Assay Protocols

Assay for Alkaline Phosphatase Activity in C3H10T1/2 Cells. The C3H10T1/2 mouse embryonic mesoderm fibroblasts (ATCC CCL-226) were cultured in DMEM media supplemented with 10% FBS and 1% Penicillin-Streptomycin-Glutamine solution (PSG, Gibco/Life technologies). The cells were seeded at 3000 cells/50 μL/well in 384-well tissue culture plates (Corning 8867BC) and grown to confluence for 48 hours at 37 ºC with 5% CO₂ in air atmosphere. To induce the differentiation, the cells were washed with 50 μL of PBS and then stimulated by addition of 40 μL of 1% Shh conditioned medium in phenol-free DMEM supplemented with 0.5% calf serum and 1% PSG. At this point compounds were added either by pinning of 100 nL/well of the compound solution in DMSO using a CyBi Well liquid handling station (CyBio, Jena, Germany) or by pipetting of 10 μL of an appropriate compound solution prepared in the media with 2.5% DMSO (final concentration 0.5% DMSO). After 48 h incubation at 37 ºC and 5% CO₂, the media were removed and the alkaline phosphatase (AP) activity was measured in the cell lysates using the CDP-Star Chemiluminescence reagent (Perkin Elmer) according to manufacturer’s protocol. The IC₅₀ values and dose response curves were determined by fitting the data with four parameter logarithmic nonlinear regression analysis using the GraphPad Prism or Genedata software. The Shh conditioned medium was collected from native-Shh overexpressing HEK 293 cells (kindly provided by James Chen, Stanford University) cultured in the LFCM media.

Real-time PCR quantification of Gli1 transcripts. The C3H10T1/2 cells were grown, treated with compounds, and stimulated as described above for the AP assay. The cells were washed
gently with PBS using a BioTek plate washer, briefly centrifuged upside down to remove remaining liquid, and lysed for 2 minutes in 10 μL/well of the Cells-to-Ct lysis buffer (Ambion/Life Technologies). After adding 1 μL/well of the stop solution, the fresh RNA lysates were used for cDNA synthesis using the Cells-to-Ct reverse transcriptase mix (Ambion/Life Technologies) according to the manufacturer’s protocol. TaqMan Gene Expression Assay (Applied Biosystems Mm00494645_m1, FAM probe) for mouse Gli1 and the mouse Actb endogenous control (Applied Biosystems, VIC/MGB probe, primer limited) were used for quantitative expression analysis. Real-time PCR was performed in the 384-well format with the Roche Probes master mix (Roche Applied Science) using the Roche LightCycler 480 Instrument.

**β-galactosidase Assay in P*ht^-/-** Cells. The Shh pathway is constitutively activated in the P*ht^-/- cell line (kindly provided by James Chen, Stanford University), which was derived from the mouse embryonic fibroblasts and carries a Shh pathway-driven β-galactosidase reporter gene.1 The P*ht^-/- cells were cultured in DMEM with 10% FBS and 1% PSG, plated at 2000 cells/well in the 384-well plate, and grown to confluence for 72 h at 37°C, 5% CO₂. After the removal of growth media, the cells were treated with compounds in DMEM with 0.5% FBS and a constant final concentration of DMSO of 0.5%. After a 30 h incubation, the wells were treated with Beta-Glo assay reagent (Promega), and after a 30 min. incubation with gentle agitation at room temperature, the plates were read with an Envision Multi-Label Reader 2102 luminometer.

**BODIPY-Cyclopamine Competition Assay.** The Smo-binding assay was conducted with BODIPY-cylopamine and Smo-overexpressing cells as previously described.2 Briefly, the HEK-293T/17 cells were plated (10,000 cells/well) in DMEM, 10% FBS, 1% PSG in 96 well black, clear bottom, poly D-lysine coated plates, and after 36 hours were transfected with the Smo-Myc expression construct using a Fugene-6 transfection reagent (Roche). After 24 h incubation at 37 ºC, 5% CO₂, the cells were washed with PBS and incubated for 30 minutes in DMEM containing 0.5% FBS, 5 nM BODIPY-cyclopamine and the Shh pathway inhibitors. After the PBS wash, the cells were fixed for 30 min. with 4% formaldehyde, washed, and blocked with 1% FBS in PBS. After immunostaining (Anti-C-Myc antibody, Roche #1667149 followed by Alexa Fluor 568 donkey anti-mouse IgG (H + L), Invitrogen #A10037), the nuclei were stained with 10 μM Hoechst 33342, and the cells were analyzed using an Image Express Micro high content microscopy system (Molecular Devices) with FITC/GFP settings for BODIPY and Texas Red/Rhodamine settings for the antibody.

---

1 Taipale, J.; Chen, J. K.; Cooper, M. K.; Wang, B.; Mann, R. K.; Milenkovic, L.; Scott, M. P.; Beachy, P. A. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature* 2000, 406, 1005–9.

2 a) Chen, J. K.; Taipale, J.; Cooper, M. K.; Beachy, P. A. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Genes Dev* 2002, 16, 2743–8; b) Chen, J. K.; Taipale, J.; Young, K. E.; Maiti, T.; Beachy, P. A. Small molecule modulation of Smoothened activity. *Proc. Nat. Acad. Sci. USA* 2002, 99, 14071–6.
Induction of Shh pathway in C3H10T1/2 cells by SAG

Supplemental Figure 1. C3H10T1/2 cells were treated for 24 hours with a range of concentrations of SAG and the levels of the Gli transcript were measured by qPCR as described in the assay protocol and the data were fitted using GraphPad Prism software. The EC₅₀ = 2.6 nM; 20 nM SAG concentration marked by the vertical dotted line approximates minimal concentration of the agonist that produces near maximal induction of the Gli1 transcription and was used in the SAG rescue experiments.
Double-titration experiment

Supplemental Figure 2. Double-titration experiment with the Smo-agonists SAG and purmorphamine (PUR) in the presence of cyclopamine (A, D), robotnikinin (I) (B, E), and 25 (C, F). Gli1 mRNA levels were measured by qPCR using Actb as internal control.
BODIPY-Cyclopamine Competition Assay Results

A.

Supplemental Figure 3. (A): BODIPY-cyclopamine competition assay. HEK-293 cells expressing Smo-myc were incubated with BODIPY-cyclopamine (5 nM) in the presence of increasing concentrations of Shh pathway active compounds, immunostained with anti-Myc antibodies and counterstained with Hoechst 33342 as described in the Assay Protocol and imaged by fluorescence microscopy. (B): Percentage of cells displaying co-staining of the Smo-Myc with BODIPY-cyclopamine was determined using Multi Wavelength Cell Scoring algorithm of the MetaXpress imaging software (Molecular Devices) and the data were fitted using GraphPad Prism. Both 25 and 29 competed with BODIPY-cyclopamine for Smo-Myc binding.
Preparation of 25 (BRD-0607) and 29 (BRD-6851)

Synthesis of 29\(^a\)

\[
\begin{align*}
\text{Ar-} & \quad \text{NH}_2 \\
\text{OH} & \quad \text{HOC-CH=CH-} \\
\text{Ar} & \quad \text{NH} \\
\text{CO}_2- & \quad \text{CO}_2- \\
\end{align*}
\]

\(^a\) Reagents and conditions: a) EDCI, HO\(_2\)t, (i-Pr\(_2\)NEt, DCM; b) EDCI, cat. DMAP, DCM, 49\% over 2 steps; c) 10 mol\% Grubbs II cat., toluene, 0.01 M, 69\%; d) TFA, Et\(_3\)SiH, DCM; e) 4-chlorobenzylamine, EDCI, (i-Pr\(_2\)NEt, DCM (46\% over 2 steps).

General Details

All reagents and solvents were purchased from commercial vendors and used as received. NMR spectra were recorded on a Bruker 300 MHz or Varian UNITY INOVA 500 MHz spectrometer as indicated. Proton and carbon chemical shifts are reported in parts per million (ppm; \(\delta\)) relative to tetramethylsilane, CDCl\(_3\) solvent, or d\(_6\)-DMSO \((\text{H} \delta 0, \text{C} \delta 77.16, \text{or} \text{C} \delta 39.5, \text{respectively})\). NMR data are reported as follows: chemical shifts, multiplicity (obs. = obscured, app = apparent, br = broad, s = singlet, d = doublet, t = triplet, m = multiplet, comp = complex overlapping signals); coupling constant(s) in Hz; integration. Unless otherwise indicated, NMR data were collected at 25°C. Flash chromatography was performed using 40-60 um Silica Gel (60 Å mesh) on a Teledyne Isco Comiblash R\(_f\) system. Tandem liquid chromatography/mass spectrometry (LCMS) was performed on a Waters 2795 separations module and Waters 3100 mass detector. Analytical thin layer chromatography (TLC) was performed on EM Reagent 0.25 mm silica gel 60-F plates. Visualization was accomplished with UV light and aqueous potassium permanganate (KMnO\(_4\)) stain followed by heating. High-resolution mass spectra were obtained with an Agilent 1290 Infinity separations module and 6230 time-of-flight (TOF) mass detector operating in ESI\(^+\) mode. Compound purity and identity were determined by UPLC-MS (Waters, Milford, MA). Purity was measured by UV absorbance at 210 nm. Identity was determined on a SQ mass spectrometer by positive electrospray ionization. Mobile Phase A consisted of either 0.1% ammonium hydroxide or 0.1% trifluoroacetic acid in water, while mobile Phase B consisted of the same additives in acetonitrile. The gradient ran from 5\% to 95\% mobile Phase B over 0.8 minutes at 0.45 ml/min. An Acquity BEH C18, 1.7 um, 1.0 x 50 mm
column was used with column temperature maintained at 65°C. Compounds were dissolved in DMSO at a nominal concentration of 1 mg/mL, and 0.25 uL of this solution was injected.

**General procedures for amide or ester bond formation**

**Method A: EDCI, HOBt, DIPEA in an appropriate solvent**

To a flask containing the carboxylic acid (1 eq.) sealed under nitrogen was added an appropriate dry solvent (DMF or CH$_2$Cl$_2$) to give a concentration of 0.1 molar (or greater). To the resulting mixture was added diisopropylethylamine (2 eq.), the amine (1.3 eq.), HOBt (1.3 eq.) and EDCI (1.3 eq.). The reaction was stirred at room temperature until complete (typically 4-6 h). If the product had precipitated, it was filtered and dried, otherwise the mixture was diluted with ethyl acetate and washed twice with aqueous HCl, then aqueous sodium bicarbonate, then brine. The resulting solution was dried over magnesium sulfate, filtered, and concentrated.

**Method B: EDCI, DIPEA, DMAP**

The alcohol (1 eq.) and carboxylic acid (1.1 eq.) were added to a flask, sealed under nitrogen, and dissolved with dry solvent (typically CH$_2$Cl$_2$), to which was added EDCI (1.2 eq.) and DMAP (0.1 eq.). The reaction was stirred at room temperature until complete (typically 4-6 h), then it was diluted with ethyl acetate and washed twice with aqueous HCl, then twice with aqueous sodium bicarbonate, then brine. The resulting solution was dried over magnesium sulfate, filtered, and concentrated.

**General procedure for ring closing metathesis**

To a flask containing the diene (1.0 eq.) sealed under nitrogen was added an appropriate dry solvent (toluene or CH$_2$Cl$_2$) to give a concentration of 0.02 molar. Grubbs II catalyst (5 mol%) was added and the reaction was stirred at room temperature until complete (typically 8-12 h). 2-mercaptanoticinic acid (50 mol%) was then added to the reaction mixture and stirred vigorously for 2 hours at room temperature, then the mixture was washed with aqueous sodium bicarbonate, then brine. The resulting organic phase was dried with sodium sulfate and decolorized with activated carbon for 10 minutes. The mixture was then filtered through Celite and concentrated under vacuum.
Synthesis of compound 25 (BRD-0607)

(S)-4-tert-butyl 1-((R)-2-(pent-4-enamido)-3-phenylpropyl) 2-allylsuccinate: Using general method A for amide bond formation with DMF as solvent, the title compound was prepared from pent-4-enoic acid (186 µL, 1.82 mmol) and (R)-2-amino-3-phenylpropan-1-ol (250 mg, 1.65 mmol). Product was obtained and used in the next step without purification. Using general method B for ester bond formation with CH₂Cl₂ as solvent, the title compound was prepared from (S)-2-(2-tert-butoxy-2-oxoethyl)pent-4-enoic acid (390 mg, 1.82 mmol) and (R)-N-(1-hydroxy-3-phenylpropan-2-yl)pent-4-enamide (386 mg, 1.65 mmol). Product (351 mg) was obtained in 50% yield over 2 steps after column chromatography (hexanes/ethyl acetate gradient). MS (ESI⁺): 452.45 (M+Na).

 tert-butyl 2-((3R,11S,E)-3-benzyl-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)acetate: Using the general procedure for ring closing metathesis with toluene as solvent, the title compound was prepared from (S)-4-tert-butyl 1-((R)-2-(pent-4-enamido)-3-phenylpropyl) 2-allylsuccinate (350 mg, 0.82 mmol). Product (213 mg) was obtained in 65% yield following purification by column chromatography (hexanes/ethyl acetate gradient). MS (ESI⁺): 424.44 (M+Na).
2-((3R,11S,E)-3-benzyl-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)-N-(4-chlorobenzyl)acetamide (25, BRD-0607): To a solution of tert-butyl 2-((3R,11S,E)-3-benzyl-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)acetate (210 mg, 0.52 mmol) in CH$_2$Cl$_2$ (4 mL) cooled in an ice/water bath was added trifluoroacetic acid (601 µL, 7.85 mmol) and the reaction mixture was stirred for 4 h. After this time, TLC analysis indicated complete consumption of starting material. The reaction mixture was concentrated and coevaporated 3 times with toluene. Using general procedure A for amide bond formation with DMF as solvent, the title compound was prepared from 2-((3R,11S,E)-3-benzyl-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)acetic acid (181 mg, 0.52 mmol) and 4-chlorobenzylamine (70 µl, 0.58 mmol). Product (193 mg) was obtained as a white solid in 79% yield over 2 steps after column chromatography (MeOH/CH$_2$Cl$_2$ gradient). HRMS (ESI$^+$): calculated for C$_{26}$H$_{29}$N$_2$O$_4$ClNa (M+Na) 491.1714, found 491.1711; $^1$H NMR (300 MHz, MeOD): δ 7.35-7.15 (m, 11H), 5.40-5.30 (m, 2H), 5.95 (s, 1H), 4.61 (t, $J$ = 11.0 Hz, 1H), 4.50-4.40 (m, 1H), 4.30 (d, $J$ = 7.8 Hz, 2H), 3.58 (dd, $J$ = 11.0 Hz, $J$ = 2.8 Hz, 1H), 2.90-2.50 (m, 5H), 2.40-2.00 (m, 6H). $^{13}$C NMR (75 MHz, d$_6$-DMSO): δ 174.6, 170.8, 170.1, 138.7, 138.1, 131.2, 129.6, 128.9, 128.7, 128.2, 128.1, 126.2, 64.6, 48.0, 42.2, 41.3, 38.2, 37.0, 36.0, 35.4, 28.6.

![UV Spectrum](image)

Synthesis of compound 29 (BRD-6851)
**(R)**-2-amino-2-(4-chlorophenyl)ethanol: Lithium aluminium hydride (317 mg, 8.35 mmol) was suspended in anhydrous THF (5 mL) under nitrogen atmosphere and cooled to 0 °C. **(R)**-2-amino-2-(4-chlorophenyl)acetic acid (500 mg, 2.69 mmol) dissolved in THF (10 mL) was then added dropwise. After the addition was complete, the reaction mixture was refluxed overnight. After cooling the mixture to 0 °C, water (0.3 mL) was added slowly, followed by saturated aqueous K$_2$CO$_3$ (0.3 mL). Addition of an excess of K$_2$CO$_3$ powder was followed by filtration and washing with THF and CHCl$_3$. Evaporation of the solvents gave 510 mg of a yellow solid. Product (312 mg) was obtained in 68% yield after column chromatography (CH$_2$Cl$_2$/MeOH gradient). **MS (ESI$^+$):** 172.05 (M+H).

**(R)**-N-(1-(4-chlorophenyl)-2-hydroxyethyl)pent-4-enamide: Using general method A for amide bond formation with DMF as solvent, the title compound was prepared from pent-4-enoic acid (65 µl, 0.64 mmol) and **(R)**-2-amino-2-(4-chlorophenyl)ethanol (100 mg, 0.58 mmol). Product (148 mg) was obtained and used in the next step without purification. **MS (ESI$^+$):** 254.12 (M+H).

**(S)**-4-tert-butyl 1-((**R**)2-(4-chlorophenyl)-2-(pent-4-enamido)ethyl) 2-allylsuccinate: Using general method B for ester bond formation with CH$_2$Cl$_2$ as solvent, the title compound was prepared from (**S**)2-(2-tert-butoxy-2-oxoethyl)pent-4-enoic acid (137 mg, 0.64 mmol) and (**R**)-
N-(1-(4-chlorophenyl)-2-hydroxyethyl)pent-4-enamide (148 mg, 0.58 mmol). Product (128 mg) was obtained in 49% yield over 2 steps after column chromatography (hexanes/ethyl acetate gradient). MS (ESI$^+$): 472.31 (M+Na).

**tert-butyl 2-((3R,11S,E)-3-(4-chlorophenyl)-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)acetate:** Using the general procedure for ring closing metathesis with toluene as solvent, the title compound was prepared from (S)-4-tert-butyl 1-((R)-2-(4-chlorophenyl)-2-(pent-4-enamido)ethyl) 2-allylsuccinate (128 mg, 0.28 mmol). Product (83 mg) was obtained as a white solid in 69% yield following purification by column chromatography (hexanes/ethyl acetate gradient). MS (ESI$^-$): 420.14 (M-H).

N-(4-chlorobenzyl)-2-((3R,11S,E)-3-(4-chlorophenyl)-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)acetamide (29, BRD-6851): To a solution of tert-butyl 2-((3R,11S,E)-3-(4-chlorophenyl)-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)acetate (70 mg, 0.17 mmol) in CH$_2$Cl$_2$ (1.8 mL) cooled in an ice/water bath was added trifluoroacetic acid (191 µL, 2.49 mmol). The reaction mixture was stirred 4 h at which point TLC analysis indicated complete consumption of starting material. The reaction mixture was concentrated and coevaporated 3 times with toluene. Then, using general procedure A for amide bond formation with DMF as solvent, the title compound was prepared from 2-((3R,11S,E)-3-(4-chlorophenyl)-5,12-dioxo-1-oxa-4-azacyclododec-8-en-11-yl)acetic acid (61 mg, 0.17 mmol) and 4-chlorobenzylamine (22 µL, 0.18 mmol). Product (37 mg) was obtained in 46% yield over 2 steps after column chromatography (MeOH/CH$_2$Cl$_2$ gradient). HRMS (ESI$^+$): calculated for C$_{25}$H$_{26}$N$_2$O$_4$Cl$_2$Na
(M+Na) 511.1167, found 511.1169; $^1$H NMR (300 MHz, d$_6$-DMSO): $\delta$ 8.47 (br s, 1H), 8.28 (d, $J = 9.7$ Hz, 1H), 7.50-7.30 (m, 5H), 7.25-7.20 (m, 3H), 5.40-5.20 (m, 3H), 4.60 (t, $J = 11.2$ Hz, 1H), 4.22 (d, $J = 5.7$ Hz, 1H), 3.66 (dd, $J = 11.2$ Hz, $J = 3.2$ Hz, 1H), 2.80-2.70 (m, 1H), 2.40-1.90 (m, 8H). $^{13}$C NMR (300 MHz, d$_6$-DMSO): $^{13}$C NMR (75 MHz, d$_6$-DMSO): $\delta$ 174.5, 171.1, 170.0, 138.6, 137.9, 132.1, 131.3, 130.0, 129.0, 128.8, 128.4, 128.2, 65.1, 50.1, 42.1, 41.3, 38.2, 36.1, 35.6, 29.0.