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Agonizing Hedgehog

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An approach using 'chemical genetics' has identified small-molecule agonists of the Hedgehog signaling pathway that may lead the way to drugs for chronic degenerative diseases.

It is a rare treat when a drug discovery program teaches us something about the biology of the process that it attempts to modulate. The paper by Maria Frank-Kamenetsky and colleagues in this issue of the *Journal of Biology* [1] presents a compelling example of how the search for therapeutics can provide powerful experimental tools and insights into fundamental biology, blurring the distinction between applied and basic research. By characterizing a small group of chemically similar agonists of the **Hedgehog** signaling pathway, Frank-Kamenetsky *et al*. have been able to propose a new model for how the **Smoothened** component of the Hedgehog-receptor complex works, and to hint at the existence of natural-ligand agonists of the Hedgehog signaling pathway (see 'The bottom line' box for a summary of their work).

Hedgehog history

Signaling by the Hedgehog (Hh) family of secreted proteins plays a central role in regulating cell differentiation and tissue patterning during development [2]. The *hedgehog* gene (*hh*) was first identified by virtue of its role in the specification of positional identity during *Drosophila* embryonic segmentation, and it was subsequently found to control patterning of structures such as the eye and the abdominal cuticle. In mammals there are three *hh* homologs, called ***Sonic Hedgehog***, ***Indian Hedgehog*** and ***Desert Hedgehog*** (*Shh*, *Ihh* and *Dhh*, respectively), which have been implicated in patterning events in a range of developing tissues (see the 'Background' box) [2,3].

Recently, signaling by Hh has been shown to be important for patterning of the cerebellum, where it promotes the proliferation of granule neuron precursors. A link between Hh proteins and stem-cell proliferation has raised

The bottom line

- Frank-Kamenetsky and colleagues designed a cell-based, high-throughput assay to screen 140,000 compounds to find modulators of the Hedgehog signaling pathway.
- They identified a small group of related synthetic non-peptidyl molecules that can act as agonists of Hedgehog signals at nanomolar concentrations, having previously identified small-molecule Hedgehog antagonists.
- A range of *in vitro* and *in vivo* assays were used to show that the agonists can be used as drugs to overcome Hedgehog-signaling defects and to promote cell proliferation.
- The action of the agonist compounds is independent of the Hedgehog ligand and the inhibitory receptor Patched. Further characterization revealed that the agonists bind directly to the receptor Smoothened.
- These data give new insights into the nature of Hedgehog-Smoothened signaling and raise the possibility of analogous endogenous modulators of Hh signaling.

the enticing possibility that modulating Hh signaling might be relevant for the clinical management of certain degenerative diseases. Indeed, it was recently demonstrated that Shh might be effective in treating peripheral nerve damage or degenerative brain disorders such as **Parkinson's disease** [4,5]. Perhaps not surprisingly given its role in development, misregulated Hh signaling has also been implicated in **cancer** [3]. Specifically, medulloblastoma and basal cell carcinoma (BCC) are associated with inappropriate activation of Hh signaling [6,7]. These observations motivated Frank-Kamenetsky and colleagues to search for small-molecule modulators of the Hh pathway, with the hope that **antagonists** and **agonists** might be used as drugs to treat proliferative or degenerative diseases, and that **small molecules** might prove more amenable to pharmacological delivery than the Hh-family proteins themselves.

Sending the signal

Hedgehog signaling challenges the way we normally think about signal transduction pathways. Biology is full of examples of extracellular ligands that bind to specific cell-surface receptors, initiating a cascade of biochemical events (often involving protein kinases) that leads to the activation of a transcription factor and the induction of a set of effector genes. But Hedgehog signaling is not so simple. Even the ligand is complicated: the Hh proteins undergo unusual processing and cleavage to generate an extracellular cholesterol-linked peptide that serves as the signaling ligand [2]. And the receptor component is far from understood.

The cellular response to Hh is controlled by two transmembrane proteins, **Patched** (Ptc) and Smoothened (Smo). The Ptc protein weaves across the cell membrane twelve times and resembles some transmembrane channels. It acts as a negative regulator of the Hh signal and has been defined as a tumor-suppressor. In contrast, Smo is a proto-oncogene and activates signaling in response to Hh ligand. The Smo protein is a seven-transmembrane receptor that resembles conventional G-protein-coupled receptors (Figure 1a).

It appears that Ptc inhibits Smo, although the precise mechanisms are unclear. Hh stimulation relieves Smo from inhibition by Ptc, leading to the generation of an intracellular signal that culminates in a nuclear transcriptional response (Figure 1b). When Ptc is removed the pathway is constitutively 'on', independent of the Hh ligand, whereas certain mutations in Smo can activate Hh signaling, bypassing Ptc regulation. A heteromeric receptor model has been proposed, in which Hh interacts directly with Ptc and thereby affects the interaction of Ptc with Smo [8]. "But things look much more complicated than we had earlier thought," says Philip Beachy (Johns Hopkins University School of Medicine, Maryland, USA). "The previous models are not tenable," and he suggests that alternative models of receptor function must be considered.

The power of chemical genetics

Frank-Kamenetsky *et al.* [1] chose to use **chemical genetics** in an attempt to identify compounds that could interfere with the inhibition of Smo by Ptc, or could activate Smo independent of Ptc, or that might act downstream of Smo. In addition to making effective drug candidates, these molecules might also help to illuminate the complex mechanisms underlying signaling by Hh, Ptc, and Smo (see the 'Behind the scenes' box for more of the background to the work).

The high-throughput screen was elegantly simple. First, Frank-Kamenetsky

Background

- Mammals have three **Hedgehog (Hh)** proteins (**Sonic Hedgehog**, **Indian Hedgehog** and **Desert Hedgehog**) that are processed to generate functional extracellular ligands. Two receptors are involved in Hh signaling: **Patched (Ptc)** is a negative regulator of the Hh-triggered signaling pathway and **Smoothened (Smo)** is a positive activator (Hh, Ptc and Smo were all originally named for the cuticular phenotypes of mutant *Drosophila* larvae). When Hh ligands bind to Ptc they relieve the negative inhibition and Smoothened initiates a signaling cascade that results in the activation of nuclear transcription factors of the **Gli** family that regulate effector gene transcription (see Figure 1).

- Hh signaling has been implicated in a wide range of developmental processes. **Small-molecule modulators** of Hh signaling are potential candidates as therapeutic drugs to treat human diseases – molecules that mimic Hh signaling (**agonists**) might be used to treat degenerative disorders such as **Parkinson's disease**, whereas blockers (**antagonists**) could be used as drugs against certain types of **cancer**.

- **Chemical genetics** uses synthetic small molecules to dissect cellular functions such as signal transduction pathways. By analogy with loss- and gain-of-function mutations in genetics, the functional interactions between chemical inducers and inhibitors is used to define the hierarchical relationship between protein components of the pathway.

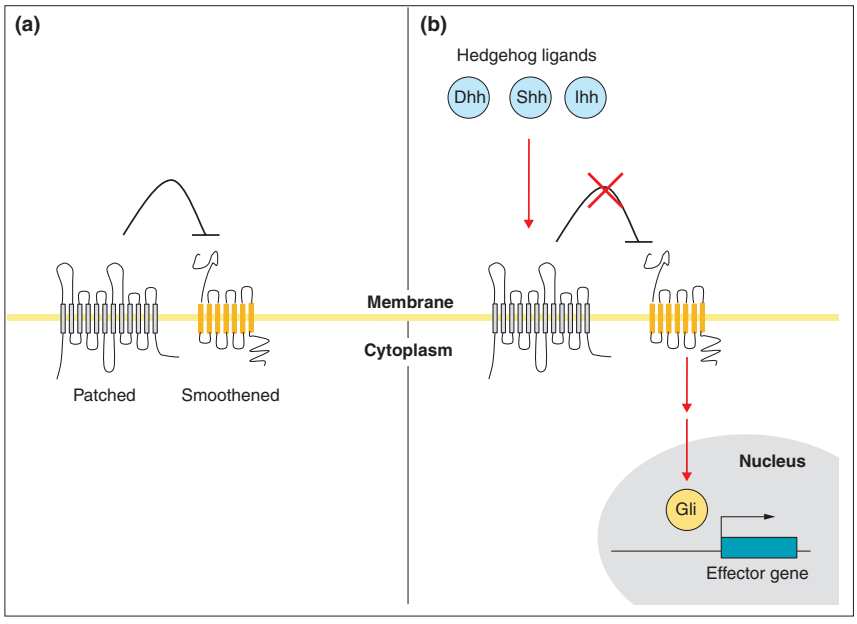

Figure 1
The Hedgehog signaling pathway. **(a)** In the absence of Hedgehog ligands, Patched protein inhibits the activity of Smoothened, which resembles G-protein-coupled receptors. **(b)** On activation by a ligand of the Hedgehog family, the inhibition of Smoothened by Patched is relieved and Smoothened is freed to trigger an intracellular signaling cascade; this ultimately leads to transcriptional regulation by transcription factors of the Gli family.

et al. identified a cell line that responds well to Hh stimulation. The introduction of a reporter gene (encoding the firefly luciferase protein) that was turned on by Hh signaling permitted screening for compounds that block or induce signaling by monitoring the expression of the luciferase protein (using a simple luminescence test). They had previously used such a screen to isolate antagonists of Hh signaling, and had demonstrated the effectiveness of some antagonists as potential anti-tumor drugs to treat BCC [7]. Their new screen of 140,000 synthetic compounds led to the discovery of a few candidate agonists that could stimulate the reporter gene and mimic Hh activity.

Once the cell-based screen was completed, the chemists took over, synthesizing 300 derivative molecules until they found a few compounds that were related to the previous 'best' agonist but that were a thousand times more effective at eliciting a cellular response, affecting cells when applied in the nanomolar range. "Getting more potent compounds was essential if we were to figure out where the agonists were acting" recalls Jefferey Porter who headed the team at Curis, Inc.

Then the cell biologists began again, studying the effects of the agonists on the proliferation of primary neonatal cerebellar granule neuron precursors. They monitored the incorporation of tritiated thymidine into cultured rat neurons (as a marker of DNA synthesis and hence proliferation) and were pleased to see that the agonists were as effective in this assay as the Hh protein itself. An assay using an explant of embryonic neural plate was used to confirm that the agonists could induce dose-dependent gene expression in neural precursors, just as the Shh protein does.

Having established the effects of the agonists in culture assays, the researchers then turned to an *in vivo* model, feeding the compounds to pregnant mice and following the effects on the phenotypes of embryos lacking *Shh* or *Smo*. The treated embryos displayed activated Hh signaling, demonstrating that the compounds were not toxic and could cross the placental barrier. The developmental defects of $Shh^{-/-}$ embryos were rescued by treatment with the agonist but the compound had no effect on Hh signaling in the absence of Smo.

"Once we knew that the agonists were targeting Smo, we wanted to investigate whether they bound to Smo directly and how they activated Hh signaling," says Porter. The cell line that was created for the screen served as a useful tool to test the effects of known antagonists on the function of the agonist. An anti-Hh blocking antibody had no effect, so the agonist must work downstream of the Hh-Ptc interaction. But the agonist was blocked by antagonists that work at the level of Smo or further downstream. "We have similar conclusions," says Beachy, whose group used photoaffinity labeling and cross-linking experiments to show that small-molecule agonists and antagonists bind directly to Smo.

Next, for Frank-Kamenetsky *et al.*, it was time for some careful biochemistry. Analysis of the expression of fusion proteins of Ptc or Smo showed that, unlike the Hh ligand itself, the agonist had no effect on the stability of the Ptc protein. In contrast, both Hh and the agonist could increase the stability of the Smo receptor. Immunoprecipitation experiments with radiolabeled agonist showed that the agonist must bind directly to Smo receptors, and that Hh-signaling inhibitors compete with the agonist for binding. Pharmokinetic analysis provided evidence for a single binding site competition model. Finally, Frank-Kamenetsky *et al.* exploited an oncogenic, constitutively active mutant form of Smo

(Smo^{act}); the agonists bound equally well to mutant and wild-type forms, whereas the antagonist bound less well to the mutant form.

Learning lessons from drugs

Frank-Kamenetsky *et al.* have demonstrated convincingly that high-throughput chemical genetics can be used to isolate modulators of a developmental signaling pathway. The agonists that they have generated are efficient and apparently non-toxic mimics of Hh signals and are promising candidates for drugs for regenerative medicine. The authors work at the biotechnology company Curis Inc. (Cambridge, USA), so finding new drugs is obviously their primary objective. But the agonists also provide useful tools for probing the complex Hh-Ptc-Smo signaling pathway.

"A lot of our biological insight is driven by having specific chemical inhibitors, and many drugs are used as tools to dissect signaling systems as a substitute for genetic studies," says Arnon Rosenthal (Rinat Neuroscience Corp., Palo Alto, USA). Beachy agrees: "these compounds, first the plant-derived inhibitor cyclopamine and more recently the agonists, have really helped us to understand Smoothened function." Recent work from Beachy's lab [9,10], demonstrating that Ptc suppresses Smo in catalytic manner, has led to speculation that Ptc may function as a transporter protein pumping natural small-molecule Smo modulators across the cell membrane. Rosenthal adds that "good basic research always leads to better medicines, since the more we understand about the mechanisms operating in the body, the better able we are to modulate them rationally."

The data presented by Frank-Kamenetsky *et al.* [1] led them to propose a new model for Hh signaling based on the classic 'ternary complex model' that was developed to describe ligand-induced conformational changes of G-protein-coupled receptors [11]. According to this model, active and inactive conformations of Smo are selected by the binding of agonists or antagonists at independent sites. Furthermore, the model predicts that Smo binds to a novel effector molecule

Behind the scenes

Journal of Biology asked Jeff Porter, group leader at Curis Inc., to comment on the background to the project to search for small-molecule modulators of Hedgehog signaling.

What prompted you to set up a screen for agonists of the Hedgehog pathway?

There was evidence that manipulating Hedgehog signaling might be useful in a therapeutic context to treat degenerative neurological diseases. There were promising data using modified Hedgehog ligands in animal disease models, but we felt that a small molecule would be a more effective therapeutic. We had previously set up the cell-based assay to screen for antagonists of Hedgehog signaling and successfully isolated inhibitors that could inhibit tumor growth. So we adapted the assay to find Hedgehog agonists.

What was your initial reaction to the results, and how were they received by others?

This was a kind of 'black box' screen - we were looking for a change in a biological readout, in contrast to traditional pharmacological screens that focus on a particular target. So, we couldn't be sure what type of molecule we would get; we could have predicted that we'd find inhibitors of Patched or stabilizers of Gli. I was surprised and excited when we realized that our agonists were targeting Smoothened. Once we had nailed down the target, there was a lot of interest in these compounds and what they can teach us about Hedgehog signaling.

How long did the project take?

We began the screen in late 1999. It took a few months to get the initial compounds, and then we began the process of making slow improvements by chemical modification. Without the improvements we couldn't have figured out what was going on. We had to establish the techniques and acquire the reagents necessary to characterize the agonists in detail. The potent compound derivatives were also key to the success of the cell-free binding assays - they look simple but the binding experiments were very tricky.

What are the next steps?

We want to figure out the mechanisms of Smo regulation and signal transduction - how Ptc talks to Smo and how Smo talks to Gli. And, of course, we are also testing these molecules as potential therapeutics in animal models of disorders of the central nervous system. The preclinical results are promising: the compounds show low toxicity, they can be administered orally and cross the blood-brain barrier. These compounds might also be useful in *ex vivo* therapies to stimulate stem cell proliferation. I am optimistic that Hedgehog agonists will be tested in human trials in the future.

and it raises the possibility that endogenous ligands, analogous to the newly found agonist, may naturally regulate Smo activity.

Cheryll Tickle (University of Dundee, UK) studies the role of Hh in development and finds the possibility that there are endogenous small-molecule agonists that interact with Smoothened particularly intriguing. "This would mean that we are missing a whole layer of control of Hedgehog signaling in our current models," she notes. Porter adds, "It is tempting to speculate that endogenous molecules act directly on Smo, bypassing Ptc. That is consistent with what we know about how G-protein-coupled receptors work. But at the moment, it's pure speculation."

The study by Frank-Kamenetsky *et al.* [1] exploits a dazzling variety of experimental techniques to illustrate the path from high-throughput screening to compound characterization. Biochemists, cell biologists, pharmacologists and chemists have come together to demonstrate effectively that 'drug discovery' can combine all the excitement of 'real' scientific discovery with the satisfaction of isolating compounds that might be used for tomorrow's therapies.

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