Supporting information

Rational Design of Selective Adenine Based Scaffolds For Inactivation of Bacterial Histidine Kinases

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Contents **Page No.**

MULTIPLE SEQUENCES ALIGNMENT

Figure S1: Multiple sequence alignment of different histidine kinases (HKs); HK853 (*Thermotoga maritima*) co-crystallized with ADPβN (PDB:3DGE), WalK (*Bacillus subtilis*) co-crystallized with ATP (PDB:3SL2) and PhoQ (*Escherichia coli*) co-crystallized with AMP-PNP (PDB:1ID0) using Clustal Omega.[1, 2] Within the sequences, the different parts of the Bergerat fold (namely N, G1, G2, G3, F boxes, flexible loop) are indicated within boxes. An * (asterisk) indicates positions which have a single, fully conserved residue. A : (colon) indicates conservation between groups of strongly similar properties, roughly equivalent to scoring > 0.5 in the Gonnet PAM 250 matrix. A . (period) indicates conservation between groups of weakly similar properties, roughly equivalent to scoring =< 0.5 and > 0 in the Gonnet PAM 250 matrix.

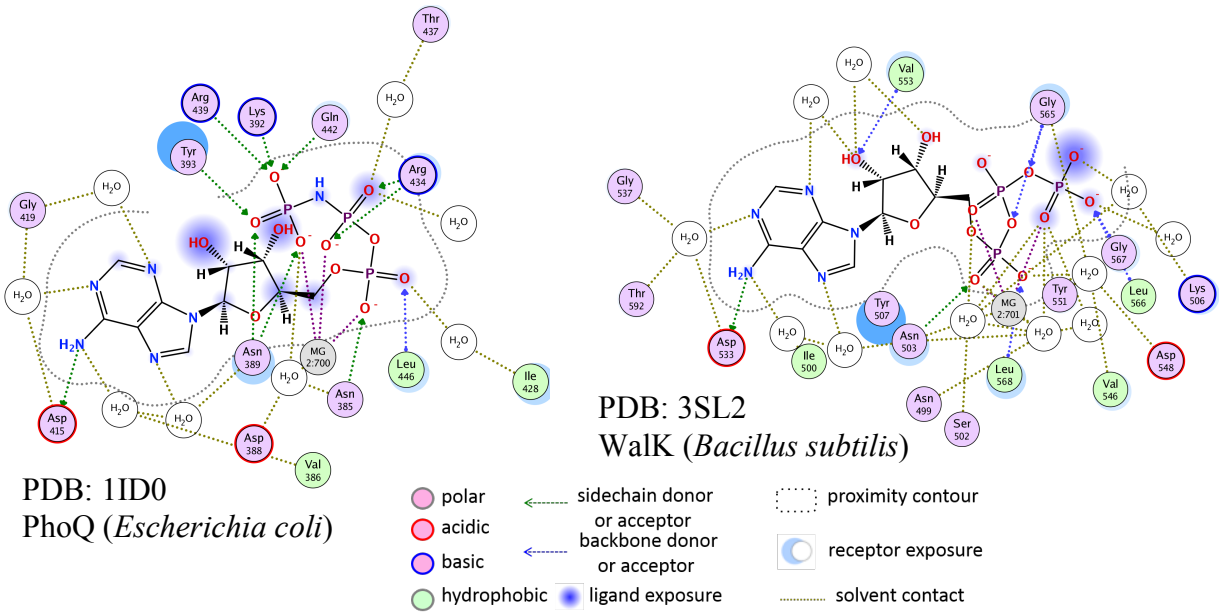


Figure S2: Ligand-protein interactions generated with MOE program for HK co-crystal structures (PDB: 1ID0, PDB: 3SL2). Legend of the possible ligand interactions is shown.

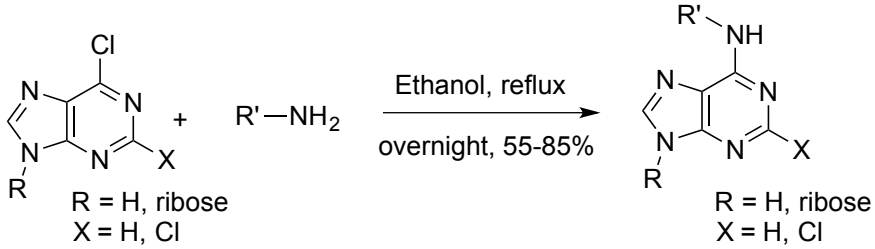
METHODS AND MATERIALS

Adenine inhibitor library

Compounds **1-11**, **13-22**, **25-27**, **30**, **S2**, guanine, guanosine, hypoxanthine, inosine, Ibrutinib, Idelalisib were purchased and used without further purification. Compounds **12**, **23-24**, **28-29**, **S1** were synthesized. Previously reported methods were followed for the syntheses of **24**,[3] **28-29**,[4] **S1**[5] and the NMR/MS spectra of these compounds matched literature. The details of new compounds are given below. All molecules were >95% purity as judged by HPLC analysis (Instrument: Agilent HPLC 1200 series, column: Agilent Eclipse XDB-C18, 5 µm, 9.4x250 mm, gradient run of 20 min with 95% water to 100% acetonitrile, modifier: 0.1% Formic acid). In addition, our scaffold was assessed for the presence of PAINs characteristics as described.[6]

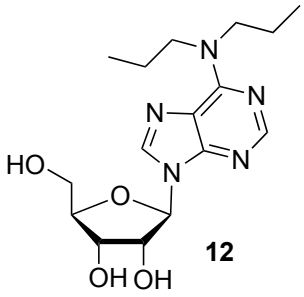
Syntheses of compounds

General scheme for N-6 substituted purines



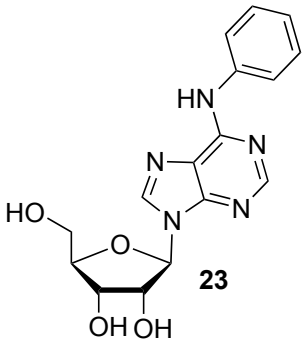
The *N*-6 substituted purines were synthesized following previous protocols.[7] In brief, to chloropurine or chloropurine riboside was added the amine (3 equiv., 3-5 mmol) and ethanol. This mixture was refluxed overnight (>16 h). After completion of reaction was confirmed by TLC, the solvent was evaporated *in vacuo* and the residue purified by column chromatography with EtOAc:MeOH (gradient solvent from 12:1 to 9:1) as solvent system. For characterization,

NMRs were taken on 500 MHz Bruker instrument equipped with a cryoprobe. ESI-MS was performed on an Agilent UPLC-QTOF instrument in positive ionization mode.



12

6-N,N dipropylaminopurine riboside (white crystalline solid, M.Pt. 136-138°C, 72%). HNMR (CD_3CN) ^{1}H NMR (500 MHz, CD_3CN) δ 8.14, 7.92 (2s, 2H, adenine ring Hs), 5.96 – 5.83 (m, 1H, H-1 of ribose), [5.78 (d, *J* = 7.1 Hz, 1H), 4.82 (td, *J* = 6.8, 5.0 Hz, 1H), 4.29 (td, *J* = 3.1, 1.5 Hz, 1H), 4.14 (d, *J* = 1.6 Hz, 1H), 3.72 – 3.62 (m, 4H), 3.52 (d, *J* = 3.15 Hz, 1H) ribose ring Hs, 2x -N-C**H$_2$**CH_2CH_3] , 1.70 (h, *J* = 7.4 Hz, 4H, 2x -N-CH_2C**H$_2$**CH_3), 0.93 (t, *J* = 7.4 Hz, 6H, 2x -N-CH_2CH_2C**H$_3$**).^{13}C NMR (126 MHz, CD_3CN) δ 155.35, 152.35, 150.25, 139.93, 121.68 (5C, adenine ring Cs), 91.43, 88.41, 74.21, 72.89 (5C, ribose ring Cs), 63.61, 11.36 (2C, -N-CH_2**C**H_2**C**H_3). Due to the fast tumbling of tertiary amines -N-**C**H_2 was not observed in the C-NMR. ESI-MS: expected (M+H) = 351.1907, found = 351.1898.



23

2-Chloro, 6-N anilinopurine riboside (while amorphous solid, 85%). HNMR (CD_3CN): ^{1}H NMR CD_3CN) δ 8.38, 8.11 (2s, 2H, adenine ring Hs), 8.26 (s, 1H, -N**H**-Ph), [7.91 – 7.80 (d, *J* = 7.9 Hz 2H), 7.39 (t, *J* = 7.4 Hz, 2H), 7.17 – 7.10 (t, *J* = 7.4 Hz, 1H) Ph ring Hs] , 5.87 (d, *J* = 6.8 Hz, 1H, anomeric H-1 of ribose), [4.80 (t, *J* = 6.0 Hz, 1H), 4.33 (s, 1H), 4.16 (s, 1H), 3.87 – 3.63 (m, 1H), 3.56 (s, 1H), ribose-ring Hs]. ^{13}C NMR (126 MHz, CD_3CN) δ 153.79, 152.72, 149.62, 142.26 (5C, adenine ring Cs), 139.82, 129.64, 124.60, 122.17, 119.45 (4C, Ph-ring Cs), 91.40 (1C, C-1 of ribose), 88.32, 74.75, 72.71, 63.46 (4C, ribose ring Cs). ESI-MS: expected (M+H) = 343.1281, found = 343.1289.

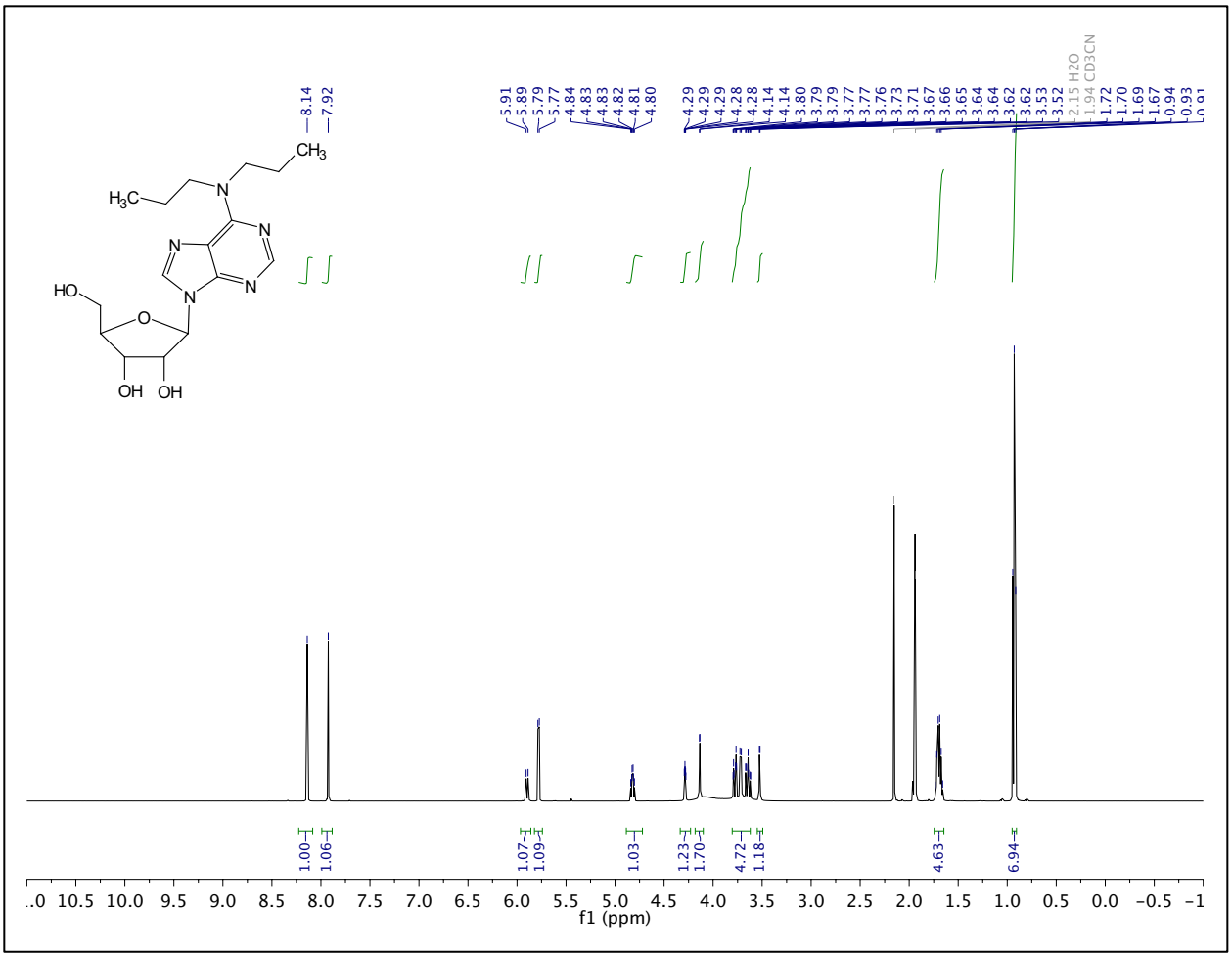


Fig. ^{1}HNMR of **12**

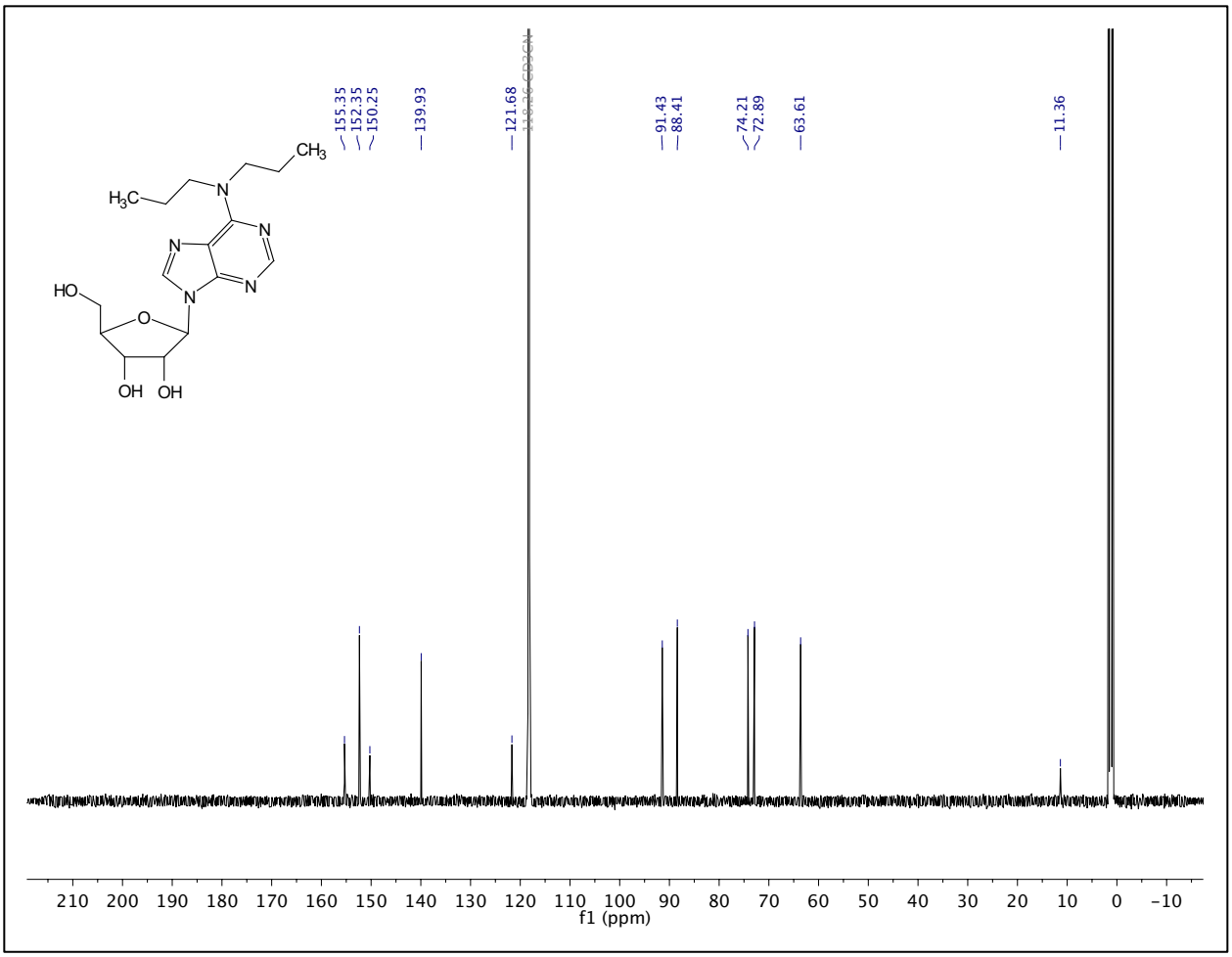


Fig. ^{13}CNMR of **12**

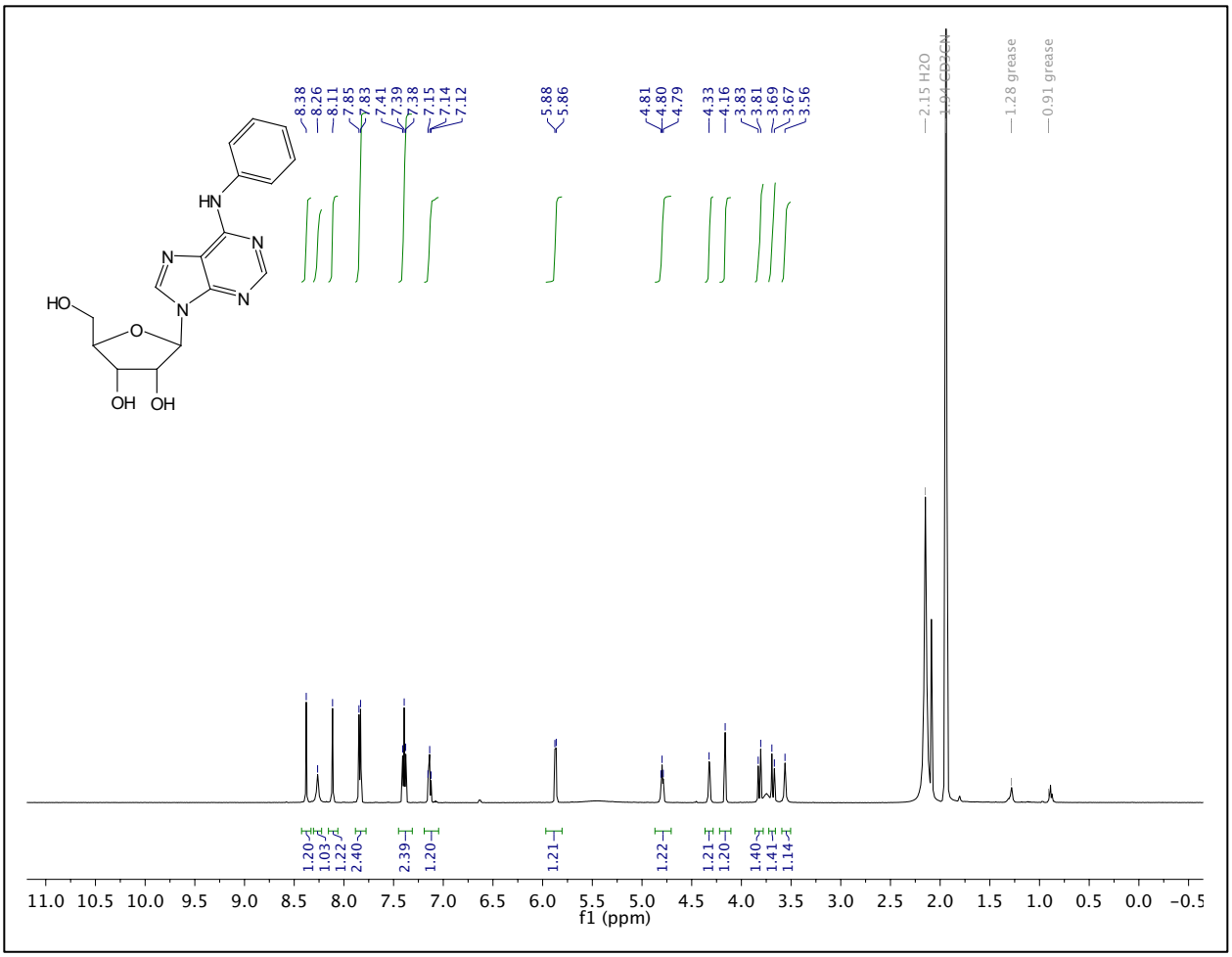


Fig. ^{1}HNMR of **23**

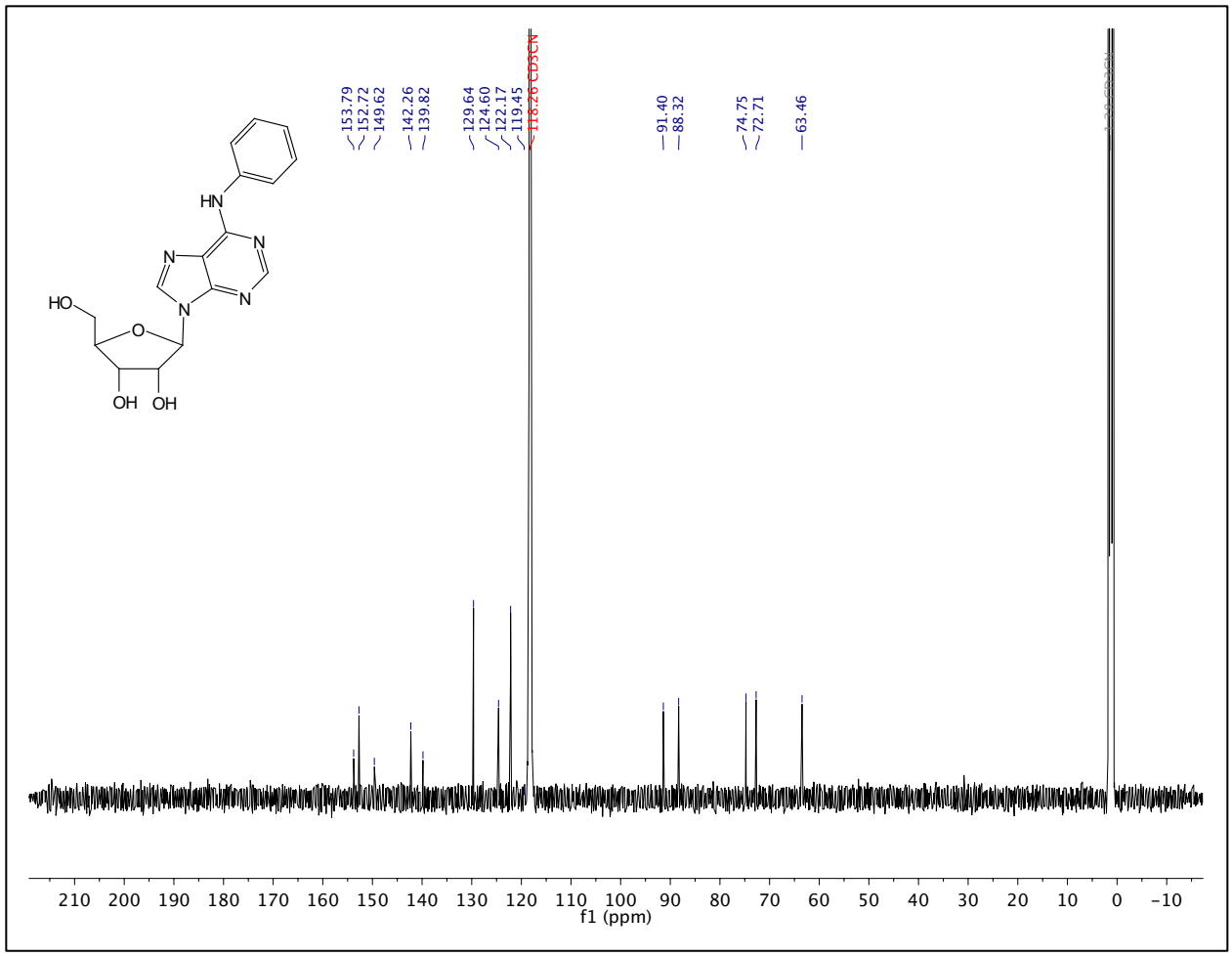


Fig. ^{13}CNMR of **23**

Protein Production

HK853 overexpression and purification. HK853 in the pHis-parallel vector was prepared as described previously.[8] DNA was transformed into competent BL21(DE3)-pLysS Rosetta *E. coli* cells. Transformed *E. coli* cells were plated overnight on lysogeny broth (LB) agar containing 100 μg mL^{-1} ampicillin (amp) and 34 μg mL^{-1} chloramphenicol (Cm). A single colony was transferred to 100 mL sterile LB media in a 250-mL flask supplemented with antibiotics and incubated at 37 °C overnight at 220 rpm. At OD_{620} of 0.4–0.6, 15 mL was transferred to 1 L sterile LB broth containing antibiotics in 2.8-L baffled flasks. Cultures were grown by shaking at 220 rpm at 37 °C to an OD ~0.6. After equilibrating to 20 °C for 3 h, HK853 overexpression was induced with 0.22 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) (Calbiochem) and incubation at 20 °C for 16 h at 220 rpm. Cells were collected by centrifugation at 8000 *x g* for 20 min, resuspended in 10 mL of buffer (25 mM Tris-HCl, pH 8, 500 mM NaCl, 10% glycerol, and 2 mM DTT), and quickly frozen on dry ice for storage at –80 °C.

For purification, each pellet from 1 L of culture was resuspended in a total volume of ~50 mL lysis buffer (25 mM Tris-HCl, pH 8, 500 mM NaCl, 10% glycerol, and 2 mM DTT) containing 20 units Deoxyribonuclease I (Sigma) and four Complete Mini EDTA-free protease inhibitor tablets (Roche). Resuspended cells were lysed by a Branson Sonifier 250 with 1/8-inch

tapered microtip (power setting 3.5, duty cycle 30%) for 1 h 20 min on ice. Lysate was centrifuged at 14,000 x *g* for 40 min at 4 °C. The supernatant was collected and filtered (0.22 µm). Using an AKTApurifier (GE Healthcare) at 4 °C, HK853 was purified from lysate by nickel affinity on a nickel-nitriloacetic acid column (Ni-NTA; Qiagen). Ni-NTA buffer was 25 mM Tris-HCl, pH 8, 500 mM NaCl, 10% glycerol, and 2 mM DTT. An elution gradient of 5 mM imidazole (buffer A) to 1 M imidazole (buffer B) was used to elute His-tagged protein. Eluted HK853 was concentrated for size exclusion chromatography on a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) using 10 mM Tris-HCl, pH 7.6, 0.1 mM EDTA, 0.5 M NaCl, 12% glycerol, and 2 mM DTT. This buffer was also used for storage of protein at –80 °C, in which protein was flash frozen on dry ice/isopropanol. Protein concentration was determined using the DC Protein Assay (Bio-Rad).

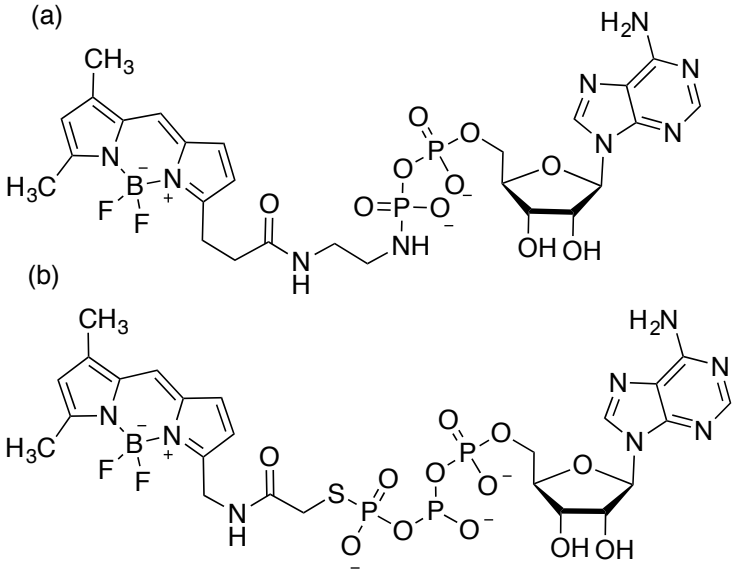


Figure S3: Probes used in inhibition assays. (a) ADP-BODIPY, (b) BODIPY-ATPγS.

***Reaction buffer*.** Used in all assays, the reaction buffer was composed of 50 mM Tris-HCl, pH 7.8, 200 mM KCl, 5 mM $MgCl_2$.

Protein Storage Buffer. Buffer for the storage of protein was prepared as 10 mM Tris-HCl, pH 8, 0.1 mM EDTA, 0.5 M NaCl, 12% glycerol, 2 mM DTT.

Determination of protein concentration. Protein stock concentrations were determined by a DC Protein Assay (Bio-Rad) according to the instruction manual and with BSA as a standard. The concentrations of at least two dilutions of protein stock were determined and averaged. Where indicated, protein concentration was also determined using a Implen's Nanophotometer spectrophotometer (Thermo Fisher Scientific) at 280 nm and Beer's Law,

$$A=\varepsilon c\ell \qquad \text{(Equation 1)}$$

where A is absorbance, ε is the protein extinction coefficient ($M^{-1}cm^{-1}$), c is concentration (M), and ℓ is pathlength (cm).

Determination of nucleotide and adenine concentration. After preparing nucleotide working stock solutions in water (or adenine in DMSO), concentrations were confirmed using Beer's Law (Equation 1) by measuring the absorbance on a Implen's Nanophotometer (adenine extinction

coefficient of 15,400 M^{-1} cm^{-1} at 259 nm). For higher concentrations (*i.e.*, millimolar), dilutions (usually 1:100 and 1:1000) were measured and the final concentration averaged. Nucleotide solutions were always prepared fresh.

SDS-PAGE. 2X SDS-PAGE sample loading buffer contained 125 mM Tris, pH 6.8, 20% glycerol, 4% SDS (w/v), 5% 2-mercaptoethanol, and 0.2% bromophenol blue (w/v). Tris-glycine stacking gels were prepared with a 10% polyacrylamide resolving gel and 4.5% polyacrylamide stacking gel. Running parameters were 180 V, 400 mA, and 60 W for 1 h 20 min. SDS-PAGE running buffer was diluted ten-fold from Novex 10X Tris-Glycine SDS Running buffer (Invitrogen) and pre-chilled prior to electrophoresis.

Native-polyacrylamide gel electrophoresis (Native-PAGE). Native-PAGE sample loading buffer contained 40 mM Tris, pH 7.5, 8% glycerol, and 0.08% Bromophenol blue (w/v). Native-PAGE gels were 7.5% polyacrylamide Tris-glycine resolving gels. Running parameters were 180 V, 400 mA, and 60 W for 1 h 20 min. The pre-chilled electrophoresis running buffer was 83 mM Tris, pH 9.4, and 33 mM glycine.

Gel fluorescence detection. After SDS-PAGE, gels were washed three times with MQ water. They were scanned on a Typhoon Variable Mode Imager 9500 (GE) using 526-nm (short-pass filter) detection for BODIPY (λex: 504 nm, λem: 514 nm).

Coomassie staining. Each step was carried out at room temperature (RT) with an orbital shaker. After SDS-PAGE, gels were washed three times with MQ water and submerged in enough coomassie stain (0.1% (w/v) Coomassie Brilliant Blue R-250, 10% acetic acid, 40% methanol) to cover the gel and incubated for 10 min. Stain was removed, and destain (10% acetic acid, 40% methanol) was added to gel and incubated 30 min. After removing destain, gel was washed in water overnight. After staining, they were scanned on a Typhoon Variable Mode Imager 9500 (GE) using coomassie stain settings.

Silver staining. Native-PAGE gels were silver stained. All steps were carried out at RT with an orbital shaker. The gels were stained using a Pierce™ Silver Stain Kit (ThermoFisher Scientific) following manufacture's protocol. After staining/destaining, they were scanned on a Typhoon Variable Mode Imager 9500 (GE) using silver stain settings.

Inhibition of HK853 Activity

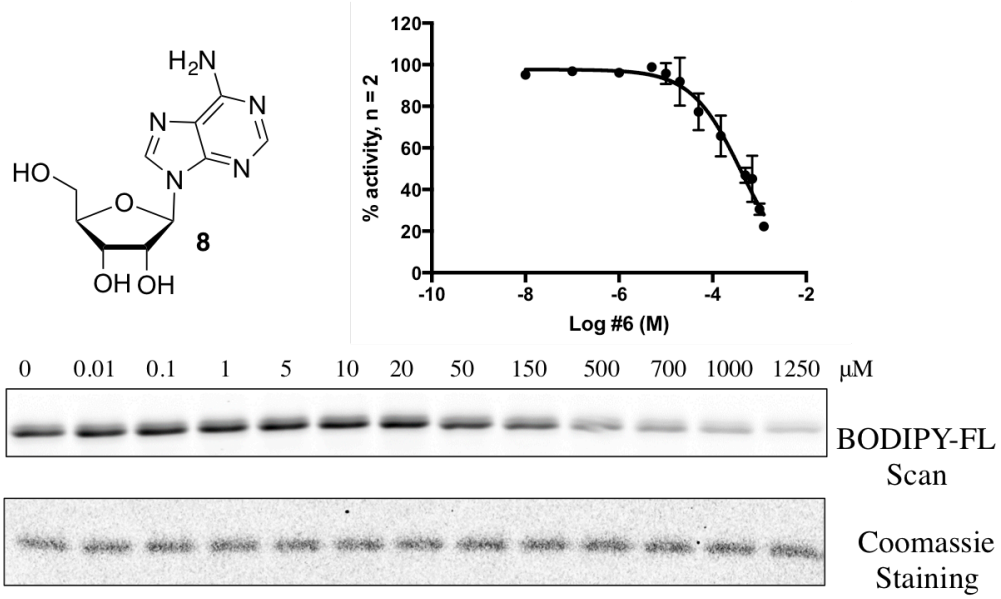
BODIPY-ATPγS competition screening was performed at inhibitor concentrations that did not cause aggregation. Triton X-100 was premixed with reaction buffer to yield 0.1% (v/v) in final 25-μL reactions. In reaction buffer, 1 μM HK853 was preincubated with test compounds (final concentration, 0.01−1250 μM) in 24 μL for 30 min. 1 μL BODIPY-ATPγS was added to bring the final 25-μL reactions to 0.96 μM HK853 and 2 μM BODIPY-ATPγS in the presence of competitors and 5% DMSO. Samples were mixed and incubated in the dark at RT for 1 h before quenching with 8.6 μL 4× SDS-PAGE sample loading buffer and loading 15 μL on a 10% stacking gel. After SDS-PAGE, in-gel fluorescence detection elucidated HK853 activity, and coomassie staining of the gels ensured even protein loading. Integrated density values of the fluorescent gel bands were normalized as "% Activity" with respect to a control that contained no inhibitor. Data were plotted in GraphPad Prism with relation to the log of molar inhibitor to determine IC_{50} values (Equation 2).

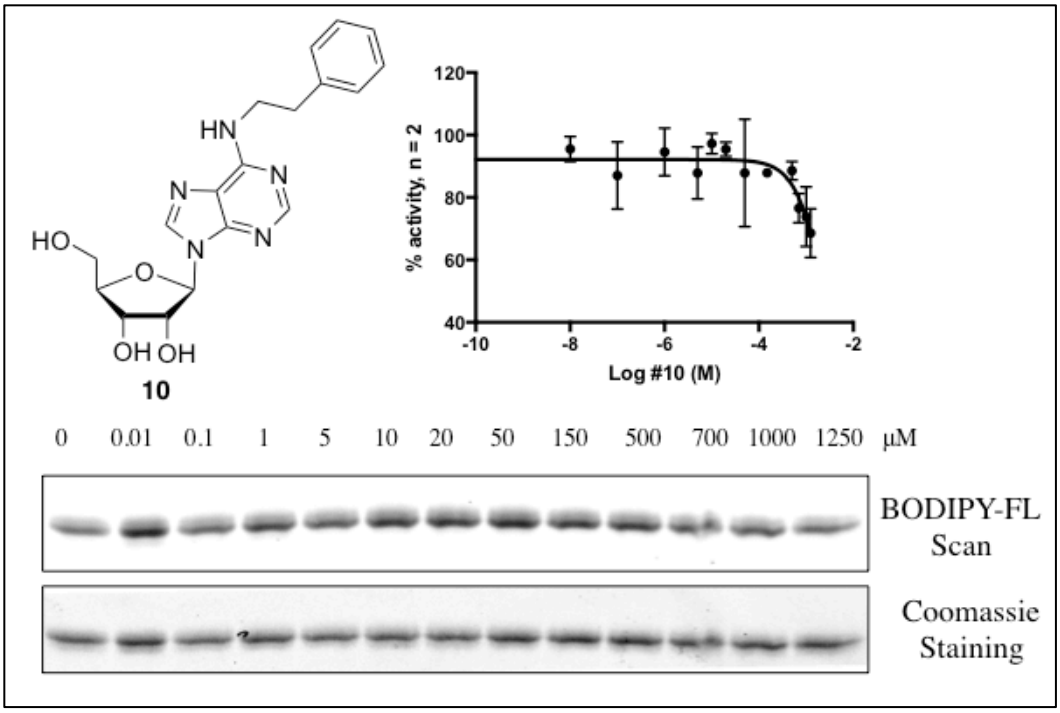
Data analysis. Integrated density measurements of in-gel fluorescence and phosphorescence were performed in ImageJ.[9] Data were prepared and analyzed in GraphPad Prism (version 7.0 for

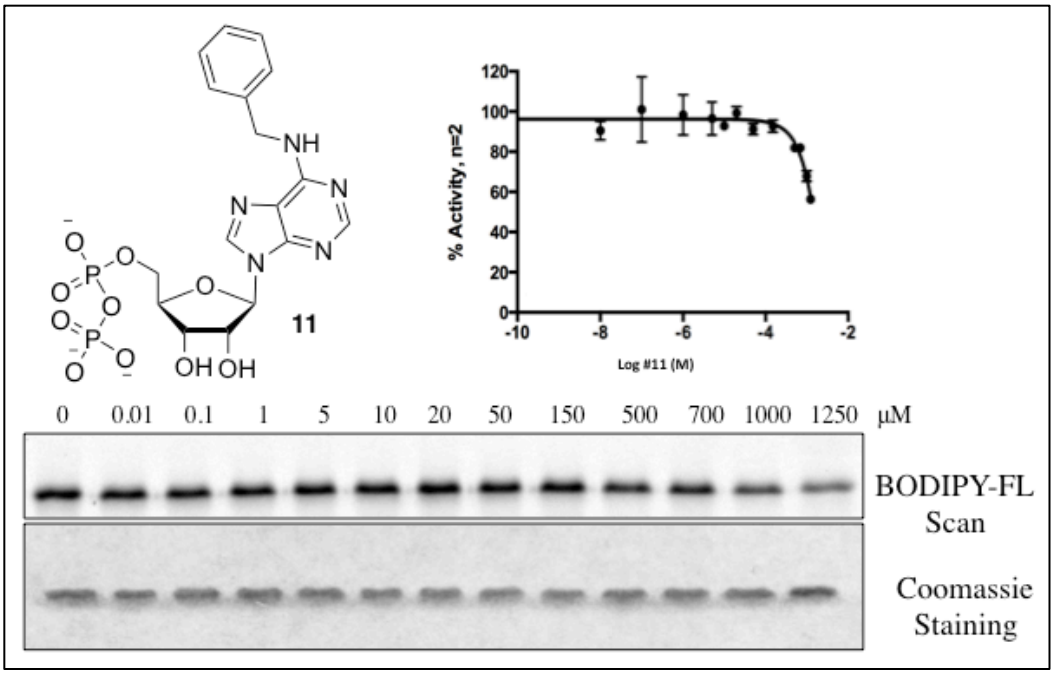
Mac, GraphPad Software, San Diego, California USA, www.graphpad.com). For all DRCs (control FP competition and activity assays), data were fit to a four-parameter logistic equation,

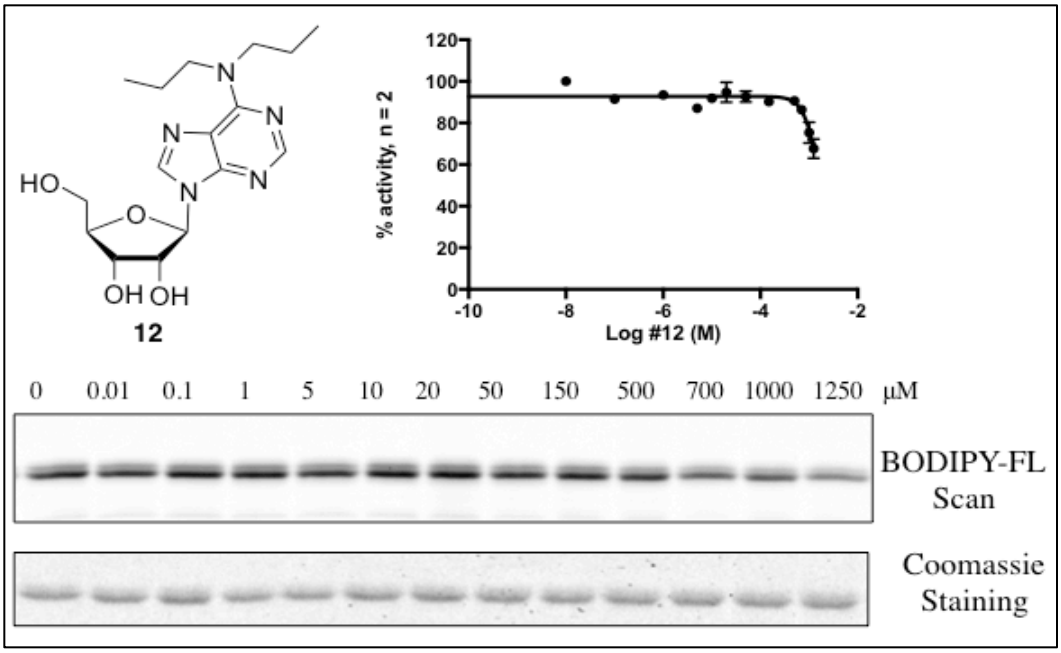
$$y = Bottom + \frac{(Top - Bottom)}{1+10^{\left((LogIC_{50} - x) * HillSlope\right)}} \qquad \text{(Equation 2)}$$

where *y* is the response, *Bottom* and *Top* are plateaus in the units of the y-axis, *x* is the log of the molar concentration of inhibitor, *HillSlope* is the slope of the curve, and IC_{50} is the concentration of compound required for 50% inhibition (a response half way between *Bottom* and *Top*). Some compounds exhibited incomplete DRCs because going to higher concentrations would increase the required DMSO or cause protein aggregation. Visually, this meant there was no curve plateau for the "Bottom" value. However, IC_{50} values were desirable for purposes of comparison to other compounds. As a result, IC_{50} values were estimated by constraining the bottom of the curve to "0."




HO
HN
N
N
N
N
O
OH OH
10
% activity, n = 2
120
100
80
60
40
-10
-8
-6
-4
-2
Log #10 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining


NH
N
N
N
N
O
O
O
O
O
O
O
O
O
OH OH
11
% Activity, n=2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #11 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining


HO
OH OH
12
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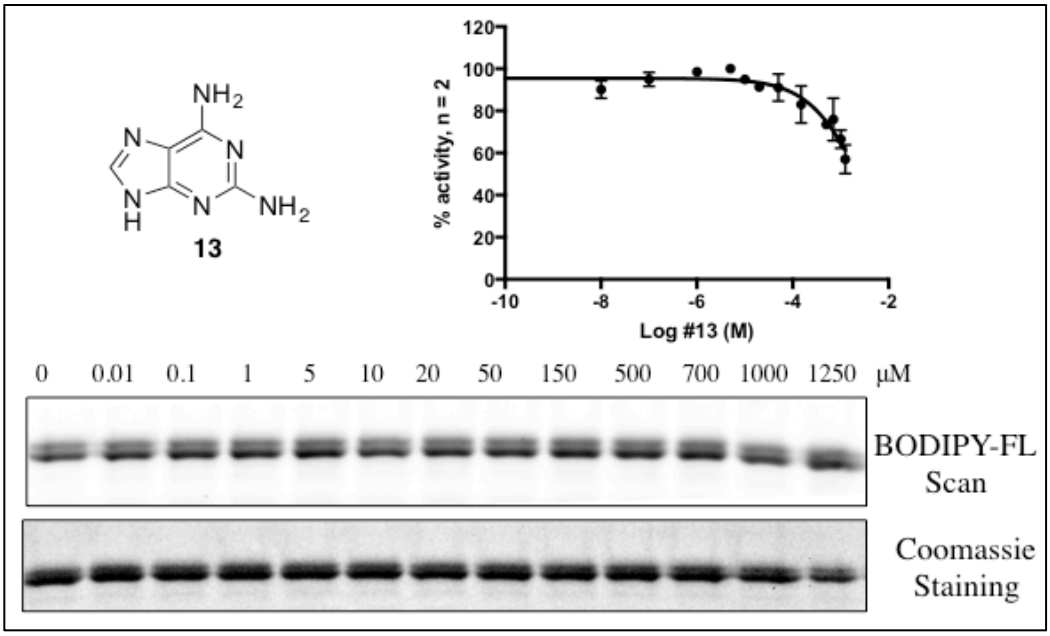
NH2
NH2
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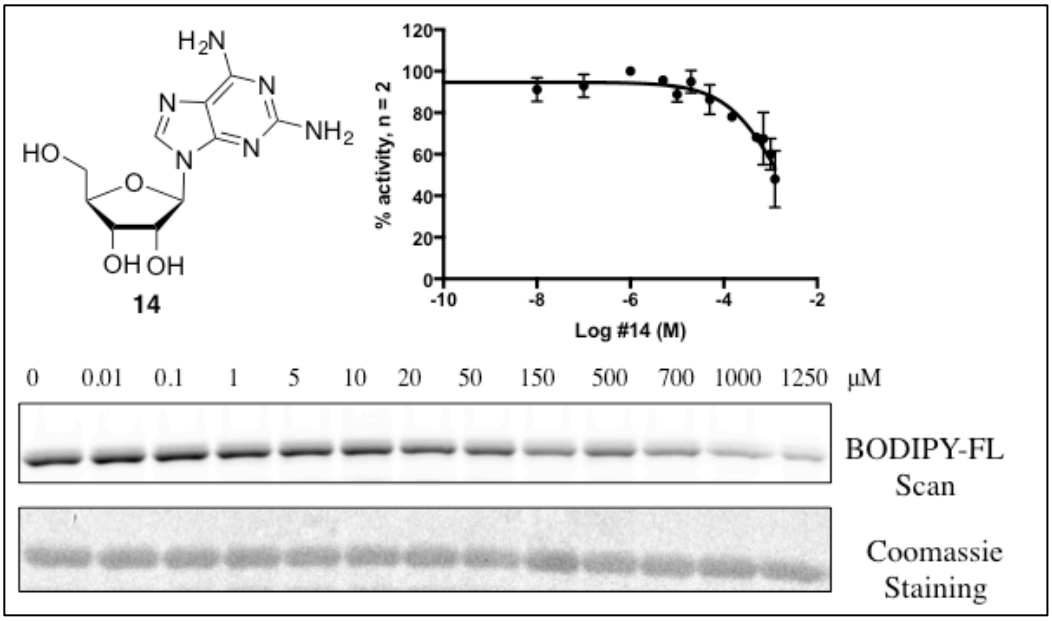
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N
NH2
HO
N
N
O
OH OH
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100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #14 (M)
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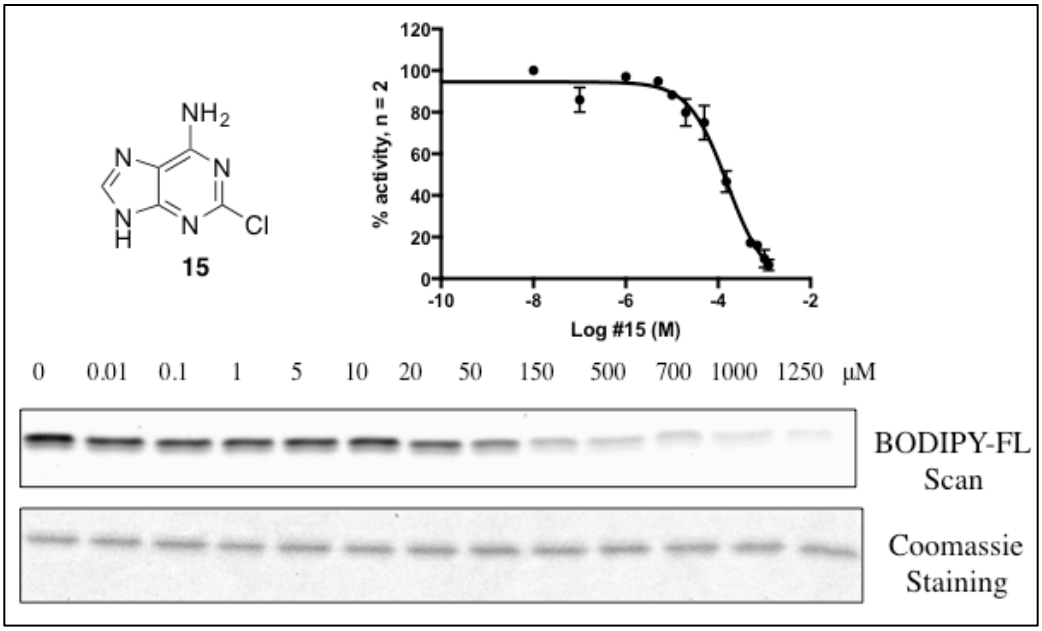
BODIPY-FL Scan
Coomassie Staining


NH2
N
N
N
H
N
Cl
15
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80
60
40
20
0
-10
-8
-6
-4
-2
Log #15 (M)
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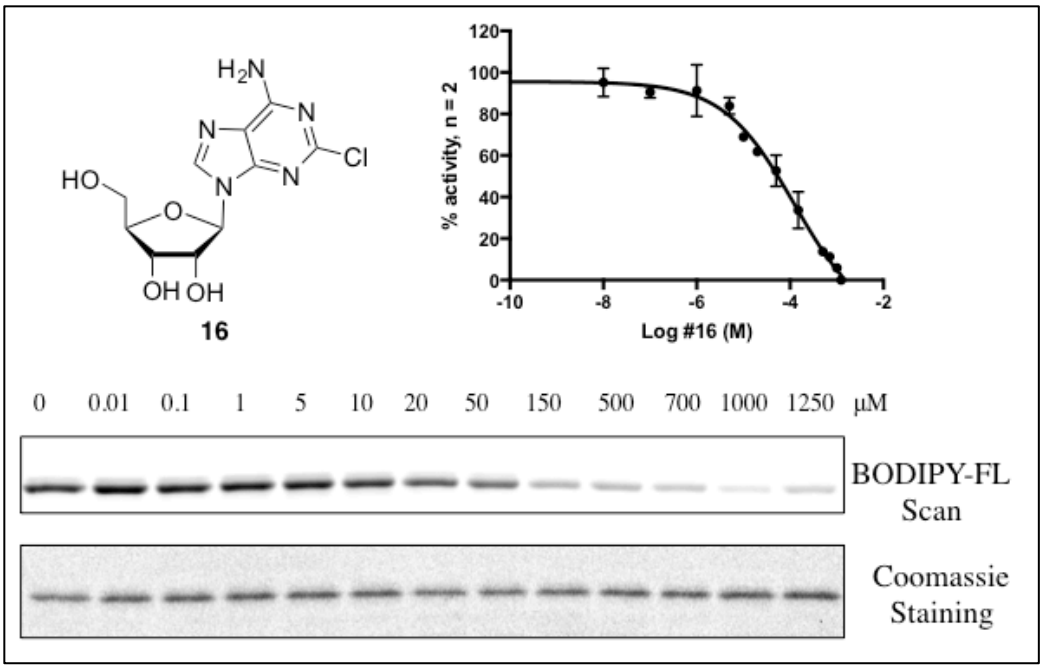
BODIPY-FL Scan
Coomassie Staining


H2N
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N
Cl
HO
N
O
OH OH
16
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100
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60
40
20
0
-10
-8
-6
-4
-2
Log #16 (M)
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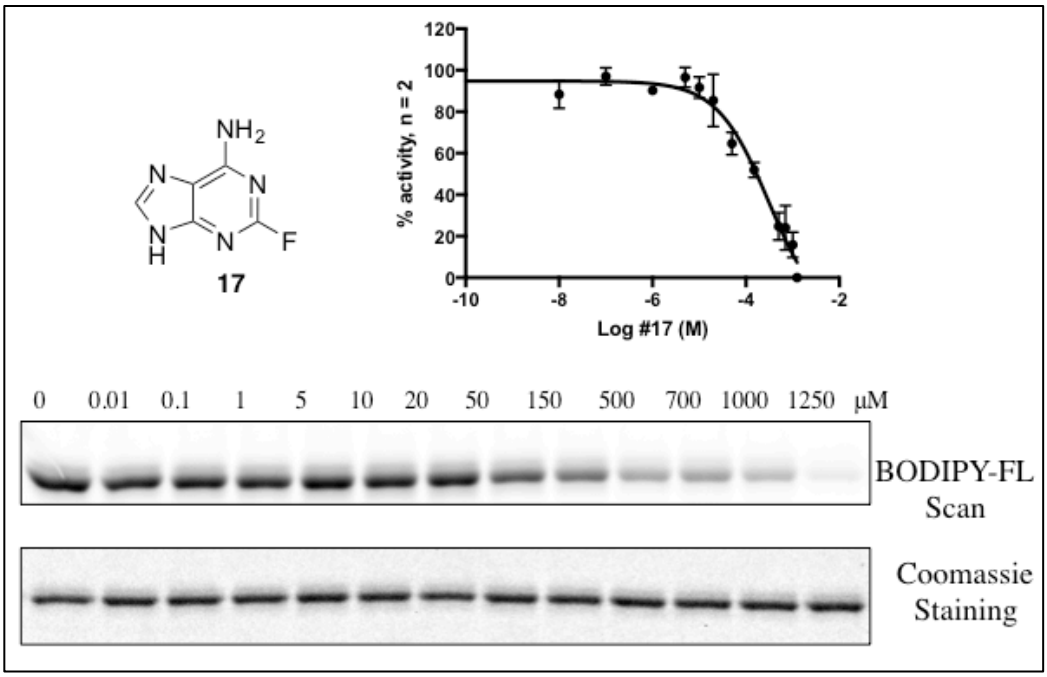
BODIPY-FL Scan
Coomassie Staining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N
N
H
N
F
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100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #17 (M)
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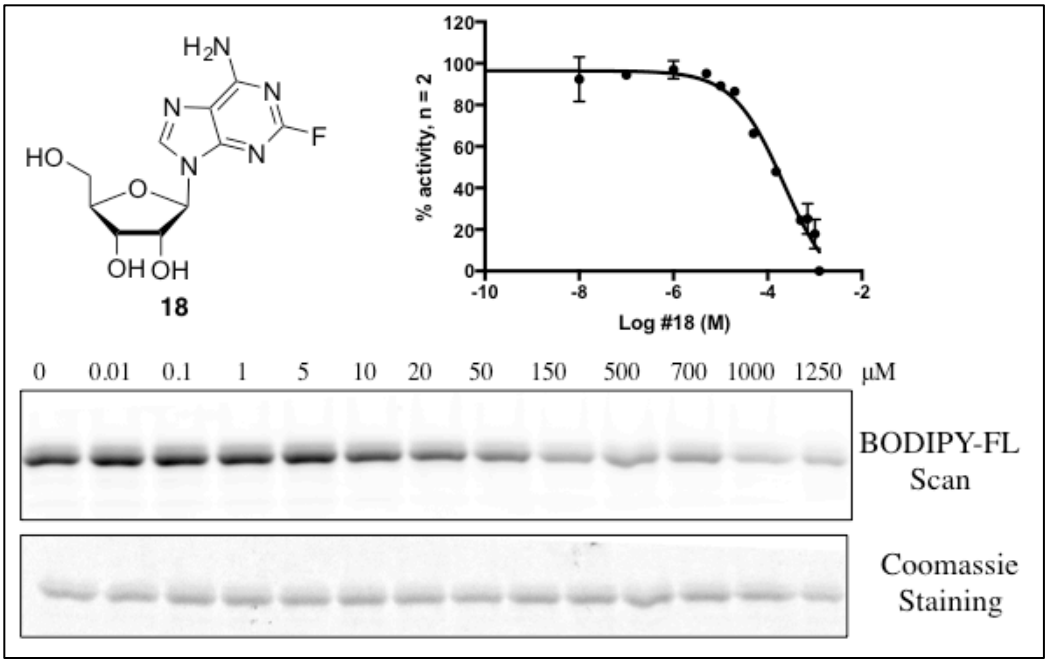
BODIPY-FL Scan
Coomassie Staining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Log #18 (M)
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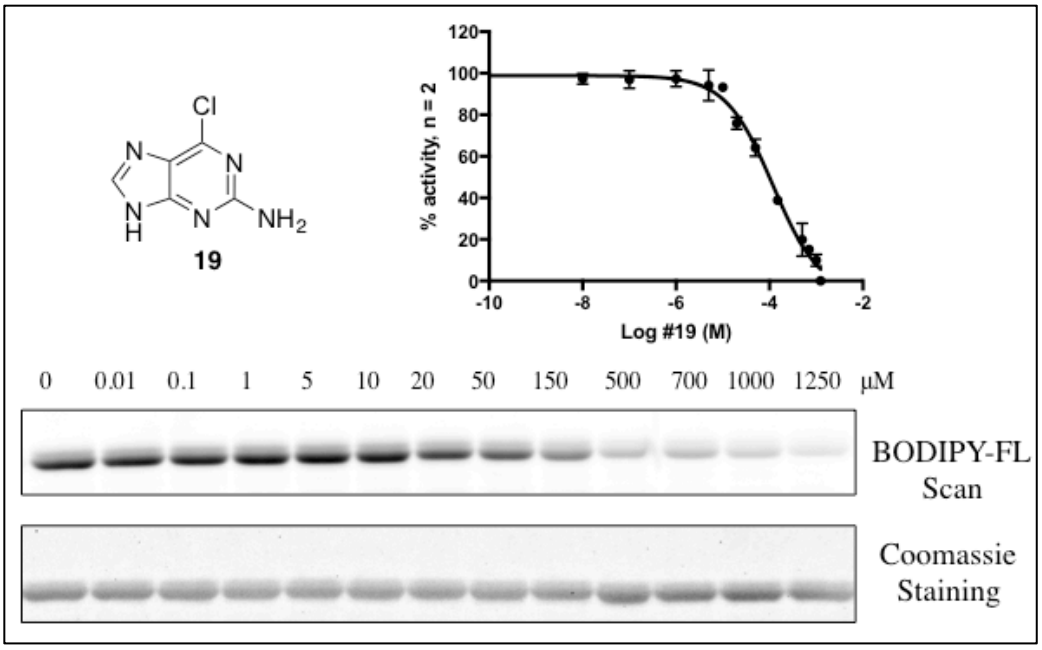
BODIPY-FL Scan
Coomassie Staining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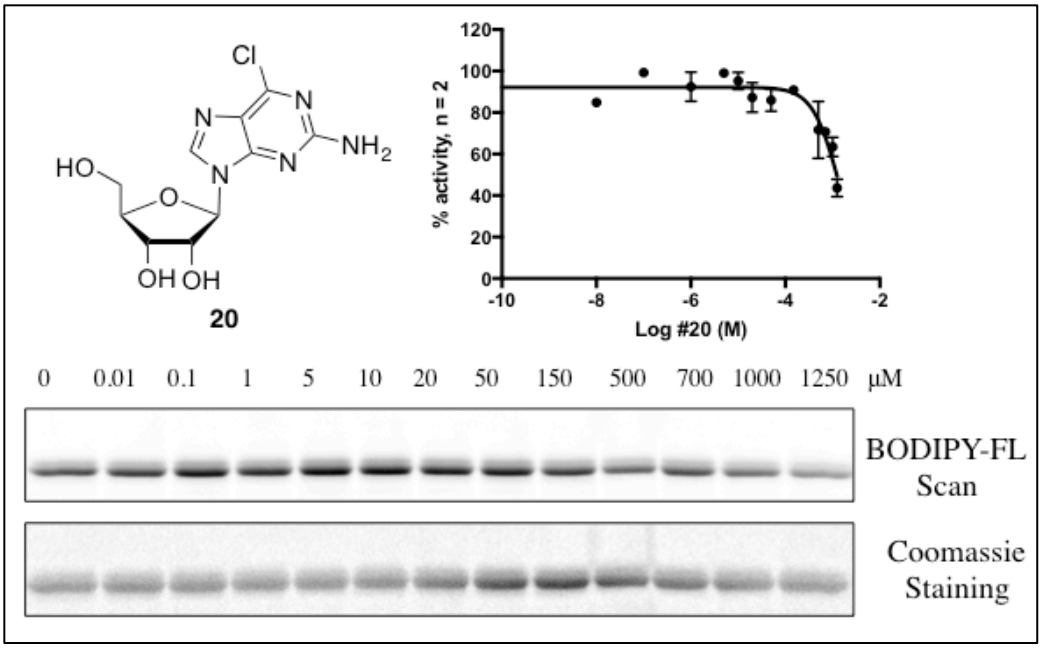
BODIPY-FL Scan
Coomassie Staining


Cl
N
N
NH2
HO
N
N
O
OH OH
20
120
100
80
60
40
20
0
% activity, n = 2
-10
-8
-6
-4
-2
Log #20 (M)
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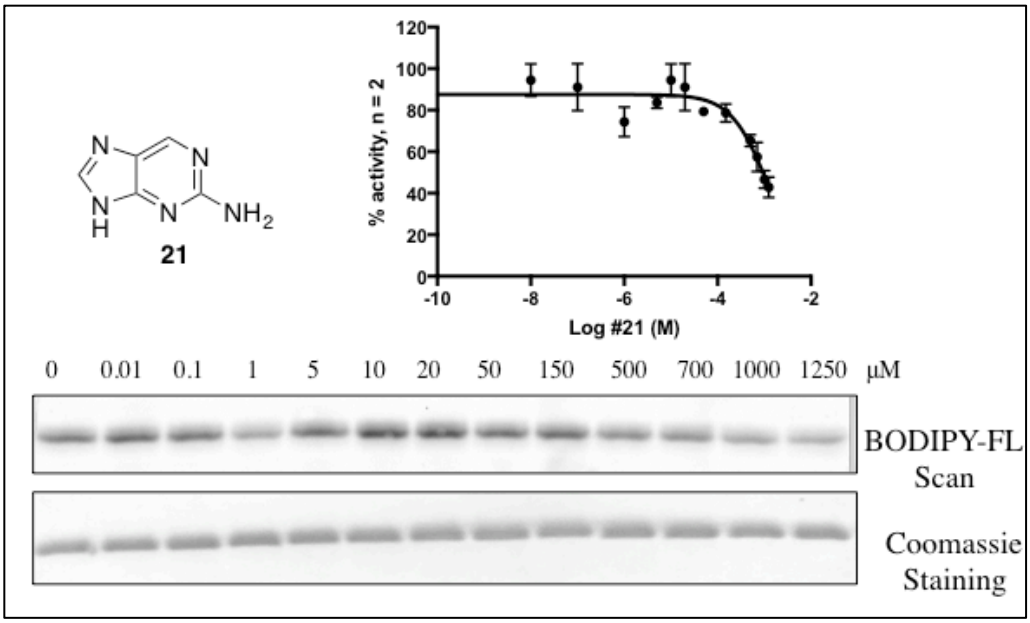
BODIPY-FL Scan
Coomassie Staining


N
N
H
N
N
NH2
21
120
100
80
60
40
20
0
% activity, n = 2
-10
-8
-6
-4
-2
Log #21 (M)
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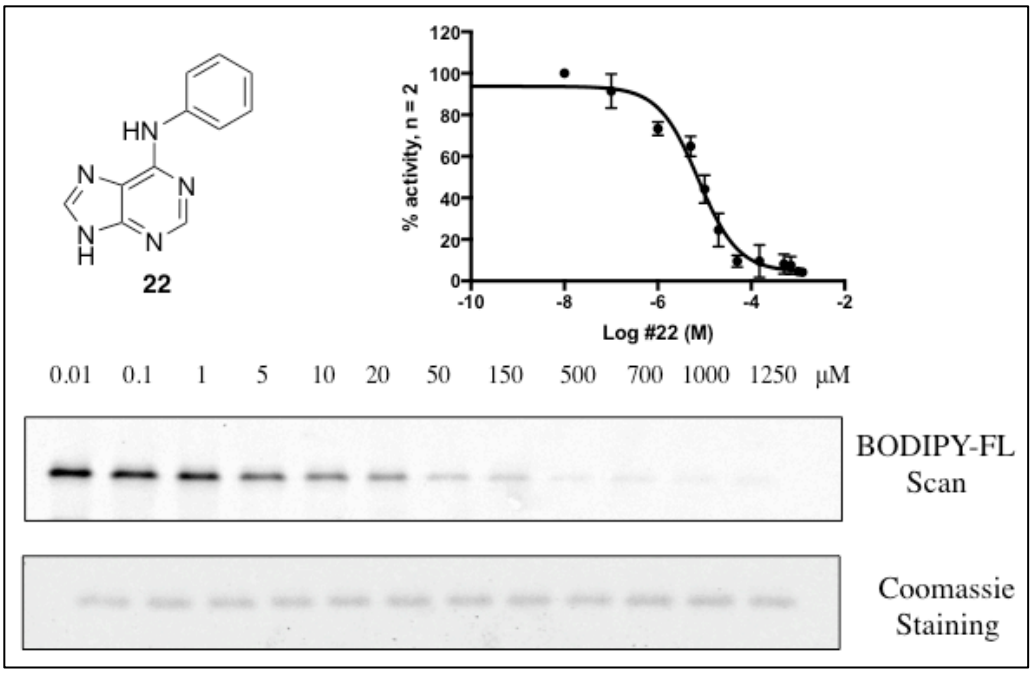
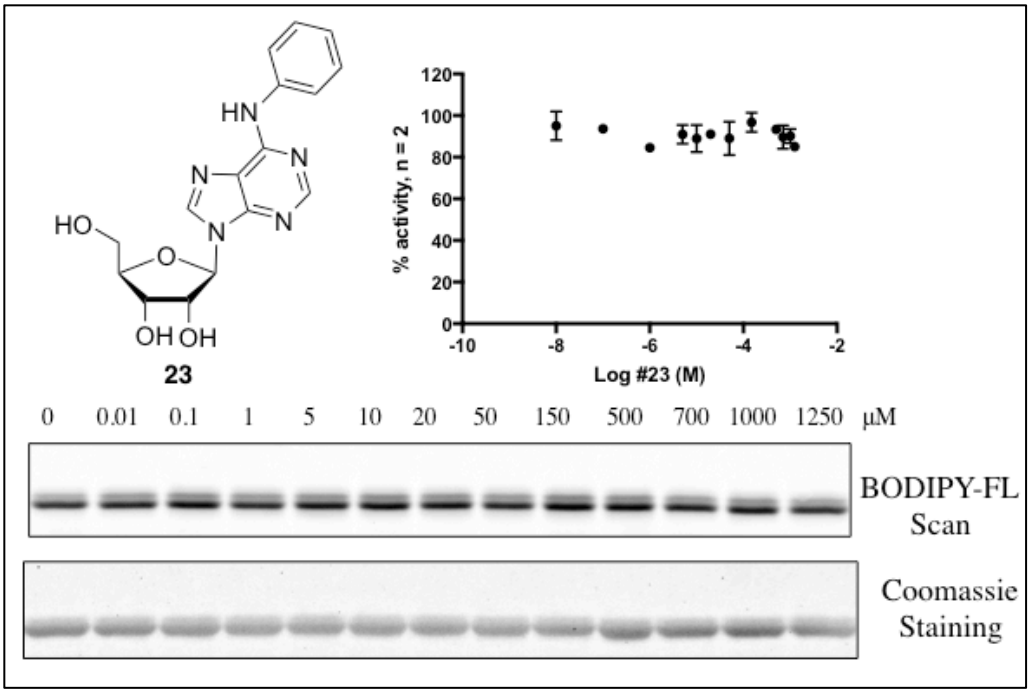
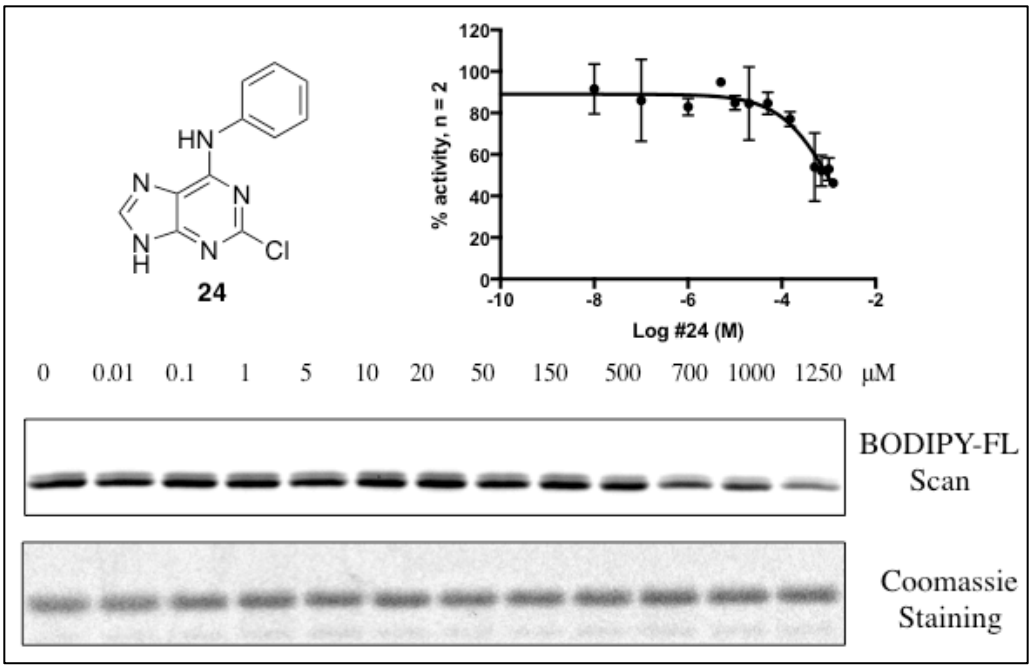
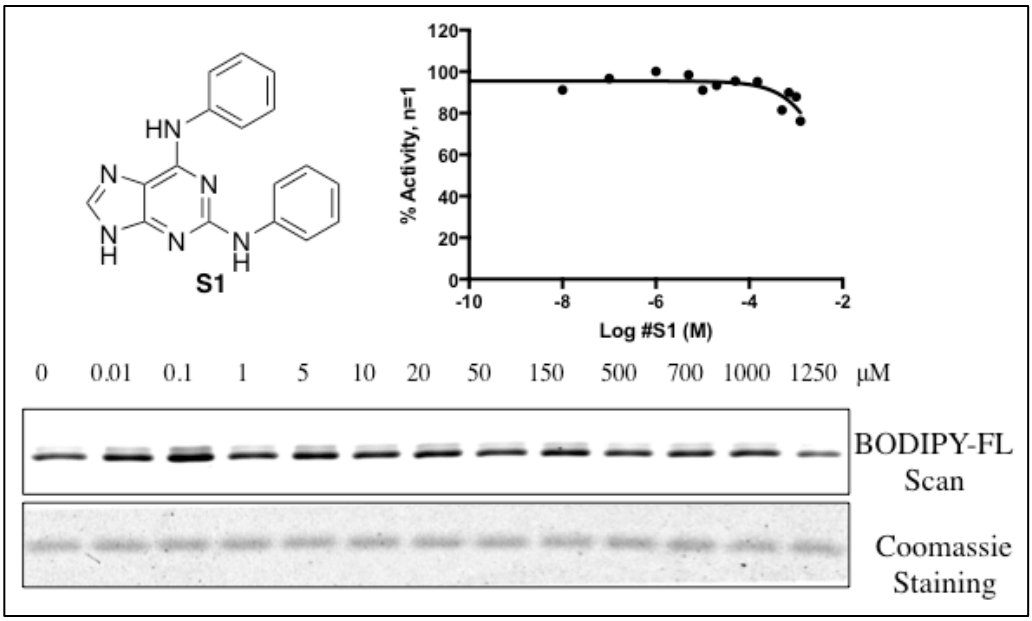
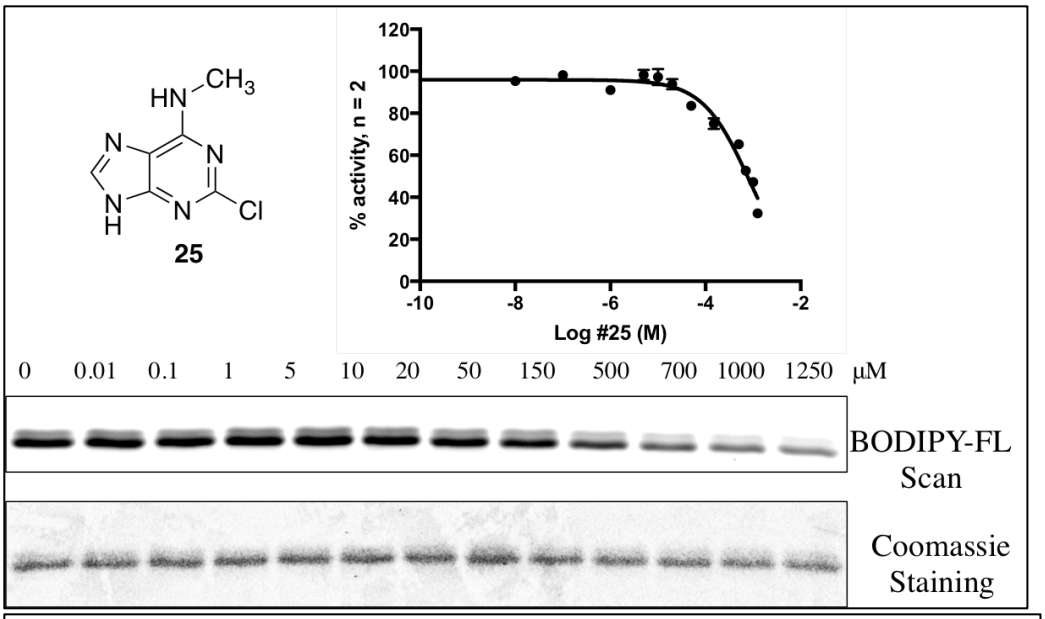
BODIPY-FL Scan
Coomassie Staining

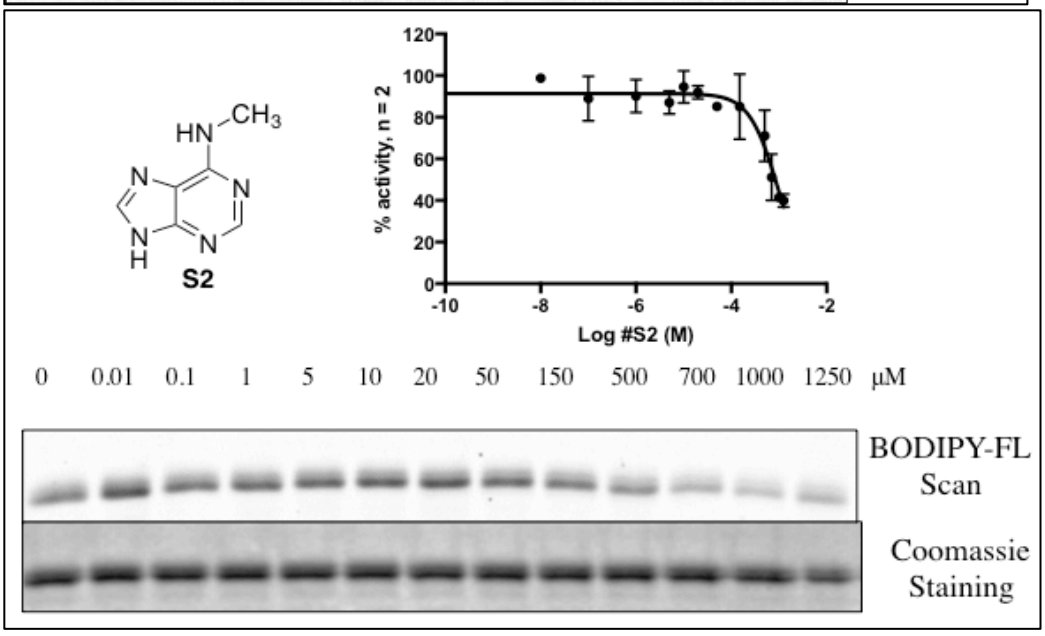

HN
N
N
N
H
N
22
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #22 (M)
0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

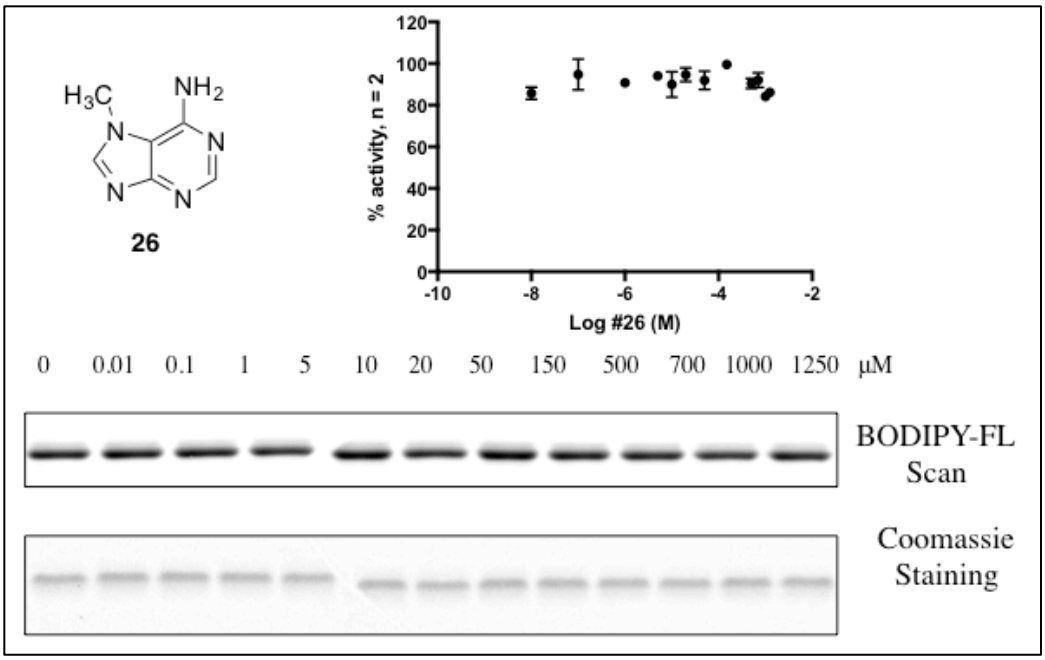

HN
N
N
N
N
HO
O
OH OH
23
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #23 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

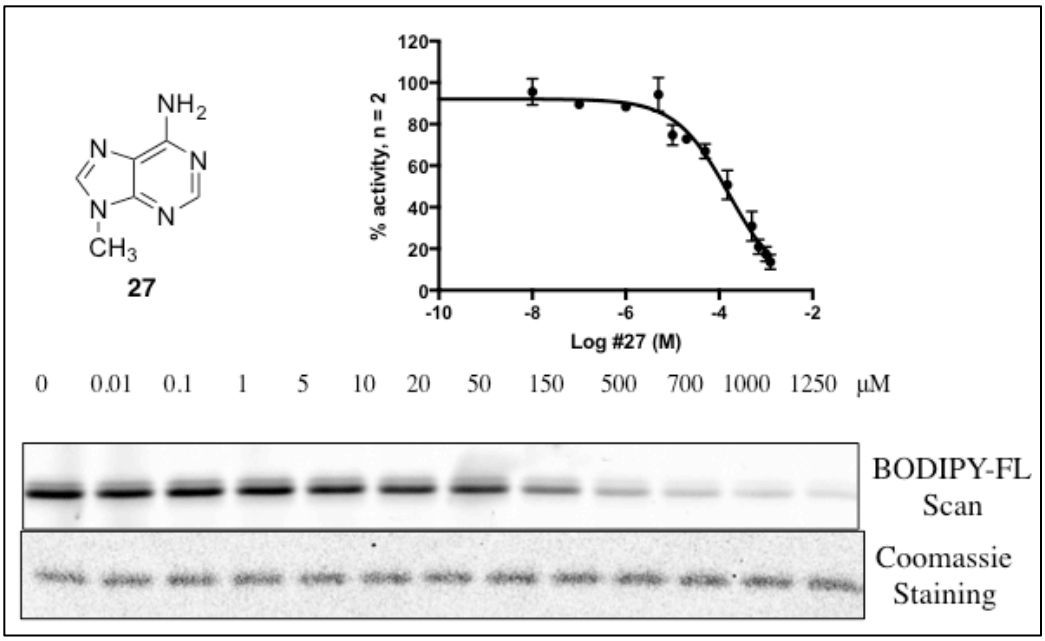

HN
N
N
N
N
H
Cl
24
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #24 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

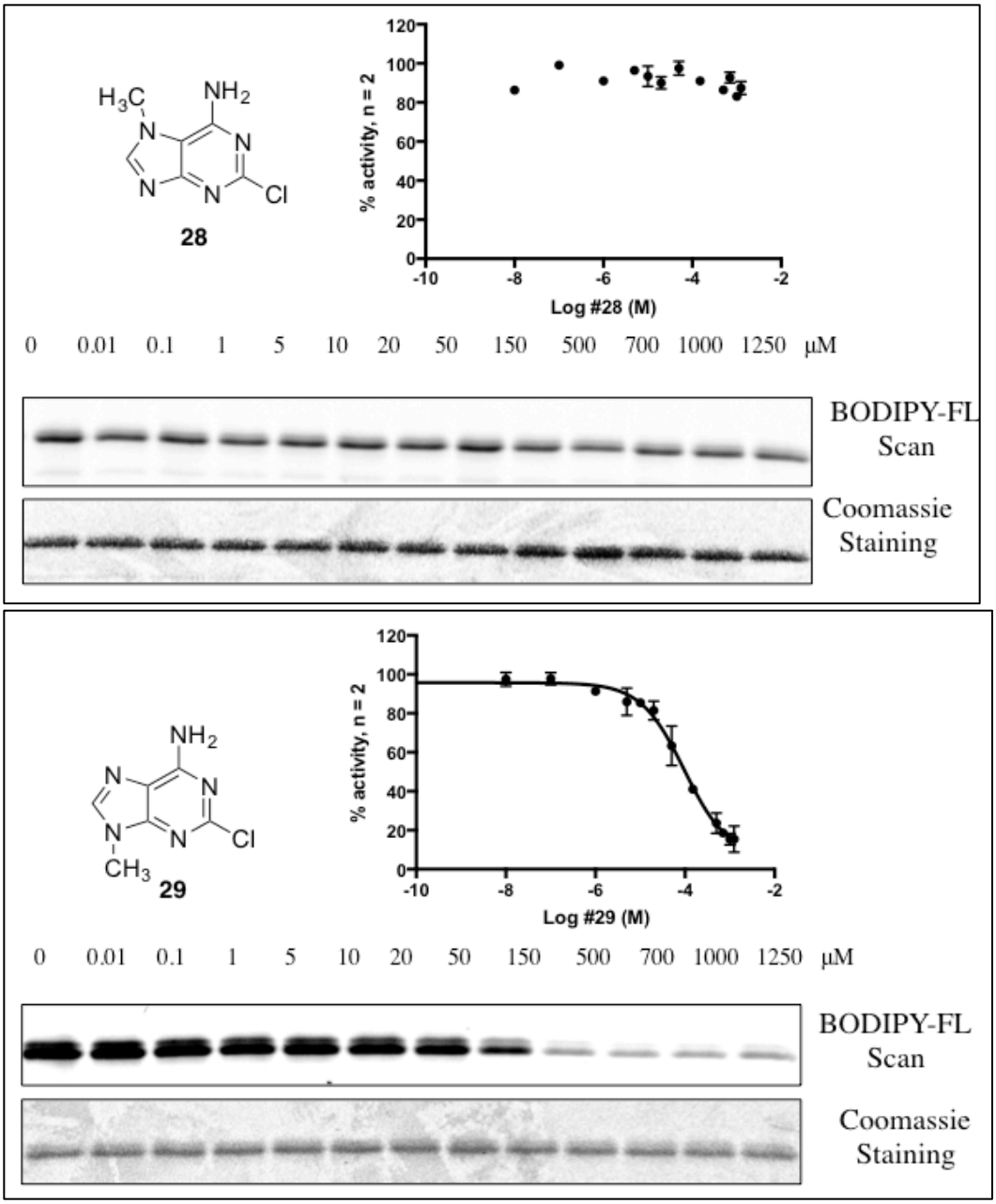

HN
N
N
N
N
H
N
H
S1
% Activity, n=1
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #S1 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

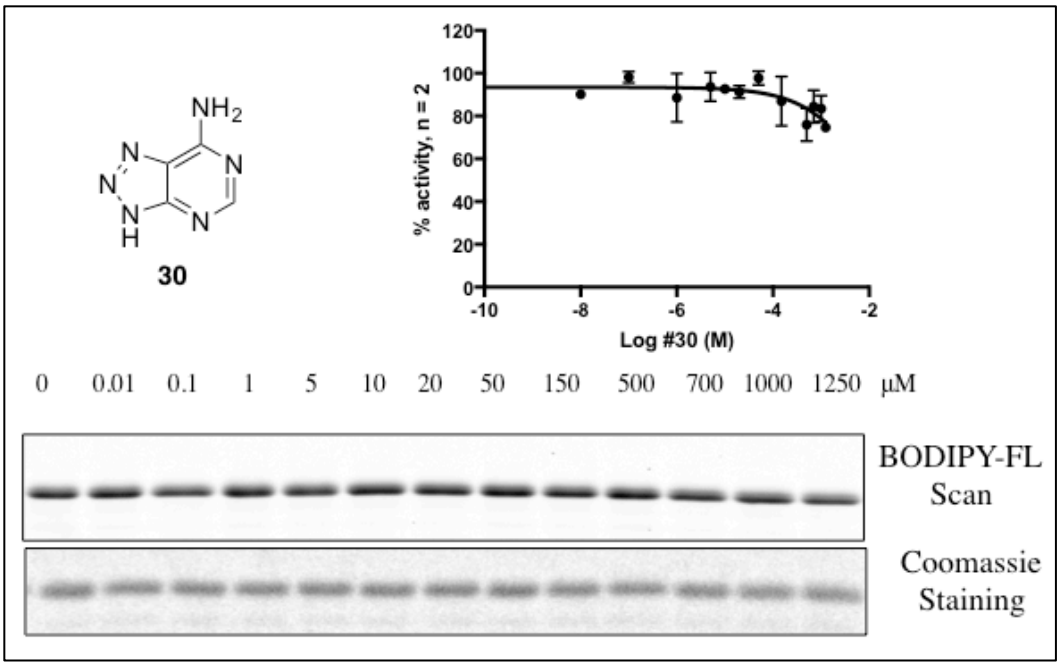

HN
CH3
N
N
N
N
H
Cl
25
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #25 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

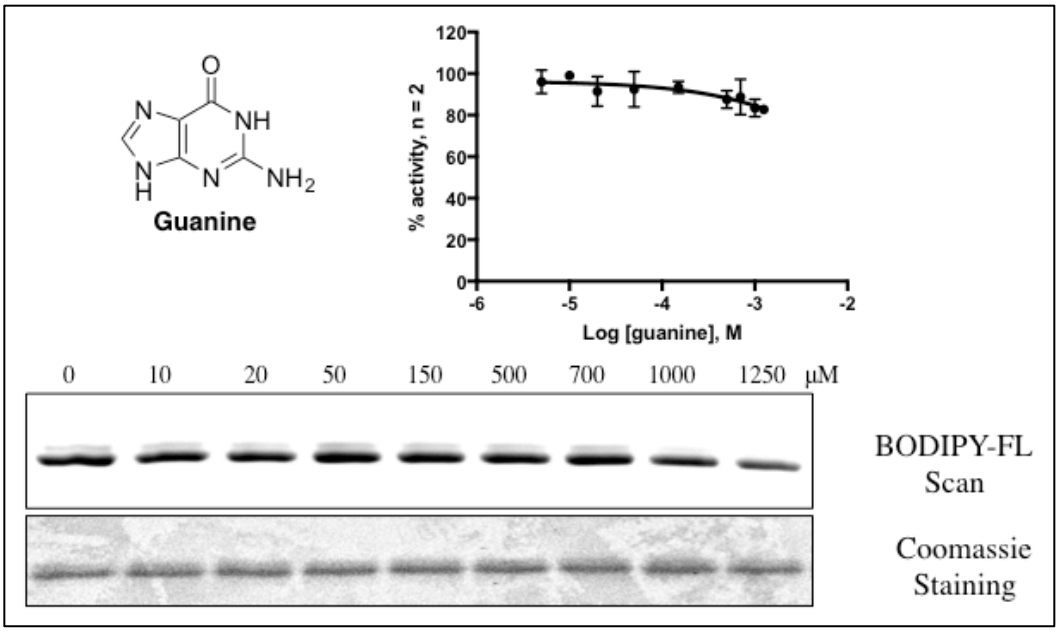

HN
CH3
N
N
N
N
H
S2
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #S2 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

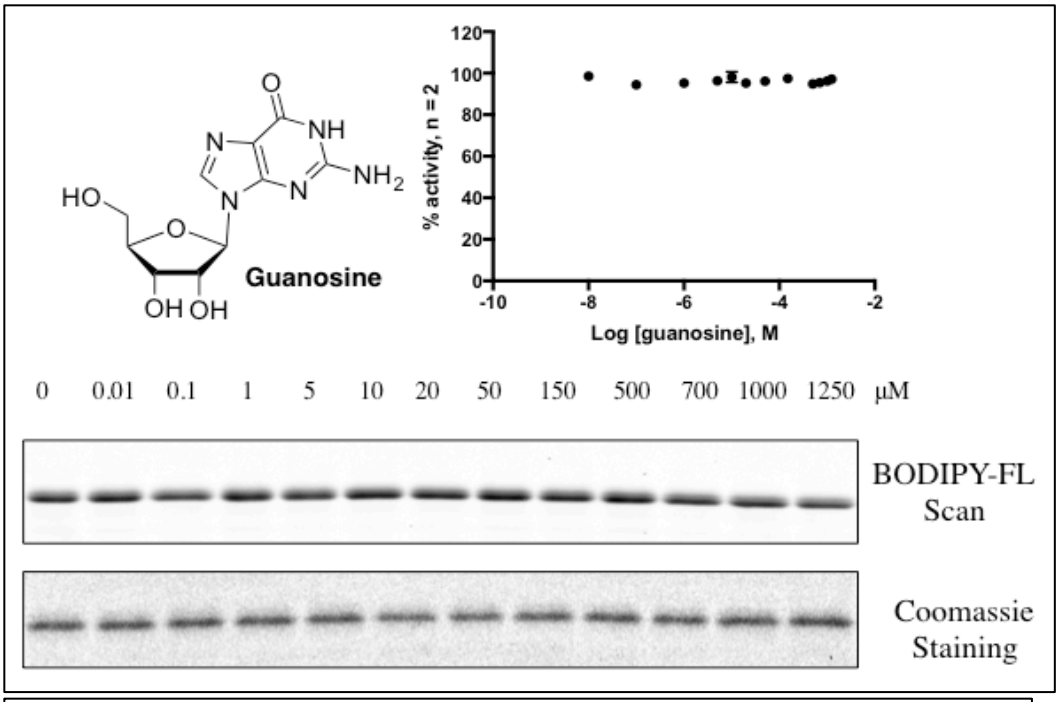

H3C
NH2
N
N
N
N
26
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #26 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

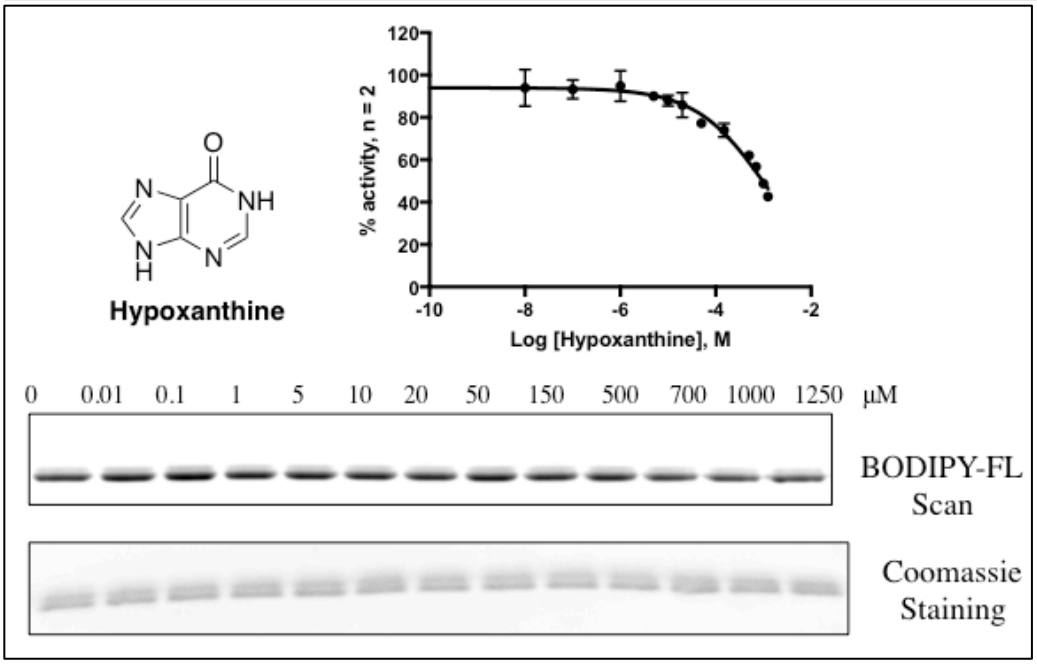

NH2
N
N
N
N
CH3
27
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #27 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

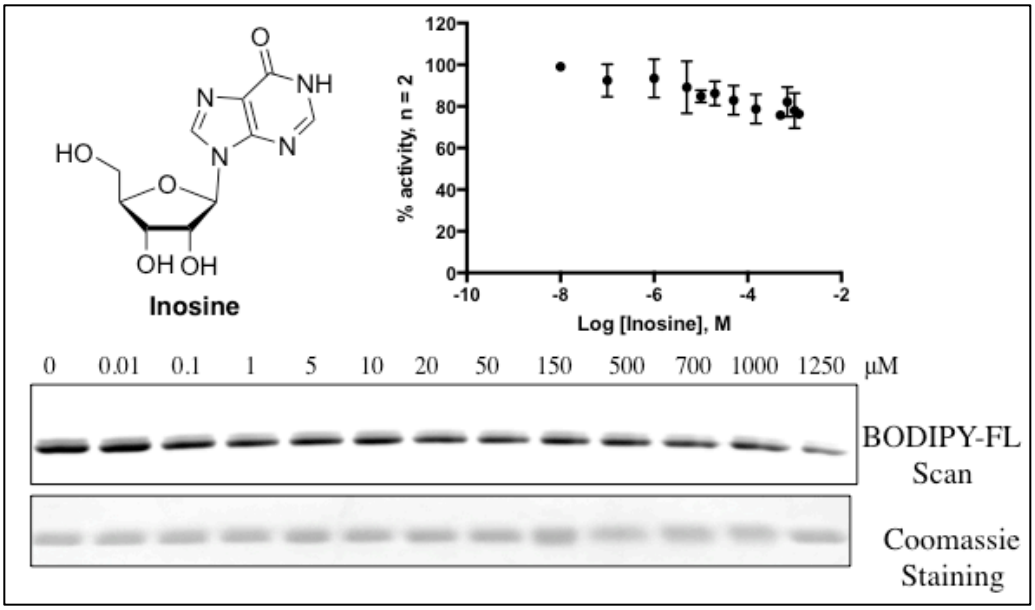

H3C
NH2
N
N
N
N
Cl
28
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #28 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining
NH2
N
N
N
N
Cl
CH3
29
% activity, n = 2
120
100
80
60
40
20
0
-10
-8
-6
-4
-2
Log #29 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining


NH2
N
N
N
N
H
30
120
100
80
60
40
20
0
% activity, n = 2
-10
-8
-6
-4
-2
Log #30 (M)
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining


O
NH
N
N
N
H
NH2
Guanine
120
100
80
60
40
20
0
% activity, n = 2
-6
-5
-4
-3
-2
Log [guanine], M
0 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining


Guanosine
% activity, n = 2
Log [guanosine], M
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining


Hypoxanthine
% activity, n = 2
Log [Hypoxanthine], M
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining


Inosine
% activity, n = 2
Log [Inosine], M
0 0.01 0.1 1 5 10 20 50 150 500 700 1000 1250 µM
BODIPY-FL Scan
Coomassie Staining

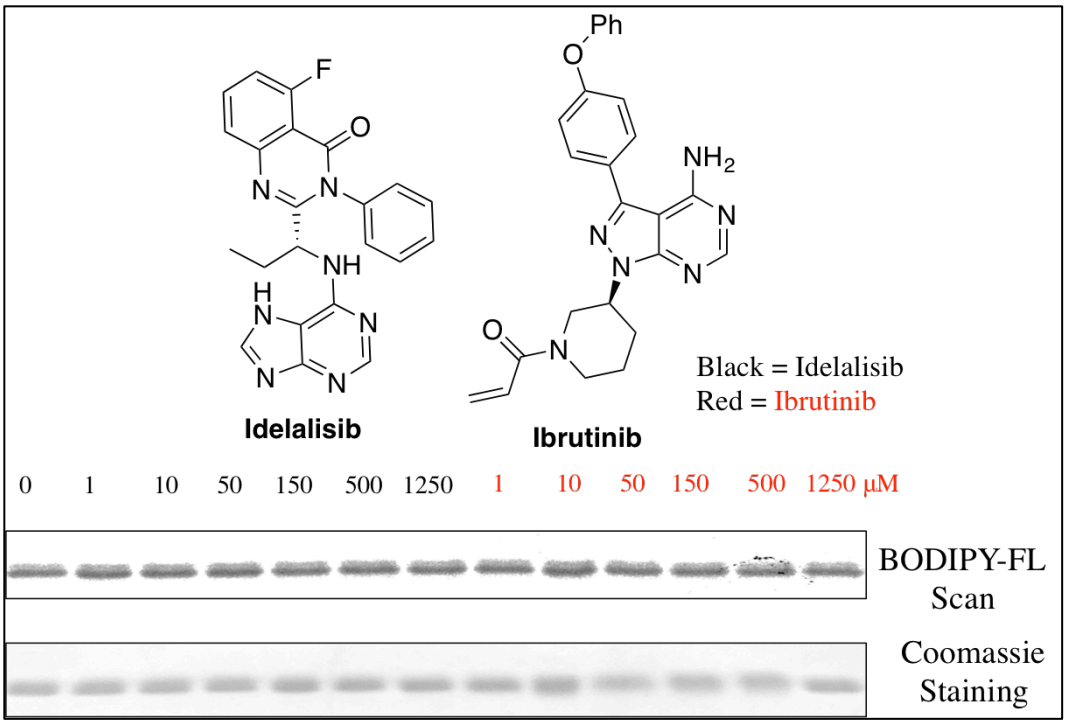
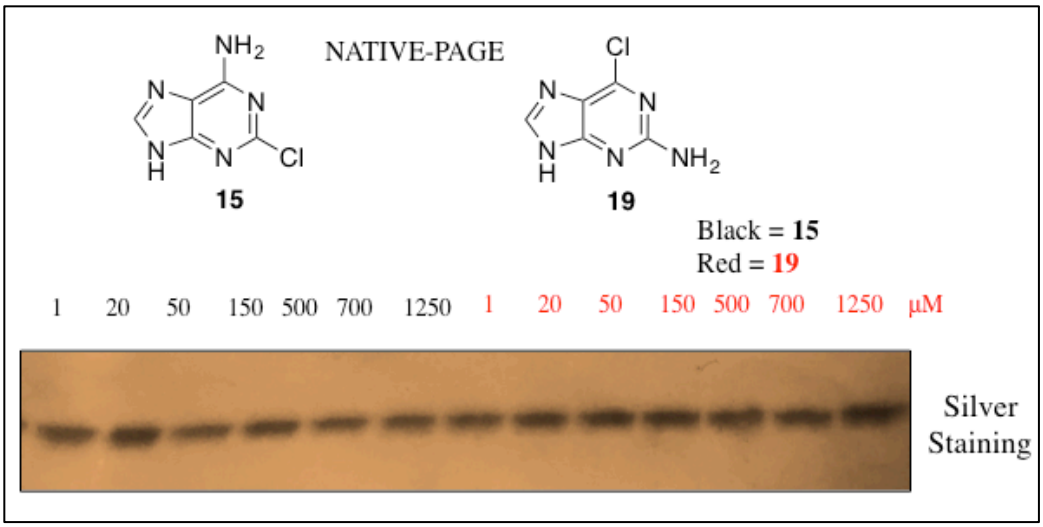

Idelalisib
Ibrutinib
Black = Idelalisib
Red = Ibrutinib
0 1 10 50 150 500 1250 1 10 50 150 500 1250 µM
BODIPY-FL Scan
Coomassie Staining

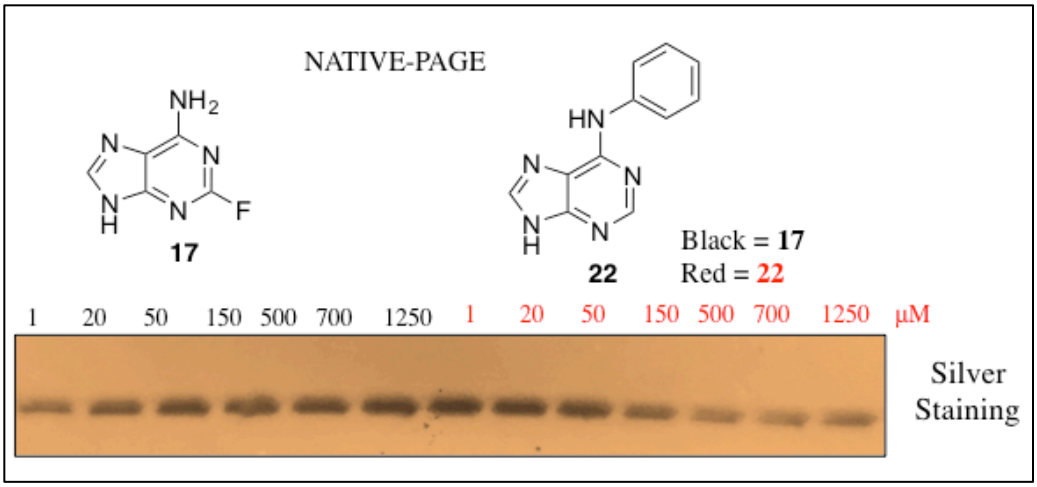
Table S1. IC_{50} values of adenine compounds on HK853 inhibition.

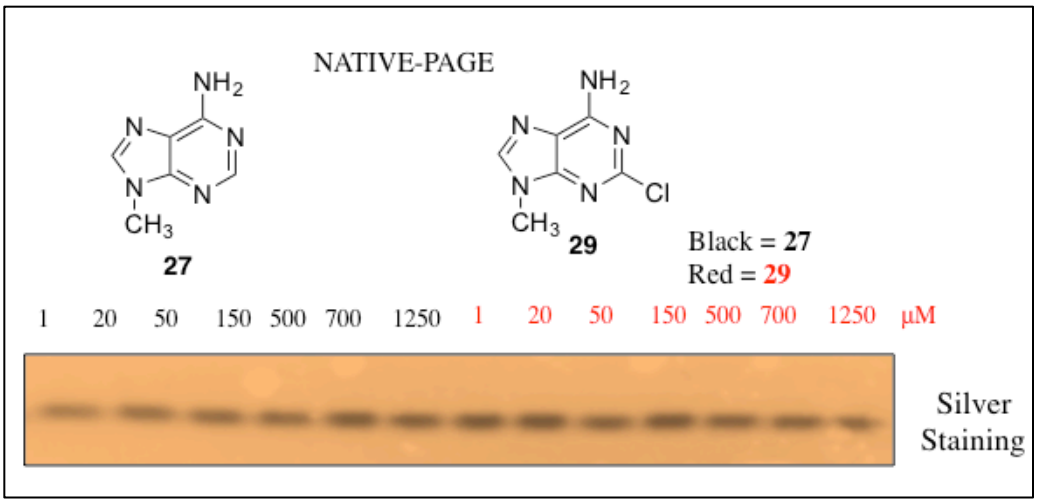
Cmpd #	*IC_{50} values (μM) (95% confidence interval), n = 2*	*Cmpd #*	*IC_{50} values (μM) (95% confidence interval), n = 2*
8	407 (308-536)	**23**	Not Converged
10	1790 (1368-2343)	**24**	1283 (835-2566)
11	1585 (1217-2065)	**25**	837 (700-1004)
12	2683 (991-7257)	**26**	Not Converged
13	2623 (1671-5487)	**27**	164.2 (116.7-226.5)
14	1786 (1244-3256)	**28**	Not Converged
15	156 (107.4-327.2)	**29**	95.4 (65.6-190.5)
16	126 (56.0-187.7)	**30**	13660 (2419-18930)
17	310 (110-1897)	**Guanine**	28060 (21710-7.5x10^6)
18	218 (100-3312)	**Guanosine**	Not Converged
19	118 (77-260)	**Hypoxanthine**	1199 (944.9-1576)
20	1363 (1117-2128)	**Inosine**	Not Converged
21	1207 (917-1950)	**S1**	7276 (Ambiguous)
22	7.3 (4.57-104)	**S2**	954 (787-1221)

HK853 Aggregation Analysis

To analyze the propensity for compounds to cause aggregation, each was mixed at six concentrations (0−1250 μM) with purified 0.5 μM HK853 in 25 μL of 20 mM HEPES buffer (5% (v/v) DMSO final). After incubating at RT for 30 min, 8.6 μL native-PAGE sample loading buffer was added, and 15 μL was loaded onto a 7.5% polyacrylamide gel. Proteins were resolved by native-PAGE and silver staining. Compound-induced aggregation was detected by the disappearance of the dimeric HK853 band. NH125 (Tocris Bioscience) was used as a positive aggregation control.







MOLECULAR DOCKING STUDIES

All molecular modeling operations were performed using SYBYL X Surflex-Dock through the University of Minnesota's Supercomputing Institute. The protein used for docking was HK853 co-crystallized with ADP (PDB:3DGE). The receptor was prepared by removing co-crystallized ligands and water molecules followed by the addition of hydrogens using the Prepare Protein Structure tool. Atom types were assigned using the AMBER method and a staged-minimization was performed on the hydrogens. The compounds were prepared prior to docking using the "Sanitize" protocol in the Ligand Structure Preparation tool found in SYBYL, which removed all counter ions and energy was minimized. The docking was done with the surflex-dock default values for threshold, bloat, number of poses. Visualization of docked poses was done using PyMOL and interactions of the protein-ligand with CCG MOE software.[10]

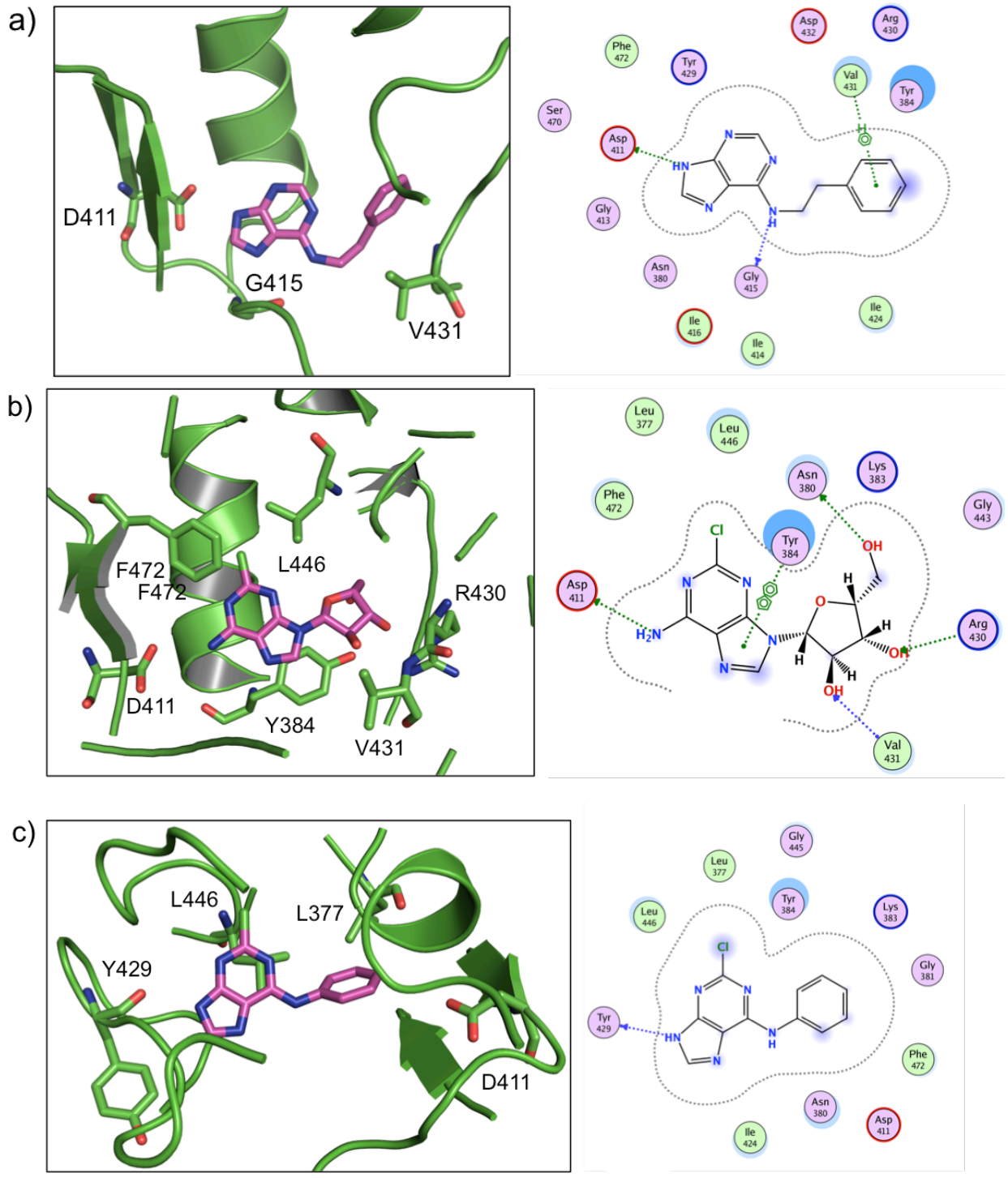


Figure S4: Docking pose of compounds, a) **3**, b) **16**, c) **24**. The left image shows the docking pose of the compound in the receptor cavity. For clarity purposes, only the receptor cavity (green ribbons) and not the full protein, along with the important residues (sticks) participating in the ligand-protein binding, are shown. The ligands are shown as sticks with C = magenta, N = blue, Cl = green. The right image was produced with PyMOL after the docking was performed in SYBYL Surflex-Dock. The images are ligand-protein interactions generated with MOE program.

Legend of the possible ligand interactions generated by MOE.

Table S2. Results from docking studies of adenine inhibitors into HK853 receptor cavity.

Cmpd #	*Docking score*	*Crash*	*Polar*	*Ligand similarity*
1	8.13	-0.3	1.35	0.28
3	8.23	-0.6	3.01	0.01
8	8.17	-0.5	5.16	0.554
13	5.05	-3.96	4.55	0.31
14	5.5	-4.5	4	0.3
15	6.67	-1.1	1.9	0.3
16	7.9	-0.9	3.9	0.6
20	6.23	-0.12	1.9	0.2
22	7.92	-0.9	1.9	0.3
24	5.1	-1.7	1.56	0.18

D411N MUTANT PROTEIN SYNTHESIS AND CD ANALYSIS

Table S3. Mutant primers used in PCR site-directed mutagenesis to generate HK853 D411N.

Primer	*Sequence*	*Comments*
KEW026	TGAGAAAGACGGTGGTGTGCTGATCATCGTGGAG**GAT**AATG	Wild-type, sense, Asp
KEW027	GGTCCGGGATGCCGATACCATT**ATC**CTCCACGATGATCAG	Wild-type, antisense, Asp
FHJ086	TGAGAAAGACGGTGGTGTGCTGATCATCGTGGAG**AAT**AATG	Mutant, sense, **Asn**
FHJ087	GGTCCGGGATGCCGATACCATT**AAT**CTCCACGATGATCAG	Mutant, antisense, **Asn**

Table S4. Final protein properties of the HK853 D411N mutant. Gray residues represent a polyhistidine tag coded by the p-His-parallel vector. The red residue is the mutated residue. Values for pI and extinction coefficient are estimated.

Protein	***Sequence***	***MW (kDa)***	***pI***	***ε ($M^{-1}cm^{-1}$)***
HK853 D411N	MSYYHHHHHHDYDIPTTENLYFQGAMENVT ESKELERLKRIDRMKTEFIANISHELRTPLTAI KAYAETIYNSLGELDLSTLKEFLEVIIDQSNH LENLLNELLDFSRLERKSLQINREKVDLCDL VESAVNAIKEFASSHNVNVLFESNVPCPVEA YIDPTRIRQVLLNLLNNGVKYSKKDAPDKYV KVILDEKDGGVLIIVENNGIGIPDHAKDRIFE QFYRVDSSLTYEVPGTGLGLAITKEIVELHG GRIWVESEVGKGSRFFVWIPKDRAGEDNRQ DN	32.4	5.27	27,390

PCR site-directed mutagenesis for generation of HK853 D411N construct.[11] The DNA synthesized for wild-type HK853 was used as a template. Sense and antisense primers that originally coded for D411 were altered to asparagine (Table S3). Primers were ordered from New England Biolabs. Two reactions were prepared in PCR tubes: 2.5 ng HK853 wild-type DNA template, 2.5 µL 2.5 mM dNTPs, 2.5 µL 10 X Pfu buffer, 14.65 µL nuclease-free water, and 0.25 µL 100 X Pfu. To one tube, 2.5 µL of 5 µM mutant sense primer (FHJ086) and 2.5 µL of 5 µM outermost wild-type antisense primer were added. To the other, 2.5 µL of 5 µM outermost wild-type sense primer and 2.5 µL of mutant antisense primer (FHJ087) were added. The final reaction volumes were 25 µL. The PCR reaction was 95 °C for 60 s; 30 cycles of 95°C for 30 s, 56 °C for 120 s, and 72 °C for 90 s; and 72 °C for 360 s. To amplify the mutated template, 0.5 µL of product from both the first and second tubes were mixed with 5.0 µL 2.5 mM dNTPs, 5.0 µL of 5 µM outermost sense primer, 5.0 µL of 5 µM outermost antisense primer, 5.0 µL of 10 X Pfu buffer, 28.5 µL nuclease-free water, and 0.5 µL 100 X Pfu to a total volume of 50 µL. The same PCR method was run. PCR product was purified, digested, and ligated into the p-His-parallel vector as before.[12] The DNA sequence was confirmed as successful through sequencing at the Indiana Molecular Biology Institute. Additionally, transformation of p-His-parallel-HK853 D411A into *E. coli* strain BL21 (DE3)Rosetta, pLysS, and subsequent protein overexpression and purification were performed as described for wild-type HK853.[8]

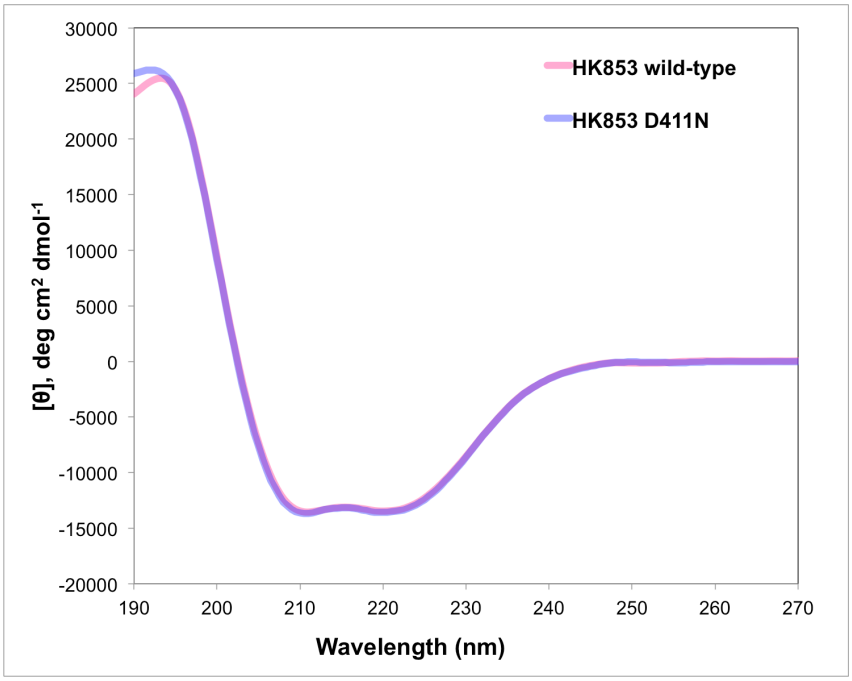


Figure S5. Analysis of HK853 proteins by CD spectroscopy**.** CD spectra of HK853 wild-type (pink) and D411N (blue) proteins scanned from 190–270 nm shown as mean residue ellipticity.

Table S5. Estimated secondary structure of HK853 proteins from CD spectra (n=3).

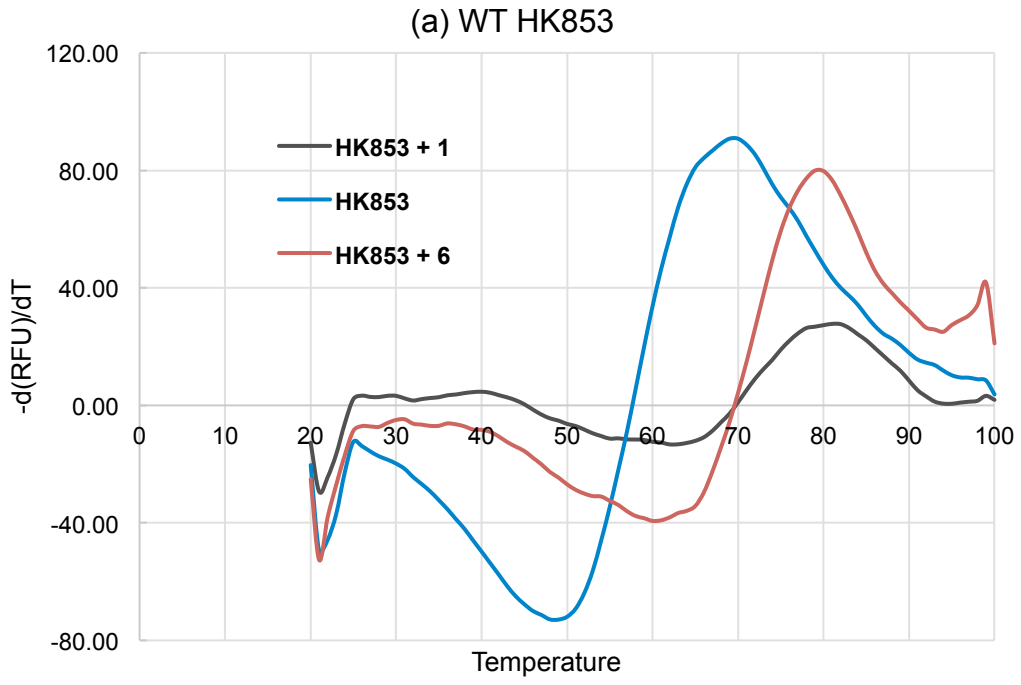
Construct	***% α-Helix***	***% β-Strand***	***% Turns***
Wild-Type	42.1 ± 0.3	14.7 ± 0.3	16.6 ± 0.1
D411N	41.9 ± 0.4	14.6 ± 0.2	16.4 ± 0.3

Circular dichroism (CD) spectroscopy of HK853 proteins. Previous CD methods were used as guidelines for this procedure.[13, 14] Using a Jasco J-715 CD spectropolarimeter, CD spectra were acquired for purified HK853 wild-type and D411N proteins. Proteins were exchanged into 10 mM potassium phosphate, pH 7.5, four times using 0.5-mL 10K Amicon Ultra centrifugal filters (Millipore). The Bio-Rad DC Protein Assay was used to determine protein concentrations, which were 0.111 mg/mL for HK853 wild-type and 0.110 mg/mL for HK853 D411N. Buffer and protein solutions were filtered with 0.22-µm Ultrafree-MC centrifugal filters (Millipore) to ensure the removal of any particulates that could interfere with CD readings. Protein solutions were loaded into a 0.1-cm quartz cuvette (Hellma), and spectra were obtained at 25 °C. Each spectrum was measured in triplicate with the following parameters: standard (100 mdeg) sensitivity, 190–270 nm range, 0.5 nm data pitch, continuous scanning mode, scanning speed of 100 nm/min, response of 1 s, 1.0-nm bandwidth, and an accumulation of 4 scans. Spectra were smoothed using a Savitsky-Golay filter (15-point smoothing window). Averaged buffer spectra were subtracted from the protein spectra. The CD data in millidegrees were used to calculate mean residue ellipticity, [θ], according to the following equation: [θ]= (millidegrees)/(path length in mm x concentration in M x number of amino acid residues).[14] The final units for mean residue ellipticity were deg cm^{-1} $dmol^{-1}$. Additionally, data from each CD spectrum (in millidegrees) were submitted to Dichroweb for secondary structure analysis using SELCON3 and reference set

4.[15-17] Values for helices, strands, and turns from each spectrum were averaged, and error was reported as the standard deviation. NRMSD values for Dichroweb results ranged from 0.037 to 0.052.

THERMAL SHIFT ASSAYS-DIFFERENTIAL SCANNING FLUORIMETRY (DSF) STUDIES

All DSF measurements were done on 96-well PCR plates on a Bio-Rad MyiQ2 instrument using HEX filter for relative fluorescence quantification. The DSF protocol was developed based on published methods.[18] The final volume of each reaction was 25 µL and the reagents were kept on ice. The wild-type HK853 or the mutant HK853 protein solutions were prepared in reaction buffer to attain a final concentration of 2-5 µM in the reaction. Next, the 1000x SYPRO orange dye (Thermo Fisher) was diluted in water and added to the protein to be finally at 5x concentration. From a 25 mM DMSO stock solutions of inhibitors, 1 µL was added to obtain a final concentration of 1 mM (for negative control, 1 µL of DMSO was added). The reagents were pipetted carefully to mix them properly. The well plate was then sealed with optical PCR seals (Bio-Rad) and centrifuged briefly to ensure the reagents are settled at the bottom of the plate and to remove bubbles. The PCR plate was then run on the instrument for the "melt curve" analysis from 20 °C to 95 °C at 1 °C min^{-1}. The curves were processed in the iQ5 software to obtain the melting temperatures and the graphs were plotted using Microsoft Excel.



18, 19

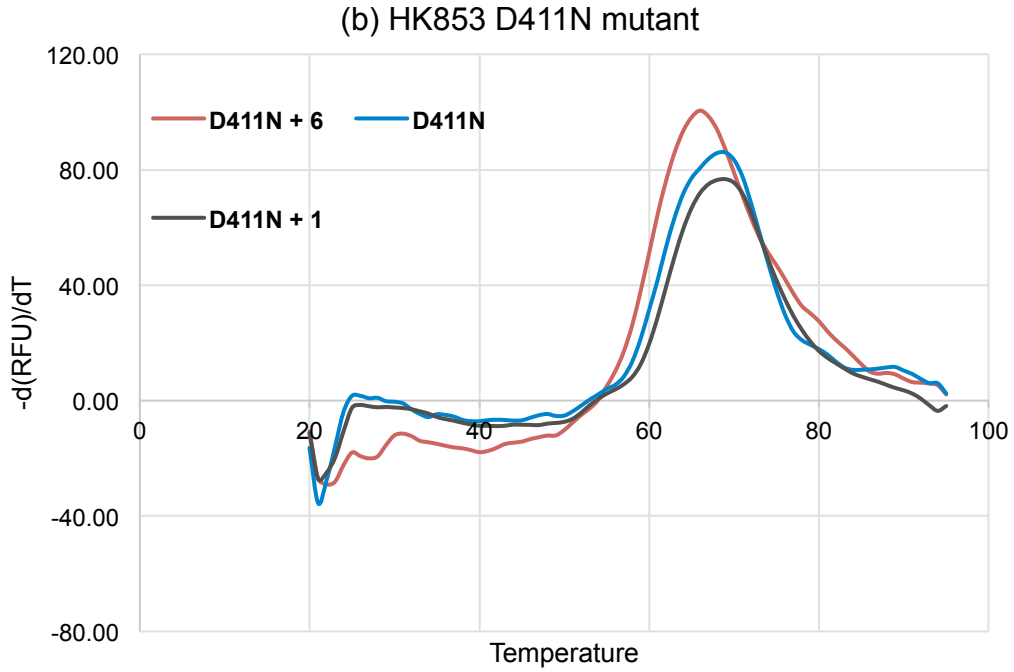


Figure S6. DSF curves of (a) WT and (b) mutant HK853 proteins in +/– ligands. Data is plotted based on n = 3.

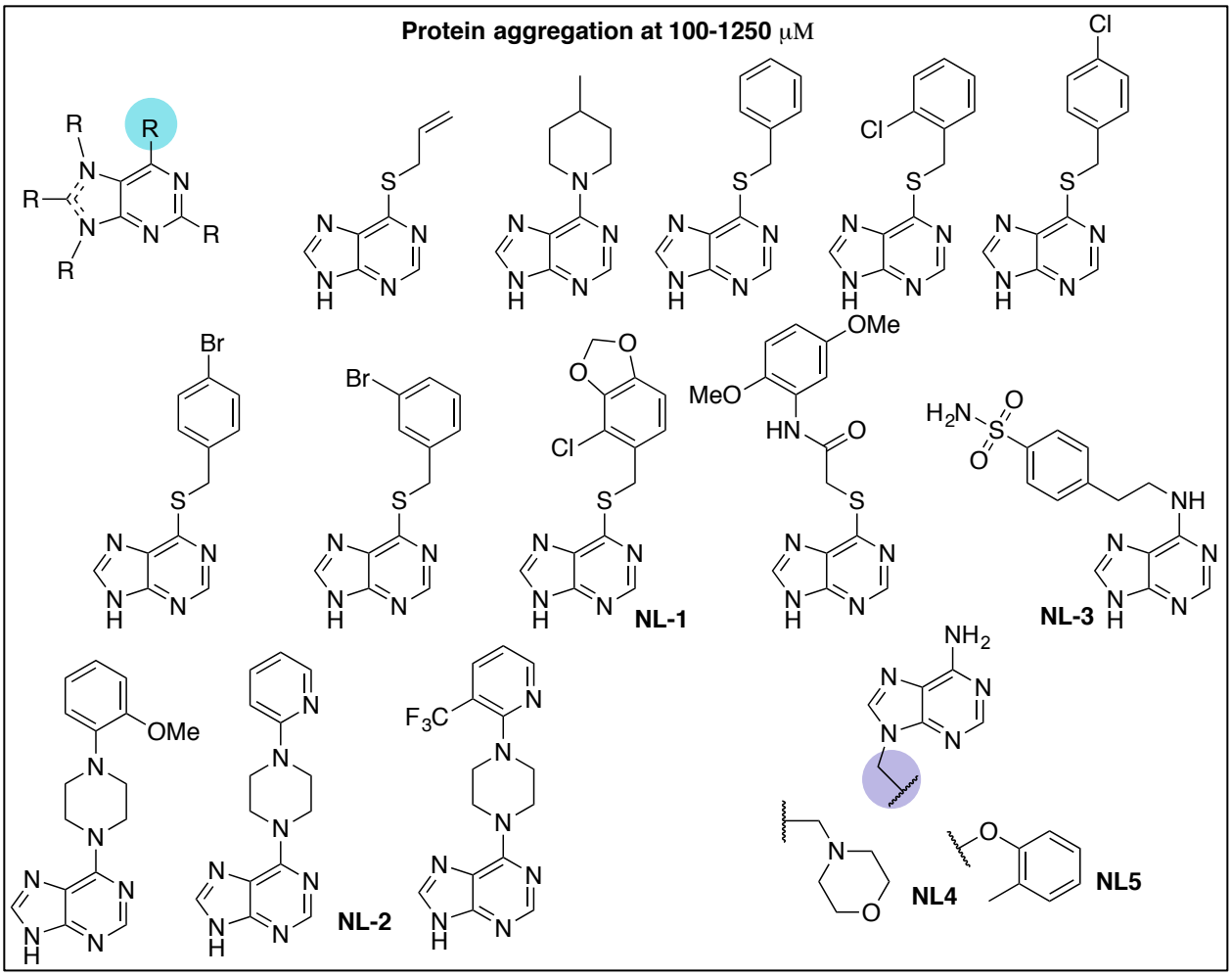


Figure S7. Structures of adenine-based non-lead compounds from the HT screen. These compounds were found as "hits" from the screen, but were not pursued further as they caused protein aggregation at amounts of 100-1250 μM.[20]

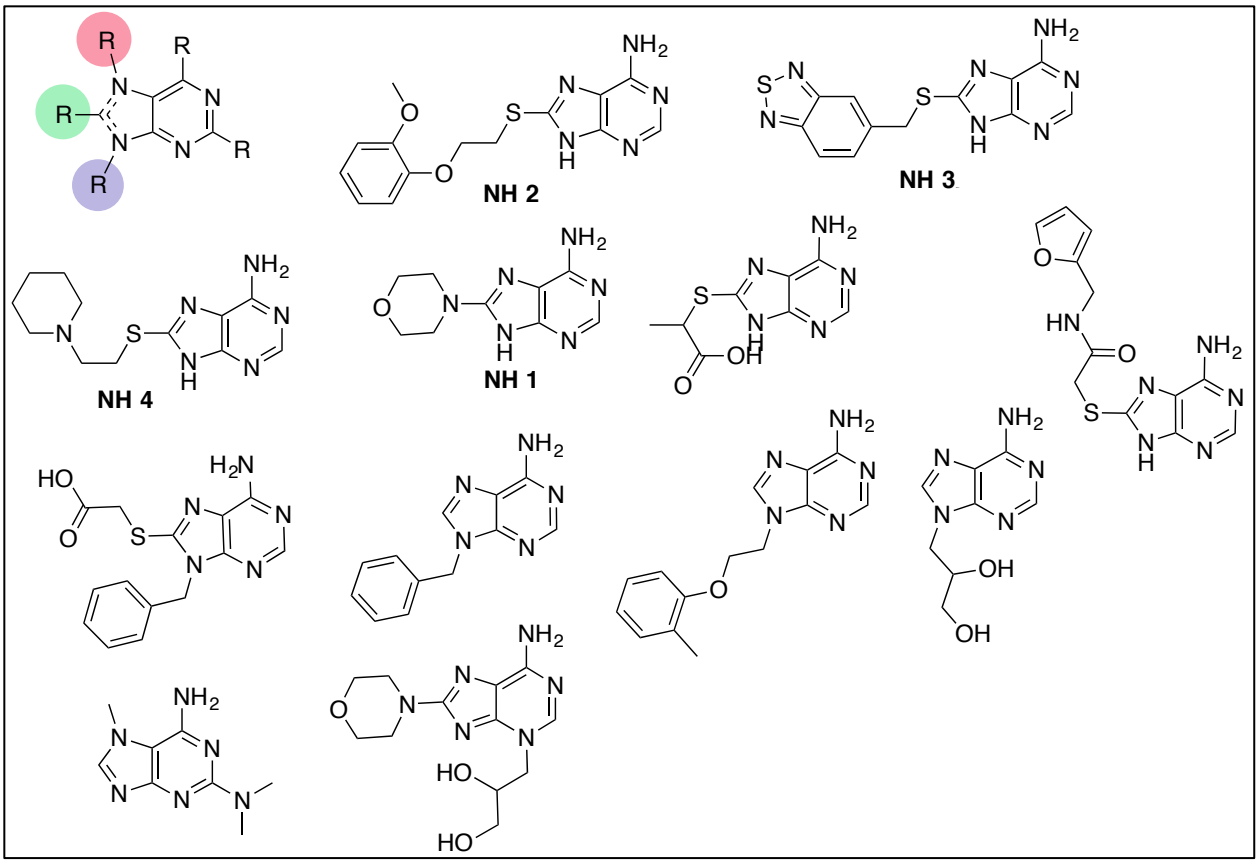


Figure S8. Structures of adenine-based compounds that were not hits (binding to HK853 <30%) from the HT screen.

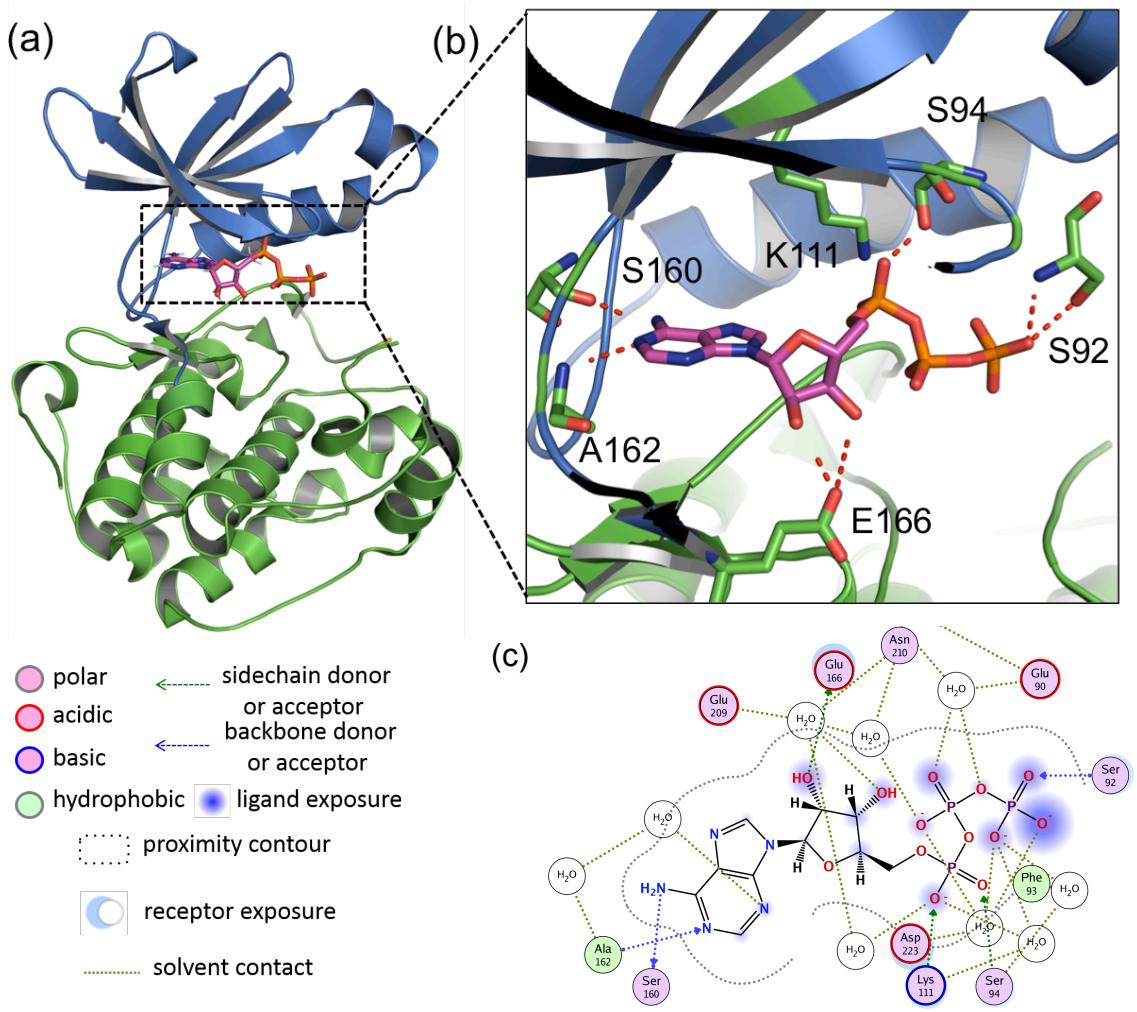


Figure S9. (a) PDK1 (Ser/Thr Kinase) co-crystallized with ATP (PDB:4XX9) showing the N-lobe (blue ribbon) and C-lobe (green ribbon), (b) expanded view of ADP-hinge region showing important residues, (c) Interactions of the ligand (ADP) with the kinase, obtained using MOE program. The legend of interactions in also shown here.

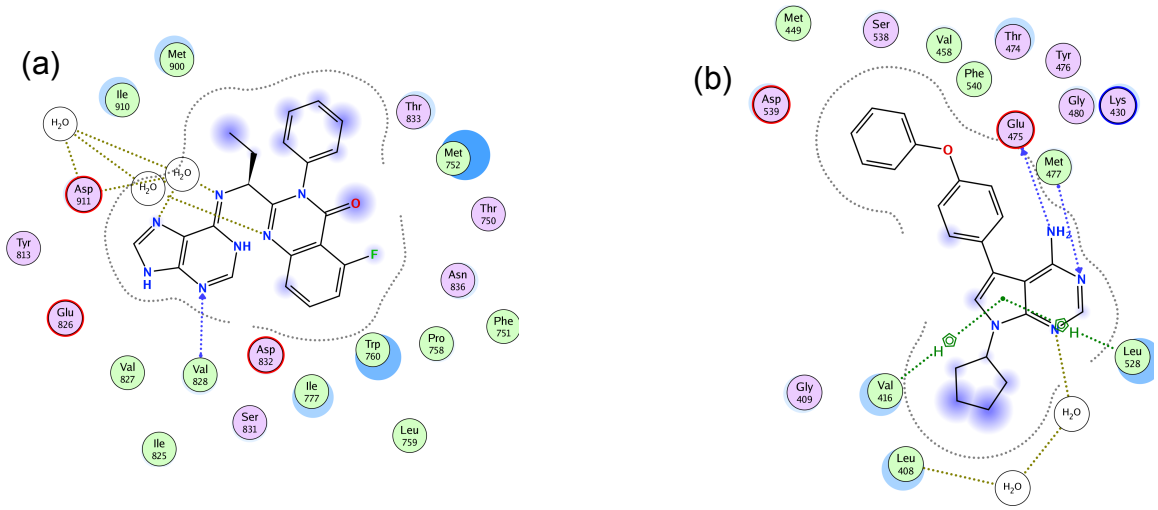


Figure S10. (a) Interactions of Idelalisib with PI3Kδ (PDB:4XE0) obtained using MOE program, (b) Interactions of B43 with BTK (PDB:3GEN) obtained using MOE program.

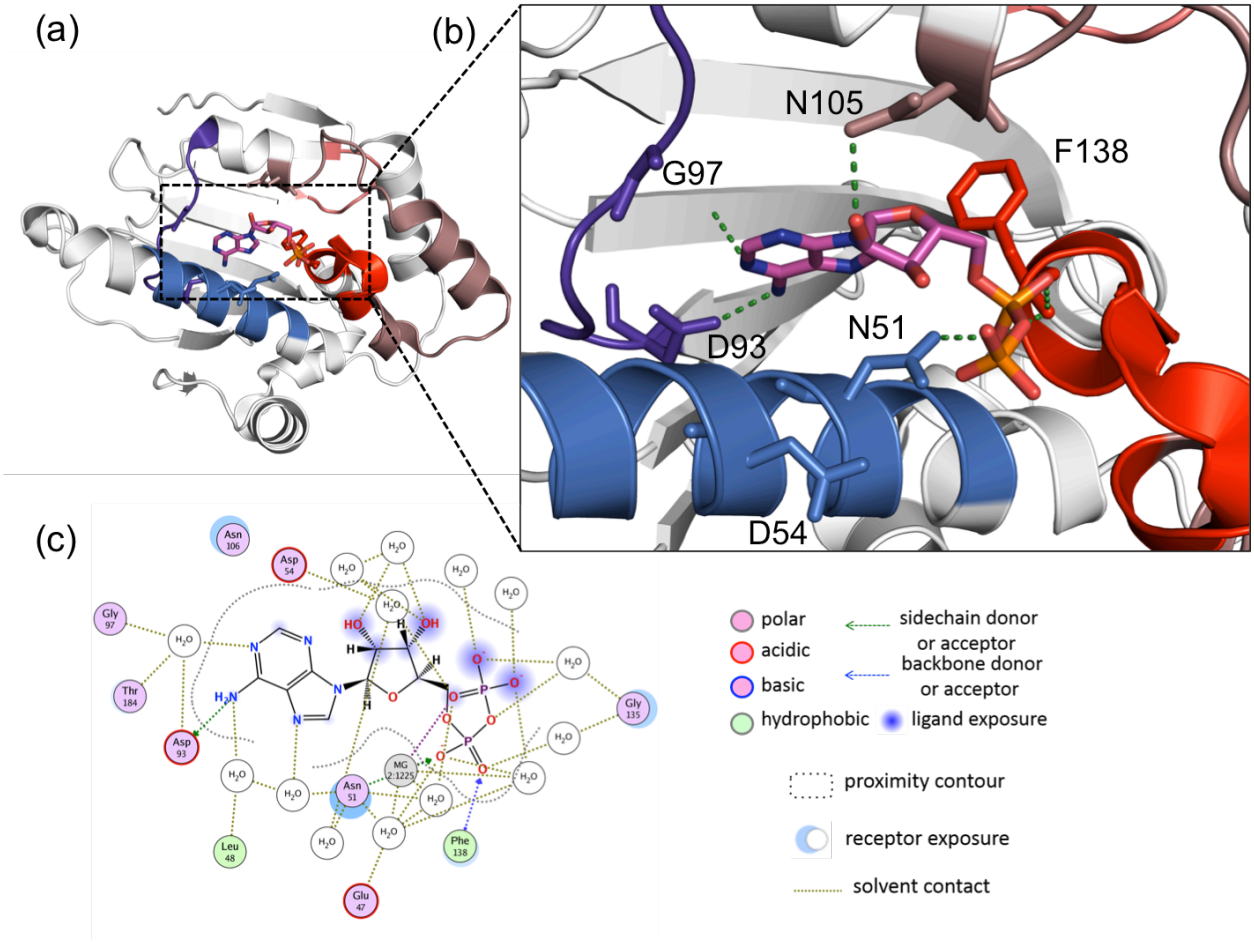


Figure S11. (a) HSP90 co-crystallized with ADP (PDB:2XK2), (b) expanded view of ADP-binding showing important residues, (c) Interactions of the ligand (ADP) with the protein, obtained using MOE program. The legend of interactions in also shown here.

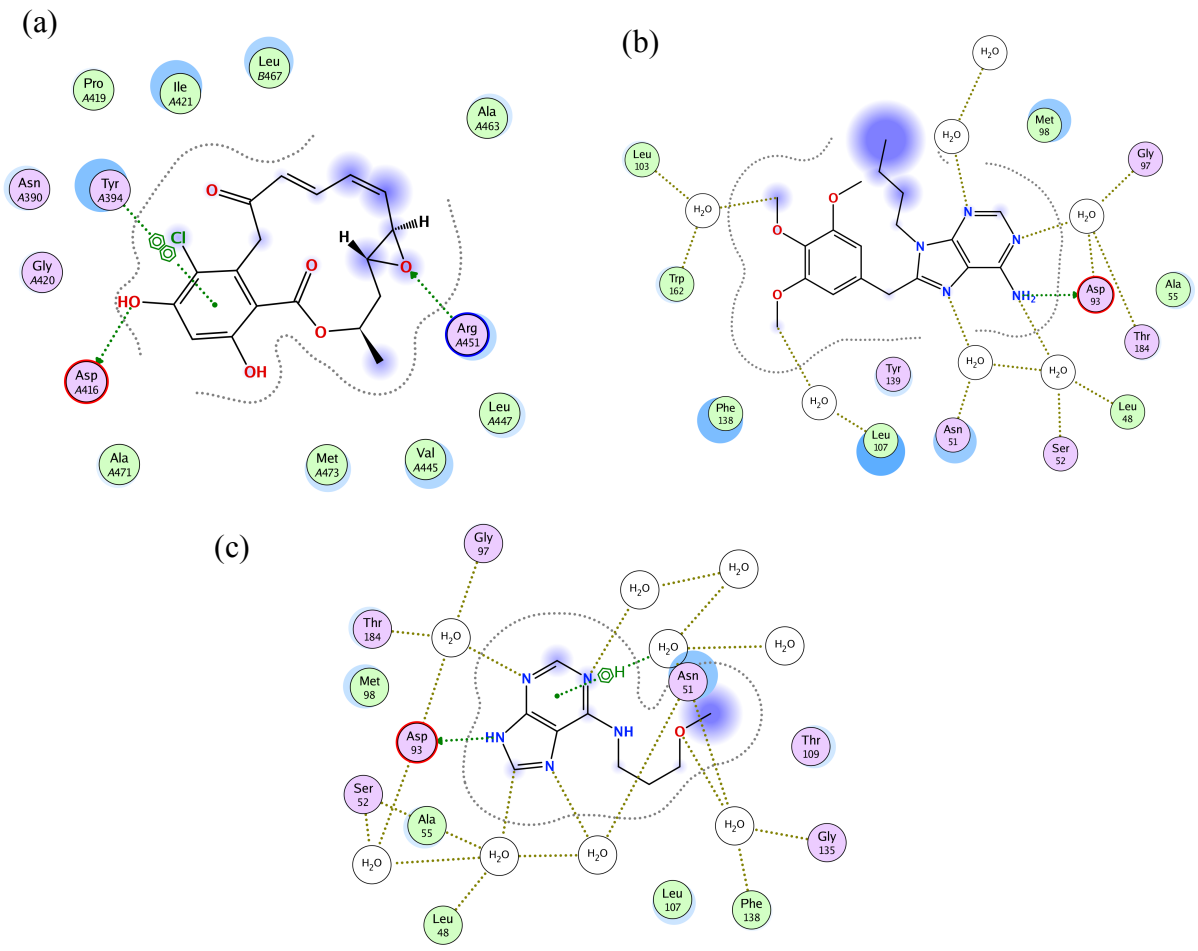


Figure S12. Ligand protein interactions from co-crystal structures of: (b) **Radicicol** with PhoQ (PBD:3CGY), (c) **PU3** with HSP90 (PDB: 1UY6), (c) **VF3** with HSP90 (PDB:2YEH). Images generated using MOE program.

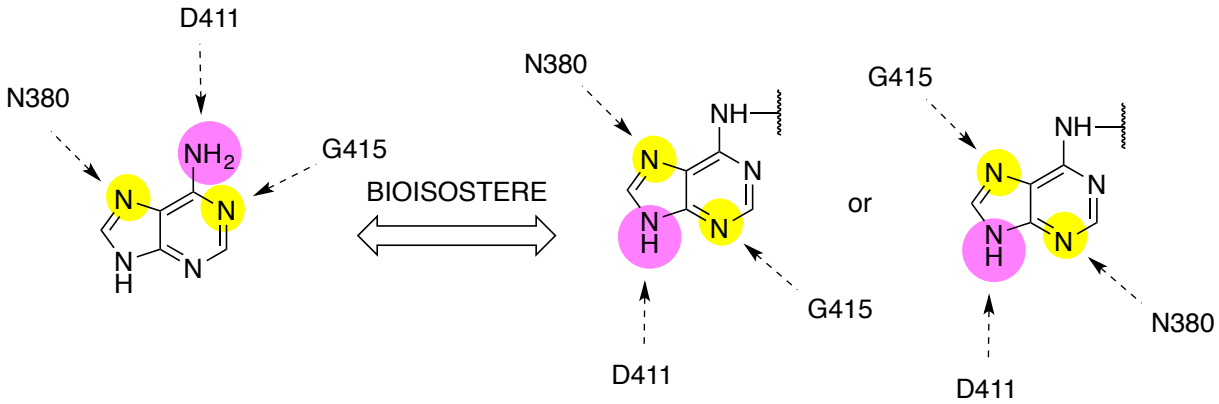


Figure S13. Bioisostere fragments within the adenine scaffold.

HSP90 INHIBITION ASSAYS

The HSP90 inhibition assays were performed with a commercially available fluorescence polarization based Hsp90α Assay Kit from BPS Biosciences according to the instructions provided.[21] The measurements were recorded on a Tecan infinite 500 instrument in Greiner 96 Flat Bottom Black Polystyrol plates at excitation between 475-495 nm and emission between 518-538 nm. The adenine compounds as listed in the scheme below were tested at a concentration of 1.25 mM (n = 3) in the assay. The compounds, which showed inhibition higher than 45% were further tested, dose response curves plotted with 8 concentrations (1-1250 μM) and their IC_{50} values were calculated using Equation 2 with GraphPad Prism software. For many compounds, the curves did not reach a plateau (weak inhibitors) and the IC_{50}s could not be accurately calculated. To validate the accuracy of the Hsp90α assay, a positive control compound, Geldanamycin was purchased and its IC_{50} was also calculated and found to be consistent with literature.[21]

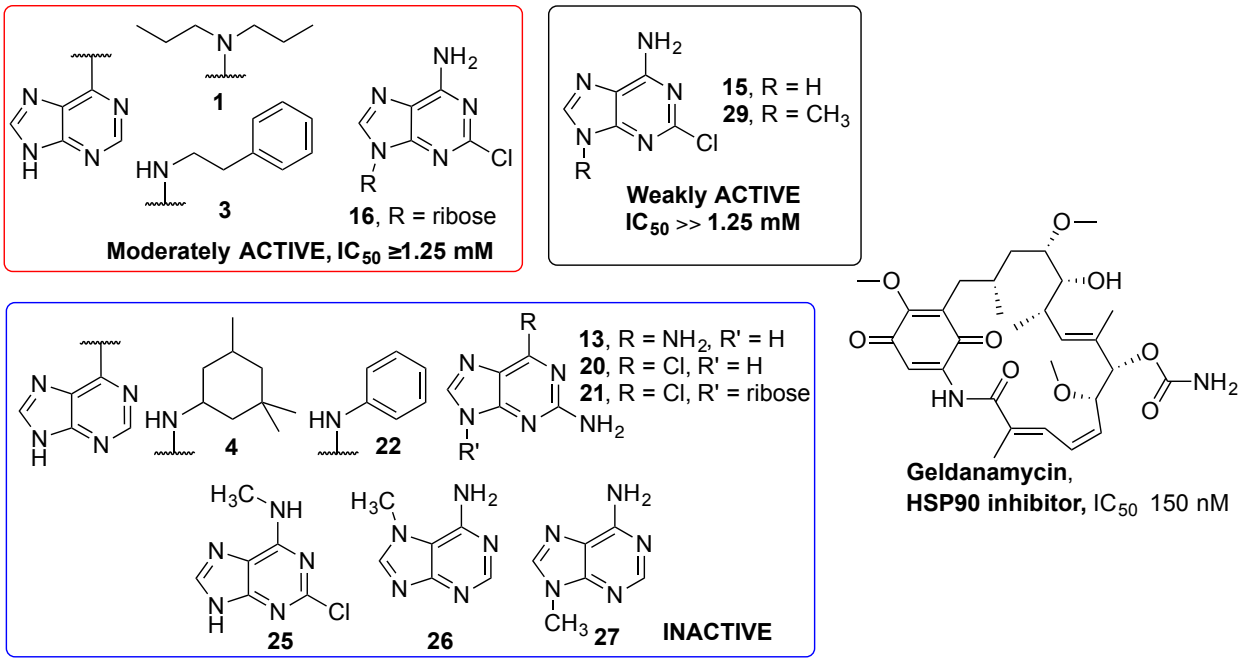
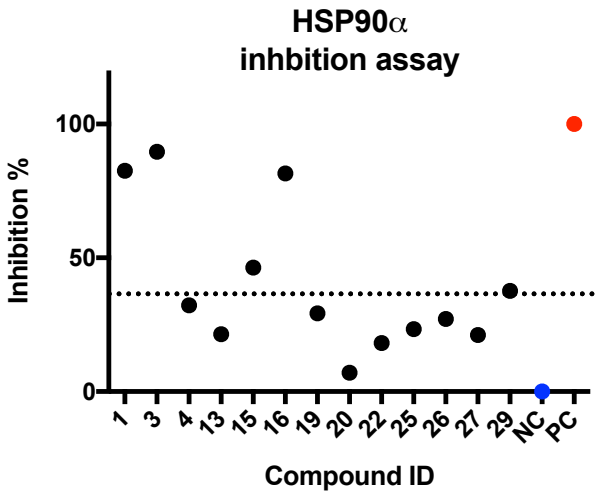
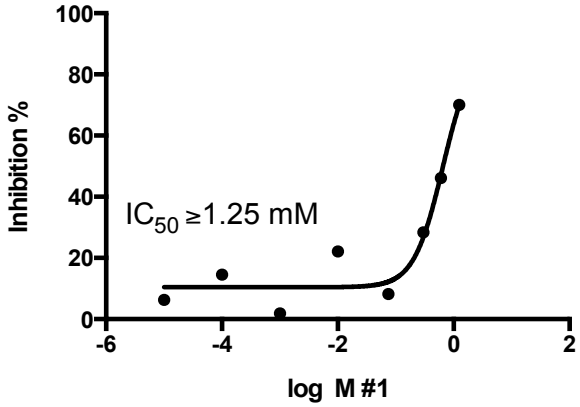


Figure S14. The structures of adenine compounds that were tested for HSP90 inhibition. These compounds are categorized into three groups based on their activity. The structure of known HSP90 inhibitor, Geldanamycin.


HSP90α
inhbition assay
Inhibition %
100
50
0
1
3
4
13
15
16
19
20
22
25
26
27
29
NC
PC
Compound ID


Inhibition %
100
80
60
40
20
0
IC50 ≥1.25 mM
-6
-4
-2
0
2
log M #1

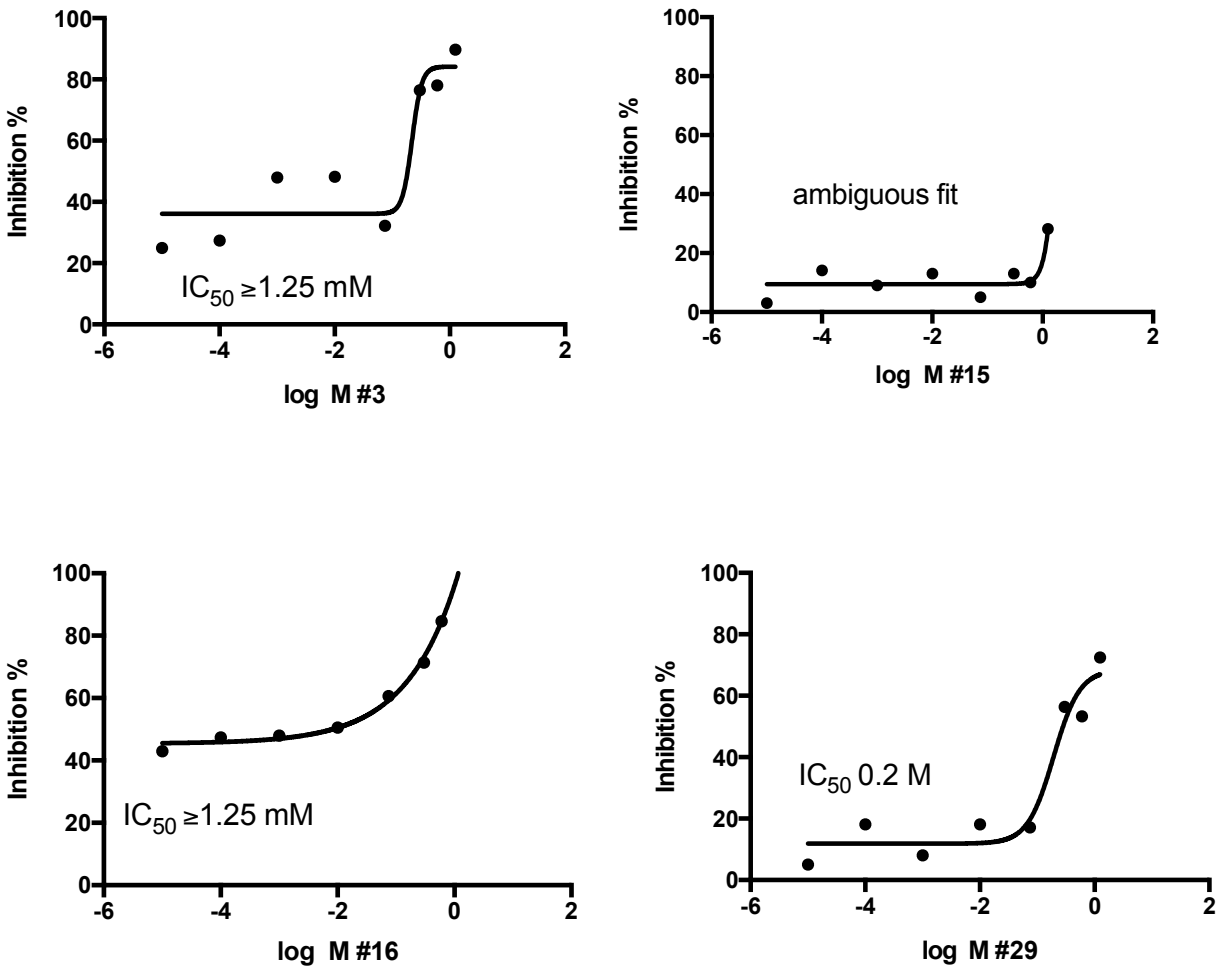


Figure S15. HSP90α inhibition assay results with various adenine compounds and their dose-response curves. PC = positive control, Geldanamycin, NC = DMSO.

Compound No.	HK853 inhibition (μM)	HSP90 inhibition (μM)	Fold difference
1	49.6	>1250	>25
3	145	>1250	>9
16	126	>1250	>10
29	95.4	20,000	2096

Table S6. Comparison of inhibitory activities of adenine compounds in HKs vs. HSP90

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