Hedgehog signal transduction by Smoothened: Pharmacologic evidence for a 2-step activation process

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The Hedgehog (Hh) signaling pathway controls growth, cell fate decisions, and morphogenesis during development. Damage to Hh transduction machinery can lead to birth defects and cancer. The transmembrane protein Smoothened (Smo) relays the Hh signal and is an important drug target in cancer. Smo enrichment in primary cilia is thought to drive activation of target genes. Using small-molecule agonists and antagonists to dissect Smo function, we find that Smo enrichment in cilia is not sufficient for signaling and a distinct second step is required for full activation. This 2-step mechanism—localization followed by activation—has direct implications for the design and use of anticancer therapeutics targeted against Smo.

The Hedgehog (Hh) signaling pathway, studied first for its role in animal development, has become an attractive target for anticancer drugs (1). Mutations that lead to constitutive Hh signaling cause Gorlin’s syndrome, a familial cancer syndrome characterized by such neoplasms as basal cell carcinomas (BCCs) and medulloblastomas. Persistent activation of this pathway also has been implicated in the pathogenesis of sporadic human cancers that develop in different organ systems (1). Several inhibitors of Hh signaling have now reached clinical testing. The first Hh inhibitor used in human patients has shown promising activity against advanced BCCs and is being tested in trials of colon and ovarian cancer. The direct protein target for these inhibitors is the 7-pass transmembrane Smoothened (Smo) (1). A single Smo gene is required in animals for transduction of the Hh signal in receiving cells. Smo is a human proto-oncogene; activating mutations in Smo have been found in BCCs and medulloblastomas (reviewed in ref. 1).

In the absence of an Hh ligand, the activity of Smo is inhibited by the Hh receptor Patched1 (Ptc1) (2, 3). Binding of one of the secreted protein ligands, Sonic Hh (Shh), Indian Hh, or Desert Hh, to Ptc1 unleashes Smo activity. This is followed by the inactivation of Suppressor of Fused (SuFu), a negative regulator of the pathway, leading to activation or derepression of target gene transcription through 3 Gli transcription factors (Gli1–3).

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Despite the central importance of Smo in Hh signaling, the biochemical steps that lead to Smo activation in mammals are incompletely understood. Two types of observations have shed light on the mechanism of Smo activation, one involving small-molecule regulation of Smo function and one involving intracellular transport of Smo. By analogy to kinetic models of G protein–coupled receptor (GPCR) function, Smo is thought to exist in 2 states, active and inactive (4, 5). Ptc1 may regulate the distribution of Smo between these states through a small molecule (6). Small-molecule Smo inhibitors would function by either stabilizing the inactive state or destabilizing the active state. Circumstantial evidence for such molecules comes from the frequency with which small-molecule modulators of Smo function, including cyclopamine, SAG, and SANT, have been found in chemical screens (7, 8). Despite the structural similarity of Smo to GPCRs and evidence for the coupling of Smo to a G protein (9), an endogenous ligand for Smo has not been discovered. Smo mutations that lead to constitutively activated Smo protein, such as the SmoM2 allele found originally in BCCs, would alter the equilibrium in favor of the active state and allow Hh signaling even in the absence of Shh (4).

Analysis of Smo also has focused on subcellular localization at the primary cilium. Hh signaling in mammals appears to be orchestrated at the primary cilium, and Smo enrichment at primary cilium is correlated with activation of signaling (10, 11). It has been suggested that Smo may be activated in the specialized lipid environment of the ciliary membrane, or that it is able to engage downstream pathway components, such as SuFu and the Gli proteins, at cilia (12). The precise relationship between ciliary localization and activation of Smo is not clear, however. Although it remains possible that Smo can be active outside cilia, a case in which mammalian Hh target genes are active in the absence of Smo at cilia has not been described. In the present work, we focused on the fate of Smo at cilia.

Using previously described small-molecule agonists and antagonists of Smo, we sought to dissect the activation pathway of Smo in cultured fibroblasts. We found that Smo localization at cilia is not sufficient for activation, and we provide evidence that the accumulation of Smo at cilia is followed by an essential second activation step. The 2 steps can be pharmacologically separated, because different inhibitors can selectively block each step. This work provides a framework for understanding both Smo activation and the mechanism of action of Smo inhibitors, an emerging class of anticancer agents.

Results

Endogenous Smo accumulates at primary cilia only after the addition of Shh to cells (13). But while studying GFP-tagged Smo introduced into NIH 3T3 cells by transient transfection, we made the unexpected observation that wild-type Smo (Smo-GFP) and a constitutively active mutant Smo (SmoM2-GFP) exhibited different behaviors. We introduced into NIH 3T3 cells by transient transfection, we made the unexpected observation that wild-type Smo (Smo-GFP) and a constitutively active mutant Smo (SmoM2-GFP) exhibited different behaviors. We introduced these constructs into NIH 3T3 cells (14). Using previously described small-molecule agonists and antagonists of Smo, we sought to dissect the activation pathway of Smo in cultured fibroblasts. We found that Smo localization at cilia is not sufficient for activation, and we provide evidence that the accumulation of Smo at cilia is followed by an essential second activation step. The 2 steps can be pharmacologically separated, because different inhibitors can selectively block each step. This work provides a framework for understanding both Smo activation and the mechanism of action of Smo inhibitors, an emerging class of anticancer agents.


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were the sole determinant of activity, then both overproduced proteins should increase target gene transcription by similar amounts; however, SmoM2-GFP is a much more effective inducer of target gene transcription than the wild-type protein (Fig. 1B) (4). Moreover, cells overexpressing Smo-GFP can be further activated by the addition of Shh without a corresponding change in the amount of Smo-GFP at cilia (Fig. 1).

Increasing the level of Smo protein in cells can cause constitutive ciliary localization without fully activating signaling, probably because Ptc1 can inhibit Smo even when it is localized in cilia. This interpretation is consistent with a recent report suggesting that defects in the dynein that transports proteins out of cilia leads to constitutive ciliary Smo localization without pathway activation (14). The data from that study suggest that a low level of Smo cycles through cilia in the absence of ligand; thus, steady-state levels of Smo at cilia can be increased by increasing total levels of Smo in the cell or by decreasing the export of Smo from cilia.

Because localization of Smo to cilia is not sufficient for maximal target gene transcription, a second step must be necessary to activate ciliary Smo. A kinetic scheme for Smo activation is proposed in Fig. 5. In this model, the movement of Smo into and out of the cilium (controlled by steps R1 and R2) is accompanied by a rate-controlling activation step (R3). The biochemical nature of step R3 is unknown, but it could be a posttranslational modification, protein interaction, or conformational change (5). Thus, Smo exists in 3 states: a cytoplasmic inactive state (Smo1), a ciliary inactive state (Smo2), and a ciliary active state (Smo3). This scheme is inspired by studies demonstrating that the activation pathway of most GPCRs is not a single-step conversion from an inactive state to an active state, but rather a multistep process comprising several intermediary states, each with distinct effects on downstream signaling proteins (15, 16). In the case of GPCRs, these states were elucidated using agonists and antagonists that stabilize intermediate states.

To test the hypothesis that Smo is activated through a multistep process, we investigated whether existing Smo antagonists can be exploited to lock Smo in different states. For all of the experiments described in Figs. 2–4, we examined only endogenous Hh pathway proteins in NIH 3T3 cells, thereby avoiding artifacts that can be associated with tagged or overproduced proteins, such as the ciliary localization seen with overproduced Smo shown in Fig. 1. We used the compounds SANT1, SANT2, and cyclopamine. The plant alkaloid cyclopamine directly binds and inhibits Smo (4, 17). Like cyclopamine, the SANT1 and 2 molecules are inhibitors of the Hh pathway. They can compete with a fluorescent cyclopamine derivative for binding to formaldehyde-fixed, Smo-overproducing cells (8). Thus, all 3 molecules either bind to a single site on Smo or bind to different sites that have a negative allosteric interaction.

We studied the effects of these 3 compounds on Smo localization using quantitative fluorescence microscopy. NIH 3T3 cells were treated with Shh in the presence of increasing concentrations of SANT1, SANT2, and cyclopamine (Fig. 2A–C). SANT1 and 2 blocked the Shh-induced enrichment of Smo in cilia, with IC50 values (~5 and 13 nM, respectively) close to the Kd values of both compounds (1.2 and 12 nM) for binding to Smo (Fig. 2B) (8). In contrast, cyclopamine did not inhibit the accumulation of Smo at cilia, even when used at concentrations (5–10 μM) > 10-fold above its Kd for Smo (Fig. 2C) (17). Despite these striking differences in effect on Smo localization, all 3 compounds inhibited Hh signal transduction and target gene activation. Ptc1 is a direct Shh target gene, and its transcriptional activation is commonly used as a metric for Shh signaling. All 3 compounds blocked Shh-induced Ptc1 protein accumulation in the same NIH 3T3 cells used to assay Smo localization in cilia (Fig. 2D). These data support the kinetic scheme shown in Fig. 5. SANTs 1 and 2 lock Smo in the cytoplasmic inactive state (Smo1), whereas cyclopamine locks Smo in the ciliary, inactive state (Smo2). In this view, all 3 compounds are inhibitors, but cyclopamine and the SANTs have opposite effects on Smo enrichment in cilia.

An alternative explanation for these observations is that SANTs 1 and 2, but not cyclopamine, induce the degradation of Smo. Assessment of Smo protein levels in NIH 3T3 cells after the drug treatments demonstrated the opposite effect, however (Fig. 2D). Cyclopamine, which allows substantial accumulation of Smo in cilia, caused a major decrease in total Smo protein levels. Although SANTs 1 and 2 had a less pronounced effect on the total amount of Smo, they did selectively reduce the levels of fully glycosylated Smo. Smo protein runs as a doublet on SDS/PAGE gels, and the upper bands (denoted as “top” in Fig. 2D) have been characterized as fully glycosylated post-Golgi protein forms (17). Thus, SANTs 1 and 2 may retard the movement of Smo through the secretory pathway by blocking transport through the endoplasmic reticulum (ER) or the Golgi apparatus. In contrast, cyclopamine had a smaller effect on levels of fully glycosylated Smo than on total Smo. Together, these findings support the idea that the 3 inhibitors stabilize biochemically distinct states of the Smo protein.

Our results differ from a previously published report concluding that Smo translocation to cilia is inhibited by cyclopamine (11). The reason for this difference is unknown, but there are several possible explanations. First, we examined the behavior of endogenous Smo, whereas previous studies examined overex-
experiments. SANTs 1 and 2 functioned identically in our assays, we focused on their effects on Smo activity are not mediated by Ptc1. Because the compounds’ ability to act on cells that lack Ptc1 confirms that Smo at primary cilia (Fig. 3A). Cyclopamine induced a substantial decrease in Smo protein levels (Fig. 2C). If this effect were more pronounced in other cell lines or under other conditions, this could explain why cyclopamine has been previously reported to inhibit Smo transport to cilia.

To confirm that the inhibitor effects were not unique to NIH 3T3 cells, we evaluated the effects of SANT1, SANT2, and cyclopamine on Smo localization in mouse embryonic fibroblasts (MEFs) that lack Ptc1 protein. Ptc1−/− cells exhibit constitutive activated target gene expression and constitutive enrichment of Smo at primary cilia (Fig. 3A). Consistent with our findings in NIH 3T3 cells, SANTs 1 and 2, but not cyclopamine, caused a loss of Smo from primary cilia in MEFs. As seen with NIH 3T3 cells, all 3 inhibitors blocked the constitutive transcription of target genes seen in these cells (data not shown). The compounds’ ability to act on cells that lack Ptc1 confirms that their effects on Smo activity are not mediated by Ptc1. Because SANTs 1 and 2 functioned identically in our assays, we focused on the comparison of SANT1 and cyclopamine in subsequent experiments.

The experiments presented up to this point explored the effects of SANTs and cyclopamine on Smo localization induced by Shh. Our proposed model also makes specific predictions about the effects of the inhibitors on Smo localization in cilia in the absence of Shh. If cyclopamine indeed binds and locks Smo in a state localized in cilia (Smo2; Fig. 5), the drug should cause the accumulation of Smo in cilia even in the absence of Shh. The alternative possibility is that our model is incorrect and that Smo exists in only 2 states: an active, ciliary state and an inactive, cytoplasmic state. In this case, cyclopamine would affect Smo activity without influencing its localization and so should have no effect on Smo in the absence of Shh.

Consistent with the 3-state model for Smo activation, cyclopamine alone induced dose-dependent enrichment of Smo in cilia (Fig. 4A and B). SANT1 did not have this effect (Fig. 4A). As controls for the specificity of the cyclopamine effect, cyclopamine from 2 distinct sources had similar effects, and tomatidine, a hydrophobic amine that does not bind to Smo and does not influence Hh signaling, had no effect (18). This allays any concerns that hydrophobic amines, such as tomatidine and cyclopamine, may have nonspecific effects on protein trafficking because of their propensity to accumulate in vesicles with acidic contents. Further evidence of the specificity of this effect is that cyclopamine’s EC50 for Smo transport into cilia is roughly equal to its IC50 for inhibition of Shh-induced target gene transcription (Fig. 4C). These pharmacologic dose data support the idea that both effects are mediated through the same target protein, Smo.

If cyclopamine induces the accumulation of Smo in primary cilia by a direct interaction with Smo, then it should be able to bind Smo protein that is located at cilia. To show a direct interaction with Smo, then it should be able to bind Smo protein that is located at cilia.
interaction between ciliary Smo and cyclopamine, we used BODIPY-cyclopamine, a fluorescent derivative of cyclopamine that binds Smo and inhibits Hh signaling (17). When added at trace concentrations (5 nM) well below its IC_50 (150 nM) for Hh pathway inhibition, BODIPY-cyclopamine showed striking co-localization with a tdTomato-tagged Smo protein localized in primary cilia of live cells (Fig. 4D). This interaction is specific because a 100-fold excess of unlabeled cyclopamine can inhibit the ciliary binding of BODIPY-cyclopamine without causing a corresponding change in levels of tdTomato-Smo in primary cilia (Fig. 4D).

Because the binding of cyclopamine and SANT1 to Smo is mutually exclusive (8), our model predicts that SANT1 and cyclopamine, both inhibitors of signaling, should compete with each other for induction of ciliary Smo accumulation. On the other hand, if the effect of one of these inhibitors were indirect—mediated by a different protein—then we would not see a competitive interaction. To test this, we incubated cells in cyclopamine at a saturating concentration of 5 μM (∼10× K_D), in combination with increasing concentrations of SANT1 (Fig. 4A and E). SANT1 inhibited cyclopamine-induced ciliary Smo accumulation with an IC_50 of ∼200 nM. This value is consistent with a competitive interaction between cyclopamine and SANT1 and supports our model of cyclopamine and SANT1 competing with each other to stabilize 2 different states of Smo, located in the cilia and the cytoplasm, respectively.

Thus far, we have focused on the analysis of Smo inhibitors. Smo agonist (SAG) is a small molecule that directly binds Smo, causes its accumulation in cilia, and potently activates target gene transcription (EC_50 = 5 nM) (7, 8, 13). Thus, SAG would be predicted to increase the active Smo3 state in cilia and should compete with SANT1 for induction of ciliary Smo. When we incubated cells with 100 nM SAG (> 10× K_D), increasing concentrations of SANT1 inhibited ciliary Smo accumulation with an IC_50 of ∼10 nM (Fig. 4A and F) (9). Again, the concept that Smo exists in 2 states in cilia, Smo2 and Smo3, is supported by the finding that cyclopamine and SAG, which have opposite effects on target gene induction, can both induce Smo movement to cilia. It is important to exclude the possibility that SANT1 affects Smo, not by stabilizing the Smo3 state, but more indirectly, by causing the movement of Ptc1 out of cilia (13). Double-labeling of Ptc1 and Smo in SAG-activated NIH 3T3 cells clearly showed that both Smo and Ptc1 are simultaneously present at cilia, supporting the model that Smo3 is a Ptc1-insensitive state in the primary cilium (Fig. S2).

In summary, we have shown that SANT1 can block ciliary localization of Smo induced by Shh, which acts on Ptc1, and by cyclopamine or SAG, which act directly on Smo. This supports our suggestion that SANT1 traps Smo in a state, perhaps one that cannot progress through the secretory pathway, before the step in which these other molecules influence Smo. Interestingly, the IC_50 of SANT1 in the presence of SAG (∼10 nM) was lower than its IC_50 in the presence of cyclopamine (∼200 nM), even though both SANT1 and cyclopamine were present in ∼10-fold excess over their respective K_D for association with Smo (7, 8). This would be the expected result if SANT1 directly competed with cyclopamine for transport to cilia but worked against SAG indirectly (or noncompetitively) by simply excluding Smo from the ciliary compartment in which SAG can act.

Discussion
Smo is a central transducer of the Hh signal and an important anticancer drug target. Elucidating the biochemical pathway for Smo activation is central to uncovering how Smo inhibitors work, to both facilitate the design of better antagonists and cope with drug resistance mechanisms that will undoubtedly emerge. Here we present a kinetic framework for the activation pathway of Smo comprising 2 pharmacologically separable steps: the trafficking of Smo protein to the primary cilium and a second activation step that allows Smo to engage the downstream signaling machinery in cilia. We found that Smo translocation to cilia can be clearly separated from activation, because cyclopamine blocks target gene activation but induces Smo accumulation in cilia. The inhibitor SANT1 blocks both ciliary transport and activation, suggesting that ciliary transport is a prerequisite for the activation step. SANT1 may function by retarding the movement of Smo through the secretory pathway or by influencing its interactions with a protein, such as β-arrestin or Grk2, that has been implicated in Smo transport to cilia (19).

Our model suggests that a low level of Smo is constantly trafficking through cilia, with entry and exit rates R1 and R2 (14). The low basal level of Smo in cilia (Smo2) does not activate downstream signaling, because a second, rate-determining step prevents significant conversion of Smo2 into the active Smo3 (R3 ≪ R2 in the absence of ligand). Increasing total Smo protein in cells (by, e.g., transient transfection, as shown in Fig. 1) raises the concentration of all 3 states of Smo without changing the rate constants controlling steps R1–4, leading to ciliary localization of Smo and a modest degree of pathway activation (Fig. 1A). Although R3 is not zero in the absence of ligand, it is much smaller than R1, so the effect of increasing total Smo in the cell on ciliary Smo accumulation is much more pronounced than the effect on target gene activation.

Ptc1 is a 12-pass transmembrane protein that serves as a Shh-binding receptor. In the absence of Shh, Ptc1 effectively prevents Smo from activating target gene transcription and concomitantly prevents Smo accumulation in cilia (13). Which step in Smo activation is blocked by Ptc1? Although we cannot exclude the possibility of an additional effect on Smo transport, Ptc1 must inhibit the second activation step (R3), because overproduced Smo localized in cilia is still not fully active and...
thus is mostly in the postulated Smo2 state (Fig. 1). The IC_{50} (~10 nM) of SANT-1 for ciliary Smo is very similar in the presence of Shh (which inhibits Ptc1) or SAG (which stabilizes Smo3), suggesting that Ptc1 and SAG regulate steps in Smo activation after the ciliary transport step. Inactivation of Ptc1 by Shh leads to increased conversion of Smo2 to the active Smo3.

Fig. 4. Cyclopamine can induce and SANT1 can block the accumulation of Smo in primary cilia. (A) Confocal images (shifted overlays) of NIH 3T3 cells treated for 7 h with the indicated combinations of SANT1 (100 nM), cyclopamine (5 μM), or SAG (100 nM). (B) The mean Smo fluorescence at cilia increases with increasing doses of cyclopamine from 2 different sources (#1 and #2), but not with the inactive alkaloid tomatidine. (C) Cyclopamine concentrations required to induce Smo in cilia of untreated NIH 3T3 cells (black circles) are similar to those required to inhibit the Shh-stimulated Gli-luciferase reporter (red squares) in ShhL2 cells. (D) Confocal images (shifted overlay) of cilia (arrows) from live NIH 3T3 cells transfected with tdTomato-tagged Smo (red) and treated for 50 min with BODIPY-cyclopamine (green) alone (Top) or in combination with a 100-fold excess of unlabeled cyclopamine (Bottom). (E and F) SANT1 can inhibit the accumulation of Smo in cilia of NIH 3T3 cells induced by either 5 μM cyclopamine (E) or 100 nM SAG (F). All points in the graphs show mean (± SEM) values.
This also will lead to the accumulation of Smo in cilia, especially if R4 (the rate of active Smo3 exit) is slower than R2 (the rate of inactive Smo2 exit) (Fig. 5). Whereas Ptc1 controls R3, SAG likely acts at a more distal step to promote activation by binding to Smo3, because high Ptc1 can effectively prevent SAG from activating the pathway, presumably by reducing the Smo3 pool (8). When considering these possibilities, it is important to keep in mind that we could not measure Smo2 and Smo3 separately in cilia; our fluorescence microscopy–based assay measured total Smo at cilia, the sum of Smo2 and Smo3.

Activating mutations of Smo, such as SmoM2, have been isolated from patients with BCC (20). Because such mutations lead to Shh-independent, high-level activation of the protein, they can be predicted to preferentially stabilize the Smo3 state over the Smo2 state (4). The SmoM2 mutation is less likely to promote the trafficking step, because SANT1 binds and inhibits Smo and SmoM2 with equal potency (8), and because both Smo and SmoM2 are detected at roughly equal levels in cilia when overproduced in cells (Fig. 1). In contrast, cyclopamine has a higher affinity for Smo than for SmoM2 (4). Based on our model, this is because the M2 mutation likely favors the Smo3 conformation, which has a lower affinity for cyclopamine.

An important idea that emerges from our model is that Smo inhibitors fall into 2 categories, “cyclopamine-like” inhibitors that affect the activation step and “SANT1-like” inhibitors that influence the trafficking of Smo to cilia. In fact, fundamental mechanistic differences between these 2 Smo inhibitors have been postulated based on differences in their abilities to inhibit SmoM2– or SAG-induced signaling (8). When Smo inhibitors are used in the clinic, resistance will arise, and our model may be useful for analyzing and overcoming resistance mechanisms. The widespread use of tyrosine kinase inhibitors (TKIs) in treating cancer has highlighted the importance of second site mutations in the kinase target that decrease its affinity for the drug (21). A mutation like SmoM2 would be one mechanism of resistance to a cyclopamine-like drug in Hh-driven tumors. But, based on our model, such tumors may remain sensitive to a SANT1-like molecule. Increased activity of cellular pathways that promote delivery of proteins to the cilia might be a resistance mechanism for a SANT1-like inhibitor. An analogy for this is the finding that increased delivery of HER3 to the plasma membrane provides a mechanism by which breast cancer cells evade TKIs (22). In analogous cases of heightened Smo transport, high-affinity cyclopamine-like inhibitors would be useful in fully suppressing the Smo2→Smo3 step. Thus, these 2 classes of inhibitors may show a lack of cross-resistance and occupy complementary roles in the clinic.

In summary, using a set of small-molecule modulators of Smo, we have provided support for a 2-step model for regulation of Smo in which transport to the primary cilium is followed by a second rate-determining step necessary to activate downstream events leading to target gene induction. Such a multistep activation mechanism is commonly used strategy in signal transduction pathways both to ensure fidelity and to endow a system with combinatorial control. Given that Hh signaling often controls irreversible cell fate decisions, gating Smo activation at 2 steps is a good strategy, because relying solely on localization in cilium would leave the pathway sensitive to stochastic fluctuations in Smo protein levels. In addition, mechanisms that control Smo trafficking to cilia may provide a way for other intrinsic or extrinsic signals to regulate the responsiveness of a cell to a fixed amount of Shh. Uncovering the molecular machinery of Smo trafficking and the biochemical nature of the second activation step are important goals for future research. Materials and Methods Methods used for microscopy, image analysis, and Hh reporter assays have been described previously (13). Detailed descriptions of the constructs, antibodies, cell lines, and methods used in this study are provided in SI Materials and Methods.

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