Bioorganic & Medicinal Chemistry 21 (2013) 7004–7010

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Synthetic trimethyllysine receptors that bind histone 3, trimethyllysine 27 (H3K27me3) and disrupt its interaction with the epigenetic reader protein CBX7

Sara Tabet, Sarah F. Douglas, Kevin D. Daze, Graham A. E. Garnett, Kevin J. H. Allen, Emma M. M. Abrioux, Taylor T. H. Quon, Jeremy E. Wulff, Fraser Hof *

Department of Chemistry, University of Victoria, Victoria, BC V8W 3V6, Canada

ARTICLE INFO

Article history:
Received 25 June 2013
Revised 30 August 2013
Accepted 7 September 2013
Available online 19 September 2013

Keywords:
Post-translational modifications
Methylation
Epigenetics
Protein–protein interactions
Supramolecular chemistry
Calixarenes

ABSTRACT

Post-translational modifications act as 'on' or 'off' switches causing downstream changes in gene transcription. Modifications such as trimethylation of lysine 27 on histone H3 (H3K27me3) cause repression of transcription and stable gene silencing, and its presence is associated with aggressive cancers of many types. We report here macrocyclic host-type compounds that can bind H3K27me3 preferentially over unmethylated H3K27, and characterize their binding affinities and selectivities using a convenient dye-displacement method. We also show that they can disrupt the protein–protein interaction of H3K27me3 with the chromobox homolog 7 (CBX7), a methyllysine reader protein, using fluorescence polarization. These results show that sub-micromolar potencies are achievable with this family of host compounds, and suggest the possibility of their use as new tools to induce the disruption of methyllysine-mediated protein–protein interactions and to report on lysine methylation in vitro.

1. Introduction

Post-translational histone modifications play an important role in many diseases, and the 'histone code' is a metaphor that explains their functions in terms of the 'writers,' 'erasers,' and 'readers' of each modification type. The diverse modifications of N-terminal histone tails include acetylation (of Lys), phosphorylation (of Ser and Thr), methylation (of Lys and Arg), lysine ubiquitylation and SUMOylation, and even noncovalent post-translational modification in the form of *cis–trans* proline isomerisation.[1–5] Such histone modifications act on chromatin by directly influencing the interactions of histones with DNA and/or by acting as specific recruitment sites for other regulatory proteins. Different modifications are directly responsible for a variety of downstream signalling outcomes that include transcriptional activation or repression, chromatin remodelling, and DNA repair and recombination.[6–8] Lysine 27 of histone 3 can be trimethylated, generating an epigenetic mark (H3K27me3) that is the focus of intense current interest in biomedical research due to its importance as a signalling element in multiple metastatic cancers.[9–12] Histones bearing the H3K27me3 mark are generally associated with gene silencing by the combined action of two multiprotein complexes, called polycomb repressive complexes 1 and 2 (PRC1/PRC2).[13,14] The canonical description of the polycomb silencing pathway starts with 'writer' proteins EZH1/EZH2, which are histone methyltransferases and components of PRC2, installing the H3K27me3 mark. Subsequent recruitment of PRC1 to the locus is driven by an H3K27me3-'reader' protein that is a modular component of PRC1. The ultimate result of PRC1/PRC2 action is DNA methylation and stable gene silencing.[13] The *Drosophila* parent of the H3K27me3 reader module is called polycomb (the namesake of the entire pathway); in humans there are five paralogs of polycomb called chromobox homolog (CBX) 2, 4, 6, 7, and 8. Each of these reader proteins is functionally distinct.[13–17] The paralog whose function has been most carefully studied is CBX7. CBX7 is specifically associated with the silencing of the tumor suppressors $p16^{INK4a}$ and $p14^{ARF}$ that are upstream controllers of Rb- and p53-mediated apoptosis, respectively.[18–21] As with many epigenetic pathways, the functional outcome of signalling by CBX7 is highly context dependent.[13] CBX7 is consistently shown to be strongly proliferative in castration-resistant prostate cancer cell lines, embryonic and adult stem cells, and in hematopoiesis/lymphomagenisis.[15,17,20–23] CBX7 is upregulated in prostate cancer upon progression from the androgen dependent state to the more aggressive androgen-independent state.[21]

Abbreviations: CBX7, chromobox homolog 7; H3K27, histone H3 lysine 27; H3K27me3, histone H3 lysine 27 trimethylated.

* Corresponding author.
E-mail address: fhof@uvic.ca (F. Hof).

0968-0896/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.bmc.2013.09.024

The molecular basis for targeting the H3K27me3–CBX7 complex is most clearly demonstrated by mutagenesis studies that show the complete abrogation of proliferative signal when a single H3K27me3-binding residue of CBX7 is mutated.[17,21,22] We have been pursuing chemical agents for the disruption of the H3K27me3–CBX7 complex. The histone's trimethyllysine residue is a perfectly defined and potent hot spot for this protein–protein interaction, since CBX7 does not bind at all to unmethylated histone 3.[24] In the natural protein–protein complex, the trimethyllysine residue is recognized and bound by an aromatic cage motif in CBX7, which is a rigid pocket defined by Phe11, Trp32, and Trp35, (Fig. 1B, PDB code 2L1B).[22] Multiple cation–pi contacts between these pi-rich side chains and the methylated ammonium ion of Kme3 combine to drive complexation.[25]

We have previously shown that rigid, macrocyclic synthetic compounds based on *para*-sulfonatocalix[4]arene (PSC) are potent and selective binders of trimethyllysine as a free amino acid.[26,27] These pocket-like macrocycles bind the methylated side chain of Kme3 via multiple charge–charge and cation–pi contacts.[27] Other macrocycles can also bind Kme3 as the free amino acid[28] and within histone-tail peptide sequences[29,30], representing an increasing interest in using structured macrocycles to target post-translational modifications directly. We have also shown, using a FRET-based protein biosensor, that some simple calixarene-based agents can disrupt the interaction of H3K27me3 with CBX7.[31] We report here a set of macrocyclic compounds that constitute a new family of H3K27me3-targeting compounds that, unlike previously reported macrocycles, are easily modified to tune affinities and selectivities. We report on a method for characterizing the direct binding of such compounds to H3K27me3 using a competitive fluorescence-based dye-displacement assay. Finally, we demonstrate their disruption of in vitro H3K27me3–CBX7 binding using a fluorescence polarization assay that is amenable to high-throughput screening. The resulting structure–activity relationships uncover unanticipated aspects of molecular recognition for these macrocyclic agents.

2. Materials and methods

2.1. Synthesis—general

All reagents for synthesis were purchased from Aldrich and used as obtained. Lucigenin dye was purchased from Invitrogen or Sigma and used as obtained. ESI-MS was performed on a Finnigan LCQ MS. The syntheses of compounds **1**–**4** and **8** and **9** have been previously published.[26,31,32] New hosts were made using **2** as starting material. All calixarenes and peptides were purified by HPLC (or HPLC-MS) on a preparative Apollo C18 column (Alltech, 5 μm, 22 × 250 mm) or preparative Luna C-18 column (Phenomenex, 5 μm, 21.2 × 250 mm), detecting at 280 nm. Compounds were purified by running a gradient from 90:10 0.1% TFA in H_2O:0.1% TFA in MeCN to 10:90 0.1% TFA in H_2O:0.1% TFA in MeCN over 35 min.

2.2. Synthesis of calixarenes

2.2.1. 5-(4-Methylphenyl)-25,26,27,28-tetrahydroxy-11-17-23-trisulfonatocalix[4]arene (5)

Compound **2** (0.1011 g, 0.1360 mmol), 4-methylphenylboronic acid (0.0204 g, 1.1 equiv, 0.1496 mmol), $Pd(OAc)_2$ (0.0061 g, 20 mol %) and sodium carbonate (0.0548 g, 3.8 equiv, 0.517 mmol) were dissolved in 5 mL of deionized water inside a microwave vial, sealed, and heated to 150 °C under microwave irradiation for 5 min with cooling air and stirring on. HPLC purification and evaporation of solvents in vacuo afforded a white powder in 47.5% yield (0.0489 g). Mp: 240 °C (dec). IR (KBr pellet): 3350br, 1474s, 1457s, 1264w, 1211s, 1155s, 1113s, 1040s, 886w, 816w, 783 m, 668 m, 654 m, 626 m, 545 m. ^{1}H NMR (500 MHz, D_2O): δ 7.83 (d, J = 2.4 Hz, 2H), 7.75 (d, J = 2.4 Hz, 2H), 7.60 (s, 2H), 7.19 (s, 2H), 6.80 (d, J = 7.6 Hz, 2H), 5.70 (s, 2H), 4.06 (s, br, 8H), −0.68 (s, 3H). ^{13}C NMR (125 MHz, D_2O): δ 152.1, 150.8, 146.5, 136.8, 136.4, 134.3, 133.9, 129.0, 128.8, 128.5, 128.4, 127.8 126.9, 126.8, 126.7, 126.5, 125.1, 30.7, 16.0. HR-ESI-MS: 753.07738 ($[M-H]^-$, $C_{35}H_{29}O_{13}S_3^-$; calcd 753.07758).

2.2.2. 5-(4-Methoxyphenyl)-25,26,27,28-tetrahydroxy-11-17-23-trisulfonatocalix[4]arene (6)

Compound **2** (0.1080 g, 0.1454 mmol), 4-methoxyphenylboronic acid (0.0244 g, 1.1 equiv, 0.1606 mmol), $Pd(OAc)_2$ (0.0061 g, 20 mol %) and sodium carbonate (0.0551 g, 3.8 equiv, 0.519 mmol) were dissolved in 5 mL of deionized water inside a microwave vial and irradiated to 150 °C for 5 min with cooling air and stirring on. HPLC purification and evaporation of solvents in vacuo afforded a white powder in 43.9% yield (0.0491 g). Mp: 240 °C (dec). IR (KBr pellet): 3245br, 1473s, 1457s, 1260w, 1239s, 1213s, 1180s, 1155s, 1114s, 1040s, 883w, 830w, 811w, 785m, 657m, 626m, 604m, 549m. ^{1}H NMR (500 MHz, D_2O): δ 7.78 (d, J = 2.4 Hz, 2H), 7.71 (d, J = 2.0 Hz, 2H), 7.27 (s, 2H), 7.06 (d, J = 8.5 Hz, 2H), 6.03 (d, J = 7.7 Hz, 2H),), 4.06 (s, br, 8H), 1.60 (s, 3H). ^{13}C NMR (125 MHz, D_2O): δ 157.4, 151.8, 150.9, 146.9, 136.6, 136.2, 134.5, 131.6, 128.7, 128.5, 128.2, 128.0, 127.1, 127.0, 126.7, 126.5, 113.7, 52.8, 30.9, 30.6. HR-ESI-MS: 769.07107 ($[M-H]^-$, $C_{36}H_{31}O_{15}S_3^-$; calcd 769.07249).

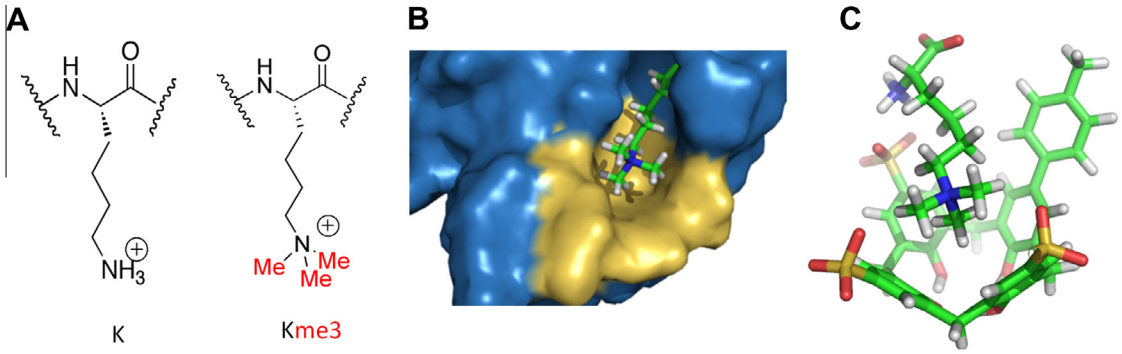


Figure 1. (A) Unmethylated (K) and trimethylated (Kme3) states of lysine. (B) H3K27me3 bound to CBX7 (PDB ID: 2L1B). Aromatic residues that make cation–pi interactions with the Kme3 side chain are colored yellow. (C) Trimethyllysine bound in the *p*-sulfonatocalix[4]arene cavity of Host 5; model generated using MMFFaq energy minimization in Spartan.

2.2.3. 5-(2,3-Dimethoxyphenyl)-25,26,27,28-tetrahydroxy-11-17-23-trisulfonatocalix[4]arene (7)

Compound **2** (0.041 g, 0.055 mmol), 2,3-dimethoxyphenylboronic acid (0.010 g, 1 equiv, 0.055 mmol), tetrabutylammonium bromide (0.0089 g, 0.5 equiv, 0.028 mmol), $Pd(OAc)_2$ (0.0025 g, 20 mol %) and sodium carbonate (0.026 g, 3.8 equiv, 0.209 mmol) were dissolved in 5 mL of deionized water inside a microwave vial and irradiated at 150 °C for 5 min with cooling air and stirring on. The aqueous solution was washed with CH_2Cl_2 (2 × 20 mL) then EtOAc (1 × 25 mL) and concentrated. HPLC purification and evaporation of solvents in vacuo afforded a white powder in 42% yield (0.018 g). Mp: 245 °C (dec). IR (KBr pellet): 3366br, 1465s, 1261w, 1213s, 1160s, 1118s, 1042s, 889w, 784m, 661m, 625m, 559m. ^{1}H NMR (500 MHz, D_2O): δ 7.64 (d, J = 2.1 Hz, 2H), 7.62 (d, J = 2.0 Hz, 2H), 7.37 (s, 2H), 7.10 (s, 2H), 6.96 (t, J = 7.8 Hz, 1H), 6.82 (d, J = 7.8 Hz, 1H), 6.76 (d, J = 6.9 Hz, 1H), 3.99 (m, br, 8H), 3.70 (s, 3H), 2.48 (s, 3H). ^{13}C NMR (125 MHz, D_2O): δ 153.3, 152.3, 150.9, 147.8, 145.2, 135.4, 135.3, 134.4, 131.4, 130.0, 129.1, 128.5, 128.2, 128.0, 126.6, 126.5, 126.4, 124.9, 122.3, 112.1, 59.6, 56.0, 30.7, 30.5. HR-ESI-MS: 799.07935 ([M−H]$^-$, $C_{36}H_{31}O_{15}S_3^-$; calcd 799.08303).

2.3. Synthesis of peptides

All reagents used for peptides synthesis were purchased from ChemImpex or Sigma Aldrich. All Histone 3 peptides (H3K27 = Ac-AARKSAPY-C(O)NH_2, H3K27me3 = Ac-AARKme3SAPY-C(O)NH_2, FITC-H3K27me3 = FITC-βAla-AARKme3SAPY-C(O)NH_2) were synthesized using the standard Fmoc solid-phase peptide synthesis protocol[33] as implemented on a CEM Liberty 1 microwave-based peptide synthesizer on Rink amide resin (ChemImpex). All sequences had a tyrosine introduced at the C-terminus to facilitate UV detection during HPLC purification. All peptides were purified as described under Section 2.1 and used without desalting.

2.4. Protein expression

CBX7 chromodomain was expressed and purified as previously reported,[24] using Addgene plasmid 25241 deposited by C. Arrowsmith, Structural Genomics Consortium, Toronto, Canada.

2.5. K_d Determination—direct titrations for calixarene–dye affinities

Samples for the direct titration were prepared in NUNC 96 black-well plates with an optically clear bottom, and were composed of 0.01 M of phosphate buffer at pH 7.4, 500 nM of lucigenin, and varying concentrations of hosts (0–5 μM) made up with distilled water to a final volume of 200 μL. Emission spectra from 445–645 nm using a SpectraMax® M5/M5e Microplate Reader were collected at λ_{exc} 369 nm. Host **5** required the use of a smaller concentration of lucigenin of 250 nM and therefore a smaller range of concentrations of hosts (0–1.5 μM) due to very strong binding and inability to fit curves at high concentrations. All experiments were performed in duplicate. Calixarene-LCG K_{Ind} values were determined by plotting emission intensity (dF_{obs}) as a function of calixarene concentration (H) and fitting the data to the following expression[34] using origin:

$$dF_{obs} = (F_{max} - F_{min}) * ((D + Ht + (1/K_i) - sqrt((D + Ht + (1/K_i))(\hat{2}) - (4 * Ht * Ht))))/(2 * D) \quad (1)$$

where; y equals the change in fluorescence ($dF_{obs} = F_{obs} - F_{min}$) and × equals the total host concentration Ht ([calixarene]t = 0–5 μM). Parameters, F_{min} and K_i where adjustable where F_{min} equals the minimum fluorescence of dye when saturated with host. D and F_{max} were treated as constants, at 0.5 μM and the maximum fluorescence of dye without presence of host, respectively.

2.6. K_d Determination—competition experiments for calixarene–peptide affinities

Samples for the dye displacement assay were prepared in NUNC-96 black well plates with optically clear bottom, and were composed of 0.01 M of phosphate buffer at pH of 7.4, 500 nM of lucigenin, 1.25 μM of calixarene, and varying peptide concentrations (H3K27 or H3K27me3, 0–2 mM) made up with distilled water to a final volume of 200 μL. Emission spectra were collected as above. All experiments were performed in duplicate. Calixarene–peptide K_d values were determined by plotting emission intensity (dF_{obs}) as a function of peptide concentration $[G]_t$ and fitting the data with the program Origin using an expression and accompanying cubic equations initially derived by Nau and coworkers.[35–37] (These expressions were subsequently adapted and shown to be useful also for UV–vis data.[38])

Equations used for fitting of dye displacement data:

$$dF_{obs} = Ei + ((Ehi - Ei) * ((K_i * H)/(1 + (Ki * H)))) \quad (2)$$

Fitting was achieved by iterative nonlinear least squares regression using the cubic step equation:

$$(a * H * H * H + b * H * H + c * H + d)/(3 * a * H * H + 2 * b * H + c)$$

$$a = K_i * K_g;$$

$$b = K_i + K_g + K_i * K_g * It + K_i * K_g * \times - K_i * K_i * Ht;$$

$$c = 1 + K_i * It + K_g * \times - (K_i + K_g) * Ht;$$

$$d = -Ht;$$

where y is $dF_{obs} = F_{obs} - F_{min}$ (change in emission intensity) and × (incorporated in the term H as shown above) equals the total guest concentration [G]t, which was varied from 0–1.5 mM for H3K27 and 0–0.5 mM for H3K27me3. K_i (association constant for host–dye complex) was determined by previous 1:1 direct titration (see above) and held constant, and Ehi (the inherent emission intensity of the calixarene–dye complex) was set to 0. It (total concentration of dye) and Ht (total concentration of host) were held constant during the titration at [lucigenin]t = 0.5 μM and [calixarene]t = 1.25 μM. The adjustable parameters during fitting were K_g (association constant of the calixarene–guest complex) and Ei (emission intensity of the uncomplexed dye). H (free concentration of host) was defined as above and used for iterative fitting with an initial guess of $H = H_t$.

2.7. Fluorescence polarization assay

The conditions for FP displacement assay were adapted from those used in an earlier report of a direct peptide-protein titration (see Supplementary information).[24] The peptides FITC–H3K27me3, H3K27, H3K27me3 and bovine serum albumin (Sigma) were resuspended in deionized H_2O and the calixarenes were dissolved in water containing 4 equiv of NaOH to neutralize residual acid from HPLC purifications. The competitive assay was performed in black, 96-well plates (NUNC-black well plates with optically clear bottom) in a buffer containing 20 mM Tris–HCl pH 8.0, 250 mM NaCl, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, and 0.01% Tween. Constant concentrations of CBX7 and FITC–K27me3 were used at 8.68 μM and 500 nM, respectively. Calixarene concentrations were varied from 0–10 mM, and samples were made up to a final volume of 100 μL. Plates were incubated for 15 min in darkness prior to reading with a SpectraMax M5 plate reader

(Molecular Devices) with λ_{exc} 450 nm, λ_{obs} 530 nm, and an instrument cutoff of 515 nm. The parallel and perpendicular intensities of emission were adjusted for background of the buffer, giving millipolarization (mP) values for each reading that were determined and normalized to percentage of complex formed. Values were graphed using XLfit (IDBS) and fitted using a sigmoidal curve function from which IC_{50} values were determined. Experiments were performed in triplicate for two independent trials and the IC_{50} values reported are the averages of all values.

3. Results and discussion

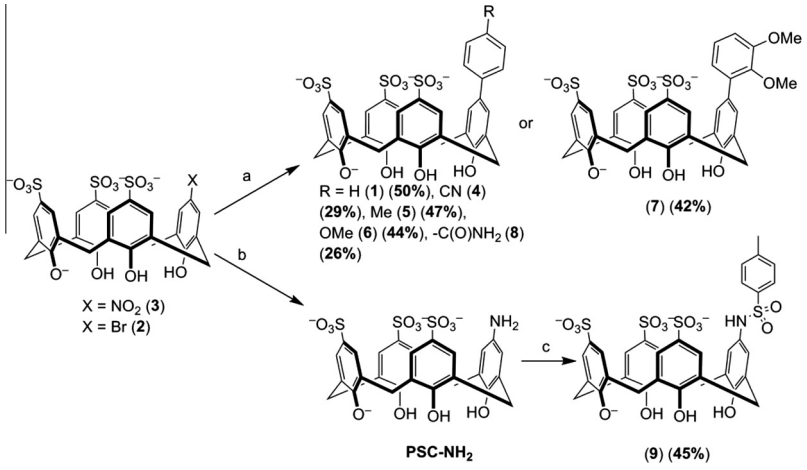
3.1. Synthesis of compounds

Our earlier trimethyllysine-targeting compounds[31] were based on a synthetic pathway that created calixarene derivatives functionalized at the 'lower rim.' We found these compounds difficult to make in a manner that allows the rapid buildup of diversity. They are also inherently poor at tuning affinities for trimethyllysine-containing peptides and proteins, simply because the lower rim is distal to the actual binding surface of the macrocycle. We recently developed a new and facile synthetic route to functionalization at the 'upper rim' and showed using simple amino acids that groups introduced at this position are in direct contact with the bound Kme3 residue, and therefore have a strong influence on binding affinities and selectivities.[26] These syntheses rely on intermediates **2** (aryl bromide) and PSC-NH_2 (aniline), that have been desymmetrized using selective, high-yielding reactions, and that are well suited to further elaboration by Pd-mediated cross coupling or formation of sulfonamides. The syntheses of compounds studied as H3K27me3-binders in this report are shown in Scheme 1. Biaryl-substituted compounds, such as **4**, were prepared by Suzuki coupling in water using 'ligand-free' reaction conditions that also included $Bu_4N^+ Br^-$ as a phase-transfer agent. The sulfonamide derivative **9** was prepared from PSC-NH_2 using optimized, buffered reaction conditions as reported previously.[26] All of the compounds prepared and tested here are characterized by having a desymmetrized upper rim bearing three sulfonates and a single, appended aromatic substituent that can modify interactions with the H3K27me3 target.

3.2. Dye displacement assay for histone tail binding

A dye-displacement assay was used to measure the binding of each compound with unmethylated and trimethylated peptides representing H3K27me3 (Ac-AARKme3SAPY-C(O)NH_2) and unmethylated H3K27 (Ac-AARKSAPY-C(O)NH_2). Previous studies have shown that the dye lucigenin is a promiscuous binder of many different sulfonated calixarenes,[39,40] and we found it to be generally well suited to use in a dye-displacement format with each of these compounds. We sought to create a rapid, quantitative assay for H3K27me3 (and H3K27) binding according to the schematic in Fig. 2. The first step is a direct titration of calixarene into lucigenin; upon binding, fluorescence decreases and the dissociation constant of the dye for host (K_{Ind}) is determined by curve fitting of emission intensity as a function of concentration (Fig. 2B and Supplementary Fig. in SI). The second step is a titration in which a starting solution of calixarene and dye is titrated with a histone peptide of interest. The addition of the peptide causes dye displacement, and the fluorescence increase upon release of dye is fitted to determine the calixarene–peptide dissociation constant K_d (Fig. 2C and Figures in SI). Each of these titration steps was carried out rapidly for multiple compounds at a time using 96-well microplates.

The affinities of each compound for H3K27 and H3K27me3 are reported in Table 1. Data were reproducible from replicate to replicate, and satisfactory fits for all curves were obtained (see SI), except for those of compound **9**, which is a very weak binding compound (see below), and compound **3**, which itself contains a *p*-nitrophenol (PNP) chromophore in its structure. The K_d values for the target histone tail H3K27me3 range from 0.34 to 10 μM. For comparison, the reported in vitro binding affinities of naturally evolved Kme3-reader proteins for trimethyllysine histone elements are in the range of 0.7–110 μM.[24,25,41] All compounds showed a preference for the methylated peptide H3K27me3 over unmethylated H3K27, with the magnitude of selectivity ranging from 3- to 26fold. Selectivity data for natural proteins is hard to obtain from the literature because affinities of reader proteins for unmethylated peptides are often not reported, or are too weak to measure under the conditions of a given biological assay. Some reported trimethylated/unmethylated selectivities exist, and seem to point to a general limit of >100fold for trimethyllysine/lysine selectivities.[25,42] One exception is the PHD domain of reader protein CHD4, which binds trimethylated and unmethylated H3K9 partners with nearly equal affinities.[43] In any case, we can say with some confidence that most of the naturally evolved proteins have better selectivities for methylated over unmethylated partners than do these synthetic compounds. The slight erosion of selectivity of the aryl-substituted compounds relative to PSC seems to arise more generally from an increased affinity for unmethylated H3K27 (relative to PSC) than from any general change in affinities



Scheme 1. Synthesis of sulfonato-calix[4]arenes **1**–**9**. Reagents and conditions: (a) Ar-$B(OH)_2$, Na_2CO_3, TBAB, $Pd(OAc)_2$, μw, 2 h, 150 °C b) NaOH then RaNi, MeOH/H_2O (1:1), H_2, overnight (c) TsCl, H_2O, 100 mM Na_2HPO_4, pH 8, overnight.

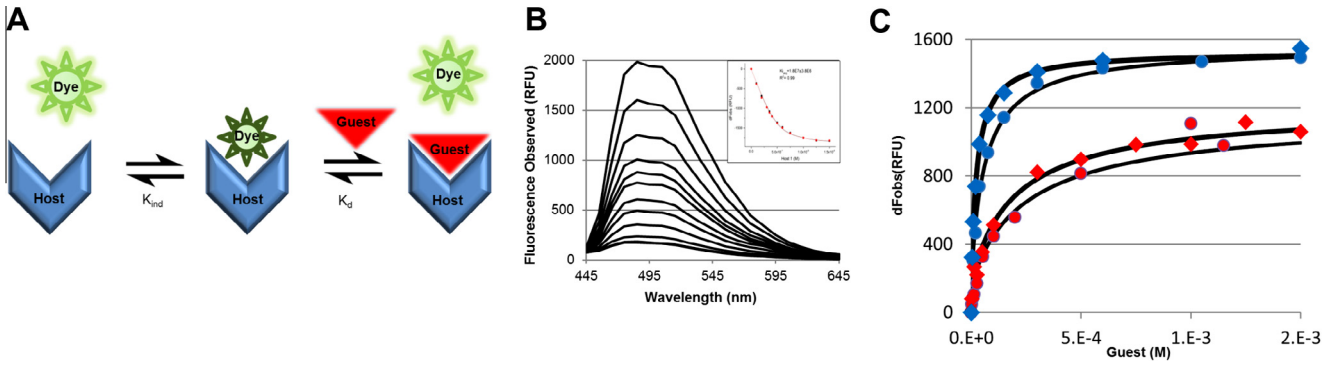


Figure 2. Indicator displacement assay for calixarene-peptide binding. (A) Schematic of the indicator displacement assay showing first the quenching of the dye upon addition of the host (governed by the dissociation constant of the host to dye (K_{ind})) followed by the competitive addition of an unmethylated or trimethylated peptide (guest) causing the release of the dye (governed by the host-guest dissociation constant (K_d)). (B) Direct fluorescence titration of LCG (0.5 μM) with host **1** in 0.01 M phosphate buffer at pH 7.4, λ_{ex} = 369 nm. The inset shows the fitted titration curve for the calculation of the dissociation constant K_{ind} at 485 nm. (C) Exemplary dye-displacement assay curves done using 0.01 M phosphate buffer at pH 7.4, λ_{ex} = 369 nm, λ_{em} = 485 nm. 500 nM lucigenin dye, 1.25 μM of host 6 ♦, host 8 ● and varying concentrations of unmethylated (red) or trimethylated peptide (blue) showing the different selectivities of each host to a particular peptide. For clarification a semi-log plot of these data can be found in Supplementary information.

Table 1
Activities of trimethyllysine-targeting compounds as determined by dye displacement assay and FP protein–protein disruption assay[a]

Calix[4]arene	Substituents	K_{Ind}[b] for LCG (μM)	K_d[c] for H3K27 (μM)	K_d[c] for H3K27me3 (μM)	H3K27me3/H3K27 selectivity	IC_{50} for H3K27me3-CBX7 disruption (μM)
PSC[d]	X= SO_3^-	n.d.	220 ± 7[d]	5.4 ± 0.1[d]	40	2500 ± 900
Host 1	X= Ph	0.055 ± 0.011	19.0 ± 4.4	0.75 ± 0.18	25	510 ± 50
Host 2	X= Br	0.085 ± 0.025	13.3 ± 7.6	0.70 ± 0.17	19	470 ± 60
Host 3	X= NO_2	0.460 ± 0.044	30 ± 60	10 ± 13	3	>5000
Host 4	X= Ph(CN)	0.050 ± 0.020	11.3 ± 2.9	0.88 ± 0.12	13	370 ± 40
Host 5	X= Ph(Me)	0.0132 ± 0.013	2.7 ± 1.0	0.34 ± 0.03	8	230 ± 20
Host 6	X= Ph(OMe)	0.120 ± 0.009	20 ± 9.3	0.78 ± 0.22	26	360 ± 30
Host 7	X= Ph(2,3-OMe)	0.103 ± 0.017	25 ± 14	2.13 ± 0.53	12	1700 ± 700
Host 8	X= Ph($CONH_2$)	0.124 ± 0.019	23 ± 16	1.86 ± 0.64	12	590 ± 40
Host 9	X= $NHSO_2Ph(Me)$	1.91 ± 0.29	>100	>100	n.d.	>2000

[a] All K_d values are averages from duplicate determinations. IC_{50} values from fluorescence polarization assay are averages of triplicate determinations.
[b] Conditions for direct titration of calixarene into LCG: [LCG] = 0.5 μM with host at varying concentrations between 0–5 μM in 0.01 M phosphate buffer at pH 7.4, λ_{ex} 369 nm, λ_{em} 485 nm done in duplicates.
[c] Conditions for the competitive titration of peptide into host/LCG solution: [LCG] = 0.5 μM, [host] = 1.25 μM with varying peptide concentrations of [H3K27] = 0–1.5 mM and [H3K27me3] = 0–0.5 mM in 0.01 M sodium phosphate buffer at pH 7.4, λ_{ex} 369 nm, λ_{em} 485 nm.
[d] Data for PSC taken from reference. [31] Literature values were determined by isothermal titration calorimetry (K_d values) or FRET assay for H3K27me3-CBX7 disruption (IC_{50} value).

for the targeted H3K27me3. The compounds all make complexes with Kme3 in which the quaternary ammonium ion is buried deep within the macrocycle's binding pocket, and the appended aromatic rings are in close contact with the trimethyllysine side chain's methylene groups, as previously confirmed for representative members of this class by ^{1}H NMR.[26] This structural model can explain the increased affinities for H3K27 upon introduction of aryl groups, because the aryl groups make similar favourable contacts with the methylenes of both trimethyllysine and unmethylated lysine.

We also looked for the expected connections between ring electronics and strength of cation–pi interactions; that is, that more electron-rich rings (e.g. methoxy-substituted **6**) would form stronger cation–pi interactions with the lysine/trimethyllysine side chains of the histone peptides than would a more electron-poor ring (e.g. cyano-substituted **4**). These trends are not present in the data for any aryl-appended host. For example, MeO-substituted **6** and CN-substituted **4** have equal affinities for H3K27me3, and similar affinities for H3K27. But strong effects do exist for substituents on the main macrocycle—Compound **3** (nitro-) differs from compound **2** (bromo-) by only a single group, but has ~15fold lower affinity for H3K27me3 and has the lowest H3K27me3/H3K27 selectivity of any compound in this group. This suggests that a strong cation–pi interaction does exist for the aryl groups of the main macrocycle that does not exist for the aryl groups appended to the upper rim. Further, compound **7** (2,3-dimethoxy–) is significantly weaker than is compound **6** (4-methoxy–), which is in this case best explained by the conformational differences induced by the presence of an *ortho*-substituted biaryl linkage in **7** that is not present in **6**. Compound **9**—the only member that bears a sulfonamide linked aryl group—has very low affinities for either methylated or unmethylated target. One possibility is that the sulfonamide linker disrupts binding. But we also see evidence of self-inclusion in the ^{1}H NMR spectrum of **9** (a strongly upfield-shifted CH_3 resonance that indicates inclusion of the methyl group in the binding pocket of another copy of the calixarene) that offers an alternative explanation for the poor activity of **9**.

3.3. Fluorescence polarization assay for H3K27me3–CBX7 disruption

Fluorescence polarization (FP) assays have been used extensively to measure the affinities of histone peptides for their reader proteins.[24,25,42] They are also amenable for testing for protein interaction disruptors.[44] In our implementation, we used a direct titration of recombinant CBX7 chromodomain into FITC-labeled H3K27me3 to establish selective protein–peptide binding, and to determine the concentration of protein at which 90% of full FP

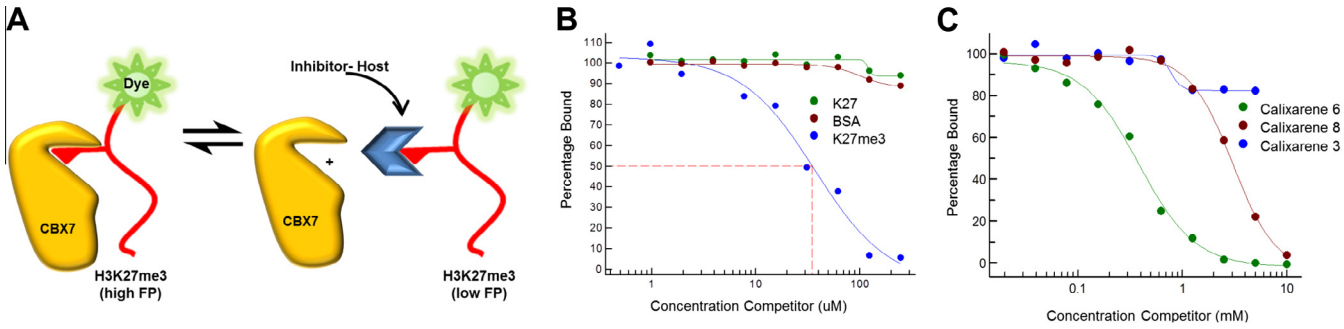


Figure 3. (A) Schematic of fluorescence polarization assay showing a dye-labeled H3K27me3 peptide and a CBX7 protein with high fluorescence polarization (see Supplementary information). Addition of a synthetic host that binds the peptide disrupts the complex and causes a return to low fluorescence polarization. (B) Determination of specificity for FP competition assay. FITC-H3K27me3 Peptide and CBX7 constant at concentrations of 500 nM and 8.68 μM were exposed to nonfluorescently labeled H3K27me3 (blue) and H3K27 (green) and BSA (red) at concentrations ranging from 0–250 mM in buffer (20 mM Tris–HCl pH 8.0, 250 mM NaCl, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 0.01% Tween). (C) FP competition assay with calixarenes. Competition assays were performed with FITC-H3K27me3 peptide and CBX7 constant at concentrations of 500 nM and 8.68 μM. Calixarenes were re-suspended in 4 equiv NaOH and were tested from 5 mM (calixarene **3**-blue) or 10 mM (calixarenes **6** green and **8** red) to 0 mM in buffer (20 mM Tris–HCl pH 8.0, 250 mM NaCl, 1 mM DTT, 1 mM benzamidine, 1 mM PMSF, 0.01% Tween).

response was achieved. Titration of inhibitor compounds into a pre-formed complex of CBX7 and FITC-H3K27me3 caused a decrease in FP that demonstrates disruption of the reader protein–H3K27me3 complex. It is unconventional that this particular assay involves disruption of the protein–peptide complex with an agent that binds to the dye-labelled peptide, as opposed to an agent that binds to the unlabelled protein. But the FP signal of the peptide–calixarene complex is significantly lower than that arising from the peptide–protein complex, due to the fact that the calixarenes (~1 kDa) are lower molecular weight than the CBX7 protein domain (7.8 kDa), so the assay functions well. Control titrations with unlabeled H3K27me3 (positive control), as well as with unmethylated H3K27 and BSA (negative controls), demonstrate the specificity of the observed disruption. Fig. 3B and C show exemplary data for the disruption of the interaction between CBX7 and the peptide upon back-titration with different control compounds (B) and with calixarenes of differing potency (C). Again, all compounds were conveniently tested in 96-well plate format with good run-to-run reproducibility. In competition assays such as these, the IC_{50} values depend strongly on the concentrations of fluorescently labelled peptide and/or the protein competing with the inhibitor for the peptide's binding site, and their magnitudes only approximate K_d values. Satisfyingly, the IC_{50} values determined using this disruption FP assay fall into three categories—low, medium, and high—that agree reasonably well with the rank ordering of direct H3K27me3 binding determined by dye displacement assay.

4. Conclusion

The calixarenes that we report have a unique ability to bind to post-translational modifications, as opposed to binding within the ordered binding pockets of protein targets. This novel feature offers an effective route to the disruption of protein–protein interactions that are triggered by post-translational modifications. It also opens the door to their further development as agents for the characterization and analysis of post-translational modification pathways. The compounds presented here could, in principle, be active against many different trimethyllysine-containing targets—a feature that distinguishes them from small molecules that target concave binding pockets with some inherent selectivity. The broad selectivity of the calixarenes will in fact facilitate their use as biochemical reagents for the study of multiple pathways, and the promise of using supramolecular reagents for the construction of novel biochemical assays has already been established.[36,37,40] There is also hope for improving their specificity further to the point of being useful for studies into the chemical biology of modification pathways. The structure–function relations identified here have provided us with a basic understanding of the molecular recognition determinants for these synthetic receptors. Also, the modular synthesis that we use to produce them suggests that more analogs, designed based on those lessons, can be made and tested in order to create more potent and more site-selective analogs. The flexible synthesis also offers the chance to identify agents with selectivities for other post-translational modification states,[30] and that would potentially disrupt other classes of reader protein interactions. Given the extreme rarity of chemical agents of any kind that can disrupt the complexes of methyllysine–reader proteins,[45–49] these compounds, though unconventional in their approach, represent a jumping off point for new bioorganic and biochemical studies of post-translational methylation pathways.

Acknowledgments

This work was supported by Genome B.C., West Coast Ride to Live, Prostate Cancer Canada, and NSERC. K.D.D. thanks Ride to Live—Vancouver Island and the Prostate Cancer Foundation of BC for fellowship support. J.E.W. and F.H. are both Canada Research Chairs and Scholars of the Michael Smith Foundation for Health Research.

Supplementary data

Supplementary data (titration curves, characterization data, and supplementary isothermal titration calorimetry experiments) associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2013.09.024.

References and notes

1. Nelson, C. J.; Santos-Rosa, H.; Kouzarides, T. *Cell* **2006**, *126*, 905.
2. Dilworth, D.; Gudavicius, G.; Leung, A.; Nelson, C. J. *Biochem. Cell Biol.* **2012**, *90*, 55.
3. Musselman, C. A.; Lalonde, M. E.; Cote, J.; Kutateladze, T. G. *Nat. Struct. Mol. Biol.* **2012**, *19*, 1218.
4. Chi, P.; Allis, C. D.; Wang, G. G. *Nat. Rev. Cancer* **2010**, *10*, 457.
5. Ruthenburg, A. J.; Li, H.; Patel, D. J.; David Allis, C. *Nat. Rev. Mol. Cell. Biol.* **2007**, *8*, 983.
6. Kouzarides, T. *Cell* **2007**, *128*, 693.
7. Jenuwein, T.; Allis, C. D. *Science* **2001**, *293*, 1074.
8. Sawan, C.; Herceg, Z. In Adv. Genet.; Zdenko Herceg and Toshikazu, U., Ed.; Academic Press, 2010; Vol. 70, pp. 57.
9. Qi, W.; Chan, H.; Teng, L.; Li, L.; Chuai, S.; Zhang, R.; Zeng, J.; Li, M.; Fan, H.; Lin, Y.; Gu, J.; Ardayfio, O.; Zhang, J.-H.; Yan, X.; Fang, J.; Mi, Y.; Zhang, M.; Zhou, T.; Feng, G.; Chen, Z.; Li, G.; Yang, T.; Zhao, K.; Liu, X.; Yu, Z.; Lu, C. X.; Atadja, P.; Li, E. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 21360.
10. Verma, S. K.; Tian, X.; LaFrance, L. V.; Duquenne, C.; Suarez, D. P.; Newlander, K. A.; Romeril, S. P.; Burgess, J. L.; Grant, S. W.; Brackley, J. A.; Graves, A. P.;

Scherzer, D. A.; Shu, A.; Thompson, C.; Ott, H. M.; Aller, G. S. V.; Machutta, C. A.; Diaz, E.; Jiang, Y.; Johnson, N. W.; Knight, S. D.; Kruger, R. G.; McCabe, M. T.; Dhanak, D.; Tummino, P. J.; Creasy, C. L.; Miller, W. H. *ACS Med. Chem. Lett.* **2012**, *3*, 1091.
11. McCabe, M. T.; Ott, H. M.; Ganji, G.; Korenchuk, S.; Thompson, C.; Van Aller, G. S.; Liu, Y.; Graves, A. P.; Iii, A. D. P.; Diaz, E.; LaFrance, L. V.; Mellinger, M.; Duquenne, C.; Tian, X.; Kruger, R. G.; McHugh, C. F.; Brandt, M.; Miller, W. H.; Dhanak, D.; Verma, S. K.; Tummino, P. J.; Creasy, C. L. *Nature* **2012**, *492*, 108.
12. Knutson, S. K.; Wigle, T. J.; Warholic, N. M.; Sneeringer, C. J.; Allain, C. J.; Klaus, C. R.; Sacks, J. D.; Raimondi, A.; Majer, C. R.; Song, J.; Scott, M. P.; Jin, L.; Smith, J. J.; Olhava, E. J.; Chesworth, R.; Moyer, M. P.; Richon, V. M.; Copeland, R. A.; Keilhack, H.; Pollock, R. M.; Kuntz, K. W. *Nat. Chem. Biol.* **2012**, *8*, 890.
13. Gieni, R. S.; Hendzel, M. J. *Biochem. Cell Biol.* **2009**, *87*, 711.
14. Sauvageau, M.; Sauvageau, G. *Cell Stem Cell* **2010**, *7*, 299.
15. O'Loghlen, A.; Muñoz-Cabello, A. M.; Gaspar-Maia, A.; Wu, H.; Banito, A.; Kunowska, N.; Racek, T.; Pemberton, H. N.; Beolchi, P.; Lavial, F.; Masui, O.; Vermeulen, M.; Carroll, T.; Graumann, J.; Heard, E.; Dillon, N.; Azuara, V.; Snijders, A. P.; Peters, G.; Bernstein, E.; Gil, J. *Cell Stem Cell* **2012**, *10*, 33.
16. Morey, L.; Pascual, G.; Cozzuto, L.; Roma, G.; Wutz, A.; Benitah, S. A.; Di Croce, L. *Cell Stem Cell* **2012**, *10*, 47.
17. Klauke, K.; Radulović, V.; Broekhuis, M.; Weersing, E.; Zwart, E.; Olthof, S.; Ritsema, M.; Bruggeman, S.; Wu, X.; Helin, K.; Bystrykh, L.; de Haan, G. *Nat. Cell. Biol.* **2013**, *15*, 353.
18. Aguilo, F.; Zhou, M.-M.; Walsh, M. J. *Cancer Res.* **2011**, *71*, 5365.
19. Maertens, G. N.; El Messaoudi-Aubert, S.; Racek, T.; Stock, J. K.; Nicholls, J.; Rodriguez-Niedenführ, M.; Gil, J.; Peters, G. *PLoS One* **2009**, *4*, e6380.
20. Gil, J.; Bernard, D.; Martinez, D.; Beach, D. *Nat. Cell. Biol.* **2004**, *6*, 67.
21. Bernard, D.; Martinez-Leal, J. F.; Rizzo, S.; Martinez, D.; Hudson, D.; Visakorpi, T.; Peters, G.; Carnero, A.; Beach, D.; Gil, J. *Oncogene* **2005**, *24*, 5543.
22. Yap, K. L.; Li, S.; Muñoz-Cabello, A. M.; Raguz, S.; Zeng, L.; Mujtaba, S.; Gil, J.; Walsh, M. J.; Zhou, M.-M. *Mol. Cell* **2010**, *38*, 662.
23. Bracken, A. P.; Helin, K. *Nat. Rev. Cancer* **2009**, *9*, 773.
24. Kaustov, L.; Ouyang, H.; Amaya, M.; Lemak, A.; Nady, N.; Duan, S.; Wasney, G. A.; Li, Z.; Vedadi, M.; Schapira, M.; Min, J.; Arrowsmith, C. H. *J. Biol. Chem.* **2011**, *286*, 521.
25. Hughes, R. M.; Wiggins, K. R.; Khorasanizadeh, S.; Waters, M. L. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 11184.
26. Daze, K. D.; Ma, M. C.; Pineux, F.; Hof, F. *Org. Lett.* **2012**, *14*, 1512.
27. Beshara, C. S.; Jones, C. E.; Daze, K. D.; Lilgert, B. J.; Hof, F. *ChemBioChem* **2010**, *11*, 63.
28. Gamal-Eldin, M. A.; Macartney, D. H. *Org. Biomol. Chem.* **2013**, *11*, 488.
29. Ingerman, L. A.; Cuellar, M. E.; Waters, M. L. *Chem. Commun.* **2010**, *46*, 1839.
30. James, L. I.; Beaver, J. E.; Rice, N. W.; Waters, M. L. *J. Am. Chem. Soc.* **2013**, *135*, 6450.
31. Daze, K. D.; Pinter, T.; Beshara, C. S.; Ibraheem, A.; Minaker, S. A.; Ma, M. C. F.; Courtemanche, R. J. M.; Campbell, R. E.; Hof, F. *Chem. Sci.* **2012**, *3*, 2695.
32. Daze, K. D.; Hof, F. *Acc. Chem. Res.* **2013**, *46*, 937.
33. Amblard, M.; Fehrentz, J.-A.; Martinez, J.; Subra, G. *Mol. Biotechnol.* **2006**, *33*, 239.
34. Hirose, K. *J. Incl. Phenom. Macro. Chem.* **2001**, *39*, 193.
35. Bakirci, H.; Nau, W. M. *Adv. Funct. Mat.* **2006**, *16*, 237.
36. Hennig, A.; Bakirci, H.; Nau, W. M. *Nat. Meth.* **2007**, *4*, 629.
37. Bailey, D. M.; Hennig, A.; Uzunova, V. D.; Nau, W. M. *Chem. Eur. J.* **2008**, *14*, 6069.
38. Hargrove, A. E.; Zhong, Z.; Sessler, J. L.; Anslyn, E. V. *J. New Chem.* **2010**, *34*, 348.
39. Minaker, S. A.; Daze, K. D.; Ma, M. C. F.; Hof, F. *J. Am. Chem. Soc.* **2012**, *134*, 11674.
40. Guo, D.; Uzunova, V. D.; Su, X.; Liu, Y.; Nau, W. M. *Chem. Sci.* **2011**, *2*, 1722.
41. Nielsen, P. R.; Nietlispach, D.; Mott, H. R.; Callaghan, J.; Bannister, A.; Kouzarides, T.; Murzin, A. G.; Murzina, N. V.; Laue, E. D. *Nature* **2002**, *416*, 103.
42. Bernstein, E.; Duncan, E. M.; Masui, O.; Gil, J.; Heard, E.; Allis, C. D. *Mol. Cell Biol.* **2006**, *26*, 2560.
43. Mansfield, R. E.; Musselman, C. A.; Kwan, A. H.; Oliver, S. S.; Garske, A. L.; Davrazou, F.; Denu, J. M.; Kutateladze, T. G.; Mackay, J. P. *J. Biol. Chem.* **2011**, *286*, 11779.
44. Roehrl, M. H. A.; Wang, J. Y.; Wagner, G. *Biochem.* **2004**, *43*, 16056.
45. Gao, C.; Herold, J. M.; Kireev, D.; Wigle, T.; Norris, J. L.; Frye, S. *J. Am. Chem. Soc* **2011**, *133*, 5357.
46. Herold, J. M.; Wigle, T. J.; Norris, J. L.; Lam, R.; Korboukh, V. K.; Gao, C.; Ingerman, L. A.; Kireev, D. B.; Senisterra, G.; Vedadi, M.; Tripathy, A.; Brown, P. J.; Arrowsmith, C. H.; Jin, J.; Janzen, W. P.; Frye, S. V. *J. Med. Chem.* **2011**, *54*, 2504.
47. James, L. I.; Barsyte-Lovejoy, D.; Zhong, N.; Krichevsky, L.; Korboukh, V. K.; Herold, J. M.; MacNevin, C. J.; Norris, J. L.; Sagum, C. A.; Tempel, W.; Marcon, E.; Guo, H.; Gao, C.; Huang, X. P.; Duan, S.; Emili, A.; Greenblatt, J. F.; Kireev, D. B.; Jin, J.; Janzen, W. P.; Brown, P. J.; Bedford, M. T.; Arrowsmith, C. H.; Frye, S. V. *Nat. Chem. Biol.* **2013**, *9*, 184.
48. Kireev, D.; Wigle, T. J.; Norris-Drouin, J.; Herold, J. M.; Janzen, W. P.; Frye, S. V. *J. Med. Chem.* **2010**, *53*, 7625.
49. Wagner, E. K.; Nath, N.; Flemming, R.; Feltenberger, J. B.; Denu, J. M. *Biochemistry* **2012**, *51*, 8293.