Supplemental Information

Chemical synthesis

Commercially obtained reagents were used as received. All reagents and anhydrous solvents used for Grignard reaction were freshly distilled. Progress of reactions was monitored by TLC performed on Analtech 250 micron silica gel GF plates visualized with 254 nm UV light and also by mass spectrometry using a Waters single-quadrupole LCMS. All compounds were purified on Biotage Isolera Four Flash Chromatography system using SNAP cartridges. Melting points were determined on a Mel-Temp manual melting point apparatus with a Fluke 51II thermocouple. ^{1}H and ^{13}C NMR spectra were recorded on Bruker NMR spectrometers operating at 600 and 800 MHz in $CDCl_3$, DMSO-d_6, and MeOH-d_4 with TMS as internal standard. Chemical shift values are reported in δ ppm units. Mass spectra were recorded on a Micromass AutoSpec Ultima Magnetic sector mass spectrometer in positive ESI mode.

The following compounds 2a-2c, 3a-f and 4a-4i, 5a-l, 6a-c, 7a-d were prepared by reductive cyclization method as described previously (Illendula et al., 2015).

5-methoxy-2-phenyl-1H-benzo[d]imidazole **(2a**) CAS Registry Number 79877-53-5

2-(furan-2-yl)-5-methoxy-1H-benzo[d]imidazole **(2b)** CAS Registry Number 517901-94-9

2-(5-methoxy-1H-benzo[d]imidazol-2-yl)phenol (**2c**) CAS Registry Number 939752-51-9

2-(pyridin-2-yl)-1H-benzo[d]imidazole (**3a**) CAS Registry Number 1137-68-4

5-ethoxy-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**3b**) CAS Registry Number 84123-79-5

5-fluoro-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**3c**) CAS Registry Number 875468-81-8

5-methyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**3d**) CAS Registry Number 7471-12-7

5-phenyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**3e**) CAS Registry Number 14060-65-2

2-(pyridin-2-yl)-5-(trifluoromethyl)-1H-benzo[d]imidazole (**3f**) CAS Registry Number 1256094-37-7

4-methoxy-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**4a**) CAS Registry Number 68118-46-7

4-ethoxy-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**4b**) CAS Registry Number 1256094-25-3

4-methyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**4c**) CAS Registry Number 68118-45-6

2-(pyridin-2-yl)-4-(trifluoromethyl)-1H-benzo[d]imidazole (**4d**) CAS Registry Number 1256094-38-8

4,5-dimethyl-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**4e**) CAS Registry Number 89481-12-9

5,6-difluoro-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**4f**) CAS Registry Number 1256094-32-2

5-methoxy-2-(6-methoxypyridin-2-yl)-1H-benzo[d]imidazole (**4g**) CAS Registry Number 1256094-58-2

5-methoxy-2-(6-methylpyridin-2-yl)-1H-benzo[d]imidazole (**4h**) CAS Registry Number 67273-50-1

2-(6-fluoropyridin-2-yl)-5-methoxy-1H-benzo[d]imidazole (**4i**) CAS Registry Number 1256094-60-6

5-isopropoxy-2-(pyridin-2-yl)-1H-benzo[d]imidazole **(5a**) CAS Registry Number 1256094-26-4

2-(pyridin-2-yl)-5-(trifluoromethoxy)-1H-benzo[d]imidazole **(5b)** CAS Registry Number 1256094-31-1

5-(methylthio)-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**5c**) CAS Registry Number 1256094-29-7

2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxylic acid (**5d**) CAS Registry Number 669070-64-8

2-(pyridin-2-yl)-1H-benzo[d]imidazole-5-carboxamide (**5e**) CAS Registry Number 1256094-43-5

N,N-dimethyl-2-(pyridin-2-yl)-1H-benzo[d]imidazol-5-amine (**5f**) CAS Registry Number 889664-39-5

2-(pyridin-2-yl)-5-(pyrrolidin-1-yl)-1H-benzo[d]imidazole (**5g**) CAS Registry Number 1256094-40-2

5-(piperidin-1-yl)-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**5h**) CAS Registry Number 1256094-41-3

4-(2-(pyridin-2-yl)-1H-benzo[d]imidazol-5-yl)morpholine (**5i**) CAS Registry Number 1256094-42-4

4,6-dichloro-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**5j**) CAS Registry Number 1256094-36-6

5-chloro-6-methoxy-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**5k**) CAS Registry Number 1256094-35-5

4,5,6-trifluoro-2-(pyridin-2-yl)-1H-benzo[d]imidazole (**5l**) CAS Registry Number 1256094-33-3

5-methoxy-2-(5-methoxypyridin-2-yl)-1H-benzo[d]imidazole (**6a**) CAS Registry Number 1256094-47-9

5-methoxy-2-(5-(2-methoxyethoxy)pyridin-2-yl)-1H-benzo[d]imidazole (**6b**)
CAS Registry Number 1256094-49-1

5-methoxy-2-(4-methoxypyridin-2-yl)-1H-benzo[d]imidazole (**6c**) CAS Registry Number 1256094-56-0

5-methoxy-2-(3-methoxypyridin-2-yl)-1H-benzo[d]imidazole (**7a**) CAS Registry Number 1256094-51-5

2-(3-methylpyridin-2-yl)-5-(trifluoromethoxy)-1H-benzo[d]imidazole (**7b**) CAS Registry Number 1256094-53-7

5-(trifluoromethoxy)-2-(3-(trifluoromethyl)pyridin-2-yl)-1H-benzo[d]imidazole (**7c**) CAS Registry Number 1256094-55-9

2-(3-fluoropyridin-2-yl)-5-(trifluoromethoxy)-1H-benzo[d]imidazole (**7d**) CAS Registry Number 1256094-54-8

Synthesis of 3,5-disubsituted pyridine-2-aldehydes and benzimidazoles

The following substituted pyridine-2-aldehydes, were prepared as described previously (Harriman et al., 2013, Zhang et al., 2005, Griffith et al., 1992, Smith et al., 2013, Anderson et al., 2006).

3-methoxypicolinaldehyde; CAS Registry Number 1849-53-2

3,5-dimethoxypicolinaldehyde; CAS Registry Number 1256790-69-8

3methyl 2-formylnicotinate; CAS Registry Number 25230-59-5

2-formylnicotinic acid; CAS Registry Number 23590-67-2

2-formylnicotinamide; CAS Registry Number 951924-56-4

3-(dimethylamino) picolinaldehyde; CAS Registry Number 1780942-71-3

3,4-dimethoxypicolinaldehyde; CAS Registry Number 142470-53-9

3-(pyrrolidin-1-yl) picolinaldehyde; CAS Registry Number 1707358-09-5

5-hydroxy-3-methoxypicolinaldehyde; CAS Registry Number 1289039-21-9

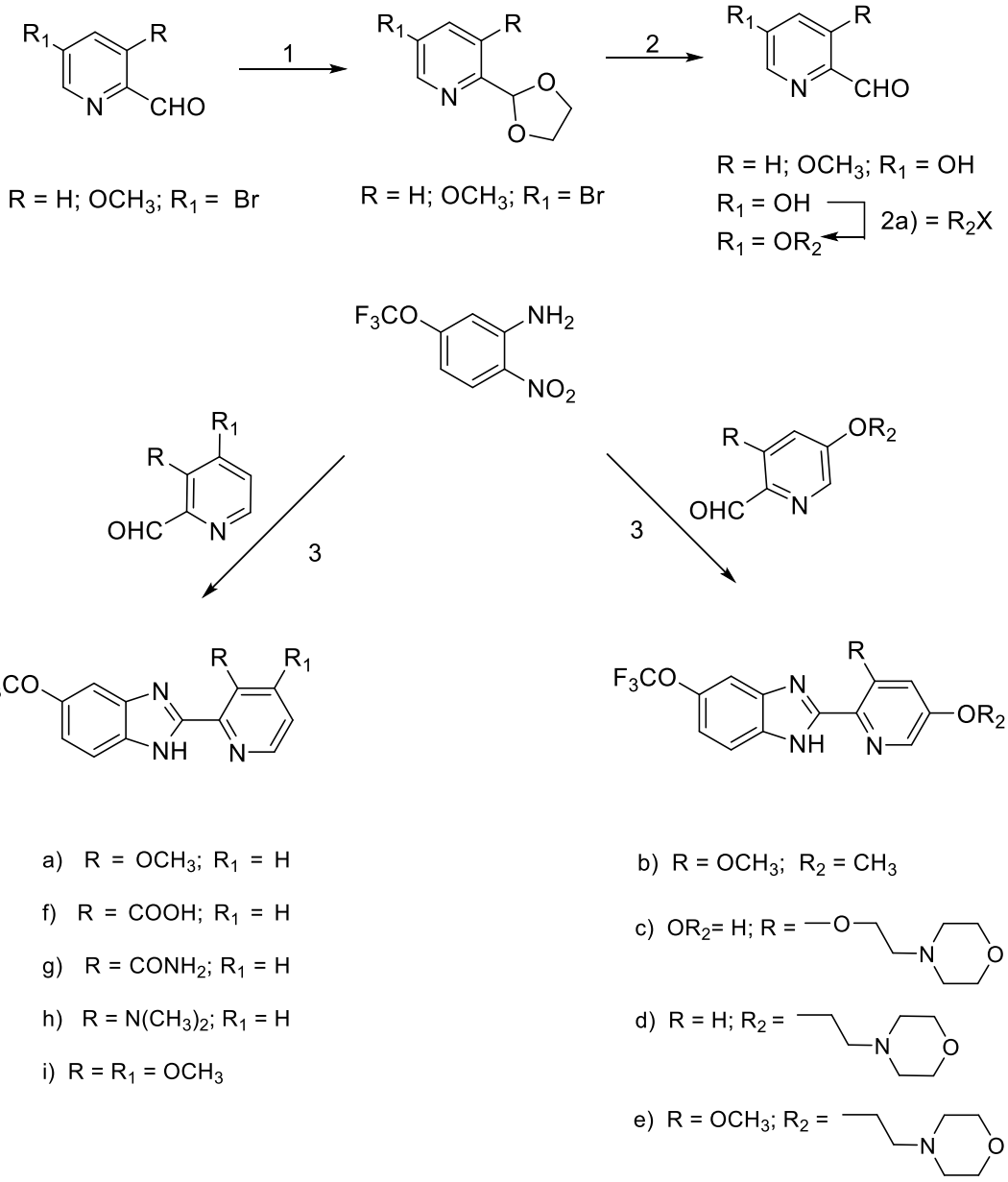
General procedure for the synthesis of substituted benzimidazoles (Scheme 1, a-i)

To a solution of appropriately substituted aldehyde (1.0 mmol) and 2-nitro-5-(trifluoromethoxy)aniline (1.0 mmol) in ethanol (4 mL) and DMSO (0.2 mL, 5%), a sodium dithionite solution (0.52 g, 3 mmol in 3 mL water) was added (Illendula et al., 2015). The reaction mixture was refluxed for 8 hours or completion of the reaction as assessed by TLC. The solvent was removed under reduced pressure, diluted with water, and neutralized with aqueous NH_4OH solution. After usual work up the crude reaction mixtures were purified by flash chromatography by using appropriate solvent systems and evaporation of the solvents led to viscous compounds or solids. The slowly solidifying

precipitate/viscous compounds were dissolved in a minimal amount of dichloromethane and triturated with hexanes, stirring overnight at r.t to get a clear solids. The solid thus obtained was filtered, and dried under vacuum to give substituted benzimidazoles.

Methyl 2-(5-(trifluoromethoxy)-1H-benzo[d]imidazol-2-yl)nicotinate (Pearson et al., 1991) CAS Registry Number 136556-21-3

Scheme I



a) R = OCH_3; R_1 = H

f) R = COOH; R_1 = H

g) R = $CONH_2$; R_1 = H

h) R = $N(CH_3)_2$; R_1 = H

i) R = R_1 = OCH_3

b) R = OCH_3; R_2 = CH_3

c) OR_2= H; R =

d) R = H; R_2 =

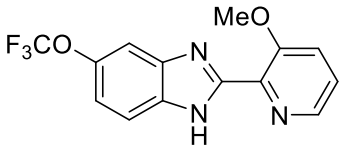
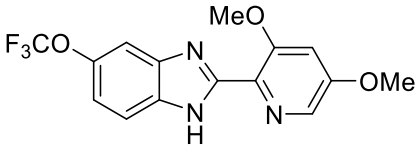
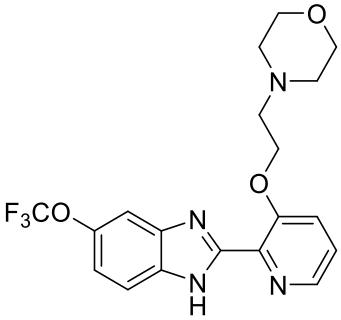
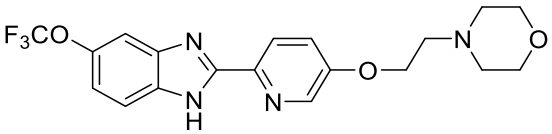
e) R = OCH_3; R_2 =

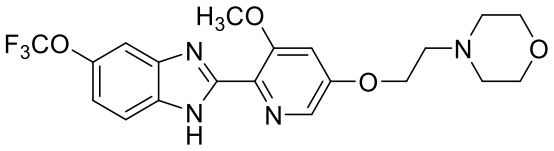
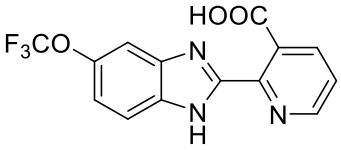
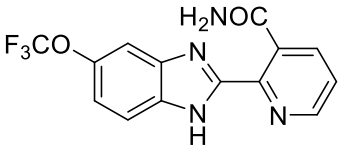
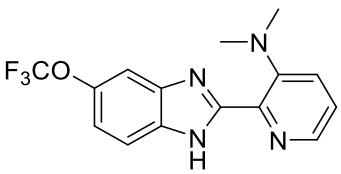
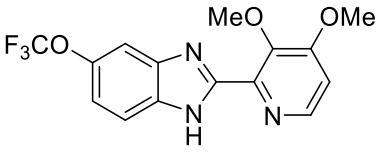
1. Ethane-1,2-diol, TsOH, toluene, reflux, 8h
2. $Pd_2(dba)_3$, Tetramethyl tBuXPhos, KOH, dioxane/water, reflux, 16h

2a. R2-Cl, K_2CO_3, DMF, 70°C, 8h

3. Aldehyde, $Na_2S_2O_4$, EtOH/water, 80°C, 8h

Physical properties and spectral data

Name	Structure	MP (°C)	^{1}H-NMR	^{13}C-NMR	HRMS
AI-10-104 (a)		165-167	^{1}H-NMR (800 MHz, MeOD): δ4.10 (3H, s), 7.22-7.23 (1H, d, *J* = 8.00 Hz), 7.51-7.53 (1H, dd, *J* = 8.00, 8.00 Hz), 7.61 (1H, s), 7.69-7.71 (1H, d, *J* = 8.00 Hz), 7.74-7.75 (1H, d, *J* = 8.00 Hz), 8.37-8.38 (1H, d, *J* = 8.00 Hz)	^{13}C-NMR (800 MHz, MeOD): δ56.48, 118.04, 121.49, 121.70, 122.96, 124.23, 127.44, 137.47, 142.74, 146.63, 152.66, 156.44	m/z $[M+H]^+$ calcd for $C_{14}H_{10}F_3N_3O_2$ 310.0798; found: 310.0797
AI-12-16 (b)		164-166	^{1}H-NMR (600 MHz, MeOD): δ3.98 (3H, s), 4.08 (3H, s), 7.15-7.17 (1H, d, *J* = 8.6 Hz), 7.20 (1H, s), 7.54 (1H, s), 7.65-7.67 (1H, d, *J* = 8.5 Hz), 8.07 (1H, s)		m/z $[M+H]^+$ calcd for $C_{15}H_{12}F_3N_3O_3$ 340.0904; found: 340.0905
AI-14-55 (c)		>250	^{1}H-NMR (800 MHz, MeOD): δ2.63 (4H, s), 2.96-2.97 (2H, t), 3.68-3.69 (4H, t), 4.46-4.47 (2H, t), 7.26-7.28 (1H, d, *J* = 8,4 Hz), 7.54-7.56 (1H, dd, *J* = 8.40, 8.40 Hz), 7.63 (1H, s), 7.66-7.77 (2H, d, *J* = 8.00 Hz), 8.41-8.42 (1H, d, *J* = 8.00 Hz)	^{13}C-NMR (800 MHz, MeOD): δ54.99, 58.29, 67.54, 67.87, 117.30, 118.21, 120.43, 121.70, 122.96, 123.27, 124.23, 127.49, 138.32, 143.47, 146.67, 152.58, 155.63	m/z $[M+H]^+$ calcd for $C_{19}H_{19}F_3N_4O_3$ 409.1482; found: 409.1485
AI-12-126 (d)		135-137	^{1}H-NMR (800 MHz, MeOD): δ2.63 (4H, s), 2.86-2.87 (2H, t), 3.74-3.75 (4H, t), 4.29-4.30 (2H, t), 7.20-7.21 (1H, d, *J* = 5.36 Hz), 7.50-7.73 (3H, m), 8.23-8.24 (1H, d, *J* = 8.40 Hz), 8.44 (1H, d, *J* = 2.72 Hz)	^{13}C-NMR (800 MHz, MeOD): δ55.24, 58.65, 67.28, 67.79, 106.02, 112.27, 113.63, 117.49, 118.36, 120.55, 121.67, 122.74, 122.94, 123.80, 134.63, 135,85, 139.62, 141.55, 143.56, 145.04, 146.28, 154.73, 157.86	m/z $[M+H]^+$ calcd for $C_{19}H_{19}F_3N_4O_3$ 409.1482; found: 409.1482

AI-14-91 (e)		94-96	^{1}H-NMR (800 MHz, MeOD): δ2.62 (4H, s), 2.84-2.86 (2H, t), 3.71- 3.73 (4H, m), 4.07 (3H, s), 4.30-4.32 (2H, t), 7.17-7.18 (1H, d, *J* = 8.4 Hz), 7.20-7.21 (1H, d, *J* = 2.24 Hz), 7.55 (1H, s), 7.67-7.68 (1H, d, *J* = 8.4 Hz), 8.08-8.09 (1H, d, *J* = 1.4 Hz)	^{13}C-NMR (800 MHz, MeOD): δ55.24, 56.61, 58.69, 67.49, 67.78, 106.99, 109.42, 116.87, 117.71, 120.44, 121.70, 122.96, 124.23, 130.15, 131.00, 146.41, 152.93, 157.52, 159.07	m/z $[M+H]^+$ calcd for $C_{20}H_{21}F_3N_4O_4$ 439.1588; found: 439.1593
AI-12-150 (f)		235-238	^{1}H-NMR (800 MHz, MeOD): δ7.33-7.34 (1H, dd, *J* = 1.4, 8.72 Hz), 7.66 (1H, s), 7.68-7.70 (1H, dd, *J* = 8.00, 8.00 Hz), 7.78-7.79 (1H, d, *J* = 8.00 Hz), 8.65-8.66 (1H, dd, *J* = 1.44, 8.00 Hz), 8.89-8.90 (1H, dd, *J* = 1.1, 5.4 Hz)	^{13}C-NMR (800 MHz, MeOD): δ109.53, 117.62, 119.55, 120.32, 121.59, 122.86, 124.13, 126.62, 130.47, 136.54, 138.16, 142.56, 145.82, 147.43, 152.94, 153.34 169.01	m/z $[M+H]^+$ calcd for $C_{14}H_8F_3N_3O_3$ 324.0591; found: 324.0592
AI-12-149 (g)		>250	^{1}H-NMR (800 MHz, MeOD): δ7.22 (1H, s), 7.57-7.69 (3H, m), 8.06-8.07 (1H, m), 8.80-8.81 (1H, d, *J* = 8.00 Hz)	^{13}C-NMR (800 MHz, MeOD): δ104.25, 111.93, 115.75, 116.98, 118.87, 120.14, 121.40, 122.66, 124.04, 132.27, 136.76, 144.80, 149.91, 151.85, 173.04	m/z $[M+H]^+$ calcd for $C_{14}H_9F_3N_4O_2$ 323.0750; found: 323.0753
AI-14-18 (h)		189-191	^{1}H-NMR (800 MHz, MeOD): δ2.77 (6H, s), 7.24-7.25 (1H, dd, *J* = 1.5, 8.60 Hz), 7.44-7.46 (1H, dd, *J* = 8.32, 8.32 Hz), 7.61 (1H, s), 7.69-7.70 (1H, dd, *J* = 1.2, 8.30 Hz), 7.73-7.74 (1H, d, *J* = 8.70 Hz), 8.30-8.31 (1H, dd, *J* = 1.3, 4.4 Hz)	^{13}C-NMR (600 MHz, MeOD): δ43.97, 109.37, 118.04, 120.43, 121.70, 122.96, 124.23, 126.65, 127.78, 140.72, 142.93, 146.58, 150.90, 154.37	m/z $[M+H]^+$ calcd for $C_{15}H_{13}F_3N_4O$ 323.1114; found: 323.1116
AI-14-72 (i)		96-99	^{1}H-NMR (800 MHz, MeOD): 3.97 (3H, s), 4.04 (3H, s), 7.22-7.24 (2H, m), 7.62 (1H, s), 7.74-7.75 (1H, d, *J* = 8.00 Hz), 8.39-8.34 (1H, d, *J* = 5.4 Hz)	^{13}C-NMR (800 MHz, MeOD): δ56.91, 61.85, 110.55, 118.13, 120.43, 121.70, 122.96, 124.23, 141.91, 146.66, 146.88, 147.72, 152.41, 161.72	m/z $[M+H]^+$ calcd for $C_{15}H_{12}F_3N_3O_3$ 340.0904; found: 340.0904

Pharmacokinetics analysis of AI-12-126 and AI-14-91.

Test Compound Formulation

All dosing solutions were formulated in-house and sterile filtered through 0.22-micron sterile filters prior to use. All solutions were stable over two weeks at 25°C as assessed by HPLC.

AI-12-126 and AI-14-91

Dosing solutions of 25 mg/mL in 0.1 M Captisol (Ligand Pharmacueticals, La Jolla, CA, USA) were prepared with the *in situ* formed hydrochloride salt of each of the respective agents.

Pilot Pharmacokinetic Studies

The University of Kansas Institutional Animal Care and Use Committee approved the AI-12-126 study and appropriate guidelines for the use of animals were observed during all aspects of the study. Prior to the study, mice were fasted at least three hours and water was available *ad libitum*. Animals were housed on a 12-hour light/dark cycle at 72-74°C and 30-50% relative humidity.

For IP dosing 24-28 gm male C57BL/6 mice (Harlan Laboratories, USA) were manually restrained and injected in the peritoneal cavity midway between the sternum and pubis and slightly off the midline of the mouse. A 1-cc syringe with a 27-gauge needle was used for each injection. Blood was collected from the animals according to scheduled time points. Animals were anesthetized with isoflurane and blood drawn via cardiac puncture. Blood was immediately transferred to 1.5 mL heparinized microcentrifuge tubes and centrifuged at 4000 rpm for ten minutes. Plasma was then transferred to clean tubes and frozen. Due to exsanguination, the animals did not wake from the anesthesia and death was ensured while under anesthesia by thoracotomy. This method is consistent

with the recommendations of the AVMA Guidelines on Euthanasia for use of exsanguination as a means of euthanasia.

The University of Virginia Institutional Animal Care and Use Committee approved the AI-14-91 study and appropriate guidelines for the use of animals were observed during all aspects of the study. Animals were housed on a 12-hour light/dark cycle at 72-74°C and 30-50% relative humidity.

Drug was administered by IP and gavage dosing. For IP dosing 19-21 gm female Balb C mice (Charles River Laboratories, USA) were manually restrained and injected in the peritoneal cavity midway between the sternum and pubis and slightly off the midline of the mouse. A 1-cc syringe with a 27-gauge needle was used for each injection. For gavage dosing, a 1 cc syringe and a 22-gauge gavage needle were used. Blood was collected in a terminal bleed at the time of euthanasia from two mice per drug delivery method at the following time points post-treatment: 0, 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hours. Animals were euthanized by an overdose IP injection of 6mg ketamine and 0.6mg xylazine. As soon as mice entered agonal breathing blood was collected from the chest cavity in a 1cc syringe following the severing of the aortic arch. Harvested blood was immediately transferred to microcentrifuge tubes containing 50ul of 0.5M EDTA solution and centrifuged at 4000 rpm for ten minutes. Plasma was then transferred to clean tubes and placed at -80C. Due to exsanguination, the animals did not wake from the anesthesia and death was ensured while under anesthesia by thoracotomy. This method is consistent with the recommendations of the IACUC Guidelines on Euthanasia.

Noncompartmental pharmacokinetic analysis of the mean test compound plasma concentration-time data was conducted using PK Solutions 2.0 (Summit Research Services, Montrose, CO, USA).

Bioanalysis

Materials – Methyl-t-butyl ether (MtBE) and HPLC grade acetonitrile (ACN) were obtained from Fisher (NJ, USA). Trifluoroacetic acid (TFA) and formic acids was from Fluka (St. Louis, MO, USA). Dimethylsulfoxide (DMSO) was obtained from Sigma (St. Louis, MO, USA) and was Hybri-Max grade.

General Equipment - A VX-2500 multi-tube vortexer from VWR (West Chester, PA, USA) and an accuSpin Micro 17 centrifuge from Fisher (NJ, USA) were used in the sample preparation. Solvent evaporation was carried out on a CentriVap concentrator from Labconco (Kansas City, MO, USA) with a Büchi V-800 vacuum controller (Switzerland). Samples were analyzed on an Applied Biosystems 3200 QTRAP (Grand Island, NY, USA) operated in positive ion mode with a Shimadzu SCL-10A vp controller, SIL-20AC autosampler, LC-20AD pumps and CTO-20A column oven (Kyoto, Japan).

AI-12-126 – An LC/MS/MS method was developed for AI-12-126 using an internal standard of similar structure (AI-4-70). LC/MS chromatography conditions included a Zorbax SB C18 2.1 x 50 mm at 45°C with a mobile phase flow rate of 0.30 mL/min. The gradient elution consisted of solvents A and B with A) 5/95/0.1 and B) 95/5/0.1; acetonitrile/water/formic acid. The gradient consisted of 5% B for one minute, 5 - 95% B in three minutes, 95 - 5% B in 0.5 minutes, 5% B for three mintues, and 95% B for one minute. Samples were held at 8°C and 5 µL were injected. The retention times of AI-12-126 and AI-4-70 are 3.53 and 3.52 minutes, respectively.

Mass spectrometry conditions consisted of a curtain gas of 10 xx, a temperature of 700°C, GS1 & GS2 of 40 and 45 xx, respectively. Monitored AI-12-126 transitions were 409.047/114.20 and AI-4-70 transitions were 254.2/212.2, DP = 51/50, EP = 4.5/8, CEP = 60/16, CE = 37/47, and CXP = 4/2 for AI-12-126/AI-4-70, respectively. Two sets of standards and samples, a high concentration and a low concentration range were run. Extraction of plasma samples was conducted on 50 µL of the respective samples after adding 10 µL of AI-12-126 spiking solution (10X, standards), vortexing, and

allowing the sample sit at room temperature for five min. To each sample 10 µL of 250 ng/mL AI-4-70 was then added (5X), the sample was vortexed for five min after which time 250 µL MtBe was added. The two-phase mixture was vortexed for five min then centrifuged at 13,000 rpm for five min. 240 µL of the MtBE layer was transferred to clean tubes and evaporated to dryness. The resulting residue was reconstituted in 50 µL of 50/50 ACN/H2O and vortexed followed by centrifugation at 13,000 rpm for five min. 40 µL of the supernatant was transferred to autosampler vials with polypropylene inserts and analyzed.

The validation sets consisted of blank plasma and a standard curve with the range of 1, 5, 10, 25, 50, 100, 250, 500, and 1000 ng/mL AI-12-126 with 50 ng/mL AI-4-70 ($r > 0.9996$).

AI-14-91 - An LC/MS/MS method was developed for AI-14-91 using external standards. LC/MS chromatography conditions included a Zorbax SB C18, 5 µm, 2.1 x 50 mm at 50°C with a mobile phase flow rate of 0.20 mL/min. The gradient elution consisted of solvents A and B with A) 10 mM ammonium acetate pH 8.0, and B) acetonitrile. The gradient consisted of 5-95% B in three minutes, 95% B for 1.5 minutes, 95-5% B in 0.5 minutes, and 5% B for 3.5 minutes. Samples were held at 8°C and 5 µL were injected. The retention time of AI-14-91 is 3.0 minutes.

Mass spectrometry conditions consisted of a curtain gas of 12 xx, a temperature of 650°C and GS1 & GS2 of 40 xx. Monitored AI-14-91 transitions were 439.2/114.20, DP = 51, EP = 5.5, CEP = 18, CE = 35, CXP = 3.5. Two sets of standards and samples, a high concentration and a low concentration range were run. Following the addition of 50 µL of external standard, the standards prepared in plasma and samples were charged with acetonitrile, vortexed, centrifuged and the supernatant transferred to clean tubes, which were then dried under vacuum. The standards and samples were reconstituted in 50 µL 50/50 acetonitrile/water, vortexed, centrifuged, and the supernatant transferred

to autosampler vials for analysis. The validation sets consisted of blank plasma and a standard curve with the range of 5, 10, 25, 50, 100 and 250 ng/mL AI-14-91 ($r > 0.9992$).

Differentiation of HPC-7 cells.

IC_{50} determination

10,000 HPC-7 cells in 100 µl culture medium (IMDM media supplemented with $NaHCO_3$,10% FBS, 1% Pen-Strep, 0.15 mM monothiolglycerol [MTG SIGMA, M6145],10 % stem cell factor (SCF) conditioned media) were plated into a 96-well plate with inhibitor concentrations between 0.1 nM and 1 mM concentration. After 48 hours, 10 µl of MTT reagent (5µg/µl) was added into each well and incubated for four hours at 37 °C in a CO_2 incubator. Subsequently, 100 µl dissolving reagent (10%SDS + 0.01 M HCl) was added to each well. The following day absorbance was measured at 590 nM to determine the survival rate at each drug concentration. Concentration of the drug that gave 50% cell survival was defined as IC_{50} for each drug.

HPC-7 cell differentiation

$5*10^5$ HPC-7 cells were plated into each well of a 12-well plate in 1 ml of IMDM media supplemented with $NaHCO_3$,10% FBS, 1% Pen-Strep, 0.15 mM monothiolglycerol (MTG SIGMA, M6145),10 % stem cell factor conditioned media. Cells were differentiated by the addition of mGM-CSF (# 415-ML), mG-CSF (# 414-CS) and mIL-3 (# 403-ML) from R&D Systems. Each factor was added at 20 ng/ml concentration and was changed every 3 days with fresh media. After 10 days of differentiation, cells were washed with PBS and blocked with 10% FBS containing PBS for 30 min. Subsequently, cells were incubated with CD11b-APC (ebioscience-M1/70-cat# 101211) and Gr1-FITC (ebioscience-RB6-8C5-cat# 108405) for 30 minutes in the dark. Finally, cells were washed with 500 µl cold PBS twice and used for flow cytometry analysis immediately.

References for Supplementary Information

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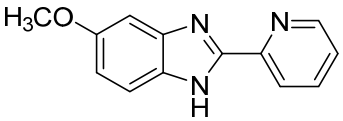
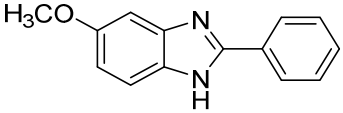
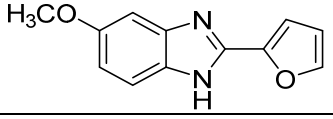
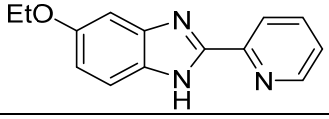
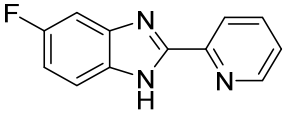
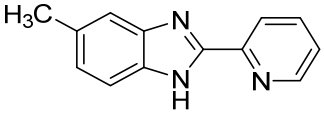
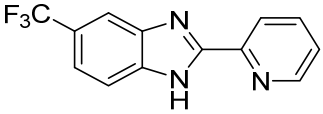
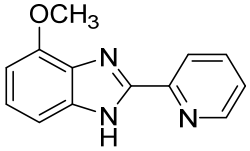
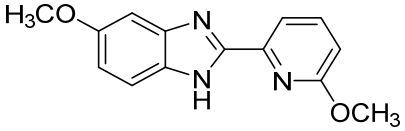
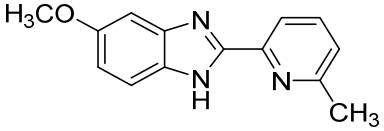
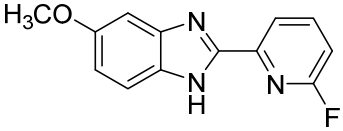
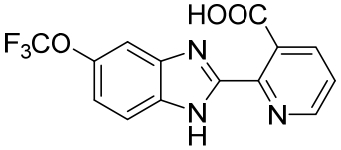
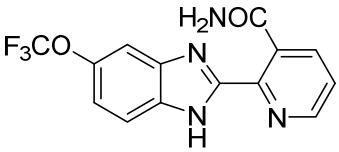
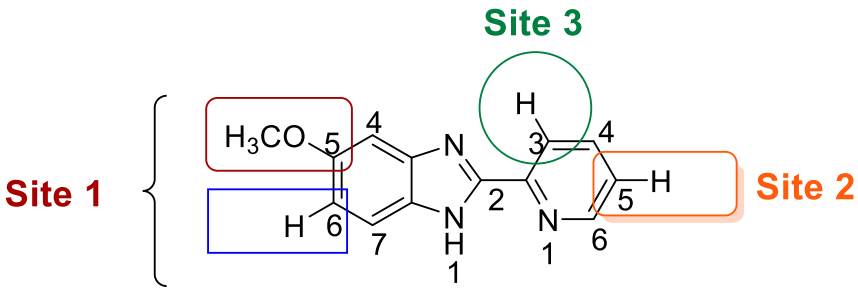
Supplementary Table 1

Compound ID	Structure	FRET IC_{50} (μM)
AI-4-57		34.3 ±0.3
2a		Not active
2b		Not active
3b		≥ 34
3c		≥ 34
3d		Not active
3f		Not active
4a		> 34
4g		Not active
4h		Not active
4i		Not active
AI-12-150 (f)		48.9 ±1.6

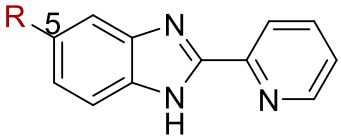
AI-12-149 (g)		Not active

Supplemental Information Figure 1

Design of library to further explore the SAR.

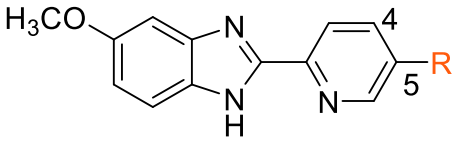


AI-4-57



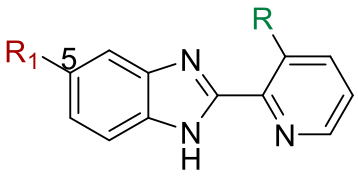
Site 1

5a) 5-isopropoxy
5b) 5-OCF_3
5c) 5-SCH_3
5d) 5-COOH
5e) 5-$CONH_2$
5f) 5-$N(CH_3)_2$
5g) 5-pyrrolidine
5h) 5-piperidine
5i) 5-morpholine
5j) 4,6-di-Cl
5k) 5-Cl, 6-OCH_3
5l) 4,5,6-tri-F



Site 2

6a) 5-OCH_3
6b) 5-$(OCH_2)_2OCH_3$
6c) 4-OCH_3

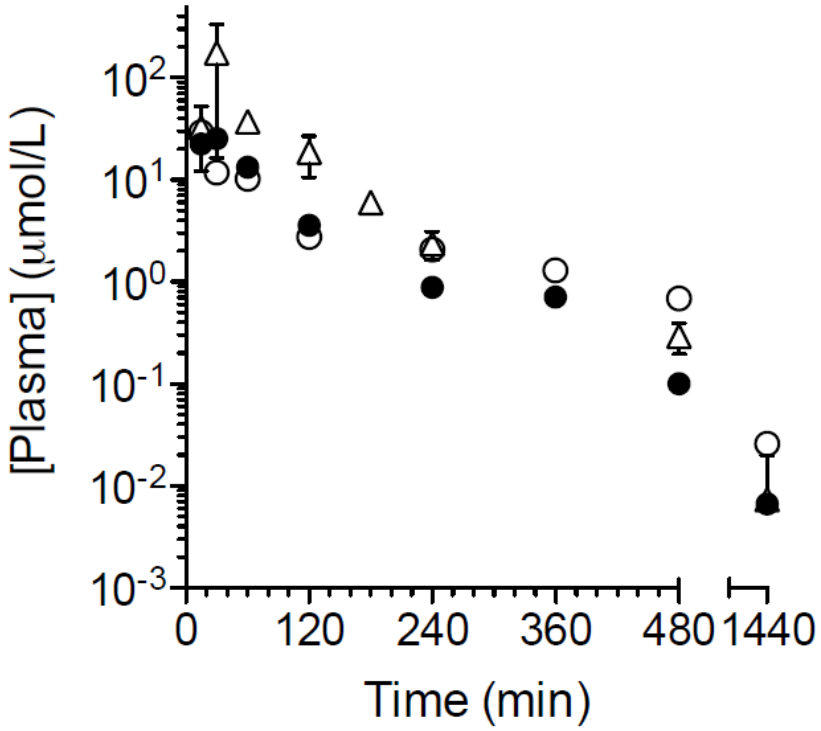


Site3

7a) R_1 = R = OCH_3
7b) R_1 = OCF_3; R = CH_3
7c) R_1 = OCF_3; R = CF_3
7d) R_1 = OCF_3; R = F

Supplemental Information Figure 2

Analysis of pharmacokinetics of AI-12-126 and AI-14-91 in mice.

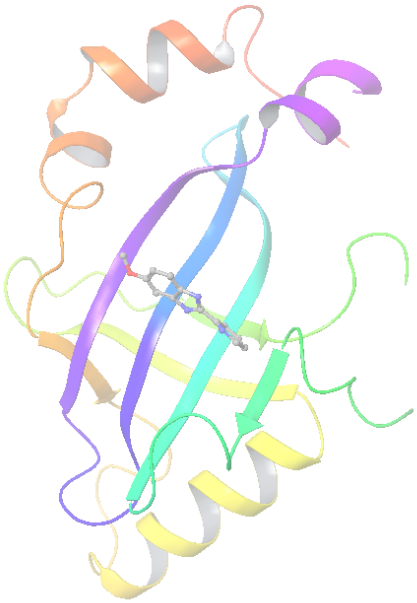


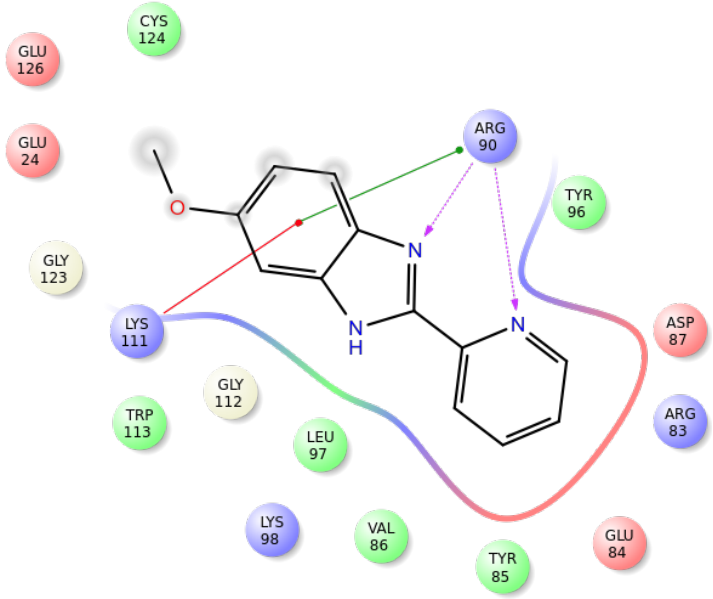
Plasma concentration data as a function of time for AI-12-126 (△, mean ± sd, n =3) or AI-14-91 (mean, n =2) from oral gavage (○) and intraperitoneal (●) dosing.

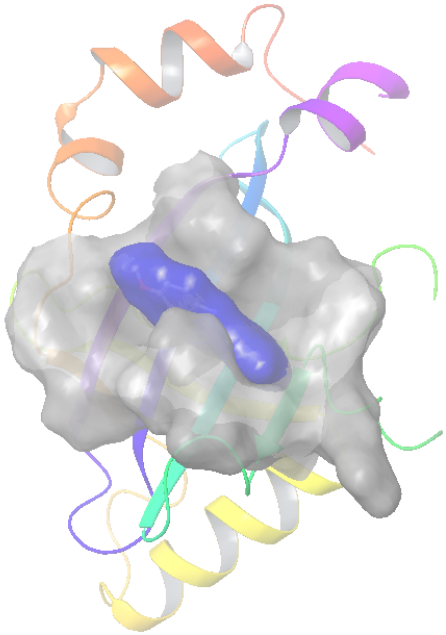
	AI-12-126 Intraperitoneal	AI-14-91 Oral Gavage	AI-14-91 Intraperitoneal
Half life (min)	179	203	243
AUC(0-inf) (µmol·min/L)	8.14	2.22	2.08
Mean Residence Time (min)	74.6	180.4	90.6
Volume of Distribution (mL)	193.9	754.0	958.7
Clearance (mL/min)	0.75	2.57	2.74

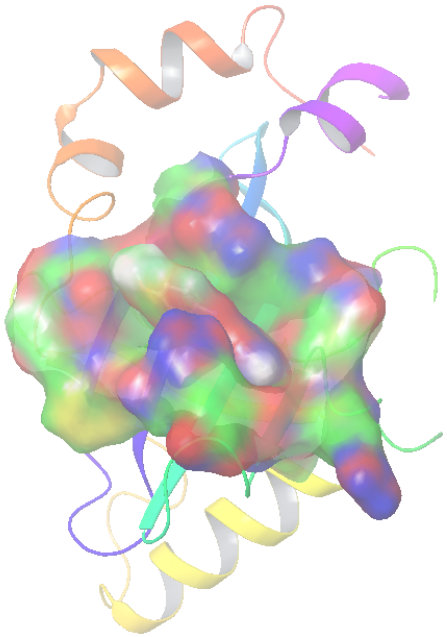
Supplemental Information Figure 3

Second orientation of AI-4-57 seen upon GLIDE docking.



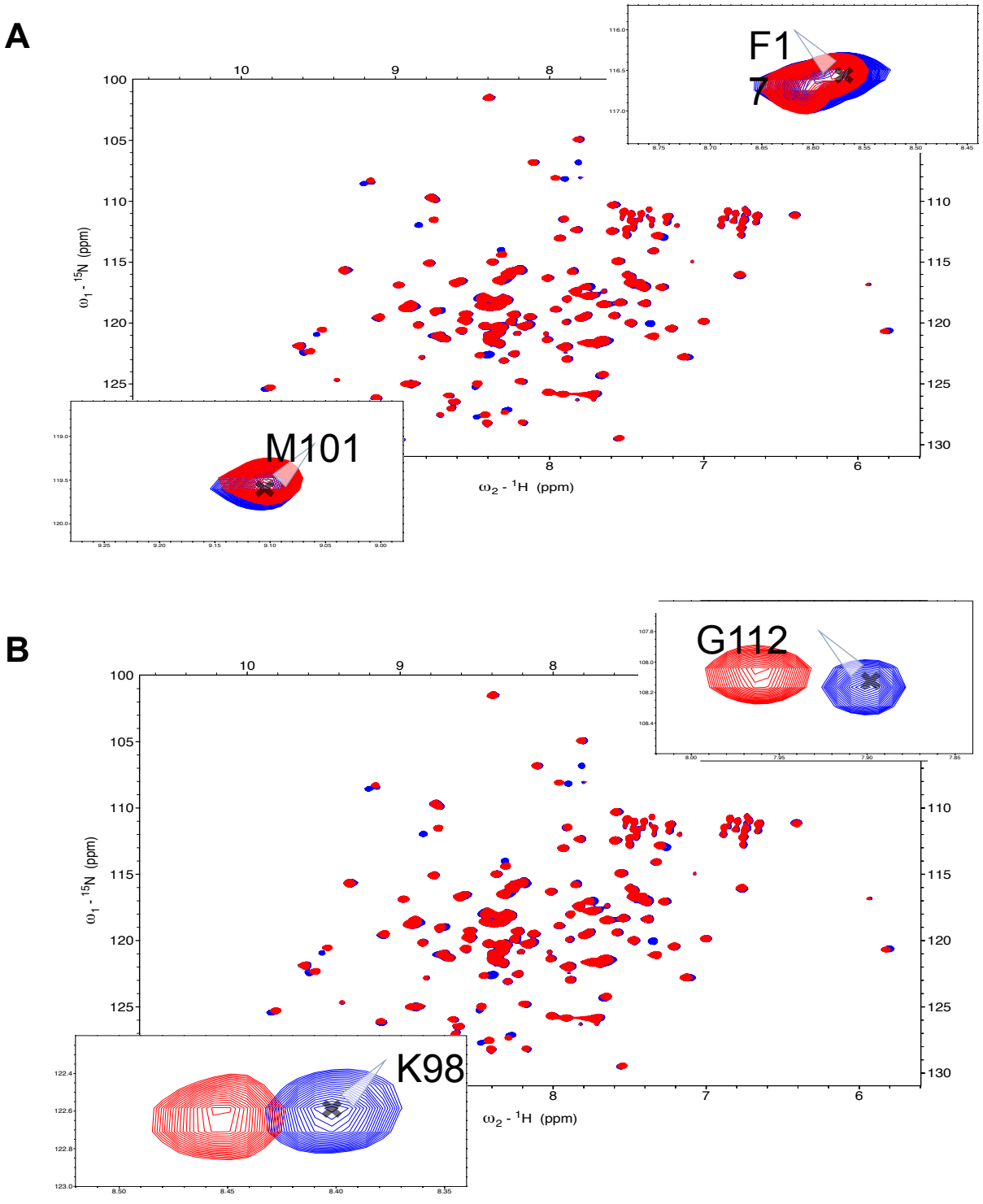






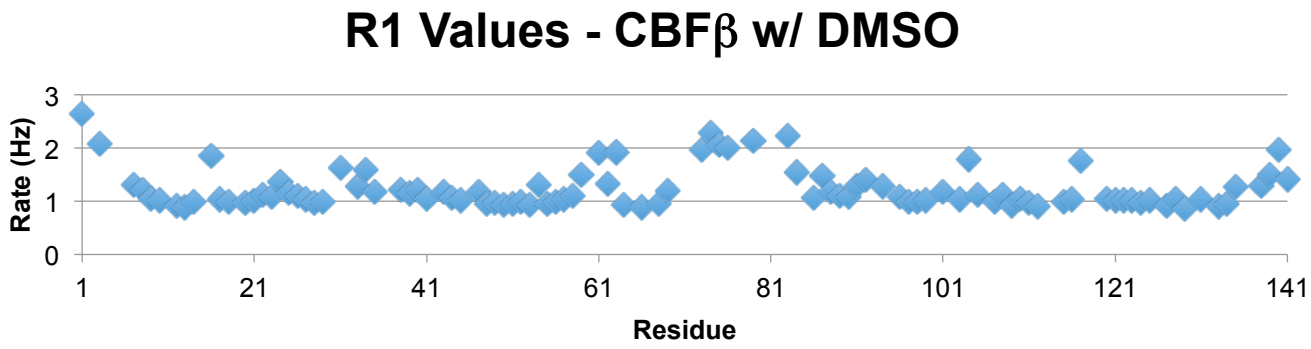
Supplemental Information Figure 4

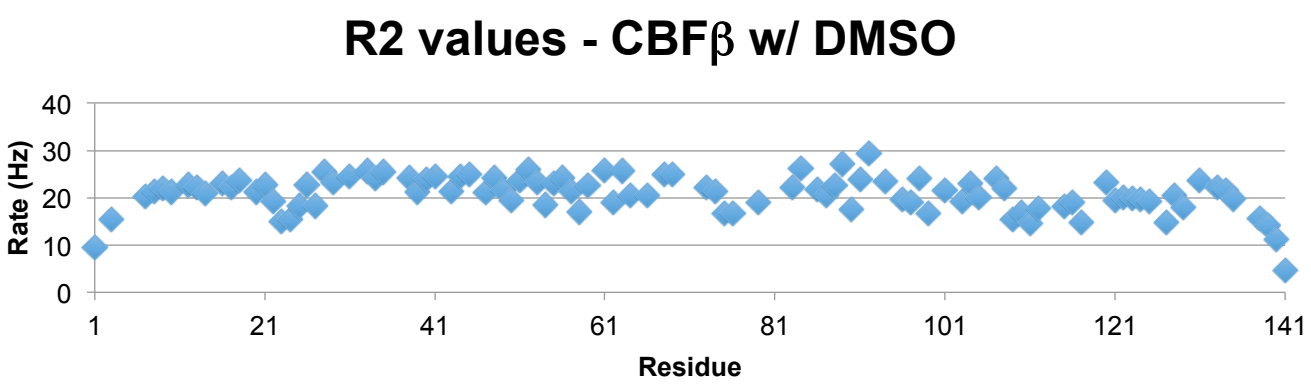
NMR chemical shift changes in CBFβ upon AI-4-57 binding. A. ^{15}N-^{1}H HSQC spectrum of CBFβ (red) and CBFβ+AI-4-57 (blue). Insets show two residues on the Runt domain binding interface that undergo chemical shift changes.
B. ^{15}N-^{1}H HSQC spectrum of CBFβ (red) and CBFβ+AI-4-57 (blue). Insets show two residues in the compound binding site that undergo chemical shift changes.

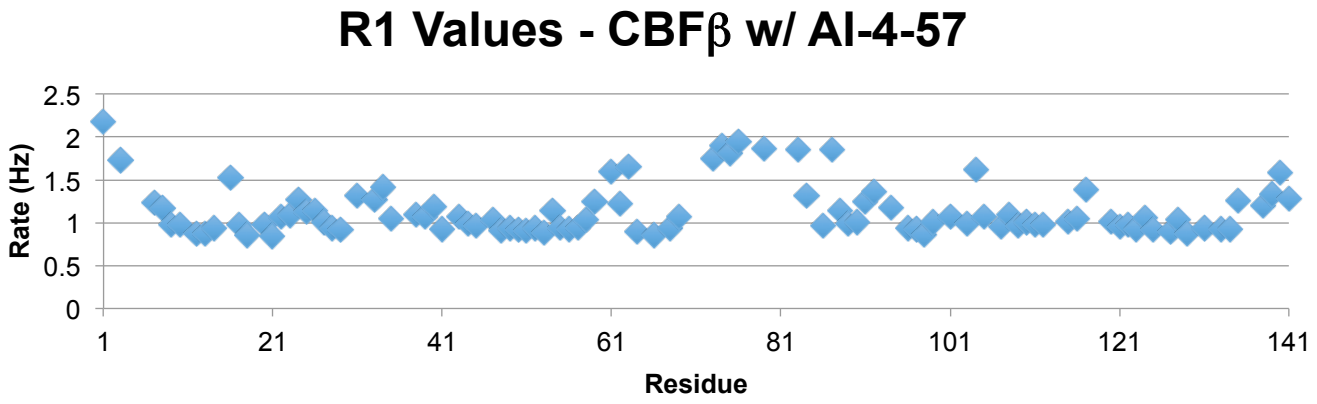


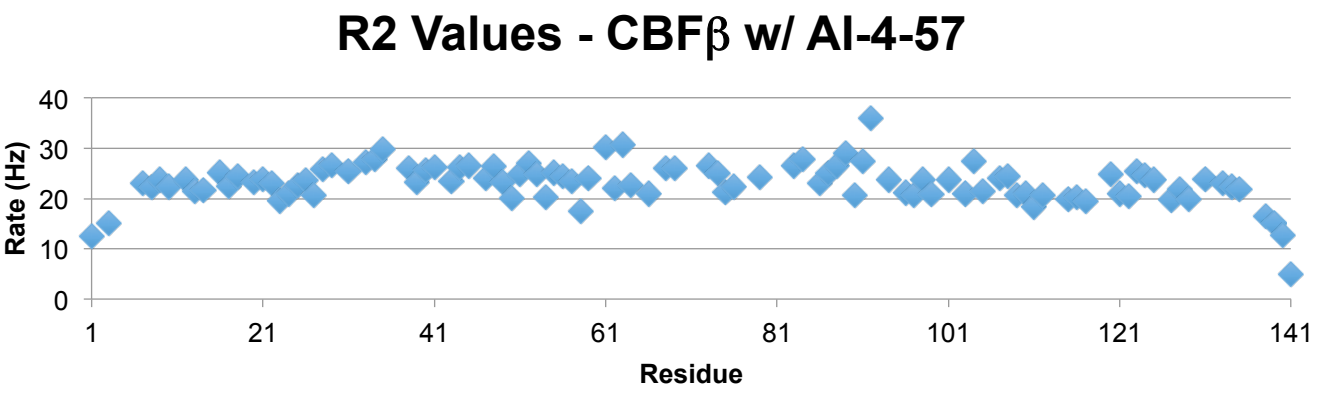
Supplemental Information Figure 5

^{15}N R1, R2 data for CBFβ alone and CBFβ+AI-4-57





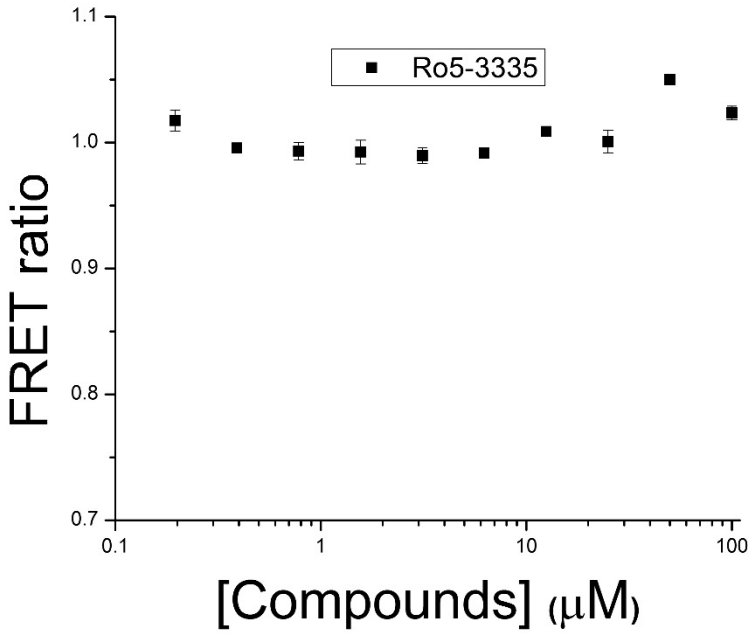




Supplemental Information Figure 6

A. FRET assay data for Ro-3335. B. Results of DiscoveRx Bromoscan bromodomain inhibition assays for Ro-3335.

a



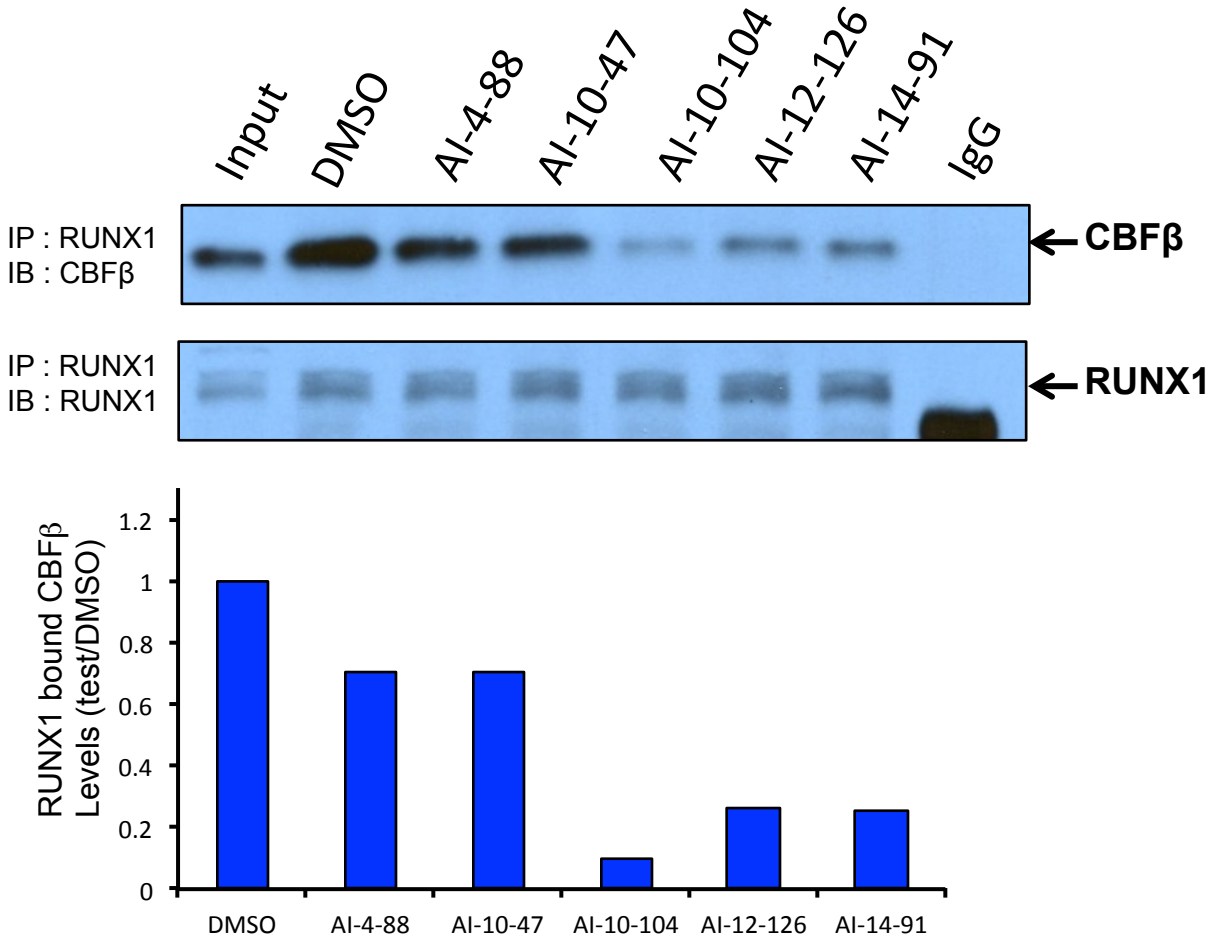
b

Target	Ro5-3335
Gene Symbol	%Ctrl @ 1000nM
ATAD2A	94
ATAD2B	100
BAZ2A	93
BAZ2B	97
BRD1	84
BRD2(1)	100
BRD2(2)	90
BRD3(1)	98
BRD3(2)	100
BRD4(1)	91
BRD4(2)	80
BRD7	91
BRD9	100
BRDT(1)	100
BRDT(2)	100
BRPF1	88
BRPF3	82
CECR2	90
CREBBP	99
EP300	89
FALZ	87
GCN5L2	82
PBRM1(2)	88
PBRM1(5)	98
PCAF	96
SMARCA2	62
SMARCA4	100
TAF1(2)	77
TAF1L(2)	88
TRIM24(PHD,Bromo.)	95
TRIM33(PHD,Bromo.)	95
WDR9(2)	62

%Ctrl Legend

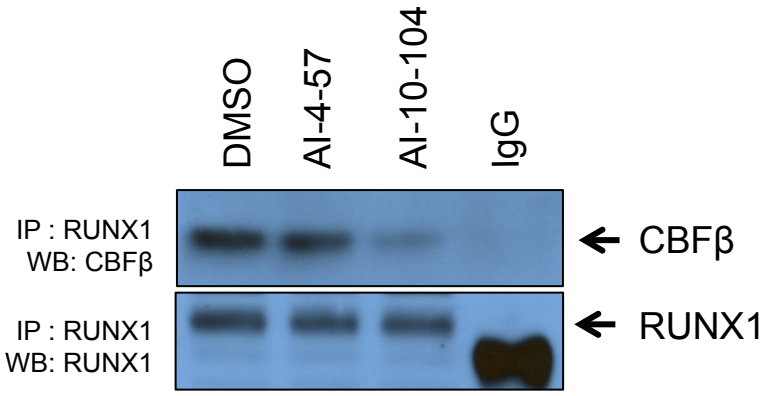
Supplemental Information Figure 7

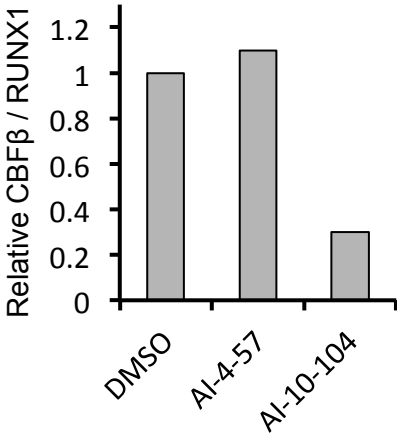
Replicate of Co-IP experiment with inhibitors (related to Figure 4)



Supplemental Information Figure 8

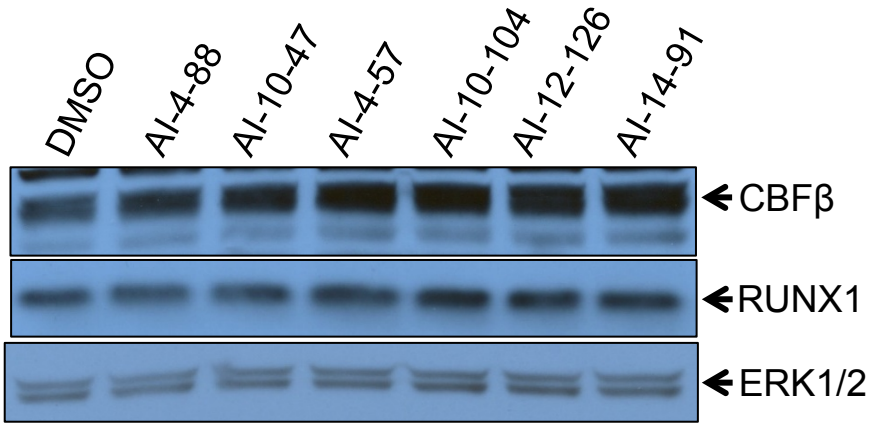
Comparison of effects of AI-4-57 and AI-10-104 on CBFβ-RUNX1 binding by coIP (related to Figure 4).

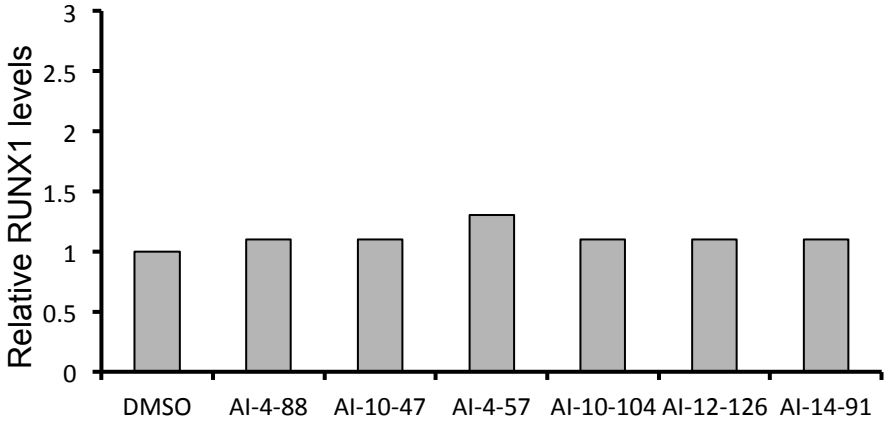


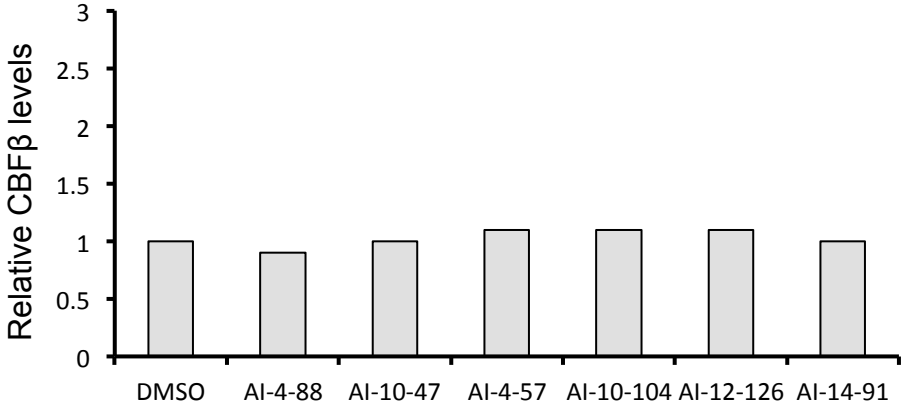


Supplemental Information Figure 9

The levels of CBFβ and RUNX1 protein were not affected by treatment with inhibitors (related to Figure 4).

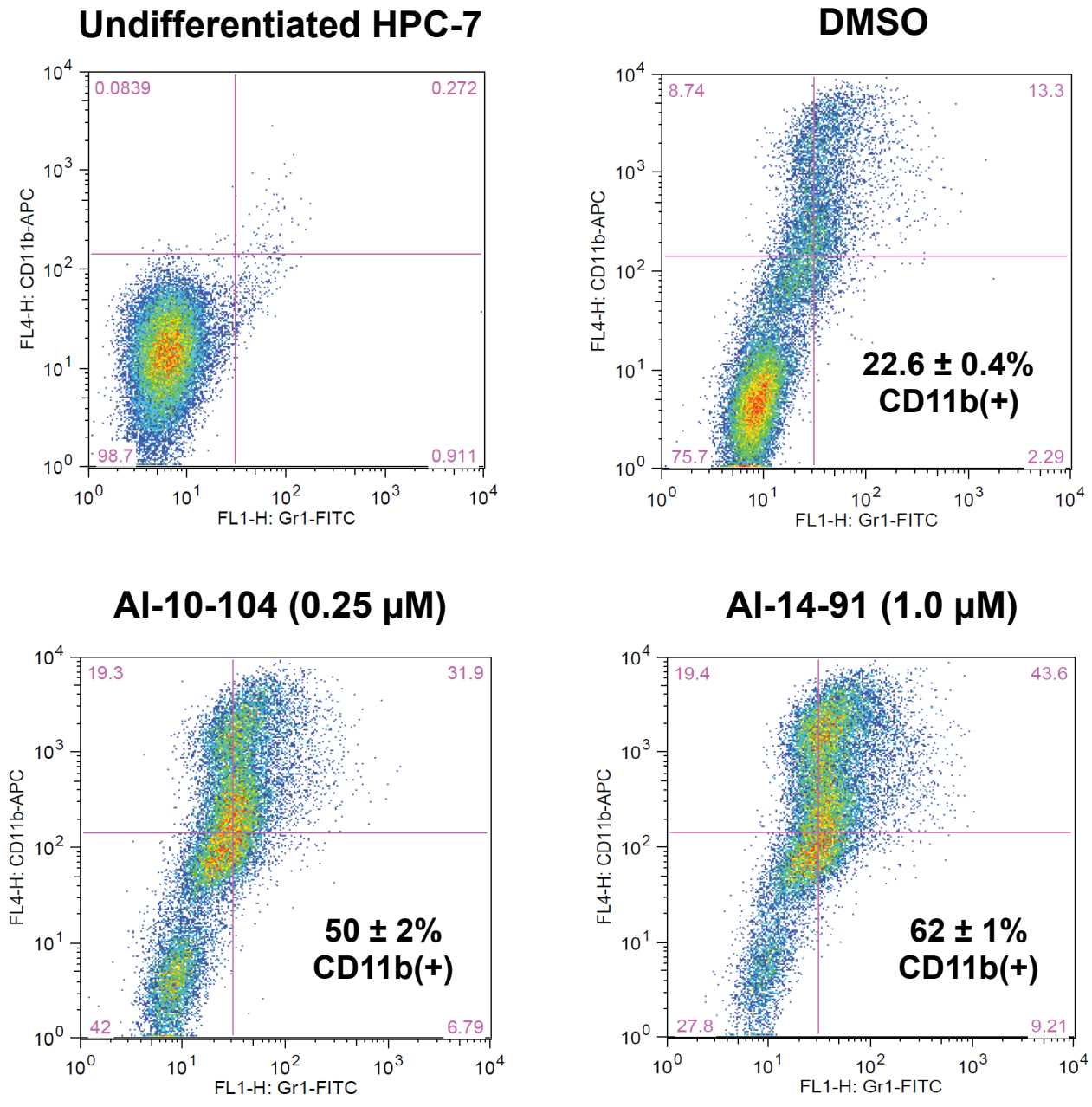






Supplemental Information Figure 10

Effects of CBFβ inhibitors on the myeloid differentiation of HPC-7 cells. HPC-7 cells were differentiated in the presence of CBFβ inhibitors for 10 days. Three different mouse stimulating factors (mGM-CSF, mG-CSF and mIL-3) were used during the differentiation. 1/10 of IC_{50} value was indicated next to the drug name which was replaced with fresh media every 3 days during the experiment. Untreated HPC-7 in stem cell media was used as a negative control. Since drugs were prepared in DMSO at 10 mM stock, DMSO was diluted 10,000 times in the control experiment as an average. CD11b and Gr1 antibodies were used to quantify the amount of myeloid differentiation. CD11b levels in the differentiated cells were measured and error bars calculated from two independent experiments.



Supplemental Information Figure 11

Effects of CBFβ inhibitors on colony formation of normal hematopoietic cells. **A.** Colony forming assays were performed on cord blood (CB) cells from healthy donors after 48 h culture in presence or absence of 10 μM of the indicated compounds. The percent change in colony forming unit (CFU) relative to DMSO control is represented. Each symbol represents CB sample. **B.** Images of the colonies from the assays with inhibitors. **C.** Results of colony assays with 20 μM of the indicated inhibitors. Data shows individual data points for 2 CB samples performed in duplicate. Error bars represent the SEM.