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Perspective

Hedgehog-Gli Signaling Pathway Inhibitors as Anticancer Agents

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Introduction

Cancer drug discovery has undergone a paradigm change over the past few years, from predominantly cytotoxic agent-based therapy to therapy aimed at genetic and molecular targets, thanks to a growing understanding of the genes and pathways responsible for cancer initiation and progression and to new drug discovery technologies. The success of drugs like trastuzumab, imatinib, gefitinib, and erlotinib has demonstrated that the targeting of specific oncogenic signal transduction pathways can be clinically useful.[1] One such pathway, the Hedgehog-glioma-associated oncogene homologue zinc finger protein (Hh-Gli[a]) signaling pathway, has attracted drug discovery scientists for the past decade. Hh-Gli signaling plays an important role in the embryonic patterning and development of many tissues and somatic structures as well as maintaining and repairing mature tissues in adults.[2−4] Uncontrolled activation of the Hh-Gli pathway has been implicated in several cancers, including medulloblastoma, rhabdomyosarcoma, melanoma, basal cell carcinoma, and breast, lung, liver, stomach, prostate, and pancreatic cancers.[2,5−8] Inhibition of the aberrant Hh-Gli pathway (Figure 1) has thus emerged as an attractive target for anticancer therapy.[9−11] One Hh pathway inhibitor has shown promising results in phase I clinical trials and is proceeding to phase II[12] studies, and two other compounds have entered phase I clinical trials.[13,14] In this article, we review the medicinal

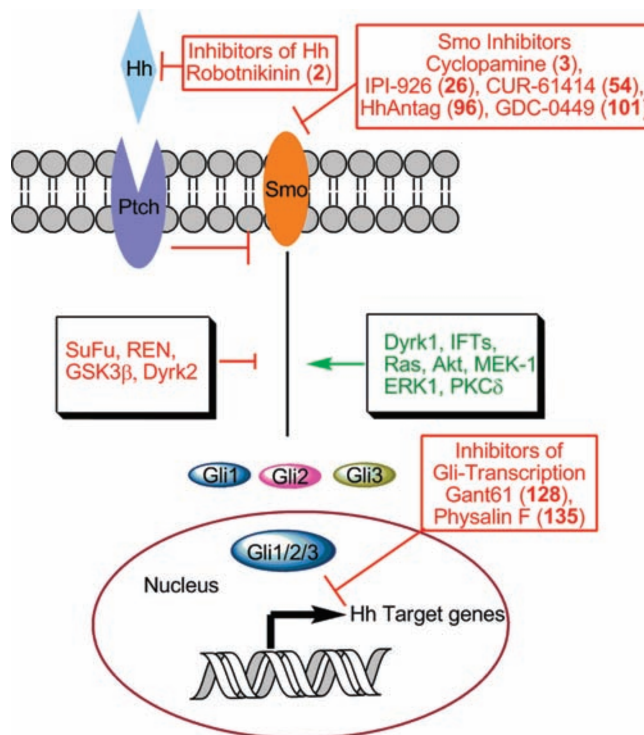

Figure 1. Hedgehog pathway activators and inhibitors. The Hh receptor Ptch is a transmembrane protein that inhibits the activity of Smo in the absence of Hh. Binding of Hh results in activation of Smo, which then modulates Gli transcription factors to initiate transcription of Hh target genes. Proteins involved in modulating signal relay between Smo and Gli are as indicated. Pathway suppressors are indicated in red and activators in green. The molecules in this pathway that are targets for chemical inhibitors include Smo and Gli.

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[a] Abbreviations: Hh, Hedgehog; Gli, glioma-associated oncogene homologue zinc finger protein; Shh, Sonic hedgehog; Dhh, Desert hedgehog; Ihh, Indian hedgehog; Smo, Smoothened; Ptch, Patched; Fu, Fused; SuFu, Suppressor of Fused; Cos2, Costal-2; PKA, protein kinase A; GSK3, glycogen synthase kinase 3; CK1, casein kinase 1; BCC, basal cell carcinoma; GS, Gorlin syndrome; Adh7, alcohol dehydrogenase 7; Raldh2, retinal dehydrogenase 2; MTP, microsomal triglyceride transfer protein; PTHrP, parathyroid hormone-related peptide; BMP 2, bone-morphogenetic protein-2; Dyrk, dual specificity tyrosine phosphorylation regulated kinase; IFT, intraflagellar transport protein; Ras, rat sarcoma; Akt, protein kinase B; MEK, mitogen-activated protein/extracellular signal-regulated kinase; ERK, extracellular regulated kinases; PKC, protein kinase C.

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chemistry efforts to identify and design inhibitors of Hh-Gli signaling and present a perspective of future developments in this dynamic field. We also present a brief overview of the role of Hh-Gli signaling pathway in normal development and cancer.

Hedgehog Pathway Components

The hedgehog (*Hh*) gene was first identified during a search for embryonic lethal mutants of *Drosophila melanogaster*, which found that mutation of *Hh* resulted in altered segment patterning of the larva.[15] Subsequently the gene was identified in many other invertebrates and vertebrates, including humans. Three mammalian counterparts of the *Hh* gene, termed Sonic hedgehog (*Shh*), Dessert hedgehog (*Dhh*), and Indian hedgehog (*Ihh*), were identified by combined screening of mouse genomic and cDNA libraries.[16] Hh undergoes multiple processing events, including autocatalytic cleavage of the C-terminal domain combined with addition of a cholesterol moiety at the cleavage site, and an N-terminal palmitoylation, to generate the active ligand.[17−19]

The receptor of secreted Hh protein is the multipass transmembrane protein Patched (Ptch). Of the two vertebrate homologues of Ptch (Ptch1 and Ptch2), the role of Ptch1 is better understood. In the absence of Hh ligand, Ptch inhibits the activity of the downstream effector Smoothened (Smo). The binding of Hh inactivates Ptch, resulting in activation of Smo.[20] In *Drosophila*, a complex of proteins comprising Fused (Fu), Suppressor of Fused (SuFu), and Costal-2 (Cos2) mediates signaling downstream of Smo and is aided by several kinases, such as protein kinase A (PKA), glycogen synthase kinase 3 (GSK3), and casein kinase 1 (CK1). Mammalian homologues of Fu and Cos2 have not yet been identified, suggesting that the signaling mechanisms differ in mammals and *Drosophila*.[21,22] Several mammalian-specific kinases that are required for Shh signaling have been identified.[23−25] These proteins modulate the function of Gli (Ci in *Drosophila*), the only transcription factor identified to date that operates directly downstream of Hh.

The first vertebrate *Gli* gene to be discovered was human *Gli1*, which was amplified about 50-fold in a malignant glioma.[26] Vertebrates have three Gli proteins (Gli1, Gli2, and Gli3), all of which have five highly conserved tandem zinc fingers, a fairly conserved N-terminal domain, several potential PKA sites, and a number of additional small conserved regions in the C-terminal end. Despite these similarities, the functions of the Gli subtypes differ. Both Gli2 and Gli3 contain activation and repressor domains. Consequently, in the absence of upstream Hh signal, full-length Gli3 and, to a lesser extent, Gli2 are constitutively cleaved to generate a truncated repressor form.[27−29] Hh signaling inhibits this cleavage, resulting in full-length Gli2 and Gli3, which have activator function. Gli1, in contrast, does not undergo proteolytic cleavage and acts as a constitutive activator.[27] The transcription of *Gli1* gene is initiated by Hh and is also controlled by Gli3.[27] Target genes of the Hh pathway other than *Gli1* include *Ptch*, several *Wnt* and *TGFβ* superfamily proteins, cell cycle proteins such as cyclin D, and stem-cell marker genes such as *NANOG* and *SOX2*.[30,31] Investigators are now attempting to comprehensively identify the Gli1-target genes.[32,33]

Hh Signaling in Development and Tissue Maintenance

The Hh signaling pathway is crucial for proper embryonic development.[34] It is also essential for restraining growth in the nervous system and other tissues and in maintenance of stem cells in adults.[35−37] The expression and roles of Hh in vertebrate tissues/organs have been extensively described in the recent reviews.[34,38]

Two of the functions of Hh in vertebrate embryonic development are both crucial and relatively well understood: neural tube differentiation and anteroposterior limb patterning. The predominant mechanism of Hh signaling in these functions is paracrine signaling, in which the Hh molecules act in a gradient fashion. For example, in vertebrate limb buds, exposure to different concentrations of Shh modulates patterning of the interdigital mesenchyme, which influences the proper growth of digits in a specific pattern.[39] In neural tube development, Shh produced by the floor plate causes dorsoventral patterning, the specification of ventral cell populations, and general cellular proliferation in the brain.[40] Holoprosencephaly, a disorder involving the development of forebrain and midface in which ventral cell types are lost, is caused in humans by mutations that lead to loss of Shh activity.[41]

Another important feature of Shh signaling is that the Gli subtypes have both unique and overlapping functions. While ectopic expression of *Gli1* in the midbrain and hindbrain of transgenic mice results in expression of some ventral cell types, mice homozygous for a mutation in the region encoding the zinc finger domain of Gli1 develop normally.[42,43] However, *Gli1*/*Gli2* double mutant mice have phenotypes with severe multiple defects, including variable loss of the ventral spinal cord, and smaller lungs; therefore, Gli2 plays a more important role in spinal cord and lung development than does Gli1. In contrast, *Gli1*/*Gli3* double mutant mice did not have these phenotypes.[43] Gli2 and Gli3 have both been implicated in skeletal development, with each subtype serving specific functional roles.[44,45] Gli2 mutant mice exhibit severe skeletal abnormalities including cleft palate, tooth defects, absence of vertebral body and intervertebral discs, and shortened limb and sternum.[45] Gli3 appears to be the major mediator of Shh effect in the limbs, as *Gli1*/*Gli2* double mutant mice had a normal digit number and pattern while *Gli3* mutant mice showed polydactyly.[43,46]

Genetic analyses of *Gli* mutants revealed that the requirement for *Gli* subtypes development is quite divergent even among vertebrates. In zebrafish, both *detour* (*dtr*) mutations (encoding loss-of-function alleles of *Gli1*) and *you-too* (*yot*) mutations (encoding C-terminally truncated Gli2) have defects in body axis formation and expression of Hh-target genes in the brain,[47] suggesting divergent requirements for Gli1 and Gli2 in mouse and zebrafish.

In adults, the Hh pathway is essential for restraining growth in the nervous system and other tissues and in maintenance of stem cells. Zhang and Kalderon have shown that Hh acts specifically on stem cells in *Drosophila* ovaries and that these cells cannot proliferate in the absence of Hh.[48] Other studies showed that Hh signaling in the postnatal telencephalon both promotes proliferation and maintains populations of neural progenitors, suggesting that Shh signaling in the mammalian telencephalon may participate in the maintenance of a neural stem cell niche.[35] The role of Hh in proliferation of adult neural progenitor cells was confirmed by a study in which Shh was overexpressed and proliferation was inhibited by using a Smo antagonist.[49]

Hh Signaling in Cancer

Hh genes have the ability to induce tissue proliferation. This function is important in embryogenesis and tissue maintenance, but inappropriate activation of the pathway can result in tumorigenesis.[50] Tumors in about 25% of all cancer deaths are estimated to involve aberrant Hh pathway activation.[4] Tumorigenesis or tumor growth can result from abnormal up-regulation

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of Hh ligand or from deregulation of the expression or function of downstream components by, for example, loss of *Ptch*,[51−53] activating mutations of *Smo*,[54] loss of *SuFu*,[55] amplification or chromosomal translocation of *Gli1*,[26] or *Gli2* gene amplification or stabilization of Gli2 protein.[56]

The first Hh pathway gene found to be amplified in cancers was *Gli1*, which was expressed at high levels in human glioblastoma and derived cell lines.[26] Subsequently, Gli1 was found to be consistently expressed in a variety of glial tumors, and Gli1 overexpression was shown to induce central-nerves system hyperproliferation.[57] Gli1 overexpression has also been observed in a panel of brain tumors ranging from low-grade to high-grade in a study that identified *Gli1* expression as the only reliable marker of Hh pathway activity.[31] Further, cell proliferation in primary cultures of many of these tumors was inhibited by *Gli1* small-interfering RNA.[31] *Gli1* expression was correlated with tumor grade in PDGF-induced gliomagenesis in mice.[58] Hh signaling components other than Gli1 also contribute to tumorigenesis in specific subsets of glioblastomas. In PDGF-induced tumors, expression level of *Shh* was correlated with the tumor grade. However, other studies found only a subset of gliomas to contain high levels of *Shh*.[31]

Another cancer with defects in Hh pathway regulation is basal cell carcinoma (BCC). Human *Ptch* was first identified by virtue of its mutation in patients with Gorlin syndrome (GS), a genetic disease that gives rise to sporadic BCC.[59] The mutations of *Ptch* identified in BCC include deletions producing truncated proteins and insertion or nonsense mutations accompanied by loss of heterozygosity (LOH) or mutations in the other allele.[51,52] These mutations inhibit the ability of Ptch to suppress Smo, resulting in constitutive Hh signaling. While *Ptch1* abnormalities are detected in the majority of BCC patients, it is now clear that a subset of BCC is also driven by a mutation in *Smo* that decreases its sensitivity to inhibition by Ptch.[54] In addition, overexpression of Gli1 protein causes BCC-like tumors in mice, establishing the importance of Gli1 transcription in BCC tumorigenesis.[60] The level of *Gli1* transcript can be used to discriminate BCC from certain other skin tumors.[61] However, blocking of Gli-based transcription has not yet been shown to arrest BCC growth.

Medulloblastoma, the most common malignant pediatric brain tumor, is linked with mutations in *Ptch* and *Smo* and mutations in other Hh pathway genes such as *SuFu* and *Gli*.[62] Inactivation of the *Ptch* locus by deletion and mutation has been found in about 10% of sporadic medulloblastomas.[53] Shh pathway involvement in these tumors was further confirmed by studies in which treatment of murine medulloblastomas with Smo inhibitors inhibited cell proliferation and reduced tumor growth in mice.[63−65] Taylor et al.[55] identified *SuFu* as a tumor-suppressor gene whose mutation predisposes individuals to medulloblastoma. They found that a subset of children with medulloblastoma carry germline and somatic mutations in *SuFu*, accompanied by loss of heterozygosity of the wild-type allele.[55] Several of these mutations encoded truncated SuFu proteins that are unable to export Gli protein from the nuclei. In addition, the tumor-suppressor REN has also been linked with medulloblastoma in which the allelic deletion and reduced expression of REN are frequently observed. It is suggested that it inhibits medulloblastoma growth by negatively regulating the Hh pathway.[66−68]

Hh has also been shown to be an early and late mediator of pancreatic cancer tumorigenesis. *Shh* was not detected in normal adult human pancreata but was aberrantly expressed in 70% of pancreatic adenocarcinoma specimens.[69] Participation of Shh

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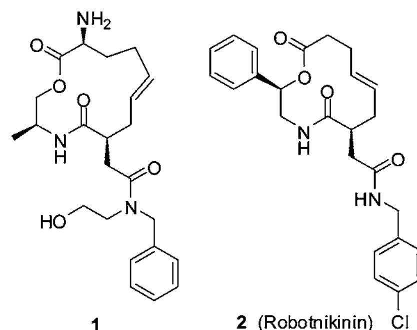

Figure 2. Shh inhibitors.

signaling has been indicated at multiple stages of pancreatic carcinogenesis and is accompanied by multiple oncogenic factors, including *K-Ras*, one of the most frequently mutated genes in pancreatic cancer.[70,71] Activated Hh signaling was detected in cell lines established from primary and metastatic pancreatic adenocarcinomas, and the Smo inhibitor cyclopamine induced apoptosis in a subset of the pancreatic cancer cell lines both in culture and in mice.[72]

Numerous studies indicate that Hh signaling is involved in prostate cancer. Sanchez and others reported the expression of Shh-Gli pathway components in adult human prostate cancer.[8] Treatment of primary prostate tumor cultures and metastatic prostate cancer cell lines with Smo inhibitors blocked the pathway and proliferation. Increased expression of Shh in prostate cancer cells up-regulates *Gli1* expression and dramatically accelerates the growth of prostate tumor xenografts.[73] Elevated Shh activity distinguished metastatic from localized prostate cancer, and manipulation of this pathway modulated the invasiveness and metastasis of these tumors.[72,74]

Hh signaling has also been implicated in various other cancers, such as lung, colorectal, bladder, endometrial, ovarian, and esophageal carcinomas and rhabdomyosarcoma.[75−83] The role of Hh-Gli signaling pathway in cancer and its potential as therapeutic target have been reviewed in more detail in recent articles.[10,11,30,84]

Small Molecule Inhibitors of the Hh-Gli Pathway

The aberrant activation of Hh-Gli signaling in several cancers has made it an attractive target for anticancer drug discovery. Here, we summarize the medicinal chemistry efforts to discover Hh-Gli pathway inhibitors. The compounds known to date (January 2009) are classified as inhibitors of Shh, Smo, class IV alcohol dehydrogenase (alcohol dehydrogenase 7, Adh7), and Gli transcription.

Shh Inhibitors

The first small molecule inhibitor that blocks the Shh signaling by binding to Shh protein was recently reported by Stanton et al.[85] A macrocycle **1** (Figure 2) was discovered by screening a 10 000 small molecule diversity-oriented library in small-molecule microarry for binding to bacterially expressed Shh N-terminus fragment (ShhN). The hit compounds were further evaluated in Shh-LIGHT II cells, a clonal NIH 3T3 cell lines stably incorporated with a Gli-responsive firefly luciferase reporter. The activity was measured as relative luciferase activity after incubation with the compound in the presence of N-palmitoylated ShhN. The structure−activity relationship (SAR) studies led to robotnikinin (**2**), a 12-membered macrocycle with a 3.1 μM K_d for the ShhN binding and a dose dependent inhibitor of ShhN-induced pathway activation in Shh-LIGHT

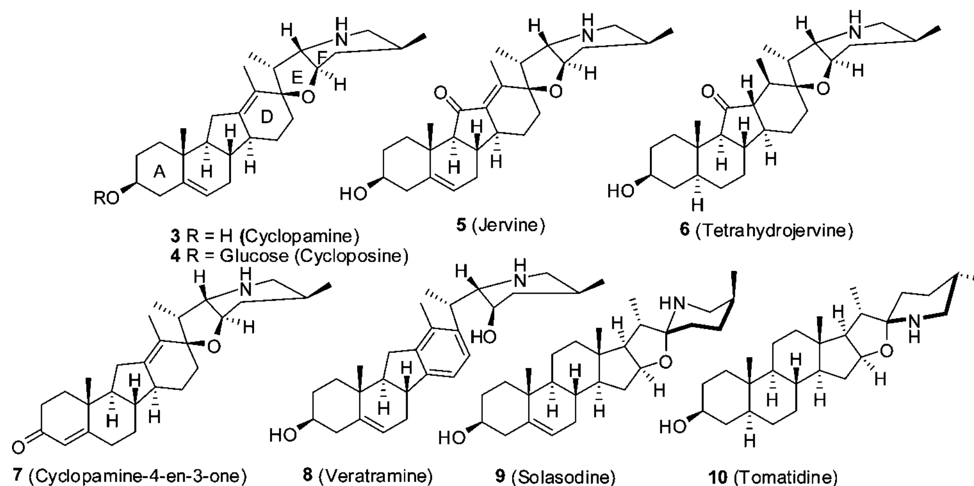

Figure 3. Cyclopamine derivatives and related steroidal alkaloids.

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II cells. It did not show any substantial cytotoxicity in cell viability assay. In a mouse *Ptch1*$^{-/-}$ cell line, in which the *Ptch1* alleles were replaced with a β-galactosidase (β-gal) reporter, compound **2** did not inhibit the β-gal levels, thus indicating that it inhibits the Shh pathway upstream of Ptch1. It also inhibited alkaline phosphatase induction in a dose-dependent manner in C3H10/T1/2 cells in the presence of ShhN, which was rescued by purmorphamine,[86] a Smo agonist (C3H10/T1/2 cells differentiate to osteoblasts upon treatment with N-palmitoylated ShhN, an event measured by alkaline phosphatase as a marker of the transformation[87,88]). Compound **2** inhibited Shh-induced expression of Gli1 and Gli2 in primary human keratinocytes and human-derived skin tissue.[85]

Smoothened Inhibitors

The Hh pathway is initiated by binding of Hh to the transmembrane protein Ptch, which releases the suppression of Smo and initiates a cascade of events resulting in expression of Hh-responsive genes (Figure 1). Cyclopamine (**3**), the prototype inhibitor of the Shh pathway, is currently in preclinical and clinical studies as an anticancer agent.[89] Cyclopamine inactivates Smo by binding to its heptahelical bundle.[74,90] A number of Smo inhibitors have now been reported and can be classified as cyclopamine analogues or synthetic Smo antagonists.

Cyclopamine Analogues

Cyclopamine (**3**), a teratogenic steroidal alkaloid derived from the plant *Veratrum californicum*, has been known since the 1960s. It was first reported to block the Shh signaling pathway in 1998 by Cooper et al.[91] The alkaloids cyclopamine and jervine (**5**) (Figure 3) from this plant were associated with holoprosencephaly and other teratogenic effects in lambs born to pregnant ewes in the 1960s.[92,93] Incardona et al.[94] further showed that cyclopamine-induced teratogenesis is independent of cholesterol metabolism and results from inhibition of Shh signaling.

Incardona et al.[95] also studied the SAR of cyclopamine derivatives and related steroidal alkaloids (Figure 3) in a chick embryo neural plate explant assay by measuring Shh inhibition as loss of HNF3β, a floor plate marker. For cyclopamine to be active, its E ring should be perpendicular to rings A−D. Opening of this ring, as in veratramine (**8**), resulted in much weaker activity. Oxidation of cyclopamine to cyclopamine-4-en-3-one (**7**) improved potency by at least 2-fold in the explant assay. Jervine (**5**), a C11 ketone analogue of cyclopamine, was 5- to 10-fold less potent, although it was metabolically more stable in vivo. Reduction of jervine to tetrahydrojervine (**6**) further decreased the activity by 3-fold. The glycoalkaloid analogue of cyclopamine, cyloposine (**4**), was equipotent to jervine (**5**). The steroidal alkaloids solasodine (**9**) and tomatidine (**10**), with E rings coplanar to the A−D rings, were 15- to 20-fold less active.[95]

The modification of cyclopamine at the 3-position (Figure 4) by addition of a carbamate group (**11**, **12**) decreased activity 100-fold. The secondary amine in the ring F of cyclopamine and jervine tolerates modifications (Figure 4), if its basicity is preserved. A small- to medium-size substitution at this position (**13**−**15**) improved the inhibitory activity, which is lost by large-size substitution, possibly because of cell impermeability or steric hindrance. Any branched substitution (**16**) close to the cyclopamine scaffold also decreases the activity. Oxidation of the hydroxyl at the 3-position in the N-substituted derivatives improved activity by 2-fold over that of the corresponding hydroxyl analogues (**14** vs **13**). Compound **14** (3-keto-*N*-aminoethylaminocaproyldigyrocinnamoyl (KAAD)-cyclopamine) was identified as the most potent inhibitor in this series.[96] It showed 10- to 20-fold better potency but similar or lower toxicity compared to cyclopamine. Taipale et al.[97] reported that the mechanism of action of cyclopamine and its analogues is through reversal of oncogenic activation of the Hh pathway by antagonism of Smo. Photoaffinity studies with a fluorescent-tagged derivative of cyclopamine confirmed the direct binding of cyclopamine to the heptahelical bundle of Smo, inducing a conformation that was structurally similar to that induced by Ptch.[90] Compound **14** is currently in preclinical development.[89]

Researchers at Infinity Pharmaceuticals and Johns Hopkins carried out further SAR studies of cyclopamine by making changes in rings A, D, and F (Figure 5) to overcome poor aqueous solubility and chemical instability in acid.[98−100] Tremblay et al.[99] synthesized the D-homocyclopamine analogue **17** (IPI-611), which was more stable than cyclopamine at low pH, but its hydrochloride salt was less soluble in water and showed weaker activity than cyclopamine. Oppenaeur oxidation of **17** to the corresponding conjugated enone yielded a potent compound, **18** (IPI-269609/IPI-609),[98−100] that inhibited differentiation of C3H10/T1/2 cells to osteoblasts (a Shh dependent event[87,88] measured by alkaline phosphatase assay) with an EC_{50} of 200 nM. It also showed improved aqueous solubility and stability.[98−100] Compound **18** can be prepared from cyclopamine with an overall yield of 25−30%.[100] Removal of the keto group and saturation of the 4,5-double bond (**19**) decreased activity.[98]

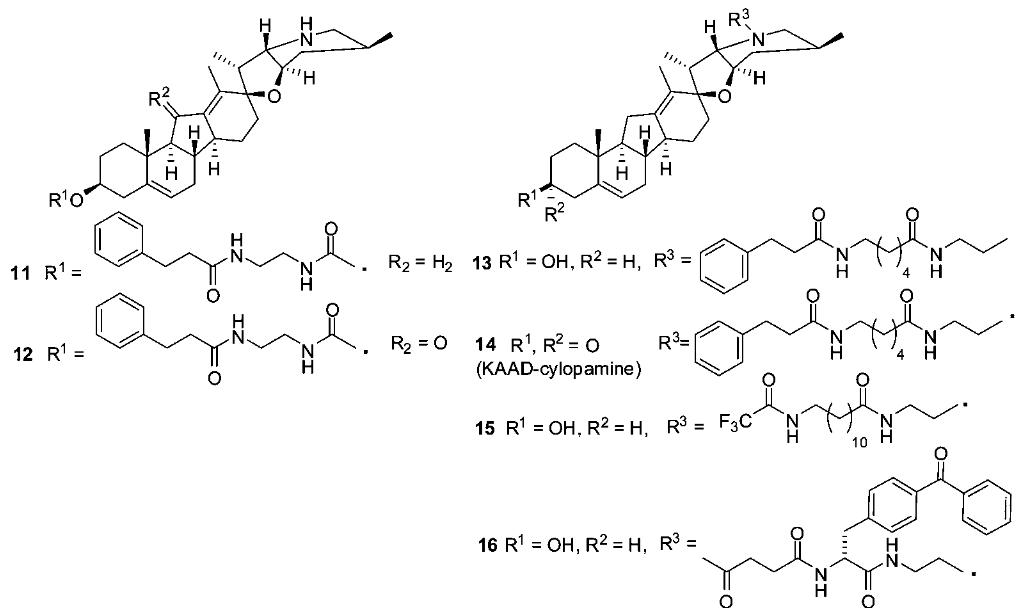

Figure 4. Cyclopamine analogues with substitution at 3-position or secondary amine in F ring.

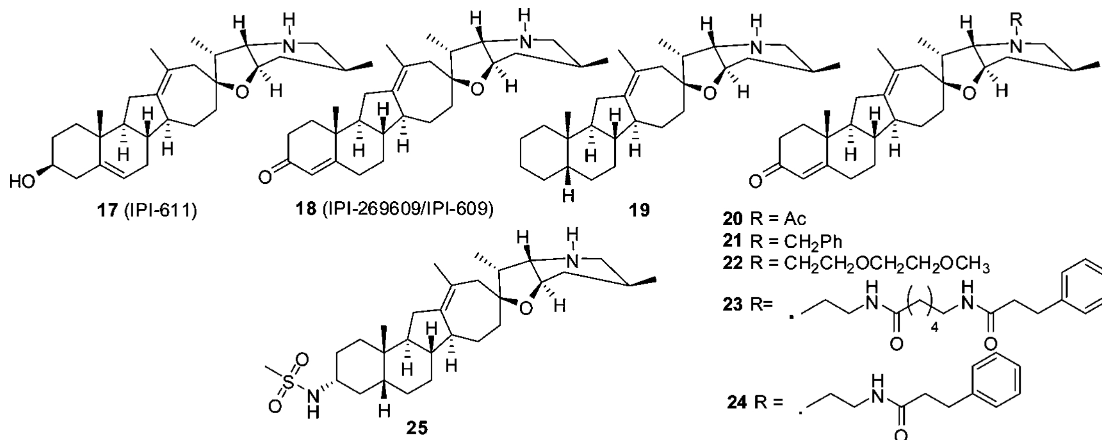

Figure 5. D-Homocyclopamine analogues.

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The *N*-acetamide derivative, **20**, showed activity similar to that of the parent compound, contrary to earlier reports,[96] suggesting importance of basicity of the nitrogen. Bulky groups such as benzyl (**21**) reduced potency, while the glycolate analogue (**22**) displayed activity similar to that of **18**. Compound **23**, with a side chain similar to that of **14** (KAAD-cylopamine), showed a 20-fold increase in activity over that of **18**, while its truncated analogue **24** was 10-fold more potent. However, **23** and **24** are less soluble than **18**.[100] Replacement of the 3-keto group with a methanesulfonamide group afforded **25** that potently inhibits C3H10/T1/2 cell differentiation and cell growth of human multiple myeloma, acute myeloid leukemia, myelodysplastic syndrome, non-Hodgkin's lymphoma, Hodgkin's lymphoma, and pre-B cell acute lymphoblastic leukemia cell lines. It also suppressed tumor growth in pancreatic cancer and medulloblastoma mouse models and showed clear tumor regression in a medulloblastoma model at higher doses.[98]

Compound **18** showed inhibitory effect in a Gli-responsive reporter assay to a degree comparable to that of cyclopamine. It diminishes the migration and colony formation of pancreatic cancer cells, suppressed growth of a subcutaneous xenograft of pancreatic cancer E3LZ10.7, and abrogates metastasis in orthotopic pancreatic cancer xenografts of E3LZ10.7 and Capan-1 cell lines. The results indicated that Smo inhibitors could be effective in inhibiting metastasis in pancreatic cancers.[101] Pretreatment with **18** diminishes tumorigenicity in vivo.[101] The hydrochloride salt of **18** formulated in water containing 30% of 2-hydropropyl-β-cylodextrin displayed 80% oral bioavailability and a plasma half-life of 3.2 h in CD-1 mice.[100]

Infinity Pharmaceuticals designed **26** (IPI-926)[102] (structure not disclosed) as a second-generation cyclopamine analogue that is orally bioavailable, has a long half-life in plasma (10−24 h in multiple species) and tumors, and is active in vivo. Once-daily oral administration of **26** at 40 mg/kg resulted in complete tumor regression in a murine medulloblastoma model.[102] Compound **26** also delays or prevents tumor recurrence after completion of chemotherapy in a small-cell lung cancer xenograft model.[103] It entered phase I clinical trials for advanced and/or metastatic solid tumor malignancies in September 2008.[13]

The cyclopamine lactam analogues (Figure 6) have also been reported to inhibit differentiation of C3H10/T1/2 cells to osteoblasts in a alkaline phosphatase assay.[104] The replacement of the ring A of **18** with a γ-lactam ring and a trans A/B ring junction gave compound **27** with potent activity ($IC_{50} < 20$ nM) in alkaline phosphatase assay. Change in the A/B ring junction to cis configuration (**28**) resulted in >5-fold decrease in the activity. Expansion of ring A to a seven membered ring (**29**) restored the activity, although it was still 2- to 5-fold less active

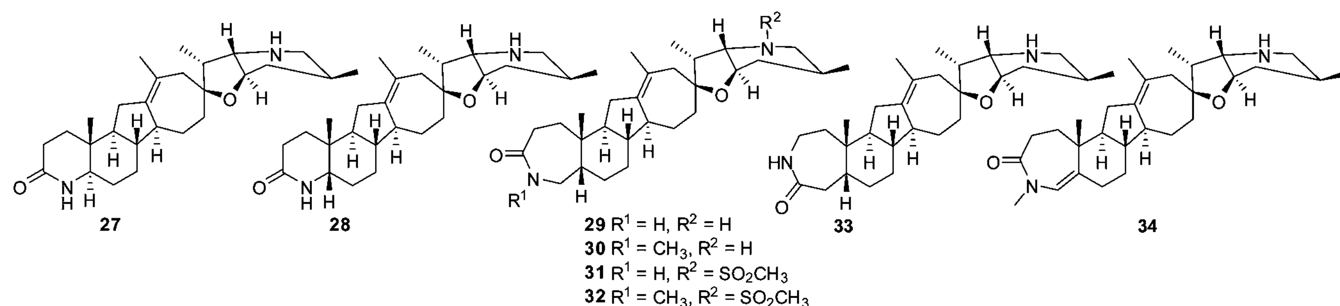

Figure 6. Cyclopamine lactam analogues.

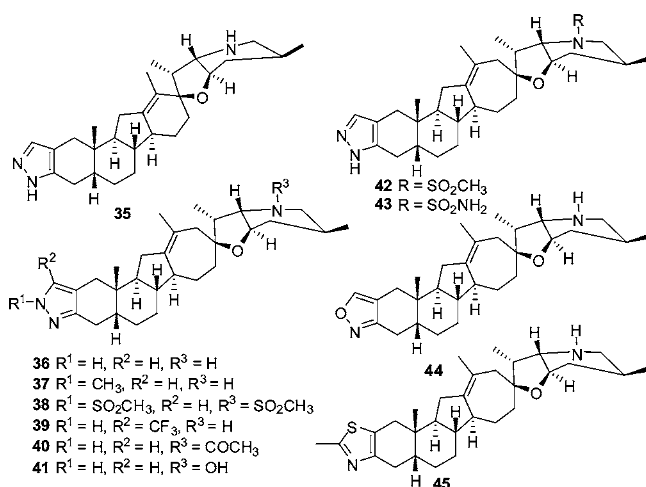

Figure 7. Cyclopamine analogues with a fused heterocyclic ring.

than **27**. Reversing the amide group of **29** (**33**) resulted in a significant loss in activity. The ring A methylamide **30** was equipotent to **29**, while the ring F methanesulfonamide analogue **31** showed improvement in activity to match the potency of **27**. However, incorporating both the methyl group in ring A and the methanesulfonyl group in ring F together resulted in significant loss in activity in **32**. An unsaturated analogue (**34**) of **30** showed significant loss in activity. Compound **29** effectively reduced the Gli-responsive luciferase reporter activity in pre-B cell acute lymphocytic leukemia cell lines and inhibited growth of primary cultures derived from patients with multiple myeloma, acute myeloid leukemia, myelodysplastic syndrome, non-Hodgkin's lymphoma, and Hodgkin's disease. It was suggested that **29** affects a tumor−stroma interaction in human pancreatic xenograft in mice, as it down-regulated the mRNA levels of murine Gli1 but not of human Gli1. It showed $ED_{50} < 7.5$ mg/kg with once daily oral dose in an allograft model of medulloblastoma from a transgenic mouse with loss of function mutations in *Ptch1* and *Hypermethylated in Cancer* (*Hic1*).[104]

Infinity Pharmaceuticals also reported cyclopamine analogues that have a heterocyclic ring fused to ring A (Figure 7).[105] Compound **35** showed potent inhibition ($IC_{50} < 100$ nM) in an alkaline phosphatase assay using C3H10/T1/2 cells in the presence of Shh-N. Ring D expansion further improved the activity to yield the most potent compound, **36** ($IC_{50} < 20$ nM). Any further substitution on the pyrazole ring (**37**−**39**) or ring F (**40**−**43**), however, decreased the activity. Replacement of the pyrazole ring with an oxazole (**44**) decreased the activity 1- to 5-fold, and replacement of the ring with a 2-methylthiazole ring (**45**) decreased activity more than 5-fold compared to that of **36**. Compound **36** significantly suppressed the growth of human pancreatic cancer xenografts in mice at 40 (mg/kg)/day.[105]

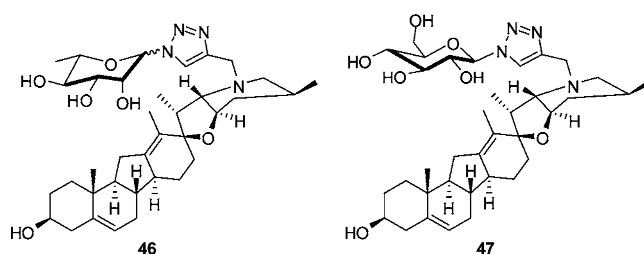

Figure 8. Carbohydrate cyclopamine analogues. Compound **46** is a 89/11 mixture of α/β.

Cyclopamine is poorly soluble in water, hindering its development as a drug. Zhang et al.[106] recently reported carbohydrate−cyclopamine analogues (Figure 8) aimed at improving solubility and introducing structural diversity. They utilized click chemistry (copper-catalyzed azide−alkyne coupling) to synthesize a library from an alkyne-modified cyclopamine and diverse azidosugars. Compound **46** showed activity against a lung cancer cell line (A549) comparable to that of cyclopamine in a standard viability assay, with an IC_{50} of 33 μM compared to 49 μM for cyclopamine; however, its aqueous solubility was superior. Another β-linked analogue (**47**) was less potent than cyclopamine. They also suggested that α-linked L-pyranose might be essential for activity.[106]

In an effort to decrease the adverse effects of cyclopamine, Kumar et al.[107] synthesized prodrugs of cyclopamine (Figure 9) by coupling cyclopamine to the peptide carriers that are proteolytically removed in cancers. They designed the peptide carriers as substrates of the tissue-specific serine protease prostate-specific antigen (PSA), which is expressed at high levels in prostate but not in other organs. Two such carriers, His-Ser-Ser-Lys-Leu-Gln (HSSKLQ) and Ser-Ser-Lys-Tyr-Gln (SSKYQ), were coupled with cyclopamine to yield conjugates **48** and **49**, respectively.[107]

Compounds **48** and **49** are converted to cyclopamine in the presence of PSA, with half-lives of 3.2 and 22 h, respectively. Both showed minimal activity in the DU145 prostate cancer cell line, which lacks PSA, but addition of PSA to the medium resulted in a 7-fold increase in efficacy over that of cyclopamine. In vivo studies of these compounds are ongoing.[107]

Noncyclopamine-Scaffold Compounds

Several pharmaceutical companies have identified new Smo inhibitors with druglike properties by optimization of high-throughput screen hits. One such small molecule, **101** (GDC-0449, Curis and Genentech, Figure 14), is currently in phase I/II clinical trials for advanced BCC and solid epithelial tumor.[108] Development of another candidate, **54** (CUR-61414, Figure 11), was suspended after phase I.[89]

The structurally diverse compounds **50** (SANT-1), **51** (SANT-2), **52** (SANT-3), and **53** (SANT-4) (Figure 10) were discovered

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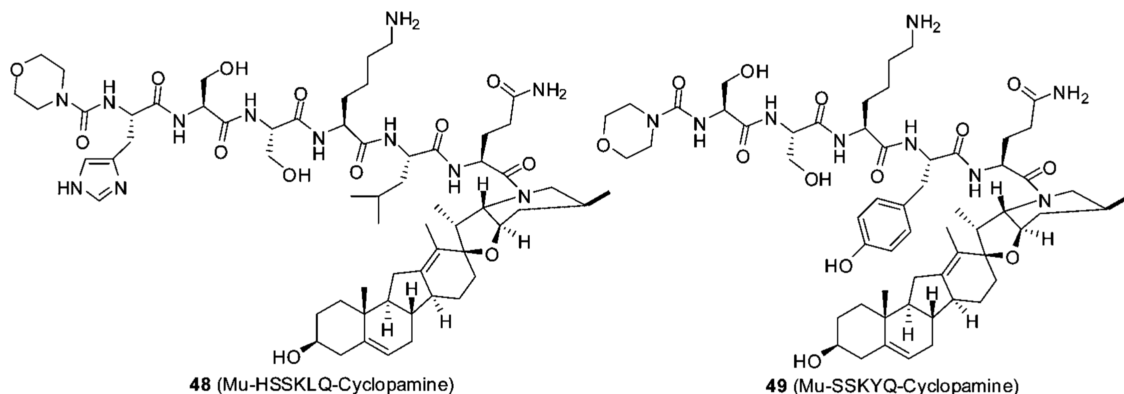

Figure 9. Cyclopamine prodrugs.

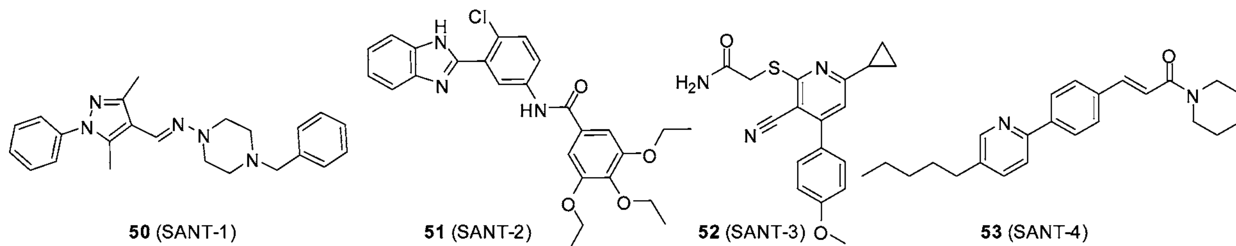

Figure 10. Compounds discovered by screening with a Shh-reporter.

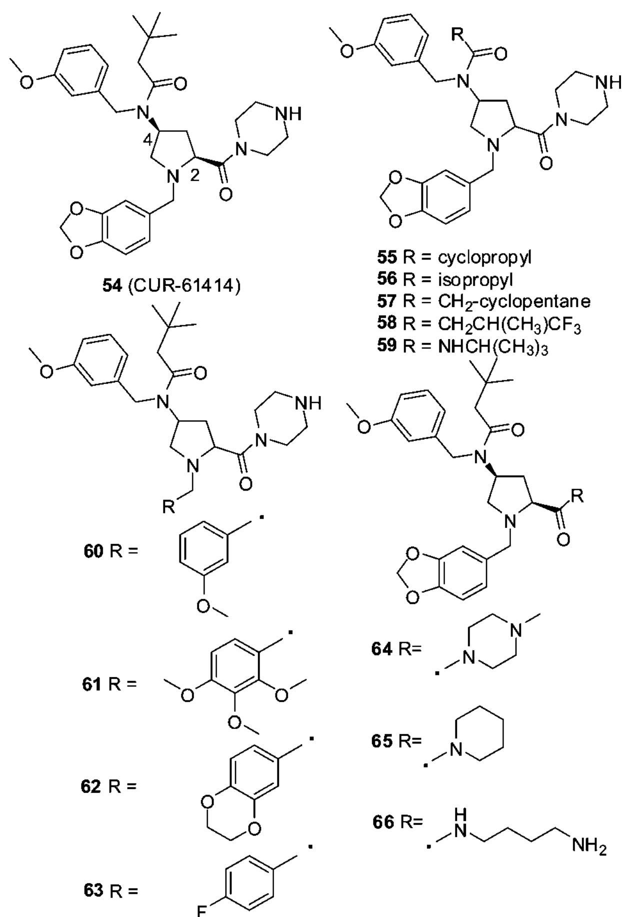

Figure 11. Aminoproline Smo inhibitors.

by Chen et al.[109] by screening 10 000 small molecules for Hh signaling inhibition (IC_{50} values for **50**−**53** were 20, 30, 100, and 200 nM, respectively, in a Shh-LIGHT II assay). These molecules have different mechanisms of action.[109]

Aminoprolines

Another high-throughput screen of a library of 100 000 small molecules using a Hh-reporter cell line (s12) led to discovery of the lead compound **54** (Figure 11), an aminoproline with an IC_{50} of 100−200 nM, compared to 500−700 nM for jervine (**5**).[110] Compound **54** showed a physicochemical profile suitable as a drug candidate and had no toxicity in preclinical testing. It suppressed proliferation and induced apoptosis in BCC cells without affecting normal skin cells.[110] Curis prepared a library of derivatives of compound **54** to elucidate the SAR of the amide group at the 4-postion of aminoproline and at the substitution at the N1 position of the pyrrolidine ring and the piperazine ring. The cis isomers exhibited more than 100-fold greater activity than the trans isomers. A cyclopropyl or isopropyl substitution of the amide at the 4-position of the proline in **55** and **56**, respectively, resulted in similar potency (IC_{50} < 100 nM). Compounds **57** and **58**, with a cyclopentane ring and $CH(CH_3)CF_3$ respectively, showed weaker activity than did **55** and **56**. Replacement of amide with urea (**59**) also decreased activity. 3,4,5-Trimethoxybenzyl (**61**) or (2,3-dihydrobenzo[*b*][1,4]-dioxin-6-yl)methyl (**62**) substitutions at the 1-position of proline were better tolerated than benzo[*d*][1,3]dioxol-5-ylmethyl (**54**) or 3-methoxybenzyl (**60**). The piperazine ring on proline carbamide is essential for activity. Its replacement with a methylpiperazine (**64**), piperidine (**65**), or aliphatic amine (**66**) decreases activity by several-fold.[111] Clinical trials of **54** were halted after a phase I study as a topical agent in patients with BCC.[89]

Quinazolinones and Quinazolines

Brunton et al. at Evotec and Curis increased the potency of the screening hit **67** (IC_{50} = 1.4 μM) to the nanomolar level by lead optimization. They carried out SAR studies in a reporter assay focusing on the urea group, the 4-fluorophenyl, and the quinazolinone ring (Figure 12). The bis-desmethyl analogue of **67** (**68**) showed a 2-fold improvement in activity, indicating that both methyl groups are not necessary. The methyl derivative

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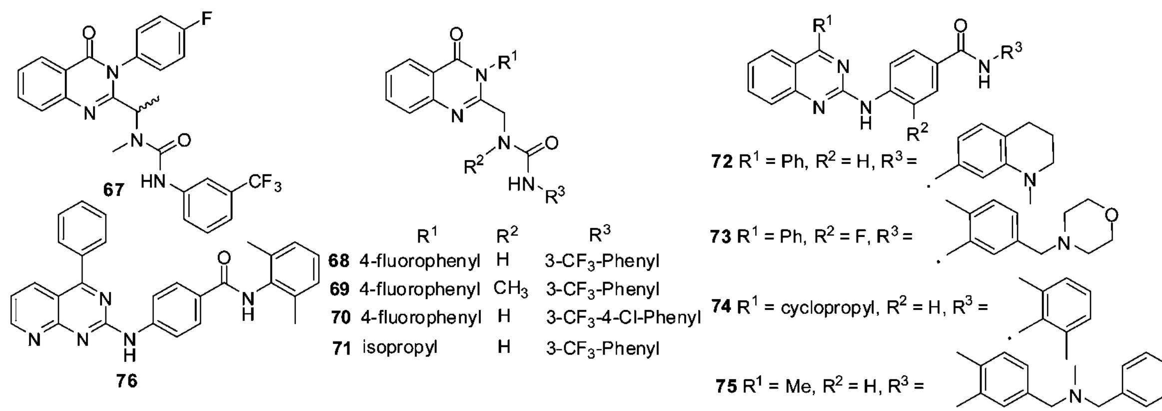

Figure 12. Quinazolinones and quinazolines.

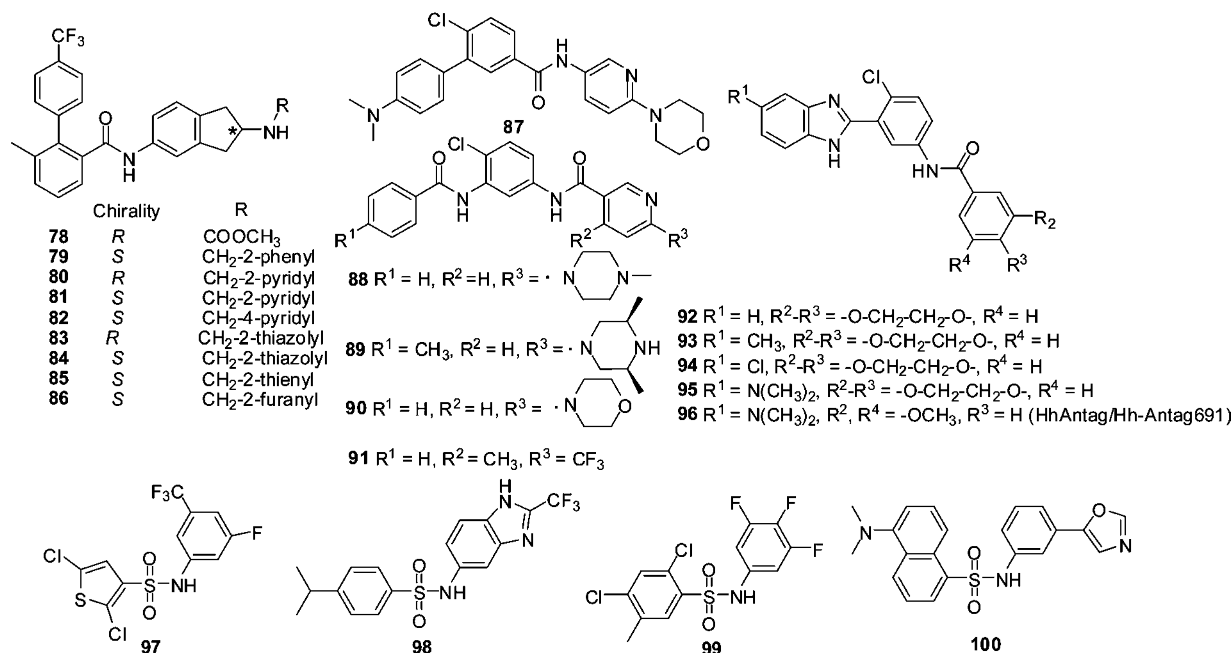

Figure 13. Biarylcarboxamide, bisamide, benzimidazole, and sulfonamide derivatives.

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69 was 3-fold less active than **67**. The 3-trifluoromethyl-4-chlorophenyl-substituted desmethyl analogue **70** showed a 20-fold improvement in activity over **67**, exhibiting an IC_{50} of 70 nM. Replacement of the 4-fluorophenyl with an isopropyl group in **71** decreased activity 3-fold, indicating the importance of the phenyl ring.[112]

Exelixis has described quinazolines (**72**−**75**) and pyridopyrimidine (**76**) (Figure 12).[113] The tetrahydroisoquinoline analog **72** exhibited an IC_{50} of 5.8 nM in a reporter assay in the Shh LIGHT II cell line. The cyclopropyl-substituted **74** was most potent, with an IC_{50} of 2.8 nM. Replacement of the quinazoline with pyridopyrimidine yielded the equipotent **76**, with an IC_{50} of 3.6 nM.[113] Exelixis and Bristol-Myers Squibb are co-developing **77** (XL139/BMS-833923)[14,114] (structure not disclosed) as an inhibitor of the Hh pathway. Compound **77** is in phase I clinical trials for advanced or metastatic cancers.[14,114]

Biarylcarboxamide, Bisamide, Benzimidazole, and Sulfonamide Derivatives

Peukert et al. from Novartis reported a series of biarylcarboxamides (Figure 13) based on an initial micromolar screening hit **78**, which was originally designed as a microsomal triglyceride transfer protein (MTP) antagonist.[115,116] These compounds were subjected to several screens to identify ones that bind directly to Smo. SAR studies revealed that benzylamines (**79**) were more potent than carboxamides or sulfonamides. The *S*-enantiomers (**81** and **84**) were significantly more potent Smo inhibitors than *R*-enatiomers (**80** and **83**) which had more potent MTP inhibitory activity. The trifluoromethyl group is essential for activity. The phenyl-substituted **79** was 5- to 7-fold more potent than pyridyl analogues **81** and **82**. The five-membered hetrocyclic analogues **84**−**86** showed the most potent activity in TM3Hh12 cells in a transcription assay with approximately 10-fold selectivity over MTP inhibition and also showed direct Smo binding in membranes from CHO-K1 cells stably expressing HA-tagged mouse or human Smo.[115]

Biarylcarboxamides, such as **87**,[117] and structurally similar bisamide compounds (**88**−**91**)[118] also are active in the Gli-responsive luciferase reporter assay.

Curis has reported benzimidazole derivatives (**92**−**96**) (Figure 13) that are structurally similar to **51**.[119] SAR study showed that addition of a methyl or chloro group at the 5-position of the benzimidazole ring of **93** or **194**, respectively, resulted in activity 50-fold greater than that of **92**. Replacement of the methyl group in **93** with a *N,N*-dimethyl (**95**) resulted in a further 10-fold increase in activity, to $IC_{50} < 1$ nM. Compound **96**

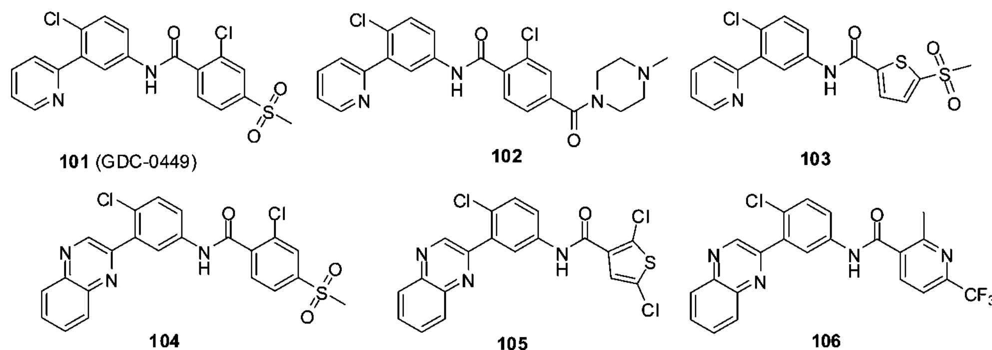

Figure 14. Pyridyl and quinoxaline Smo inhibitors.

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(HhAntag/Hh-Antag691)[120] inhibited Shh activity with an EC_{50} of 40 nM compared to 500 nM for cyclopamine in a Gli-responsive luciferase reporter assay, although the two compounds exhibited similar (IC_{50} = 10−30 μM) inhibition of growth of medulloblastoma cultures.[65,119] Twice-daily oral administration of **96** at 100 mg/kg for 2 weeks eliminated medulloblastomas that spontaneously form in *Ptch1*$^{+/-}$*p53*$^{-/-}$ mice,[65] suggesting that **96** may penetrate the blood−brain barrier, at least partially. No side effects were observed during a 50-day study. Compound **96** inhibited the expression of several genes including *Gli1* and *Gli2* in the tumors of the mice[65] and eliminated medulloblastoma allografts with an active Shh pathway but was ineffective against allografts with an inactive Shh pathway, thus showing specificity for medulloblastomas in which the Shh pathway is up-regulated.[121] Compound **96** (100 mg/kg twice daily for 4 days) also decreased the volume of medulloblastoma allografts from another mice that had *Cxcr6* mutation, overexpression of Shh pathway target genes, and low levels of *Ptch1*.[122] However, treatment of young mice (postnatal days 10−14, a period of extremely rapid bone growth) with two doses of 100 mg/kg of **96** within 24 h resulted in permanent defects in bone structure and growth, despite restoration of the somatic Hh reporter activity 48 h after withdrawing **96**. The inhibition of Hh pathway by **96** resulted in differentiation of chondrocytes, expansion of hypertrophic zone, and breakdown in columnar organization leading to permanent defects in joint structures. The complete inhibition of the Hh pathway in tumor cells is essential for tumor elimination while even a transient exposure of proliferating chondrocytes to **96** can result in terminal differentiation, thus suggesting that these bone defects occurs within the therapeutic window in the young mice. This finding raises a concern about using **96** in pediatric patients, although it is not known if the bone defect will occur in humans also.[123] An earlier study in adult mice did not show any major side effects,[65] but the transient inhibition of Hh pathway might also compromise the regenerative capacity of some adult tissues.[123]

Compounds that have a urea, thiourea, or sulfonamide linker in place of an amide linker were also synthesized and evaluated.[119] Sulfonamides **97−100** (Figure 13) also showed potent activity.[124]

Pyridyl and Quinoxaline Compounds

Genentech and Curis have reported pyridyl (**101−103**)[125] and quinoxaline (**104−106**)[126] compounds (Figure 14). Compound **101** is currently in a phase I study for locally advanced or metastatic solid tumors and phase II clinical trials for advanced BCC, metastatic colorectal cancer, and advanced ovarian cancer.[12,108,127] Compound **101** (IC_{50} = 3 nM in a Gli-responsive luciferase reporter assay) is an once-daily orally active, potent

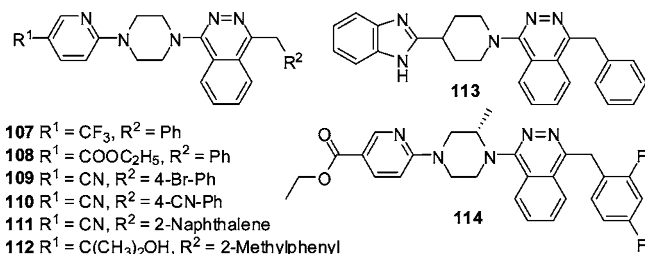

Figure 15. Phthalazine Smo inhibitors.

inhibitor of Smo that has an extremely long half-life (10−14 days) with sustained high-micromolar plasma concentrations. Daily dosages of 150, 270, and 540 mg of **101** were tested in phase I studies in patients with BCC or pancreatic, colorectal, or adenocystic carcinomas. It showed at least 2-fold down-regulation of *Gli1* expression in skin punch biopsies, irrespective of the dosage level. Two of nine patients with BCC on treatment with **101** had a confirmed response, four had a confirmed partial response, two had stable disease, and one had progressive disease. No dose-limiting toxicity was observed. The side effects included fatigue, asymptomatic hyponatremia, dysgeusia, and alopecia.[12,128]

Phthalazine Analogues

Novartis reported phtalazine analogues (**107−114**, Figure 15) as Smo antagonists.[129] The compounds were evaluated for inhibition of Gli-responsive luciferase activity in TMHh12 cells in the presence of a small molecule Smo agonist. Compounds that showed a shift in IC_{50} with increase in concentration of Smo agonist were judged to be directly interacting with Smo. The compounds were also screened in a Smo binding assay to determine IC_{50} for displacement of a radiolabeled small molecule Smo agonist from a filter-bound mouse and human Smo.[129]

Triazole Derivatives

Merck had discovered triazole derivatives as 11β-hydroxysteroid dehydrogenase type-1 inhibitors for metabolic disorders.[130] Some of these derivatives, **115−120** (Figure 16), also showed Smo antagonist activity in the Shh-Light II reporter assay and Smo binding assay and suppressed medulloblastoma xenograft growth in mice models.[131]

Smo Intracellular-Loop Analogues

Smo resembles a G-protein-coupled receptor (GPCR) in general topology. Remsberg et al.[132] designed structural analogues of the Smo intracellular loop. Unlike inhibitors designed for GPCRs using this strategy that were often nonspecific (because of significant sequence homology in the intracellular loop), the peptides derived from the Smo intracellular loop were

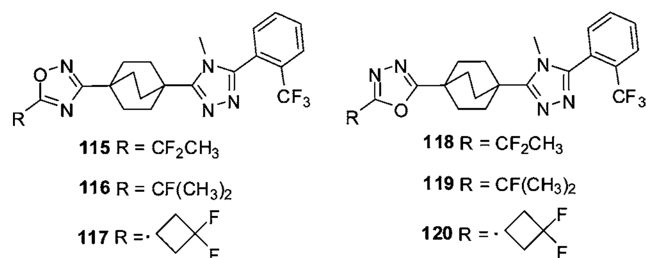

Figure 16. Phthalazine Smo inhibitors.

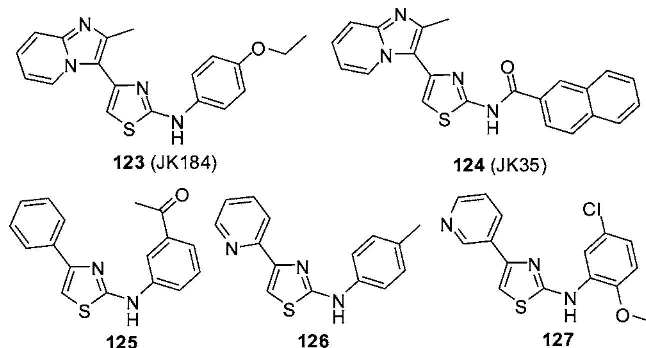

Figure 17. 2-Aminothiazoles.

expected to be specific because of the unique structure of Smo, which is conserved among species.[132] The peptide analogues of the intracellular loop of Smo were evaluated for growth inhibition in the MCF-7 breast cancer cell line. The second and third loop derivatives were further optimized by C-terminal and N-terminal truncation to identify a 10-amino-acid derivative of the N-terminal half of the second intracellular loop, **121** (SMOi2−12, palmitoyl-LTYAWHTSFK), with a nanomolar IC_{50}. The retroinverso peptide of **121** with all-D-amino acids and reverse sequence, **122** (SMOi2−20, Ac-KFSTHWAYTLK-e-Pal (all-D-)), was metabolically more stable and 200-fold more potent than **121**, with an IC_{50} of 0.3 nM in an MTT assay in SK-Mel2 melanoma cells.[132]

Inhibitors of Adh7

Inhibitors of class IV alcohol dehydrogenase 7 (Adh7) were also reported as the Hh signaling antagonists, though the exact mechanism of action of these compounds for the Hh pathway is not understood. The Hh signaling has been shown to be affected by the deletion mutation of retinal dehydrogenase 2 (Raldh2).[133] Raldh2 converts retinal, which is produced by alcohol dehydrogenase from retinol, to retinoic acid. Thus, Hh signaling can be partially affected by Adh7.[134]

A high-throughput screen of 20 000 compounds using a transcription assay driven by Gli-responsive elements in the presence of Shh N-terminus led to identification of 2,4-disubstitutued thiazole compounds as Hh pathway inhibitors (e.g., **123** and **124**, Figure 17). 2-Aminothiazole **123** (JK184) inhibited the Shh-N-mediated Gli-transcription with an IC_{50} of 30 nM. Inhibition of the pathway was confirmed also by RT-PCR, in which **123** showed suppression of *Gli1* and *Ptch1* expression. In a cytotoxicity assay, compound **123** inhibited the growth of cell lines with abnormally activated Hh signaling, including the pancreatic cancer cell lines L3.6pl, Panc 05.04, and BxPC3 and the medulloblastoma cell lines D283 med and Daoy, with GI_{50} of 3−21 nM, while showing no toxicity to normal human dermal fibroblast cells.[134,135] Compounds with arylamide substitution at the 2-position of thiazole (**124**) were less active than **123**. Compound **123** induced 30−50% inhibition of tumor growth in xenograft models of human L3.6sl and BxPC3 pancreatic cancers when administered orally, but its efficacy was comparable to that of cyclopamine when it was administered subcutaneously, indicating poor oral bioavailability (16%, $t_{1/2}$ = 1.3 h). It did not compete with BODIPY-cyclopamine for binding with Smo, indicating that it acts by a mechanism other than the direct Smo binding. Compound **123** was shown to inhibit the class IV alcohol dehydrogenase, Adh7.[134] Hh pathway antagonists structurally similar to **123** were also discovered by screening another chemical library.[136]

Licentia Oy screened a diversity-oriented library of 6000 small molecules in a Gli-responsive luciferase reporter assay in the Shh-LIGHT2 cell line. They identified 12 compounds with IC_{50} values ranging from 100 nM to 1 μM, including **125**−**127** (Figure 17), which share the 2-aminothiazole structure with **123** and **124**.[124]

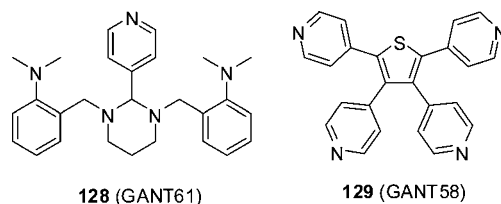

Figure 18. Small molecule Gli-transcription inhibitors from a high-throughput screen.

Inhibitors of Gli-Mediated Transcription

Most of the medicinal chemistry efforts involving the Hh-Gli pathway have focused on targeting Smo. However, some cancers have alternative mechanisms for activating the Hh-Gli signaling through effectors that are downstream of Smo, thus rendering Smo inhibitors ineffective. For example, mutation or overexpression of *SuFu*,[21,55,137] *REN*,[66,67] *Gli1*,[6,30,138] and *Gli2*[56,139] has been shown to activate this pathway. Thus, inhibitors of Gli-transcription, the final event in the pathway, would have broader applicability in cancers, irrespective of the component responsible for the activation of the Hh-Gli signaling.

Lauth et al.[140] reported two small-molecule inhibitors of the Gli-transcription, **128** (GANT61) and **129** (GANT58) (Figure 18), discovered in a screen based on inhibition of Gli1-transcription in HEK293 cells transiently transfected with plasmid cDNAs encoding Gli1 and a Gli-responsive luciferase reporter. Since they block the reporter signal induced by exogenously up-regulated Gli1, they are expected to be active against cancers in which Gli1 is overexpressed.[74,138] Both compounds also inhibited endogenous Hh signaling at an IC_{50} of 5 μM in an NIH 3T3 cell line in which the Gli reporter was stably incorporated and induced with a Smo agonist. Unlike cyclopamine, compounds **128** and **129** decreased the expression of *Gli1* and *Hip1* in Sufu$^{-/-}$ cells, indicating that they act downstream of SuFu. Gli1-positive 22Rv1 prostate cancer xenografts in mice were eradicated by subcutaneous injection of 50 mg/kg of compound **128** every second day for 18 days.[140]

Natural Products for Inhibition of Gli-Transcription

Hosoya et al.[141] designed a cell-based assay to determine Gli1-mediated transcription (Gli-responsive luciferase reporter assay under presence of *exogenously* overexpressed Gli1) and cell viability (using a fluorimetric microculture cytotoxicity assay) to screen a library of 94 natural products and 192 plant extracts for ability to inhibit the Gli1-transcription without affecting cell viability. Cyclopamine was inactive in this assay

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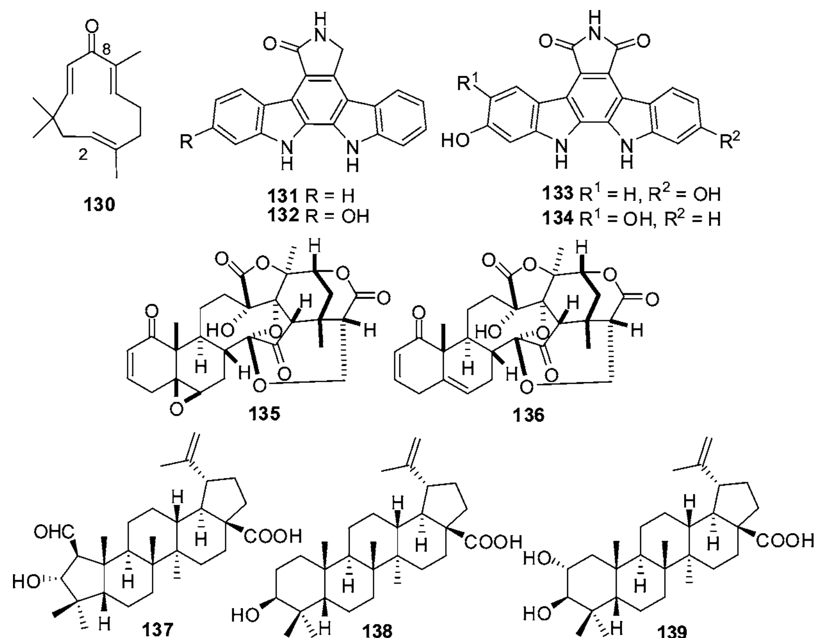

Figure 19. Natural product inhibitors of Gli-transcription.

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at 40 μM, indicating that the assay selectively targeted signaling downstream of Smo. Active compounds were also evaluated for inhibition of Gli2-transcription activity. Three novel classes of inhibitors were identified (Figure 19).[141]

The sesquiterpene zerumbone (**130**), isolated from *Zingiber zerumbet*, inhibited Gli1- and Gli2-transcription with IC_{50} values of 7.1 and 0.91 μM, respectively, showing 7-fold selectivity for Gli2 over Gli1. The enone moiety of **130** is important for the inhibition. Other terpenoids, flavonoids, phenylpropanoids, and their glycosides showed weak or no inhibition. Staurosporinone (**131**) was most active among the bisindole alkaloids **131**−**134**, inhibiting Gli1- and Gli2-transcription with IC_{50} values of 1.8 and 2.7 μM, respectively. The extract of *Physalis minima* was the most active of 15 plant extracts. The 13,14-*seco*-16,24-cyclosteroids physalin F (**135**) and physalin B (**136**) isolated from this plant extract inhibited Gli1-mediated transcription with IC_{50} values of 0.66 and 0.62 μM, respectively, without affecting the cell viability. Both compounds showed 2- to 2.5-fold selectivity for Gli1 over Gli2. Compounds **131**, **135**, and **136** had 3- to 5-fold greater cytotoxicity in the PANC1 human pancreatic carcinoma cell line, which has activated Hh-Gli signaling, than in the C3H10/T1/2 cell line.[141]

The same group also evaluated the active component of an extract of *Zizyphus cambodiana* (Rhamnaceae). The pentacyclic triterpenes colubrinic acid (**137**), betulinic acid (**138**), and alphitolic acid (**139**) inhibited Gli1-mediated transcription with IC_{50} values of 38, 32, and 42 μM, respectively. All three compounds showed cytotoxicity against the PANC1 and DU145 cell lines, and compound **137** also inhibited *Ptch* expression in PANC1 in a dose-dependent manner. Compounds **137** and **138** induced apoptosis and decreased the expression of *Bcl2*, an antiapoptotic target gene of Gli1-transcription.[142]

Triarylpyrazolines

Pyrazoline compounds **140** and **141** (Figure 20) down-regulated expression of *Ptch1* and *Gli1* in the non-small-cell lung carcinoma (NSCLC) cell lines H460 and A549 at 30 μM concentration.[143] These compounds also induced apoptosis in NSCLC, melanoma, mesothelioma, sarcoma, human hepatocellular carcinoma, colon, prostate, pancreatic, breast, gastric, nasopharyngeal, and glioma cell lines. Alteration of the length of the carbon chain (**142**) or replacement of the hydroxyl group on the amide substituent with a carboxylic acid (**143**) or alkyl (**144**) group decreased or eliminated the cytotoxicity. Compound **145**, with isopropyl groups on the pyrazole ring in place of phenyl, showed loss of the cytotoxicity. Compound **141** suppressed the growth of NSCLC H460, melanoma LOX, and NSCLC A549 in mouse xenograft models.[143]

We further analyzed the SAR of **141** analogues by synthesizing **146**−**151** (unpublished results, Figure 20). Removal of the phenolic hydroxyl of **141** (**146**) substantially decreased the inhibitory activity for Gli1-transcription in C3H10/T1/2 cells that were transiently co-transfected with plasmid cDNAs encoding Gli1 and a Gli-responsive luciferase reporter. Aromatization of the pyrazole ring (**147**) resulted in loss of activity. Alteration of the linker element in **141** by reversing the amide group afforded an equipotent compound **148**. The methoxy analogue **149** and the aromatized analogues **150** and **151** showed weaker inhibition. We also have replaced the pyrazoline moiety with another scaffold and will describe the results in a forthcoming report.

Conclusion and Perspective

Involvement of the Hh-Gli pathway in many cancers has motivated pharmaceutical companies and academic drug discovery groups to develop inhibitors for this pathway.[145] These discovery efforts have led to several lead compounds and clinical candidates (Table 1). Cyclopamine proved to be an excellent prototype Hh pathway inhibitor and helped in elucidating the biology of the pathway. A part of the drug discovery efforts have focused on cyclopamine analogues that have resulted in one compound with an improved profile that is currently in phase I clinical trials.[13] High-throughput screening, the other main focus of the drug discovery efforts, and subsequent medicinal chemistry studies have also led to discovery of a diverse set of small molecules targeting Smo. Table 2 enlists the screening assays used for evaluation of compounds presented in this review. Compound **54** was the first Smo inhibitor to enter the clinical trials as a topical agent in patients with BCC, but trials were subsequently halted after the phase I study.[89] Results

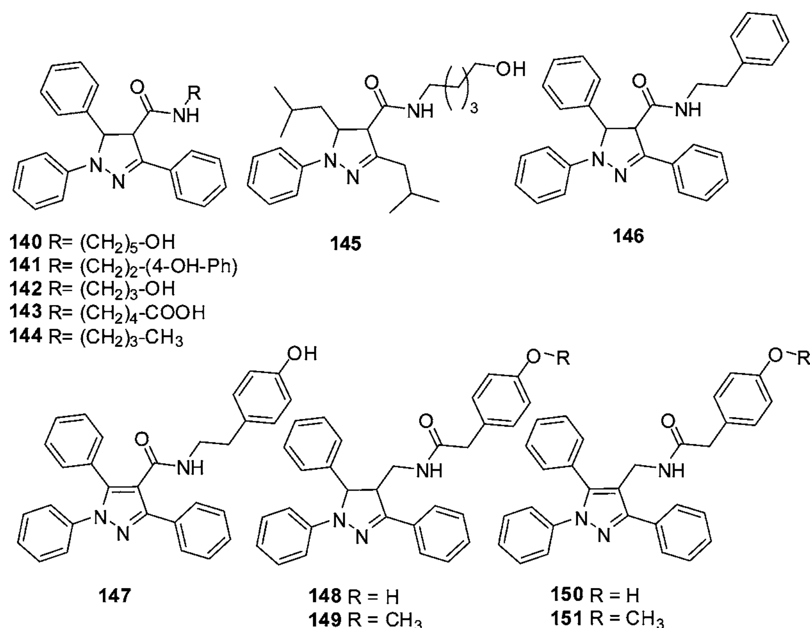

Figure 20. Triarylpyrazolines. Compound **140**−**146** and **148**−**149** are racemic trans isomers.

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Table 1. Hh-Gli Pathway Inhibitors in Clinical and Preclinical Studies[a]

compd	target	stage of development
cyclopamine (**3**)[144]	Smo	preclinical/clinical
KAAD-cyclopamine (**14**)[97]	Smo	preclinical
18[101]	Smo	preclinical
26[13]	Smo	phase I
54[89]	Smo	halted after phase I
77[14,114]	Smo	phase I
96[65,123]	Smo	preclinical
101[108]	Smo	phase I/II
128[140]	Gli	preclinical

[a] As of December 2008.

Table 2. Assay Methods Used in Each Discovery Program

assay method	compd
chick embryo neural plate explant	**3**−**10**[95]
Shh-responsive luciferase reporter assay in a colonal cell line derived from NIH-3T3	**11**−**16**[96]
alkaline phostphatase	**1**−**2**,[85] **17**−**24**,[100] **27**−**34**,[104] **35**−**45**[105]
Shh-LIGHT-II	**1**−**2**,[85] **50**−**53**,[109] **72**−**76**,[113] **115**−**120**,[131] **125**−**127**[124]
Gli-responsive luciferase reporter (no exogenous *Gli*)	**54**−**66**,[110,111] **67**−**71**,[112] **78**−**86**,[115] **87**,[117] **88**−**91**,[118] **92**−**96**,[119] **101**−**103**,[125] **104**−**106**,[126] **107**−**114**,[129] **123**−**124**,[134] **128**−**129**[140]
Smo binding	**78**−**86**[115]
Gli-responsive luciferase reporter with exogenous *Gli1* or *Gli2*	**130**−**139**,[141,142] **140**−**145**,[143] **146**−**151**

of the phase I trial of another small molecule Smo inhibitor **101** have provided a proof-of-concept for Hh-Gli pathway inhibitors as anticancer agents (six of nine patients with BCC showed a confirmed response or a confirmed partial response, and two had a stable disease).[128,146]

Most drug discovery efforts till now have been focused on targeting Smo. However, recently compounds targeting Gli-mediated transcription,[140−143] a downstream event in the Hh-Gli pathway, have also been reported and can be good prototypes for medicinal chemistry studies. Furthermore, inhibitors targeting the downstream events could provide a broader spectrum of activity against cancers where Hh-Gli pathway components downstream of Smo including *SuFu*,[21,55,137] *REN*,[66,67] *Gli1*,[6,30,138] and *Gli2*,[56,139] (Figure 1) have been implicated in deregulation. Smo inhibitors might not be effective in such cases; for example, Gli proteins in isolated *SuFu*(−/−) mouse embryonic fibroblasts, having cell-autonomous downstream activation of the Hh signaling, showed a constitutive Gli activity that could not be inhibited by cyclopamine, as activation occurs downstream of Smo.[147,148] Drugs specifically designed to modulate these downstream components would provide personalized medicine for the patients with specific mutations in this pathway.

The Hh-Gli pathway has been reported to play a role in metastasis.[74,81,149] Gli1 has been shown to induce expression of *Snail*, resulting in reduced expression of proteins such as E-cadherin that maintains epithelial organization, thus resulting in a metastatic phenotype that can be inhibited by cyclopamine.[74] *Gli1* and *Snail* were also significantly overexpressed in 50% and 75% of the metastatic foci, respectively, in samples obtained from patients with disseminated pancreatic cancer.[150] The local or systemic interference in Hh-Gli signaling was reported to inhibit melanoma growth and prevent metastasis in mice.[149] Therefore, inhibitors of the Hh-Gli pathway may be effective in preventing metastasis.

The Hh-Gli pathway is essential in development and tissue maintenance; therefore, great care should be taken to assess the safety of drugs that act through this pathway. Phase I clinical study of **101** indicates a satisfactory safety profile in adults,[12] but permanent defects in bone structure were observed in young mice (10−14 days old) exposed to another Smo inhibitor (**96**),[123] though no serious side effects were observed in adult mice.[65] This would also underline, as mentioned previously, the importance of designing inhibitors of downstream events of this pathway especially for pediatric therapeutics. Gli1-transcription, one such downstream event, plays an important role in several cancers[6,30,139] and has been suggested as a potential target for development of novel therapies.[121,138] *Gli2* expression has also

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been implicated in the tumorigenicity of human glioma stem cells.[30] *Gli1* knockout mice have been reported to show no obvious abnormalities, whereas *Gli2* and *Gli3* knockout mice show abnormal skeletal growth and embryonic or perinatal lethality.[5,43,45,138] Gli2 is an important factor for expression of parathyroid hormone-related peptide (PTHrP) in the developing growth plate, while Gli1 and Gli3 do not stimulate the PTHrP promoter.[44] An important mediator of bone differentiation, bone-morphogenetic protein (BMP)-2 is regulated by Gli2 but not by Gli1.[151] Runx2, an essential transcription factor for bone formation, has a downstream function of BMP2 signaling and is up-regulated by Gli2 in C3H10/T1/2 cells[152] that express very little endogenous Gli1. Therefore, selective inhibition of Gli1-transcription might eliminate the permanent bone defects observed with a Smo inhibitor.[145]

The significance of the Hh-Gli pathway as an anticancer drug target has been supported by numerous experiments using homogeneous cancer cell cultures with aberrant Hh-Gli signaling. Recent evidence has shown the importance of the paracrine mechanism for up-regulating the Hh signaling in a tumor animal model.[120] This observation raises the need to consider the stroma−tumor interactions in Hh-Gli signaling in the experimental models.[153] Other observations also suggest that maintenance of the Hh-Gli pathway in cancers needs such paracrine regulation. For example, in a human pancreatic xenograft mouse model, compound **29** only down-regulated the murine *Gli1* mRNA levels but not the human *Gli1*.[104] Nolan-Stevaux et al.[154] have also suggested that paracrine signaling in adjacent mesenchymal cells via secreted Shh ligand is responsible for pancreatic ductal adenocarcinoma progression. Such stroma−tumor interactions have been well demonstrated in many faces of cancer progression, as pioneered by Judah Folkman's work in tumor angiogenesis. Secondary screens that mimic the paracrine Hh environment could be used to further evaluate hit compounds. Coculture of cancer cells and stroma cells have been previously used to study microenvironments involving paracrice Hh controlled angiogenesis.[155] The *functional* role of the Hh-Gli pathway in tumor progression needs to be further studied for creating the next generation of lead compounds with better safety and clinical efficacy.

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