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Review

Modulators of the hedgehog signaling pathway

Philipp Heretsch, Lito Tzagkaroulaki, Athanassios Giannis *

Institut für Organische Chemie, Universität Leipzig, 04103 Leipzig, Germany

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ABSTRACT

Since its discovery by C. Nüsslein-Volhard and E. F. Wieschaus, hedgehog (hh) signaling has come a long way. Today it is regarded as a key regulator in embryogenesis where it governs processes like cell proliferation, differentiation, and tissue patterning. Furthermore, in adults it is involved in the maintenance of stem cells, and in tissue repair and regeneration. But hh signaling has a second—much darker—face: it plays an important role in several types of human cancers where it promotes growth and enables proliferation of tumor stem cells. The etiology of medulloblastoma and basal cell carcinoma is tightly linked to aberrant hh activity, but also cancers of the prostate, the pancreas, the colon, the breasts, rhabdomyosarcoma, and leukemia, are dependent on irregular hh activity. Recent clinical studies have shown that hh signaling can be the basis of an important new class of therapeutic agents with far-reaching implications in oncology. Thus, modulation of hh signaling by means of small molecules has emerged as a valuable tool in combating these hh-dependent cancers. Cyclopamine, a unique natural product with a fascinating history, was the first identified inhibitor of hh signaling and its story is closely linked to the progress in the whole field. In this review we will trace the story of cyclopamine, give an overview on the biological modes of hh signaling both in untransformed and malignant cells, and finally present potent modulators of the hh pathway—many of them already in clinical studies. For more than 30 years now the knowledge on hh signaling has grown steadily—an end to this development is far from being conceivable.

1. Introduction[1]

In the late 1970s C. Nüsslein-Volhard and E. F. Wieschaus at the *European Molecular Biology Laboratory* studied mutations of the fruit fly *Drosophila melanogaster* and identified more than 50 different genes that have a direct effect on embryonic development. One of these genes, when it was mutated, caused the larvae to grow a coat of spines at their undersides. The similarity in appearance of these larvae with a hedgehog led to the term *hedgehog*-gene (*hh*).[2] For their landmark research in the field of genetics of early embryonic development Nüsslein-Volhard and Wieschaus together with E. B. Lewis were awarded with the Nobel Prize in medicine in 1995.[3] In 1993 the three paralogous genes in vertebrates were identified and termed *Sonic hh* (*Shh*), after Sega's video game hero, *Indian hh* (*Ihh*), and *Desert hh* (*Dhh*), both after existing hedgehog species.[4–7]

The *hedgehog*-genes are highly conserved from fruit fly to human and today are regarded as key regulators of embryonic development.[8] They govern processes such as cell proliferation, differentiation, and tissue patterning. In insects hh signaling controls correct segmentation and development of the wings, in vertebrates it induces left–right-asymmetry and correct formation of limbs, skeleton, muscles, skin, eyes, lungs, teeth, nervous system, intestines, and differentiation of sperm and cartilage. In adult

Abbreviations: AKT, protein kinase B; ALP, alkaline phosphatase; BCC, basal cell carcinoma; BCR-ABL1, breakpoint cluster region-Abelson murine leukemia; β-TrCP, β-transducin repeat containing protein; BMP, bone morphogenic protein; Boc, biregional cell adhesion molecule; BODIPY, boron-dipyrromethene; 2BP, 2-bromo palmitate; CDO, ciliary dysfunction-only; CFP, cyan fluorescent protein; CK1ε, casein kinase 1ε; CML, chronic myeloic leukemia; Cul1, Cullin1; Dhh, desert hedgehog; Disp, dispatched; Dyrk, dual-specifity tyrosine-(Y)-phosphorylation-regulated kinase; Eg5, kinesin family member 11; ERK, extracellular signal-regulated kinase; Fn, fibronectine; FGF, fibroblast growth factor; FRET, fluorescent resonance energy transfer; GANT, Gli antagonist; Gas1, growth arrest specific 1; GFP, green fluorescent protein; Gli, glioma-associated oncogene; GPI, glycosidyl-phosphatidylinositol; GSK3β, glycogen synthase kinase-3β; hh, hedgehog; Hhat, hedgehog acyl transferase; Hhip, hedgehog interacting protein; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; HPI, hedgehog pathway inhibitor; Ihh, Indian hedgehog; Ig, immunoglobulin; IFT, intraflagellar transport; IGF, insulin-like growth factor; KAAD, 3-keto-*N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl); KIF7, kinesin family member 7; 14LDM, 14-α-lanosterol demethylase; MAPK/MEK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; PAT, palmitoyl transferase; PKA, protein kinase A; PKC, protein kinase C; Ptch, Patched; Ras, rat sarcoma; REN, rennin; SAG, Smo agonist; SANT, Smo antagonist; Shh, sonic hedgehog; Shh-N, sonic hedgehog N-terminal peptide; SMM, small-molecule microarrays; Smo, smoothened; SSD, sterol sensing domain; SuFu, suppressor of fused; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor; Wnt, wingless; YFP, yellow fluorescent protein.

* Corresponding author. Tel.: +49 341 9736531; fax: +49 341 9736599.
E-mail address: giannis@uni-leipzig.de (A. Giannis).

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organisms this pathway has important functions in the maintenance of stem cells, as well as in tissue repair and regeneration.

Recent research has revealed the darker side of hh signaling: in mammals it is linked to the etiology of basal cell carcinoma (BCC) and medulloblastoma. Additionally, cancers of the pancreas, prostate, lung, and breast are dependent on irregular hh activity. Inhibition of hh pathway activity by the means of small molecules has therefore led to new therapeutic strategies. Contrarily, stimulators of the hh pathway may find applications as angiogenesis inducers, as promoters of wound healing, and as valuable tools in the field of stem cell research.

This review will discuss the hh signaling pathway, different models of hh involvement in cancer, its modulators (i.e., inhibitors and activators), putting an emphasis on cyclopamine and related molecules, as well as recent and possible future applications of hh modulation in the therapy of inflammatory and proliferative diseases. An exhaustive presentation of all known hh inhibitors is beyond the scope of this article and the interested reader is referred to several reviews on this field.[9–15]

2. The hedgehog signaling pathway

In order to understand the modes of action of different types of hh pathway modulators a basic knowledge of the components of hh signaling and their interplay is necessary.

The hh pathway was discovered in *D. melanogaster*, but it was found to be relatively conserved in different species including humans.[16,17] In mammals the *hh*-genes encode for three unique proteins, Shh, Ihh, and Dhh.

2.1. Maturation of the hedgehog proteins

The hh proteins have to undergo a maturation process before their active forms can be released from the cell and activate hh signaling in auto-/juxta- or paracrine manner (see Fig. 1). Hh proteins are known to be covalently modified by lipid moieties, which membrane-anchoring properties are not consistent with passive models of protein mobilization within tissue.[18] After translation, the N-terminal signal sequence is first removed from the ~45 kDa precursor polypeptides and then an autocatalytic cleavage between GlyCys residues, that are part of an absolutely conserved GlyCysPhe tripeptide, forms the N-terminal signaling domain. Additionally, this signaling domain is modified in this process at the C-terminal glycine by cholesterol and hence gives a ~19 kDa segment with which all known signaling activities are associated. The cholesterol moiety is, paradoxically, not required for signal transduction but acts as a lipid anchor that restricts spatial mobility and results in the association of the precursor protein with the plasma membrane. It has been shown in vitro that other steroidal compounds can substitute for cholesterol in the process-

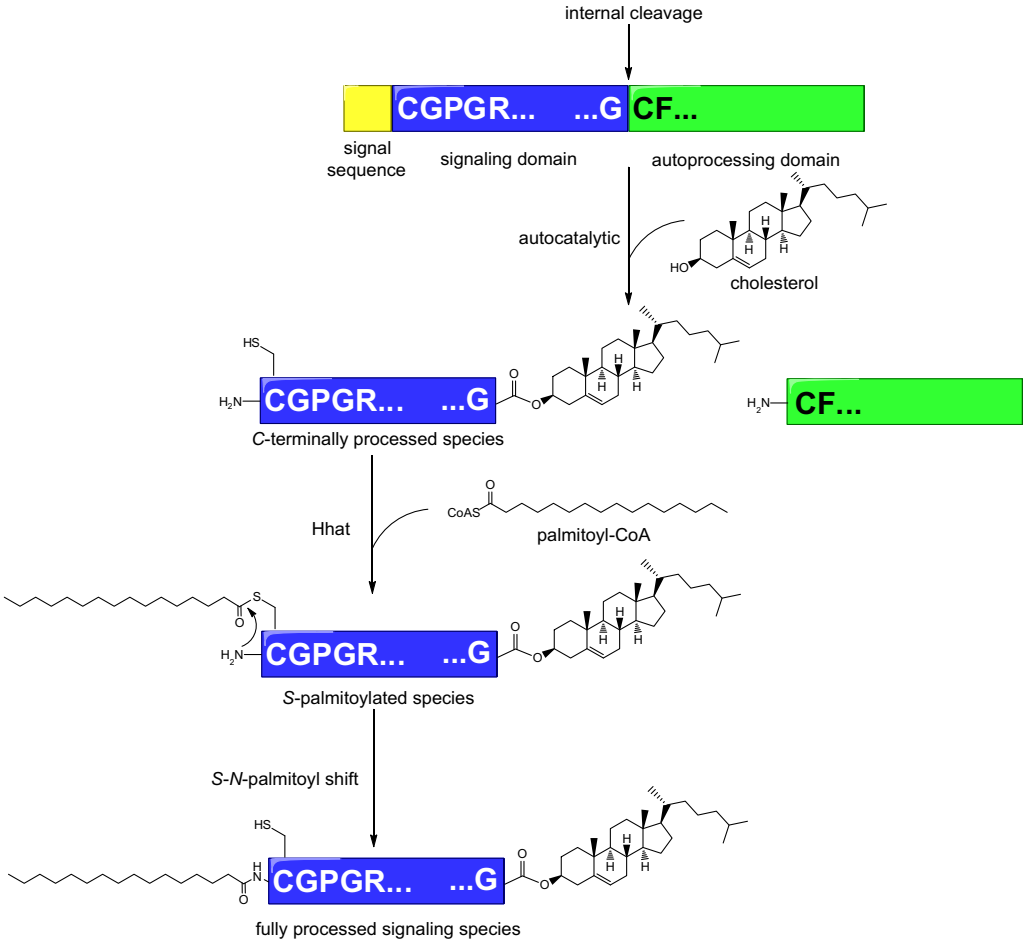

Figure 1. Maturation of the hh protein. hh-Genes encode precursor polypeptides of ~45 kDa which undergo both N-terminal signal sequence trimming and acylation as well as internal proteolysis at a conserved sequence. Endoproteolytic cleavage at the GlyCysPhe sequence is catalyzed by the processing activity associated with the C-terminal domain and produces a ~19 kDa segment. During cleavage, the signaling domain is modified at its C-terminal Gly by cholesterol, the N-terminal Cys then becomes palmitoylated by Hhat.

ing of hh proteins. Additionally, it has been speculated that in vivo not only cholesterol, but other endogenous steroids are possibly attached in the hh maturation process.[18]

At the plasma membrane, finally, the N-terminal cysteine is palmitoylated by the enzyme Hhat (hedgehog acyl transferase) to form the fully active hh signaling protein.[19] The cholesterol modification is not a necessity for the attachment of palmitate to the hh protein, but the highly conserved N-terminal CysGlyProGlyArg-sequence is an absolute requirement. Unlike most known palmitoylated proteins, the palmitate moiety is attached via an amide bond to the N-terminus. The fatty palmitoylation is proposed to occur via a thioester intermediate involving the side chain of the N-terminal cysteine, followed by a spontaneous rearrangement (*S*-*N*-acyl shift) to form the amide bond. This final modification contributes critically to full signaling potency of hh proteins, though experiments indicate that this enhancement of signaling activity is largely attributed to general hydrophobic effects rather than exclusive specificity for palmitate.[18] Secretion of mature hh proteins is dependent on dispatched (Disp), a 12-transmembrane protein. Although, both Patched, the hh receptor, and dispatched, contain a sterol sensing domain (SSD), these SSDs do not aid binding of the cholesterol adduct of the hh protein. The dependence of cholesterol in the maturation process of hh proteins played an important role in the discovery of the mode of action of the first hh inhibitor cyclopamine (see Section 4).

2.2. The hedgehog signaling pathway in vertebrates

The mature hh proteins are ligands of the membrane bound receptors Patched1 and Patched2 (12-transmembrane proteins termed Ptch)[20,21] and activate the hh signaling pathway, that is, they have a direct influence on the transcription of hh-response-genes. There are further regulators on the cell surface that enhance or reduce binding of hh ligands to Ptch. A negative regulator is the Hhip protein that competes with Ptch for ligand binding. Contrary to this, positive regulators like immunoglobulin (Ig)/fibronectine (Fn)-repeat-containing proteins Cdo and Boc, as well as glycosidylphosphatidylinositol (GPI)-anchored membrane-bound protein Gas1 enhance binding of hh ligands to Ptch.

Remarkably, all of the key components of the hh pathway are enriched in the cilia (see Fig. 2). Two transmembrane proteins, Ptch1 and smoothened (a seven-transmembrane protein termed Smo, which resembles G-protein-coupled-receptors and acts downstream of Ptch1) show dynamic, hh-dependent trafficking in the cilia.

Cilia are tail-like projections of the cell membrane that can be found on nearly every eukaryotic cell in a single copy. They are involved in sensing mechanical and chemical signals and act as communication hubs for signaling and control cell differentiation and polarity.[22,23] In the absence of hh ligands, membrane bound protein Ptch1 is found at the base of the primary cilium near the basal body.[24] Smo is usually not associated with the cilium because the dynein retrograde intraflagellar transport (IFT) motor prevents enrichment of Smo in the cilium.[25] Instead, Smo exists in three different states: an internalized inactive form (SmoA), that is, in equilibrium with an inactive cilium-bound form (SmoB), and an active form (SmoC), that is, generated from SmoB.[26,27] Immunoprecipitation assays have shown that Smo forms a dimer. Furthermore, a fluorescent resonance energy transfer (FRET) analysis of Smo proteins bearing yellow fluorescent protein (YFP) and cyan fluorescent

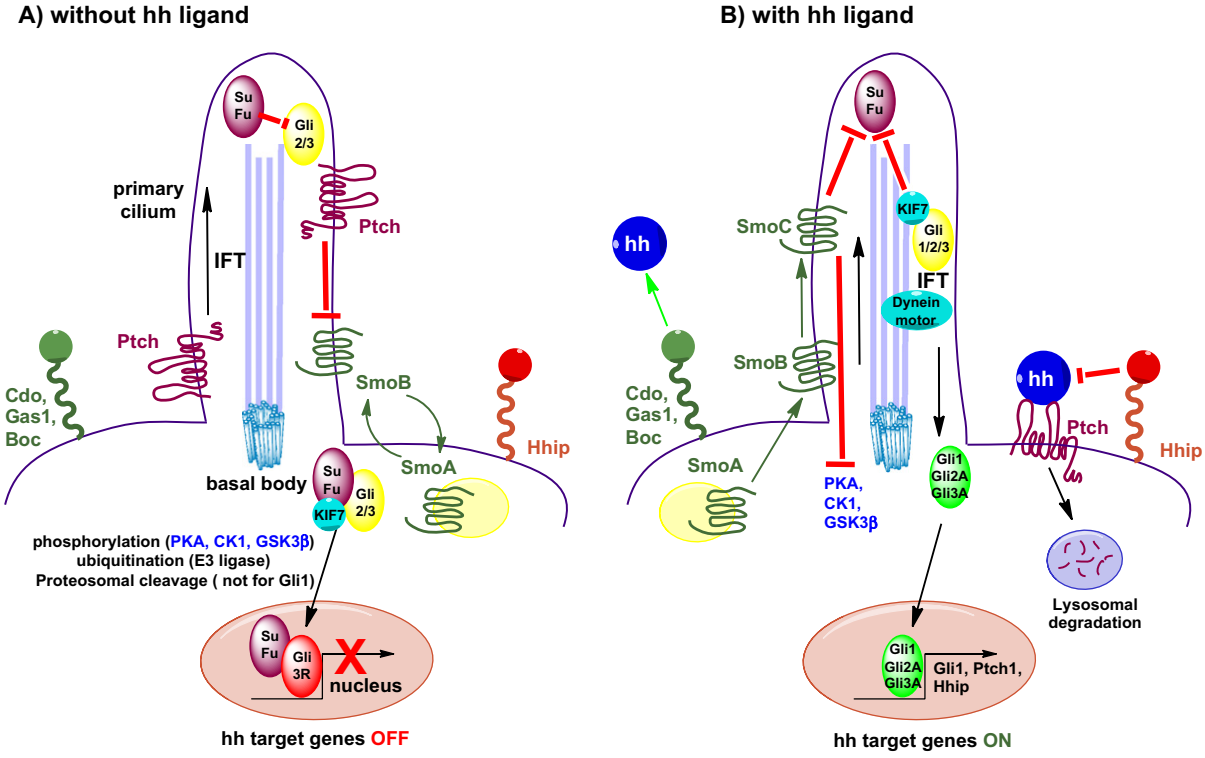

Figure 2. The hh signaling pathway. (A) In the absence of a hh ligand Ptch is located at the ciliary membrane, where it inhibits the formation of active SmoC from ciliary membrane located SmoB (itself in equilibrium with internalized inactive SmoA by the dynein retrograde intraflagellar transport (IFT) machinery) by an unknown mechanism. Without SmoC the Gli2/3 transcription factors are stepwise labeled by phosphorylation and ubiquitination events for proteosomal cleavage into a truncated repressor form (Gli3 to Gli3-R) or complete degradation (Gli2). Gli3-R travels into the nucleus, presumably by the retrograde IFT machinery[37,38] and inhibits gene transcription of hh-target-genes. Gli1 (not shown) is always active and activates transcription of genes of hh components to keep the pathway functional. (B) In the presence of a hh ligand it binds to Ptch, that is, inhibited by internalization and lysosomal degradation. Binding can be repressed by Hhip and supported by Cdo, Gas1 and Boc. Without Ptch active SmoC is formed at the ciliary membrane where it inhibits kinases PKA, CK1 and GSK3β. Now the Gli transcription factors Gli2/3 can be processed to their active forms (Gli2/3-A) that are transported to the nucleus where they finally activate gene transcription of the hh-target-genes. KIF7 as a typical motor protein acts as an anterograde motor in the cilium.

protein (CFP) at their C-terminal cytoplasmatic tails, has shown that Smo is a conformational switch, changing between two forms referred to as the 'open' and active (also referred to as SmoC) and the 'closed' and inactive form (SmoB). Phosphorylation events at arginine rich regions at the intracellular part of Smo may attribute to this behavior.[28]

At the base of the primary cilium, Ptch inhibits the activation of SmoB into SmoC by an unknown mechanism. Ptch inhibits Smo in a catalytic (though not enzymatic) way since one molecule of Ptch can inhibit several molecules of Smo. The idea of a stoichiometric model in which Ptch binds to Smo has been abandoned. Instead, endogenous small molecules that modulate Smo activity and that are gated by Ptch are discussed (e.g., oxysterols), but no such small molecules have been identified yet.[29]

In the absence of active SmoC in the ciliary membrane, the Gli family of latent Zn-finger transcription factors, in a complex with SuFu (the suppressor of protein fused, an important negative regulator of mammalian hh signaling),[30] are proteosomically processed. This is sequentially accomplished by protein kinase A (PKA), GSK3β, and CK1ε-mediated phosphorylation of full-length Gli2/3, which creates a binding site for the adapter protein β-TrCP. Then, the Gli/β-TrCP complex becomes subject to ubiquitination mediated by the Cul1-based E3 ligase, which finally results in partial proteosomal degradation to form Gli3-R[31] or complete degradation in the case of Gli2.[32,33] The Gli3-R factor prevents transcription of the hh-response-genes. Whether cytoplasmic nuclear shuttling of Gli is also dependent on SuFu is not clear yet. Contrary to this, Gli1 only occurs as a full-length transcriptional activator of genes of hh components to keep the pathway functional.[34] However, in vivo the transcription repressor Gli3-R is the predominant species.

Upon binding a hh ligand, Ptch translocates out of the primary cilium and is degraded by lyosomes. Active SmoC can now be generated and inhibits PKA which no longer phosphorylates full-length Gli and as a result prevents β-TrCP/Cul1 binding and processing, and eventually proteosomal Gli cleavage. Activated Gli transcription factors (GliA) then bind to Gli promoters in the nucleus and stimulate the transcription of hh-response-genes. Direct targets for GliA are three genes in the pathway themselves: *Gli1*, *Ptch1*, and *Hhip*.[35,36] Since the products of these genes are positive or negative regulators of hh signaling, this leads to feedback loops that enhance or reduce hh response. It is not known whether the set of target genes is the same for the GliR and GliA transcription factors. While in *D. melanogaster* cilia are not required for hh signaling, in vertebrates they play a role of paramount importance (vide supra). This may be due to the role of KIF7, a kinesine that tethers the hh signaling to the cilium and is only present in vertebrates. KIF7, as a typical motor protein, acts as an anterograde motor in the cilium.

Finally, it is important to emphasize that the hh signaling pathway is tightly connected to many other signaling pathways like Wnt/β-catenin, TGF-β/BMP, Notch and FGF pathways, all of them deeply involved in processes of tissue morphogenesis and homeostasis, organogenesis, and stem cell renewal in adults.

3. The hedgehog signaling pathway in cancer

Three basic models are discussed for hh pathway activity in cancer in literature (see Fig. 3):[39–45]

Type 1 cancers (ligand independent, mutation driven) were discovered first. The Gorlin syndrome belongs to this category. Patients with Gorlin syndrome have a high incidence of basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma. The molecular basis for the development of this type of malignancies can be numerous. Inherited inactivating mutations in *Ptch1* (i.e., *Ptch1* loss of heterozygosity and/or inactivating mutations), lead to constitutively activated hh signaling in the absence of a ligand. Gorlin patients are excellent candidates for therapy applying inhibitors at the level of Smo or below. Loss of function mutations have also been described for SuFu. An activating mutation in Smo results in ligand-independent constitutive hh pathway activation. Additionally, gene amplification and translocation of positive-acting components in the pathway like Gli belong to this category. It is important to realize that the therapy of type 1 cancers by inhibition of constituents of the hh pathway can only be successful, if these are situated downstream of the acquired mutation. We will show later that most inhibitors target the protein Smo. However, inhibitors downstream of Smo (e.g., on transcriptional level) would obviously be the only effective means to generally address hh-dependent cancers of type 1.

Type 2 cancers are hh ligand dependent and auto- or juxtacrine. A hh ligand is both produced and responded to by the same tumor cells. Only recently it was demonstrated conclusively in human cancer cell lines of the colon that this type of hh signaling exists.[46]

Type 3 cancers are hh ligand dependent and paracrine. Recently, evidence accumulated that in most cancers aberrant hh signaling is linked to an overproduction of hh ligand by the tumor cells. Hh proteins may stimulate stroma cells near the tumor (endothelial cells, epithelial cells, fibroblasts, and immune cells) in a paracrine manner, which results in an indirect support of tumor growth through mechanisms originating in the stroma cells. Such mechanisms include the support of tumor cells including tumor stem cells, stimulation of tumor angiogenesis, effects on the extracellular matrix, and secretion of components of molecular signaling pathways involving insulin-like growth factor (IGF) and Wnt.[47,48] A variant of this type of hh signaling is the so-called 'reverse paracrine' signaling (type 3b), whereby hh ligands are secreted from stromal cells to receiving cells in a tumor.

4. Cyclopamine and the hedgehog signaling pathway

During their studies on hh signaling in the 1990s, P.A. Beachy and co-workers searched for a simple way to control this pathway that did not involve gene knockout—a task difficult and lengthy to perform. The use of small molecules as biochemical probes presented an easier and faster alternative. However, a molecule that was able to influence hh signaling was not known at that time.

Finally, Beachy and co-workers succeeded in combining their knowledge on hh signaling with an apparently different scientific field to solve this problem: they remembered a strange incident in the 1950s in Idaho. In decade after World War II a random batch of lambs in sheep herds was born with severe craniofacial defects. Up to 25% of newborn lambs of sheep grazing in the mountains of central Idaho were affected. The severity of this malformation termed holoprosencephaly varied from the extreme of cyclopia, that is, the existence of only one eye placed directly on the forehead, accompanied with malformations of the brain, to only a slightly shortened upper jaw. The more severe malformations also included incompletely or totally undivided cerebral hemispheres, olfactorical and optical nerves. Finally, these sheep herders asked the *Department of Agriculture* for help, when the disease had taken endemic proportions and the economic loss was not tolerable anymore. One of the scientists sent, Lynn F. James, lived with the sheep for three summers and discovered that in times of drought the sheep moved to higher grounds and grazed on the abundantly growing flower *Veratrum californicum*. Richard F. Keeler of the *Poisonous Plant Research Laboratory* later figured out the connection between the consumption of *V. californicum* by pregnant sheep at the 14th day of gestation and the occurrence of cyclopia in their offspring.[49–51]

On the basis of these initial studies an extraction method was developed, that allowed the alkaloids of *V. californicum* to be

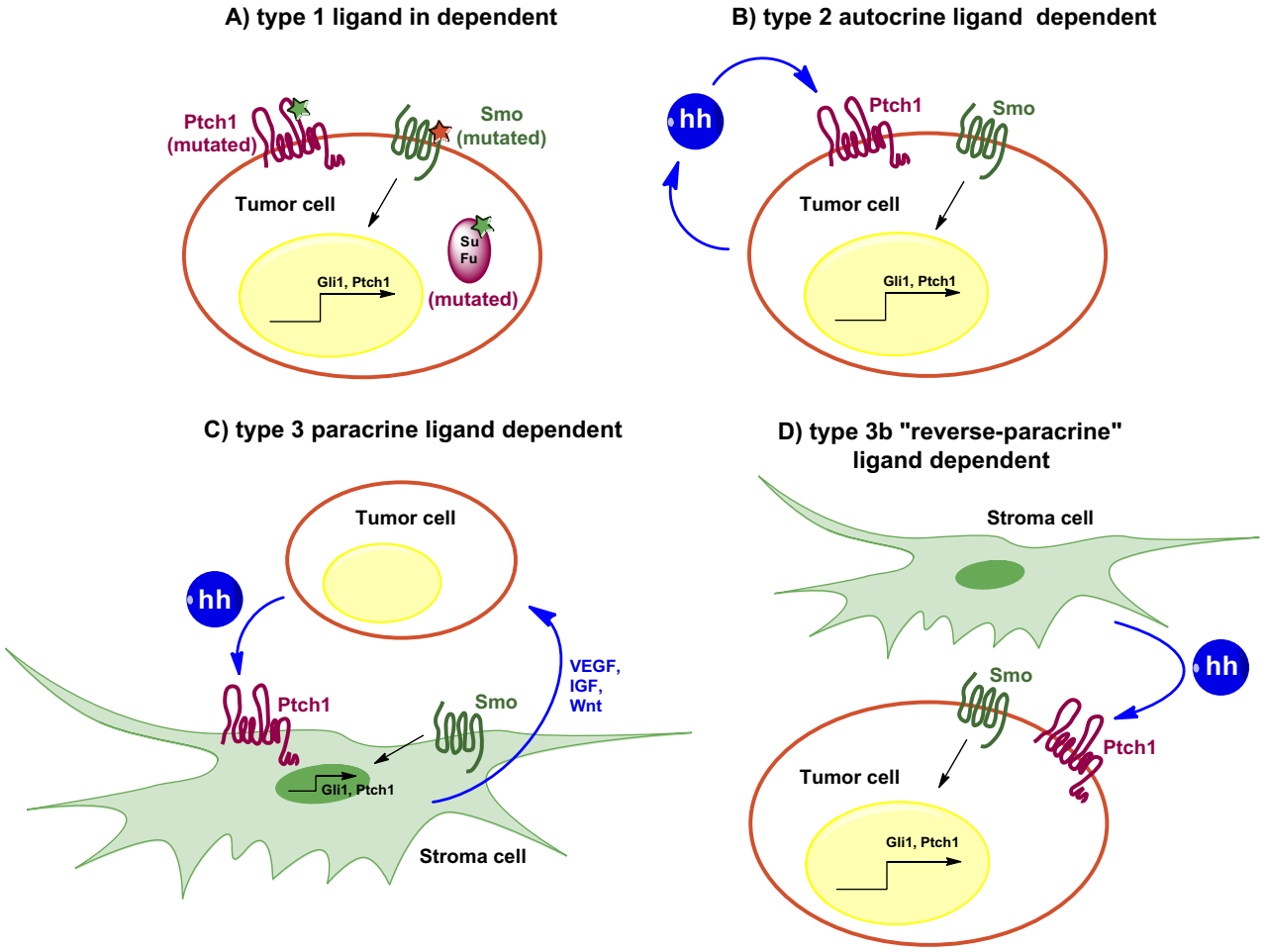

Figure 3. The hh signaling pathway in cancer. (A) Type 1 cancers are hh ligand independent. Hh pathway is overly active due to an activating or inactivating mutation in one of its constituents. (B) In type 2 (autocrine) tumors the same cell produces and accepts hh ligands. (C) Type 3 (paracrine) tumors secrete hh ligands into the stroma. Stroma cells respond by forming a growth promoting environment for the tumor. (D) In type 3b (reverse-paracrine) tumors the reverse situation can be observed: stroma cells secrete hh ligands that promote the growth of tumor cells.

isolated for investigation of their ability to induce cyclopia in embryos. It was shown that only three compounds posses this ability in varying potency, namely the known alkaloid jervine, cycloposine (3-glucosyl cyclopamine) and a third most active steroidal alkaloid that was termed cyclopamine (see Fig. 4).[52,53]

Because Beachy and co-workers knew that in severe cases of the Smith-Lemli-Opitz syndrome (a defect in cholesterol biosynthesis caused by a deficiency of 7-dehydrocholesterol reductase) also holoprosencephaly was observed,[54] they proposed that cyclopamine acts as an inhibitor of cholesterol biosynthesis—an idea further supported by structural similarities of these molecules. However, in 1998 Beachy and co-workers could show that this first assumption was incorrect.[55,56] Instead, cyclopamine interacts with the protein Smo, induces its accumulation in the primary cilium and inhibits its activity by a conformational change into the 'closed' form even with hh ligands being present.[57,58]

The maturation process of the hh protein is dependent on cholesterol (vide supra). Although in embryos the same phenotype is observed both in cases of cholesterol biosynthesis deficiencies and cyclopamine intoxication the reason for this is not a lack of correctly modified hh proteins. It has been shown, that in sterol depleted cells a diminished hh response already occurs at sterol levels still sufficient for normal autoprocessing of the hh protein. Instead, sterol depletion affects the activity of Smo. This effect could be mediated either through direct interaction of cholesterol with Smo or through an impact on membrane properties, for example, trafficking.[59,60]

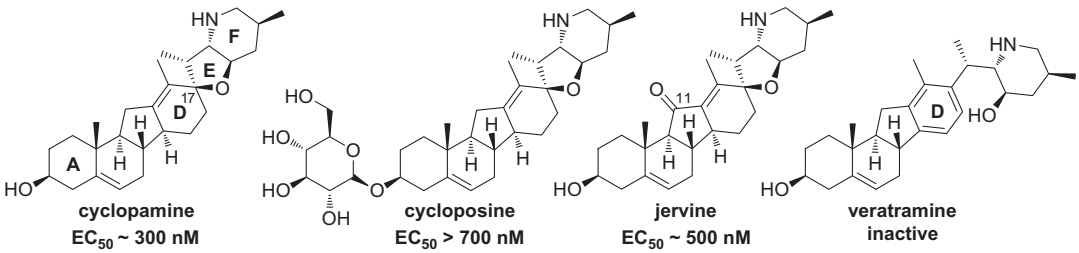

Figure 4. Structures of *veratrum* alkaloids cyclopamine, cycloposine, jervine, and veratramine.

Cyclopamine itself is currently used in clinical trials in the therapy of BCC (see Section 7) though its pharmacological and physicochemical properties are not ideal: cyclopamine exhibits only a moderate inhibition of transcription of the hh-target-genes *Gli1* and *Ptch1*, with an EC_{50} of 300 nM. Additionally, it has very poor aqueous solubility and is unstable to low pH values. However, it was shown recently, that cyclopamine does not degrade to veratramine[61] as it has been postulated by Keeler.[62] Veratramine, a natural occurring C-*nor*-D-*homo*-steroid with an aromatic D-ring and without the furane E-ring, has no hh inhibitory effect. Instead, cyclopamine isomerizes to give the C17-epimer and another non-aromatic elimination product—both of these show no hh inhibitory properties. To overcome the problems associated with cyclopamine several other inhibitors of hh signaling, in particular of Smo, have been synthesized in recent years. Some of them bear structural elements or are chemical derived from cyclopamine, others have completely different chemical scaffolds.

Recently, our group succeeded in a biomimetic synthesis of cyclopamine starting from abundantly available dehydroepiandrosterone. This new approach will facilitate the synthesis of cyclopamine-based Smo antagonists and furthermore allow deeper insights into cyclopamine's structure–activity relationship.[63,64]

5. Further inhibitors of the hedgehog signaling pathway

Although Smo has been termed the hh pathways most 'drugable' target, and a majority of the known inhibitors currently available address Smo, other components of the hh pathway can be inhibited with the same result. Among these are targets upstream of Smo, more precisely the Shh ligand itself and also targets downstream of Smo like the Gli transcription factors. Selected examples for all these strategies are presented (see Fig. 5).

For the discovery of new inhibitors of hh signaling several assays are available. The most common one is using Shh-LIGHT2 cells which represent a clonal mouse embryonic fibroblast cell line (NIH 3T3 stably incorporating a Gli1-dependent firefly luciferase reporter and a constitutive *Renilla* luciferase reporter).[65] The binding of a Shh-N protein to Ptch releases Gli transcription factors which in turn lead to the expression of luciferase. After lysis of the cells the enzyme can be detected by reaction with luciferin and luminescence measurement. An antagonist, that is, able to bind to Smo will decrease Gli1-dependent expression of luciferase and will therefore lower the luminescence. Analysis of constitutive *Renilla* luminescence is used to normalize for any potential unspecific Gli-reporter gene luminescence. The Smo agonist SAG (see Fig. 14) is frequently used in this assay to give an up-regulated (positive) readout both for comparison and determination of inhibitory strength of potential antagonists.[66]

A cell-based assay not dependent on the transfection of a reporter construct uses a hh-dependent phenotypic readout in mouse mesoderm fibroblast C3H10T1/2 cells.[67] In the presence of a Smo agonist C3H10T1/2 progenitor cells differentiate into osteoblasts, mediated by the hh signaling pathway. The enzymatic activity of alkaline phosphatase (ALP) is a marker for this hh induced process and can be readily measured. The ALP expression level in cells is influenced by increasing concentrations of hh pathway inhibitors.

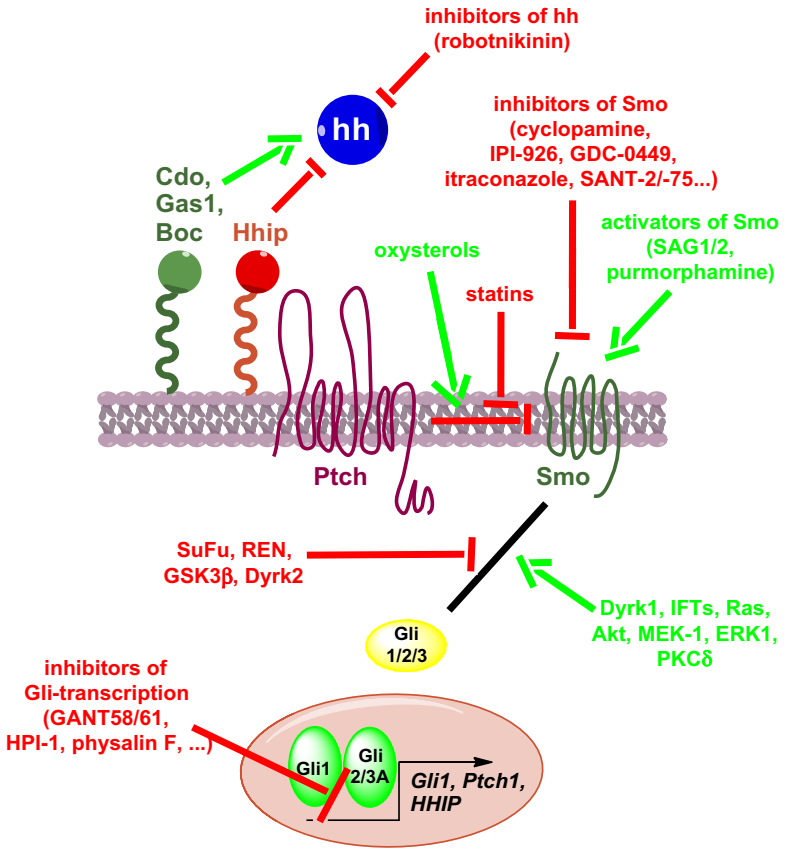

Figure 5. Mechanisms of regulation and modulation of the hh signaling pathway. The hh pathway bears several regulative elements, for example, at the stage of hh binding, and activation/deactivation of the Gli transcription factors. Small molecular inhibitors, either natural products or synthetic compounds, are known for Smo, Gli/DNA binding in the nucleus, and also for the hh ligand. Additionally, Smo agonsists are available. It is known that these agonists and also different antagonists bind to distinct sites of Smo. The inhibitory effect of Ptch on Smo can also be modulated. It is enhanced by statins and reduced by oxysterols.

5.1. Inhibitors of smoothened

Since cyclopamine was the first inhibitor of hh signaling to be discovered large efforts have been conducted to improve the deficits of this natural product mentioned before. One of the first semisynthetic cyclopamine-based inhibitors was KAAD-cyclopamine (3-keto-*N*-(aminoethyl-aminocaproyl-dihydrocinnamoyl)cyclopamine, see Fig. 6). It displays both higher potency, with an IC_{50} of 20 nM, and reduced cytotoxicity. Although, its stability with special regard to the unconjugated double bond in the B-ring remains unexplored.

Later, *Infinity Pharmaceuticals* could overcome cyclopamine's acid lability by derivatization of the D-ring. Therefore, natural cyclopamine was protected and subjected to the Simmons–Smith cyclopropanation reaction. Then, BF_3-promoted ring expansion by rearrangement gave D-*homo*-cyclopamine that was further modified to yield IPI-269609.[68] This analog both possesses better solubility (by the factor 20) and an enhanced acid stability (tested in a human liver microsomes assay) in comparison with natural cyclopamine. A number of D-*homo*-cyclopamine derivatives were then accessed by variations of the A-ring. Among these, three lead compounds (IPI-926, WO2008109184, and WO2008109829, see Fig. 7) emerged and IPI-926 was found to have the most improved pharmaceutical properties, highest potency, and a favorable pharmacokinetic profile relative to cyclopamine and IPI-269609.[69] IPI-926 has progressed into human clinical trials phase 1/2 (see Section 7.1).

Cyclopamine-based antagonists of Smo bind to a distinct site of Smo, that is, different from the one that SANT-2 (see Fig. 8) as well as the agonist SAG bind to. This has been established by competition experiments using fluorescent-labeled BODIPY-cyclopamine: KAAD-cyclopamine, for example, reduces bound BODIPY-cyclopamine to near background levels at 200 nM while SANT-2, though a potent inhibitor of Smo, has no influence on bound BODIPY-cyclopamine.

In a high-throughput screen based on murine 10T1/2 (S12) embryonic fibroblast cells containing a plasmid with a luciferase reporter gene downstream of the Gli binding site, a hit-to-lead optimization identified Smo-antagonist GDC-0449.[70] This bisarylcarboxamide exhibited excellent potency also in an analogs human Gli luciferase assay and a low clearance with high absorption in rats and dogs. GDC-0449 has progressed into human clinical trials phase 2 (see Section 7.2).

Another screen in a Shh-LIGHT2 cell assay of 10,000 small molecules gave among others the Smo antagonists SANT-2. Both inhibitors, although identified in different screens conducted by different groups, share many structural features.

Based on the scaffold of Smo agonist SAG (see Section 6) by only changing the aminomethyl into a more bulky aminopropyl group the potent Smo antagonist SANT-75 was synthesized.[71] An IC_{50} value of 20 nM was determined using Shh-LIGHT2 cells, competitive experiments with BODIPY-cyclopamine showed, that SANT-75 abrogates cyclopamine/Smo binding in a dose dependent manner. FRET-experiments could confirm that SANT-75 overrides Shh induced conformational change of Smo and locks Smo conformation in the inactive 'closed' form. By using a novel Gli-GFP transgenic zebrafish assay the same group also succeeded in establishing a fast in vivo detection of hh activity in living embryos.

A recently conducted screen of drugs previously tested in humans identified itraconazole (Sporanox®, *Johnson&Johnson*), a systemic antifungal with a triazole core that has been used in the therapy of fungal infections in humans since 1988, as an antagonist of hh signaling (IC_{50} of 800 nM, see Fig. 9).[72] Antiangiogenic properties of itraconazole have been described before.[73,74] Itraconazole is a potent inhibitor of ergosterol biosynthesis in fungi acting on 14-α-lanosterol demethylase (14LDM). Although itraconazole inhibits also cholesterol biosynthesis in humans at high dosage, this is not the mechanism of action on hh signaling, since related fluconazole, though also an inhibitor of sterol biosynthesis, both in fungi and to a weaker extent in humans, lacks any hh inhibitory activity. Further experiments revealed itraconazole to be an inhibitor downstream of Ptch (by using Ptch loss-of-function cells) and eventually act on Smo (since itraconazole's inhibitory action was bypassed using cells expressing SmoA1, a constitutively active oncogenic variant of murine Smo). The ability to suppress pathway

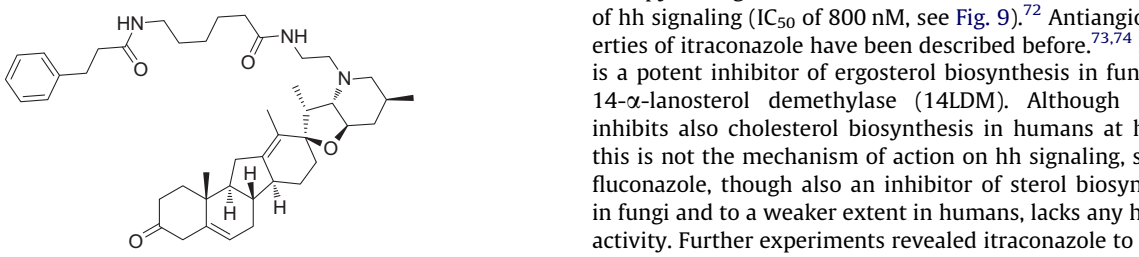

Figure 6. Structure of KAAD-cyclopamine.

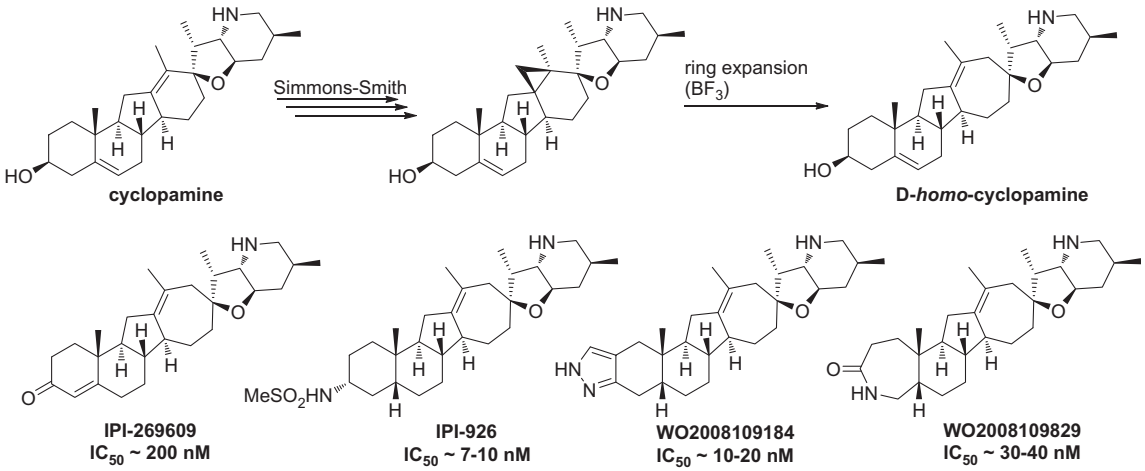

Figure 7. Synthesis of D-*homo*-cyclopamine derivatives from cyclopamine and some of the most potent analogs.

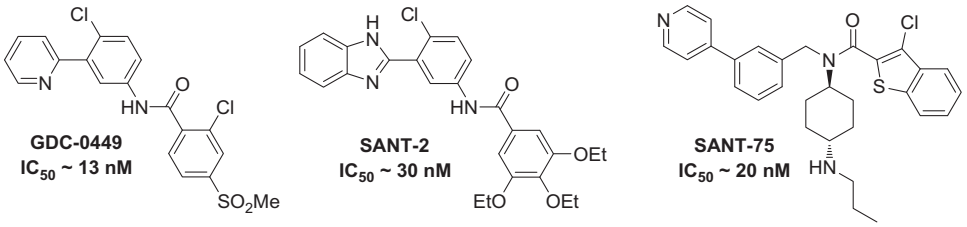

Figure 8. Structures of further Smo antagonists.

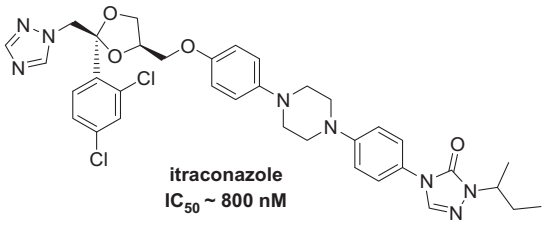

Figure 9. Structure of Smo antagonist and approved antimycotic itraconazole.

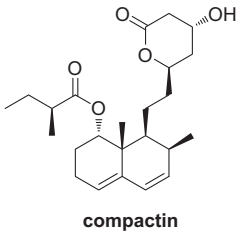

Figure 11. Structure of the statin compactin.

activity from Smo overexpression but not from SmoA1 suggests that itraconazole may be an inverse agonist of Smo. Treatment with itraconazole dramatically reduced Shh-induced accumulation of Smo in the primary cilium, similar to SANT-2, but in contrast to cyclopamine, which induced Smo accumulation in the primary cilium. Large synergistic effects were observed using both KAAD-cyclopamine and itraconazole (IC_{50} for KAAD-cyclopamine in combination with itraconazole was 2 nM), again suggesting that itraconazole binds to a distinct site from cyclopamine and its derivatives.

5.2. Inhibitors upstream of smoothened

In a screen of bacterially expressed sonic hedgehog N-terminal peptide (ShhN) by small-molecule microarrays (SMMs), containing a collection of more than 10,000 diversity-oriented synthesis compounds and natural products, a number of structurally related macrocycles emerged as positive hits. Binding to ShhN was proven by surface plasmon resonance. Several related macrocycles were synthesized and tested in Shh-LIGHT2 cells and eventually robotnikinin emerged as an optimized structure (see Fig. 10). Robotnikin is a moderately active hh inhibitor at the level of Shh and shows no cytotoxicity at relevant inhibitory concentrations.[75,76]

An approach using antibodies to inactivate the Shh ligand has been successfully conducted with the Shh monoclonal antibody 5E1.[77]

Statins are a class of drugs that reduce serum cholesterol by HMG-CoA reductase inhibition. HMG-CoA reductase converts 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate, which is the rate-limiting step in cholesterol biosynthesis. Therefore, statins are usually used to combat coronary heart diseases induced by hypercholesterolemia. A recent study revealed that in pregnant women of first trimester that were exposed to lipophilic statins like cerivastatin, simvastatin, lovastatin or atorvastatin, in many cases birth defects like holoprosencephaly were observed. It was concluded that the inhibition of cholesterol biosynthesis eventually led to down-regulation of the cholesterol dependent hh pathway.[78]

In an approach to inhibit maturation of the hh protein by compactin (see Fig. 11) induced cholesterol depletion it was shown that even at low cholesterol levels the hh protein still matures correctly. Additionally, cholesteroylation is not a necessity for the hh ligand to be fully active (vide supra). Therefore it was concluded that the inhibition of response to the hh protein is a more probable cause of the malformations associated with cholesterol biosynthetic disorders than is inhibition of hh autoprocessing.[79] Statins seem to enhance the inhibitory effect of Ptch on Smo. A combination therapy of cancer using a hh pathway antagonist and an additional statin could therefore provide benefits.

Since inhibition of cholesterol biosynthesis does not target the hh protein directly, another approach to interfere with the hh maturation process would be the inhibition of hh plamitoylation by Hhat. Only N-terminal palmitoylated hh proteins become fully active signaling species. Therefore, selective inhibitors of Hhat can emerge as a new possibility to combat hh-dependent cancers of

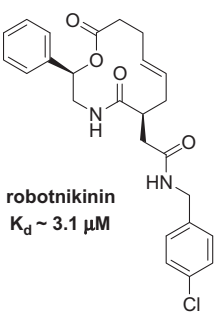

Figure 10. Structure of the Shh protein inhibitor robotnikinin.

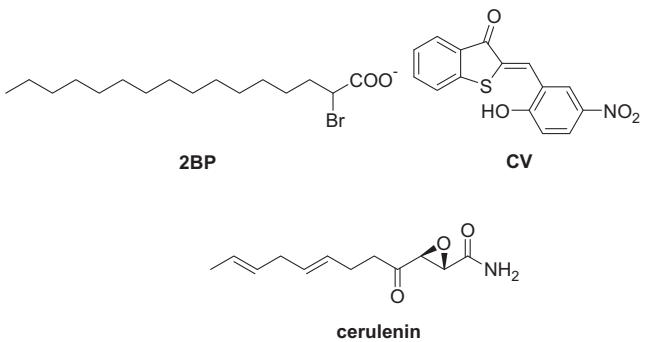

Figure 12. Structures of known unspecific palmitoylation inhibitors.

type 2 and 3 (see Section 3). Known inhibitors of the human palmitoyl transferases PAT1 and PAT2, respectively, are the natural product cerulenin,[80] and the synthetic compounds 2-bromo palmitate (2BP), and 2-(2-hydroxy-5-nitro-bezylidene)-benzo[*b*]tiophen-3-one (CV)[81,82] (see Fig. 12), with 2BP being an irreversible inhibitor, and cerulenine and CV being reversible ones. It should be emphasized, that none of these molecules exerts specificity for the palmitoylation enzymes. Cerulenin, for example, is additionally an inhibitor of HMG-CoA-reductase and therefore also inhibits cholesterol biosynthesis. 2BP is also an inhibitor of fatty acid oxidation. This highlights the need for new and specific inhibitors of Hhat to be used as inhibitors of the crucial step of hh protein maturation.

5.3. Inhibitors downstream of smoothened

Most inhibitors of hh signaling target Smo. However, hh-dependent cancers belonging to type 1 (see Section 3) have alternative mechanisms to affect Gli-mediated effector gene transcription. This is the case in mutation and overexpression of SuFu, REN, Gli1, and Gli2. Therefore, inhibitors of the Gli-factors should have a broad applicability in cancers, irrespective of the component responsible for hh signaling activation. With GANT58, a tetrapyridyl thiophene, and GANT61, a hexahydropyrimidine (see Fig. 13), two small-molecule inhibitors of hh signaling downstream of Smo targeting Gli-transcription have been described.[83] Both were 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. Both compounds also inhibited endogenous hh signaling at an IC_{50} of 5 μM each in a NIH 3T3 cell line in which the Gli reporter was stably incorporated and induced with SAG. For GANT61 the mechanism of inhibition has been elucidated to include interference with DNA binding of Gli1.

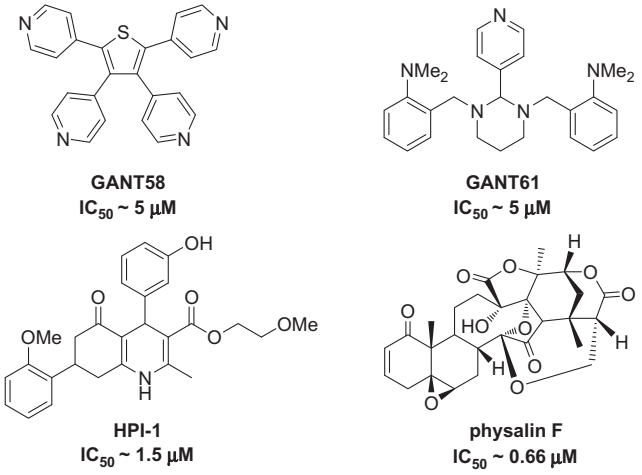

Figure 13. Structures of the synthetic Gli-antagonists GANT58, GANT61, HPI-1, and natural product physalin F.

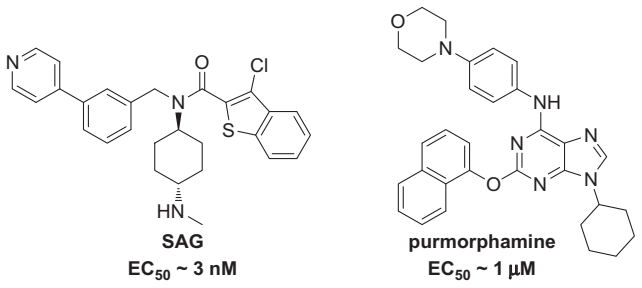

Figure 14. Structure of the synthetic Smo-agonists SAG and purmorphamine.

Four new Gli antagonists have been identified in a large-scale, high-throughput screening by Chen et al.[84] Among them, the compound HPI-1 displays the highest potency for hh inhibition (IC_{50} ~1.5 μM). HPI-1 was found neither to block BODIPY-cyclopamine/Smo binding in Smo expressing cells, nor to competitively interact with SAG, suggesting that it acts downstream of Smo. HPI-1 suppresses hh-target-gene expression induced by loss of SuFu and/or Gli protein overexpression. It has been speculated, that HPI-1 may target a primary cilium-independent process such as a posttranslational modification of the Gli protein and/or interaction between the transcription factors and a co-factor. HPI-1 activity is due at least in part to an increase in Gli repressor levels, since it uncouples Shh signaling from Gli2 processing. However, the ability of HPI-1 to inhibit hh pathway activation induced by overexpressed Gli1 and to increase Gli1 stability indicates that this compound must antagonize Gli activator function in a more direct manner. The partial resistance of Gli2 ΔPKA to HPI-1 further suggests that this compound acts through a mechanism, that is, potentiated by Gli phosphorylation.

A screen of natural products and plant extracts gave several hits for inhibition of Gli1 with the physalins, 13,14-*seco*-16,24-cyclosteroids, as the most active compounds.[85] Physalin F reduces Gli1-mediated activity with an IC_{50} of 0.66 μM. Additionally, Gli2-mediated transcription is inhibited with an IC_{50} of 2.6 μM as has been proven in a separate cell-based assay. Inhibition of Gli function seems to be an indirect antagonizing effect through a PKC/MAPK pathway blockade.

To conclude, HPI-1 differs mechanistically from GANT58, GANT61, and physalin F. Furthermore, it has striking structural similarities to the known kinesin Eg5 inhibitor dimethylenastron.[86] At least in part, inhibitory activity may therefore arise from the inhibition of the correct formation of the mitotic spindle or related processes, for example, the antero- and retrograde IFT of the Gli-factors.

6. Activators of the hh signaling pathway

During a high-throughput screening in Shh-LIGHT2 cells the compound SAG was discovered (see Fig. 14). SAG activates hh signaling by binding to Smo and induces Gli activity well above the level of 2 nM ShhN induction. Using FRET-experiments it has been confirmed that SAG binds to the heptahelical bundle of Smo and locks the conformation of Smo in the active 'open' form.

Initially, from a library screen of more than 50,000 compounds, the synthetic purine derivative purmorphamine was identified to induce osteogenesis of C3H10T1/2 cells.[87] Mouse embryonic mesoderm fibroblast C3H10T1/2 cells are multipotent mesenchymal progenitor cells which can differentiate into various mesenchymal cells and have been widely used as a model system for studies of osteoblast differentiation. For the library screen the expression of the osteogenesis marker gene *ALP* (*alkaline phosphatase*) was assayed. A subsequent gene expression profile study showed, that purmorphamine upregulates *Gli1* and *Ptch1*, but not *Shh*, *Ihh*, and *Dhh* and therefore is a hh pathway agonist.[88] The molecular mechanism of purmorphamine was then elucidated.[89] Purmorphamine activity is independent of Ptch1 (since $Ptch^{-/-}$ MEFs were unchanged in their activity when treated with purmorphamine). Also in $Smo^{-/-}$ MEFs it had no measurable activity. In Shh-LIGHT2 cells, treated with KAAD-cyclopamine, purmorphamine showed a 10-fold higher EC_{50}, indicating that Smo inhibitors counteract the activity of this hh pathway agonist. Finally, purmorphamine blocks BODIPY-cyclopamine binding in a Smo binding assay involving HEK 293T cells which overexpress Smo. Taken together, pur-

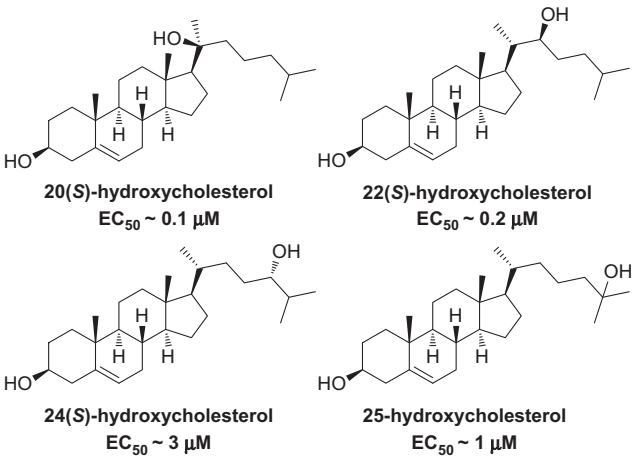

Figure 15. Structure of some naturally occurring oxysterols as agonists of hh signaling.

morphamine is a structurally novel agonist of hh signaling that targets Smo at its cyclopamine binding site.

Also oxysterols have an agonistic effect on hh signaling. Although they have been thoroughly investigated, their mechanism of action remains unknown. Oxysterols reduce the inhibitory effect of Ptch on Smo, typical members of this class have EC_{50}-values ranging from 0.1 to 3 μM (see Fig. 15).[90]

7. Medical relevance and applications of hedgehog signaling inhibitors

Since aberrant activation of the hh pathway leads to malignancies, including basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, leukemia, prostate, pancreatic, colorectal, and breast cancer, inhibition of hh signaling provides a route to novel anticancer therapies. Hh pathway inhibitors proved to be effective in in vitro studies on cancer cell lines and in animal disease models. Importantly, the anticancer activity of such inhibitors was recently demonstrated in clinical trials. In this section, some important developments in this field are presented. Finally, preclinical and recently published results of clinical studies with Smo inhibitors used as anticancer drugs will be discussed.

7.1. Treatment of pancreatic cancer with cyclopamine and its derivatives

More than 90% of pancreatic cancers are ductal adenocarcinomas. They are the fourth most common cause of cancer-related mortality in both females and males in the USA. At metastatic stages, pancreatic cancer can almost never be controlled by any of the available drugs, and the 5-year survival rate is estimated to be <2%. Even in cases with early stage, localized disease, where surgical resection with curative intention can be done, the majority of patients develop local recurrence or metastases in distant organs, and finally die.[91] Intensive efforts have been undertaken in recent years to develop therapeutic strategies that directly target the spread of metastatic tumors, and it is anticipated that such strategies will have tremendous clinical impact.

Recently, a study involving global sequencing analysis identified the hh signaling pathway as one of the core signaling pathways that undergoes somatic alterations in nearly all pancreatic cancers.[92] Inhibition of hh signaling with cyclopamine has enhanced the survival rate in a genetically engineered mouse model of pancreatic cancer and has abrogated the systemic metastases arising from orthotopic xenografts.[93] This study also provides evidence that hh signaling is a valid target for the development of novel therapeutics for pancreatic cancer and is worth to be evaluated in a clinical setting. The derivative IPI-269609 (see Section 5.1) was developed to overcome some of cyclopamine's deficits. IPI-269609 profoundly inhibited systemic metastases in orthotopic xenografts established from human pancreatic cancer cell lines. IPI-926, a novel semisynthetic cyclopamine analog, was subsequently synthesized (see Section 5.1). By using a highly invasive and lethal genetically engineered model of pancreatic cancer, Olive et al.[94] showed that IPI-926 increases survival when used in combination with gemcitabine in an otherwise gemcitabine-resistant mouse model. This study clearly shows that inhibition of the hh pathway in the stroma of malignant tumors results in a striking reduction in the dense fibrotic reaction that accompanies these tumors. IPI-926 also increases tumor neovascularization, thereby facilitating the distribution of gemcitabine to malignant cells. This study suggests that patients with locally advanced pancreatic cancer are the most likely to benefit from a therapeutic inhibition of the hh pathway.

7.2. A hedgehog pathway inhibitor for treatment of basal cell carcinoma and medulloblastoma

Basal-cell carcinoma is the most common skin cancer in the USA and constitutes approximately 80% of all non-melanoma skin cancers. This disease has an estimated annual incidence of 0.1–0.5% and is largely caused by exposure to UV radiation.[95] Surgery cures most cases of basal cell carcinoma, but in a few patients there is progression to life-threatening, inoperable, locally advanced, or metastatic tumors. Inhibitors of the hh pathway at the level of Smo represent valuable strategies for the treatment of this type of cancer. A cream containing cyclopamine was applied to basal cell carcinoma in patients who were scheduled to have their tumors excised.[96] All of the tumors treated with cyclopamine regressed rapidly. Histological and immunohistochemical analyzes showed inhibition of the proliferation and highly efficient induction of the differentiation and apoptosis of the tumor cells. By transient inhibition of hh signaling in vivo a rational approach to treating BCC is provided. Recently, 33 patients with metastatic or locally advanced basal cell carcinoma received GDC-0449 orally (see Section 5.1). Of these 33 patients, 18 had an objective response to GDC-0449. According to assessment by imaging and/or physical examination, 11 patients had a response, of which two showed a complete response and 16 a partial response. The other 15 patients had either stable disease (11 patients) or progressive disease (4 patients). No dose-limiting toxic effects were observed during the study period. In conclusion, GDC-0449 appears to have antitumor activity in locally advanced or metastatic basal-cell carcinoma. These findings also confirm the involvement of the hh pathway in basal cell carcinoma and suggest that inhibition of this pathway could be useful for the treatment of inoperable tumors.

GDC-0449 has also been used in the therapy of medulloblastoma in one patient.[97] Medulloblastoma is the most common embryonal tumor in children. The median age at diagnosis is 5 years, with the age range extending into young adulthood. Therapy consists of surgical resection followed by radiation therapy and chemotherapy. Current therapies have serious short-term and long-term adverse effects, including neurocognitive deficits, endocrinopathies, sterility, and the risk of secondary high-grade glioma or meningioma.[98] In the above-mentioned study, a 26-year-old man had metastatic medulloblastoma that was refractory to multiple therapies. Treatment with GDC-0449 resulted in a rapid but transient regression of the tumor and a dramatic reduction of the symptoms. Molecular analyzes of tumor specimens obtained before treatment suggested that there was an activation of the hh pathway, with loss of heterozygosity and somatic mutation of the gene *Ptch1*. The mutational status of hh signaling genes in

the tumor was determined after disease progression to evaluate the mechanism of resistance in the medulloblastoma patient.[99] Finally, an amino acid substitution at a conserved aspartic acid residue of Smo was observed that had no effect on hh signaling, but disrupted the ability of GDC-0449 to bind to Smo and act as an inhibitor of the hh pathway. A mutation altering the same amino acid in a mouse model also arose in a GDC-0449-resistant medulloblastoma.

These findings clearly show that acquired mutations in a serpentine receptor that has features of a G-protein-coupled receptor can serve as a mechanism of drug resistance in human cancer. Furthermore, the demonstration that these mutations do not have an impact on hh signaling continues to support the rationale for targeting this pathway, but also highlights the need to identify second-generation Smo inhibitors capable of overcoming acquired resistance and to identify inhibitors that target signaling molecules downstream of the Smo receptor.[84]

7.3. Treatment of leukemia with hedgehog pathway antagonists

Recently it was demonstrated that hh signaling is essential for the maintenance of cancer stem cells in multiple myeloma[100] as well as in chronic myeloid leukemia (CML). For this reason inhibitors of the hh pathway could be valuable tools for treatment of such neoplastic diseases. For example, pharmacological inhibition of hh signaling by cyclopamine impairs not only the propagation of CML driven by wild-type BCR-ABL1, but also the growth of imatinib-resistant mouse and human CML. These data indicate that hh activity is required for the maintenance of normal and neoplastic stem cells of the hematopoietic system and raise the possibility that drug resistance associated with imatinib treatment of CML might be avoided by targeting this essential stem cell maintenance pathway.[101,102]

8. Conclusion

With P. A. Beachy's initial report on cyclopamine to be an inhibitor of the hedgehog signaling pathway, discovered by C. Nüsslein-Volhard and E. F. Wieschaus, a story that began in the 1950s with unsettling observations in Idaho culminated into a breakthrough in the search for a novel and selective anticancer therapy. Now clinical studies show that the hedgehog pathway can be the basis of an important new class of therapeutic agents with far-reaching implications in oncology. Based on the interdisciplinary interplay between chemistry, biology, and medicine, not only cyclopamine's development into an anticancer therapeutic was fostered, but also several synthetic modulators became available that selectively access various points of the hedgehog signaling pathway. In this review we tried to trace the story of cyclopamine, to give an overview on the biological modes of hedgehog signaling both in untransformed and malignant cells, and finally to present potent modulators—many of them already in clinical studies. For more than 30 years now the knowledge on hedgehog signaling grows steadily—but an end to this development is far from being conceivable.

Acknowledgement

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References and notes

1. Portions of this review have been previously published: Heretsch, P.; Tzagkaroulaki, L.; Giannis, A. *Angew. Chem., Int. Ed.* **2010**, *49*, 3418.
2. Nüsslein-Volhard, C.; Wieschaus, E. F. *Nature* **1980**, *287*, 795.
3. Nüsslein-Volhard, C. *Angew. Chem., Int. Ed.* **1996**, *35*, 2176.
4. Riddle, R. D.; Johnson, R. L.; Laufer, E.; Tabin, C. *Cell* **1993**, *75*, 1401.
5. Echelard, Y.; Epstein, D. J.; St-Jacques, B.; Shen, L.; Mohler, J.; McMahon, J. A.; McMahon, A. P. *Cell* **1993**, *75*, 1417.
6. Krauss, S.; Concordet, J.-P.; Ingham, P. W. *Cell* **1993**, *75*, 1431.
7. Chang, D. T.; López, A.; von Kessler, D. P.; Chiang, C.; Simandl, B. K.; Zhao, R.; Seldin, M. F.; Fallon, J. F.; Beachy, P. A. *Development* **1994**, *120*, 3339.
8. Varjosalo, M.; Taipale, J. *Genes Dev.* **2008**, *22*, 2454.
9. Mahindroo, N.; Punchihewa, C.; Fujii, N. *J. Med. Chem.* **2009**, *52*, 3829.
10. Stanton, B. Z.; Peng, L. F. *Mol. BioSyst.* **2010**, *6*, 44.
11. Tremblay, M. R.; Nesler, M.; Weatherhead, R.; Castro, A. C. *Expert Opin. Ther. Patents* **2009**, *19*, 1039.
12. Firestone, A. J.; Chen, J. K. *ACS Chem. Biol.* **2010**, *5*, 15.
13. Mas, C.; Ruiz i Altaba, A. *Biochem. Pharmacol.* **2010**, *80*, 712.
14. Tremblay, M. R.; McGovern, K.; Read, M. A.; Castro, A. C. *Curr. Opin. Chem. Biol.* **2010**, *14*, 1.
15. Peukert, S.; Miller-Moslin, K. *Chem. Med. Chem.* **2010**, *5*, 500.
16. Huangfu, D.; Anderson, K. V. *Development* **2006**, *133*, 3.
17. Hausmann, G.; von Mering, C.; Basler, K. *PLoS Biol.* **2009**, *7*, e1000146.
18. Mann, R. K.; Beachy, P. A. *Annu. Rev. Biochem.* **2004**, *73*, 891.
19. Buglino, J. A.; Resh, M. D. *J. Biol. Chem.* **2008**, *283*, 22076.
20. Goodrich, L. V.; Johnson, R. L.; Milenkovic, L.; McMahon, J. A.; Scott, M. P. *Genes Dev.* **1996**, *10*, 301.
21. Stone, D. M.; Hynes, M.; Armanini, M.; Swanson, T. A.; Gu, Q.; Johnson, R. L.; Scott, M. P.; Pennica, D.; Goddard, A.; Phillips, H.; Noll, M.; Hooper, J. E.; de Sauvage, F.; Rosenthal, A. *Nature* **1996**, *384*, 129.
22. Goetz, S. C.; Anderson, K. V. *Nat. Rev. Genet.* **2010**, *11*, 331.
23. Plotnikova, O. V.; Golemis, E. A.; Pugacheva, E. N. *Cancer Res.* **2008**, *68*, 2058.
24. Rohatgi, R.; Milenkovic, L.; Scott, M. P. *Science* **2007**, *317*, 372.
25. Corbit, K. C.; Aanstad, P.; Singla, V.; Norman, A. R.; Stainier, D. Y.; Reiter, J. F. *Nature* **2005**, *437*, 1018.
26. Rohatgi, R.; Milenkovic, L.; Corcoran, R. B.; Scott, M. P. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 3196.
27. Kim, J.; Kato, M.; Beachy, P. A. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 21666.
28. Zhao, Y.; Tong, C.; Jiang, J. *Nature* **2007**, *450*, 252.
29. Rohatgi, R.; Scott, M. P. *Nat. Cell. Biol.* **2007**, *9*, 1005.
30. Pearse, R. V., II; Collier, L. S.; Scott, M. P.; Tabin, C. J. *Dev. Biol.* **1999**, *212*, 323.
31. Wen, X.; Lai, C. K.; Evangelista, M.; Hongo, J.-A.; de Sauvage, F. J.; Scales, S. J. *Mol. Cell. Biol.* **2010**, *30*, 1910.
32. Pan, Y.; Bai, C. B.; Joyner, A. L.; Wang, B. *Mol. Cell Biol.* **2006**, *26*, 3365.
33. Wang, B.; Fallon, J. F.; Beachy, P. A. *Cell* **2000**, *100*, 423.
34. Sasaki, H.; Nishizaki, Y.; Hui, C.; Nakafuku, M.; Kondoh, H. *Development* **1999**, *126*, 3915.
35. Oliver, T. G.; Grasfeder, L. L.; Carroll, A. L.; Kaiser, C.; Gillingham, C. L.; Lin, S. M.; Wickramasinghe, R.; Scott, M. P.; Wechsler-Reya, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 7331.
36. Regl, G.; Neill, G. W.; Eichenberger, T.; Kasper, M.; Ikram, M. S.; Koller, J.; Hinter, H.; Quinn, A. G.; Frischauf, A.-M.; Aberger, F. *Oncogene* **2002**, *21*, 5529.
37. Haycraft, C. J.; Banizs, B.; Aydin-Son, Y.; Zhang, Q.; Michaud, E. J.; Yoder, B. K. *PLoS Genet.* **2005**, *1*, 480.
38. Liu, A.; Wang, B.; Niswander, L. A. *Development* **2005**, *132*, 3103.
39. Taipale, J.; Beachy, P. A. *Nature* **2001**, *411*, 349.
40. di Magliano, M. P.; Hebrok, M. *Nat. Rev. Cancer* **2003**, *3*, 903.
41. Rubin, L. L.; de Sauvage, F. J. *Nat. Rev. Drug Discov.* **2006**, *5*, 1026.
42. Scales, S. J.; Sauvage, F. J. *Trends Pharmacol. Sci.* **2009**, *30*, 303.
43. Dlugosz, A. A.; Talpaz, M. *N. Engl. J. Med.* **2009**, *361*, 1202.
44. Yang, L.; Xie, G.; Fan, Q.; Xie, J. *Oncogene* **2009**, *29*, 469.
45. Teglund, S.; Toftgård, R. *Biochim. Biophys. Acta* **2010**, *1805*, 181.
46. Varnat, F.; Duquet, A.; Malerba, M.; Zbinden, M.; Mas, C.; Gervaz, P.; Ruiz i Altaba, A. *EMBO Mol. Med.* **2009**, *1*, 338.
47. Curran, T.; Ng, J. M. Y. *Nature* **2008**, *455*, 293.
48. Yauch, R. L.; Gould, S. E.; Scales, S. J.; Tang, T.; Tian, H.; Ahn, C. P.; Marshall, D.; Fu, L.; Januario, T.; Kallop, D.; Nannini-Pepe, M.; Kotkow, K.; Marsters, J. C., Jr.; Rubin, L. L.; de Sauvage, F. J. *Nature* **2008**, *455*, 406.
49. Binns, W.; James, L. F.; Keeler, R. F.; Balls, L. D. *Cancer Res.* **1968**, *28*, 2323.
50. Keeler, R. F. *J. Agric. Food Chem.* **1969**, *17*, 473.
51. James, L. F.; Panter, K. E.; Gaffield, W.; Molyneux, R. J. *J. Agric. Food Chem.* **2004**, *52*, 3211.
52. Keeler, R. F. *Phytochemistry* **1968**, *7*, 303.
53. Keeler, R. F. *Teratology* **1970**, *3*, 169.
54. Edison, R.; Muenke, M. *Congenit. Anom.* **2003**, *43*, 1.
55. Cooper, M. K.; Porter, J. A.; Young, K. E.; Beachy, P. A. *Science* **1998**, *280*, 1603.
56. Incardona, J. P.; Gaffield, W.; Kapur, R. P.; Roelink, H. *Development* **1998**, *125*, 3553.
57. Taipale, J.; Chen, J. K.; Cooper, M. K.; Wang, B.; Mann, R. K.; Milenkovic, L.; Scott, M. P.; Beachy, P. A. *Nature* **2000**, *406*, 1005.
58. Wang, Y.; Zhou, Z.; Walsh, C. T.; McMahon, A. P. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 2623.
59. Eaton, S. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 437.
60. Khaliullina, H.; Panáková, D.; Eugster, C.; Riedel, F.; Carvalho, M.; Eaton, S. *Development* **2009**, *136*, 4111.
61. Wilson, S. R.; Strand, M. F.; Krapp, A.; Rise, F.; Petersen, D.; Krauss, S. *J. Pharm. Biomed. Anal.* **2010**, *52*, 707.
62. Keeler, R. F. *Phytochemistry* **1969**, *8*, 223.
63. Giannis, A.; Heretsch, P.; Sarli, V.; Stößel, A. *Angew. Chem., Int. Ed.* **2009**, *48*, 7911.
64. Heretsch, P.; Rabe, S.; Giannis, A. *Org. Lett.* **2009**, *11*, 5410.

65. Frank-Kamenetsky, M.; Zhang, X. M.; Bottega, S.; Guicherit, O.; Wichterle, H.; Dudek, H.; Bumcrot, D.; Wang, F. Y.; Jones, S.; Shulok, J.; Rubin, L. L.; Porter, J. A. *J. Biol.* **2002**, *1*, 10.1.
66. Miller-Moslin, K.; Peukert, S.; Jain, R. K.; McEwan, M. A.; Karki, R.; Llamas, L.; Yusuff, N.; He, F.; Li, Y.; Sun, Y.; Dai, M.; Perez, L.; Michael, W.; Sheng, T.; Lei, H.; Zhang, R.; Williams, J.; Bourret, A.; Ramamirthy, A.; Yuan, J.; Guo, R.; Matsumotu, M.; Vattay, A.; Maniara, W.; Amaral, A.; Dorsch, M.; Kelleher, J. F. *J. Med. Chem.* **2009**, *52*, 3954.
67. Dywer, J. R.; Sever, N.; Carlson, M.; Nelson, S. F.; Beachy, P. A.; Parhami, F. *J. Biol. Chem.* **2007**, *282*, 8959.
68. Tremblay, M. R.; Nevalainen, M.; Nair, S. J.; Porter, J. R.; Castro, A. C.; Behnke, M. L.; Yu, L.-C.; Hagel, M.; White, K.; Faia, K.; Grenier, L.; Campbell, M. J.; Cushing, J.; Woodward, C. N.; Hoyt, J.; Foley, M. A.; Read, M. A.; Sydor, J. R.; Tong, J. K.; Palombella, V. J.; McGovern, K.; Adams, J. *J. Med. Chem.* **2008**, *51*, 6646.
69. Tremblay, M. R.; Lescarbeau, A.; Grogan, M. J.; Tan, E.; Lin, G.; Austad, B. C.; Yu, L.-C.; Behnke, M. L.; Nair, S. J.; Hagel, M.; White, K.; Conley, J.; Manna, J. D.; Alvarez-Diez, T. M.; Hoyt, J.; Woodward, C. N.; Sydor, J. R.; Pink, M.; MacDougall, J.; Campbell, M. J.; Cushing, J.; Ferguson, J.; Curtis, M. S.; McGovern, K.; Read, M. A.; Palombella, V. J.; Adams, J.; Castro, A. C. *J. Med. Chem.* **2009**, *52*, 4400.
70. Robarge, K. D.; Brunton, S. A.; Castanedo, G. M.; Cui, Y.; Dina, M. S.; Goldsmith, R.; Gould, S. E.; Guichert, O.; Gunzner, J. L.; Halladay, J.; Jia, W.; Khojasteh, C.; Koehler, M. F. T.; Kotkow, K.; La, H.; LaLonde, R. L.; Lau, K.; Lee, L.; Marshall, D.; Marsters, J. C., Jr.; Murray, L. J.; Qian, C.; Rubin, L. L.; Salphati, L.; Stanley, M. S.; Stibbard, J. H. A.; Ubhayaker, S.; Wang, S.; Wong, S.; Xie, M. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 5576.
71. Yang, H.; Xiang, J.; Wang, N.; Zhao, Y.; Hyman, J.; Li, S.; Jiang, J.; Chen, J. K.; Yang, Z.; Li, S. *J. Biol. Chem.* **2009**, *284*, 20876.
72. Kim, J.; Tang, J. Y.; Gong, R.; Kim, J.; Lee, J. J.; Clemons, K. V.; Chong, C. R.; Chang, K. S.; Fereshteh, M.; Gardner, D.; Reya, T.; Liu, J. O.; Epstein, E. H.; Stevens, D. A.; Beachy, P. A. *Cancer Cell* **2010**, *17*, 388.
73. Chong, C. R.; Xu, J.; Lu, J.; Bhat, S.; Sullivan, D. J., Jr.; Liu, J. O. *ACS Chem. Biol.* **2006**, *2*, 263.
74. Xu, J.; Dang, Y.; Ren, Y. R.; Liu, J. O. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 4764.
75. Stanton, B. Z.; Peng, L. F.; Maloof, N.; Nakai, K.; Wang, X.; Duffner, J. L.; Taveras, K. M.; Hyman, J. M.; Lee, S. W.; Koehler, A. N.; Chen, J. K.; Fox, J. L.; Mandinova, A.; Schreiber, S. L. *Nat. Chem. Biol.* **2009**, *5*, 154.
76. Peng, L. F.; Stanton, B. Z.; Maloof, N.; Wang, X.; Schreiber, S. L. *Bioorg. Med. Chem. Lett.* **2009**, *19*, 6319.
77. Ericson, J.; Morton, S.; Kawakami, A.; Roelink, H.; Jessel, T. M. *Cell* **1996**, *87*, 661.
78. Edison, R. J.; Muenke, M. *N. Engl. J. Med.* **2004**, *350*, 1579.
79. Cooper, M. K.; Wassif, C. A.; Krakowiak, P. A.; Taipale, J.; Gong, R.; Kelley, R. I.; Porter, F. D.; Beachy, P. A. *Nat. Genet.* **2003**, *22*, 508.
80. Lawrence, D. S.; Zilfou, J. T.; Smith, C. D. *J. Med. Chem.* **1999**, *42*, 4932.
81. Jennings, B. C.; Nadolski, M. J.; Ling, Y.; Beckham Baker, M.; Harrison, M. L.; Deschenes, R. J.; Linder, M. E. *J. Lipid Res.* **2009**, *50*, 233.
82. Ducker, C. E.; Griffel, L. K.; Smith, R. A.; Keller, S. N.; Zhuang, Y.; Xia, Z.; Diller, J. D.; Smith, C. D. *Mol. Cancer Ther.* **2006**, *5*, 1647.
83. Lauth, M.; Bergström, Å.; Shimokawa, T.; Toftgård, R. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 8455.
84. Hyman, J. M.; Firestone, A. J.; Heine, V. M.; Zhao, Y.; Ocasio, C. A.; Han, K.; Sun, M.; Rack, P. G.; Sinha, S.; Wu, J. J.; Solow-Cordero, D. E.; Jiang, J.; Rowitch, D. H.; Chen, J. K. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 14132.
85. Hosoya, T.; Arai, M. A.; Koyano, T.; Kowithayakorn, T.; Ishibashi, M. *ChemBioChem* **2008**, *9*, 1082.
86. Müller, C.; Gross, D.; Sarli, V.; Gartner, M.; Giannis, A.; Bernhardt, G.; Buschauer, A. *Cancer Chemother. Pharmacol.* **2007**, *59*, 157.
87. Wu, X.; Ding, S.; Ding, Q.; Gray, N. S.; Schultz, P. G. *J. Am. Chem. Soc.* **2002**, *124*, 14520.
88. Wu, X.; Walker, J.; Zhang, J.; Ding, S.; Schultz, P. G. *Chem. Biol.* **2004**, *11*, 1229.
89. Sinha, S.; Chen, J. K. *Nat. Chem. Biol.* **2006**, *2*, 29.
90. Corcoran, R. B.; Scott, M. P. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 8408.
91. Lohr, J. M. *Expert Rev. Anticancer Ther.* **2007**, *7*, 533.
92. Jones, S.; Zhang, X.; Parsons, D. W.; Lin, J. C.-H.; Leary, R. J.; Angenendt, P.; Mankoo, P.; Carter, H.; Kamiyama, H.; Jimeno, A.; Hong, S.-M.; Fu, B.; Lin, M.-T.; Calhoun, E. S.; Kamiyama, M.; Walter, K.; Nikolskaya, T.; Nikolsky, Y.; Hartigan, J.; Smith, D. R.; Hidalgo, M.; Leach, S. D.; Klein, A. P.; Jaffee, E. M.; Goggins, M.; Maitra, A.; Iacobuzio-Donahue, C.; Eshleman, J. R.; Kern, S. E.; Hruban, R. H.; Karchin, R.; Papadopoulos, N.; Parmigiani, G.; Vogelstein, B.; Velculescu, V. E.; Kinzler, K. W. *Science* **2008**, *321*, 1801.
93. Feldmann, G.; Habbe, N.; Dhara, S.; Bisht, S.; Alvarez, H.; Fendrich, V.; Beaty, R.; Mullendore, M.; Karikari, C.; Bardeesy, N.; Ouellette, M. M.; Yu, W.; Maitra, A. *Gut* **2008**, *57*, 1420.
94. Olive, K. P.; Jacobetz, M. A.; Davidson, C. J.; Gopinathan, A.; McIntyre, D.; Honess, D.; Madhu, B.; Goldgraben, M. A.; Caldwell, M. E.; Allard, D.; Frese, K. K.; DeNicola, G.; Feig, C.; Combs, C.; Winter, S. P.; Ireland-Zecchini, H.; Reichelt, S.; Howat, W. J.; Chang, A.; Dhara, M.; Wang, L.; Rückert, F.; Grützmann, R.; Pilarsky, C.; Izeradjene, K.; Hingorani, S. R.; Huang, P.; Davies, S. E.; Plunkett, W.; Egorin, M.; Hruban, R. H.; Whitebread, N.; McGovern, K.; Adams, J.; Iacobuzio-Donahue, C.; Griffiths, J.; Tuveson, D. A. *Science* **2009**, *324*, 1457.
95. Rubin, A. I.; Chen, E. H.; Ratner, D. *N. Engl. J. Med.* **2005**, *353*, 2262.
96. Tabs, S.; Avci, O. *Eur. J. Dermatol.* **2004**, *14*, 96.
97. Rudin, C. M.; Hann, C. L.; Laterra, J. *N. Engl. J. Med.* **2009**, *361*, 1173.
98. Crawford, J. R.; MacDonald, T. J.; Packer, R. J. *Lancet Neurol.* **2007**, *6*, 1073.
99. Yauch, R. L.; Dijkgraaf, G. J. P.; Alicke, B.; Januario, T.; Ahn, C. P.; Holcomb, T.; Pujara, K.; Stinson, J.; Callahan, C. A.; Tang, T.; Bazan, J. F.; Kan, Z.; Seshagiri, S.; Hann, C. L.; Gould, S. E.; Low, J. A.; Rudin, C. M.; de Sauvage, F. J. *Science* **2009**, *326*, 572.
100. Peacock, C. D.; Wang, Q.; Gesell, G. S.; Corcoran-Schwartz, I. M.; Jones, E.; Kim, J.; Devereux, W. L.; Rhodes, J. T.; Huff, C. A.; Beachy, P. A.; Watkins, D. N.; Matsui, W. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 4048.
101. Zhao, C.; Chen, A.; Jamieson, C. H.; Fereshteh, M.; Abrahamsson, A.; Blum, J.; Kwon, H. Y.; Kim, J.; Chute, J. P.; Rizzieri, D.; Munchhof, M.; VanArsdale, T.; Beachy, P. A.; Reya, T. *Nature* **2009**, *458*, 776.
102. Dierks, C.; Beigi, R.; Guo, G. R.; Zirlik, K.; Stegert, M. R.; Manley, P.; Trussell, C.; Schmitt-Graeff, A.; Landwerlin, K.; Veelken, H.; Warmuth, M. *Cancer Cell* **2008**, *14*, 238.