Cell Signaling

DOI: 10.1002/anie.200805666

The Imidazopyridine Derivative JK184 Reveals Dual Roles for Microtubules in Hedgehog Signaling**

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The Hedgehog (Hh) signaling pathway is a critical regulator of embryonic patterning, and aberrant Hh pathway activation in children and adults has been implicated in several cancers, including basal cell carcinomas, medulloblastomas, small-cell lung cancer, pancreatic adenocarcinomas, and prostate tumors.[1] Small molecules that block this developmental pathway are therefore potential chemotherapeutic agents, and their discovery and mechanistic characterization are critical steps toward realizing effective anticancer therapies. One molecule reported to potently inhibit the Hh pathway is the imidazopyridine derivative JK184, which can bind and inhibit alcohol dehydrogenase 7 (Adh7).[2] How JK184 and Adh7 regulate Hh target gene expression is not fully understood, although interference with retinoic acid biosynthesis and signaling has been proposed as one possibility.[2] Herein we report our studies of JK184 and its mechanism of action. We demonstrate that JK184 is a potent inhibitor of microtubule assembly and that microtubule-depolymerizing agents can either negatively or positively regulate the Gli family of transcription factors, dependending on the mechanism by which the pathway is activated.

Hh pathway activation during embryogenesis involves the binding of secreted proteins, such as Sonic Hedgehog (Shh) to the transmembrane receptor Patched1 (Ptch1), thereby inhibiting its repression of a second membrane-localized receptor called Smoothened (Smo; Figure 1).[1] Through mechanisms that are not yet clear but involve the microtubule-based primary cilium,[3–6] Smo activation then inhibits the proteolytic processing of the Gli2 and Gli3 transcription factors into C-terminally truncated repressors and promotes their stabilization as full-length forms. The full-length proteins are further modified to activate the expression of Hh target genes such as Gli1, which is not subject to proteolytic processing and reinforces Hh pathway activation through a positive feedback loop. Smo may also modulate the function of Suppressor of Fused (Su(fu)), which binds and negatively regulates the Gli proteins.

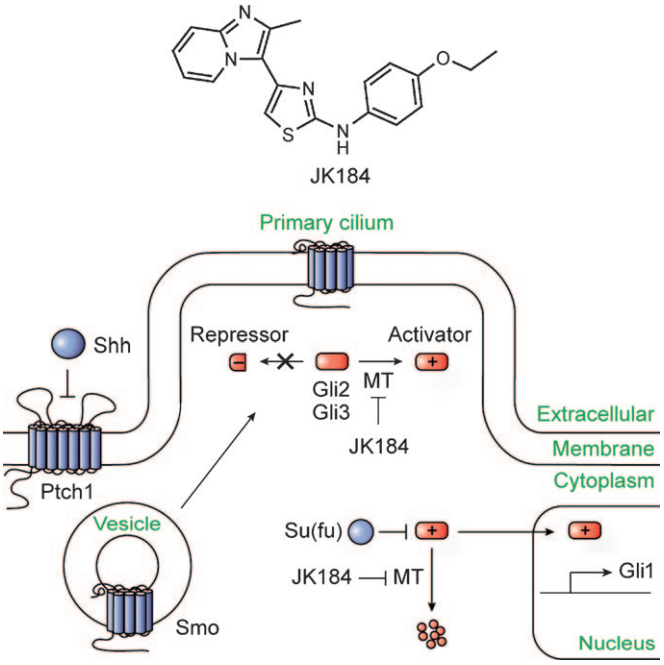

Figure 1. Schematic representation of the Hh signaling pathway in its activated state, with Gli transcription factors depicted in red, regulatory proteins in blue, and subcellular compartments in green. Two JK184-sensitive, microtubule (MT)-dependent steps indicated by our studies are shown. In the absence of Hh ligand, Ptch1 is localized to the primary cilium and suppresses Smo trafficking to this organelle, thus leading to the cilia-dependent proteolysis of Gli proteins into transcriptional repressors. Upon Hh pathway activation, Ptch1 exits and Smo accumulates in the primary cilium, Gli processing is inhibited (black cross), and full-length Gli proteins are converted into transcriptional activators.

In contrast to embryonic Hh signaling, oncogenic Hh target gene expression can be initiated at several points within the pathway. Some tumors require Hh ligand through autocrine or paracrine mechanisms,[7,8] and others arise from pathway-activating mutations in downstream effectors. For example, medulloblastomas can result from inactivating mutations in Ptch1,[9] activating mutations in Smo,[10] or loss of Su(fu)[11] function. Compounds that directly inhibit Smo and exhibit antitumor activities have been described, such as the natural product cyclopamine and the synthetic inhibitor HhAntag.[12,13] However, these molecules are most active against tumors that require Hh ligands or lack Ptch1 function,

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[**] We thank Dr. G. Heffner, B. Moree, and Prof. Dr. A. F. Straight for technical assistance. This work was supported by the Stanford Interdisciplinary Translational Research Program, the Sidney Kimmel Foundation for Cancer Research, the Astellas USA Foundation, and the Brain Tumor Society/Rachel Molly Markoff Foundation.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.200805666.

and inhibitors that act downstream of Smo may be efficacious against a broader spectrum of Hh pathway-dependent tumors. Since JK184 cannot compete with the binding of cyclopamine to Smo and has been reported to inhibit Adh7,[2] we sought to further investigate the mechanism by which it suppresses Hh signaling.

We first mapped the activity of JK184 with respect to known Hh signaling proteins. Our studies utilized an NIH-3T3 cell line that was stably transfected with Gli-dependent firefly luciferase and constitutive *Renilla* luciferase reporters (Shh-LIGHT2 cells[14]), which can be stimulated with Shh-conditioned medium or the Smo agonist SAG.[15,16] JK184 was able to block firefly luciferase expression induced by either reagent with a median inhibitory concentration (IC_{50}) of approximately 300 nM (Figure 2a). We next characterized the epistatic relationships between JK184, Su(fu), and the Gli proteins. Murine embryonic fibroblasts (MEFs) derived from mice lacking *Su(fu)* exhibit constitutive Hh pathway activation,[17] which can be measured with the Gli-dependent firefly luciferase reporter. We transfected *Su(fu)*$^{-/-}$ MEFs with the reporter constructs and cultured them in the presence of varying concentrations of JK184. As with the Shh- and SAG-treated Shh-LIGHT2 cells, the imidazopyridine derivative JK184 was able to block Hh target gene expression in this context with nanomolar potency (Figure 2b), thus demonstrating that JK184 acts downstream of Su(fu). Yet, when we activated Hh target gene expression in Shh-LIGHT2 cells by infecting them with retroviral constructs for the expression of FLAG-tagged Gli1 or Gli2, we observed that JK184 actually potentiated the transcriptional activities of these overexoressed factors. (Figure 2c).

These results suggest that the target of JK184 might promote and inhibit distinct aspects of Hh signal transduction. Hh pathway regulators with these attributes have been previously described, such as protein kinase A and casein kinase 1.[18–21] These kinases are required for both Smo activation upon Shh/Ptch1 binding and the proteolytic processing of Gli proteins to form transcriptional repressors. In addition, ciliary function is required for the formation of both Gli activators and repressors,[3,4,6] and the *Drosophila* Gli ortholog, Cubitus interruptus (Ci), is regulated in part by the microtubule cytoskeleton.[22] We therefore hypothesized that JK184 might affect microtubules in Hh-responsive mammalian cells, either throughout the cell or specifically within the primary cilia.

To ascertain the effects of JK184 on the microtubule cytoskeleton, we treated NIH-3T3 cell lines with the compound and then probed them with antibodies against α-tubulin and N-acetylated α-tubulin, which label microtubules throughout the cytoplasm and in the primary cilium, respectively. JK184 caused depolymerization of the cytoplasmic microtubule network, and although primary cilia were still visible, some exhibited abnormal morphologies (Figure 3a). The compound also induced nuclear fragmentation, which is consistent with disruption of microtubule-dependent chromosomal segregation. These phenotypes are similar to those observed with the microtubule-depolymerizing agent nocodazole (Figure 3a). We further established the ability of JK184 to inhibit microtubule assembly by testing its activity

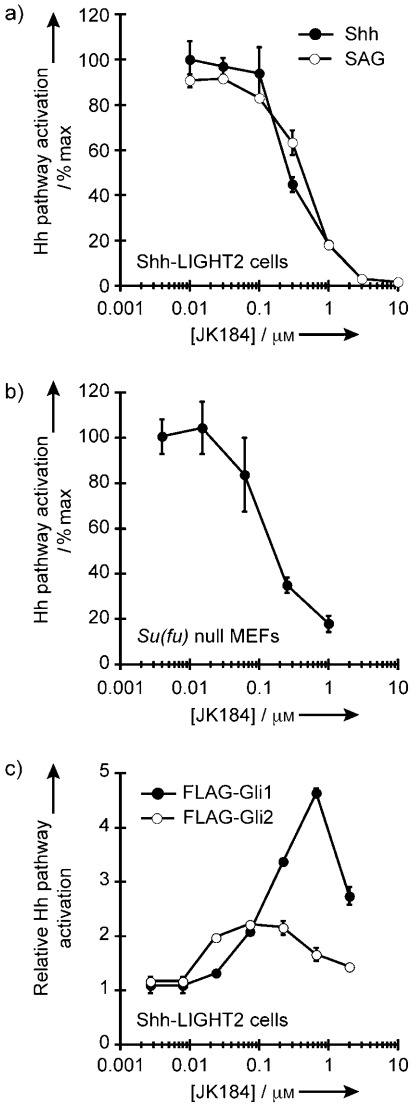

Figure 2. JK184 has both inhibitory and stimulatory effects on Hh pathway activity and acts at the level of the Gli transcription factors. a) JK184 inhibits Hh pathway activation in Shh-LIGHT2 cells stimulated with either Shh-conditioned media or the Smo agonist SAG. b) JK184 inhibits constitutive pathway activation in *Su(fu)*$^{-/-}$ MEFs transfected with a Gli-dependent firefly luciferase reporter. c) JK184 potentiates Hh pathway activity in Shh-LIGHT2 cells infected with retroviral constructs expressing either FLAG-Gli1 or FLAG-Gli2. Data represent the mean ± standard deviation of at least triplicate samples.

on purified components in vitro. Microtubule polymerization can be initiated by the addition of guanosine triphosphate (GTP), thereby producing filaments that can be separated from tubulin monomers by ultracentrifugation.[23] Consistent with our immunocytochemistry analyses, we observed that JK184 increases the amount of soluble tubulin in the presence of GTP with a potency consistent with its IC_{50} in Shh signaling assays (Figure 3b). These findings confirm that JK184 directly targets tubulin subunits to prevent their polymerization into filaments.

To demonstrate that microtubule disassembly can account for the Hh pathway phenotypes of JK184, we next inves-

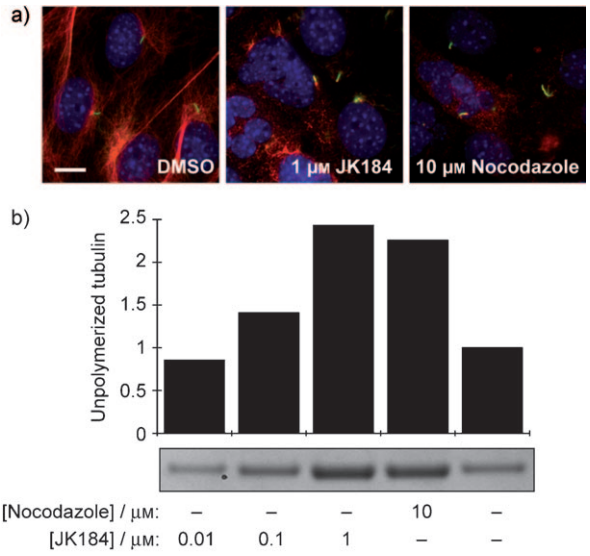

Figure 3. JK184 is a microtubule-depolymerizing agent. a) Cells cultured in the presence of JK184 exhibit depolymerized microtubules (red) and fragmented nuclei (blue), as visualized by anti-α-tubulin and DAPI staining, respectively. Primary cilia (green) are still present, as indicated by N-acetylated a-tubulin staining, although some appear to have structural defects. These cytoskeletal perturbations are similar to those observed with nocodazole, a known microtubule-disrupting agent. Scale bar: 10 μm. b) JK184 also inhibits in vitro GTP-dependent tubulin polymerization in a dose-dependent manner. Soluble tubulin levels after GTP-initiated polymerization are shown by Coomassie staining and quantified.

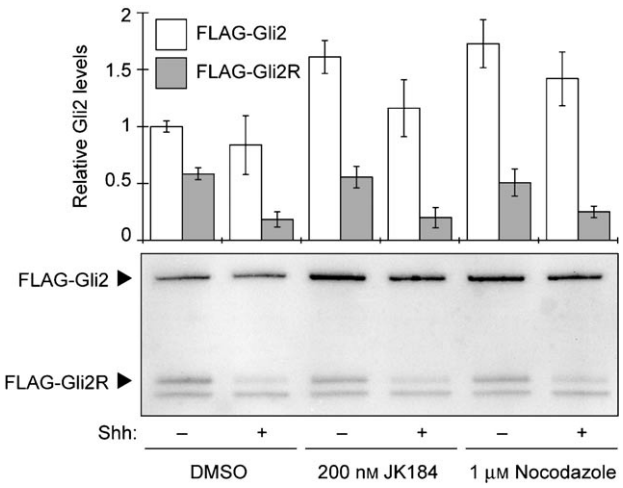

Figure 4. JK184 and nocodazole increase cellular levels of full-length Gli2 but do not significantly alter Shh-regulated Gli2 repressor (Gli2R) formation, as determined with a clonal NIH-3T3 cell line that stably expresses FLAG-tagged Gli2. A representative western blot depicting FLAG-Gli2 and FLAG-Gli2R levels is shown, as well as quantitative data representing the mean of three independent experiments ± standard deviation.

tigated the effects of nocodazole on Hh target gene expression in various cellular contexts. This antimitotic agent inhibited Shh- and SAG-induced firefly luciferase expression in Shh-LIGHT2 cells, decreased the constitutive Gli reporter activity in *Su(fu)*$^{-/-}$ MEFs, and potentiated exogenous Gli1 and Gli2 activity in Shh-LIGHT2 cells (Figure S1 in the Supporting Information). Colchicine, a structurally unrelated microtubule-depolymerizing agent that acts through a distinct mechanism, had similar effects in these assays (Figure S2 in the Supporting Information). Thus, it is likely that JK184 exerts its effects on the Hh pathway by destabilizing microtubules, and its inhibition of Adh7 is an ancillary activity.

Having established that JK184 is a microtubule-destabilizing agent, we sought to determine how microtubule depolymerization might perturb Hh pathway activation. Our epistatic analyses indicated that JK184, nocodazole, and colchicine act downstream of Su(fu), thus suggesting that they perturb Hh target gene expression at the level of the Gli transcription factors. To investigate the mechanisms by which JK184 and nocodazole might alter Gli activity, we evaluated their effects on cellular levels of full-length Gli2 and Gli2 repressor (Gli2R). Since antibodies that recognize endogenous Gli2 are not yet available, we established a clonal NIH-3T3 cell line that stably expresses FLAG-tagged Gli2. FLAG-Gli2R is constitutively formed in these cells, and Shh treatment inhibits this proteolytic processing step (Figure 4). In comparison to a DMSO control, both JK184 and nocodazole increased FLAG-Gli2 levels under basal and Shh-stimulated conditions. In contrast, neither compound significantly altered FLAG-Gli2R formation. FLAG-Gli2 levels also consistently decreased upon Shh stimulation even though FLAG-Gli2R formation was suppressed, thus suggesting that Hh pathway activation may promote the degradation of full-length Gli2.

The maintenance of Shh-dependent Gli processing and accumulation of full-length Gli2 in cells treated with JK184 or nocodazole is perhaps counterintuitive, since Hh target gene expression is dictated by the cellular balance of Gli activators and repressors. This apparent paradox can be explained by functional differences between full-length Gli2 and its activator form, which cannot be resolved by gel electrophoresis. In this model, JK184 and nocodazole would disrupt microtubule-dependent processes that are required for the conversion of endogenous full-length Gli proteins into transcriptional activators but for not Gli processing, thereby causing accumulation of full-length, non-activated Gli2. Consistent with this idea, certain ciliary defects are associated with a loss of Gli activator function but maintenance of Hh ligand-sensitive Gli processing,[24] and primary cilia in cells treated with JK184 exhibit some structural abnormalities. Overexpressed transcription factors could at least partially bypass this activation step, thus explaining the inability of JK184, nocodazole, or colchicine to block Hh target gene expression mediated by exogenous Gli1 or Gli2. The potentiation of overexpressed Gli1 and Gli2 by microtubule depolymerizing agents further indicates that Gli activator function is restricted by other microtubule-dependent mechanisms. We postulate that the degradation of Gli activators is also microtubule-dependent.

Taken together, our observations demonstrate that JK184 modulates Hh pathway activity by depolymerizing microtubules and that microtubule-dependent signaling events both positively and negatively regulate Gli function. Our findings raise questions about the potential of JK184 as a targeted

antitumor agent, since this compound will generally block mitosis rather than selectively inhibit Hh pathway-dependent cell proliferation. There may even be physiological conditions in which JK184 potentiates Hh target gene expression. Nevertheless, the ability of JK184 to potently, rapidly, and reversibly perturb microtubule-dependent mechanisms could make it a valuable probe for studying how cells control Hh pathway activity during ontogeny and oncogenesis.

Received: November 20, 2008
Revised: January 24, 2009
Published online: February 16, 2009

Keywords: bioorganic chemistry · hedgehog pathway · inhibitors · microtubules · signal transduction

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