established AlphaScreen-based high-throughput screening assay against an in-house compound library, DCSM06 was identified as a novel SMARCA2-BRD inhibitor with an IC_{50} value of 39.9±3.0 μmol/L. Surface plasmon resonance demonstrated the binding between SMARCA2-BRD and DCSM06 (K_d=38.6 μmol/L). A similarity-based analog search led to identification of DCSM06-05 with an IC_{50} value of 9.0±1.4 μmol/L. Molecular docking was performed to predict the binding mode of DCSM06-05 and to decipher the structural basis of the influence of chemical modifications on inhibitor potency. DCSM06-05 may be used as a starting point for further medicinal chemistry optimization and could function as a chemical tool for SMARCA2-related functional studies.

Keywords: AlphaScreen; high-throughput screening; SMARCA2; bromodomain; small molecule inhibitor

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Introduction

The switch/sucrose non-fermenting (SWI/SNF) complexes are evolutionarily conserved nucleosome remodeling factors that use the energy derived from ATP hydrolysis to remodel chromatin[1]. SWI/SNF complexes contain the evolutionarily conserved subunit SMARCA2 (SWI/SNF related, Matrix associated, Actin dependent Regulator of Chromatin, subfamily A, member 2), which forms the catalytic core of the remodeling complexes[2, 3].

SMARCA2 has distinct domain structures containing various motifs, including an ATP domain, a bromodomain (BRD) and a chromodomain, that contribute specifically to chromatin-remodeling activities[4, 5]. The BRD is a protein module of ~110 amino acids that contains a conserved structure composed of four α helices linked by flexible loops; this domain specifically recognizes acetylated lysine residues (KAc) on protruding histone tails[6-8]. Emerging evidence demonstrates that bromodomains are involved in a plethora of biological processes, including transcriptional regulation, the formation of scaffolding proteins and signal transduction[9-11]. Recent findings indicated that dysregulation of SMARCA2 occurs in approximately 10%–20% of solid tumors, including lung cancer, prostate cancer and

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gastric cancer[12]; thus, this target has gained considerable attention in both academia and industry[13, 14]. Although many potent and specific inhibitors targeting other BRDs have recently been discovered and widely applied to cancer treatments and biological research[15-19], hit discovery targeting SMARCA2-BRD is particularly lacking. To date, only compound PFI-3 has been identified as a *bona fide* inhibitor targeting the SMARCA2 bromodomain[20]. There is still an urgent need to develop inhibitors of novel chemotypes that may function as chemical probes for *in vivo* SMARCA2-related mechanism studies.

In the present work, we have developed an optimized AlphaScreen HTS assay for the discovery of small-molecule inhibitors targeting the SMARCA2-BRD and histone H4 interface. The high Z′ factors and signal-to-background ratio at different dimethyl sulfoxide (DMSO) concentrations indicate that the assay is robust and reproducible. Based on this platform, we performed a high-throughput screen against an in-house compound library containing *ca.* 20 000 diverse compounds, leading to the identification of a novel SMARCA2-BRD inhibitor, DCSM06. Through similarity-based analogue searching, we discovered the more potent derivative DCSM06-05, which may provide a novel chemotype for further *in vivo* SMARCA2-related functional studies.

Materials and methods

Protein expression and purification

The human SMARCA2 bromodomain (1373 to 1493) DNA sequence was cloned into a pET28a vector. The fusion protein was expressed with an N-terminal 6×His-tag in *Escherichia coli* BL21 (DE3) cells. When the OD_{600} reached 0.6–0.8 at 37 °C, cell cultures were then induced with 0.4 mmol/L isotropy-β-*D*-thiogalactoside (IPTG) overnight (14–16 h) at 16 °C. The cell pellets were collected and sonicated in pre-cooled lysis buffer (50 mmol/L HEPES pH 8.0, 150 mmol/L NaCl, 1 mmol/L DTT and 10 mmol/L imidazole). After centrifugation at 18 000 r/min for 30 min at 4 °C, the supernatant was loaded onto nickel affinity columns (HisTrap FF, GE Healthcare), and the recombinant protein was eluted with 350 mmol/L imidazole. Then, the protein was further purified by gel-filtration chromatography using a Superdex 200 10/300 GL column (GE Healthcare) in storage buffer containing 25 mmol/L HEPES pH 8.0, 150 mmol/L NaCl, and 1 mmol/L DTT. The protein was concentrated to 5 mg/mL and flash-frozen in liquid nitrogen for further use.

Protein thermal shift assay

Protein thermal shift assays were performed as previously described[21]. In each well, 5 μmol/L SMARCA2-BRD protein and 5×SYPRO Orange (Invitrogen) were mixed in a total volume of 20 μL containing 25 mmol/L buffer and 100 mmol/L NaCl. The assay plate was heated from 25 °C to 95 °C at a ramp rate of 0.05 °C/s. The changes in SYPRO Orange fluorescence intensity were monitored and collected for further analysis. The melting temperature (Tm) was calculated with the Boltzmann fitting method in the Protein Thermal Shift Software v1.2. Different buffer salts, including MES (pH 5.5–6.8), BIS-TRIS (pH 5.8–8.0), PIPES (pH 6.1–7.9) and MOPS (pH 6.5–8.0), were tested for buffer optimization. The experiments were performed in triplicate and independently repeated three times.

Peptide synthesis

The peptide abbreviated as "H4 peptide" was synthesized by Shanghai China Peptide Corporation with the sequence SGRG-K (Ac)-GG-K (Ac)-GLG-K (Ac)-GGA-K (Ac)-RHRKVGG-K-Biotin[6]. The peptide was purified by reverse phase preparative HPLC using a Waters C-18 reverse phase column. The molecular weight and purity of the peptide were validated by using an API150-ESI mass spectrometry system and HPLC. Fractions were lyophilized into white powder for long-term storage.

Molecular library

A ligand database containing *ca.* 200 000 diverse structures (each with more than 10 mg of stored compound) was extracted from the SPECS Company (SPECS_SC_10mg_Dec2016). The database was filtered by Lipinski's rule and the pan-assay interference compounds (PAINS) rule[22] using Pipeline Pilot, version 7.5 (Pipeline Pilot; Accelrys Software Inc, San Diego, CA, USA). The remaining molecules were clustered into 20 000 groups according to their structural differences using the Cluster Molecules component of Pipeline Pilot, version 7.5. Then, we selected 20 000 stable and structurally representative drug-like compounds from each group to build an in-house molecular library for the assays in this study. All compounds dissolved in DMSO were stored at 4 °C for long-term storage.

AlphaScreen high-throughput screening assay

As shown in Table S1, all reagents were diluted with 1×assay buffer and allowed to equilibrate to room temperature prior to addition to plates. A total of 2.5 μL of assay buffer or compounds was pre-plated into 384-well plates (OptiPlate, PerkinElmer). Then, 2.5 μL of 200 nmol/L SMARCA2-BRD protein was transferred into the assay plate. Plates were sealed and incubated at room temperature for 20 min, and 5 μL of biotinylated peptide H4 was added to a final concentration of 100 nmol/L. Plates were sealed and incubated at room temperature for another 30 min. Then, 5 μL of nickel-chelate acceptor beads (PerkinElmer) and 5 μL of streptavidin-conjugated donor beads (PerkinElmer) were mixed and added under subdued light. Plates were sealed and incubated at room temperature for 60 min, and signals were read on a Multilabel Reader (EnVision, PerkinElmer) using a 680 nm dichroic AlphaScreen™ mirror for excitation and a 570 nm cutoff filter for emission. The compound PFI-3 was used as the positive control.

Z′ factor and S/B calculation

The *Z′* factor is commonly used as an indicator of high-throughput screening assay performance and is calculated as

follows:

$$Z'=1-3(\delta_p+\delta_n) / |(\mu_n-\mu_p)|$$

In this formula, the means and standard deviations of the positive (p) and negative (n) controls are denoted as μ_p, δ_p and μ_n, δ_n respectively[23, 24]. DMSO and PFI-3 (40 μmol/L) are the negative and positive controls, respectively, and were included on each plate to calculate the *Z'* factor. The S/B value is the ratio of the mean of the negative controls to the mean of the positive controls in the reactions treated with 40 μmol/L PFI-3.

Surface plasmon resonance (SPR)-based binding assays

The SPR binding assays were performed on a Biacore T200 instrument (GE Healthcare) at 25 °C. SMARCA2-BRD protein was covalently immobilized on a CM5 chip using a standard amine-coupling procedure in 10 mmol/L sodium acetate (pH 5.0). The chip was first equilibrated with HBS buffer (10 mmol/L HEPES pH 7.4, 150 mmol/L NaCl, and 0.1% (*v*/*v*) DMSO) overnight. The compound was serially diluted with HBS buffer and injected for 120 s to allow binding; this was followed by a 120 s dissociation step. The K_d values of the compound (representing binding to SMARCA2-BRD) were determined using Biacore T200 evaluation software (GE Healthcare).

Similarity-based analogue searching

The prepared SPECS library was searched in Pipeline Pilot, version 7.5 (Accelrys Software Inc, San Diego, CA, USA) to investigate preliminary structure-activity relationships (SAR). Derivatives of interest were identified and purchased for biological activity tests.

Molecular modeling

The molecular modeling software Maestro (Schrödinger LLC 2015, USA) was selected to conduct molecular docking experiments. PFI-3 and DCSM06-05 were prepared with LigPrep and Advanced Conformational Search to generate protonation states and conformations. The crystal structure of SMARCA2-BRD bound to its inhibitor PFI-3 (from the PDB database, PDB ID: 5DKC) was chosen as the molecular docking receptor. The protein was prepared with the Protein Preparation Wizard Workflow, with a pH value of 7.4. SP and XP modes of induced-fit docking were used for the experiment. The docking center was set to the PFI-3 binding site. Other parameters were set as the default.

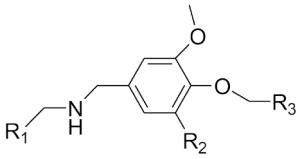
Results

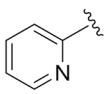
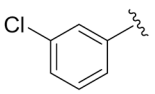
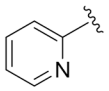
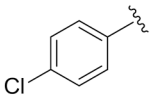
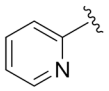
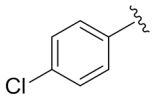
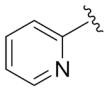
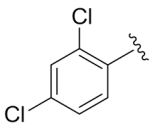
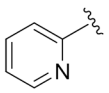
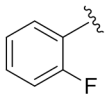
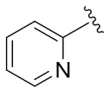
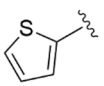
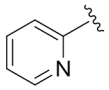
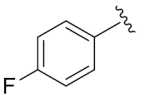
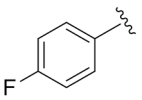
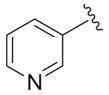
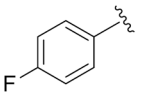
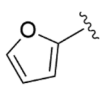
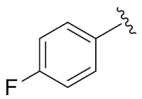
Optimization of physicochemical conditions

Buffer conditions–including the pH value, salt concentration, and detergent percentage–were optimized in order to select the buffer in which SMARCA2-BRD is most stable and to improve the detection limit for high-throughput screening assays. Protein thermal shift assays were carried out to investigate the stability of SMARCA2-BRD under different pH conditions (5.5 to 8.0). We observed a relative increase in the Tm value when using HEPES buffer at pH 8.0 (Figure 1A and Figure S1A). Thus, HEPES buffer pH 8.0 was selected for the development of our AlphaScreen assay.

Previous studies have demonstrated that the application of additives could prevent polypeptide aggregation, thus reducing nonspecific signals and ensuring the solubility and stability of components[25]. Therefore, we studied different additives during our AlphaScreen assay development. The SMARCA2-BRD AlphaScreen normalized signal was plotted as a function

Table1. Inhibitory activities of DCSM06 derivatives.



Compound ID	R1	R2	R3	IC_{50} (μmol/L)
DCSM06-01		H		9.7±1.4
DCSM06-02		H		13.2±2.9
DCSM06-03		Cl		35.2±3.2
DCSM06-04		Cl		9.1±1.8
DCSM06-05		H		9.0±1.4
DCSM06-06		H		17.8±1.9
DCSM06-07		H		16.7±2.3
DCSM06-08		H		[a]N.D.
DCSM06-09		H		81.3±3.1
DCSM06-10		Cl		73.3±4.1

[a]N.D. represents not determined.

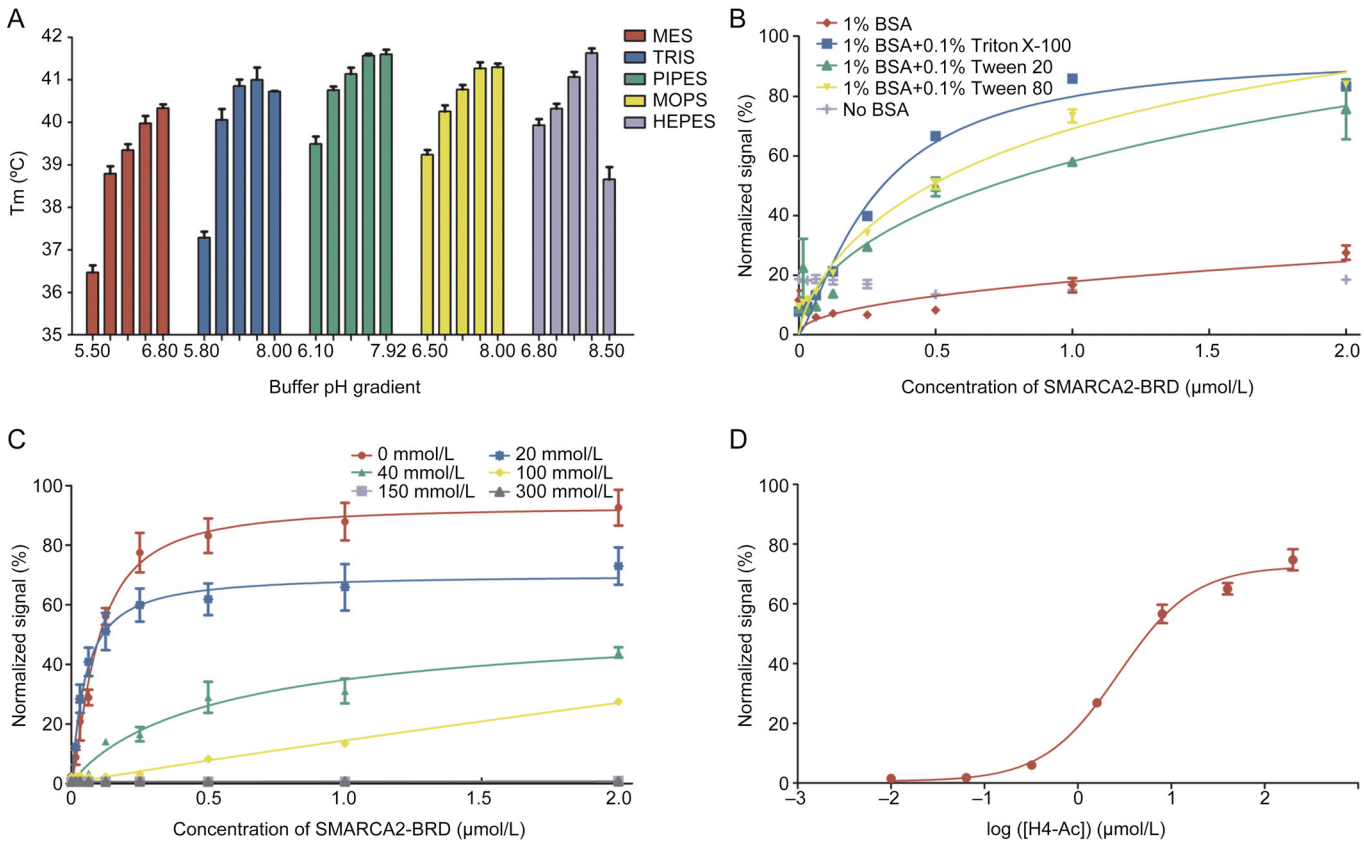


Figure 1. AlphaScreen assay development and optimization. (A) The thermostability of SMARCA2-BRD in different buffers. (B) AlphaScreen saturation binding curves with increasing concentrations of SMARCA2-BRD in the absence or presence of different detergents. (C) AlphaScreen saturation binding curves with different salt concentrations. (D) AlphaScreen saturation binding curves with increasing concentrations of H4 peptide in optimized buffer.

of protein titration (from 0.01 to 2 μmol/L) in the absence or presence of different detergents (Figure 1B). Of these detergents, 1% (*w/v*) BSA and 0.01% (*v/v*) Triton X-100 proved to be most efficient at elevating the signal-background ratio and decreasing non-specific binding. Hence, both 1% (*w/v*) BSA and 0.1% (*v/v*) Triton X-100 were added to the AlphaScreen assay buffer.

Salt concentration is another important component of biological assays that must be optimized to maintain ionic strength and satisfy the specific requirements of individual proteins. Thus we studied a range of different NaCl concentrations (0 mmol/L–300 mmol/L) (Figure 1C). As the salt concentration increased, the signal-background ratio of the AlphaScreen signal decreased significantly. With these results in mind, the optimal AlphaScreen assay buffer was selected using 25 mmol/L HEPES pH 8.0, 1% (*w/v*) BSA, and 0.01% (*v/v*) Triton X-100. Replacement of HEPES pH 8.0 with more acidic buffer salts led to decreased SMARCA2-BRD binding activity, which is consistent with previous observations (Figure S1B). Under the optimized conditions described above, the relative low concentrations (200 nmol/L SMARCA2-BRD and 100 nmol/L biotinylated H4 peptide) that provided reliable AlphaScreen signals were selected for future application (Figure 1D).

Evaluation of DMSO tolerance and *Z*′ factor

DMSO is a widely applied solvent typically used to dissolve small-molecule compounds. Thus, DMSO tolerance is one of most important factors influencing the stability of high-throughput screens. Therefore, we studied various concentrations (0%–10%) of DMSO in our assay. As illustrated in Figure 2A–2B, although increased DMSO concentrations led to lower signal-to-background ratios (S/B), at 8% DMSO, the S/B was still greater than 10-fold, and the K_d values were stable. This indicated that our AlphaScreen assay platform was highly robust and qualified for inhibitor screening.

In addition, the Z′ factor was evaluated to analyze data variation and signal dynamic range in this assay[26]. We evaluated the effects of DMSO concentration (0%, 2% and 4%) on the Z′ factor; these concentrations are commonly used in HTS assays (Figure 3C–3E). Generally, high-throughput screens with a Z′ factor value greater than 0.5 are considered to be highly qualified systems[27, 28]. The Z′ factors in our AlphaScreen assays were greater than 0.7, even in the presence of 4% DMSO. These results clearly revealed that our AlphaScreen assay is robust, reproducible, and appropriate for HTS with DMSO concentrations up to 4%. The feasibility of this high-throughput method was further verified by testing the inhibitory activities of the positive compound PFI-3; the reported IC_{50} value for this compound was 0.154±0.030 μmol/L, which

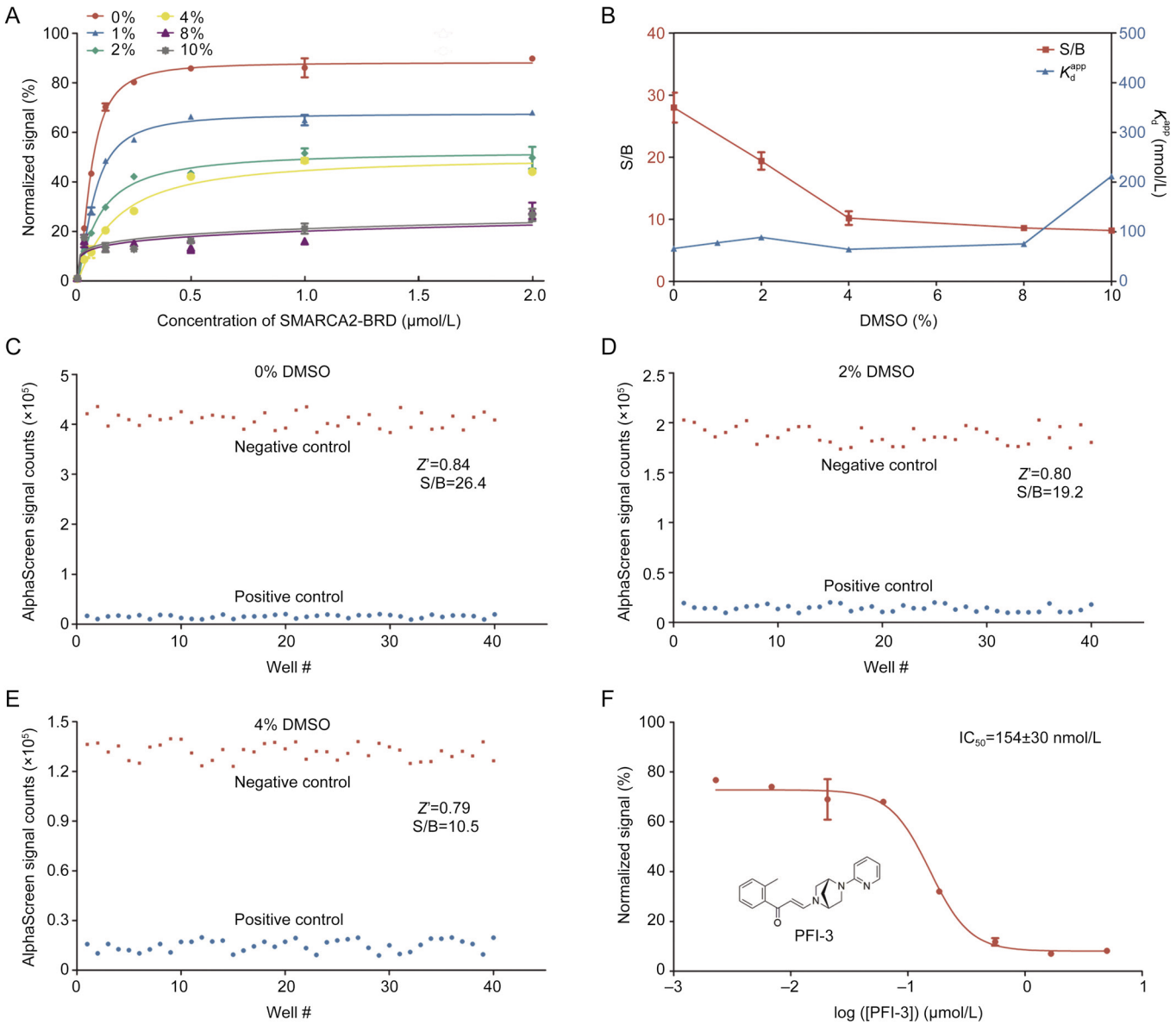


Figure 2. Evaluation of DMSO tolerance in the optimized AlphaScreen binding assay. (A) AlphaScreen saturation binding curves in the absence or presence of different DMSO concentrations. (B) Signal-background ratio and apparent K_d values at different DMSO concentrations. (C–E) Evaluation of *Z'* factors for the AlphaScreen high-throughput screening assay in the absence or presence of 0%, 2%, and 4% DMSO. The AlphaScreen normalized signals from both negative and positive controls are shown in a scatter plot. (F) IC_{50} curve for PFI-3. All experiments were performed at least three times. The data were analyzed in GraphPad Prism 5.0.

is in line with previous reports (Figure 3F)[29].

High-throughput screening against an in-house compound library

We established our in-house compound library containing *ca*. 20 000 compounds of diverse chemical scaffolds through filtering and optimization using the Lipinski rules of five and the PAINS filter in Pipeline Pilot, version 7.5 (Pipeline Pilot; Accelrys Software Inc., San Diego, CA). We performed a primary screen at a single concentration (200 μmol/L), and then, compounds with inhibitory activity >90% were identified for further evaluation.

After a primary AlphaScreen-based high-throughput screen, we identified 8 hits with novel scaffolds, the IC_{50} values of which are shown in Figures 3A and S2. Because fluorescence quenching agents, light scattering agents (insoluble compounds), singlet oxygen quenching agents and biotin mimetics commonly interfere with AlphaScreen signals and result in false positives, the AlphaScreen TruHits kit was used to identify false positives in the screening process. As shown in Figure 3B, three of eight compounds, including DCSM11, DCSM22, and DCSM50, displayed greater than 50% inhibition at a 100 μmol/L concentration. Thus, these compounds were excluded from further validation steps. Among the remaining hit compounds, DCSM06 showed the greatest inhibitory activ-

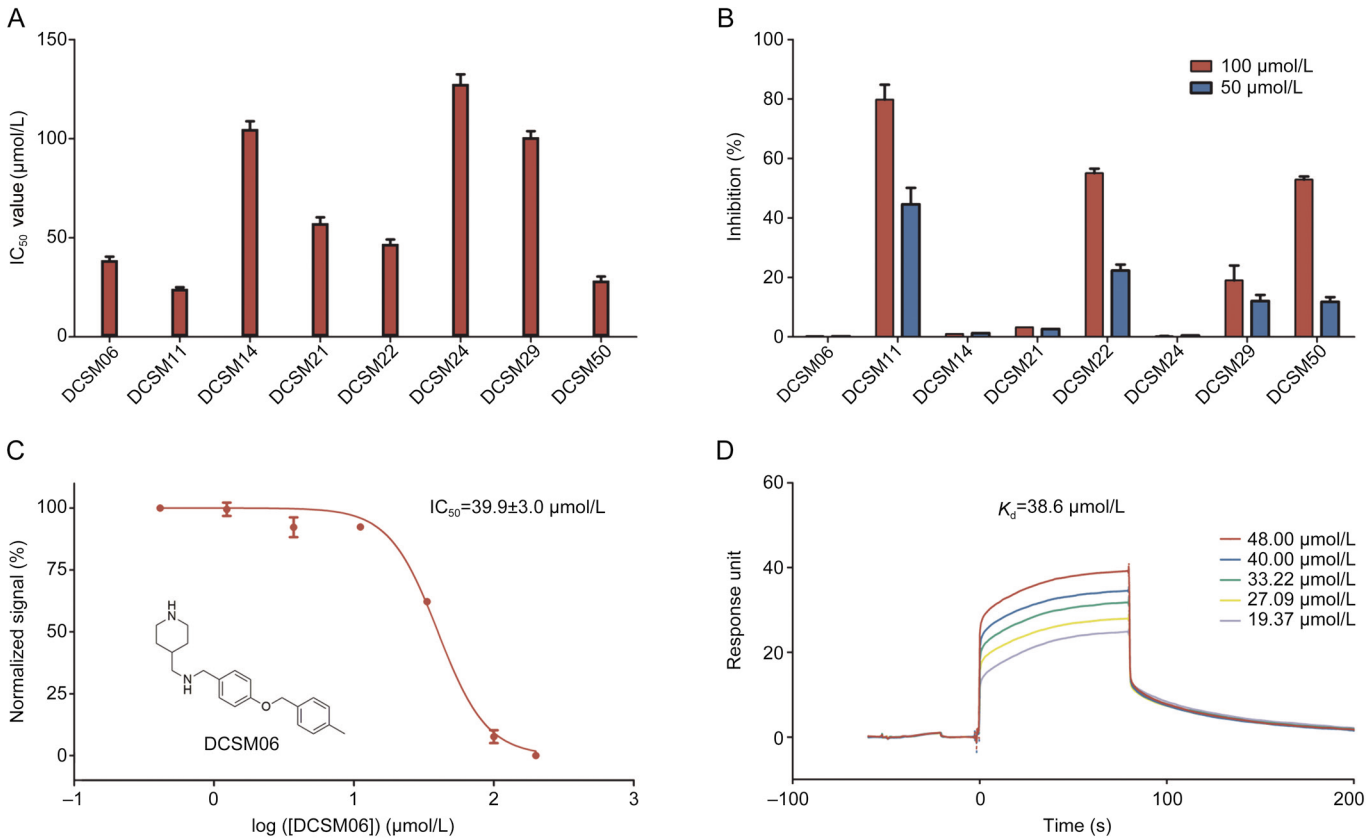


Figure 3. Discovery and validation of novel SMARCA2-BRD inhibitors. (A) Primary AlphaScreen high-throughput screening results. (B) AlphaScreen TruHits kit validation. (C) Inhibitory activities of DCSM06. (D) SPR assay demonstrated binding between SMARCA2-BRD and DCSM06.

ity, with an IC_{50} value of 39.9±3.0 μmol/L (Figure 3C).

SPR-based binding assay

Surface plasmon resonance (SPR), one of most useful biophysical methods for hit validation, was used to demonstrate the binding between DCSM06 and SMARCA2-BRD[30]. As shown in Figure 3D, DCSM06 directly bound to SMARCA2-BRD with an equilibrium dissociation constant (K_d) of 38.6 μmol/L, which was consistent with the IC_{50} value of 39.9±3.0 μmol/L. These results suggest that DCSM06 binds to SMARCA2-BRD *in vitro*.

Similarity-based analogue searching

To further study the structure activity relationship (SAR), we used Pipeline Pilot to identify several derivatives, and we purchased these derivatives from the SPECS Company. The structures and their inhibitory activities against SMARCA2-BRD are summarized in Table 1 and Figure S2. Of these, the derivative DCSM06-05 displayed the best inhibitory activity, with an IC_{50} value of 9.0±1.4 μmol/L and a K_d value of 22.4 μmol/L based on SPR binding assays (Figure S3, Figure 4A–4B). Thus, DCSM06-05 was chosen for further analyses.

Docking simulations and SAR analysis

To reveal the binding modes and mechanisms of interaction between DCSM06 derivatives and SMARCA2-BRD, the crystal structures of DCSM06-05 and SMARCA2 (PDB ID: 5DKC) were selected for a molecular docking study using the molecular modeling software Maestro (Schrödinger LLC 2015, USA). First, we re-docked the positive control compound, PFI-3, into the SMARCA2-BRD binding pocket to validate the docking model. As illustrated in Figure S4, the model of PFI-3 docking (in yellow) successfully reproduced the real binding mode of this inhibitor (in green). After the reliability and practicability of the docking model were validated, we docked DCSM06-05 into the SMARCA2 binding pocket . The binding results suggested that DCSM06-05 displayed a similar binding mode to PFI-3. Further, similar hydrogen bonds stabilized the DCSM06-05 docking conformation, though the precise interaction details need further experimental validation. As shown in Figure 4D, DCSM06-05 forms π-π stacking interactions with F1409, which is a universal feature of inhibitors binding to this site in this model. The 2-pyridine (R1) group contributes significantly to improvements in inhibitory activity, and any chemical modifications to this group may reduce the potency of inhibition. This demonstrates that a hydrogen bond interaction between the nitrogen of the inhibitor's pyridine group and F1409 is preferred to ensure enhanced binding affinities in this series of inhibitors. Moreover, the benzene ring docks into a shallow hydrophobic pocket formed by P1413 and

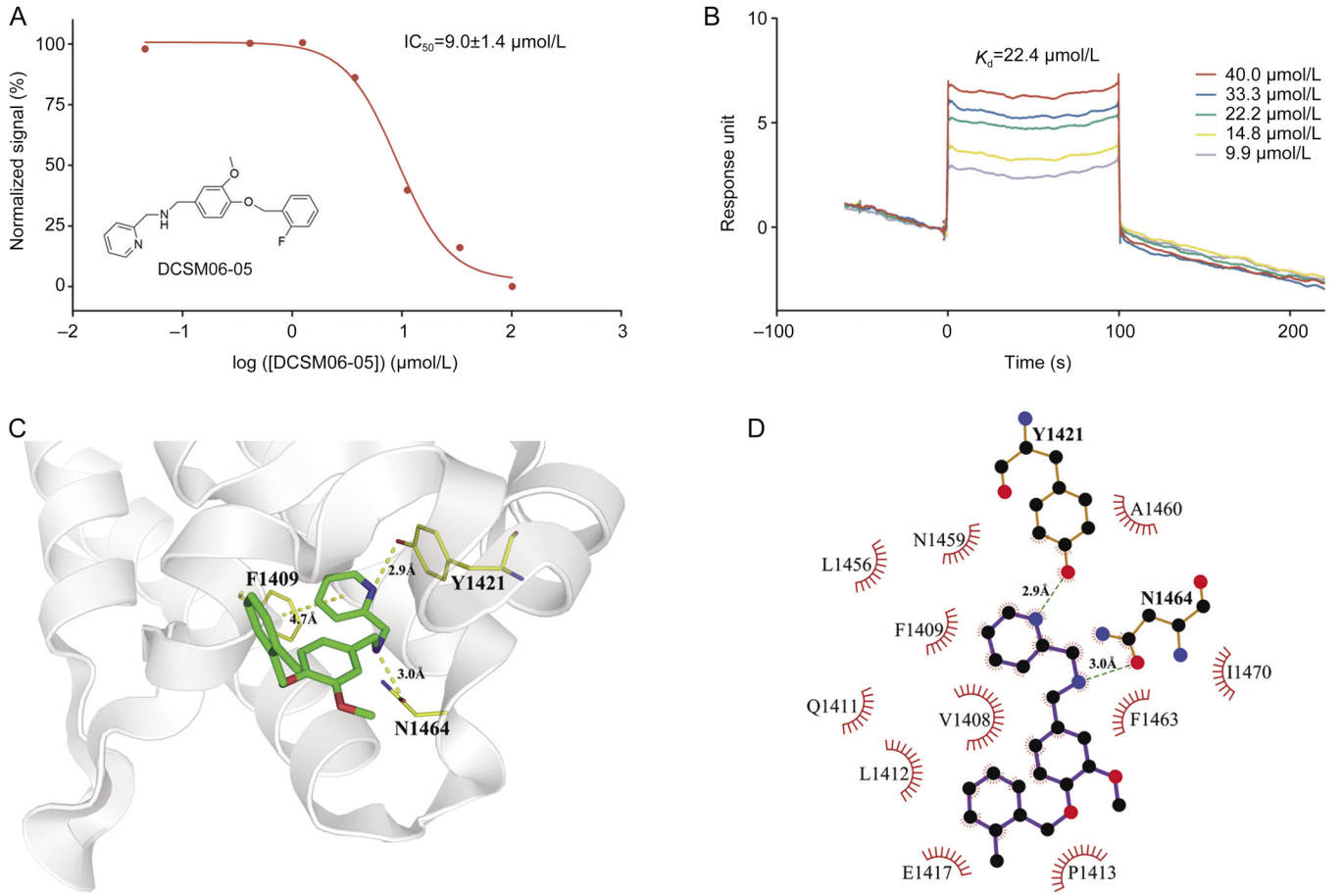


Figure 4. Hit validation and analysis of the mechanism of action of the derivative DCSM06-05. (A) Inhibitory activities of DCSM06-05. (B) SPR assay demonstrated binding between SMARCA2-BRD and DCSM06-05. (C) Molecular docking analysis elucidated key interactions with SMARCA2-BRD. (D) Schematic diagram showing interactions between SMARCA2-BRD and DCSM06-05. Hydrogen bonds are shown in green dashed lines, and residues involved in these interactions were illustrated in the LigPlot$^+$ program[33].

I1470, and methoxy substitution can create a hydrogen bond with F1409 in a similar manner as PFI-3. Additionally, slight differences in halogen substitutions in the R3 region do not appear to have a major influence on the inhibitory activity of these compounds. Collectively, the docking results suggest that DCSM06-05 can bind within the H4 peptide-binding site of SMARCA2-BRD in a manner quite similar to the binding of PFI-3. These results help us to decipher the structural basis of the improved potency of DCSM06-05.

Discussion

Bromodomains specifically recognize acetylated lysine residues on histones and play a pivotal role in downstream gene transcription, DNA repair and chromatin remodeling. The therapeutic targeting of bromodomains has recently generated great interest in industry and academia[31, 32]. In sharp contrast to the rapid discovery and development of drugs targeting BET family proteins, very little investigation into non-BET family bromodomains has occurred. PFI-3 was the only potent chemical probe to be discovered as a SMARCA2-BRD interactor, limiting further biological studies. There is still an urgent need to develop potent SMARCA2-BRD inhibitors of novel chemotypes.

In this study, we developed a robust and reproducible AlphaScreen-based HTS assay targeting SMARCA2-BRD. The high Z' factors (>0.7) at different DMSO concentrations demonstrated the robustness and reliability of this AlphaScreen HTS assay. Based on this platform, we conducted a pilot screen against an in-house compound library, leading to the identification of DCSM06, which has an IC_{50} value of 39.9±3.0 μmol/L. Through 2D similarity-based analogue searching, DCSM06-05 was identified as a more potent inhibitor targeting SMARCA2-BRD with an IC_{50} value of 9.0±1.4 μmol/L. Further molecular docking analyses provided a detailed mechanism of action for DCSM06-05 and provided a preliminary structure-activity relationship that could be exploited for further modification.

Considering all these data together, this robust AlphaScreen-based HTS assay may be a reliable platform that could be used to speed up hit discovery targeting SMARCA2-BRD and other non-BET family proteins. Further, DCSM06-05 may be a starting point for further medicinal chemistry optimization and

could be used as an efficient chemical probe to uncover the elusive role of SMARCA2 in physiological and pathological processes.

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Author contribution

Cheng LUO, Hui-fang CHAI and Kai-xian CHEN designed the research; Wen-chao LU, Tian LU and Jie HAN performed the biological experiments; Jun-chi HU and Hao JIANG performed computational analysis; Hong DING Yuan-yuan ZHANG, Li-yan YUE and Shi-jie CHEN analyzed and interpreted the data. Tian LU, Jun-chi HU and Wen-chao LU wrote the manuscript; Cheng LUO, Hua-liang JIANG, and Kai-xian CHEN reviewed the manuscript. All authors read and approved the manuscript.

Supplementary information

Supplementary information is available at the website of Acta Pharmacologica Sinica.

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