Supplemental Information

The Hedgehog Pathway Effector Smoothened Exhibits Signaling Competency in the Absence of Ciliary Accumulation

Chih-Wei Fan, Baozhi Chen, Irene Franco, Jianming Lu, Heping Shi, Shuguang Wei, Changguang Wang, Xiaofeng Wu, Wei Tang, Michael G. Roth, Noelle S. Williams, Emilio Hirsch, Chuo Chen, and Lawrence Lum
Figure S1

A

<table>
<thead>
<tr>
<th>Pathway Test</th>
<th>Compounds</th>
<th>Inhibitors</th>
<th>FL/RL Test</th>
<th>IC50 Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hh (2.5μM)</td>
<td>200K</td>
<td>1600</td>
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<tr>
<td>Dose-response (0.8, 2.5, 7.5μM)</td>
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<td>94</td>
<td>FL &lt; 6x SD, FL &lt; 1x SD</td>
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<tr>
<td>Wnt (2.5μM)</td>
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<td>81</td>
<td>FL/RL &gt; 80% control</td>
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<tr>
<td>Notch (2.5μM)</td>
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<td>77</td>
<td>FL/RL &gt; 80% control</td>
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<tr>
<td>Exogenous Hh (2.5μM)</td>
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<td>FL/RL &lt; 5x SD</td>
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<tr>
<td>IC50</td>
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<td>7</td>
<td>Hh IC50 &lt; 0.2μM</td>
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</tbody>
</table>

B

Novel Hh pathway antagonists

IC50 (μM) Hh: 0.0076 Wnt: >10
IC50 (μM) Hh: 0.053 Wnt: >10
IC50 (μM) Hh: 0.062 Wnt: >10
IC50 (μM) Hh: 0.073 Wnt: >10
IC50 (μM) Hh: 0.11 Wnt: >10
IC50 (μM) Hh: 0.16 Wnt: >10
IC50 (μM) Hh: 0.20 Wnt: >10

C

Acetylated tubulin (NIH3T3 cells)

DMSO
IHR-1
IHR-2
IHR-3
IHR-4
IHR-5
IHR-6
IHR-7
SANT1

D

3T3-ShhFL cells
2.5μM compound

- IHR-1
- IHR-2
- IHR-3
- IHR-4
- IHR-5
- IHR-6
- IHR-7
- SANT1

Hh pathway response (3T3-ShhFL cells)
Wnt pathway response (L-Wnt-STF cells)

Cmpd [μM]
Noramlized Hh pathway response
IC50 (μM)

Known Smo antagonists

Cyclopamine
SANT1

- Cyclopamine
- SANT1
Figure S1, related to Figure 1. (A) The UTSW chemical library was screened in a cell line that reports cell-autonomous, ligand-mediated Shh pathway response with a pathway responsive firefly luciferase (FL) reporter (GliBS reporter). A dose-response test was performed to identify potent compounds and to eliminate those with cytotoxicity (indicated by change in activity of a control Renilla luciferase (RL) reporter). Compounds with specific Hh pathway activity were revealed following testing in cells that report Wnt and Notch signaling. Compounds with activity against Hh pathway response were separated from those that potentially block Shh protein production by testing their activity against exogenously provided Shh protein in NIH3T3 cells transfected with the GliBS and control Renilla luciferase reporters. Seven inhibitors (IHR-1 to -7) with IC50s against Hh pathway response less than 0.2 μM were further evaluated. Concentration of compounds used in each assay is indicated on the left and criteria used for selecting compounds of interest are indicated to the right of the flow chart. Standard deviation (SD) calculations are mean centered. (B) IHR compounds are specific for Hh response. IC50 plots for IHR compounds in Hh and Wnt pathway response. Hh pathway response with compounds was measured in 3T3-ShhFL. Wnt pathway activity was measured in L-Wnt-STF cells. Cyclopamine and SANT1 are known Smo antagonists. (C) IHR compounds do not affect ciliogenesis. NIH3T3 cells were treated with IHR compounds and immunostained for acetylated tubulin to visualize primary cilia. (D) Ptc1 expression induced by Shh is inhibited by the IHR compounds. cDNA generated from 3T3-ShhFL cells treated with IHR compounds or the Smo antagonist SANT1 was used to determine relative abundance of Ptc1 mRNA. The assay is linear across a broad range of cDNA concentrations used as template. (E) IHR compounds inhibit Hh-induced expression of alkaline phosphatase. Lysate from C3H10T1/2 cells treated with conditioned medium containing ShhN protein (ShhN CM) in the absence or presence of various IHR compounds or the Smo antagonist SANT1 for 5 days were analyzed for alkaline phosphatase activity levels. Data are mean ± SEM from three measurements. (F) Flow cytometric quantification of Bodipy-cyclopamine labeled cells in the presence of various IHR compounds, or SANT1 (inhibitor of Wnt/β-catenin pathway activity). Results are normalized to Bodipy signal from Smo-transfected cells treated with Bodipy-cyclopamine. (G) Flow cytometric quantification of Bodipy-cyclopamine labeled cells in the presence of SAG. Same experiment as in "F" except with SAG.
Figure S2, related to Figure 1. (A) Hh induced suppression of Gli proteolytic processing is dependent upon Smo. (B) IHR-1 blocks Smo-mediated disengagement of Gli2 proteolytic processing. NIH3T3 cells stably expressing Gli2-FLAG stimulated with ShhN CM were additionally treated with indicated known Smo antagonists (SANT1, Vismodegib) or IHR-1. (C) IC50 of IHR-1 in PTCH1-/- MEFs. Known Smo antagonists (cyclopamine, SANT1, Vismodegib) or IHR-1 were dosed in PTCH1-/- MEFs transfected with GliBS reporter and a control reporter used to normalize for transfection efficiency. Data are mean+SEM from three measurements.
Figure S3, related to Figures 1 and 2. (A) IHR-Cy3 is active. 3T3-ShhFL cells were treated with increasing amounts of IHR-Cy3 and Hh pathway response determined by luciferase activity. (B) IHR-Cy3 blocks ShhN CM induced Smo accumulation in the primary cilia. % of cells with Smo localized to the primary cilium was quantified (N=100). Data are mean+SEM of three fields. (C) IHR-Cy3 blocks SAG induced Smo accumulation in the primary cilia. % of cells with Smo localized to the primary cilium was quantified (N=100). Data are mean+SEM of three fields. (D) IHR-1 activity is supported by a para substitution pattern centered on the central aromatic ring. IC50 of IHR-1, meta-IHR-1, and ortho-IHR-1 for Hh pathway response was determined in 3T3-ShhFL cells. Data are mean+SEM from three measurements. (E) IHR-Cy3 binding to Smo is specific. IHR-Cy3 (5μM) binds to cells expressing Smo and not Fzd4. Furthermore, this binding can be competed away with IHR-1 and not meta-IHR-1 or ortho-IHR-1 molecules (all tested at 10μM). (F) SAG is able to achieve maximal activity in the presence of IHR-1. Assays were performed in NIH-3T3 cells transfected with the GliBS reporter. Data are mean+SEM from three measurements.
Figure S4, related to Figure 2. (A) The ability of IHR-NAc to compete with the binding of Bodipy-cyclopamine to Smo was tested as described in Figure S1F. Bodipy-cyclopamine (5nM) and IHR-NAc (1μM). (B) Assignment of Smo subcellular distributions was achieved using EndoH sensitivity. Lysate from cells transiently transfected with Smo or SmoM2 DNA were treated with EndoH, an enzyme that recognizes simple sugar modifications found in secreted pre-medial Golgi proteins but not complex sugar modifications found in proteins that have traversed the medial Golgi. (C) IHR-NAc but not IHR-1 is able to promote SmoM2 exit from the ER. Cos7 cells transfected with either Smo-myc or SmoM2-myc DNA were subjected to cell surface biotinylation using a cell membrane impermeable biotinylation reagent. Biotinylated Smo protein was isolated using streptavidin cross-linked agarose beads and detected with an anti-myc antibody. (D) IHR-NAc induces accumulation of mature SmoM2 by facilitating SmoM2 exit from the ER. Cos-7 cells transiently transfected with SmoM2 DNA were pulse-labeled with S35-methionine and -cysteine. Immature SmoM2 (pre-medial Golgi) was then chased in the presence or absence of IHR-NAc.
Figure S5, related to Figure 3. Control for Sufu immunoprecipitation. Immunoprecipitated material from Sufu KO MEFs using an anti-Sufu antibody show no reactivity to protein detected by an anti-Sufu antibody in wt MEF-derived samples.
Figure S6, related to Figure 5. (A) The IHR-1 derivative IHR-NAc has improved capacity as compared to IHR1 for inhibiting SmoM2-induced disruption of Gli3 proteolytic processing. Lysate from NIH3T3 (with or without ShhN conditioned medium) or NIH3T3 cells stably expressing SmoM2 treated with IHR1 or IHR-NAc were analyzed by Western blotting for Gli3 and Gli3 repressor (Gli3R). (B) IHR-NAc but not IHR-1 blocks SmoM2-induced depletion of Gpr161 from the primary cilium. % of IMCD3 cells with Gpr161 localized to the primary cilium (labeled with an acetylated tubulin antibody) is indicated for each chemical condition (N=100). Data are mean+SEM of three fields.
Supplemental Experimental Procedures

All reactions were performed in glassware under a positive pressure of argon. TLC analyses were performed on EMD 250 µm Silica Gel 60 F254 plates and visualized by quenching of UV fluorescence ($\lambda_{\text{max}} = 254$ nm) or by staining with ceric ammonium molybdate. 1H and spectra were recorded on a Varian Inova-400 instrument. Chemical shifts for 1H spectra are reported in ppm ($\delta$) relative to the residual 1H signals of the solvent (CD$_3$OD: $\delta$ 3.31; DMSO-$d_6$: $\delta$ 2.50) and the multiplicities are presented as follows: s = singlet, d = doublet, t = triplet, m = multiplet.

![Chemical structure](image)

To a suspension of acid 1 (1.91 g, 10.0 mmol) in DCM (20 mL) and DMF (0.020 mL) was added oxalyl chloride (0.875 mL, 10 mmol) at 0 °C under Ar. After 0.5 h stirring at room temperature, a solution of 2 (0.54 g, 5.0 mmol) in DCM (25 mL) and NEt$_3$ (3.5 mL, 25 mmol) was added. The reaction was stirred overnight and concentrated. After washing the resulted solid by EtOAc (2 mL×2), compound 3 was collected as white solid (1.93 g, 85%).

![Chemical structure](image)

To a suspension of acid 1 (98 mg, 0.5 mmol) in DCM (2 mL) and DMF (0.020 mL) was added oxalyl chloride (0.044 mL, 0.5 mmol) at 0 °C under Ar. After 10 min stirring at room temperature, a solution of 4 (0.104 g, 0.5 mmol) in DCM (1 mL) and NEt$_3$ (0.35 mL, 2.5 mmol) was added. After 3h at room temperature, the solvent was removed by evaporation. The residue was dissolved in 20% TFA/DCM (1.0 mL). After 1 h at room temperature, the reaction was concentrated and gave crude 5 (138 mg) which was used without further purification.

![Chemical structure](image)

To a suspension of acid 6 (136 mg, 0.5 mmol) in DCM (2 mL) and DMF (0.020 mL) was added oxalyl chloride (0.044 mL, 0.5 mmol) at 0 °C under Ar. After 20 min stirring at room
temperature, a solution of crude 5 above in DCM (1 mL) and NEt₃ (0.35 mL, 2.5 mmol) was added. After 3h at room temperature, the reaction was concentrated. The resulted solid was washed by EtOAc (0.5 mL×2) and gave 7 as a white solid (200 mg, 73%). ¹H NMR (400 MHz, DMSO-d6) δ 10.56 (s, 1H), 10.47 (s, 1H), 9.67 (s, 1H), 7.75-7.38 (m, 10H), 1.46 (s, 9H).

![Image of compounds with structures](image)

Compound 7 (70 mg, 0.13 mmol) was dissolved in 20% TFA/DCM (0.5 mL). After 20 min, the solvent was removed by evaporation. The deprotected product was used without further purification.

To a suspension of acid 8 (32 mg, 0.13 mmol) in DCM (2 mL) and DMF (0.010 mL) was added oxalyl chloride (0.013 mL, 0.14 mmol) at 0 °C under Ar. After 10 min stirring at room temperature, a solution of deprotected product of 7 above in DCM (1 mL) and NEt₃ (0.07 mL, 0.5 mmol) was added. After 5h at room temperature, the reaction was concentrated. The resulted solid was washed by EtOAc (0.5 mL×2) and gave 9 as a white solid (46 mg, 40%). ¹H NMR (400 MHz, DMSO-d6) δ 10.56 (s, 1H), 10.49 (s, 1H), 10.14 (s, 1H), 7.85-7.43 (m, 10H), 6.76 (t, 1H, J = 5.6 Hz), 3.61-3.55 (m, 2H), 2.91-2.83 (m, 2H), 2.30 (t, 2H, J = 7.2 Hz), 1.77-1.71 (m, 2H), 1.35 (s, 9H).
Compound 9 (20 mg, 0.032 mmol) was dissolved in 20% TFA/DCM (0.5 mL). After 20 min, the reaction was concentrated. The deprotected product was used without further purification.

To a solution of Cy3-Osu (27 mg, 0.03 mmol) in DMF (0.40 mL) was added a solution of deprotected product of 9 above in Py. (0.30 mL) under Ar. After stirring overnight at 40 °C, the product was purified by RP-TLC (30% TFA-H2O) and gave red solid 10 (6.0 mg, 15%). **IHR-Cy3**: 1H NMR (400 MHz, CD3OD) δ 8.56 (t, 1H, J = 13.2 Hz), 7.97-7.93 (m, 2H), 7.93-7.88 (m, 3H), 7.68 (m, 3H), 7.70-7.62 (m, 5H), 7.62-7.59 (m, 1H), 7.52-7.49 (m, 2H), 7.45-7.37 (m, 3H), 6.52 (t, 2H, J = 13.2 Hz), 4.26-4.12 (m, 4H), 3.53-3.46 (m, 1H), 3.15-3.08 (m, 2H), 2.38 (t, 2H, J = 7.2 Hz), 2.24 (t, 1H, J = 7.6 Hz), 2.18 (t, 2H, J = 7.2 Hz), 1.78 (s, 6H), 1.77 (s, 6H), 0.93-0.87 (m, 3H).

Compound 7 (44 mg, 0.082 mmol) was dissolved in 20% TFA/DCM (0.5 mL). After 30 min, the solvent was removed by evaporation. The residue was dissolved in Py. (1 mL) which was
followed by the addition of AcCl (0.009 mL, 0.13 mmol). After stirring at room temperature overnight, the reaction was concentrated and the residue was washed by EtOAc (0.5 mL×2). Compound 11 was collected as white solid (34 mg, 88%). $^1$H NMR (400 MHz, DMSO-$d_6$) $\delta$ 10.56 (s, 1H), 10.50 (s, 1H), 10.21 (s, 1H), 7.85-7.40 (m, 10H), 2.05 (s, 3H).