

Targeting Hedgehog — a Cancer Stem Cell Pathway

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Abstract

The Hedgehog (Hh) pathway has been implicated in a wide variety of human tumors, and early clinical trials with pathway antagonists have validated Hh signaling as a *bona fide* anticancer target. Despite these encouraging results, several issues surrounding the basic biology of the Hh pathway in human cancers remain unclear. These include the influence of specific oncogenic events on Hh signal transduction, the precise mode of Hh signaling (i.e., autocrine or paracrine) that occurs within human tumors, and the best means to inhibit aberrant pathway activity in the clinical setting. The cancer stem cell (CSC) hypothesis may explain a number of clinical phenomena, such as unchecked self-renewal and the development of metastatic disease, and to some extent, the Hh signaling pathway has been implicated in all of these processes. Therefore, Hh pathway inhibitors may also represent some of the first agents to formally examine the CSC hypothesis in the clinical setting. The diverse nature of Hh signaling in human cancers suggests that disease-specific factors must be carefully considered to identify the optimal use of novel pathway inhibitors. *Clin Cancer Res*; 16(12); 3130–40. ©2010 AACR.

Development and Hedgehog Pathway Signal Transduction

The Hedgehog (Hh) signaling pathway was initially identified in *Drosophila* as a critical mediator of segmental patterning during embryonic development, and it regulates the proliferation, migration, and differentiation of target cells in a spatial, temporal, and concentration dependent manner (1–3). Hh signaling is conserved in vertebrates and highly active during mammalian development, especially within the neural tube and skeleton, but subsequently silenced in most adult tissues. However, some postnatal organs, such as the central nervous system and the lung, rely on continued Hh signaling for tissue homeostasis and repair following injury (4–6).

Pathway activation is initiated by binding of one of the three secreted and lipid-modified ligands found in mammals, Sonic (SHh), Desert (DHH), and Indian Hedgehog

(IHh), to Patched (Ptch1), a 12-pass transmembrane-spanning receptor (Fig. 1A, B). In the absence of ligand, Ptch constitutively represses the activity of Smoothened (Smo), a 7-pass transmembrane-spanning protein with homology to G-protein-coupled receptors. Following Hh ligand binding to Ptch, the repression of Smo is released, and the expression and/or post-translational processing of the three Gli zinc-finger transcription factors is modulated. Gli1 acts as a transcriptional activator and Gli3 as a repressor, whereas Gli2 can either activate or repress gene expression depending on post-transcriptional and post-translational modifications (7). The balance between the activating and repressive forms of the Glis results in the expression of target genes, including *Ptch1* and *Gli1* (8, 9).

Within this simplified schema, several other cellular components are required for Hh pathway activity. These include proteins involved in Hh ligand modification and cell surface binding [Hedgehog interacting protein (Hip), Hedgehog acyltransferase (Hhat), growth arrest-specific 1 (Gas1), and CDO], and Gli processing and localization [Suppressor of fused (SuFu), Protein kinase A (PKA), glycogen synthase kinase 3 β (GSK3 β), casein kinase 1 (CK1), G-protein-coupled receptor kinase 2 (GRK2), and β TRCP; refs. 10–14]. Moreover, increasing evidence has suggested that the subcellular localization of Hh pathway components is a major regulator of its activity. The examination of developmental defects arising in mice showed that mutations within the intraflagellar transport proteins Kif3a and IFT88 produce patterning defects that mimic Hh loss-of-function mutations (15). These proteins are

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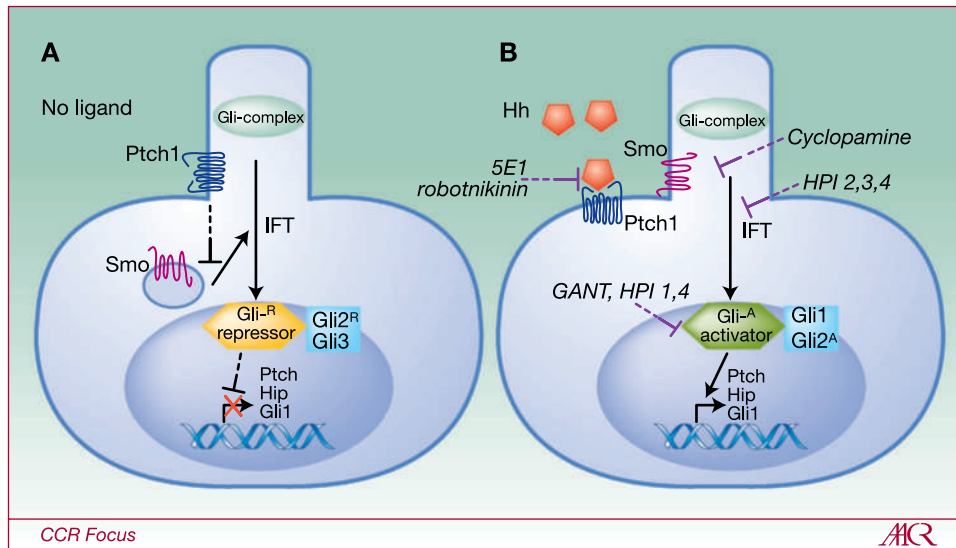


Fig. 1. Hh signaling. A schematic of Hh pathway signal transduction derived from developmental and cancer models. A, in the absence of Hh ligand, Ptch is located in the cilium and blocks Smo entry. Gli transcription factors exist in repressor forms that prevent transcription of target genes. B, three mammalian homologs of Hh (SHh, IHh, DHh) bind Ptch at the cell surface and allow it to move out of the primary cilium. Smo is derepressed and moves into the primary cilium where it can activate Gli transcription factors. During this process, the Gli transcription factors are processed to activator forms and translocated to the nucleus to induce the transcription of Hh target genes. Antibodies against the Hh ligands (5E1) and robotnikinin block pathway activation by preventing the interaction of Hh ligand with Ptch. Cyclopamine and novel antagonists of Smo directly bind and inhibit its function. Compounds such as HPI 2,3,4 block the transport of components in the signaling cascade. Direct Gli antagonists, such as GANT, block binding of Gli transcription factors to DNA.

required for the assembly and maintenance of primary cilia that are present on most cells of the body during interphase and involved in a wide variety of cellular processes including mechanosensation and the transduction of several signaling pathways. A number of studies have subsequently shown that pathway components translocate during activation, and in the absence of ligand, Ptch, but not Smo, is located within the primary cilia (16–20). Upon ligand binding, Ptch moves out and Smo moves into primary cilia to interact with Glis and their associated proteins that subsequently enter the nucleus to regulate gene expression (Fig. 1B).

Studies from a variety of experimental systems have identified the major components involved in Hh signal transduction, but extension of these results to human cancers should be approached with caution for several reasons. Many genetic studies have determined the role of specific pathway components by examining the effects of mutations on normal developmental programs, but the precise similarities between development and carcinogenesis are not fully understood. Meanwhile, detailed biochemical and cell biology studies have typically studied immortalized and nonmalignant embryonic fibroblasts (e.g., NIH 3T3 or CH310T1/2 cells) that exhibit robust pathway activity compared with most cancers. Additionally, these cells are mesenchymal in origin and can differentiate into mature cartilage, bone, and adipose cells. Thus, their relevance to Hh signaling within cancers derived from epithelial or hematopoietic tissues is unclear. Both

genetic and epigenetic events underlie the formation and progression of human cancers, but the influence of these factors on Hh pathway signaling is largely unknown either in general or within specific tumors. Activating mutations in *KRAS* are found in a wide variety of epithelial carcinomas and may cause Gli1 activation and Hh target gene expression in a Smo-independent manner (21, 22), therefore, oncogenic events may directly induce pathway activity. The Hh signaling pathway has also been found to interact with other signaling pathways commonly activated in human cancers, such as PI3K/AKT (23, 24), and it is possible those also alter classical Hh signal transduction. It is also unclear whether primary cilia are absolutely required for Hh signaling in human cancers because they are absent in *Drosophila* cells that are fully capable of pathway activation and may or may not be found at a high frequency within primary human basal cell carcinomas (BCC; ref. 25). Moreover, recent studies examining the role of primary cilia and aberrant Hh signaling in the formation of BCC and medulloblastoma have shown that mutations within Smo are not tumorigenic in the absence of cilia (25, 26). However, a constitutively activated form of Gli2 paradoxically induced tumor formation when cilia formation was blocked. Therefore, Hh signaling seems to be complex in cancers, and although several aspects of Hh signal transduction remain unclear, a better understanding of the mechanisms mediating pathogenic pathway activity within human cancer cells may identify additional therapeutic strategies.

Table 1. Cancers associated with Hedgehog signaling

Tumor Type	Mutation-Ligand Mediated (Paracrine-autocrine)	Stroma Involved in Signaling?	Required for CSC?	Type of Data	Ref.
Multiple myeloma	Ligand-autocrine Paracrine (stroma tumor)	Stroma to tumor	Yes	P	52, 61
Medulloblastoma	Mutation Ligand	NR	NR	MM P CT	26, 42, 82, 83, 96, 99
Upper GI	Ligand	NR	NR	P	36
Pancreatic	Ligand-autocrine Epithelial cell Intrinsic Paracrine (to stroma)	Data support intrinsic requirement and paracrine signaling to signaling to stroma	Yes (60)	MM, P	22, 53, 60, 97, 100–103
Ewings Sarcoma	Non Smo, direct activation Gli1	NR	NR	P	86, 87
Melanoma	Nonligand, nonmutation	NR	NR	MM P	24, 104
Glioma	Ligand-paracrine (exogenous to tumor)	Possibly	Yes	P	39, 40, 105
Breast	Ligand, both autocrine and paracrine	Possibly	Yes	MM P	59, 106
Ovarian	Ligand	NR	NR	P	107, 108
Prostate	Ligand-autocrine	NR	Yes	MM P	38, 109
Small cell lung	Ligand	NR	No	MM P	5
Lymphoma	Ligand-autocrine and paracrine	Stroma signals to tumor	NR	P	52, 110, 111
Leukemia/CML	Upregulation of Smo. No mutation Ligand-NR	No	Yes	MM P	56–58
BCC	Mutations in Ptch and Smo Ligand driven mouse model	No	NR	MM, P, CT	25, 29, 30, 80

NOTE: A summary of selected references describing aberrant Hh signaling by tumor type, the mode of Hh signaling [mutational, ligand-mediated (paracrine or autocrine), involvement of stroma, and activity in CSC], and the experimental systems examined [mouse models, preclinical (includes human cancer cells lines, xenografts, primary patient samples) and clinical trials].

Abbreviations: NR, not reported; MM, mouse model; P, preclinical; CT, clinical trial.

Hedgehog Signaling in Cancer

The identification of somatic *PTCH1* mutations in patients with Gorlin syndrome and basal cell nevus syndrome first implicated a role for the Hh pathway in cancer as these individuals are highly predisposed to developing advanced BCC, medulloblastoma, and rhabdomyosarcoma (27, 28). Additional evidence that aberrant Hh pathway activity may play a causal role in human cancers was provided by the identification of *PTCH1* and *SMO* mutations in sporadic BCCs and medulloblastomas (29–31). Definitive proof that aberrant Hh signaling can induce cancers has come from transgenic mouse studies in which conditional loss of function of *Ptch* or gain of function of *Smo* or the *Glis* can recapitulate medulloblastoma and BCC (reviewed in ref. 32). Other Hh pathway components may also be genetically altered in human cancers including *SUFU* mutations in medulloblastoma, *GLI1* and *GLI3* mutations in pancreatic adenocarcinoma, as well as *GLI1* gene amplification in glioblastoma (33–35).

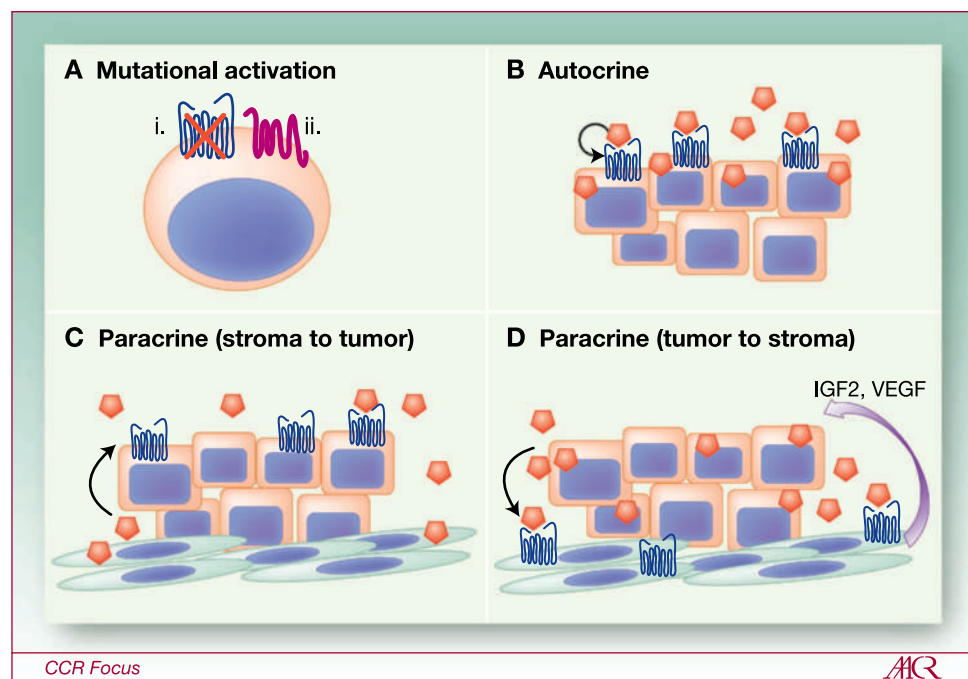
In contrast to tumors harboring activating mutations, several human cancers display aberrant pathway activity in response to increased levels of HH ligand (Table 1). Within these ligand-dependent tumors, several modes of pathway activation have been described (Fig. 2). The simplest is autocrine and/or juxtacrine signaling in which tumor cells both produce and respond to HH ligand (Fig. 2B). This mode of pathogenic HH activity has been described in a wide variety of human tumors including small cell lung, pancreatic, colon, and metastatic prostate cancers as well as glioblastomas and melanomas (5, 24, 36–40). Increased HH pathway activity either from mutational activation or

autocrine signaling may induce the expression of genes affecting proliferation (*Cyclin D1*, *Cyclin D2*, *N-Myc*, *Igf2*, *Hes1*), cell survival (*Bcl2*), angiogenesis (*Vegf*), and genetic instability (*p53*; refs. 41–51). Moreover, HH ligand blocking antibodies (in the case of ligand-dependent signaling), pharmacologic SMO antagonists, or small interfering RNA (siRNA) directed against *SMO* or *GLI1* have been shown to inhibit tumor growth.

Hh signaling may act in a paracrine fashion during embryonic development in which cells secreting Hh ligands are distinct from those responding with pathway activation, and several recent studies have shown that pathologic Hh signaling may also occur in a similar manner that involves the tumor microenvironment (52–54). In a recent report examining B-cell lymphomas and multiple myeloma, HH ligand was found to be primarily produced by stromal cells derived from the bone marrow, spleen, or lymph nodes, rather than tumor cells (Fig. 2C; ref. 52). However, the activation of HH signaling within tumor cells was found to act as an important survival factor because treatment with SMO antagonists induced cell death and decreased *BCL2* expression *in vitro* as well as inhibited tumor growth *in vivo*. This effect was specifically mediated by HH signaling as the expression of *GLI1* or a constitutively activated form of *SMO* abrogated these effects.

An alternative mode of paracrine Hh signaling has been recently described in which tumor cells secrete HH ligands that induce pathway activity within stromal cells (Fig. 2D; refs. 53–55). In a study examining human pancreatic and colon primary tumors and cell lines grown as xenografts in mice, the expression of HH ligand by tumor cells did not correlate with their expression of target genes, but rather was associated with canonical pathway activity by tumor

Fig. 2. Models of Hh signaling in cancer. A, non—ligand-mediated signaling (mutational activation). Mutations in *PTCH1*, *SMO*, *SUFU*, or amplification of *GLI1* have all been reported in human cancers. (i) Loss of *PTCH1* activity may increase Hh pathway activity. (ii) Overexpression or activating mutations in *SMO* are also depicted. B, ligand-mediated autocrine signaling. Tumor cells produce Hh ligand that stimulates Hh pathway activity in tumor cells. C, ligand-mediated paracrine signaling. Nonmalignant stromal cells produces Hh ligand required by tumor cells for growth and survival. D, ligand-mediated paracrine signaling. Tumor cells produce Hh ligand that activates Hh signaling in nonmalignant stromal and endothelial cells; this results in the production of unknown factors within the microenvironment that ultimately support tumor cell growth and survival as well as angiogenesis.



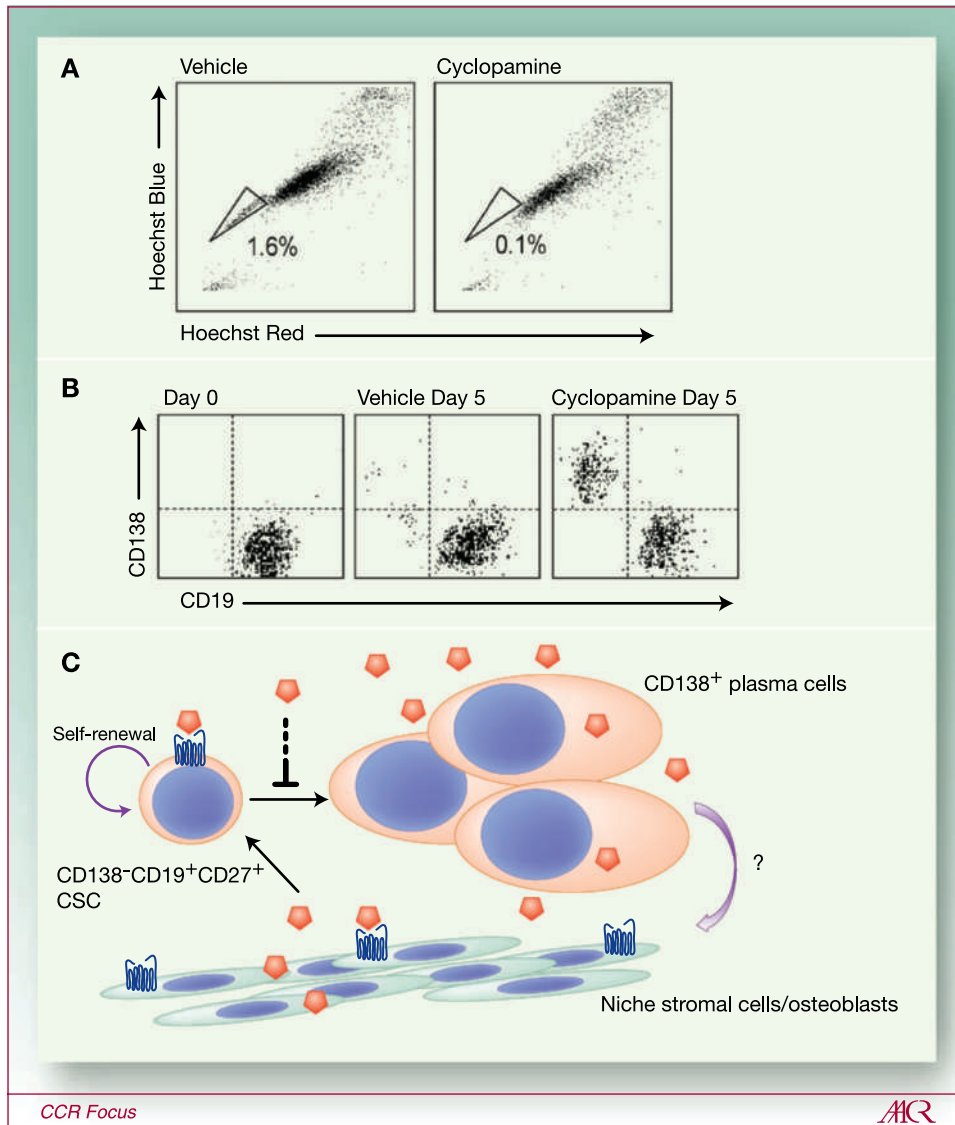


Fig. 3. Hh signaling in multiple myeloma. Regulation of multiple myeloma CSC by the HH signaling pathway. A, the inhibition of HH signaling inhibits multiple myeloma CSC displaying the side population phenotype by, B, inducing terminal plasma cells differentiation of multiple myeloma CSC as indicated by the expression of CD138. C, multiple modes of signaling seem to be active in multiple myeloma. Experimental data suggest that differentiated plasma cells can produce the ligand necessary for CSC survival and proliferation. Blocking signaling leads to CSC differentiation. Normal bone marrow stromal cells can also produce ligand and signal to myeloma cells to support their growth and survival. A possible role for tumor-to-stroma paracrine signaling may also take occur. A and B are reproduced with permission from Peacock et al. (61).

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infiltrating stromal cells derived from the murine host (53). Decreased tumor growth was observed following administration of a pharmacologic SMO antagonist or HH-neutralizing monoclonal antibody and correlated with decreased expression of mouse *Gli1* expression, whereas human *GLI1* expression remained unchanged. Further studies using a transgenic mouse model of pancreatic cancer similarly failed to identify canonical Hh pathway activity within epithelial tumor cells despite expression of a constitutively activated form of *Smo* in these cells (54). These results strongly suggest that tumor derived HH ligand primarily induces pathway activity within infiltrating stromal cells that in turn influences tumor growth. Although the precise factors generated by stromal cells that modulate tumor growth are unknown, HH pathway activation may induce the secretion of soluble factors, such as insulin growth factor 2 (IGF2) or vascular endothelial

growth factor (VEGF), that influence tumor cell proliferation and survival (43, 48, 49).

Conflicting data about the precise mode of Hh signaling in specific tumors, such as pancreatic and colon carcinoma (see below), may be due to differences in experimental systems. Given the spatial restriction of Hh signaling during normal development, the study of human tumors may require three-dimensional models to precisely determine the role HH plays in cancer biology and the antitumor effects of pathway inhibitors. It is also likely that the exact role of Hh signaling will be dependent on the specific tumor type as well as distinct clinical and biological factors, such as disease stage or genetic lesions. For example, intact Hh signaling, specifically *Smo* expression, is required for myeloid leukemia arising from the *BCR-ABL*, but not the *MLL-AF9*, fusion gene (56–58). Although delineating disease specific information will likely require extensive

studies, it is likely required to optimize the clinical use of Hh pathway inhibitors.

Hedgehog Signaling in Cancer Stem Cells

Emerging data from many human tumors including glioblastoma, breast cancer, pancreatic adenocarcinoma, multiple myeloma, and chronic myeloid leukemia (CML) have suggested that Hh signaling regulates cancer stem cells (CSC; refs. 39, 40, 56, 59–62). CSCs have been functionally defined by their capacity to undergo self-renewal and give rise to differentiated progeny that recapitulates the original tumor in an ectopic setting (63). Self-renewal is required for maintenance of the malignant clone, and reports examining mouse models of CML have provided evidence that Hh signaling regulates this property (56, 62). In these studies, the loss of Hh signaling by genetically disrupting *Smo* resulted in the inhibition of BCR-ABL expressing leukemic stem cells and prolonged survival. Active Hh signaling pathway has also been identified in glioblastoma CSCs, and pathway inhibition with cyclopamine or siRNA directed against pathway components results in the loss of tumorigenic potential (39, 40). In breast cancer, pathway activation in CSCs using Hh ligand and *GLI1* or *GLI2* expression or inhibition with cyclopamine or siRNA directed against *GLI1* or *GLI2* alters the expression of *BMI-1*, a central regulator of self-renewal in normal stem cells, and tumorigenic potential *in vitro* and *in vivo* (59). In multiple myeloma, CSCs that phenotypically resemble normal memory B cells have been found to display relatively higher levels of Hh signaling than the mature plasma cells constituting the tumor bulk (61). Pathway activation with SHH ligand resulted

in CSC self-renewal and expansion, whereas the SMO antagonist cyclopamine or the ligand-neutralizing antibody 5E1 induced terminal differentiation and loss of clonogenic growth potential (Fig. 3A and B). Thus, HH signaling may dictate CSC fate decisions that include self-renewal and differentiation possibly by generation of a malignant niche (64). Data from multiple myeloma demonstrate that Hh signaling can act through multiple signaling modes within the same cancer and can mediate interactions between CSCs, differentiated tumor cells and the microenvironment (Fig. 3C).

In addition to tumor formation, CSCs have been implicated in disease progression and the development of metastasis in solid tumors (Fig. 4; refs. 65, 66), and Hh signaling may play a critical role in this process similar to the Notch and Wnt pathways in cancer (67, 68). In colon carcinomas derived from primary clinical specimens, Hh signaling has been found to be preferentially activated within CSCs as evidenced by relatively higher expression of *GLI1*, *GLI2*, *PTCH1*, and *HIP* within this cellular compartment (37). Moreover, the relative expression of these pathway components as well as the target gene *SNAIL1*, which is associated with the epithelial-to-mesenchymal transition (EMT) and implicated in metastasis, increases in CSCs with disease stage. Inhibition of Hh pathway activity with cyclopamine or siRNA against *SMO*, *GLI1*, and *GLI2*, or expression of the repressive form of *GLI3* reduced tumor cell proliferation and induced apoptosis. Moreover, cyclopamine reduced tumor regrowth *in vivo* and small hairpin RNA (shRNA) directed against *SMO* eliminated the formation of metastatic disease. The relationship between EMT and clonogenic growth potential has also been examined in pancreatic CSCs, and cyclopamine

Fig. 4. Hh signaling induces EMT and metastasis formation. Cells undergoing EMT under the influence of Hh signaling and become more motile and invasive as they acquire mesenchymal cell properties. This allows cells to escape from the primary tumor and circulate to distant sites. Once established at a distant site, Hh may be required for the clonogenic growth and self-renewal.

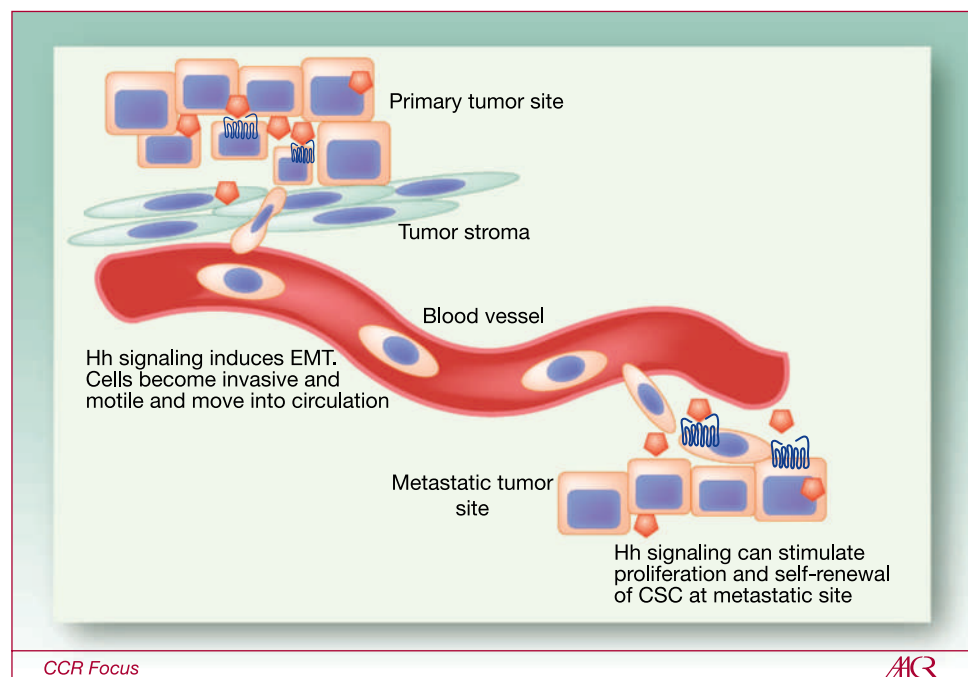


Table 2. Novel Hedgehog pathway antagonists in clinical testing

Compound	Company	Disease (Phase)	Reference
GDC-0449	Genentech/Curis	BCC (II), pancreatic cancer (I), SCLC (II), medulloblastoma (II), glioblastoma (II), colorectal cancer (II), ovarian cancer (II), breast cancer, gastric cancer (II), gastroesophageal cancer (II)	74, 80, 112
IPI-926	Infinity Pharmaceuticals	Solid tumors (I)	79, 97, 112
PF-04449913	Pfizer	Myeloid malignancies (I/II)	112
XL139/BMS833923	Exelixis/Bristol-Myers Squibb	Solid tumors (I), multiple myeloma (I/II), SCLC (I), gastric cancer (I), esophageal cancer (I)	83, 112
LDE225	Novartis	Solid tumor (I), medulloblastoma (I), BCC (I)	112

NOTE: A summary of Hh pathway antagonists currently undergoing clinical testing in humans. For each compound the company involved in development, the tumor types being tested (and the phase of testing), and associated references are listed. Abbreviation: SCLC, small cell lung cancer.

has been found to inhibit each of these functional properties and the formation of metastatic disease (60).

Clinically Targeting Aberrant Hedgehog Signaling

Initial evidence that Hh signaling could be pharmacologically inhibited arose from the identification of cyclopamine, a steroidal alkyloid derived from *Veratrum californicum*, as an active compound capable of producing congenital defects in sheep (69). Recognition that *SHh* loss-of-function mutations recapitulate the neural defects seen with *Veratrum* teratogenicity led to findings that cyclopamine inhibits pathway activity by directly binding and inactivating Smo (70–72). Several pathway antagonists have been subsequently identified in compound screens that inhibit Gli-dependent reporter activity (73–77). Despite the unbiased nature of these screens against any one component of the Hh signaling pathway, the vast majority of compounds have been found to bind to and inhibit Smo despite being structurally distinct from one another and cyclopamine (reviewed in ref. 78). It is possible that these whole cell screens have primarily identified SMO antagonists because of its location on the cell surface, the central role it plays in Hh signal transduction, and the relative ease with which inhibitors of other 7-pass transmembrane proteins have been identified. An alternative approach to identifying novel Hh inhibitors has involved chemically modifying cyclopamine to improve its specificity, bioavailability, and pharmacokinetics (79).

The first clinical data reported using a SMO antagonist have involved GDC-0449 that was originally identified in a chemical compound screen and further modified and

developed through a collaborative agreement by Curis and Genentech (74). The initial phase I trial examining the safety and tolerability of GDC-0449 enrolled a total of 68 solid tumor patients (80). Early data suggested efficacy in patients with advanced BCC, presumably because of the high frequency of aberrant Hh signaling, and the trial was expanded to further examine this group of patients. Of a total of 33 patients with locally advanced or metastatic BCC, 55% showed clinical responses that included two complete remissions. Although efficacy data have not yet been reported from the other 35 patients without BCC enrolled in the trial, a published abstract indicated that a single patient with adenocystic carcinoma experienced stable disease (81). Reported toxicities were mild, and the most common events were mild loss of taste, hair loss, weight loss, and hyponatremia. Importantly, biopsies of noninvolved skin showed *in vivo* inhibition of HH signaling evidenced by downregulation of *GLI1* expression following drug administration. A transient subjective tumor response was also reported in a single medulloblastoma patient treated with GDC-0449 under compassionate use (82). Early results from another SMO antagonist, BMS-833923 (XL139), codeveloped by Exelixis and Bristol-Myers Squibb have been recently reported and shown clinical benefit in patients with medulloblastoma and basal cell nevus syndrome (83). On the basis of the safety and efficacy data generated in the early phase clinical trial, multiple phase II trials examining GDC-0449 in combination with standard agents in solid tumors have been initiated (Table 2). Moreover, early phase clinical testing of alternative SMO antagonists have been undertaken by several other companies including Exelixis/Bristol-Myers Squibb, Novartis, Infinity, and Pfizer (Table 2). Although all these agents target SMO,

they are chemically distinct and no comparative studies have been carried out. It is possible that differences in SMO binding may lead to efficacy against some mutations that may arise during treatment (82), or that differences in pharmacokinetic properties may influence tissue distribution that provides more favorable inhibition of specific tumors, such as those in the central nervous system, or attenuation of side effects.

These early results provide critical proof-of-concept that inhibiting HH pathway activity has antitumor activity in humans, but they also generate several questions. For example, the majority of BCC tumor specimens from patients treated with GDC-0449 displayed increased levels of *GLI1* expression consistent with the nature of the disease, but slightly more than half of the patients experienced clinical responses despite circulating levels of drug that were well above the concentration required to inhibit Hh signaling *in vitro* (74, 80). Additionally, the examination of pretreatment specimens for the presence of primary cilia or HH pathway activity within tumor stromal cells may have correlated with responses and validated the importance of these factors in the clinical setting. Similarly, serial tumor biopsies were not required, but it would have been interesting to correlate the degree of intratumoral pathway inhibition with clinical responses to draw a conclusive relationship between pathway activity and human cancers. Finally, the rapid loss of response and finding of an acquired mutation in SMO in the medulloblastoma patient treated with GDC-0449 suggest that loss of response in BCC patients may have occurred in a similar manner (82, 84), and isolation of tumor tissue at disease progression could have explored this possibility.

Alternative Strategies to Inhibit Hedgehog Signaling

Initial results with SMO antagonists are encouraging, but the development of alternative means to inhibit Hh signaling may be desirable for several reasons. First, several genetic lesions acting downstream of SMO have been detected in human cancers, such as mutations or loss of heterozygosity of the negative pathway regulators *SUFU* or *REN^{KCTD11}* or amplifications in *GLIs* (33, 35, 85). Moreover, *GLI1* activity may be induced in a SMO-independent manner by transforming events, such as EWS-FLI in Ewing sarcoma or mutant KRAS in pancreatic cancer (21, 22, 86, 87). Because Hh signal transduction ultimately activates the *GLI* transcription factors, inhibitors modulating *GLI* may be useful, similar to therapeutic strategies being developed to target Wnt/ β -catenin signaling in cancer (68). A screen for compounds capable of inhibiting *GLI*-mediated transcription identified *Gli*-antagonist (GANT)-58 and GANT-61 (88). Both of these compounds act downstream of Smo and SuFu and inhibit tumor growth both *in vitro* and *in vivo*. The precise inhibitory mechanism of these compounds is unclear, but GANT-61 limits *GLI1* DNA binding. A screen designed to identify molecules

capable of inhibiting *Gli1* transcriptional activity without targeting Smo identified four distinct Hh pathway inhibitors (HPI; ref. 89). Each of these compounds inhibits *Gli* at a level epistatic to SuFu, but their precise mechanisms are unknown. HPI-1 inhibits both *Gli1* and *Gli2*, perhaps by altering their post-translational modification, HPI-4 seems to partially inhibit ciliogenesis, and HPI-2 and 3 may inhibit the conversion of *Gli2* from a repressive to an activated form. Although less specific, *GLI* activity may also be inhibited by modulating the activity of other pathways known to interact with Hh signaling, such as phosphoinositide 3-kinase (PI3K)/AKT, mitogen-activated protein kinase (MAPK), and transforming growth factor β (TGF- β ; refs. 23, 24, 90–92).

Mutations in Hh pathway components have not been described in most cancers displaying aberrant signaling. Therefore, the inhibition of ligand binding may inhibit pathway activation, and the ligand neutralizing monoclonal antibody 5E1 has been shown to have clear antitumor activity in several diseases (see above). More recently, robotnikinin, was identified as a compound that binds to SHh and blocks its ability induce pathway activity at the level of Ptch (93). It is also possible that specific on-target side effects attributable to the inhibition of Hh pathway activity in some cells, such as defects in bone growth seen in young mice treated with a Smo antagonist (94), may be avoided by blocking the activity of a specific Hh ligand.

Clinical Translation of Hedgehog Inhibitors

It is likely that the effective clinical use of Hh inhibitors will require a precise understanding of the role that the pathway plays in a specific tumor. In cancers with mutational pathway activation affecting all tumor cells, pathway inhibition may impact the growth and survival of all tumor cells evident as reduction in disease burden as observed in BCC patients receiving GDC-0449 (80). Alternatively, Hh may be required early in carcinogenesis or in the formation of precancerous lesions, but once tumors are established, it may not be needed for tumor maintenance or progression. In these cases, Hh inhibitors may be active chemo-preventative agents in high-risk patients, but would be ineffective in treating established disease. Alternatively, the Hh pathway may be activated in response to chemotherapy or during regrowth of minimal residual disease, perhaps by regulating CSCs (95). In this case Hh inhibitors would be most effective as maintenance therapy following tumor debulking with surgery or cytotoxic chemotherapy. Hh signaling has also been implicated in mediating chemoresistance (96, 97), therefore, inhibitors may act as potent chemo-sensitizers and require the simultaneous administration of cytotoxic chemotherapy.

Given the diverse biological effects of Hh activity, accurately measuring the activity of Hh inhibitors may also require context-dependent indicators of clinical response. The Hh pathway may regulate EMT and the formation of metastatic disease (37, 38, 60). In this case,

treatment with Hh inhibitors may not result in clinically significant inhibition of the primary tumor, but could prevent the formation of new metastasis and prolong progression free survival. Similarly, if Hh signaling is primarily active in CSCs, clinical responses to Hh inhibitors may be delayed in patients with extensive disease. Effective inhibition of CSCs should lead to improved overall survival, but most early phase trials are not designed or powered to detect differences in long-term outcomes (98). Therefore, reliable biomarkers that accurately quantify CSCs may be useful endpoints within early clinical testing. Finally, patient selection is likely to be a critical factor in establishing the clinical efficacy of Hh pathway antagonists similar to most targeted therapies. Because mutations seem to drive only a minority of Hh-related cancers, selection of patients will not be possible by simply genotyping tumor specimens, but better understanding of the specific role the Hh signaling pathway plays in each tumor type is likely to be valuable during the development of these novel agents. It is clear that several issues in the precise role of the Hh signaling pathway in human cancer remain unresolved, including the precise mechanisms of signal transduction, the exact mode of signaling between tumor cells and the microenvironment, and the role of signaling in the regulation of CSCs.

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Disclosure of Potential Conflicts of Interest

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