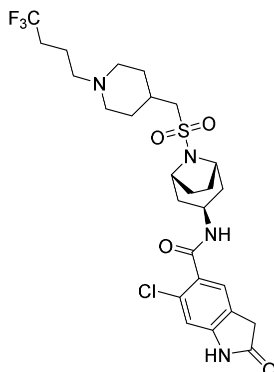
Supporting information

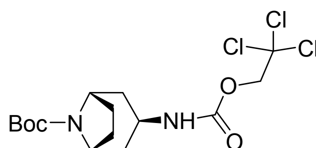
Compound synthesis

Synthesis of EPZ031686, 6-chloro-2-oxo-N-((1S,3r,5R)-8-((1-(4,4,4-trifluorobutyl)piperidin-4-yl)methylsulfonyl)-8-aza-bicyclo[3.2.1]octan-3-yl)indoline-5-carboxamide



Step 1

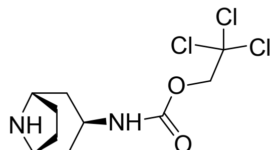
tert-butyl (1R,3S,5S)-3-[[(2,2,2-trichloroethoxy)carbonyl]amino]-8-azabicyclo[3.2.1]octane-8-carboxylate



Into a 250-mL round-bottom flask, was placed water (120 mL). This was followed by the addition of tert-butyl (1R,5S)-3-amino-8-azabicyclo[3.2.1]octane-8-carboxylate (2 g, 8.84 mmol, 1.00 equiv), sodium bicarbonate (1.92 g, 22.85 mmol, 2.59 equiv). To the mixture was added 2,2,2-trichloroethyl chloroformate (2.28 g, 10.76 mmol, 1.22 equiv) dropwise with stirring at 0°C. The resulting solution was stirred for 18 h at 20°C. The resulting solution was extracted with 3x150 mL of ethyl acetate and the organic layers combined and dried over anhydrous sodium sulfate and concentrated under vacuum. This resulted in 4.16 g (crude) of tert-butyl (1R,5S)-3-[[(2,2,2-trichloroethoxy)carbonyl]amino]-8-azabicyclo[3.2.1]octane-8-carboxylate as a white solid. ^{1}H NMR(300 MHz, $CDCl_3$): δ 4.75(s, 2H), 4.25 (s, 2H), 4.00-3.90(m, 1H), 2.29-2.00(m, 4H), 2.89-2.71(m, 4H), 1.45(s, 9H) *ppm*.

Step 2

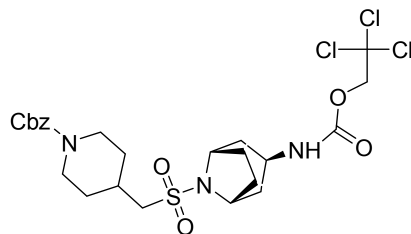
2,2,2-trichloroethyl (1S,3r,5R)-8-aza-bicyclo[3.2.1]octan-3-ylcarbamate



Into a 100-mL round-bottom flask, was placed dichloromethane (20 mL), tert-butyl (1R,3S,5S)-3-[[(2,2,2-trichloroethoxy)carbonyl]amino]-8-azabicyclo[3.2.1]octane-8-carboxylate (2 g, 4.98 mmol, 1.00 equiv). Then hydrogen chloride gas was introduced into mixture. The resulting solution was stirred for 12 h at 80°C. The resulting mixture was concentrated under vacuum. This resulted in 1.7 g (crude) of 2,2,2-trichloroethyl N-[(1R,3S,5S)-8-azabicyclo[3.2.1]octan-3-yl]carbamate as a yellow solid. LCMS (method D, ESI): RT = 0.86 min, *m/z* =301.2 $[M+H]^+$.

Step 3

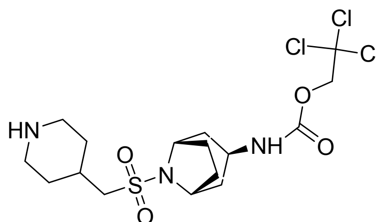
benzyl 4-(((1S,3r,5R)-3-((2,2,2-trichloroethoxy)carbonylamino)-8-aza-bicyclo[3.2.1]octan-8-ylsulfonyl)methyl)piperidine-1-carboxylate



Into a 100-mL round-bottom flask, was placed dichloromethane (30 mL), 2,2,2-trichloroethyl N-[(1R,3S,5S)-8-azabicyclo[3.2.1]octan-3-yl]carbamate (2.4 g, 7.96 mmol, 1.00 equiv), TEA (3.2 g, 31.62 mmol, 3.97 equiv). Then benzyl 4-[(chlorosulfonyl)methyl]piperidine-1-carboxylate (4 g, 12.05 mmol, 1.51 equiv) was added by dropwise at 0^oC. The resulting solution was stirred for 12 h at 10^oC. The resulting mixture was washed with 3x30 mL of water and 1x30 mL of brine. The mixture was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was applied onto a silica gel column with dichloromethane/methanol (20:1). This resulted in 2.8 g (59%) of benzyl 4-[[(1R,3S,5S)-3-[[(2,2,2-trichloroethoxy)carbonyl]amino]-8-azabicyclo[3.2.1]octane-8-sulfonyl]methyl]piperidine-1-carboxylate as a yellow solid. ^1H NMR(300 MHz, $CDCl_3$): δ 7.41-7.30 (m, 5H), 5.15 (s, 2H), 4.75 (s, 2H), 4.30-4.15(m, 4H), 4.05-3.90 (m, 1H), 2.95-2.76 (m, 4H), 2.35-2.10 (m, 4H), 2.10-1.90 (m, 5H), 1.57(s, 1H), 1.40-1.20(m, 3H) *ppm*. LCMS (method D, ESI): RT = 1.15 min, *m/z* =596.1 $[M+H]^+$.

Step 4

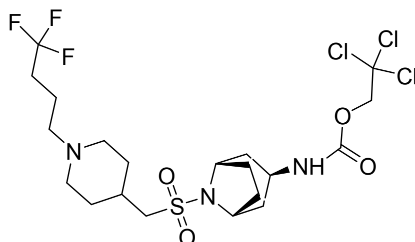
2,2,2-trichloroethyl (1S,3r,5R)-8-(piperidin-4-ylmethylsulfonyl)-8-aza-bicyclo[3.2.1]octan-3-ylcarbamate hydrochloride salts



Into a 250-mL round-bottom flask, was placed benzyl 4-[[(1R,5S)-3-[[(2,2,2-trichloroethoxy)carbonyl]amino]-8-azabicyclo[3.2.1]octane-8-sulfonyl]methyl]piperidine-1-carboxylate (1.5 g, 2.51 mmol, 1.00 equiv). This was followed by the addition of hydrochloric acid (12 N, 140 mL) at 10^oC. The resulting solution was stirred for 12 h at 50^oC. The resulting mixture was concentrated under vacuum. This resulted in 1.1 g (88%) of 2,2,2-trichloroethyl N-[(1R,5S)-8-[(piperidin-4-ylmethane)sulfonyl]-8-azabicyclo[3.2.1]octan-3-yl]carbamate hydrochloride as a yellow solid. LCMS (method D, ESI): RT = 0.67 min, *m/z* =462.0 $[M+H]^+$.

Step 5

2,2,2-trichloroethyl (1S,3r,5R)-8-((1-(4,4,4-trifluorobutyl)piperidin-4-yl) methylsulfonyl)-8-aza-bicyclo[3.2.1]octan-3-ylcarbamate

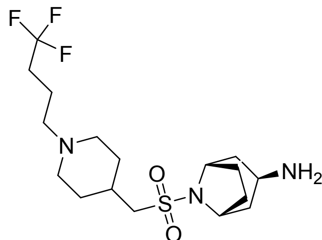


Into a 100-mL round-bottom flask, was placed dichloromethane (40 mL). This was followed by the addition of methanol (20 mL), 2,2,2-trichloroethyl N-[(1R,5S)-8-[(piperidin-4-ylmethane)sulfonyl]-8-azabicyclo[3.2.1]octan-3-yl]carbamate hydrochloride (300 mg, 0.60 mmol, 1.00 equiv), 4,4,4-trifluorobutanal (227 mg, 1.80 mmol, 3.00 equiv). Then $NaBH_3CN$ (303 mg, 4.81 mmol, 8.00 equiv) was added into by batchwise. To the mixture was added acetic acid (1 mL). The resulting solution was stirred for 6 h at 10^oC. The resulting mixture was concentrated under vacuum. The resulting solution was extracted with 3x100 mL of dichloromethane and the organic layers combined. The resulting mixture was washed with 1x50 mL of brine. The mixture was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was purified by flash chromatography with eluent (PE/EtOAc=2/1 to 100% EtOAc). This resulted in 295 mg (86%) of 2,2,2-trichloroethylN-[(1R,5S)-8-([[1-(4,4,4-trifluorobutyl)piperidin-4-yl]methane]sulfonyl)-8-azabicyclo[3.2.1] octan-3-yl]carbamate

as yellow oil. ^{1}H NMR(300 MHz, $CDCl_3$): δ 4.75 (s, 2H), 4.30 (s, 2H), 4.02-3.95 (m, 1H), 3.10-3.02 (m, 2H), 3.00-2.95 (m, 2H), 2.58-2.50 (m, 2H), 2.32-1.78 (m, 17H), 1.60-1.48 (m, 2H) *ppm*. LCMS (method D, ESI): RT = 0.97 min, *m/z* =572.0 $[M+H]^+$.

Step 6

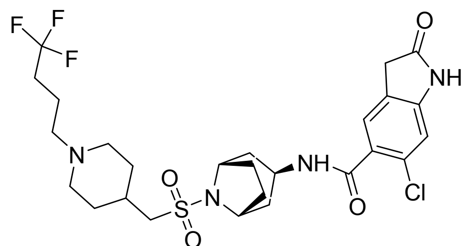
(1S,3r,5R)-8-((1-(4,4,4-trifluorobutyl)piperidin-4-yl)methylsulfonyl)-8-aza-bicyclo[3.2.1]octan-3-amine



Into a 100-mL round-bottom flask, was placed 2,2,2-trichloroethyl N-[(1R,3S,5S)-8-([[1-(4,4,4-trifluorobutyl)piperidin-4-yl]methane]sulfonyl)-8-azabicyclo[3.2.1]octan-3-yl]carbamate (50 mg, 0.09 mmol, 1.00 equiv). This was followed by the addition of acetic acid (15 mL), water (1 mL) and Zn (90 mg). The resulting solution was stirred for 12 h at 10^oC. The solids were filtered out. The pH value of the solution was adjusted to 8 with sodium carbonate (sat. aq.). The resulting solution was extracted with 3x50 mL of ethyl acetate and the organic layers combined and concentrated under vacuum. This resulted in 25 mg (72%) of (1R,3S,5S)-8-([[1-(4,4,4-trifluorobutyl)piperidin-4-yl]methane]sulfonyl)-8-azabicyclo[3.2.1]octan-3-amine as a yellow solid. LCMS (method B, ESI): RT = 1.24 min, *m/z* =398.0 $[M+H]^+$.

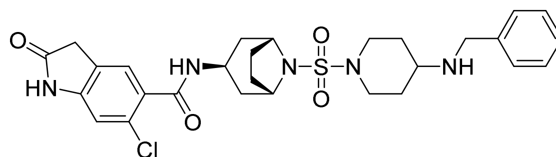
Step 7

6-chloro-2-oxo-N-((1S,3r,5R)-8-((1-(4,4,4-trifluorobutyl)piperidin-4-yl)methylsulfonyl)-8-aza-bicyclo[3.2.1]octan-3-yl)indoline-5-carboxamide



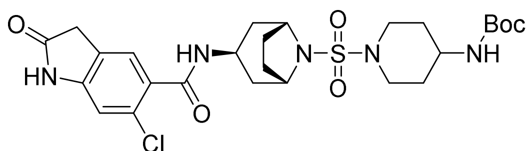
Into a 100-mL round-bottom flask, was placed N,N-dimethylformamide (10 mL), (1R,3S,5S)-8-([[1-(4,4,4-trifluorobutyl)piperidin-4-yl]methane]sulfonyl)-8-azabicyclo[3.2.1]octan-3-amine (50 mg, 0.13 mmol, 1.00 equiv), 6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid (46 mg, 0.22 mmol, 1.73 equiv), 1H-1,2,3-benzotriazol-1-ol (35 mg, 0.26 mmol, 2.06 equiv), EDCI (50 mg, 0.26 mmol, 2.07 equiv), TEA (0.3 mL). The resulting solution was stirred for 12 h at 10^oC. The solids were filtered out. The resulting mixture was diluted with 10 mL of water. The resulting solution was extracted with of 2x10 mL dichloromethane and the organic layers combined. The organic phase was dried over anhydrous sodium sulfate and concentrated under vacuum. The crude reside was purified by Prep-HPLC with the following conditions (2#-Waters 2767-2(HPLC-08)): Column, Xbridge Prep Phenyl, 5 um, 19x150 mm; mobile phase, Water with 50 mmol ammonium bicarbonate and acetonitrile (10.0% acetonitrile up to 33.0% in 2 min, up to 53.0% in 8 min, up to 100.0% in 1 min, down to 10.0% in 1 min); Detector, UV 254 nm. This resulted in 5.7 mg (8%) of 6-chloro-2-oxo-N-[(1R,3S,5S)-8-([[1-(4,4,4-trifluorobutyl)piperidin-4-yl]methane]sulfonyl)-8-azabicyclo[3.2.1]octan-3-yl]-2,3-dihydro-1H-indole-5-carboxamide as a light pink solid. ^{1}HNMR (300 MHz, CD_3OD): δ 7.32 (s, 1H), 6.95 (s, 1H), 4.30-4.10 (m, 3H), 3.35 (s, 2H), 3.10-2.90 (m, 4H), 2.50-2.40 (m, 2H), 2.40-1.90 (m, 15H), 1.85-1.70 (m, 2H), 1.51-1.35 (m, 2H) *ppm*. LCMS (method B, ESI): RT=1.67 min, *m/z* =591.1 $[M+H]^+$.

Synthesis of EPZ030456, N-[(1R,3r,5S)-8-[4-(benzylamino)piperidine-1-sulfonyl]-8-azabicyclo[3.2.1]octan-3-yl]-6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamide trifluoroacetate



Step 1

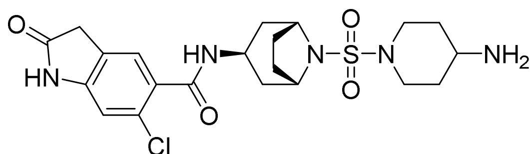
tert-butyl N-[1-[(1R,3r,5S)-3-(6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamido)-8-azabicyclo[3.2.1]octane-8-sulfonyl]piperidin-4-yl]carbamate



Into a 25-mL round-bottom flask was placed 6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxylic acid (170 mg, 0.80 mmol, 1.00 equiv), dichloromethane (10 mL), HOBT (216 mg, 1.60 mmol, 2.00 equiv), EDCI (306 mg, 1.60 mmol, 2.00 equiv), and tert-butyl N-1-[(1R,3r,5S)-3-amino-8-azabicyclo[3.2.1]octane-8-sulfonyl]piperidin-4-ylcarbamate (375 mg, 0.97 mmol, 1.20 equiv). This was followed by the addition of TEA (400 mg, 3.95 mmol, 5.00 equiv) dropwise with stirring at $0^{o}C$. The resulting solution was stirred for 2 h at room temperature. The reaction mixture was diluted with 10 mL of dichloromethane and washed with 2x5 mL of brine. The organic layer was dried over anhydrous sodium sulfate and concentrated under vacuum. The residue was chromatographed on a silica gel column with ethyl acetate/petroleum ether (3:1). This resulted in 300 mg (64%) of tert-butyl N-[1-[(1R,3r,5S)-3-(6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamido)-8-azabicyclo[3.2.1]octane -8- sulfonyl] piperidin-4-yl]carbamate as a red solid. LCMS (method C, ESI): RT =0.88 min, *m/z* = 582.0 $[M+H]^+$.

Step 2

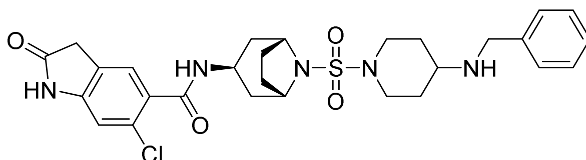
N-[(1R,3r,5S)-8-(4-aminopiperidine-1-sulfonyl)-8-azabicyclo[3.2.1]octan-3-yl]-6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamide hydrochloride



Into a 25-mL round-bottom flask was placed tert-butyl N-[1-[(1R,3r,5S)-3-(6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamido)-8-azabicyclo[3.2.1]octane-8-sulfonyl]piperidin-4-yl]carbamate (300 mg, 0.52 mmol, 1.00 equiv) and hydrogen chloride/dioxane (10 mL, saturated, this solution was made by introducing hydrogen chloride gas into 1,4-dioxane under $0^{o}C$ for 6 hours). The resulting solution was stirred for 4 h at room temperature. The mixture was then concentrated under vacuum. This resulted in 170 mg (64%) of N-[(1R,3r,5S)-8-(4-aminopiperidine-1-sulfonyl)-8-azabicyclo[3.2.1]octan-3-yl]-6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamide hydrochloride as a red solid. LCMS (method A, ESI): RT =0.96 min, *m/z* = 482.0 $[M+H]^+$.

Step 3

N-[(1R,3r,5S)-8-[4-(benzylamino)piperidine-1-sulfonyl]-8-azabicyclo[3.2.1]octan-3-yl]-6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamide; trifluoroacetic acid



Into a 25-mL round-bottom flask was placed N-[(1R,3r,5S)-8-(4-aminopiperidine-1-sulfonyl)-8-azabicyclo[3.2.1]octan-3-yl]-6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamide hydrochloride (50 mg, 0.10 mmol, 1.00 equiv), methanol (5 mL), and benzaldehyde (12.3 mg, 0.12 mmol, 1.20 equiv). The mixture was stirred for 0.5 h at 20 ^{o}C. To the above $NaBH_3CN$ (7.3 mg, 0.12 mmol, 1.20 equiv) was added in batches. The resulting solution was stirred for 1 h at $70^{o}C$. The reaction mixture was then concentrated under vacuum and thecrude product purified by Prep-HPLC with the following conditions: Column: X Select C18, 19*250 mm, 5 um; Mobile Phase A: Water/0.05% TFA, Mobile Phase B: ACN; Flow rate: 30 mL/min; Gradient: 5%B to 36%B in 12.5 min; Detector: 254nm. This resulted in 28 mg (42%) of N-[(1R,3r,5S)-8-[4-(benzylamino)piperidine-1-sulfonyl]-8-azabicyclo[3.2.1]octan-3-yl]-6-chloro-2-oxo-2,3-dihydro-1H-indole-5-carboxamide trifluoroacetateas a white solid. ^1H NMR (400 MHz, CD_3OD) : 7.53-7.49 (m, 5H), 7.33 (s, 1H), 6.97 (s, 1H), 4.29 (s, 2H), 4.16 (s, 3H), 3.91 (d, *J*=12.8Hz, 2H), 3.57 (s, 2H), 3.41-3.37 (m, 1H), 2.89 (t, *J*=10.8Hz, 2H), 2.37-2.21 (m, 4H), 2.20-2.08 (m, 4H), 2.05-1.96 (m, 2H), 1.80-1.70 (m, 2H) *ppm*. LCMS (method A, ESI): RT =1.31 min, *m/z* = 572.2 $[M+H]^+$.

ADME methods

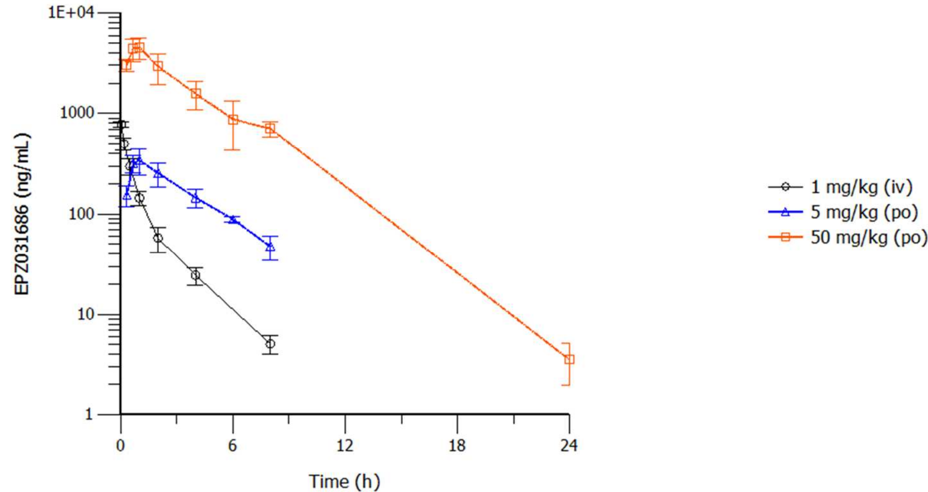
Plasma protein binding. Plasma protein binding was assessed by equilibrium dialysis utilizing the HT-dialysis cell format with a cellulose semi-permeable membrane (molecular weight cut-off of 5000 Da). Solutions of **EPZ030456** and **EPZ031686** (1 µM, 0.5% final DMSO concentration) were prepared in isotonic phosphate buffer (pH 7.4) and 100% pooled male CD-1 mouse plasma. The buffer solution was added to one side of the membrane and the plasma solution to the other side. Incubations were performed in duplicate for 16 h, at 37°C, in order to allow the compounds to reach equilibrium. Haloperidol was incubated in parallel as the control compound for each species. At the end of the incubation time, samples were taken from both sides of the membrane. Following protein precipitation, the samples were analyzed by LC-MS/MS using two sets of calibration standards for protein free (7 points) and protein containing solutions (6 points). Samples were quantified using standard curves prepared in the equivalent matrix. The fraction unbound in plasma (f_u) was calculated using the following equation: $f_u^{corrected} = \frac{PF}{\{(PC - PF) \times V_{Correction}\} + PF}$

Where, PC = sample concentration in protein containing side, PF = sample concentration in protein free side, and $V_{Correction}$ = correction factor for the volume shift i.e. ratio of the volume of the protein after dialysis to that before dialysis.

Liver microsome (LM) stability. Pooled male CD-1 LM (0.5 mg/mL), 0.1 M phosphate buffer pH 7.4 and 1 µM compound (0.25% DMSO final) were pre-incubated in duplicate at 37°C prior to the addition of 1 mM NADPH to initiate the reaction. The final incubation volume was 50 µL. Negative control incubations were included where phosphate buffer was added instead of NADPH. Positive controls were diazepam and diphenhydramine. Each compound was incubated for 0, 5, 15, 30 and 45 min. The negative controls were incubated for 45 min only. The reactions were stopped by transferring 25 µL of incubate to 50 µL methanol at the appropriate time points. Termination plates were centrifuged at 2,500 rpm for 20 min at 4°C to precipitate the protein and metoprolol (internal standard) was added to the sample supernatants prior to LC-MS/MS analysis. *In vitro* $t_{½}$ values were determined by plotting the natural logarithm of the analyte/internal standard peak area ratios as a function of time, with the slope of the linear regression (-k) converted to *in vitro* $t_{½}$ value where $t_{½} = -0.693/k$. Subsequently, intrinsic CL (CL_{int}) was calculated as: (incubation volume/microsomal protein)x0.693/$t_{½}$ and scaled CL values were obtained using the well-stirred venous equilibration model.

Caco-2 permeability. Caco-2 cells (ATCC), between passage numbers 40 to 60, were seeded onto Millipore Multiscreen plates at $1x10^5$ cells/cm^2 in Dulbecco's Modified Eagle Medium. Media was changed every two or three days. On Day 20, the permeability study was performed using Hanks Balanced Salt Solution (HBSS) pH 7.4 buffer with 25 mM HEPES and 4.45 mM glucose at 37°C as the medium. Incubations were carried out in an atmosphere of 5% CO_2 with a relative humidity of 95% at 37°C. The monolayers were prepared by rinsing both basolateral and apical surfaces twice with HBSS at 37°C. Cells were then incubated with HBSS in both apical and basolateral compartments for 40 min to stabilize physiological parameters. HBSS was then removed from the apical or basolateral compartment and replaced with 10 µM test compound dosing solutions (1% final DMSO). The fluorescent integrity marker lucifer yellow was included in the dosing solution. The apical compartment inserts were then placed into 'companion' plates containing fresh HBSS. At 120 min the companion plate was removed and apical and basolateral samples diluted for analysis by LC-MS/MS. Permeability was assessed in duplicate, on two different occasions. On each plate atenolol and propanolol were run as controls. Compounds were quantified by LC-MS/MS analysis using a 5 point calibration curve. The starting concentration (C_0) was determined from the dosing solution and experimental recovery calculated from C_0 and both apical and basolateral compartment concentrations. The permeability coefficient (P_{app}) for each compound was calculated as: (dQ/dt)/(C_0xA), where dQ/dt is the rate of permeation of the drug across the cells, C_0 is the donor compartment concentration at time zero and A is the area of the cell monolayer.

Mouse pharmacokinetic studies. The study was performed in accordance with the AAALAC International guidelines standards. Male CD-1 mice (25-29 g; Beijing SBF Bio-Technology Ltd. n=3/group) were treated with a single dose of **EPZ031686** at 1 mg/kg by intravenous tail vein injection (5% *N*-Methyl-2-pyrrolidone, 5%Solutol in saline, pH 7.0) and 5 and 50 mg/kg by oral gavage administration (30% PEG400, 10%HP-β-cyclodextrine, pH 7.0). Animals were weighed prior to dose administration. Approximately 30 µL of blood were taken from animals via the dorsal metatarsal vein at pre-specified time intervals (9 time-points) for up to 24 hours post-dose. Blood samples were transferred into K_2-EDTA tubes and stored at -70 ± 10°C prior to protein precipitation and LC-MS/MS analysis. Standard calibration curves were constructed by analyzing a series of control plasma aliquots containing 100 ng/mL dexamethasone as internal standard and 0.5 to 2000 ng/mL **EPZ031686**. Five levels of quality controls were also included in the analysis (1.0 to 1600 ng/mL). The concentration of **EPZ031686** in each unknown sample was determined by solving the linear calibration curve equation for each corresponding drug/internal standard ratio. Data were analyzed using noncompartmental methods (Phoenix WinNonlin 6.3.0).



Supplementary Figure 1. Mean total blood concentration-time profiles of EPZ031686 after an i.v. dose of 1 mg/kg and p.o. doses of 5 mg/kg and 50 mg/kg in male CD-1 mice, n=3, mean ± SD.

Trimethyl-MEKK2-In-Cell Western Assay

Materials: 293T/17 adherent cells were purchased from ATCC (American Type Culture Collection), Manassas, VA, USA. MEM/Glutamax medium, Optimem Reduced Serum medium, penicillin-streptomycin, 0.05% trypsin and 1x D-PBS were purchased from Life Technologies, Grand Island, NY, USA. PBS-10X was purchased from Ambion, Life Technologies, Grand Island, New York, USA. PBS with Tween 20 (PBST (10x)) was purchased from KPL, Gaithersburg, Maryland,USA. Tet System FBS- approved FBS US Source was purchased from Clontech, Mountain View, California, USA. Odyssey blocking buffer, 800CW goat anti-rabbit IgG (H+L) antibody, 680CW Goat anti-mouse IgG (H+L) and Licor Odyssey infrared scanner were purchased from Licor Biosciences, Lincoln, NE, USA. Tri-methyl-Lysine [A260]-MEKK2 antibody, MEKK2 and SMYD3 plasmids were made at Epizyme. Anti-flag monoclonal mouse antibody was purchased from Sigma, St. Louis, MO, USA. Methanol was purchased from VWR, Franklin, MA, USA. 10% Tween 20 was purchased from KPL, Inc., Gaithersburg, Maryland, USA. Fugene was purchased from Promega, Madison, WI, USA. The Biotek ELx405 was purchased from BioTek, Winooski, Vermont, USA. The multidrop combi was purchased from Thermo Scientific, Waltham, Massachusetts, USA. 293T/17 adherent cells were maintained in growth medium (MEM/Glutamax medium supplemented with 10% v/v Tet System FBS and cultured at 37 °C under 5% CO_2.

Cell treatment, In Cell Western (ICW) for detection of trimethyl-lysine-MEKK2 and MEKK2. 293T/17 cells were seeded in assay medium at a concentration of 33,333 cells per cm^2 in T150 flasks and incubated at 37 °C under 5% CO_2. Plasmids were prepared for delivery to cells by first mixing 1350 µL Opti-MEM with Fugene (81 µL) in a sterile Eppendorf and incubated for five minutes at room temperature (RT). MEKK2-Flag (13.6 µg/T150) MEKK2 p3XFlag-CMV-14 with C-3XFlag and SMYD3 (0.151 µg/T150) SMYD3 p3XFlag-CMV-14 without C-3XFlag plasmids were aliquotted to sterile microfuge tube. The gene ID for MEKK2 and SMYD3 is NM_006609.3 and Q9H7B4, respectively. Entire volume of Opti-MEM/Fugene mixture was then mixed with DNA plasmid, and incubated for 15 minutes at RT. The medium on 293T/17 cells was refreshed, and the DNA/Fugene complex was added aseptically to each flask and incubated at 37 ºC for 5 hours. Cells were washed once with PBS and trypsinized 0.05% for three minutes, then mixed with room temperature MEM+10% Tet system FBS followed by counting using the Vi-cell. Cells were seeded at 100,000 cells/mL in 50 µL of MEM/10%Tet FBS/Pen/Strep to a 384-well black/clear poly-D-lysine coated plate containing test agent diluted in DMSO. The final top concentration of test compound was 40 µM. The total concentration of DMSO did not exceed 0.2% (v/v). Plates were incubated for 30 minutes at RT in low-airflow area, followed by incubation at 37 °C under 5% CO_2 for 24 hours. Medium was aspirated from all wells of the assay plates prior to fixation and permeabilization with ice cold (-20 ºC) methanol (90 µL/well) for ten minutes. Plates were rinsed with PBS three times on BioTek ELx405. PBS was removed with a final aspiration, and Odyssey blocking buffer (50 µL/well) was added to each well and incubated for one hour at RT. Primary antibody solution was prepared (anti-trimethyl-MEKK2 plus mouse anti-Flag antibody in diluent (Odyssey Blocking buffer + 0.1% Tween 20)) and 20 µL per well was dispensed using the Multidrop Combi. Assay plates were then sealed with foil, and incubated overnight at 4º C. Plates were washed five times with PBS-Tween (1X) on the Biotek ELx405 plate-washer and blotted on paper towels to remove excess reagent. Detection antibody solution (IRDye 800 CW goat anti-rabbit IgG diluted 1:400 in diluent (Odyssey Blocking buffer + 0.1% Tween 20), plus IRDye 680CW goat anti-mouse IgG at 1:500 in diluent (Odyssey Blocking buffer + 0.1% Tween 20) was added (20 µL/well) and incubated in the dark for one hour at RT. Plates were then washed four times with PBS-T (1X) on ELx405. A final rinse with water was performed (115 µL/well x three washes on the

ELx405). Plates were left to dry in the dark. The Odyssey Imager was used to measure the integrated intensity of 700 and 800 wavelengths at resolution of 84 µm, medium quality, focus offset 4.0, 700 channel intensity = 3.5 to measure the MEKK2-flag signal, 800 channel intensity = 5 to measure the Trimethyl-MEKK2 signal of each well.

Calculations: First, the ratio for each well was determined by:

$$\left(\frac{Trimethyl\ MEKK2\ 800nm\ value}{flag\ tagged\ MEKK2\ 700nm\ value}\right)$$

Each plate included fourteen control wells of DMSO only treatment (Minimum Inhibition) as well as fourteen control wells for maximum inhibition (Background). The average of the ratio values for each control type was calculated and used to determine the percent inhibition for each test well in the plate. Cytotoxic compound concentrations were identified as wells which had less than 30% of the average DMSO Flag-tagged MEKK2 signal, and were excluded from the calculation of the IC_{50} value. Reference compound was serially diluted two-fold in DMSO for a total of nine test concentrations, beginning at 40 µM. Percent inhibition was calculated (below).

$$\text{Percent Inhibition} = 100\text{-}\left(\left(\frac{\text{(Individual Test Sample Ratio)}-\text{(Background Avg Ratio)}}{\text{(Minimum Inhibition Ratio)}-\text{(Background Average Ratio)}}\right)*100\right)$$

Non-linear regression curves were generated to calculate the IC_{50} and dose-response relationship using triplicate wells per concentration of compound. The IC_{50} determinations were validated when the ratio of the upper 95% confidence limit over the lower 95% confidence limit was less than 3 and the two independent IC_{50} determinations were within three-fold of each other.

Protein purification

Full length SMYD3 (1-428) was cloned into a plasmid containing an N-terminal TEV protease cleavable His tag and a SUMO/ULP cleavage site. The isoform of SMYD3 containing Lys at position 13 was used for the biochemical assay while SMYD3 isoform containing Asn at position 13 was utilized for x-ray crystal structures. Both SMYD3 proteins were produced in *E. coli* using IPTG to induce expression at reduced temperatures (16ºC) for 16 hours. After harvest by centrifugation, the resulting cell pellets were resuspended, sonicated, and centrifuged to remove cell debris. The supernatant was passed over a nickel affinity column and SMYD3 was eluted from the column with buffers containing 100-200 mM imidazole. After incubation with ULP to cleave the affinity tag, the protein was passed over a second nickel affinity column and was collected in the flow-through. SMYD3 was further purified using a Q Sepharose anion exchange column. The resulting Asn13 SMYD3 was greater than 95% pure as measured by SDS-page gel. Lys13 SMYD3 was further purified using an S200 size-exclusion column with a final purity of 89% as measured using an Agilent Bioanalyzer.

Biochemical methylation assay

SMYD3 methyltransferase activity was measured by detecting the transfer of a tritiated methyl group from ^{3}H-S-(5'adenosyl)-L-methionine (SAM, American Radiolabeled Chemicals, Inc.) to MEKK2. N-terminally GST-tagged MEKK2 protein was purchased from Life Technologies. Balanced assay conditions were used with substrate concentrations equal to their K_M values. Compound and SMYD3 were incubated for 30 minutes before initiating the reaction with the addition of substrates. Final concentrations in the assay were 8 nM ^{3}H-SAM, 12 nM MEKK2, and 2 % DMSO in assay buffer (25 mM Tris, pH 8.0, 1 mM TCEP, 0.005% bovine skin gelatin and 0.005% Tween-20) in a 50 µL reaction volume. Reactions were performed at room temperature and quenched during the linear portion of product formation with 10 µL of 600 µM cold SAM. For IC_{50} determinations, 0.4 nM SMYD3 was used and radiolabeled MEKK2 was detected using a SPA bead assay. To the quenched reaction, 10 µL of 10 mg/mL RNA SPA beads (Perkin Elmer) in 0.5 M citric acid was added and the plates were centrifuged at 600 rpm for 1 min and read in a Perkin Elmer TopCount NXT plate reader. Percent inhibition (% inh) was calculated using the equation

$$\%\,inh = 100 - \left(\frac{cpm_{cmpd} - cpm_{min}}{cpm_{max} - cpm_{min}}\right) \times 100$$

where cpm_{cmpd} is the cpm for a given compound concentration, cpm_{max} is the mean cpm for the DMSO controls and cpm_{min} is the mean cpm for the background controls containing 1 mM of the inhibitor S-(5'adenosyl)-L-homocysteine (SAH). IC_{50} values were determined from the fit of the percent inhibition data versus inhibitor concentration (I) using the following equation

$$\%\,inh = Min + \frac{(Max - Min)}{(1 + \left(\frac{I}{IC_{50}}\right)^{H})}$$

where max and min are the maximal and minimal percent inhibition respectively, H is the Hill coefficient and IC_{50} is the inhibitor concentration at half-maximal inhibition.

The mechanism of inhibition for EPZ030456 and EPZ031686 with respect to substrate was determined by fixing one substrate concentration equal to its K_M value and varying the second substrate and inhibitor concentrations. Assay conditions were as above except that 0.3 nM SMYD3 was used and a filterplate assay was employed to accommodate the high concentrations of MEKK2. After quenching the reaction with 10 µL of 600 µM cold SAM, 50 µL was transferred to a filterplate with 50 µL cold 20 % TCA. The filters were washed three times with 100 µL cold 10 % TCA and once with 100 µL 95 % ethanol. Filterplates were dried and read in a Perkin Elmer TopCount NXT plate reader. Compound IC_{50} values were determined as above and plotted against substrate concentration. The data were best described using the Cheng-Prusoff equations for either mixed-type or noncompetitive inhibition (1).

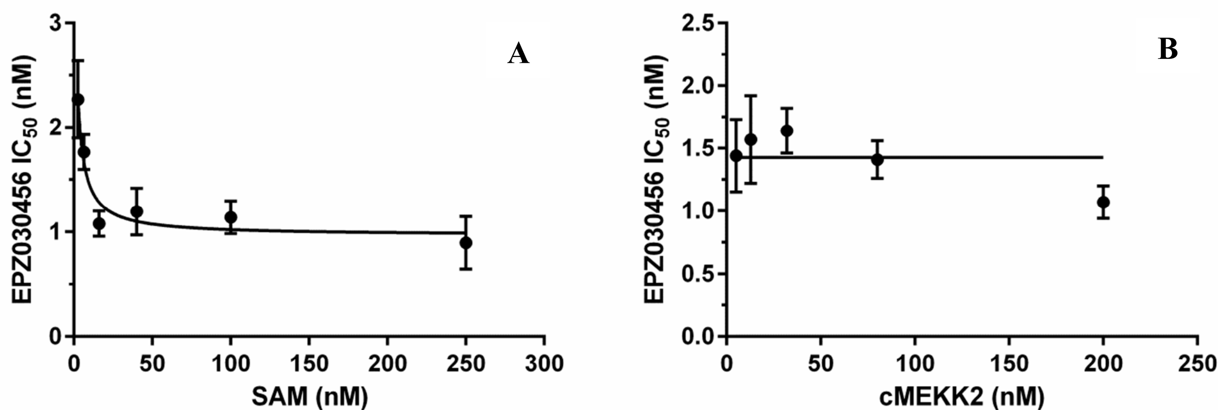
Mixed-type:

$$IC_{50}=\frac{K_M+S}{\frac{K_M}{K_i}+\frac{S}{\propto K_i}}$$

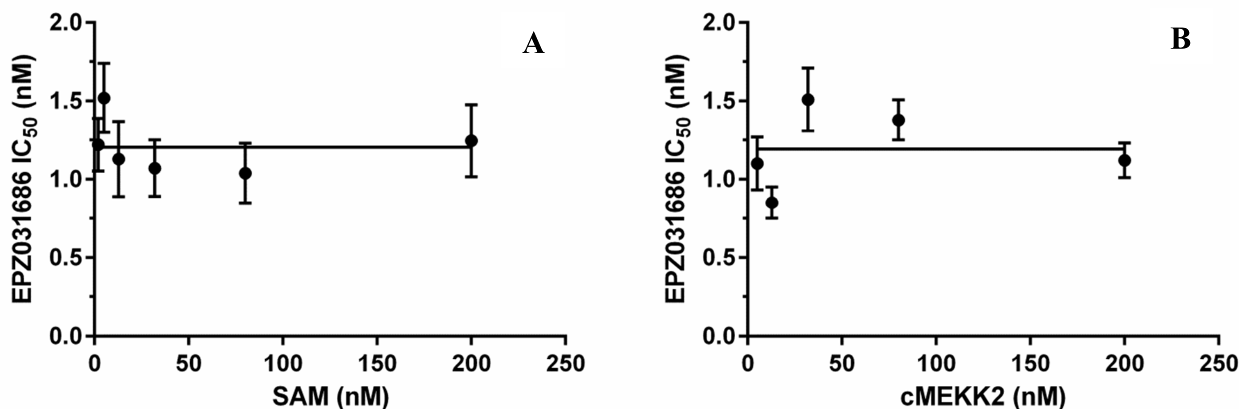
Noncompetitive:

$$IC_{50}=K_i$$

K_M is the Michaelis constant for substrate S, K_i is the inhibitor dissociation constant, and α is the coefficient applied to the K_i for differences in inhibitor affinity between binding enzyme forms after versus before substrate binding.



Supplementary Figure 2. Mechanism of inhibition of EPZ030456. Plot of the EPZ030456 IC_{50} values dependence on the concentration of A) SAM and B) MEKK2. EPZ030456 was best described by mixed-type inhibition versus SAM and noncompetitive inhibition versus MEKK2. Values for the inhibition constants are shown in Supplementary Table 1.



Supplementary Figure 3. Mechanism of inhibition of EPZ031686. Plot of the EPZ031686 IC_{50} values dependence on the concentration of A) SAM and B) MEKK2. EPZ033686 was best described by noncompetitive inhibition versus SAM and MEKK2. Values for the inhibition constants are shown in Supplementary Table 1.

Crystallography methods

Compound **1** was soaked into pre-formed crystals of SMYD3 bound to SAM. SAM was solubilized at 100 mM in DMSO and added to SMYD3 (8 mg/ml in a buffer containing 25 mM Tris, pH 8.0, 150 mM NaCl, and 2 mM TCEP) to a final concentration of 2 mM and incubated on ice for 1 hour. Vapor diffusion methods utilizing hanging drops with a 0.5 mL reservoir were used for crystallization. 2 uL of protein were added to 1 uL of reservoir solution containing 0.1M magnesium formate, 0,1M Tris, pH 8.0 and 14% (w/v) PEG3350. Crystal trays were incubated at 18ºC for 24 hours. Crystals were then incubated in a soaking solution containing 0.1M magnesium formate, 0,1M Tris, pH 8.0, 14% (w/v) PEG3350, 10 mM **1** and 10% DMSO for 24 hours prior to harvesting. Crystals were passed through a cryosolution containing 20% PEG400 and 80% soaking buffer prior to freezing in liquid nitrogen. SMYD3-**1** crystals were collected at the SSRF synchrotron source (beamline X). EPZ030456 was cocrystallized with SMYD3 after addition of 2 mM compound (solubilized at 100 mM in DMSO) and 2 mM SAM to the previously described protein buffer. Crystallization was performed in a similar manner as previously described; microseeding was required to produce crystals of EPZ030456 of sufficient quality for data collection. For both structures, data reduction and scaling were performed using HKL2000 (2). Structure determination was performed using previously solved structures of SMYD3 and visual inspection of electron density maps. Ligand dictionaries were generated using ProDrg (3) within the CCP4 software package (4) and ligand fitting was performed manually. Structure refinement was completed using iterative cycles of refinement and model building using REFMAC (5) and COOT (6), respectively. Data collection and refinement statistics are shown in Supplementary Table 2.

1. Cheng Y, Prusoff, W.H. Relationship between the inhibition constant (Ki) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **1973**, 22 (23), 3099-3108.
2. Otwinowski, Z. and W. Minor, *Processing of X-ray Diffraction Data Collected in Oscillation Mode, Methods in Enzymology*, in *Macromolecular Crystallography, Part A*, J. Charles Carter and R. Sweet, Editors. 1997, Academic Press: New York. p. 307.
3. Schuttelkopf, A.W. and D.M.F. van Aalten, *PRODRG: a tool for high-throughput crystallography of protein-ligand complexes.* Acta Crystallographica Section D, 2004. **60**(8): p. 1355-1363.
4. Winn, M.D., et al., *Overview of the CCP4 suite and current developments.* Acta Crystallographica Section D, 2011. **67**(4): p. 235-242.
5. Murshudov, G.N., A.A. Vagin, and E.J. Dodson, *Refinement of Macromolecular Structures by the Maximum-Likelihood Method.* Acta Crystallographica Section D, 1997. **53**(3): p. 240-255.
6. Emsley, P., et al., *Features and development of Coot.* Acta Crystallographica Section D, 2010. **66**(4): p. 486-501.

Supplementary Table 1. Inhibition constants for EPZ030456 and EPZ031686.

	Parameter	varied substrate[1] SAM	MEKK2
EPZ030456	Mechanism	Mixed[2]	noncompetitive[3]
	K_i (nM)	4.7 ± 1.8	1.3 ± 0.1
	αK_i (nM)	1.1 ± 0.1	NA[3]
	α	0.23 ± 0.09	NA[3]
EPZ031686	Mechanism	noncompetitive[3]	noncompetitive[3]
	K_i (nM)	1.2 ± 0.1	1.1 ± 0.1
	αK_i (nM)	NA[4]	NA[4]
	α	NA[4]	NA[4]

[1]Values are averaged from 2 independent experiments
[2]Values from fit to mixed-type inhibition equation above
[3]Values from fit to noncompetitive inhibition equation above
[4]NA = not applicable

Supplementary Table 2. Crystallographic data collection and refinement statistics for SMYD3 crystal structures

Compound	**1**	**EPZ030456**
PDB code	5CCL	5CCM
Space group	$P2_12_12_1$	$P2_12_12_1$
	58.6, 65.2, 106.0	60.5, 65.9, 107.4
	90.0, 90.0, 90.0	90.0, 90.0, 90.0
Resolution range (Å)	50.00-1.50	50.00-2.30
(Highest resolution shell)	(1.55-1.50)	(2.38-2.30)
Rmerge $_{overall}$ [1]	0.069 (0.226)	0.146 (0.480)
Completeness overall (%)	94.4 (98.6)	98.4 (90.2)
Reflections, unique	62186	19568
Multiplicity	5.0 (4.9)	5.1 (2.5)
I/σ	17.4 (5.9)	13.2 (2.2)
Rvalue $_{overall}$ (%) [2]	11.3	20.1
Rvalue $_{free}$ (%)	16.4	25.5
R.m.s. deviations from ideal values		
Bond lengths (Å)	0.015	0.006
Bond angles (º)	1.7	1.211
Φ, Ψ angle distribution for residues [3]		
In preferred regions (%)	98.1	97.1
In allowed regions (%)	1.9	2.9
Outliers (%)	0.0	0.0

[1] $R_{merge} = \Sigma_{hkl} [(\Sigma_i |I_i - \langle I \rangle|)/ \Sigma_i I_i]$
[2] $R_{value} = \Sigma_{hkl} ||F_{obs}| - |F_{calc}|| / \Sigma_{hkl} |F_{obs}|$
R_{free} is the cross-validation R factor computed for the test set of 5 % of unique reflections
[3] Ramachandran statistics as defined in COOT