SUPPORTING INFORMATION

Structure-Guided Discovery of Selective Antagonists for the Chromodomain of Polycomb Repressive Protein CBX7

Chunyan Ren[1,5], Steven G. Smith[1,5], Kyoko Yap[1], SiDe Li[1,2], Jiaojie Li[1,3], Mihaly Mezei[1], Yoel Rodriguez[1,4], Adam Vincek[1], Francesca Aguilo[1,2], Martin J. Walsh[1,2], and Ming-Ming Zhou[1,6]

[1] Department of Structural and Chemical Biology, [2] Department of Pediatrics, Icahn School of Medicine at Mount Sinai, New York, NY 10029, USA.
[3] Division of liberal arts and sciences, Gist college, Gwangju Institute of Science & Technology, Buk-gu, Gwangju 61005, Republic of KOREA
[4] Department of Natural Sciences, Hostos Community College of City University of New York, Bronx, NY 10451, USA

[5] These authors contributed equally to this work.
[6] Correspondence should be addressed to M.-M.Z. (e-mail: ming-ming.zhou@mssm.edu)

Materials and Methods

In Silico Chemical Screening

Computational screening of chemical compounds for the CBX7ChD was conducted using the NMR structure of the CBX7ChD/H3K27me2 complex (2KVM)[1]. For the virtual screening, three of these structures were selected: the one whose root-mean-square derivations (RMSDs) with the rest are the smallest (i.e. the structure in the middle of the ensemble) and a pair of structures whose RMSDs are the largest among all pairs (i.e., the two extremes). The screening was performed with a collection of approximately 100,000 chemical compounds acquired from Chembridge, Inc. Two programs were used for virtual screening: Autodock-4, combined with AutoDockTools to set up the target structure file[2] and eHiTS[3]. Autodock-4 employs a Lamarckian genetic algorithm to dock flexible ligands while eHiTS breaks each ligand into small fragments and dock those and finds the best reconstruction of the ligand from the docked fragment poses. Significantly, eHiTS varies the tautomeric state of the ligand and the protonation state of the target. The screening and the analysis of the results were driven by the script set Full-screen and the program Dockres[4]. The twenty top scoring ligands were tested for their binding to the CBX7ChD experimentally by using 2D ^{1}H-^{15}N HSQC NMR spectroscopy. Chemical structures were standardized using the OpenEye OEChem toolkit[5].

Protein Expression and Purification

CBXChD constructs were cloned into pET28a, pET28-MHL or pET28a-LIC expression vectors. For protein expression, RIPL-BL21 (DE3)-CodonPlus competent cells transformed with the CBX7ChD plasmid were grown in TB medium or M9 medium containing ^{15}N-NH_4Cl (for ^{15}N-labeled proteins) at 37°C and switched to 15°C until OD reached approximately 1.8-2.0 (for TB media) or 0.5~1 (for M9 medium). Cells were induced by 1 mM isopropyl ß-d-1-thiogalactopyranoside (IPTG) for 16-20 hours. Cell paste was collected and lysed in PBS buffer containing 500 mM NaCl and 5% glycerol using microfluidizer. The lysate was centrifuged at 18,000g for 40 mins and the supernatant passed through DE52 beads to remove RNA and DNA. The protein was purified using nickel affinity, Superdex75 (and Source 30S for CBX7ChD

used in crystallization) sequentially. His-tag might be removed using thrombin or TEV cleavage after nickel affinity column purification. The purified protein was concentrated in 30 mM HEPES buffer of pH 7.4 containing 250 mM NaCl. Detailed information about proteins used in this study was described previously in a study by Ren C. et al[6].

Fluorescence Anisotropy Binding Assay

Fluorescence anisotropy binding assay was performed with increasing ligand concentrations in the presence of 10 nM FITC-labeled probe (SETDB1-K1170me3, H3K27me3, or ANRIL-LoopC RNA) and CBX7ChD (residues 7-66) at its K_d concentration (1.4 μM, 27.7 μM, and 120 μM corresponding to each FITC probe). The assay buffer for FITC-SETDB1 and FITC-H3K27me3 is PBS with 0.005% Igepal 630. The assay buffer FITC-ANRIL-LoopC RNA is 25 mM Tris-HCl pH7.4, 100 mM NaCl and 0.005% Igepal 630. The fluorescence measurement was conducted using a Tecan Safire II microplate reader and IC_{50} was calculated using GraphPad Prism Version 5.0b software (GraphPad Software, CA). Measurements were performed in duplicate and error bars denote standard error of the mean (SEM). Detailed information about peptides, RNA, and fluorescent probes used in the study can be found in Ren C. et al[6] and Yap KL. et al[1], as well as **Supplemental Table S3**.

Protein Crystallization, X-Ray Diffraction Data Collection and Structure Determination

Purified His-tagged CBX7ChD (residues 1-71) protein of 1.5 mM in 30 mM HEPES, 200 mM NaCl pH 7.4 buffer was mixed with 9 mM MS351 (containing 8% 45% (w/v) (2-Hydroxypropyl)-β-cyclodextrin solution, Sigma H5784). Protein-compound complex was incubated at 290K for two days before drop. The crystallization condition was 0.1 M Tris, 2.0 M ammonium sulfate pH 8.0. Complex was crystallized via sitting-drop method with equal amount of protein sample and reservoir solution at 290K for more than one year. Crystals was mounted using the original crystallization reservoir solution plus 20% glycerol. X-ray diffraction data were collected at X6A beamline at the National Synchrotron Light Source, Brookhaven National Laboratory, and processed using CCP4 suite[7]. Images were processed using HKL2000[8]. Phase was solved using molecular replacement by BALBES[9]. Electron density map refinement, model building and visualization were done using Refmac[10], Phenix refinement[11], and Coot[12]. Structure figures were generated using PyMOL Molecular Graphics System V1.5.0.4 (Schrödinger, LLC).

NMR Sample Preparation and Data Collection

Protein samples of ^{15}N-labeled CBX7 or other CBX ChDs were prepared in PBS buffer of pH 7.4 containing 10% D_2O. 1.2% DMSO and 0.8% 45%V/V (2-Hydroxypropyl)-β-cyclodextrin solution (Sigma H5784) were added to help dissolve chemical compounds when needed. The same amount of DMSO and cyclodextrin was added to the free CBX7ChD sample as a control. ^{1}H-^{15}N-HSQC spectra were recorded on Bruker 500Hz NMR spectrometers at 298K. Spectra were processed using NMRPipe[13] and analyzed using NMRViewJ software[14]. Binding affinity (K_d) measurement was done using titration analysis in NMRViewJ in which well-defined chemical shift paths were plotted against ligand concentrations to yield a K_d.

Embryonic stem cell cultures and treatment with Cbx7 ligands

Mouse CCE ESCs were acquired from the American Tissue Culture Collection. All mESC cultures were established and maintained as described[15]. The formation of embryoid bodies (EBs) was performed essentially as described[15]. The ESCs or EBs were treated at the final concentrations of MS351 indicated in DMSO over a period of 18 or 36 hours and processed for total protein or RNA. Western blots were performed using monoclonal antibody 5A8A4 against *p16^{Ink4a}* of total lysates prepared of EBs treated with MS351 after 18 hours. Total RNA was extracted from CCE mESCs using standard RNA extraction methods using Trizol® (Thermo-Fisher Scientific) according the manufacturer's instructions. qPCR was conducted using primers for murine *ink4a, nog* (noggin) and *hoxd3* mRNA transcripts. TaqMan® primers (Applied

Biosystems/ Thermo-Fisher Scientific) were purchased and used for measuring transcript levels using the single tube format.

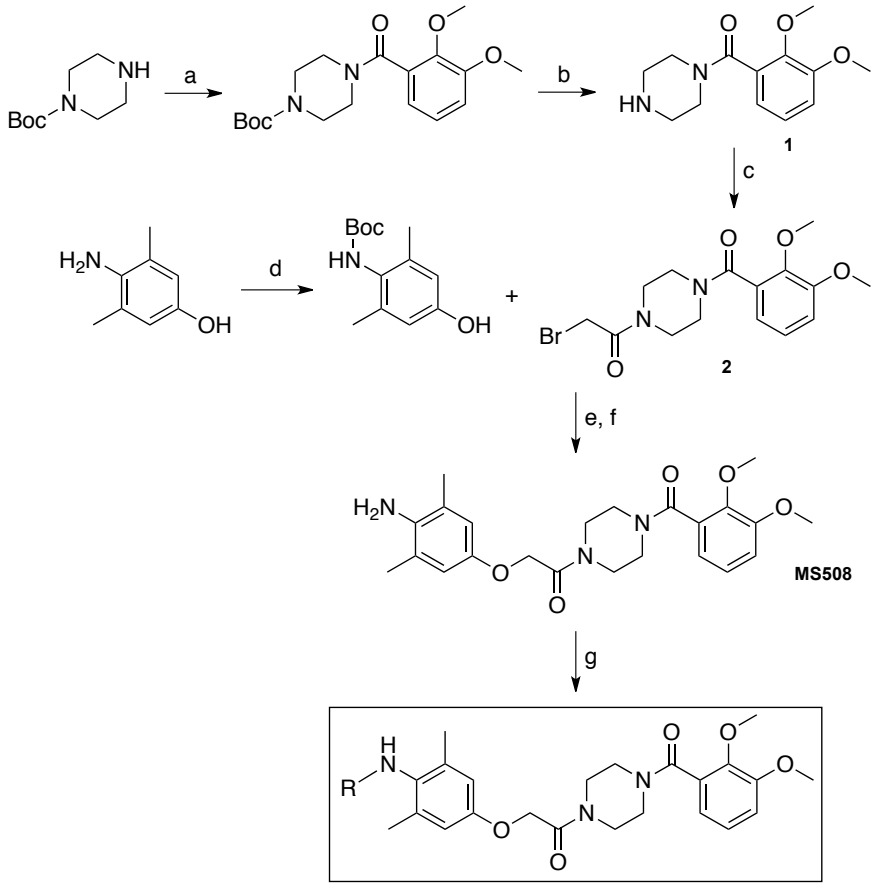
Chromatin immunoprecipitation (ChIP)
Chromatin was prepared from mouse CCE ESCs using the protocol described previously[15]. The ChIP for Cbx7 enrichment was performed with antisera directed against Cbx7 as previously described[1]. Primers used for detecting the mouse *hoxd3* promoter to produce a 319 bp fragment are indicated below: forward (5'-ggacctaaagtgagggatgg-3'); reverse (5'-tccagaaggaaa-gcaggcac-3'). DNA PCR products were visualized on 1% agarose gel perfused with 0.001% ethidium bromide and acquired digital image is shown. Control for input chromatin is shown for histone H3 enrichment for the same *hoxd3* promoter sequences.

Reverse Transcription and Real-Time Quantitative PCR (qPCR)
PC-3 prostate cancer cells were treated with compounds for 12 hrs. Total RNA was prepared using TRIzol (Life Technologies) following standard protocol. Reverse transcription was done using SuperScript III reverse transcriptase (Life Technologies). All qPCR assays were performed using SYBR® Premix Ex Taq™ II (Tli RNase H Plus, Clontech). mRNA transcription was normalized to HPRT house keeping gene and presented as 'fold change' between compound and DMSO treated samples. Results were plotted from at least three independent experiments and error bars denoted standard error of the mean (SEM).

Chemicals and General Procedures
Commercially available reagents and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI), Fluka Chemical Corp. (Milwaukee, WI), TCI America (Portland, OR), Ark Pharm (Libertyville, IL), and Acros Organics, USA (Morris Plains, NJ). They were used without any further purification. Reactions were monitored using LC/MS. LC/MS analysis was conducted on an Agilent 1200 Series HPLC with a ZORBAX 300SB-C18 column (2.1 x 150mm, 5 µm), attached to a TOF mass detector possessing an electrospray ionization source (ESI). The LC/MS utilized a gradient method with two solvents – 90% H_2O/10% Acetonitrile/0.1% Formic Acid (Solvent A) and 90% Acetonitrile/10% H_2O/0.1% Formic Acid (Solvent B), which was the eluent. A flow rate of 0.4 mL/min was used with a column temperature of 50^oC and UV detection at 210, 254, and 280 nm. The gradient was run over the course of 8 minutes, with Solvent A running from 99% to 1% and Solvent B from 1% to 99% between minutes 1-4. Purification of intermediates was performed using an Isolera Four purification system (Biotage) with prepacked SNAP silica columns. Purification of final products was performed using an Agilent 1200 Series HPLC with an Eclipse XDB-C18 column (9.4 x 250 mm, 5 µm), with the Solvent A/Solvent B system described above. Gradients for each product varied, as adjustments were necessary to properly separate products. Fractions were concentrated using a GeneVac EZ-2 and dried using a Labconco FreeZone 4.5 Plus lyophilizer.

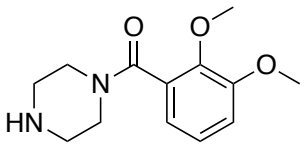
Chemical Synthetic Procedures for MS452-series Compounds
The synthesis of compounds in the MS452 series was achieved using the following steps (see **Scheme 1**). First, boc-protected piperazine was reacted with 2,3-dimethoxybenzoic acid with NBS in DCM containing pyridine at 0^oC (a), followed by a deprotection step with trifluoroacetic acid in DCM (b) to yield intermediate **1**[16]. Bromoacetyl chloride was then reacted with this intermediate in DCM containing triethylamine to yield intermediate **2** (c)[17]. The amine-containing ring was boc-protected (d)[18] and attached to intermediate **2** in a heated solution of acetone with potassium carbonate as a base (e)[19]. Following this conjugation, a deprotection reaction was run (f), yielding a compound that in three cases (MS501, MS508, MS509) was the final compound, but in all other cases, required additional reaction(s) (g)[20] to reach the final product. Pictured below is the synthesis of compounds whose parent compound is MS508, synthesis of which utilizes the starting material 4-amino-3,5-xylenol.

All intermediates were purified using Flash chromatography (Biotage) and all final products were purified using HPLC (Agilent). Details on these purification methods can be found in the "Chemicals and General Procedures" section.



Scheme 1

2,3-dimethoxyphenyl(piperazin-1-yl)methanone (1)



A mixture of 2,3-dimethoxybenzoic acid (2.184 g, 12 mmol) and triphenylphosphine (6.324 g, 24 mmol) in dichloromethane (75 mL) was cooled to 0°C. N-bromosuccinimide (4.92 g, 30 mmol) was added and the reaction mixture was stirred for 15 min. A mixture of 1-boc-piperazine (2.232 g, 12 mmol) and pyridine (2.373 g, 30 mmol) was added to the above reaction mixture at 0°C and stirring was continued for 2.5 hrs. The solvent was then evaporated from the bulk sample, washed with water and sodium bicarbonate, and extracted with ethyl acetate. The extractions were pooled and evaporated. The dried product was then re-dissolved in DCM containing 25% trifluoroacetic acid, and allowed to stir at room temperature for 2.5 hours. The reaction mixture was concentrated and purified by column chromatography – the column was first subjected to 20% ethyl acetate/80% hexanes to remove the triphenylphosphine oxide side product. After this had eluted, the same column was run in a dichloromethane/methanol solvent system, with methanol percentage slowly increasing from 0% to 10%. This purification process yielded 3.885g (>99%) of the title compound, a crunchy brown solid. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 2.57-2.63 (m, 2H), 2.72-2.74 (m, 2H), 3.04-3.06 (m, 2H), 3.50-3.59 (m, 2H), 3.70 (s, 3H), 3.81 (s, 3H), 6.72-6.73 (m, 1H), 7.06-7.11 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ = 42.4, 45.7, 46.0, 48.0, 56.2,

61.2, 113.7, 119.0, 125.0, 131.7, 144.8, 152.7, 166.6. (MS-ESI): *m/z* calculated for $C_{13}H_{18}N_2O_3$ $[M+H]^+$: 251.13, found 251.17.

2-bromo-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (2)

To a solution of 2,3-dimethoxyphenyl(piperazin-1-yl)methanone (3.500 g, 14 mmol, [14 mL of DCM]) in 90 mL of DCM at 0 °C, Et_3N (1.888 g, 2.604 mL, 18.666 mol) was added followed by slow addition of bromoacetyl chloride (2.203 g, 1.166 mL, 14 mmol). The reaction was allowed to stir under N_2 atmosphere for 30 min. Then it was partitioned between DCM and water, extracted twice with DCM, washed with brine, and dried over Na_2SO_4. A column was then run, which started at 50% ethyl acetate in hexanes. When nothing eluted, the ethyl acetate percentage was slowly ramped up to 60%, then 70-80%, at which point the desired product eluted. The fractions were pooled and the solvent was removed, yielding a sticky, foamy white solid. This purification process yielded 2.752 g (53%) of the title compound. ^{1}H NMR (600 MHz, DMSO-*d*$_6$) (Two rotameric forms) δ = 3.12-3.19 (m, 2H), 3.41-3.43 (m, 2H), 3.52-3.56 (m, 2H), 3.60-3.62 (m, 1H), 3.68-3.71 (m, 1H), 3.71 (s, 3H), 3.82 (s, 3H), 4.13-4.19 (m, 2H), 6.78-6.81 (m, 1H), 7.09-7.14 (m, 2H). ^{13}C NMR (150 MHz, DMSO-*d*$_6$) (Two rotameric forms) δ = 28.4, 41.2, 41.6, 41.9, 42.3, 46.2, 46.5, 46.6, 46.9, 56.2 (x2), 61.3 (x2), 114.0 (x2), 119.1, 125.1 (x2), 131.1, 131.2, 144.9 (x2), 152.8, 165.4, 165.5, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{15}H_{19}BrN_2O_4$ $[M+H]^+$: 371.05, found 371.06.

2-(4-amino-3,5-dimethylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (MS508)

(Example procedure of the addition of an R-group containing phenol to intermediate 2)

(1) Boc Protection – A solution of di-tert-butyl dicarbonate (4.321 g, 19.8 mmol) in anhydrous THF (60 mL) was added dropwise to 4-amino-3,5-xylenol (2.469 g, 18 mmol) in anhydrous THF (15 mL) at 0 °C. The mixture was stirred at room temperature for 24 hours. After removing THF *in vacuo*, the gold condensed mixture obtained was dissolved in ethyl acetate. The organic layer was washed three times with water and dried over $MgSO_4$, and the solvent was removed *in vacuo* to afford 3.851 g (~90%) of a white powder.

(2) Addition to Previous Intermediate (Intermediate 2) – A suspension of tert-butyl N-(4-hydroxy-2,6-dimethylphenyl)carbamate (1.053 g, 4.44 mmol) in 100 mL of acetone containing anhydrous potassium carbonate (1.842 g, 13.33 mmol) was stirred for 30 min at room temperature, followed by addition of 2-bromo-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (dissolved in acetone) (1.65 g, 4.44 mmol). The reaction mixture was then heated to 55 °C and stirred for 24 hrs.

(3) Boc Deprotection – The above product was dissolved in 70 mL of DCM and 18 mL of TFA was added. The reaction was stirred at room temperature for 2 hours. For the product to be used as an intermediate, a flash column was then run in a dichloromethane/methanol solvent system, with methanol percentage slowly increasing from 0% to 8%, yielding 1.719 g (90.6%) of a pale brown powder. (For the preparation of the final product, MS508, an HPLC column was run – see below for ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for MS508.)

"Step G" Reactions

Alkylation of final intermediate using acetic anhydride – Example: MS502

Other anhydrides used aside from acetic: isobutyric, propionic, isovaleric.

60 mg of 2-(4-amino-3-methylphenoxy)-1-[4-(2,3-dimethoxybenzyol)piperazin-1-yl]ethan-1-one (MS501) were added to a microwave reaction vessel and dissolved in 2 mL of DMF. 0.068 mL acetic anhydride (5 eq) and 0.058 mL pyridine (5 eq) were added, and the vessel was placed in

the microwave to react for 5 minutes at 75 °C. HPLC purification yielded 48.3 mg of a pure, white powder (See below for ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for MS502).

Dimethylation of free amine on intermediate[20a] – Example: MS520
To a stirred solution of 180 mg (0.421 mmol) of 2-(4-amino-3,5-dimethylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one and 168 µL (2.105 mmol) of 37% aqueous formaldehyde in 5 mL of acetonitrile was added 42 mg (0.674 mmol) of sodium cyanoborohydride. An exothermic reaction ensued, and the reaction turned a cloudy, light brown color. The reaction mixture was stirred for 15 minutes, at which point, a few drops of acetic acid were added to bring the reaction from a pH of 7 to a pH of approximately 5-6. After approximately 45 minutes of stirring, the reaction had been driven to completion. The solvent was evaporated, and the compound was purified using HPLC, yielding 75.4 mg of a pure, white powder (See below for ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for MS520).

Addition of urea functional group to free amine on intermediate[20b] – Example: MS521
500 mg (1.17 mmol) of 2-(4-amino-3,5-dimethylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one was dissolved in 4.68 mL 1M hydrochloric acid. Potassium cyanate (455 mg, 5.61 mmol, 4.8 eq) was then slowly added to the solution. Upon addition of the first small amount of KOCN, the reaction fizzed slightly. After approximately half of the KOCN had been added, the reaction became very fizzy and brown, and the product began to stick to the stir bar. After addition of the remainder of the KOCN and the passage of ~5 minutes, the fizzing stopped. The milky white solution was allowed to stand for approximately another hour, with the stir bar lightly agitating the reaction. After confirmation of the completion of the reaction, the reaction was diluted in water. All liquid was then filtered out of the mixture, leaving a sticky solid, which was then dissolved in methanol and dried. HPLC purification yielded 89.6 mg of a pure white powder (See below for ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for MS521).

Addition of dimethylcarbamyl chloride to free amine on intermediate[20d] – Example: MS525
2-(4-amino-3,5-dimethylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (107 mg, 0.250 mmol) was dissolved in 2 mL of dichloromethane, and treated at 0 °C with 70 µL triethylamine and 55.2 µL of dimethylcarbamyl chloride. The reaction was heated to 55 °C. Additional equivalents of dimethylcarbamyl chloride were added at the one, two, and three hour marks to drive the reaction to completion. The solvent was then evaporated. 16.5 mg of a pure, white powder were obtained as the final product after HPLC purification (See below for ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for MS525).

Addition of sulfonyl chlorides to free amine on intermediate – Example: MS526
2-(4-amino-3,5-dimethylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (150 mg, 0.350 mmol) was dissolved in 15 mL of dry dichloromethane, and treated at -78 °C with 172 µL triethylamine and 81 µL of methanesulfonyl chloride (both had been dissolved in 1 mL of DCM). The reaction was allowed to stir under N_2 atmosphere. After 1 hour, the desired reaction had completed, with no side product present. The reaction mixture was partitioned between DCM and water, extracted twice with DCM, washed with brine, and dried over Na_2SO_4. 55.3 mg of a pure, white powder were obtained as the final product after HPLC purification (See below for ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for MS526).

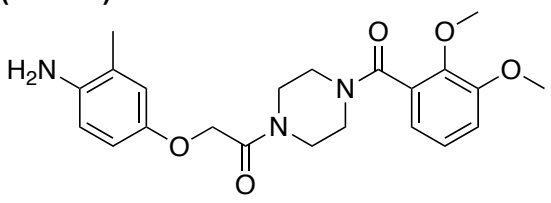
Conversion of free amine on intermediate to sulfamide[20c, 20e] – Example: MS530
The starting material, 2-(4-amino-3,5-dimethylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (180 mg, 0.420 mmol) was reacted with sulfamide (6 equiv) in refluxing 1,4-dioxane for 1.5 hours. For this specific reaction, additional equivalents of sulfamide were required (and an extra hour of heating at reflux) to drive the reaction to completion. 103.1 of a

pure, white powder were obtained as the final product after HPLC purification (See below for ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for MS530).

Final Compound Analytical Data

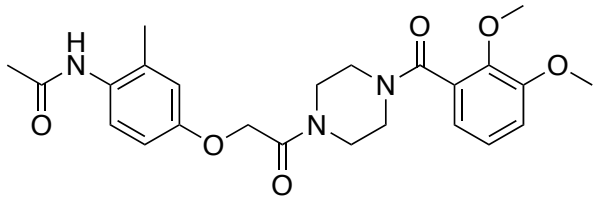
Below are the ^{1}H NMR, ^{13}C NMR, LC/MS, and purity data for each synthesized compound in the MS452 series.

2-(4-amino-3-methylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (MS501)



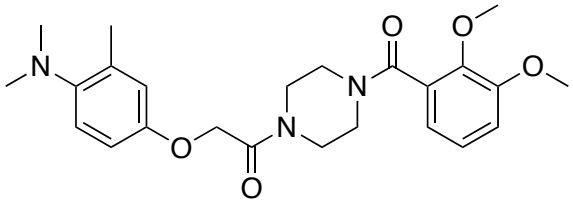
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.24 (s, 1.5H), 2.25 (s, 1.5H), 3.13-3.19 (m, 2H), 3.40 (s, 2H), 3.52-3.53 (m, 2H), 3.62-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.80-4.87 (m, 2H), 6.77-6.82 (m, 2H), 6.86-6.90 (m, 1H), 7.11-7.13 (m, 2H), 7.16-7.19 (m, 1H), 9.46 (bs, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 17.5, 41.4, 41.5, 41.7, 41.9, 44.4, 44.8, 46.7, 47.0, 56.2 (x2), 61.3 (x2), 66.3, 66.4, 113.1, 113.2, 114.0, 117.8, 119.1, 123.7, 125.1, 125.5, 131.2 (x2), 132.5, 144.9, 152.8, 157.2, 166.4, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{22}H_{27}N_3O_5$ $[M+H]^+$: 414.20, found 414.20. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)acetamide (MS502)



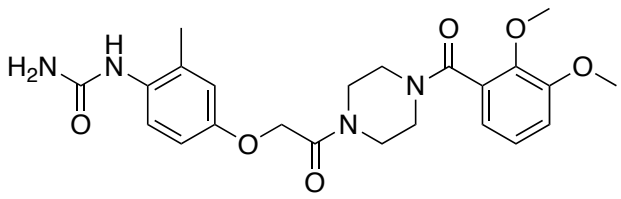
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.99 (s, 1.5H), 2.00 (s, 1.5H), 2.12 (s, 1.5H), 2.13 (s, 1.5H), 3.13-3.19 (m, 2H), 3.41 (s, 2H), 3.53-3.54 (m, 2H), 3.62-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.75-4.82 (m, 2H), 6.67-6.72 (m, 1H), 6.75 (s, 1H), 6.78-6.79 (m, 1H), 7.11 (m, 1H), 7.12-7.17 (m, 1H), 7.16 (s, 1H), 9.18-9.19 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.5, 23.5, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.0, 56.2, 61.3, 66.5, 112.2 (x2), 114.0, 116.6, 119.1, 125.1, 127.1, 130.3, 134.2, 144.9, 152.8, 155.8, 166.6, 168.6. (MS-ESI): *m/z* calculated for $C_{24}H_{29}N_3O_6$ $[M+H]^+$: 456.21, found 456.24. Purity > 99%.

1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-[4-(dimethylamino)-3-methylpenoxy]ethan-1-one (MS503)



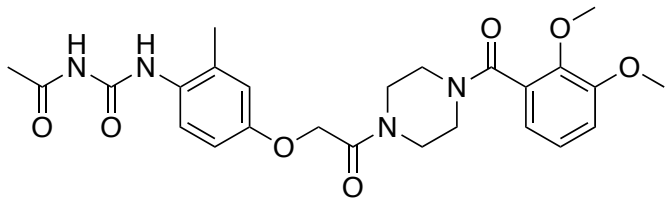
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.24 (bs, 3H), 2.55 (bs, 6H), 3.13-3.18 (m, 2H), 3.41 (s, 2H), 3.53 (s, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.71-4.78 (m, 2H), 6.70 (bs, 1H), 6.74 (bs, 1H), 6.79 (s, 1H), 6.97 (bs, 1H), 7.11 (s, 1H), 7.12-7.13 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.3, 41.4, 41.5, 41.7, 42.0, 44.6, 45.0, 46.7, 47.0, 56.2, 61.3, 66.7, 114.0, 117.7, 119.1, 125.1, 131.2 (x2), 144.9, 152.8, 163.5, 166.9. (MS-ESI): *m/z* calculated for $C_{24}H_{31}N_3O_5$ $[M+H]^+$: 442.23, found 442.24. Purity > 99%.

(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)urea (MS504)



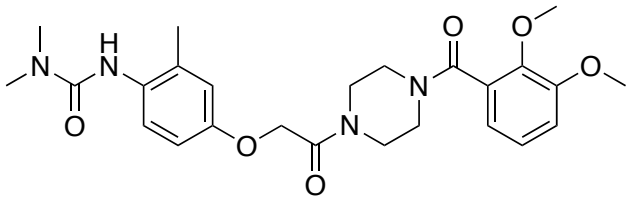
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.12 (s, 1.5 H), 2.14 (s, 1.5 H), 3.13-3.18 (m, 2H), 3.42 (s, 2H), 3.53-3.54 (m, 2H), 3.61-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.71-4.77 (m, 2H), 5.83 (s, 2H), 6.63-6.69 (m, 1H), 6.71-6.75 (m, 1H), 6.79 (s, 1H), 7.10-7.12 (m, 2H), 7.42-7.46 (t, 1H, J=10 Hz), 7.55-7.56 (m, 1 H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.5, 41.4, 41.5, 41.7, 41.9, 44.6, 44.9, 46.7, 47.1, 56.2, 61.3, 66.6, 66.7, 112.3 (x2), 114.0, 116.6, 119.1, 124.2, 125.1, 130.8, 131.2 (x2), 132.0, 144.9, 152.8, 154.0, 157.0, 166.7. (MS-ESI): *m/z* calculated for $C_{23}H_{28}N_4O_6$ $[M+H]^+$: 457.20, found 457.22. Purity > 99%.

3-acetyl-1-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)urea (MS505)



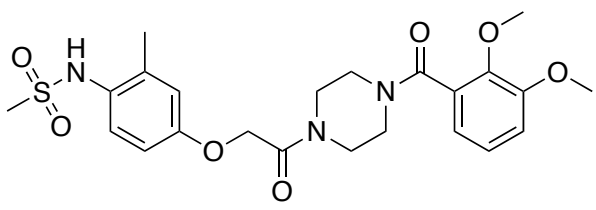
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.80-1.82 (m, 3H), 2.04 (s, 1.5H), 2.05 (s, 1.5H), 3.14-3.20 (m, 2H), 3.41-3.43 (m, 2H), 3.54-3.55 (m, 2H), 3.62-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.82-4.89 (m, 2H), 6.76-6.79 (m, 1H), 6.80-6.81 (m, 1H), 6.86-6.90 (m, 1H), 7.11 (s, 1H), 7.12 (s, 1H), 7.13 (s, 1H), 7.53 (s, 1H), 8.29-8.31 (bs, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 17.7, 26.1, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.0, 56.2 (x2), 61.3, 66.3, 66.4, 113.1, 114.0, 117.0, 119.1, 125.1, 130.6, 131.2, 132.0, 137.6, 144.9, 152.8, 152.5, 158.1, 166.4, 166.8, 166.9, 174.4. (MS-ESI): *m/z* calculated for $C_{25}H_{30}N_4O_7$ $[M+H]^+$: 499.21, found 499.23. Purity = ~96.5%.

1-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl-3,3-dimethylurea (MS506)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.09 (s, 1.5H), 2.11 (s, 1.5H), 2.89 (s, 6H), 3.13-3.19 (m, 2H), 3.42 (s, 2H), 3.54 (s, 2H), 3.61-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.74-4.80 (m, 2H), 6.64-6.70 (m, 1H), 6.73 (s, 1H), 6.78-6.79 (m, 1H), 6.99-7.03 (t, 1H, J=9.6 Hz), 7.11-7.12 (m, 2H), 7.67-7.68 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.6, 36.6, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2, 61.3, 66.5 (x2), 112.0 (x2), 114.0, 116.4, 119.1, 125.1, 128.2, 131.2 (x2), 131.9, 135.6, 144.9, 152.8, 155.5, 157.1, 166.7. (MS-ESI): *m/z* calculated for $C_{25}H_{32}N_4O_6$ $[M+H]^+$: 485.23, found 485.27. Purity = ~96%.

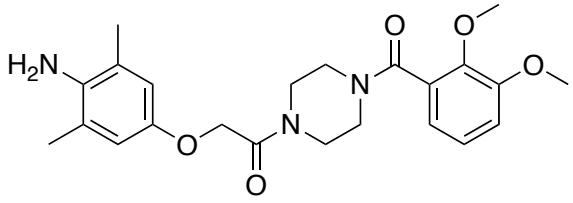
N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)methane-sulfonamide (MS507)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.25 (s, 1.5H), 2.27 (s, 1.5H), 2.90-2.91 (m, 3H), 3.14-3.19 (m, 2H), 3.41 (s, 2H), 3.53-3.54 (m, 2H), 3.62-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.78-4.84 (m, 2H), 6.71-6.76 (m, 1H), 6.79-6.80 (m, 1H), 6.84 (s, 1H), 7.11-7.12 (m, 1H), 7.13 (s, 1H), 7.15 (s, 1H), 8.86 (bs, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.9, 41.4, 41.5, 41.7, 42.0,

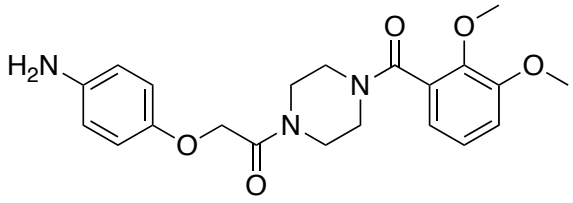
44.5, 44.9, 46.7, 47.0, 56.2, 61.3, 66.3, 66.4, 112.7, 112.8, 114.0, 119.1, 125.1, 128.9 (x2), 131.2 (x2), 137.2, 144.9, 152.8, 156.9, 166.5, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{23}H_{23}N_3O_7S$ $[M+H]^+$: 492.17, found 492.19. Purity > 99%.

2-(4-amino-3,5-dimethylphenoxy)-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (MS508)



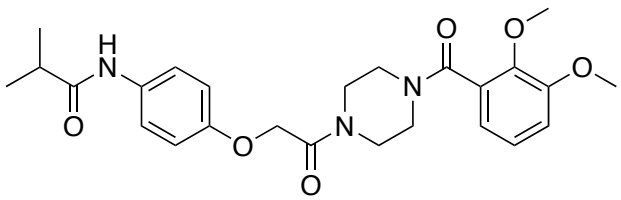
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.03 (s, 3H), 2.05 (s, 3H), 3.12-3.16 (m, 2H), 3.41 (s, 2H), 3.53 (s, 2H), 3.60-3.67 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.56-4.63 (m, 2H), 6.45 (s, 1H), 6.49 (s, 1H), 6.78-6.79 (m, 1H), 7.09-7.10 (s, 1H), 7.12-7.13 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.5, 41.4, 41.5, 41.8, 41.9, 44.7, 45.1, 46.7, 47.1, 56.2, 61.3, 67.5, 114.0, 114.9, 119.1, 122.3, 125.1, 131.2, 139.0, 144.9, 149.3, 152.7, 166.8, 166.9, 167.2. (MS-ESI): *m/z* calculated for $C_{23}H_{29}N_3O_5$ $[M+H]^+$: 428.21, found 428.21. Purity = ~99%.

2-(4-aminophenoxy)-1-[4-(2,3-dimethoxybenzyol)piperazin-1-yl]ethan-1-one (MS509)



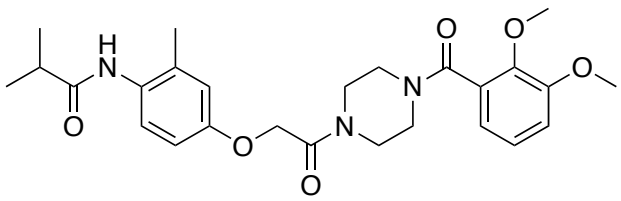
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 3.13-3.19 (m, 2H), 3.41 (s, 2H), 3.52-3.54 (m, 2H), 3.61-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 6.78-6.80 (m, 1H), 6.86-6.87 (m, 1H), 6.90-6.91 (m, 1H), 6.97-7.00 (m, 2H), 7.11 (bs, 2H), 8.49 (bs, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.0, 56.2, 61.3, 66.7 (x2), 114.0, 116.1, 119.1, 121.4, 125.1, 131.2 (x2), 144.9, 152.8, 155.2, 166.6, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{21}H_{25}N_3O_5$ $[M+H]^+$: 400.18, found 400.19. Purity = ~95%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}phenyl)-2-methylpropanamide (MS510)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.07 (m, 6H), 2.54 (s, 1H), 3.13-3.18 (m, 2H), 3.41 (s, 2H), 3.53-3.54 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.74-4.81 (m, 2H), 6.78-6.80 (m, 1H), 6.82-6.83 (m, 1H), 6.86-6.88 (m, 1H), 7.11-7.12 (m, 2H), 7.46-7.49 (m, 2H), 9.68 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 20.0, 35.2, 41.4, 41.5, 41.7, 41.9, 44.5, 44.9, 46.7, 47.0, 56.2, 61.3, 66.6, 114.0, 115.1, 119.1, 120.9, 125.1, 131.2 (x2), 133.5, 144.9, 152.8, 154.1, 166.7, 175.2. (MS-ESI): *m/z* calculated for $C_{25}H_{31}N_3O_6$ $[M+H]^+$: 470.22, found 470.25. Purity > 99%.

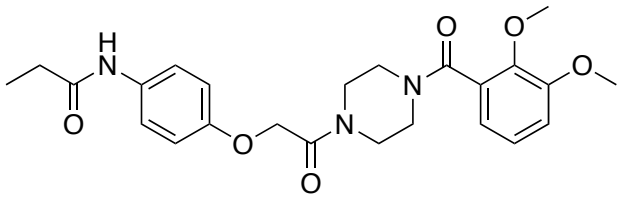
N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)-2-methylpropanamide (MS511)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.09-1.10 (m, 6H), 2.10 (s, 1.5H), 2.12 (s, 1.5H), 2.58-2.60 (m, 1H), 3.13-3.19 (m, 2H), 3.41-3.42 (m, 2H), 3.53-3.54 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.75-4.82 (m, 2H), 6.67-6.73 (m, 1H), 6.76 (s, 1H), 6.79-6.80 (m, 1H), 7.08 (s, 1H), 7.10-

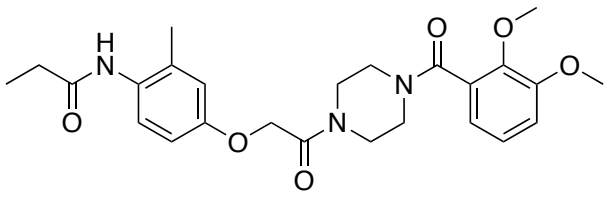
7.11 (s, 1H), 7.11 (s, 1H), 9.09-9.10 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.4, 20.1, 34.7, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2 (x2), 61.3 (x2), 66.4, 66.5, 112.2, 112.3, 114.0, 116.6, 119.1, 125.1, 127.5, 130.2, 131.2 (x2), 134.7, 144.9, 152.8, 155.9, 166.6, 175.6. (MS-ESI): *m/z* calculated for $C_{26}H_{33}N_3O_6$ $[M+H]^+$: 484.24, found 484.27. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}phenyl)propanamide (MS512)



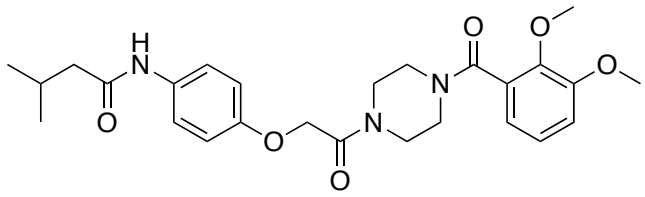
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.04-1.07 (m, 3H), 2.26-2.27 (m, 2H), 3.13-3.18 (m, 2H), 3.41 (s, 2H), 3.53-3.55 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.74-4.81 (m, 2H), 6.79 (s, 1H), 6.82-6.83 (m, 1H), 6.86-6.87 (m, 1H), 7.11-7.12 (m, 2H), 7.44-7.47 (t, 2H, J=9.6 Hz), 9.71 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 10.2, 29.8, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.0, 56.2, 61.3, 66.6 (x2), 114.0, 115.1, 119.1, 120.8, 125.1, 131.2 (x2), 133.4, 144.9, 152.8, 154.1, 166.7, 171.9. (MS-ESI): *m/z* calculated for $C_{24}H_{29}N_3O_6$ $[M+H]^+$: 456.21, found 456.24. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)propanamide (MS513)



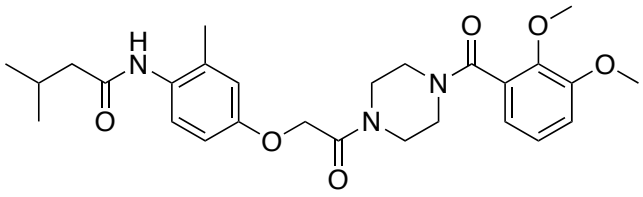
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.06-1.08 (m, 3H), 2.11 (s, 1.5H), 2.12 (s, 1.5H), 2.28-2.29 (m, 2H), 3.13-3.19 (m, 2H), 3.42 (s, 2H), 3.53-3.55 (m, 2H), 3.62-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.75-4.82 (m, 2H), 6.67-6.72 (m, 1H), 6.75 (s, 1H), 6.79-6.80 (m, 1H), 7.11 (s, 1H), 7.12-7.14 (m, 1H), 7.16 (s, 1H), 9.10-9.11 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 10.5, 18.5, 29.3, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2, 61.3 (x2), 66.4, 66.5, 112.2, 114.0, 116.6, 119.1, 125.1, 127.2, 130.3, 131.2 (x2), 134.4, 144.9, 152.8, 155.8, 166.6, 172.4. (MS-ESI): *m/z* calculated for $C_{25}H_{31}N_3O_6$ $[M+H]^+$: 470.22, found 470.23. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}phenyl)-3-methylbutanamide (MS514)



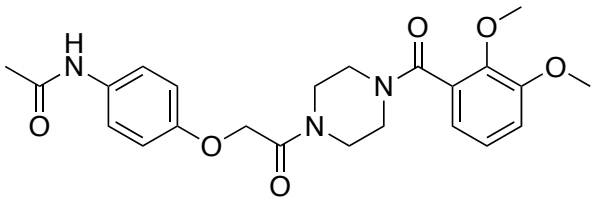
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 0.91-0.92 (m, 6H), 2.03-2.06 (m, 1H), 2.12-2.13 (m, 2H), 3.13-3.18 (m, 2H), 3.41 (s, 2H), 3.53-3.55 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.74-4.81 (m, 2H), 6.78-6.79 (m, 1H), 6.82-6.83 (m, 1H), 6.86-6.87 (m, 1H), 7.11-7.12 (m, 2H), 7.44-7.47 (m, 2H), 9.70-9.71 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 22.8, 26.1, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.0, 46.7, 47.0, 56.2, 61.3, 66.6 (x2), 114.0, 115.1, 119.1, 121.0, 131.2 (x2), 133.3, 144.9, 155.8, 154.2, 166.7, 170.6. (MS-ESI): *m/z* calculated for $C_{26}H_{33}N_3O_6$ $[M+H]^+$: 484.24, found 484.29. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)-3-methylbutanamide (MS515)



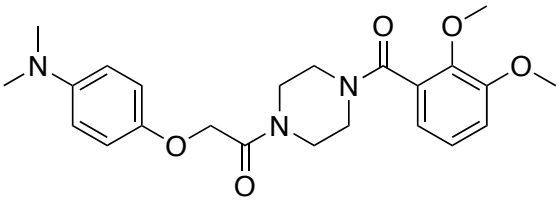
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 0.93-0.94 (m, 6H), 2.04-2.06 (m, 1H), 2.11 (m, 1.5H), 2.13 (m, 1.5H), 2.14-2.15 (m, 2H), 3.13-3.18 (m, 2H), 3.41-3.42 (m, 2H), 3.53-3.55 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.75-4.82 (m, 2H), 6.67-6.73 (m, 1H), 6.76 (s, 1H), 6.79-6.80 (m, 1H), 7.11 (s, 1H), 7.11 (s, 1H), 7.13 (s, 1H), 9.13-9.14 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.6, 22.8, 26.2, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 45.4, 46.7, 47.0, 56.2, 61.3, 66.4, 66.5, 112.2, 112.3, 114.0, 116.6, 119.1, 125.1, 127.4, 130.2, 131.2 (x2), 134.4, 144.9, 152.8, 166.6, 171.0. (MS-ESI): *m/z* calculated for $C_{27}H_{35}N_3O_6$ $[M+H]^+$: 498.25, found 498.28. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}phenyl)acetamide (MS516)



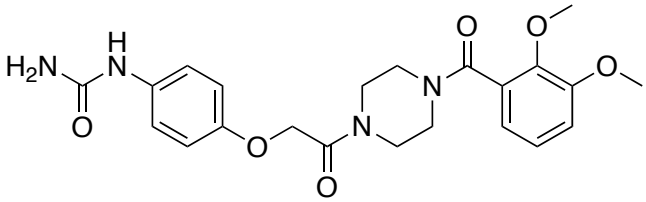
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.99 (s, 3H), 3.13-3.18 (m, 2H), 3.41 (s, 2H), 3.53-3.54 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.74-4.81 (m, 2H), 6.79 (s, 1H), 6.82-6.83 (m, 1H), 6.86-6.87 (m, 1H), 7.11 (bs, 2H), 7.42-7.46 (m, 2H), 9.79 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 24.3, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.0, 56.2, 61.3, 66.6 (x2), 114.0, 115.1, 119.1, 120.1, 125.1, 131.2 (x2), 133.4, 144.9, 152.8, 154.2, 166.7, 168.2. (MS-ESI): *m/z* calculated for $C_{23}H_{27}N_3O_6$ $[M+H]^+$: 442.19, found 442.24. Purity > 99%.

1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-[4-(dimethylamino)penoxy]ethan-1-one (MS517)



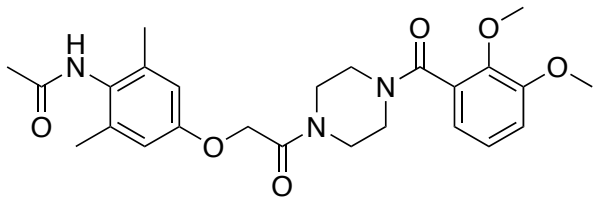
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.78-2.79 (m, 6H), 3.12-3.17 (m, 2H), 3.41 (s, 2H), 3.53 (s, 2H), 3.59-3.67 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.66-4.73 (m, 2H), 6.67-6.70 (m, 2H), 6.77 (s, 1H), 6.79 (s, 1H), 6.82-6.83 (m, 1H), 7.11-7.13 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 41.4, 41.5, 41.6, 41.7, 41.9, 44.6, 45.0, 46.7, 47.1, 56.2, 61.3, 67.2, 67.3, 114.0, 114.6, 115.9, 119.1, 125.1, 131.2 (x2), 144.9, 146.2, 150.4, 152.8, 167.0. (MS-ESI): *m/z* calculated for $C_{23}H_{29}N_3O_5$ $[M+H]^+$: 428.21, found 428.24. Purity = ~98%.

(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-phenyl)urea (MS518)



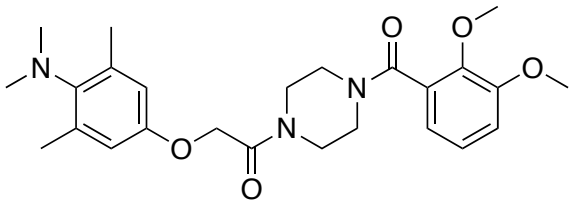
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 3.13-3.18 (m, 2H), 3.41 (s, 2H), 3.53-3.54 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.70-4.77 (m, 2H), 5.72 (s, 2H), 6.77 (s, 1H), 6.78-6.79 (m, 1H), 6.81-6.82 (m, 1H), 7.11-7.12 (m, 2H), 7.24-7.27 (m, 2H), 8.33-8.34 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 41.4, 41.5, 41.7, 42.0, 44.6, 44.9, 46.7, 47.1, 56.2, 61.3, 66.8 (x2), 114.0, 115.2, 119.1, 119.7, 125.1, 131.2 (x2), 134.6, 144.9, 152.8, 153.0, 156.6, 166.8. (MS-ESI): *m/z* calculated for $C_{22}H_{26}N_4O_6$ $[M+H]^+$: 443.19, found 443.19. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl)acetamide (MS519)



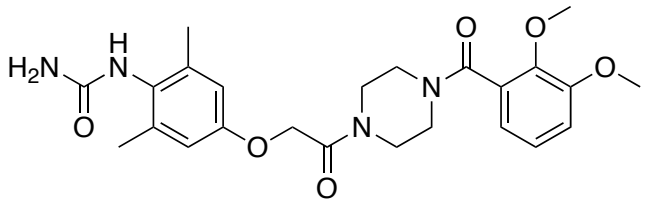
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.99-2.00 (m, 3H), 2.06 (s, 3H), 2.08 (s, 3H), 3.14-3.19 (m, 2H), 3.42 (s, 2H), 3.53-3.54 (m, 2H), 3.61-3.69 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.73-4.80 (m, 2H), 6.60 (s, 1H), 6.65 (s, 1H), 6.80 (s, 1H), 7.11-7.12 (m, 2H), 9.04-9.04 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.8, 23.0, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2, 61.3, 66.3, 66.4, 113.9 (x2), 114.0, 119.1, 125.1, 129.2, 131.2 (x2), 136.8, 144.9, 152.8, 156.3, 166.6, 168.5. (MS-ESI): *m/z* calculated for $C_{25}H_{31}N_3O_6$ $[M+H]^+$: 470.22, found 470.27. Purity > 99%.

1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-[4-(dimethylamino)-3,5-dimethylphenoxy]ethan-1-one (MS520)



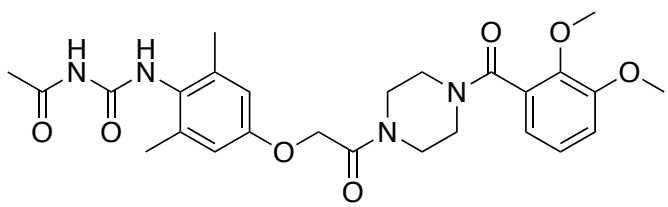
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.18 (s, 3H), 2.20 (s, 3H), 2.69-2.70 (m, 6H), 3.13-3.18 (m, 2H), 3.41-3.42 (m, 2H), 3.52-3.53 (m, 2H), 3.62-3.68 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.67-4.74 (m, 2H), 6.51 (s, 1H), 6.55 (s, 1H), 6.78-6.79 (m, 1H), 7.11-7.13 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 19.5, 41.4, 41.5, 41.7, 41.9, 43.0, 44.6, 45.0, 46.7, 47.1, 56.2, 61.3, 66.4, 66.5, 114.0, 114.8 (x2), 119.1, 125.1, 131.2 (x2), 138.0, 143.1, 144.9, 152.8, 155.0, 166.7. (MS-ESI): *m/z* calculated for $C_{25}H_{33}N_3O_5$ $[M+H]^+$: 456.24, found 456.26. Purity = 98%.

(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl)urea (MS521)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.10 (s, 3H), 2.11 (s, 3H), 3.14-3.19 (m, 2H), 3.42 (s, 2H), 3.54 (m, 2H), 3.62-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.72-4.78 (m, 2H), 5.62 (s, 1H), 6.59 (s, 1H), 6.63 (s, 1H), 7.11 (s, 2H), 7.31 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.9, 41.4, 41.5, 41.7, 42.0, 44.6, 44.9, 46.7, 47.1, 56.2, 61.3, 66.4, 66.5, 114.0, 119.1, 125.1, 130.0, 131.2 (x2), 137.4, 144.9, 152.8, 155.9, 157.5, 166.7, 166.9. (MS-ESI): *m/z* calculated for $C_{24}H_{30}N_4O_6$ $[M+H]^+$: 471.22, found 471.24. Purity > 99%.

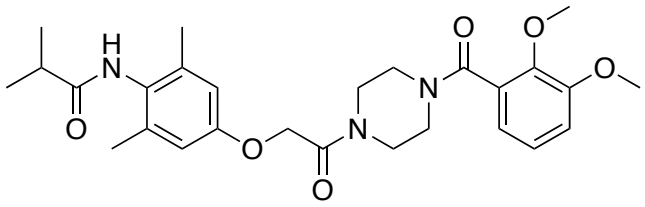
3-acetyl-1-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl)urea (MS522)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.80 (s, 3H), 2.02 (s, 3H), 2.04 (s, 3H), 3.15-3.20 (m, 2H), 3.43 (s, 2H), 3.54 (s, 2H), 3.63-3.71 (m, 2H), 3.72 (s, 3H), 3.82 (s, 3H), 6.71 (s, 1H), 6.76 (s, 1H), 6.80 (s, 1H), 7.11 (s, 2H), 7.55 (s, 1H), 8.28 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.1, 25.3, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2, 61.3, 66.4, 114.0, 114.7, 119.1, 125.1, 131.2, 137.4, 144.9, 152.8, 154.0, 157.7,

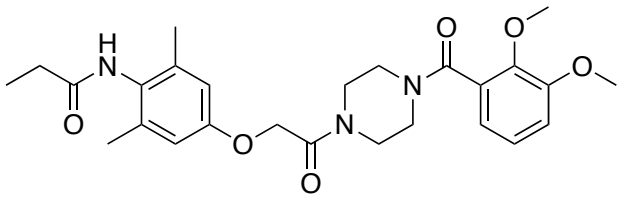
166.4, 166.8, 174.4. (MS-ESI): *m/z* calculated for $C_{26}H_{32}N_4O_7$ $[M+H]^+$: 512.23, found 513.25. Purity = 97.5%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl)-2-methylpropanamide (MS523)



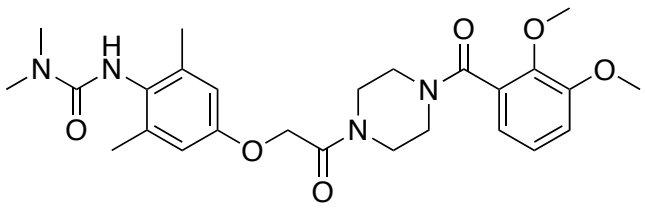
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.11 (s, 6H), 2.05 (s, 3H), 2.07 (s, 3H), 2.59 (s, 1H), 3.14-3.19 (m, 2H), 3.42 (s, 2H), 3.54 (s, 2H), 3.61-3.70 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.73-4.80 (m, 2H), 6.61 (s, 1H), 6.65 (s, 1H), 6.80 (s, 1H), 7.11 (s, 2H), 8.95 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.7, 20.2, 34.6, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2, 61.3, 66.3, 66.4, 113.9 (x2), 119.1, 125.1, 129.0, 131.2 (x2), 136.9, 144.9, 152.8, 156.3, 166.6, 175.4. (MS-ESI): *m/z* calculated for $C_{27}H_{35}N_3O_6$ $[M+H]^+$: 498.25, found 498.27. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl) propanamide (MS524)



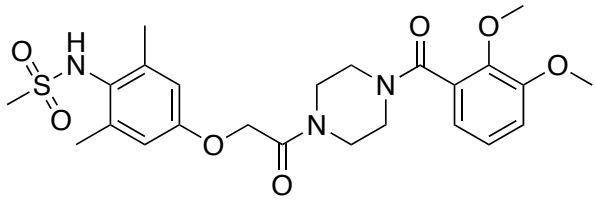
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.10 (s, 3H), 2.05 (s, 3H), 2.07 (s, 3H), 2.27 (s, 2H), 3.14-3.19 (m, 2H), 3.42 (s, 2H), 3.54 (s, 2H), 3.62-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.73-4.80 (m, 2H), 6.60 (s, 1H), 6.65 (s, 1H), 6.80 (s, 1H), 7.11 (bs, 2H), 8.98 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 10.8, 18.8, 29.1, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2, 61.3, 66.3, 66.4, 113.9 (x2), 114.0, 119.1, 125.1, 129.1, 131.2 (x2), 136.8, 144.9, 152.8, 156.3, 166.6, 166.9, 172.3. (MS-ESI): *m/z* calculated for $C_{26}H_{33}N_3O_6$ $[M+H]^+$: 484.24, found 484.27. Purity > 99%.

1-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl-3,3-dimethylurea (MS525)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.07 (s, 3H), 2.08 (s, 3H), 2.89 (s, 6H), 3.14-3.19 (m, 2H), 3.42 (s, 2H), 3.54 (s, 2H), 3.61-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.72-4.79 (m, 2H), 6.59 (s, 1H), 6.64 (s, 1H), 6.80 (s, 1H), 7.11 (s, 2H), 7.50 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.9, 36.7, 41.4, 41.5, 41.7, 42.0, 44.6, 45.0, 46.7, 47.1, 56.2, 61.3, 66.4, 66.5, 113.8, 114.0, 119.1, 125.1, 130.6, 131.2 (x2), 137.7, 144.9, 152.8, 155.9, 157.1, 166.7. (MS-ESI): *m/z* calculated for $C_{26}H_{34}N_4O_6$ $[M+H]^+$: 499.25, found 499.27. Purity > 99%.

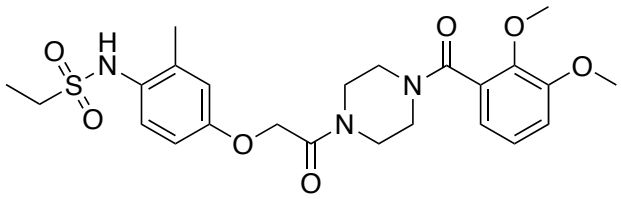
N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl)methanesulfonamide (MS526)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.27 (s, 3H), 2.28 (s, 3H), 2.99 (s, 3H), 3.14-3.19 (m, 2H), 3.41 (s, 2H), 3.54 (s, 2H), 3.62-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.75-4.82 (m, 2H), 6.63 (s, 1H), 6.67 (s, 1H), 6.80 (s, 1H), 7.11 (s, 2H), 8.65 (s, 1H). ^{13}C

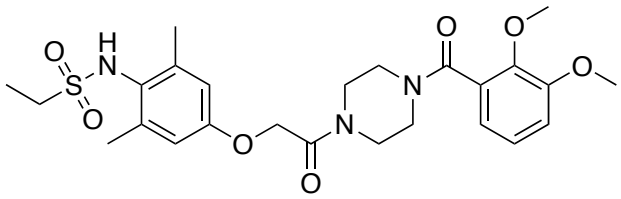
NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 19.7, 41.4, 41.5, 41.7, 42.0, 42.5, 44.5, 44.9, 46.7, 47.0, 56.2, 56.2, 61.3, 66.2, 66.3, 114.0, 114.5 (x2), 119.1, 125.1, 127.4, 131.2 (x2), 139.3, 144.9, 152.8, 156.7, 166.5, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{24}H_{31}N_3O_7S$ $[M+H]^+$: 506.19, found 506.21. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)ethane-1-sulfonamide (MS527)



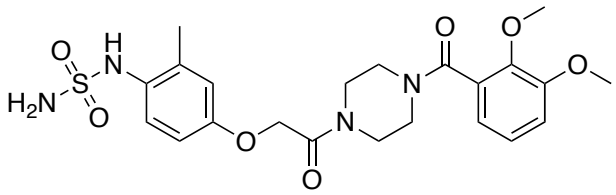
^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.24 (s, 3H), 2.26 (s, 1.5H), 2.27 (s, 1.5H), 3.00 (s, 2H), 3.13-3.19 (m, 2H), 3.41 (s, 2H), 3.53 (s, 2H), 3.62-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.77-4.84 (m, 2H), 6.71-6.73 (m, 1H), 6.74 (s, 1H), 6.79 (s, 1H), 7.11 (bs, 3H), 8.87 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 8.6, 18.9, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.4, 46.7, 47.0, 56.2, 61.3, 66.3, 66.4, 112.7, 112.8, 114.0, 117.1, 119.1, 125.1, 128.8, 131.2 (x2), 137.2, 144.9, 152.8, 156.8, 166.5, 166.9. (MS-ESI): *m/z* calculated for $C_{24}H_{31}N_3O_7S$ $[M+H]^+$: 506.19, found 506.22. Purity > 99%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl)ethane-1-sulfonamide (MS528)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.30 (s, 3H), 2.26 (s, 3H), 2.28 (s, 3H), 3.08 (s, 2H), 3.13-3.19 (m, 2H), 3.41 (s, 2H), 3.53 (s, 2H), 3.62-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.75-4.81 (m, 2H), 6.62 (s, 1H), 6.67 (s, 1H), 6.80 (s, 1H), 7.11 (s, 2H), 8.56 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 8.7, 19.7, 41.4, 41.5, 41.7, 41.9, 44.5, 44.9, 46.7, 47.1, 48.8, 56.2, 61.3, 66.2, 66.3, 114.0, 114.4, 114.5, 119.1, 125.1, 127.5, 131.2 (x2), 139.4, 144.9, 152.8, 156.7, 166.5, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{25}H_{33}N_3O_7S$ $[M+H]^+$: 520.20, found 520.22. Purity > 99%.

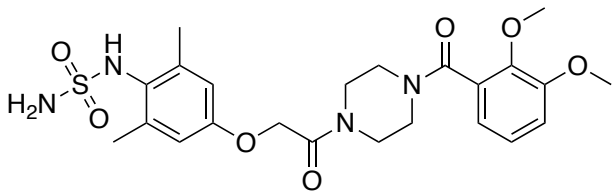
N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2-methylphenyl)aminosulfonamide (MS529)



^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.25 (s, 1.5H), 2.26 (s, 1.5H), 3.13-3.19 (m, 2H), 3.41-3.42 (m, 2H), 3.53-3.54 (m, 2H), 3.62-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.75-4.82 (m, 2H), 6.68-6.79 (m, 2H), 6.70-6.74 (m, 3H), 7.11-7.13 (m, 2H), 7.18-7.21 (t, 1H, J=9.6 Hz), 8.29 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.8, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.0, 56.2, 61.3 (x2), 66.3, 66.4, 112.4 (x2), 114.0, 116.8, 119.1, 125.1, 128.8, 130.0, 131.2 (x2), 136.8, 144.9, 152.8, 156.4, 166.6, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{22}H_{28}N_4O_7S$ $[M+H]^+$: 493.17, found 493.19. Purity = ~95%.

N-(4-{2-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]-2-oxoethoxy}-2,6-dimethylphenyl) aminosulfonamide (MS530)

^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 2.29 (s, 3H), 2.31 (s, 3H), 3.14-3.19 (m, 2H), 3.41 (s, 2H), 3.53-3.54 (m, 2H), 3.61-3.71 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.73-4.80 (m, 2H), 6.58 (s, 1H), 6.63 (s, 1H), 6.72-6.73 (m, 2H), 6.79-6.80 (m, 1H), 7.11-7.13 (m, 2H),



8.12-8.15 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 19.7, 41.4, 41.5, 41.7, 42.0, 44.5, 44.9, 46.7, 47.1, 56.2, 61.3 (x2), 66.2, 66.3, 114.0, 114.2 (x2), 119.1, 125.1, 128.5, 131.2 (x2), 139.7, 144.9, 152.8, 156.5, 166.6, 166.8, 166.9. (MS-ESI): *m/z* calculated for $C_{23}H_{30}N_4O_7S$ $[M+H]^+$: 507.18, found 507.20. Purity = ~96.5%.

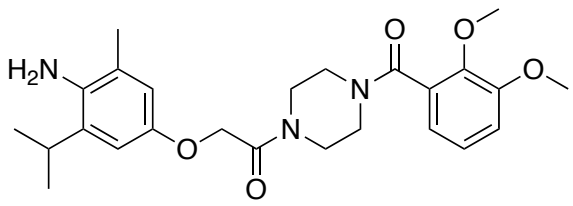
2-[4-amino-3-methyl-5-isopropylphenoxy]-1-[4-(2,3-dimethoxybenzoyl)piperazin-1-yl]ethan-1-one (MS531)

This compound required a different reactant in step d than did all other compounds in this series. The starting material for this reactant was 5-isopropyl-3-methylphenol, which then had to be nitrated and reduced prior to boc-protection in step d.

Nitration procedure: To a solution of 5-isopropyl-3-methylphenol (2.5 g, 16.6 mmol) in 3 mL ether was added a few drops of nitric acid (1.18 mL diluted with 4.7 mL of water). The reaction was cooled on ice and the remainder of the nitric acid solution was added dropwise. After an hour at 0 ^{o}C, the mixture was diluted with ether, washed with water, dried over sodium sulfate, and the solvent was evaporated. A column was then run in hexanes and ethyl acetate, running from 0% to 20% ethyl acetate. 2.711 g (71%) of the product, 3-methyl-4-nitro-5-isopropylphenol, was obtained.

Reduction procedure: 3-methyl-4-nitro-5-isopropylphenol (2.711 g, 13.9 mmol) was dissolved in 180 mL methanol. The iron powder (2.327 g, 41.7 mmol) was then added to the solution. After this addition, 180 mL NH_4Cl was added to the solution. The resulting mixture was heated to reflux for 75 minutes. The reaction mixture was filtered and then washed with a mixture of methanol and ammonium chloride. The mixture was concentrated as much as possible, and was allowed to cool slightly. Water was added, yielding a brown solution. Ethyl acetate was used to extract the desired compound from this water mixture. 1.620 g (70%) of the product, 4-amino-3-methyl-5-isopropylphenol, was obtained.

After using this starting material for the rest of the reaction scheme, the final compound MS531 was obtained:

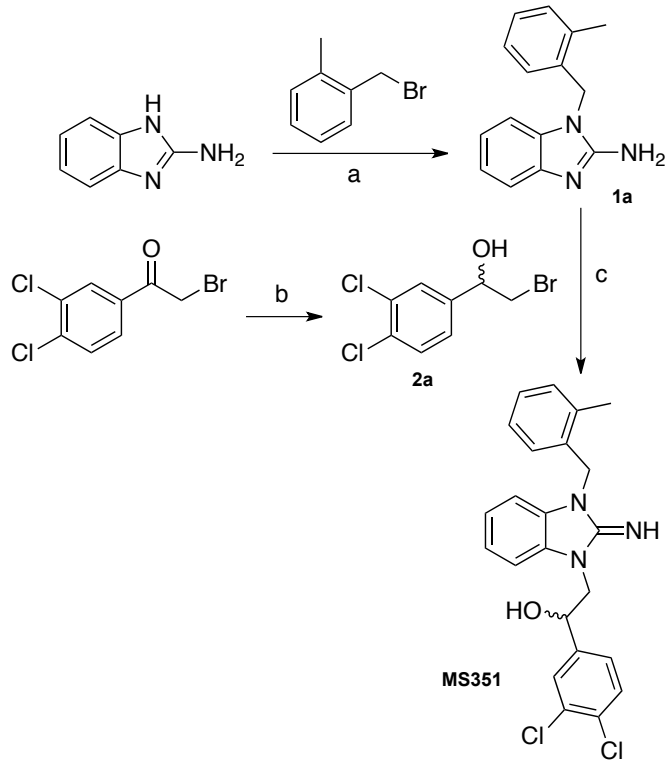


^{1}H NMR (600 MHz, DMSO-d_6) (Two rotameric forms) δ = 1.12 (s, 6H), 2.06 (s, 1.5H), 2.08 (s, 1.5H), 3.00 (s, 1H), 3.11-3.16 (m, 2H), 3.42 (s, 2H), 3.54 (s, 2H), 3.60-3.67 (m, 2H), 3.71 (s, 3H), 3.82 (s, 3H), 4.60-4.67 (m, 2H), 6.48 (s, 1H), 6.52-6.55 (m, 1H), 6.79 (s, 1H), 7.11 (bs, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) (Two rotameric forms) δ = 18.9, 23.0, 27.3, 41.4, 41.5, 41.8, 42.0, 44.7, 45.1, 46.7, 47.1, 56.2, 61.3, 67.4 (x2), 110.4 (x2), 114.0, 114.4, 119.1, 125.1, 131.2, 133.8, 144.9, 150.6, 152.7, 166.9, 167.1. (MS-ESI): *m/z* calculated for $C_{25}H_{33}N_3O_5$ $[M+H]^+$: 456.24, found 456.28. Purity = 99%.

Chemical Synthetic Procedures for MS351-series Compounds

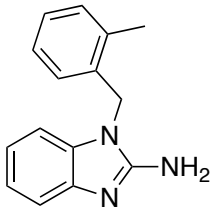
The synthesis of MS351 was achieved using the following steps (see **Scheme 2**). First, the R-group-containing benzyl bromide was reacted with the 2-aminobenzimidazole (or R-group containing 2-aminobenzimidazole) in acetone with anhydrous potassium carbonate at room temperature overnight, yielding intermediate **1a**[21]. In a separate reaction, the ketone of an R-group-containing 2-bromoacetophenone was reduced to an alcohol using sodium borohydride in methanol, yielding intermediate **2a**[22]. The final step conjugates these two intermediates in DMF

at either room temperature for 24 hours[23] or in the microwave for 4 hours at 90 degrees. All intermediates were purified using Flash chromatography (Biotage) and all final products were purified using HPLC (Agilent). Details on these purification methods can be found in the "Chemicals and General Procedures" section. Depicted below is the synthetic scheme of MS351, followed by analytical data of MS351 intermediates.



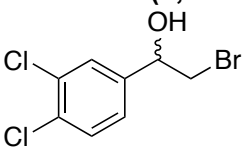
Scheme 2

1-[(2-methylphenyl)methyl]-2,3-dihydro-1H-1,3-benzodiazol-2-imine (1a)



2-methylbenzyl bromide (1.11 g, 6.0 mmol) was added to 2-aminobenzimidazole (798.8 mg, 6.0 mmol) and K_2CO_3 (166 mg, 1.2 mmol) in acetone (70 mL). The resulting solution was stirred at room temperature overnight. The crude reaction mixture was filtered and then dried under reduced pressure. A column was then run in hexanes and ethyl acetate, with ethyl acetate increasing stepwise from 70% to 100%, at which point the product eluted. 438.6 mg (30.8%) of a white solid were obtained. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 2.37 (s, 3H), 5.23 (s, 2H), 6.33-6.35 (d, 1H, J=7.8 Hz), 6.81-6.83 (t, 1H, J=7.8 Hz), 6.92-6.93 (m, 1H, J=10.8 Hz), 6.95-6.98 (dt, 1H, J=7.8, 0.6 Hz), 7.01-7.03 (t, 1H, J=7.2 Hz), 7.12-7.15 (t, 1H, J=7.2 Hz), 7.19-7.22 (m, 2H). ^{13}C NMR (150 MHz, DMSO-d_6) δ = 19.2, 43.5, 108.6, 114.9, 119.0, 121.3, 124.9, 126.3, 127.4, 130.6, 134.4, 135.1, 135.9, 142.0, 155.4, 164.3 (x2). (MS-ESI): *m/z* calculated for $C_{15}H_{15}N_3$ $[M+H]^+$: 238.13, found 238.15.

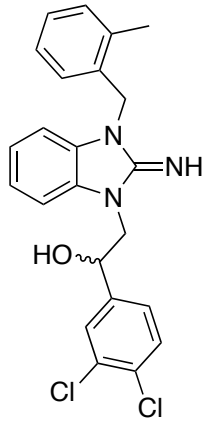
2-bromo-1-(3,4-dichlorophenyl)ethan-1-ol (2a)



$NaBH_4$ (8.5 mg, 0.224 mmol) was added portionwise to a solution of 2-bromo-3',4'-dichloroacetophenone (30 mg, 0.112 mmol) in MeOH (3 mL) at 0 °C. After gas evolution ceased, the reaction mixture was warmed to room temperature and stirred for 30 minutes. The reaction was quenched with aqueous HCl, then concentrated. The residue was partitioned between H_2O and ethyl acetate. The organic phase was separated and the aqueous phase

extracted with ethyl acetate. The combined organic extracts were dried over Na_2SO_4, then concentrated to yield the title compound (22 mg, 73%), which required no further purification. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 3.59-3.62 (m, 1H), 3.68-3.71 (m, 1H), 4.84-4.85 (m, 1H), 6.01-6.02 (m, 1H), 7.38-7.40 (dd, 1H, J=8.4, 1.8 Hz), 7.60-7.61 (m, 1H), 7.64-7.65 (m, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ = 40.3, 70.9, 127.2, 128.9, 130.3, 130.7, 131.2, 144.3. (MS-ESI): *m/z* calculated for $C_8H_7BrCl_2O$ $[M+H]^+$: 268.91, product failed to ionize on TOF.

1-(3,4-dichlorophenyl)-2-{2-imino-3-[(2-methylphenyl)methyl]-2,3-dihydro-1H-1,3-benzodiazol-1-yl]ethan-1-ol (MS351)



2-bromo-1-(3,4-dichlorophenyl)ethan-1-ol (120 mg, 0.44 mmol) was added to 1-[(2-methylphenyl)methyl]-2,3-dihydro-1H-1,3-benzodiazol-2-imine (105 mg, 0.44 mmol) in DMF (5 mL). The resulting solution was microwaved at 90 ^{o}C for 4 hours. An HPLC was run, and 12 mg of a white powder were obtained. ^{1}H NMR (600 MHz, DMSO-d_6) δ = 2.35 (s, 3H), 4.00-4.08 (m, 1H), 4.16-4.19 (m, 1H), 5.02-5.04 (m, 2H), 5.06 (s, 1H), 6.53-6.54 (m, 1H), 6.69-6.70 (d, 1H, J=7.8 Hz), 6.78-6.80 (t, 1H, J=7.2 Hz), 6.85-6.88 (t, 1H, J=7.8 Hz), 7.01-7.02 (d, 1H, J=7.8 Hz), 7.03-7.06 (t, 1H, J=7.8 Hz), 7.13-7.16 (t, 1H, J=7.2 Hz), 7.20-7.21 (d, 1H, J=7.2 Hz), 7.42-7.43 (d, 1H, J=8.4 Hz), 7.54-7.56 (d, 1H, J=8.4 Hz), 7.69 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ = 19.2, 43.0, 49.5, 70.3, 107.8, 108.2, 121.1, 121.3, 125.2, 126.3, 127.4, 128.8, 130.0, 130.6, 130.7, 131.2, 131.4, 132.1, 134.5, 136.1, 144.5, 153.9. (MS-ESI): *m/z* calculated for $C_{23}H_{21}Cl_2N_3O$ $[M+H]^+$: 426.11, found 426.11.

Chiral Separation of MS351

MS351 family compounds are obtained as formic acid salts when purified using the water/acetonitrile/formic acid solvent system. To remove this salt and obtain the free base, the powder is dissolved in water, and 10% sodium bicarbonate is added to basify the solution. The compound is extracted in ethyl acetate, which is then evaporated, before the compound is once again dissolved in water, lyophilized, and retrieved once again as a powder.

Purification of enantiomeric forms of MS351 compounds was conducted on the same HPLC (Agilent 1200), using an (R,R)-Whelk-01, 5/100 Kromasil column (25 cm x 10.0 mm; serial #40542). The solvent system was an isocratic 60% Hexanes/40% Isopropanol/0.1% Diethylamine. For the MS351 separation, two peak fractions eluted between 17 and 20 minutes (see Figure S3A). Peak purities were confirmed via LC/MS and NMR, and the fact that this purification yielded two different enantiomeric species was confirmed via an analytical reinjection into this same column with the same solvent system.

1-(3,4-dichlorophenyl)-2-{2-imino-3-[(2-methylphenyl)methyl]-2,3-dihydro-1H-1,3-benzodiazol-1-yl]ethan-1-ol (MS351; Peak1)

^{1}H NMR (600 MHz, DMSO-d_6) δ = 2.36 (s, 3H), 4.07 (m, 1H), 4.18 (m, 1H), 5.02-5.04 (m, 2H), 5.06 (s, 1H), 6.55 (m, 1H), 6.67-6.68 (d, 1H, J=7.2 Hz), 6.77-6.80 (t, 1H, J=7.2 Hz), 6.84-6.87 (t, 1H, J=7.8 Hz), 6.98-6.99 (d, 1H, J=7.2 Hz), 7.05-7.07 (t, 1H, J=7.2 Hz), 7.14-7.17 (t, 1H, J=7.2 Hz), 7.21-7.22 (d, 1H, J=7.8 Hz), 7.43-7.44 (d, 1H, J=8.4 Hz), 7.55-7.57 (d, 1H, J=8.4 Hz), 7.69 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ = 19.2, 42.6, 49.5, 70.6, 107.1, 107.4, 120.5, 120.7, 125.4, 126.3, 127.1, 127.3, 128.7, 130.0, 130.6, 130.7, 131.2, 131.7, 132.4, 134.8, 136.1, 144.8, 154.6. (MS-ESI): *m/z* calculated for $C_{23}H_{21}Cl_2N_3O$ $[M+H]^+$: 426.11, found 426.11.

1-(3,4-dichlorophenyl)-2-{2-imino-3-[(2-methylphenyl)methyl]-2,3-dihydro-1H-1,3-benzodiazol-1-yl]ethan-1-ol (MS351; Peak2)

^{1}H NMR (600 MHz, DMSO-d_6) δ = 2.36 (s, 3H), 4.06-4.08 (m, 1H), 4.18 (m, 1H), 5.00-5.03 (m, 2H), 5.07 (s, 1H), 6.56 (m, 1H), 6.69-6.70 (m, 1H), 6.79-6.81 (t, 1H, J=7.2 Hz), 6.86-6.88 (t, 1H,

J=7.2 Hz), 7.00 (m, 1H), 7.05-7.07 (t, 1H, J=7.2 Hz), 7.15-7.17 (t, 1H, J=7.2 Hz), 7.21-7.22 (d, 1H, J=7.2 Hz), 7.43-7.44 (d, 1H, J=7.8 Hz), 7.56-7.57 (d, 1H, J=8.4 Hz), 7.69 (s, 1H). ^{13}C NMR (150 MHz, DMSO-d_6) δ = 19.2, 42.7, 49.5, 70.6, 107.2, 107.5, 120.5, 120.7, 125.4, 126.3, 127.1, 127.3, 128.7, 129.9, 130.6, 130.7, 131.1, 131.7, 132.4, 134.8, 136.1, 144.7, 154.5. (MS-ESI): *m/z* calculated for $C_{23}H_{21}Cl_2N_3O$ $[M+H]^+$: 426.11, found 426.11.

References:

1. Yap, K. L.; Li, S.; Munoz-Cabello, A. M.; Raguz, S.; Zeng, L.; Mujtaba, S.; Gil, J.; Walsh, M. J.; Zhou, M. M., Molecular interplay of the noncoding RNA ANRIL and methylated histone H3 lysine 27 by polycomb CBX7 in transcriptional silencing of INK4a. *Mol Cell* **2010,** *38* (5), 662-74.

2. Morris, G. M.; Huey, R.; Lindstrom, W.; Sanner, M. F.; Belew, R. K.; Goodsell, D. S.; Olson, A. J., Autodock4 and AutoDockTools4: automated docking with selective receptor flexibility. *Journal of computational chemistry* **2009,** *16*, 2785-91.

3. Zsoldos, Z.; Reid, D.; Simon, A.; Bashir Sadjad, S.; Johnson, A. P., eHiTS: A new fast, exhaustive flexible ligand docking system. *Journal of Molecular Graphics and Modelling* **2007,** *26*, 198-212.

4. Mezei, M.; Zhou, M.-M., Dockres: a computer program that analyzes the output of virtual screening of small molecules. *Source Code for Biology and Medicine* **2010,** *5*, 2.

5. OpenEye *OEChem, version 2015.Jun.5, OpenEye Scientific Software, Inc., Santa Fe, NM, USA,* http://www.eyesopen.com*, 2015*.

6. Ren, C.; Morohashi, K.; Plotnikov, A. N.; Jakoncic, J.; Smith, S. G.; Li, J.; Zeng, L.; Rodriguez, Y.; Stojanoff, V.; Walsh, M.; Zhou, M. M., Small-Molecule Modulators of Methyl-Lysine Binding for the CBX7 Chromodomain. *Chem Biol* **2015,** *22* (2), 161-8.

7. Winn, M. D.; Ballard, C. C.; Cowtan, K. D.; Dodson, E. J.; Emsley, P.; Evans, P. R.; Keegan, R. M.; Krissinel, E. B.; Leslie, A. G.; McCoy, A.; McNicholas, S. J.; Murshudov, G. N.; Pannu, N. S.; Potterton, E. A.; Powell, H. R.; Read, R. J.; Vagin, A.; Wilson, K. S., Overview of the CCP4 suite and current developments. *Acta Crystallogr D Biol Crystallogr* **2011,** *67* (Pt 4), 235-42.

8. Otwinowski, Z.; Minor, W., Processing of X-ray diffraction data collected in oscillation mode. *Method Enzymol* **1997,** *276*, 307-326.

9. Long, F.; Vagin, A. A.; Young, P.; Murshudov, G. N., BALBES: a molecular-replacement pipeline. *Acta Crystallogr D Biol Crystallogr* **2008,** *64* (Pt 1), 125-32.

10. Murshudov, G. N.; Vagin, A. A.; Dodson, E. J., Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D* **1997,** *53*, 240-255.

11. Adams, P. D.; Afonine, P. V.; Bunkoczi, G.; Chen, V. B.; Davis, I. W.; Echols, N.; Headd, J. J.; Hung, L. W.; Kapral, G. J.; Grosse-Kunstleve, R. W.; McCoy, A. J.; Moriarty, N. W.; Oeffner, R.; Read, R. J.; Richardson, D. C.; Richardson, J. S.; Terwilliger, T. C.; Zwart, P. H., PHENIX: a comprehensive Python-based system for macromolecular structure solution. *Acta Crystallogr D* **2010,** *66*, 213-221.

12. Emsley, P.; Cowtan, K., Coot: model-building tools for molecular graphics. *Acta Crystallogr D Biol Crystallogr* **2004,** *60* (Pt 12 Pt 1), 2126-32.

13. Delaglio, F.; Grzesiek, S.; Vuister, G. W.; Zhu, G.; Pfeifer, J.; Bax, A., Nmrpipe - a Multidimensional Spectral Processing System Based on Unix Pipes. *Journal of Biomolecular Nmr* **1995,** *6* (3), 277-293.

14. Johnson, B. A., Using NMRView to visualize and analyze the NMR spectra of macromolecules. *Methods Mol Biol* **2004,** *278*, 313-52.

15. Aguilo, F.; Zhang, F.; Sancho, A.; Fildago, M.; DiCecilia, S.; Vashisht, A.; Lee, D.-F.; Chen, C.-H.; Rengasamy, M.; Andino., B.; Jahouh, F.; Roman, A.; Krig, S.; Wang, R.; Zhang, W.; Wohlschlegel., J. A.; Wang, J.; Walsh, M. J., Coordination of m6A methylation and gene transcription by zfp217 regulates pluripotency and reprogramming. *Cell Stem Cell* **2015,** *17*, 1-16.

16. Bandgar, B.; Bettigeri, S., Direct Synthesis of N-Acylalkylenediamines from Caboxylic Acids Under Mild Conditions. *Synthetic Communications* **2004,** *34* (16), 2917-2924.

17. Dutta, S.; Basak, A.; Dasgupta, S., Design and synthesis of enediyne-peptide conjugates and their inhibiting activity against chymotrypsin. *Bioorg Med Chem* **2009,** *17* (11), 3900-3908.

18. Vigroux, A.; Bergon, M.; Zedde, C., Cyclization-Activated Prodrugs: N-(Substituted 2-hydroxyphenyl and 2-hydroxypropyl)carbamates Based on Ring-Opened Derivatives of Active Benzoxazolones and Oxazolidinones as Mutual Prodrugs of Acetaminophen. *J. Med. Chem.* **1995,** *38* (20), 3983-3994.

19. Bezerra-Netto, H. J. C.; Lacerda, D. I.; Miranda, A. L. P.; Alves, H. M.; Barreiro, E. J.; Fraga, C. A. M., Design and synthesis of 3,4-methylenedioxy-6-nitrophenoxyacetylhydrazone derivatives obtained from natural safrole: New lead-agents with analgesic and antipyretic properties. *Bioorg Med Chem* **2006,** *14* (23), 7924-7935.

20. (a) Borch, R. F.; Hassid, A. I., A New Method for the Methylation of Amines. *J. Org. Chem.* **1972,** *37* (10), 1673-1674; (b) Fuchs, F.; Gilbert, D.; Koch, C.; Maskey, R.-P.; Steinbrink, S.; Boutros, M. Pyrido [2,3-D] Pyrimidines as Wnt Antagonists for Treatment of Cancer and Arthritis. 2010; (c) Klinger, A. L.; McComsey, D. F.; Smith-Swintosky, V.; Shank, R. P.; Maryanoff, B. E., Inhibition of Carbonic Anhydrase-II by Sulfamate and Sulfamide Groups: An Investigation Involving Direct Thermodynamic Binding Measurements. *J. Med. Chem.* **2006,** *49* (12), 3496-3500; (d) Kuhn, B.; Mohr, P.; Stahl, M., Intramolecular Hydrogen Bonding in Medicinal Chemistry. *J. Med. Chem.* **2010,** *53* (6), 2601-2611; (e) Maryanoff, B. E.; McComsey, D. F.; Costanzo, M. J.; Hochman, C.; Smith-Swintosky, V.; Shank, R. P., Comparison of Sulfamate and Sulfamide Groups for the Inhibition of Carbonic Anhydrase-II by Using Topiramate as a Structural Platform. *J. Med. Chem.* **2005,** *48* (6), 1941-1947.

21. Guida, X.; Jianhua, H.; Xiaomin, L., Synthesis and QSAR studies of novel 1-substituted-2-aminobenzimidazoles derivatives. *Euro. J. Med. Chem.* **2006,** *41* (9), 1080-1083.

22. Hostetler, G.; Dunn, D.; McKenna, B. A.; Kopec, K.; Chatterjee, S., Lactam and oxazolidinone derived potent 5-hydroxytryptamine 6 receptor antagonists. *Bioorg. Med. Chem. Lett.* **2014,** *24* (9), 2094-2097.

23. Roth, G. P.; Wallace, G. A.; George, D. M.; Grongsaard, P.; Hayes, M.; Breinlinger, E. C. 2-Imino-Benzimidazoles. US 2007/0232673 A1, 2007.

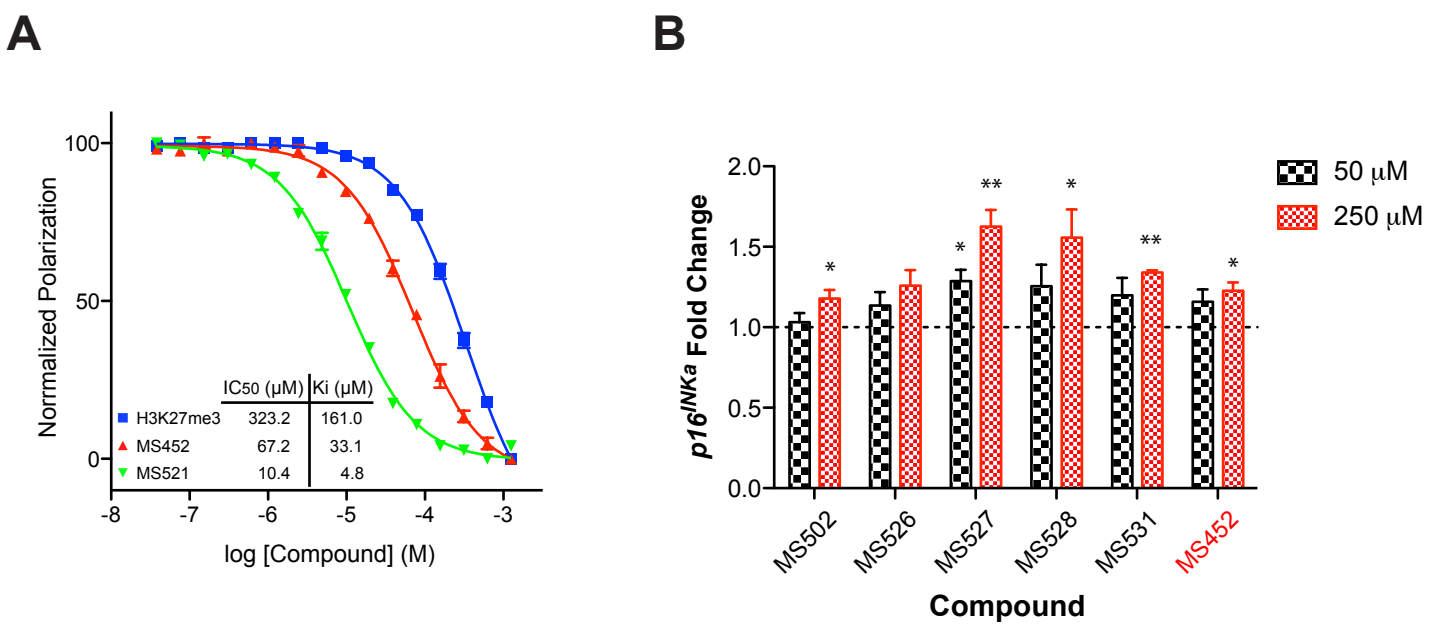


Figure S1. MS452 and its chemical analogs induce transcriptional de-repression of *p16^{INK4a}*.
(A) Fluorescence anisotropy assay determining binding affinity of H3K27me3 and SETDB1-K1170me3 peptides, or MS452 and its analog to the CBX7ChD. A FITC-SETDB1-K1170me3 peptide was used as an assay probe. See details in Experimental Methods.
(B) SAR study of MS452 and its chemical analogs, assessed by transcriptional de-repression of *p16^{INK4a}* after the 12-hr compound treatment (50 or 250 μM) in human PC3 prostate cancer cells.

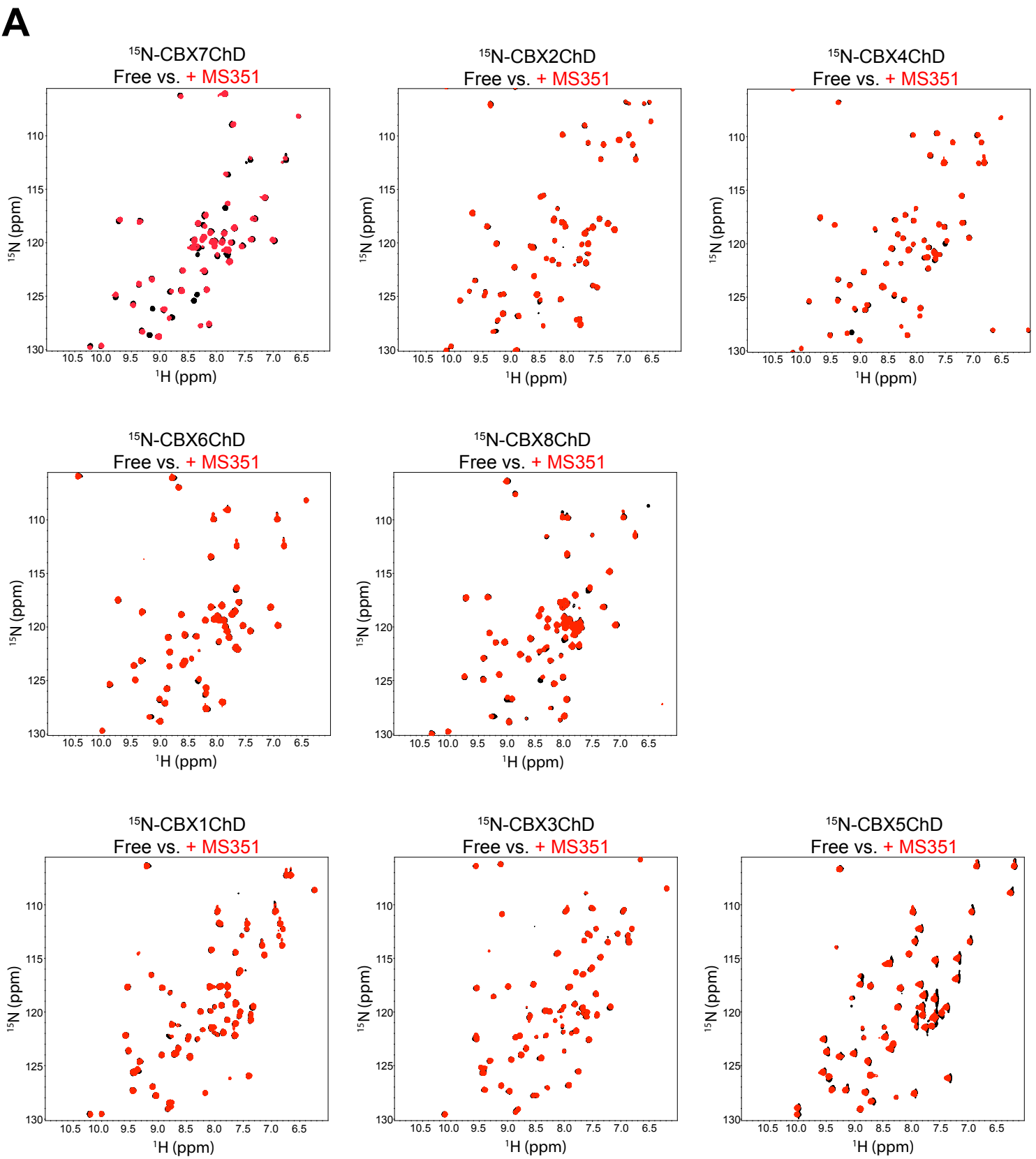


Figure S2. (A) MS351 selective binding to CBX7ChD over other CBXChDs as demonstrated by ^{1}H-^{15}N HSQC NMR spectrscopy. ^{1}H-^{15}N-HSQC spectra of ChDs of Polycomb proteins CBX2, CBX4, CBX6, CBX7, and CBX8, or heterochromatin proteins CBX1, CBX3, and CBX5, showing chemical shift perturbations of protein residues upon addition of MS351 (400 μM). Note that the ChDs of CBX-1/3/5, 2/6/7/8 used in the NMR study were 200 μM, whereas CBX74ChD was 46 μM CBX4ChD. Spectra were recorded by Bruker 600 MHz NMR spectrometer. The NMR signals of protein residues are color-coded in black and red for the free protein and in the presence of MS351, respectively.

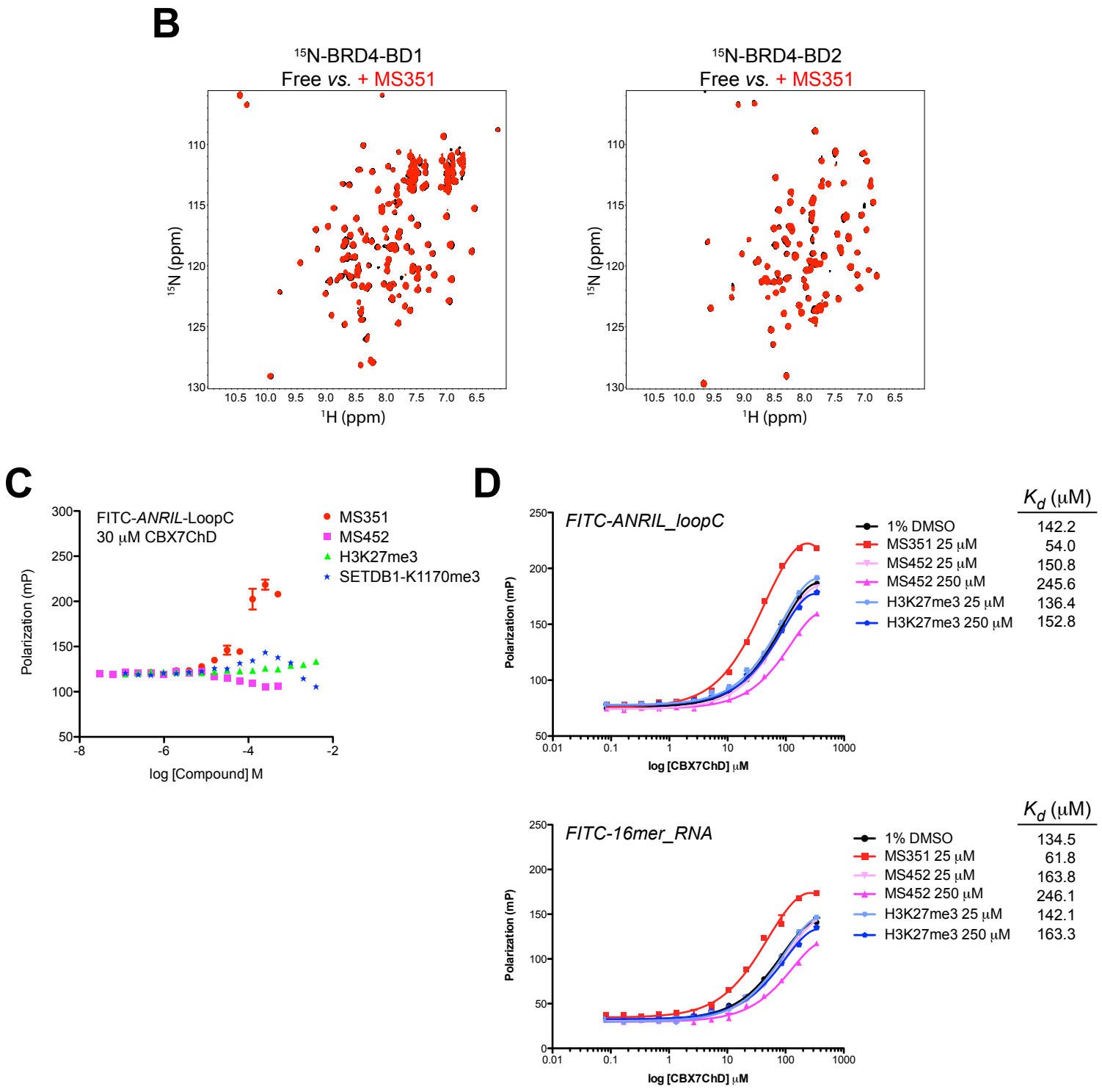


Figure S2. Characterization of MS351 binsing specificity to the CBX7ChD by NMR and fluorescnece anisotropy assays.
(B) MS351 does not bind to the first or second bromodomains (BD1 or BD2) of BRD4, as assessed by NMR. 2D ^{1}H-^{15}N-HSQC spectra of ^{15}N-labeled BRD4-BD1 or BRD4-BD2 (200 μM) was collected in the free state or in the presence of MS351 (400 μM). MS351 did not induce chemical shift perturbation or line broadening of BRD4-BDs. Spectra were recorded by Bruker 600 MHz NMR spectrometer. Black signals: protein; Red signals: protein plus MS351. Both were in 10% D_2O PBS buffer contaning 1.2%DMSO and 0.8% cyclodextrin (45%, v/v).
(C) Fluorescence anisotropy assay assessing relative binding affinity of CBX7ChD (30 μM) to MS351, MS452, H3K27me3 (aa 21-33), or SETDB1-K1170me3 (aa 1165-1174) peptide. A FITC-labeled loop C of ANRIL (10 nM) was used as an assay probe as described in detail in the Experimental Methods.
(D) MS351 enhances RNA binding to CBX7ChD, as demonstrated by fluorescence anisotropy binding assay. Fluorescence anisotropy assay assessing effects of MS351, MS452 or H3K27me3 peptide on RNA binding to the CBX7ChD. A FITC-ANRIL-loopC or

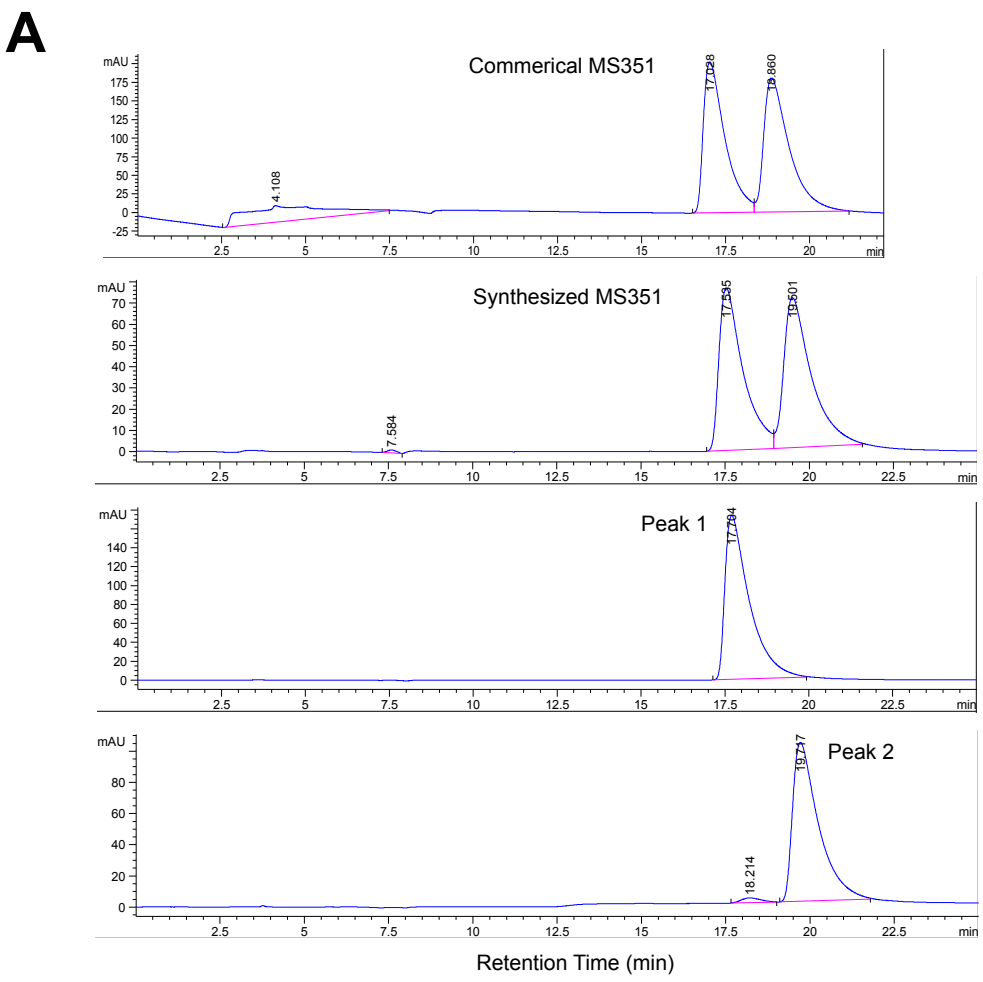
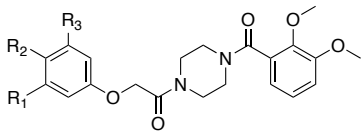


Figure S3. Separation and characterization of racemic compounds of MS351.
(A) Separation of racemic compounds of MS351. Chiral chromatography analysis of MS351, conducted in a solvent system of 60% hexanes, 40% isopropanol, and 0.1% diethylamine. Depicted are the commercially obtained MS351 (top panel), Peak 1 of the synthesized racemic MS351 (>99% ee), Peak 2 of the synthesized racemic MS351 (95% ee), and the in-house synthesized racemic MS351.
(B) Comparison of effects of racemic mixture MS351 and enantiomers of Peak 1 and Peak 2 from chiral chromatography separation, as assessed by transcriptional de-repression of *p16^{INK4a}* after the 12-hr compound treatment (1 or 5 μM) in human PC3 prostate cancer cells.

Supplemental Table S1. SAR of MS452 Series Compounds



Compound	R1	R2	R3	Ki (95% CI) (μM)	*p16*^{ink4a} De-Repression	
					50 μM	250 μM
MS452	H	H	CH_3	33.1 (29.9-36.7)	15.9 ± 7.6%	22.8 ± 5.2%*
MS501	H	NH_2	CH_3	39.3 (37.4-41.3)		
MS502	H	NH-Ac	CH_3	25.2 (17.1-37.3)		
MS503	H	$N(CH_3)_2$	CH_3	49.2 (32.4-74.5)		
MS504	H	NH-CO-NH_2	CH_3	10.0 (8.5-11.7)	0%	0%
MS505	H	NH-CO-NH-Ac	CH_3	26.5 (21.8-32.2)		
MS506	H	NH-CO-$N(CH_3)_2$	CH_3	53.3 (38.0-74.5)		
MS507	H	NH-SO_2-CH_3	CH_3	21.0 (18.4-23.9)		
MS508	CH_3	NH_2	CH_3	16.9 (13.6-20.9)	3.1 ± 5.7%	17.9 ± 5.4%*
MS509	H	NH_2	H	57.0 (42.9-76.0)		
MS510	H	NH-CO-$CH(CH_3)_2$	H	69.5 (55.5-88.0)		
MS511	H	NH-CO-$CH(CH_3)_2$	CH_3	33.0 (25.7-42.3)		
MS512	H	NH-CO-CH_2CH_3	H	60.5 (47.5-77.0)		
MS513	H	NH-CO-CH_2CH_3	CH_3	27.4 (24.7-30.5)		
MS514	H	NH-CO-$CH_2CH(CH_3)_2$	H	122.5 (80.0-187)		
MS515	H	NH-CO-$CH_2CH(CH_3)_2$	CH_3	59.5 (39.6-90.0)		
MS516	H	NH-Ac	H	40.5 (33.9-48.6)		
MS517	H	$N(CH_3)_2$	H	42.3 (35.5-50.5)		
MS518	H	NH-CO-NH_2	H	28.1 (19.3-41.0)		
MS519	CH_3	NH-Ac	CH_3	20.4 (17.6-23.8)		
MS520	CH_3	$N(CH_3)_2$	CH_3	38.5 (31.8-46.5)		
MS521	CH_3	NH-CO-NH_2	CH_3	4.8 (4.4-5.2)	0%	2.1%
MS522	CH_3	NH-CO-NH-Ac	CH_3	19.6 (15.8-24.5)		
MS523	CH_3	NH-CO-$CH(CH_3)_2$	CH_3	26.9 (23.3-30.9)		
MS524	CH_3	NH-CO-CH_2CH_3	CH_3	19.0 (16.4-22.1)		
MS525	CH_3	NH-CO-$N(CH_3)_2$	CH_3	21.9 (18.7-25.6)		
MS526	CH_3	NH-SO_2-CH_3	CH_3	11.3 (8.9-14.1)	13.5 ± 8.4%	25.9 ± 9.6%
MS527	H	NH-SO_2-CH_2CH_3	CH_3	25.0 (22.1-28.4)	28.7 ± 7.1%*	62.6 ± 10.3%**
MS528	CH_3	NH-SO_2-CH_2CH_3	CH_3	10.0 (8.7-11.6)	25.5 ± 13.4%	55.8 ± 17.4%*
MS529	H	NH-SO_2-NH_2	CH_3	21.8 (19.5-24.5)		
MS530	CH_3	NH-SO_2-NH_2	CH_3	10.1 (8.6-12.0)	0%	0%
MS531	*i*-Pr	NH_2	CH_3	38.6 (33.7-44.3)	19.8 ± 10.8%	34.0 ± 1.4%**

*: $p<0.05$; **: $p<0.01$
±: SEM

Supplemental Table S2. Crystallography Data Collection and Refinement Statistics

	MS351
Data collection	
Space group	P 31 2 1
Cell dimensions	
a, b, c (Å)	52.75, 52.75, 76.81
α, β, γ (°)	90.00, 90.00, 120.00
Resolution (Å) (highest resolution shell)	42.2-2.60 (2.64-2.60)
Measured reflections	314,235
Unique reflections	4105
Rmerge	9.9(59.3)
I/σ	29.2(5.2)
Completeness (%)	99.76(97.7)
Redundancy	12.8(13)
Refinement	
Resolution (Å)	29.41-2.60
No. reflections	3890
Rwork/ Rfree(%)	19.6/22.3
No. atoms	
Protein	659
Ligand	29
Water	19
B-factors ($Å^2$)	
Protein	22.1
Ligand	16.9
Water	20.3
RMSD	
Bond lengths (Å)	0.017
Bond angles (o)	1.844
Ramachandran plot % residues	
Favored	98.7
Allowed	1.3
Outlier	0

Supplemental Table S3. Information for proteins, peptides, and RNAs in the study

Name	Synonym	Application	Sequence	Source	notes
CBXChD proteins					
CBX7 (1-71)	CBX7 chromodomain	Crystallization	mgsshhhhhhssglvprgshMELSAIGEQVFAVESIRKKRVRKGKVEYLVKWKGWP PKYSTWEPEEHILDPRLVMAYEEKEERDRASGYRK	self-prepared	red: thrombin cleavage site
CBX7 (7-66)	CBX7 chromodomain	HSQC, FP	mgsshhhhhhssglvprgshmGEQVFAVESIRKKRVRKGKVEYLVKWKGWPPKYS TWEPEEHILDPRLVMAYEEKEERDRA	self-prepared	red: thrombin cleavage site
CBX1 (20-73)	CBX1 chromodomain	HSQC	mhhhhhhssgrenlyfqgEYVVEKVLDRRVVKGKVEYLLKWKGFSDEDNTWEPE ENLDCPDLIAEFLQSQKT	self-prepared	red: TEV cleavage site
CBX2 (9-62)	CBX2 chromodomain	HSQC	mgsshhhhhhssglvprgsEQVFAAECILSKRLRKGKLEYLVKWRGWSSKHNSWE PEENILDPRLLLAFQKKE	self-prepared	red: thrombin cleavage site
CBX3 (29-86)	CBX3 chromodomain	HSQC	mhhhhhhssgrenlyfqgEFVVEKVLDRRVVNGKVEYFLKWKGFTDADNTWEPE ENLDCPELIEAFLNSQKAGKEK	self-prepared	red: TEV cleavage site
CBX4 (8-65)	CBX4 chromodomain	HSQC	mgsshhhhhhssglvprgsEHVFAVESIEKKRIRKGRVEYLVKWRGWSPKYNTWE PEENILDPRLLIAFQNRERQEQ	self-prepared	red: thrombin cleavage site
CBX5 (18-75)	CBX5 chromodomain	HSQC	mhhhhhhssgrenlyfqgEEYVVEKVLDRRVVKGQVEYLLKWKGFSEEHNTWEP EKNLDCPELISEFMKKYKKMKE	self-prepared	red: TEV cleavage site
CBX6 (8-65)	CBX6 chromodomain	HSQC	mgsshhhhhhssglvprgsERVFAAESIIKRRIRKGRIEYLVKWKGWAIKYSTWEPE ENILDSRLIAAFEQKERERE	self-prepared	red: thrombin cleavage site
CBX8 (1-71)	CBX8 chromodomain	HSQC	mgsshhhhhhssglvprgshmMELSAVGERVFAAEALLKRRIRKGRMEYLVKWKG WSQKYSTWEPEENILDARLLAAFEEREREMELYGPKK	self-prepared	red: thrombin cleavage site
BRD4-BrD1 (44-168)	BRD4 1st bromodomain	HSQC	mhhhhhhssgvdlgtenlyfqsmNPPPPETSNPNKPKRQTNQLQYLLRVVLKTLWK HQFAWPFQQPVDAVKLNLPDYYKIIKTPMDMGTIKKRLENNYYWNAQECIQD FNTMFTNCYIYNKPGDDIVLMAEALEKLFLQKINELPTEE	self-prepared	red: TEV cleavage site
BRD4-BrD2 (331-460)	BRD4 2nd bromodomain	HSQC	mhhhhhhssgvdlgtenlyfqsmSSKVSEQLKCCSGILKEMFAKKHAAYAWPFYKPV DVEALGLHDYCDIIKHPMDMSTIKSKLEAREYRDAQEFGADVRLMFSNCYKY NPPDHEVVAMARKLQDVFEMRFAKMPDE	self-prepared	red: TEV cleavage site
Peptides					
H3K27me3	H3K27me3 (21-33)	FP	ATKAAR-Kme3-SAPATG	Genscript	
SETDB1-K1170me3	SETDB1-K1170me3 (1165-1174)	FP	RGFAL-Kme3-STHG	Genscript	
RNAs					
FAM-ANRIL-LoopC	FITC-ANRIL-LoopC	FP (fluorescence probe)	56-FAMN-rUrGrGrArGrUrUrGrCrGrUrUrCrCrA	IDT	
ANRIL-LoopC	ANRIL-LoopC	FP	rUrGrGrArGrUrUrGrCrGrUrUrCrCrA	Thermo Scientific	
FAM-Random 16mer	FITC-16mer_RNA	FP (fluorescence probe)	56-FAMN-rCrArArGrArArGrGrGrUrCrUrCrCrCrA	IDT	