

Cyclopamine and Hedgehog Signaling: Chemistry, Biology, Medical Perspectives

Philipp Heretsch, Lito Tzagkaroulaki, and Athanassios Giannis*

antitumor agents · cyclopamine · medicinal chemistry ·
natural products · signal transduction

When Odysseus left the devastated city of Troy after ten years of siege he could not foresee the perils he still had to face. The encounter with the cyclops, a giant with only one eye placed in the middle of its forehead, was doubtlessly one of the creepiest and most dangerous of his adventures. In the end, Odysseus could only escape with the help of a sheep. Whether Homers cyclops was inspired by the observation of terribly malformed neonates remains speculative. However, when sheep herders in Idaho in the middle of the 20th century faced an increasing number of cyclops-like sheep in their herds, a unique cascade of chemical, biological, and medicinal discoveries was initiated. This Minireview tells this story and shows its impact on modern biomedical research.

1. Chemistry of Cyclopamine

1.1. History of the Discovery of Cyclopamine

Veratrum californicum, also known as the California false hellebore, is a lily of the family of melanthiaceae that is native to the northern hemisphere but is especially abundant in the mountainous area of the western USA. In the middle of the 20th century, a random batch of lambs in sheep herds in the state of Idaho 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—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 included incompletely or totally undivided cerebral hemispheres as well as olfactory and optical nerves. The affected sheep herders asked the Department of Agriculture for help when the disease had reached endemic proportions and the economic loss was no longer tolerable. Lynn F. James, one of the scientists who was sent, lived with the sheep for three summers in the late 1950s 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 consump-

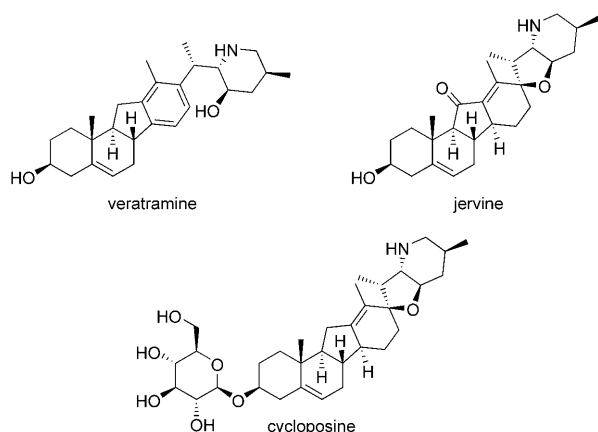
tion of *veratrum californicum* by pregnant sheep and the occurrence of cyclopia in their offspring. By feeding pregnant sheep preparations of *veratrum californicum* it was shown that these malformations were induced from the 14th day of gestation and the pregnancy was prolonged by up to 87 days (the gestation period of a healthy sheep is 147 to 152 days).^[1]

1.2. Isolation and Structural Elucidation of the Causative Agent

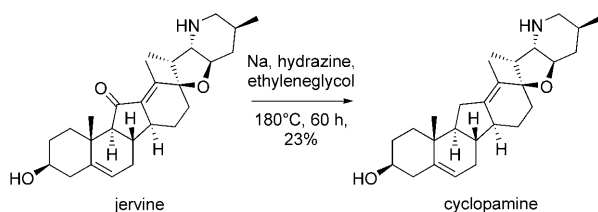
On the basis of these initial studies, an extraction method was developed (see Section 1.4.1) that allowed the alkaloids of *veratrum californicum* to be isolated for investigation of their ability to induce cyclopia in embryos of pregnant sheep. It was shown that only three compounds possessed this ability, and in various potencies, namely the known alkaloid jervine, alkaloid X (later identified as 3-glucosylcyclopamine and termed cycloposine), and a third more active steroidal alkaloid resembling jervine that induced cyclopia in the offspring of 12 of 40 pregnant sheep (Scheme 1).

In the process of elucidating the structure of the third compound it was noted that its IR and UV spectra showed similarities to the known steroidal alkaloids jervine and veratramine. The results of mass spectrometric and elementary analysis in combination with ¹H NMR spectroscopy confirmed its close resemblance to jervine. A definitive structural characterization of the new compound as 11-deoxojervine was gained from the degradation of jervine by a Wolff–Kishner reduction (Scheme 2). The product of this

[*] Dr. P. Heretsch, L. Tzagkaroulaki, Prof. Dr. A. Giannis
Institut für Organische Chemie, Universität Leipzig
Johannisallee 29, 04103 Leipzig (Germany)
Fax: (+49) 341-9736599
E-mail: giannis@uni-leipzig.de



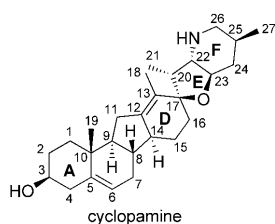
Scheme 1. Structures of veratramine, jervine, and cycloposine.



Scheme 2. Wolff-Kishner reduction of jervine.

transformation was identical in every respect to the newly isolated steroidal alkaloid. In accordance with the epic by Homer about the odyssey in which the encounter of Odysseus with the cyclops Polyphemus is described, Keeler named this new natural product cyclopamine.^[2]

Cyclopamine consists of a hexacyclic framework of four annulated carbocycles, three of them are six-membered rings (A, B, and D ring) and one is a five-membered ring (C ring). A highly substituted tetrahydrofuran forms ring E, which is spiro-connected to ring D. A piperidine ring with a secondary basic nitrogen atom forms ring F, which is annulated to ring E (Scheme 3). Cyclopamine is a so-called isosteroid (“6-6-5-6-system”). The molecular structure bears an inherent



Scheme 3. Structure of cyclopamine with ring and carbon numbering.

instability that arises from the C12–C13 double bond, which renders the furan oxygen atom allylic. Treatment of cyclopamine with Lewis acids or at pH < 2 leads to cleavage of the spiro connection of this oxygen atom to ring D. This ring aromatizes to form the toxic veratramine that no longer has any of the teratogenic effects of cyclopamine.^[1] Only a small



Philipp Heretsch was born in Lippstadt in 1982. He studied chemistry at the University of Leipzig and recently completed his PhD with Athanassios Giannis on the synthesis of cyclopamine. During his studies and his PhD research he was a fellow of the Fonds der Chemischen Industrie. In 2008 he was awarded the Procter&Gamble-Förderpreis. From summer 2010 he will be a postdoctoral associate with K. C. Nicolaou.



Lito Tzagkaroulaki was born in Greece in 1983. From 2001 to 2008 she studied medicine at the University of Athens. Since 2008 she has been conducting PhD research with Athanassios Giannis on the investigation of receptor tyrosine kinase inhibitors and modulators of hedgehog signaling.



Athanassios Giannis is a chemist and physician. He was born in Greece in 1954 and studied chemistry from 1972 to 1980 and medicine from 1978 to 1987 at the University of Bonn. He completed his PhD in 1986 with Konrad Sandhoff and habilitated in 1992 at the University of Bonn in organic chemistry and biochemistry. From 1998 to 2002 he was an associate professor at the University of Karlsruhe. Since 2002 he has been full professor for organic chemistry and natural products chemistry at the University of Leipzig. His area of research is biological- and medicinal-oriented organic chemistry.

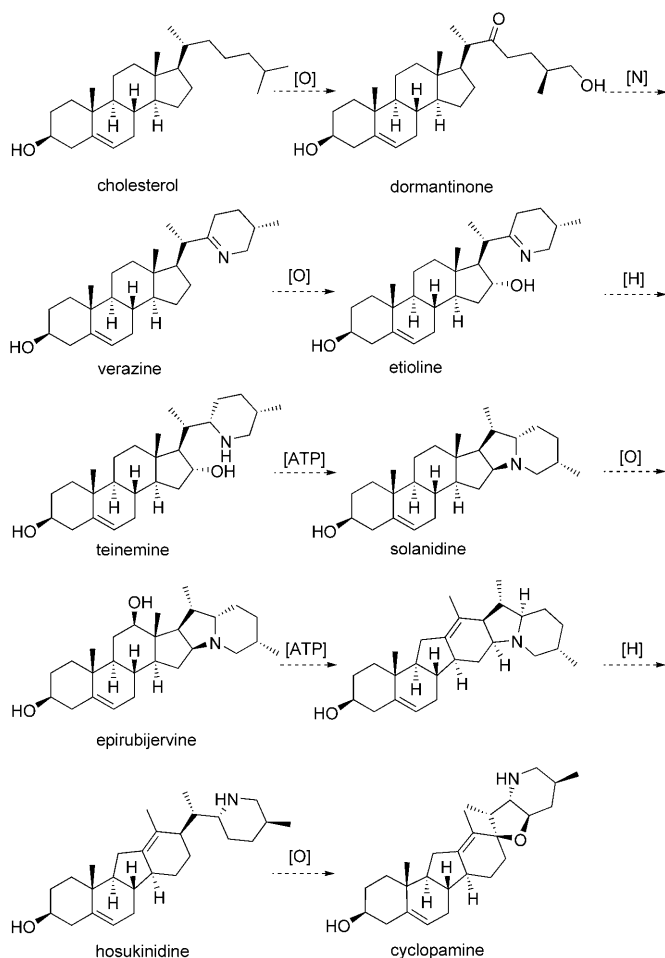
proportion of cyclopamine in *veratrum californicum* is structurally unaffected after ingestion and passage through the stomach, but this amount is sufficient to cause the malformations described.

Although a change in the grazing habits helped to eliminate the cases of cyclopia in the herds immediately, the molecular biological background and mechanism of this curious molecule remained unknown for another 25 years.

1.3. Biosynthesis of Cyclopamine

Veratrum plants contain five types of steroidal alkaloids: the solanidine alkaloids (for example, solanidine, rubijervine, and epirubijervine) and verazine alkaloids (for example, verazine and etioline), which all possess a steroid skeleton of the classical (“6-6-6-5”) type, the veratramine alkaloids (for example, veratramine), the jervine alkaloids (for example, jervine and cyclopamine), and the cevanine alkaloids that all possess a C-nor-D-homosteroid skeleton. The work of Tschesche and Kaneko^[3] in the 1960s and 1970s on *veratrum grandiflorum* allowed elucidation of the biosynthetic origin of cyclopamine and other *veratrum* alkaloids, and through

studies with ^{14}C -labeled cholesterol and acetate allowed identification of cholesterol as a common precursor. Cholesterol is transformed in the plant through a number of reactions (some have only been assumed) into the parent compound of this class of natural products: solanidine. In a series of oxidative and reductive processes, first the piperidine is synthesized and then the E ring of solanidine is formed in a reaction driven by adenosine triphosphate (ATP). A stereounspecific oxidase subsequently hydroxylates at the C12-position to form the 12 α -hydroxy product (rubijervine), which is enriched in the rhizomes, and the 12 β -hydroxy product (epirubijervine). Only the hydroxy group of the latter compound is converted into a phosphate leaving group and rearranges through a Wagner–Meerwein type reaction into the *C*-nor-*D*-homosteroid skeleton. The necessity of light for this process was discussed. Reductive opening of the pyrrolidine ring (E ring) gives husokinidine, which is finally oxidatively cyclized to form the tetrahydrofuran ring of cyclopamine (Scheme 4). Studies with ^{14}C -labeled cyclopamine showed that both jervine and veratramine are biosynthesized from cyclopamine.^[3]



Scheme 4. Biosynthesis of cyclopamine from cholesterol in *Veratrum grandiflorum*.

1.4. Accessibility of Cyclopamine

“However, we believe that with this study, the evidence is in place to justify an effort to develop a supply [of cyclopamine] so that it can be tested in humans.” In this way Philip A. Beachy commented in 2002 in an interview at the Howard Hughes Medical Institute on the necessity of making available larger amounts of cyclopamine at affordable prices.

In principle there are two ways to fulfill this task: the development of reliable extraction processes of cyclopamine from *Veratrum californicum*, and the development of a synthetic route. Both approaches have been studied since the 1960s, but only recently were both a modern extraction process and a chemical synthesis reported that meet the requirements in terms of efficiency, economy, and feasibility.

1.4.1. Extraction from *Veratrum californicum*

Keeler’s early attempts at extraction were on dried and ground roots and rhizomes of *Veratrum californicum*. This material was steeped in aqueous ammonia and extracted repeatedly with benzene. After removal of the solvent, the crystalline fraction was purified repeatedly by chromatography and recrystallization to yield the pure alkaloids cyclopamine and veratramine. This process afforded a yield of 320 mg of cyclopamine per kg of dried roots.^[2]

Recently, Cooper achieved a noticeable increase in the yield of this process by treating dried and ground roots first with aqueous ammonia, and then isolated the alkaloids by Soxhlet extraction (benzene as solvent). After removal of the solvent and two purifications by chromatography, cyclopamine was precipitated in acetone and recrystallized twice from ethanol/water to yield an average of 1.3 g of pure cyclopamine per kg of dried plants. Since dried roots of *Veratrum californicum* contain about 2.34 g of cyclopamine per kg, this process has a yield of 55 % and, therefore, can be regarded as efficient. However, problems arise from the extensive use of benzene (decaliter scale), especially when it comes to extracting large amounts of cyclopamine.^[4]

1.4.2. Synthetic Access

In the 1960s the research groups of Masamune and Johnson published the first total synthesis of jervine, and the research group of Kutney followed in the 1970s with an improved synthesis of an advanced intermediate of the original route.^[5,6] Since it had been demonstrated previously that jervine could be transformed into cyclopamine in one step and in moderate yield by a Wolff–Kishner reduction, these two syntheses also constitute the first formal route to cyclopamine. Our research group recently achieved a direct synthetic route to cyclopamine starting from commercially available dehydroepiandrosterone.^[7]

The research groups of Masamune and Johnson established a convergent access to jervine starting from the Hagemann ester—as a precursor of the *C*-nor-*D*-homosteroid—as well as (3*S*)-methylpiperidine—as a progenitor of the alkaloid substructure. The *C*-nor-*D*-homosteroid was first synthesized from the Hagemann ester in a racemic fashion by

using the Wilds–Stoutamire reaction, two Robinson annulations, as well as several redox operations. This racemic material was then resolved. The optically pure steroid was shown to be identical with material obtained in larger amounts by the known degradation and rearrangement of hecogenine. The crucial step in the synthesis was a coupling with an alkaloid precursor. Thus, enamine **2** was coupled with bromide **1**. This reaction did not proceed diastereoselectively, with the desired diastereoisomer isolated in only 5 % yield. The so obtained veratramine derivative **3** was then transformed, by manipulations of the oxidation state, into the epoxide **4**, which was then opened by intramolecular nucleophilic attack of the secondary alcoholate at the C23-position. Further manipulation of the oxidation state finally yielded jervine (Scheme 5).^[5]

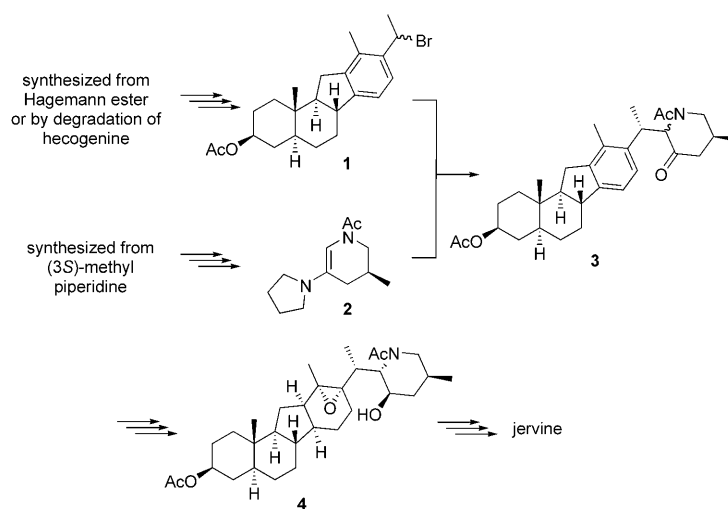
In summary, this first synthesis of cyclopamine illustrates the challenges such an endeavor poses: a convergent strategy heavily relies on an efficient and diastereoselective coupling method. This task was not addressed satisfactorily by the authors: the total yield of this synthesis starting from **1** is only about 0.001 % (estimation based on incompletely reported data). This result cannot meet modern requirements in terms of efficiency, elegance, and economy, but nevertheless represents an achievement when the limited possibilities available at that time are considered.

The formal synthesis by Kutney et al. in the 1970s generates an advanced intermediate of the Masamune/Johnson route. Again, the coupling of a lithiated picoline with a *C*-nor-*D*-homosteroid enone in a convergent fashion gives a coupling product in much better yield (62 %) than the original procedure, although as a mixture of C20 epimers. Since the subsequent hydrogenation of the pyridine nucleus to the corresponding piperidine provides (besides other isomers) the correct stereoisomer in 18 % yield, the yield of the first procedure was improved.^[6] Since the further elaboration resembles the Masamune/Johnson route, only the crucial fragment coupling was new, and thus the route was neither shorter nor much more efficient.

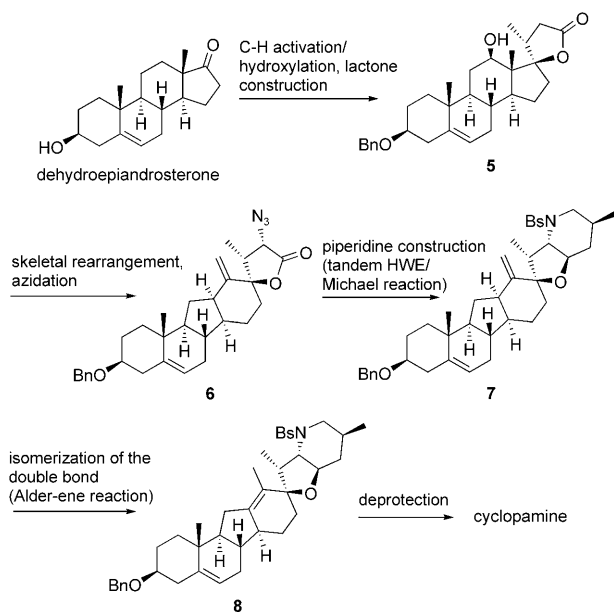
Our research group recently accomplished a biomimetic and completely diastereoselective synthetic route to cyclopamine which did not involve jervine as an intermediate. This route initially creates a 12 β -hydroxy steroid from commercially available dehydroepiandrosterone in three steps by a C–H activation and oxidation with molecular oxygen. This material is then transformed in four more steps into the lactone **5**. A subsequent Wagner–Meerwein rearrangement gives rise to the *C*-nor-*D*-homosteroid system in which the lactone is then α -azidized. The synthesis commences with the construction of the piperidine ring in seven steps to yield the cyclopamine derivative **7** with an exocyclic double bond. The decisive transformation is a tandem Horner–Wadsworth–Emmons (HWE) and intramolecular Michael reaction. The final isomerization of the double bond to the C12–C13-position is achieved using an Alder–ene reaction with *N*-sulfinylbenzene sulfonamide and subsequent desulfurization. Deprotection affords cyclopamine in 20 steps and a total yield of 1 % (Scheme 6).

This strategy uses the presumed biosynthetic concept for the creation of the *C*-nor-*D*-homosteroid skeleton by C–H activation/oxidation at the 12 β -position and ring contraction/expansion by conversion of this alcohol function into a leaving group. Thus, it can be regarded as biomimetic.^[7] Furthermore, the problem of unsatisfactory selectivity in the fragment coupling is circumvented by this linear approach. All reactions proceed under substrate control: the use of chiral auxiliaries and catalysts is therefore rendered unnecessary. This route also provides access to cyclopamine derivatives with different configurations, thus making it possible to establish fundamental structure–activity relationships. The potential of cyclopamine derivatives with an exocyclic double bond will be emphasized in particular, since these derivatives may exhibit enhanced metabolic stability and not degrade into toxic veratramine through aromatization of the D ring.

Our research group also recently published a synthetic strategy to all diastereoisomers of the piperidine alkaloid substructure of cyclopamine and all other *veratrum* alkaloids.^[8] For example, a correctly substituted piperidine lactone

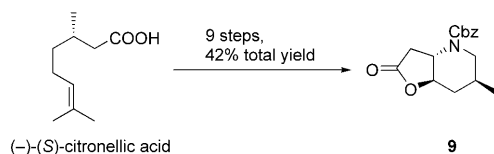


Scheme 5. Synthesis of jervine by the research groups of Masamune and Johnson.



Scheme 6. Biomimetic synthesis of cyclopamine by Giannis et al. Bn = benzyl, Bs = benzenesulfonyl.

such as **9** is synthesized in nine steps and in optically pure form with a substitution pattern that enables fragment coupling (Scheme 7). Since this synthetic route proceeds in a total yield of 42% starting from citronellic acid, it seems well suited for a new convergent approach towards cyclopamine.

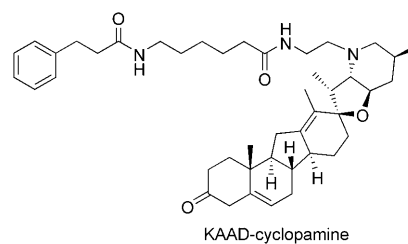


Scheme 7. Synthesis of the piperidine alkaloid substructure of cyclopamine. Cbz = benzyloxycarbonyl.

1.5. Derivatives of Cyclopamine

The improved commercial availability of cyclopamine from plant material through higher yielding extraction methods (see Section 1.4.1) has resulted in an increase in the number of semisynthetic cyclopamine derivatives. The price of cyclopamine has dropped by a factor of 50, in recent years and multigram amounts have become available. All the cyclopamine-like structures synthesized to date are semisynthetic derivatives, that is, derivatives synthesized from natural cyclopamine by chemical modification of the natural product. Since there is no facile synthetic approach (total synthesis) to cyclopamine, there are currently no completely synthetic cyclopamine derivatives.

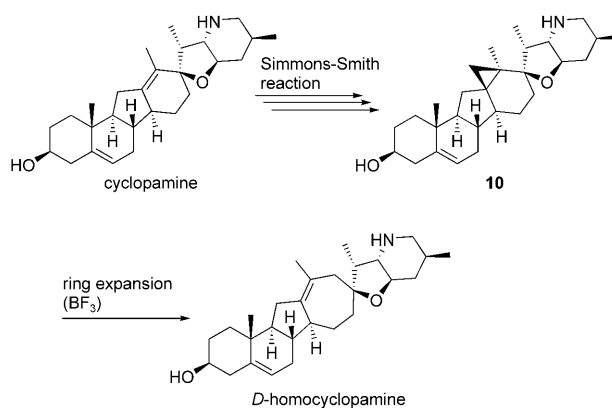
KAAD-cyclopamine (Scheme 8), the first derivative that exhibited better solubility and an increased biological potency by a factor of 10 to 20, was obtained in five steps from natural cyclopamine.^[9] To what extent the nonconjugated double



Scheme 8. Structure of KAAD-cyclopamine.

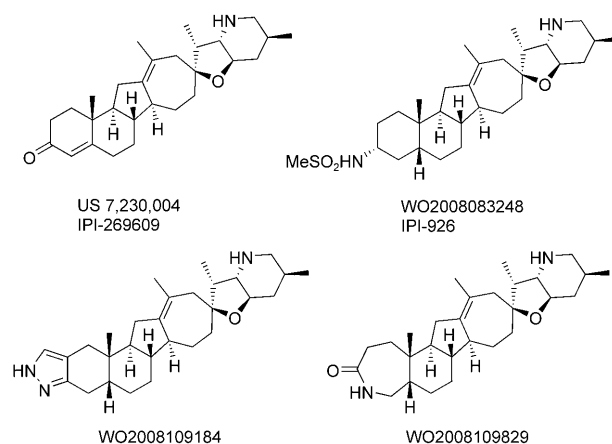
bond in the C5–C6-position is transformed to the corresponding enone under physiological conditions remains unexplored.

Infinity Pharmaceuticals published a multistep approach to *D*-homocyclopamine derivatives, that is, derivatives with a seven-membered D ring (Scheme 9). These derivatives do not



Scheme 9. D-ring expansion of cyclopamine derivatives.

inherit the instability of cyclopamine, since aromatization by cleavage of the allyl ether system cannot occur. Several derivatives of this type have been synthesized and patented, and their biological profiles have been determined—some of them are shown in Scheme 10.^[10] Carbohydrate–cyclopamine

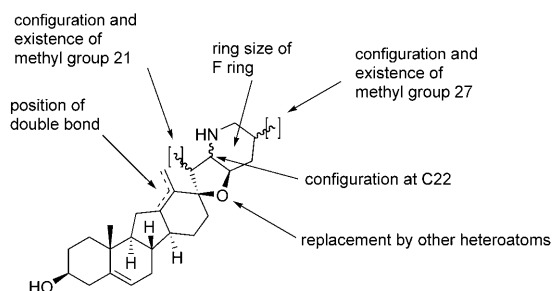


Scheme 10. Structure of some *D*-homo-ring derivatives of cyclopamine.

conjugates were prepared by using the concepts of click chemistry.^[11]

A simplified cyclopamine analogue was prepared from estrone in four steps by Winkler et al. The *C*-nor-*D*-homosteroid structure was omitted in favor of a conventional steroid system, a pyridine was used in place of the piperidine ring, and a phenol used for the homoallyl alcohol. Although these modifications have great influence on the structure, polarity, and acidity, biological activity was still evident.^[12]

The omission of the final isomerization of the double bond by an Alder–ene reaction, as used in the synthesis of cyclopamine established by our research group,^[7] would yield a cyclopamine derivative with an exocyclic double bond. Our synthetic strategy also allows the flexible introduction of methyl groups 21 and 27 with different configurations. It also allows variation of the configuration of the N-substituted center at C22, the preparation of derivatives without these methyl groups, different sizes of the F ring, and different heteroatoms in ring E. Thus, multiple analogues of cyclopamine could become available and would allow fundamental insights into structure–activity relationships from their biological potencies (Scheme 11)



Scheme 11. Possible variations in the synthesis of cyclopamine by Gianni et al.

2. Biology of Cyclopamine

In the late 1970s Nüsslein-Volhard and Wieschaus at the European Molecular Biology Laboratory in Heidelberg studied mutations in the fruit fly *Drosophila melanogaster* and identified more than 50 different genes that have a direct effect on embryonic development. One of these genes caused the larvae to grow a coat of spines on their undersides when it was mutated. The similarity of these larvae with a hedgehog led to the term *hedgehog* gene (*hh*). Christiane Nüsslein-Volhard and Eric F. Wieschaus together with Edward B. Lewis were awarded the Nobel prize in medicine in 1995 for their landmark research in the field of genetics of early embryonic development.^[13]

The *hedgehog* gene encodes for three unique proteins: Shh (Sonic hedgehog), Ihh (Indian hedgehog), and Dhh (Desert hedgehog). All of them are ligands of the membrane-bound receptor Patched (a 12-transmembrane protein) and activate the hedgehog signaling pathway, that is, they have an indirect influence on the transcription of *hedgehog-response* genes. The hedgehog signaling pathway consists of a cascade of repressive interactions. In the absence of a hh ligand, the

membrane-bound protein Patched1 (Ptch1) is found at the base of the primary cilia near the centrosome. Cilia are tail-like projections of the cell membrane that can be found in a single copy on nearly every eukaryotic cell. They are involved in sensing mechanical and chemical signals and act as communication hubs for signaling that controls cell differentiation and polarity. Ptch1 inhibits at the base of the primary cilium the translocation of the membrane-bound receptor Smoothened (Smo; a 7-transmembrane protein) from intracellular compartments to the cilium by an unknown mechanism. Thus, SuFu (the suppressor of the protein Fused), a negative regulator of hh signaling, stays active, which eventually leads to proteosomal cleavage and transformation of transcription factors Gli2 and Gli3 into their repressor forms. These Gli2/3R factors repress transcription of *hedgehog-response* genes. Many of these interactions are not well understood, although more recent studies show that translocation of Smo from endosomes into the ciliary membrane is inhibited by the influence of Ptch1 on the lipid composition of these compartments.^[14a]

The binding of a hh ligand results in the Ptch1 translocation out of the primary cilium and its degradation by lysosomes. Smo now moves into the cilium, thus stimulating the pathway and preventing cleavage of Gli2 and Gli3. Activated Gli2 (and to a lesser extent Gli3) then bind to Gli promoters in the nucleus and stimulate the transcription of *hedgehog-response* genes.^[14]

The *hedgehog* gene is now regarded as a key regulator of embryonic development and is highly conserved from fruit flies to humans. In insects, hedgehog signaling controls the correct segmentation and the development of the wings. The existence of the *Shh* gene in mammals was reported by Tabin and co-workers in 1993.^[15a] In mammals, it induces bilateral symmetry and the correct formation of limbs, skeleton, muscles, skin, eyes, lungs, teeth, nervous system, and intestines as well as the differentiation of sperm and cartilage.

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

Beachy and co-workers succeeded in combining their knowledge on two seemingly different scientific fields to solve this problem: the events in Idaho in the 1950s had remained a footnote in the history of science. It was, however, known that holoprosencephaly could be observed in severe cases of Smith–Lemli–Opitz syndrome (a defect in cholesterol biosynthesis caused by a deficiency of 7-dehydrocholesterol reductase). At first, Beachy and co-workers suspected cyclopamine to be an inhibitor of cholesterol biosynthesis (an idea further supported by the structural similarities of these molecules). However, this assumption was shown to be incorrect. In 1998, Beachy and co-workers finally identified that cyclopamine interacts with the protein Smo of the hh-signaling pathway, thereby inhibiting the activity of this pathway even when an hh ligand is present. Cyclopamine binds to Smo and inhibits its activity by a conformational

change. However, a general dependence of the hh signaling on cholesterol and its metabolites exists because of the necessity of C-terminal cholesteroylation of the hh ligand in its maturation process (additionally the hh protein is palmitoylated at the N terminus; Figure 1).^[15]

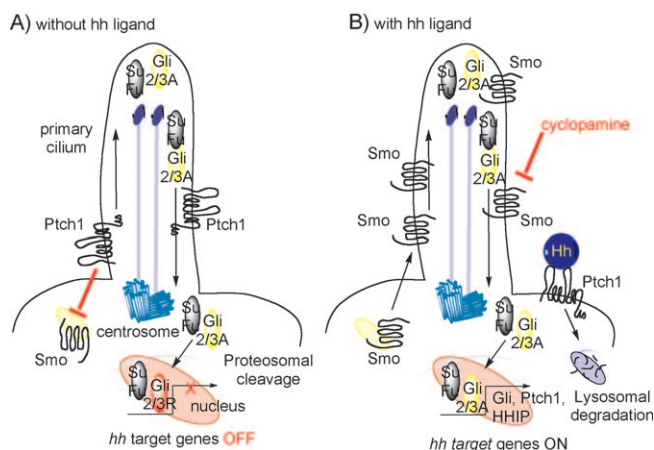


Figure 1. The hedgehog signaling pathway. A) In the absence of hh ligands, Ptch1 inhibits Smo from entering the cilium; thus, SuFu stays active, the Gli2/3 transcription factors are cleaved by proteasomes, and transcription of *hh*-target genes is prevented. B) The binding of a hh ligand results in Ptch1 being inhibited and Smo moves into the cilium, where SuFu is inhibited and the active form of the Gli2/3 factors can activate transcription of the *hh* gene in the nucleus; cyclopamine inhibits the activity of Smo and leads to deactivation of the hh path. For further explanation, see the text; scheme modified from Ref. [14b].

When pregnant sheep ingest cyclopamine, embryonic development is critically disturbed since it heavily relies on hh signaling at several stages (for example, at the 14th day of gestation), which finally results in the malformations observed in Idaho.

3. Medical Relevance of Cyclopamine and Hedgehog Signaling

The hedgehog pathway is essential in multiple developmental processes and in the regulation of stem-cell and progenitor-cell proliferation and differentiation. Since aberrant activation of the hh pathway leads to malignancies, including basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, prostate, pancreatic, colorectal, prostate, and breast cancer, inhibition of hh signaling provides a route to novel anticancer therapies. Hedgehog-pathway inhibitors (HPI) 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 basic principles of the participation of the hh pathway in cancer and the important developments in this field are presented. An exhaustive discussion is beyond the scope of this Minireview, and the interested reader is referred to several excellent reviews.^[16]

Finally, some preclinical and a few recently published results of clinical studies with Smo inhibitors used as anticancer drugs will be discussed.

3.1. Models of Cancer involving Hedgehog Signaling

Three basic models are discussed in the recent literature for the participation of the hh pathway in cancer (Figure 2)^[17]:

A) Type 1 cancers (ligand independent, mutation driven) were discovered first. Patients with Gorlin syndrome, which belongs to this category, have a high incidence of

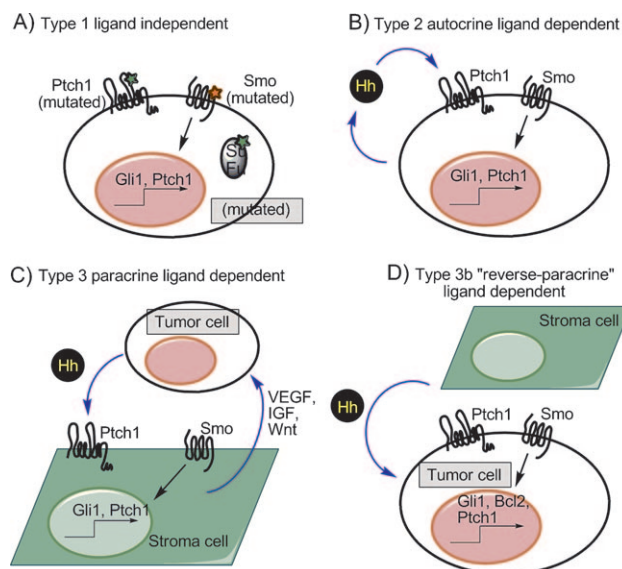


Figure 2. Models for the development of cancer that involve hh signaling; cilia are not shown for reasons of clarity. For further explanation, see the text; scheme modified from Ref. [14b].

basal cell carcinoma (BCC), medulloblastoma, and rhabdomyosarcoma. The molecular basis for the development of this type of malignancies are inherited inactivating mutations in *Ptch1* (through loss of heterozygosity and/or inactivating mutations), which lead to constitutively activated hh signaling in the absence of a ligand. Gorlin patients are excellent candidates for therapy with inhibitors of Smo or downstream components of the hh pathway.

- B) Type 2 cancers are dependent on autocrine-secreted hh ligands. The same tumor cells both produce the hh ligands and responded to them. Only recently, it was demonstrated conclusively that this type of hh signaling exists in human cancer cell lines of the colon.^[18]
- C) Type 3 cancers are dependent on paracrine-secreted hh ligands. Recent studies showed that an overproduction of hh ligands by the tumor cells occurs in most cancers linked to aberrant hh signaling. Hedgehog proteins may stimulate stromal cells near the tumor (endothelial cells, epithelial cells, fibroblasts, and immune cells). This results in an indirect support for tumor growth through mechanisms originating in the stromal 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 the molecular signaling pathways involving insulin-like growth factor (IGF) and Wnt.

- D) A variant of this type of hh signaling is the so-called “reverse-paracrine” signaling (type 3b), where hh ligands are secreted from stromal cells to receiving cells in a tumor.

3.2. Treatment of Pancreas 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 five-year survival rate is estimated to be less than 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 develop metastases in distant organs, and finally die.^[19] 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.^[20] Inhibition of hh signaling with cyclopamine has enhanced the survival rate in a genetically engineered mouse model of pancreatic cancer and abrogated the systemic metastases arising from orthotopic xenografts.^[21] This study also provides more evidence that hh signaling is a valid target for the development of novel therapeutics for pancreatic cancer and would be worth evaluating in a clinical setting.

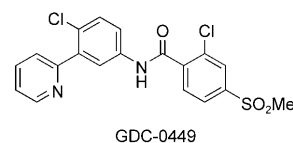
Since cyclopamine displays a low affinity for Smo, has poor oral bioavailability, suboptimal pharmacokinetics, and low metabolic stability, the derivative IPI-269609 (Scheme 10) was developed. It retains the potency of cyclopamine, but has improved physicochemical properties and is metabolically stable. IPI-269609 profoundly inhibited systemic metastases in orthotopic xenografts established from human pancreatic cancer cell lines. IPI-926 (Scheme 10), a novel semisynthetic cyclopamine analogue with substantially improved potency and a favorable pharmacokinetic profile relative to cyclopamine, was subsequently designed and synthesized. By using a highly invasive and lethal genetically engineered model of pancreatic cancer, Olive et al.^[22] showed that IPI-926 increases survival when used in combination with gemcitabine in this 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.

3.3. 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 percent of all nonmelanoma skin cancers. The disease has an estimated annual incidence of 0.1 to 0.5%^[23] and is largely caused by exposure to UV radiation. 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. For the reasons mentioned in Section 3.2, 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;^[24] all of the tumors treated with cyclopamine regressed rapidly. Histological and immunohistochemical analyses showed inhibition of the proliferation and highly efficient induction of the differentiation and apoptosis of the tumor cells. The selective and highly efficient induction of the differentiation and apoptosis of tumor cells *in vivo* by transient inhibition of hh signaling provide a rational approach to treating BCC.

Recently 33 patients with metastatic or locally advanced basal-cell carcinoma received GDC-0449 orally. GDC-0449 is an inhibitor (Scheme 12) of Smo, and structurally unrelated to



Scheme 12. Structure of GDC-0449.

cyclopamine.^[25] Of the 33 patients, 18 had an objective response to GDC-0449, and according to assessment by imaging and/or physical examination 11 patients showed a response. Of the patients who had a response, 2 had a complete response and 16 had 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 to treat inoperable tumors.

GDC-0449 has also been used in the therapy of medulloblastoma in one patient.^[26] Medulloblastoma is the most common embryonal tumor in children. The median age at diagnosis is five 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.^[27] 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 analyses 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 encoding Ptch1—a key negative regulator of hh signaling. 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.^[28] The authors identified an amino acid substitution at a conserved aspartic acid residue of Smo that had no effect on hh signaling, but disrupted the ability of GDC-0449 to bind Smo and to act as an inhibitor of the hh pathway. A mutation altering the same amino acid also arose in a GDC-0449-resistant mouse model of 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.^[29]

Taken together, these 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”.^[30]

4. Summary

The story of cyclopamine began in 1957 with an unsettling observation and could possibly have ended as a footnote in the history of science after the problem was solved. Only the interdisciplinary interplay between chemistry, biology, and medicine enabled cyclopamine to have a second coming. On the basis of the fundamental studies of Keeler and James as well as Nüsslein-Volhard and Wieschaus, Beachy was able to come to the final conclusion that initiated the development of cyclopamine as an anticancer therapeutic, the potential of which is only starting to be realized. This development has taken place over 50 years and can be regarded as a prime example of nonlinear research that rewards the persistent observer.

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