Small molecule inhibitors of Smoothened ciliary localization and ciliogenesis

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Vertebrate Hedgehog (Hh) signals involved in development and some forms of cancer, such as basal cell carcinoma, are transduced by the primary cilium, a microtubular projection found on many cells. A critical step in vertebrate Hh signal transduction is the regulated movement of Smoothened (Smo), a seven-transmembrane protein, to the primary cilium. To identify small molecules that interfere with either the ciliary localization of Smo or ciliogenesis, we undertook a high-throughput, microscopy-based screen for compounds that alter the ciliary localization of YFP-tagged Smo. This screen identified 10 compounds that inhibit Hh pathway activity. Nine of these Smo antagonists (SA1–9) bind Smo, and one (SA10) does not. We also identified two compounds that inhibit ciliary biogenesis, which block microtubule polymerization or alter centrosome composition. Differential labeling of cell surface and intracellular Smo pools indicates that SA1–7 and 10 specifically inhibit trafficking of intracellular Smo to cilia. In contrast, SA8 and 9 recruit endogenous Smo to the cilium in some cell types. Despite these different mechanisms of action, all of the SAs inhibit activation of the Hh pathway by an oncogenic form of Smo, and abrogate the proliferation of basal cell carcinoma-like cancer cells. The SA compounds may provide alternative means of inhibiting pathogenic Hh signaling, and our study reveals that different pools of Smo move into cilia through distinct mechanisms.

Results

Development of a High-Throughput Screen for Inhibitors of Smo Ciliary Localization and Ciliogenesis. We developed a microscopy-based high throughput screen for small molecules that inhibit ciliogenesis or Smo localization to cilia using IMCD3 cells stably expressing Smo-YFP, selected for their ability to produce long cilia. Because overexpression of Smo causes constitutive ciliary localization, the IMCD3 cell line exhibited constitutive localization of Smo-YFP to the primary cilium (Fig. L4) (14).

Multifactorial experimental design revealed that ciliogenesis was sensitive to cell culture conditions, cell density, and DMSO concentration. IMCD3 ciliation was optimal at a seeding density of 4 × 104 cells/well and a compound incubation time of 16–20 h. Although incubation with 1% DMSO did not cause overt signs of toxicity within 24 h, it unexpectedly increased Smo-YFP localization to cilia; thus, we used 0.1% DMSO.

We imaged cells and analyzed the proportion of cells with cilia marked with Smo-YFP, as described in SI Materials and Methods. In a preliminary trial, we identified a compound, designated SA3, which we used as a positive control during high-throughput screening (Fig. L4). The optimized assay and analysis conditions had a Z-factor of 0.6, allowing for hit identification with screening in singlicate using 10 μM compound.


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Identification of Smo and Ciliogenesis Antagonists. In an initial trial, we screened 12,250 compounds for their ability to inhibit the localization of Smo-YFP to the primary cilium. We identified 348 compounds that altered ciliary Smo-YFP fluorescence intensity by >2 SDs from the mean. We rescreened these 348 compounds and confirmed that 12 of the compounds abrogated ciliary localization of Smo-YFP in at least 60% of the cells, or 3 SD from the mean (Fig. 1B). By chromatography and mass spectrometry analysis, we confirmed the structures of these 12 compounds, 8 of which are chemically stable and do not contain obvious reactive functionalities. The other four contain potentially reactive functional groups (the α-β unsaturated ketone in SA3, sulfonamide in SA5, thiadiazole in SA10, and pentafluorobenzene sulfonamide in CA2), raising the possibility that these compounds covalently modify their targets (27). However, these compounds were stable in solution and did not induce cell death.

Loss of the ciliary Smo-YFP signal can result from inhibition of Smo-YFP movement to the cilium or loss of the cilium itself. To discriminate between these two mechanisms, we treated IMCD3 Smo-YFP cells with the 12 compounds and assessed the colocalization of Smo-YFP and acetylated tubulin, a ciliary component. Ten of the 12 compounds inhibited the localization of Smo-YFP to the primary cilium, but did not affect acetylated tubulin (Fig. S1A). We named these 10 compounds SA1–10, and the two compounds that disrupted ciliary structure CA1 and CA2 (Fig. S1B). The SA compounds fall into distinct structural clusters (i.e., SA1/2, SA3/4, and SA8/9), suggesting structure–activity relationships. To confirm the specificity of the screen, we identified five control compounds with structural similarity to the SA hits, designated NC1–5, for negative controls (Fig. S1B).

The SA compounds specifically inhibited Smo protein activity but did not block Smo transcription, translation, or the translocation of other proteins to the cilium. None of the SA compounds inhibited the ciliary localization of a GFP-tagged version of somatostatin receptor 3 (SSTR3), indicating that the compounds do not affect constitutive ciliary localization (Fig. S1C). SA treatment did not decrease Smo transcript levels or Smo protein levels (Fig. S1 E and F). Moreover, SA treatment of IMCD3 Smo-YFP cells, ASZ1 cells, and NIH 3T3 cells for 5 d did not induce caspase-3 activation, indicating that the SAs do not induce significant apoptosis (Fig. S1G).

SA1–6 Abrogate Localization of Smo to Primary Cilia of BCC-Like Cells. The ability of SA1–10 to inhibit ciliary Smo-YFP localization in IMCD3 cells raised the possibility that these SAs may interfere with the trafficking of endogenous Smo in cancer cells. ASZ1 cells are BCC-like cells derived from tumors that lost Ptch1, display constitutive localization of Smo to the cilium, and constitutively activate the Hh pathway (28, 29). Treatment of ASZ1 cells with 7 of the 10 SA compounds reduced the ciliary localization of endogenous Smo, similar to the clinical Smo antagonist LDE225 (Fig. 2A and Fig. S2); however, SA7–9 did not reduce ciliary Smo in this cell type (Fig. 2B). Given the structural similarity of SA8 and SA9, it is not surprising that these compounds exhibited similar activity.

The inability of SA8 and SA9 to inhibit the ciliary localization of Smo in ASZ1 cells could be related to differences in the levels or trafficking mechanisms of Smo in cancer cells. The Smo antagonist cyclopamine promotes ciliary localization of Smo in many cell types, but inhibits its activity (30). To determine whether SA8 and SA9 act in a cell type–specific fashion similar to cyclopamine, we treated NIH 3T3 cells with SA8 and SA9 in the absence of Hh pathway activators, and found that SA8 and SA9 promoted the localization of endogenous Smo to the primary cilium (Fig. 3A). Thus, SA8 and SA9, like cyclopamine, can promote the ciliary localization of Smo in some cell types.
Transport from the plasma membrane or through an intracellular pool of SNAP-Smo labeled with SNAP-Surface 488 (green), and cilia (acetylated tubulin; red). To determine whether the SAs disrupt trafficking of ciliary Smo at the plasma membrane or intracellular populations, we examined the effects of SA1–10 on cells constitutively expressing a SNAP-tagged version of Smo (SNAP-Smo). Smo bearing an amino-terminal SNAP tag that can be rapidly and covalently labeled with fluorescent small molecules (31). To track the lateral movement of intracellular Smo to the cilia, we blocked the cell surface SNAP-Smo, treated with SAG and SA compounds, and assessed whether SNAP-Smo was at the cilia. To track the slower movement of intracellular Smo to the cilia, we blocked the ciliary Smo localization, did not displace BODIPY-cyclopamine binding to Smo, indicating that it functions differently than the other SA compounds (Fig. 3C). One possibility is that SA10 may interact with a region of Smo distinct from the cyclopamine site. Alternatively, SA10 may inhibit the machinery that transports Smo to cilia.

**SA1–9 Interact Directly with Smo.** To test whether SA compounds antagonize the Hh pathway by interacting directly with Smo, we examined these compounds’ ability to inhibit the binding of BODIPY-cyclopamine, a fluorescent cyclopamine derivative (22). Treatment of Smo-expressing HEK293T cells with 5 μM SA1, SA3, SA4, SA8, and SA9 attenuated BODIPY-cyclopamine binding (Fig. S3A). Higher concentrations (e.g., 10 μM) of SA2, SA5, SA6, and SA7 similarly attenuated BODIPY-cyclopamine binding to Smo (Fig. S3B). SA10, despite its ability to inhibit endogenous ciliary Smo localization, did not displace BODIPY-cyclopamine binding to Smo, indicating that it functions differently than the other SA compounds (Fig. S3C). One possibility is that SA10 may interact with a region of Smo distinct from the cyclopamine site. Alternatively, SA10 may inhibit the machinery that transports Smo to cilia.

**SA1–10 Inhibit Smo-Dependent Signal Transduction.** Because Smo localization to cilia is important for vertebrate Hh signaling, we examined whether SA compounds inhibit signal transduction in Hh-responsive Shh-LIGHT2 cells and in two cell lines that display constitutive Hh pathway activation. *Pchil−/−* mouse embryonic fibroblasts (MEFs) and ASZ1 cells (19). All the SA compounds inhibited SAG activation of Hh signaling in Shh-LIGHT2 cells at an IC_{50} of 1–25 μM (22, 32) (Table 1 and Fig. S4). The SA compounds similarly inhibited the constitutive misactivation of the Hh pathway in *Pchil−/−* MEFs and ASZ1 cells. Cells lacking Pchil activity constitutively express Hh transcriptional targets, including *Pchil1* (6). MEFs derived from *Pchil1−/−* mice also display constitutive localization of ciliary Smo and express β-galactosidase under the control of the *Pchil1* promoter (32). SA treatment of *Pchil1−/−* MEFs suppressed β-galactosidase activity (Fig. S5). As with Shh-LIGHT2 cells, the IC_{50} of SA compounds in this assay were 1–25 μM (Table 1). We also evaluated the ability of the SA compounds to inhibit expression of Hh pathway target genes, *Gli1* and *Pchil1*, in ASZ1 cells. All 10 SA compounds inhibited expression of *Gli1* and *Pchil1* in ASZ1 cells (Fig. 4A).

We further assessed whether SA1–10 could inhibit the activity of an oncogenic form of Smo, SmoM2. SmoM2 contains a W535L substitution identified in BCC that causes constitutive ciliary localization and activity (32, 33). We expressed SmoM2 in MEFs derived from *Ptch1−/−* mice also display constitutive Shh-LIGHT2 cells, the IC_{50} of SA compounds in this assay were 1–25 μM (Table 1). We also evaluated the ability of the SA compounds to inhibit expression of Hh pathway target genes, *Gli1* and *Pchil1*, in ASZ1 cells. All 10 SA compounds inhibited expression of *Gli1* and *Pchil1* in ASZ1 cells (Fig. 4A).

We further assessed whether SA1–10 could inhibit the activity of an oncogenic form of Smo, SmoM2. SmoM2 contains a W535L substitution identified in BCC that causes constitutive ciliary localization and activity (32, 33). We expressed SmoM2 in MEFs and the Gli-dependent luciferase reporter in *Smo−/−* MEFs. Treatment with 25 μM SA1–10 inhibited SmoM2-induced Hh pathway activity (Fig. 4B).

Sufu acts downstream of Smo by inhibiting Gli transcription factors and negatively affecting their ability to activate the Hh transcriptional program (34). Consequently, Sufu{sup *−/−*} MEFs exhibit constitutive Hh pathway activity independent of Smo activity (35). To test whether the SA compounds act at the level of the transcriptional program (34). Consequently, Sufu{sup *−/−*} MEFs exhibit constitutive Hh pathway activity independent of Smo activity (35). To test whether the SA compounds act at the level of
Smo, we treated Smo−/− MEFs with the SA compounds. None of the SA compounds repressed Gli1 expression in these MEFs (Fig. 4C), indicating that Sufu is epistatic to SA function. Thus, it is likely that all 10 SA compounds act at or below Smo, but not at or above Sufu.

To provide insight into structure–activity relationships, we investigated the activity of SA7–10 analogues. NC1 and NC2, analogues of SA7–9, were less effective in inhibiting Gli1 expression in ASZ1 cells (Fig. 4D); similarly, NC3–5, analogues of SA10, did not inhibit Hh pathway activity in SAG-treated Shh-LIGHT2 cells (Fig. 4E). The inability of these analogues to inhibit Hh pathway activity demonstrates that small changes in chemotype can strongly reduce activity. Hh signaling shares many features with Wnt signaling (36, 37). To assess whether the SAs also affect Wnt signaling, we tested their ability to inhibit a β-galactosidase reporter under the control of canonical Wnt signals (BATgal). None of the SA compounds inhibited Wnt signaling (Fig. S1H).

**SA1–10 Inhibit Proliferation of ASZ1 BCC-Like Cells.** The Hh pathway participates in the regulation of the cell cycle. Hh stimulation of Ptch1 releases cyclin B1, a component of M-phase promoting factor (38). Hh also promotes expression of N-myc and C-myc, which induce cell cycle progression through the induction of CyclinD (38). To determine whether SA1–10 inhibit cell proliferation of Hh pathway-associated cancer cells, we arrested ASZ1 cells in low-serum medium in the presence of 25 μM SA1–10 and 10 μM bromodeoxyuridine (BrdU). After 24 h, we added 10% FBS to promote cell proliferation. SA1–10 reduced BrdU incorporation in ASZ1 cells (Fig. 4F). We further assessed whether SA1–10 inhibit ASZ1 cell proliferation at a particular cell cycle stage by determining the DNA profile of SA1–10-treated ASZ1 cells using flow cytometry. In a nonsynchronous population, SA1–10 delayed the G1-to-S transition, and SA5 also delayed the S-to-G2 transition (Fig. 4G).

**CA1 and CA2 Inhibit Ciliogenesis Through Distinct Effects on the Microtubule Cytoskeleton.** In contrast to the SA compounds, CA1 and CA2 blocked cilia formation (Fig. S1D). CA1 disrupted ciliogenesis in IMCD3 cells, but not in ASZ1 and NIH 3T3 cells (Fig. S4). We recently showed that different cell types have distinct genetic requirements for ciliogenesis, and it is possible that CA1 affects a cell type-specific aspect of ciliogenesis (39, 40). Consistent with the inability of CA1 to inhibit ciliogenesis in ASZ1 and NIH 3T3 cells, CA1 also did not inhibit Hh pathway activity in these cells (Figs. S4 and S5, and Table 1).

CA2 displayed toxicity in IMCD3 and ASZ1 cells at concentrations exceeding 10 μM and in Shh-LIGHT2 and Ptch1−/− MEFs at lower concentrations. As noted above, CA2 contains a potentially reactive pentfluorobenzene sulfonamide group, which may account for its toxicity. At subtoxic concentrations, CA2 disrupted ciliogenesis in both IMCD3 and ASZ1 cells (Fig. S4). Neither CA1 nor CA2 was able to prevent the binding of Gli1 to Smo in ASZ1 cells (Fig. S1I).

### Table 1. Half maximal inhibitory concentrations (IC50) for Hh pathway (in μM)

<table>
<thead>
<tr>
<th>Compounds</th>
<th>SA1</th>
<th>SA2</th>
<th>SA3</th>
<th>SA4</th>
<th>SA5</th>
<th>SA6</th>
<th>SA7</th>
<th>SA8</th>
<th>SA9</th>
<th>SA10</th>
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<tr>
<td>Shh-LIGHT2 cells (Gli-luciferase)</td>
<td>3.1</td>
<td>12</td>
<td>1.2</td>
<td>0.92</td>
<td>5</td>
<td>9.2</td>
<td>5.8</td>
<td>18</td>
<td>19</td>
<td>5 &gt;25</td>
</tr>
<tr>
<td>Ptc1−/− fibroblast (β-gal)</td>
<td>3.8</td>
<td>9.9</td>
<td>20</td>
<td>1.3</td>
<td>25</td>
<td>7.3</td>
<td>3.2</td>
<td>19</td>
<td>1</td>
<td>11 &gt;25</td>
</tr>
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**Fig. 4.** SA compounds inhibit Hh target gene activation, and SA1–10 reduce proliferation and delay progression through G1 of BCC-like cells. (A) Relative Gli1 and Ptch1 levels in ASZ1 cells treated with 25 μM compound, as measured by qRT-PCR. SA1–10 inhibit the expression of Gli1 and Ptch1 in ASZ1 cells. (B) Relative Gli-luciferase activity of Smo−/− MEFs expressing SmoM2 and treated with 25 μM compound, showing that SA compounds inhibit SmoM2 activity. (C) Quantitation of Gli1 levels in Sufu−/− MEFs treated with 25 μM compound, as measured by qRT-PCR. Sufu is epistatic to SA compound activity. (D) qRT-PCR measurement of relative Gli1 levels in ASZ1 cells treated with 25 μM compound. Compared with their structural analogues SA7–9, NC1 and NC2 are ineffective inhibitors of Gli1 expression. (E) Relative firefly luciferase levels of Shh-LIGHT2 cells treated with SA10 and its structural analogues NC3–5. NC3–5 did not inhibit Hh pathway activity. (F) BrdU incorporation in ASZ1 cells treated with 25 μM compound. SA1–10 reduced cell proliferation in ASZ1 cells. (G) Percentage of cells in each phase of the cell cycle as determined by flow cytometric quantitation of DNA content. ASZ1 cells treated with 25 μM SA1–10 show an increased proportion in G1 phase. Cells treated with SAS also have an increased proportion in S phase. Data in A–E are averages of triplicate measurements; data in F are the average of two independent experiments in duplicate. Asterisks indicate significance according to the Student t test (P < 0.05).
BODIPY-cyclopamine to Smo (Fig. S3D). Thus, consistent with the fact that ciliogenesis does not depend on Hh signaling, CA1 and CA2 do not target Smo.

Primary cilia comprise nine doublet microtubules that emanate from basal bodies. To investigate whether CA1 and CA2 disrupts cilia formation by affecting microtubules or basal body organization, we treated IMCD3 Smo-YFP cells with 10 μM CA1 and ASZ1 cells with 10 μM CA2 for 24 h and observed the microtubule cytoskeleton and basal body/centrosome structure. The CA1-treated cells exhibited reduced basal body γ-tubulin, dispersal of γ-tubulin into multiple foci, and a disorganized microtubule cytoskeleton (Fig. 5B). In contrast, CA2-treated cells displayed a loss of microtubules, with no effect on γ-tubulin. Given that both basal bodies and microtubules are essential for ciliogenesis, it is likely that CA1 abrogates ciliogenesis through its effect on basal body structure, whereas CA2 abrogates ciliogenesis by disrupting microtubules.

Discussion

We have developed a high-content, high-throughput screen to identify small molecules that abrogate the ciliary localization of Smo (SA1–10) or disrupt the ciliary structure (CA1 and CA2) (Fig. 5C). All of the SA compounds suppress pathological Hh pathway misactivation caused by either a loss of Ptc1 or expression of an oncogenic form of Smo. None of the SAs act epistatic to Sufu, indicating that the SA compounds interfere with Hh signal transduction downstream of Ptc1 and upstream of Sufu.

SA1–6 and SA10 inhibit localization of Smo to cilia, whereas SA8 and SA9 induce the localization of Smo to cilia in ASZ1 cells, but not in IMCD3 cells. SA1–9 inhibit Hh pathway activity through direct interactions with Smo. Thus, SA1–6 share functional characteristics with LDE225 or Vismodegib and SA8 and SA9 share functional characteristics with cyclopamine.

Differential labeling of cell surface and intracellular populations of Smo revealed that SA1–7 and SA10 specifically inhibit the translocation of intracellular Smo to the cilium. The identification of small molecules that discriminate between the movement of cell surface and intracellular Smo to the cilium indicates that the mechanisms underlying these two translocations are distinct (31, 41). Given that SA1–7 and SA10 do not inhibit the movement of cell surface Smo to the cilium but do inhibit Hh signaling in various cell types, full pathway activity may require mobilization of both Smo populations in a non-redundant manner. Alternatively, these results may indicate that the two pools of Smo exert different activities, with the intracellular population having a distinct capacity to activate downstream signaling.

To investigate whether the SA compounds resemble compounds with described biological activities, we used the similarity ensemble approach to screen the SA compounds against the 3,000 targets and 500,000 ligands of the ChEMBL database (42–44). This approach predicted that SA6 would be an Smo inhibitor, reflecting its similarity to the 1-amino-4-benzylphthalazine family of Smo inhibitors (45). The dissimilarity of the other SA compounds to known Hh pathway regulators suggests that they affect Smo in distinct ways.

Among the other compounds that bind Smo, Vismodegib, and LDE225 are similar bisaryl amides, whereas cyclopamine is a steroidal alkaloid. Despite their diverse structures, SA1–9, Vismodegib, LDE225, and cyclopamine all compete with BODIPY-cyclopamine for binding to Smo. Thus, SA1–9 expand the broad chemical palette of compounds that can bind Smo and inhibit Hh pathway activity, which may be therapeutically useful given the emergence of resistance to a Smo antagonist (46, 47). Based on the diverse structures of Smo antagonists, one might hypothesize that antagonist activity would not be tightly dependent on structure. However, analogues of SA7–10 exhibit little or no ability to inhibit Hh pathway activity. Thus, the ability of minor changes in side groups to dramatically lower activity indicates that Smo inhibitors are structurally constrained.

SA10, unlike SA1–9, Vismodegib and LDE225, does not compete with cyclopamine for Smo binding; rather, it acts upstream of or at the level of Smo through a distinct mechanism. It is possible that SA10 binds Smo at a site distinct from that of cyclopamine. Alternatively, SA10 may block Hh signaling by inhibiting the transport machinery that moves Smo to cilia.

CAs, CA1 and CA2 inhibit ciliogenesis through distinct mechanisms. CA1 reduces the localization of γ-tubulin to basal bodies,
induces the formation of multiple γ-tubulin foci, and disrupts the microtubule cytoskeleton, suggesting that CA1 distorts centrosome composition (48). In contrast, CA2 disrupts cytoplasmic microtubules microtubule cytoskeleton, suggesting that CA1 distorts centrosome

Because the Hh pathway promotes cancer growth, novel drugs that antagonize Hh signaling components, such as Smo, or disrupt the cilium could prove of therapeutic value. Loss of Ptc1 and constitutive activation of Smo cause BCC and medullo-blasto-loma, but treatment with a single Smo antagonist can fail (50). Therefore, complementary inhibitors, especially those that act through independent mechanisms, may provide synergistic clinical benefit in the treatment of Hh pathway-related cancers.

Materials and Methods

Detailed descriptions of the reagents and protocols used in this study are provided in SI Materials and Methods.

Small Molecules. SA1-10, CA1-2, and NC3-5 were purchased from ChemDiv. NC1 and NC2 were obtained from the University of California at San Francisco Small Molecules Discovery Center. LDE225 was obtained from Novartis Pharma, and Vismodegib was obtained from Genentech. Cyclopan (CalBioChem) was used as an additional inhibitor of the Hh pathway. BODIPY-cyclopamine (Medical Isotopes) was used in Smo-binding assays. Reombinant mouse Wnt3a and Dkk-1 (R&D Systems) were used to assess Wnt pathway activity.

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