Chapter 12
Cyclopamine and Its Derivatives for Cancer Therapeutics

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Introduction

In the 1950s, researchers from the United States Department of Agriculture (USDA) investigated cases of congenital cyclopia in sheep grazing in high mountain ranges in central Idaho. After nearly a decade of research, steroidal alkaloids present in the corn lily plant (*Veratrum californicum*) were found responsible for the induction of cyclopic-type craniofacial birth defects that occurred when *Veratrum* was ingested by pregnant sheep on day 14 of gestation [7]. Jervine and cyclopamine were two important teratogenic compounds isolated from *Veratrum californicum* (Fig. 12.1, compound **2** and **6**, respectively), while numerous nonteratogenic but toxic *Veratrum* alkaloids were also present such as veratramine and muldamine [42–46]. Of note, the maternal ewes do not suffer ill effects from ingestion of the plants or cyclopamine [7], with birth defects being confined to a specific window of time during fetal development [103]. Because of its steroidal structure, cyclopamine was originally proposed to antagonize putative hormones involved in regulation of specific genes [42]. The pharmacology of cyclopamine remained dormant for nearly 30 years until genetic studies revealed that mutations in the Hedgehog pathway impacted development.

The role of the hedgehog pathway in development was first discovered in the fruit fly Drosophila by Nüsslein-Volhard and Wieschaus [72] and was ultimately recognized by a Nobel Prize in 1995. Their groundbreaking mutational analysis of genes in Drosophila that control segmentation and polarity elucidated a pathway that, when mutated, resulted in larvae with spiculated cuticles on their skin, resembling the spines of a hedgehog. Subsequent identification of the specific gene products revealed a unique signaling pathway with related orthologues in vertebrate organisms [37]. Mutations generated in the Hh pathway in vertebrates resulted in animals with cyclopic features [14]. These findings were substantiated in humans,

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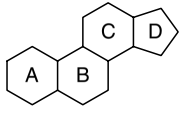
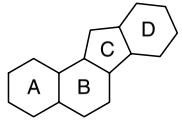
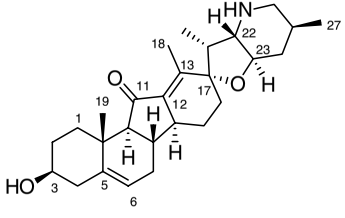
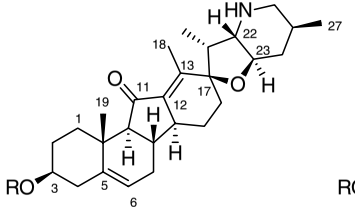


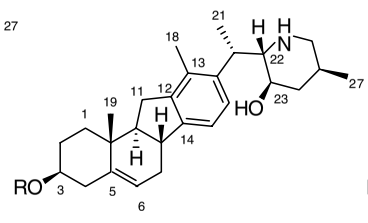
General carbon framework of steroid

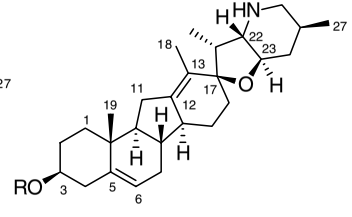


General carbon framework of C-Nor D-Homo steroid









1 Jervine (Originally proposed structure) **2** jervine (revised structure, R = H)
3 pseudojervine (R = Glu)

4 veratramine (R = H)
5 veratrosine (R = Glu)

6 cyclopamine (R = H)
7 cycloposine (R = Glu)

Fig. 12.1 General structure of steroid and C-nor D-homo steroids. Chemical structures of a subset of *Veratrum* alkaloids

where mutations in Shh were linked to holoprosencepaly, which can include cyclopic features [5, 80]. Thus, all inhibitors of the Hh pathway, whether derived from cyclopamine or not, would be expected to impact embryogenesis.

Studies of the Hh pathway in multiple developmental systems have identified key signaling proteins and genes that are regulated in response to ligand activation. Important links between Drosophila genetics and vertebrate biology led to breakthroughs in our understanding of the Hh pathway. One such link, as described above, was the discovery of the plant-derived alkaloid cyclopamine that is found in *Veratrum californicum*. Cyclopamine was subsequently found to antagonize the Hh pathway [15, 32] and to exert its inhibitory effects by binding to Smoothened (Smo) [12, 88]. The natural product cyclopamine, while not active against Drosophila Smo, has served as a powerful tool to help understand the role of the Hh pathway in many aspects of mammalian physiology and disease. While the entire book describes how the Hh pathway is involved in development and cancer, this chapter will review several aspects of the chemistry, pharmacology, and therapeutic potential of cyclopamine and its derivatives.

Cyclopamine, a Natural Steroidal Alkaloid from *Veratrum* Species: Extraction, Isolation, and Structure Elucidation

As early as in the 1870s, a number of alkaloids with the C-nor D-homo steroid skeleton have been isolated from plants of the lily family such as *Veratrum* species. Over the years, a subset of alkaloids shown in Fig. 12.1 has triggered interest because of either their relative abundance and/or their pharmacological effects. Jervine was first isolated from *Veratrum album* [108, 109]. Since this pioneering work by Wright, several extraction and isolation methods for jervine have been described [82, 83] and its structure determination has been debated over several decades. Originally, a number of proposed structures placed jervine in the category of regular steroids constructed with a 6-6-6-5 tetracyclic framework [34, 35, 106]. However, careful exploration of the chemical reactivity of the C–D ring α/β-unsaturated ketone and degradation studies provided evidence that "this alkaloid does not have a normal steroid nucleus" [22]. The structure as depicted in **1** was then proposed, but there continued to be uncertainty concerning the C17 configuration [107]. Further characterization work [54] and X-ray crystallographic data of related alkaloids [79] subsequently showed that the configuration at the C17 position was inverted in the original assignment and that structure **2** was indeed jervine's accurate molecular structure. The structure elucidation of jervine served as a cornerstone to the discovery of other *Veratrum* alkaloids.

A jervine congener, cyclopamine **6** (11-deoxojervine), was isolated independently from *Veratrum glandiforum* (aka *Veratrum album*) collected in the Tokachi district of Hokkaido in Japan [65] and from *Veratrum californicum* collected in the western US [44, 46]. As was the case originally for jervine, the C17 configuration was wrongly assigned initially but corrected when more data became available on jervine.

Early after a reliable isolation method was published for cyclopamine by the USDA research group, they also reported the isolation of the glycosylated form of cyclopamine called cycloposine **7** [47]. Several years after pioneering work by Masamune and Keeler, improved methods to extract and isolate cyclopamine were published [36, 50, 73, 86], and it is clear that *Veratrum californicum* is rich in cyclopamine from all of these reports.

Synthetic Chemistry of Cyclopamine

The amazing structural constitution of jervine and cyclopamine classified these plant natural products as C-nor D-homo steroids. Both compounds have 27 carbons arranged in four carbocycles (A–D rings) and dimethyloctahydrofuro[3,2-b] pyridine (E–F rings), the latter being spiro-connected to the D-ring at the C17 position. Stereochemical complexity in a form of ten stereogenic centers, two of which are quaternary centers, can be found in jervine and cyclopamine. Two polar functions (C3-alcohol and basic piperidine nitrogen) are on the two opposite sides of the lipophilic steroidal skeleton, which is unsaturated at two positions (B-ring and D-ring). Jervine is only different from cyclopamine by the presence of the C11-keto function, which is conjugated with the alkene on the D-ring. Relative to conventional steroids, the preparation of C-nor D-homo steroids has received far less attention from the synthetic community [11]. Nevertheless, a few strategies were exploited to access small quantities of jervine and cyclopamine (Fig. 12.2).

The rearrangement of regular steroids to C-nor D-homo system discovered by Hirschmann [31] provided the basis for the semisynthesis of jervine **2** from hecogenin **8** [67] and more recently of cyclopamine **6** from dehydroepiandrosterone **9** [24]. One of the first successful approaches to jervine started with hecogenin **8** that was converted to intermediate **11** by a six step sequence [69]. Alternatively, the same intermediate could be synthesized from Hagemann ester **10** by a series of annulation reactions [38]. Conversion of the tetracyclic intermediate **11** to jervine involves addition of the E–F ring system in multiple steps [55, 67]. Cyclopamine can be obtained from jervine through a Wolff–Kishner reduction of the C11-ketone [87]. For nearly three decades following these reports, the synthetic community paid little attention to the synthesis of in *Veratrum* alkaloids. The elucidation of the mode of action of cyclopamine coupled with its therapeutic potential revamped interest for this wonderful but complex molecule. Nowadays, many synthetic groups have active research programs aimed at the synthesis of cyclopamine. Recently, a semisynthetic approach to cyclopamine from dehydroepiandrosterone was described [24]. While this approach utilized essentially the same rearrangement pioneered by Hirschmann (*vide infra*), this key transformation was performed on the advanced intermediate **12** bearing the extremely challenging E-ring with the correct configuration at C17 (Fig. 12.2). This approach is very powerful because the spiro-γ-lactone in intermediate **12** can be readily installed and ultimately serves as a versatile building block for the construction of the piperidine ring.

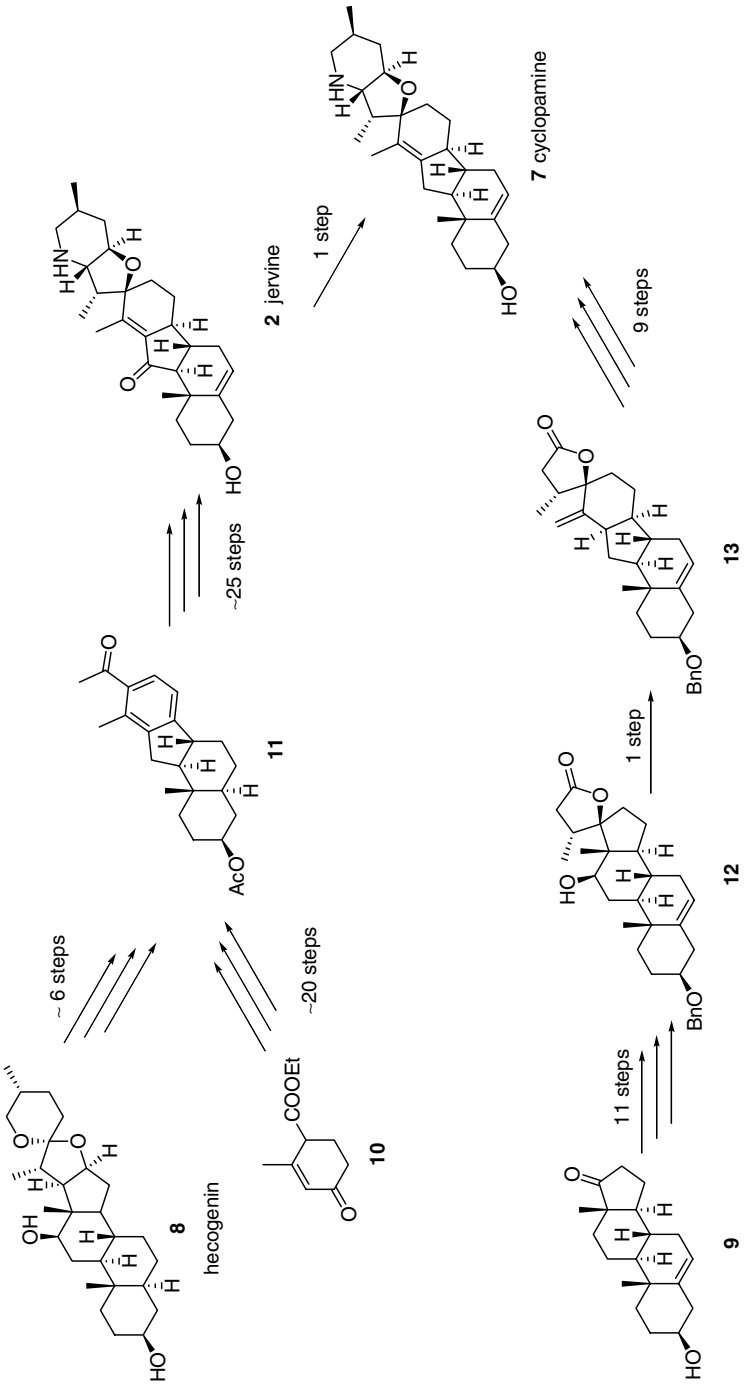


Fig. 12.2 Synthetic approaches to jervine and cyclopamine through rearrangement of C/D ring of conventional steroids to C-nor D-homo steroids or by total synthesis

Evaluation of Cyclopamine as Drug Lead: Its Drug Properties

With the emerging role of the Hh pathway in disease in the late 1990s, the need for selective modulators to test their therapeutic potential became apparent. The discovery of small molecule modulators of the Hh pathway has been comprehensively reviewed elsewhere [62, 77, 95] and the optimization of cyclopamine analogs is the focus of this book chapter. By virtue of its role in target validation of Smo and its availability from nature, cyclopamine is a very interesting starting point for the discovery of modulators of the Hh pathway. Cyclopamine can be readily isolated in high yield from *Veratrum californicum* [36, 50, 73, 86]. This plant species is naturally occurring in the western US, particularly in Utah and Idaho [49], where it was once the target for eradication due to the harmful effects on livestock. Alternatives to field cultivation can already be envisioned for *Veratrum* plant species. Successfully applied to taxol, a therapeutic compound isolated from the yew tree [52], a plant cell culture technique has been reported to generate green plants from embryonic calli of *Veratrum californicum* [61]. Most importantly, these green plants produced veratramine and traces of cyclopamine when grown in suspension media in the presence of naphthalene acetic acid. To complement the latter approach, alternative production platforms such as metabolic engineering are emerging [56] and may be applicable to *Veratrum* alkaloids. Toward that end, important steps in the biosynthetic pathway of cyclopamine have been elucidated [39, 40], which may supplement the development of in vitro techniques to produce cyclopamine. In addition to biomass production and processing, cyclopamine can be obtained by semisynthetic approaches. As one example, jervine can be converted to cyclopamine via Wolff–Kishner reduction [87] (Fig. 12.2). Another example is the single step conversion of cycloposine **7** to cyclopamine **6** by deglycosylation methods [36]. These one step transformations from other C-nor D-homo steroids provide additional source of starting materials.

While cyclopamine represents a very interesting starting point for the discovery of modulators of the Hh pathway, its clinical development was hampered mainly by poor pharmaceutical properties and suboptimal potency. Cyclopamine has low water solubility, which initially impeded the development of formulations for administration to animals. A variety of cyclopamine formulations, including ethanol [3, 50], triolein/EtOH (4:1, v/v) [6, 89], dimethylformamide [98], and dimethylsulfoxide [96], have recently been reviewed [59]. The addition of high concentrations of complexing agents such as 2-hydroxypropyl-β-cyclodextrin resulted in better aqueous formulations of cyclopamine [75, 99]. Alternatively, decreasing the concentration of 2-hydroxypropyl-β-cyclodextrin and thereby the viscosity can be achieved by using the hydrochloride salt of cyclopamine to generate chemically and physically stable formulations [21, 92]. In addition to the hydrochloride salt, water soluble tartrate salts of cyclopamine have recently been described in the literature [103, 110].

In addition to its poor aqueous solubility, cyclopamine is acid labile and readily converts to veratramine (**4**, Fig. 12.1) [48] as well as other isomeric products [104]. Although structurally related to cyclopamine, veratramine does not act as a Smo

antagonist but affects several receptors [70, 90] and causes hemolysis [2]. This liability may not be problematic for oral administration of cyclopamine in preclinical species where the stomach pH may not promote conversion, but in humans the stomach pH ranges between 1.5 and 3 [20] and gastrointestinal transit time is typically longer (20–30 h) than other laboratory animals [41].

In some of the early studies with cyclopamine in sheep, observation of the teratogenic effects required daily oral (p.o.) administration of 2–3 g/animal (average weight of animal ~50 kg; 40–60 mg/kg) [42, 45, 50]. This need for high and frequent doses could be due to poor pharmacokinetic properties and/or potency; some recent studies have shed some light on both aspects. In sheep, a short elimination half-life (1.1 ± 1 h) was measured when cyclopamine tartrate salt (1.6 mg/kg) was administered intravenously (i.v.) [103]. In rodents, the oral (p.o.) bioavailability of cyclopamine is modest (33% relative to intraperitoneal (i.p. administration [59]) and the poor pharmaceutical properties are likely responsible for this observation.

Many cellular assays have been developed to evaluate the potency of Hh pathway inhibitors. Cyclopamine inhibits Hh-dependent processes including (a) HNF3β-induction in chick embryo neural plate assay (IC_{50} of 10 nM; [33]), (b) Gli-reporter assay (IC_{50} of 300 nM; [12]), and (c) C3H10T1/2 differentiation (IC_{50} of 300–400 nM [18]). While the teratogenic effect of cyclopamine is associated with on-target activity on Smo and the Hh pathway, there is evidence that cyclopamine may not be entirely selective. High concentrations of cyclopamine (10 μM) have shown cytotoxic effect on cancer cells that do not express Smo, indicating that the observed effect was off-target. This phenomenon is not unique to cyclopamine since another small molecule Hh antagonist (Cur61614) that is not structurally related to cyclopamine has shown the same behavior [114]. The use of cancer cells grown in culture for the evaluation of Hh pathway antagonists has been extremely controversial. There have been reports in the literature revealing that cancer cell lines grown in culture lose their dependence on the Hh signaling pathway for growth [85, 101]. For this reason, in vitro assessment of a therapeutic window related to treatment with cyclopamine needs to be interpreted carefully. Recent studies have provided some insights into the toxicity of cyclopamine in vivo [59]. The observed toxicity is dependent on the route of administration and could be circumvented by infusion of the drug. This mode of administration delivers cyclopamine at steady state concentrations, whereas bolus intraperitoneal (i.p.) and oral (p.o.) dosing resulted in high and transient plasma peak concentrations that lead to severe dystonia and lethargy, respectively. The relatively rapid onset of the observed toxicity (between 2 and 6 h postdose for i.p. and p.o., respectively) suggests that off-target mechanisms are likely the cause. However, it is unclear if this toxicity is due to the parent drug or a metabolite of cyclopamine. Indeed, high plasma concentration of cyclopamine (20 μM) are well tolerated by the animals dosed i.p., whereas toxicity is observed in orally dosed animals with relatively low (2 μM) plasma concentration of cyclopamine. In summary, derivatives of cyclopamine with improved solubility, stability, and potency are highly desirable to address some of the issues outlined above.

Medicinal Chemistry of Cyclopamine Analogs

Well before the elucidation of cyclopamine's biological target, scientists have been intrigued by the structure–activity relationship (SAR) of naturally occurring jervine [9, 10] and cyclopamine, [48], in particular with respect to their teratogenic potential. However, the determination of biological effects required gram quantities of material which obviously hampered the ability to obtain SAR data. Moreover, the teratogenic potential measured in vivo encompasses both pharmacodynamic and pharmacokinetic properties of the molecules, which makes it difficult to draw conclusive SAR. With that caveat in mind, given that both jervine **2** and cyclopamine **6** are active teratogens, one may conclude that the 11-keto group is not necessary for this activity. However, this functional group plays a role in increasing the stability of D/E-ring system by reducing acid-mediated opening of the ether bridge [9]. Interestingly, cyclopamine-4-en-3-one **14** [66] (Fig. 12.3) was found to be at least twofold more bioactive than jervine as demonstrated independently by its teratogenity in hamsters [9, 23] and its inhibitory effect of Shh signaling in chick neural plate [33]. Also shown in these two assays, the reduction of C5–C6 and C12–C13 double bonds produced a weaker teratogen tetrahydrojervine **15** [68], whereas substantial activity was retained for 12β,13α-dihydrojervine **16**. Cyclo-posine **7** bearing a glucosyl group at the C3 position has also shown teratogenic activity [45], but its effects on the Shh signaling in the chick neural plate was modest [33]. It is plausible that the glycosyl group of cycloposine could be hydrolyzed to liberate cyclopamine in vivo [103], thus explaining the difference between these two results. In addition to alterations of the steroidal skeleton and substitution at the C3 position, the piperidine

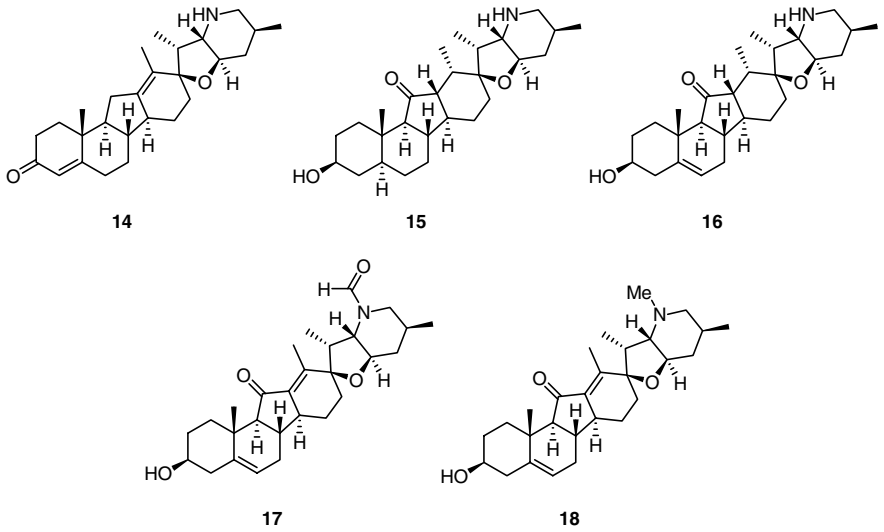


Fig. 12.3 Representative set of early analogs of cyclopamine and jervine that have shown teratogenic activity

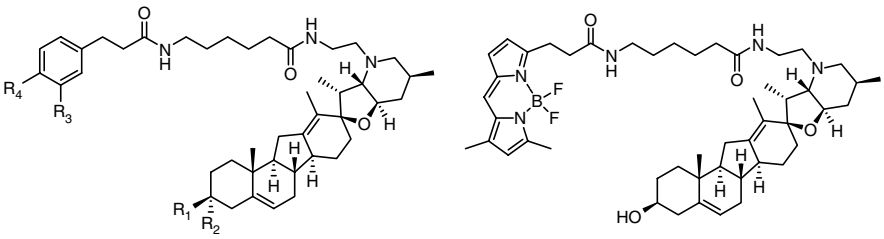


Fig. 12.4 Semisynthetic analogs and probes of cyclopamine based on the natural steroid skeleton

nitrogen was also subjected to modifications [10]. Notably, *N*-formyl (**17**) and *N*-methyl (**18**) jervine analogs were determined to be active teratogens in hamsters, while quaternization of the nitrogen or bulkier alkyl substituents almost completely eliminates the biological activity. In summary, early studies around the structure–teratogenicity relationships of jervine and cyclopamine revealed that these structures were amenable to changes while retaining the biological response. However, improvement of the drug-like properties of these compounds (*vide infra*) would require further investigations.

A synthetic analog of cyclopamine named KAAD-cyclopamine **19** (IC_{50} = 20 nM) was determined to be one order of magnitude more potent than cyclopamine (IC_{50} = 300 nM) in the Shh-Light Gli-reporter assay [13] (Fig. 12.4). This compound played a significant role in establishing Smo as the target of cyclopamine. A radiolabeled and photoaffinity derivative of KAAD-cyclopamine (**20**) (IC_{50} = 150 nM, Shh-Light) was used in the target identification studies [12] and BODIPY derivative **21** (IC_{50} = 150 nM, Shh-Light) is commonly used to assess binding affinity of Smo antagonists. A number of related analogs have also been reported in the patent literature [4]. These studies showed that the potency of cyclopamine could be improved through chemical modifications.

A diversity-oriented approach was designed to generate a focused library of carbohydrate cyclopamine conjugates with improved aqueous solubility [113]. As an example, compound **22** bearing L-rhamnose moiety has better aqueous solubility and similar anticancer activity (IC_{50} = 33 μM) relative to cyclopamine in A549 lung cancer cell line. Although no binding data on Smo were reported for these compounds, it is notable that polar solubilizing groups on the piperidine nitrogen are tolerated while KAAD-cyclopamine **20** bears highly lipophilic side-chain at the same position.

Aiming to develop a targeted therapy for prostate cancer and perhaps move away from potential on-target effects of cyclopamine on the normal stem cell niche, prodrug derivative **23** was designed and synthesized (Fig. 12.5) [53]. In this approach, a peptide carrier susceptible to selective hydrolysis by the active form of prostate-specific antigen (PSA) was grafted to cyclopamine. While the prodrug itself had no significant activity, cyclopamine was indeed released in presence of PSA and biological response was observed in DU-145 prostate cell line. The same concept,

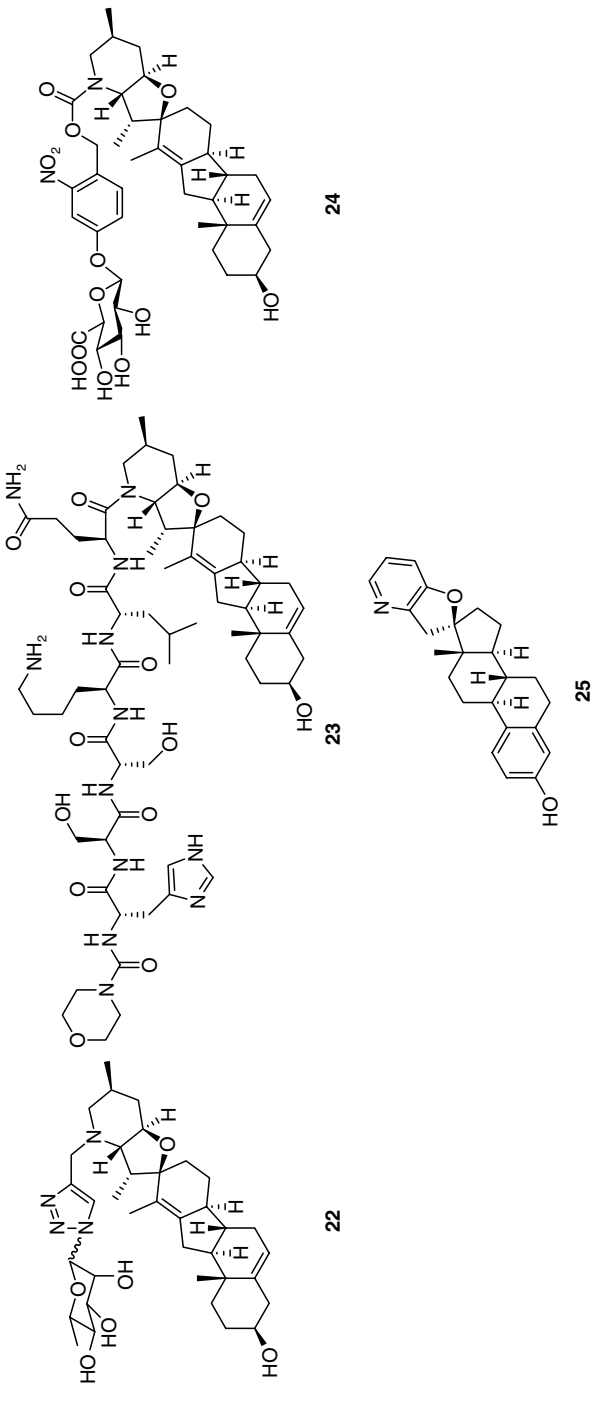


Fig. 12.5 Semisynthetic analogs of cyclopamine based on natural steroid skeletons

with potentially broader scope, led to the design and synthesis of the carbamate cyclopamine analog **24**. Carbamate prodrug **24** was not active by itself, but recapitulated cyclopamine's growth inhibition in glioma U87 cell line when exposed to β-glucuronidase, which is typically present in high levels in the tumor vicinity [26]. These two distinct approaches demonstrated that cyclopamine prodrugs offer the opportunity to selectively deliver cyclopamine to tumor environments.

Readily available steroidal synthetic starting points provided novel cyclopamine analogs that were evaluated for their effect on the Hh pathway [105]. For instance, estrone derivative **25**, which displays a simplified E/F ring system, was shown to inhibit Shh-induced proliferation of mouse granule neuron precursors as well as Shh-Light2 cells at 10 μM. This study revealed that simplification of the core structure of cyclopamine could result in analogs with desirable properties, but more work needs to be done to understand the consequence of these simplifications on the binding to Smo. As a complementary approach, the synthesis of cyclopamine from dehydroepiandrosterone (Fig. 12.2) could deliver very interesting analogs, particularly on the E/F rings, which would be otherwise challenging to obtain from the natural product [28, 29].

Discovery and Development of IPI-926, a Semisynthetic Cyclopamine Derivative in Clinical Trials

The first strategy to improve the pharmaceutical properties of cyclopamine was to address the acid lability. The approach was to alter the influence of the D-ring allylic ether on the cleavage of the spirotetrahydrofuran E-ring, with minimal change to the D-ring geometry. It was originally hypothesized that the oxygen of the allylic ether could direct a chemo- and stereo-selective cyclopropanation of the D-ring double bond. Serendipitously, it was found that the D-ring could be expanded synthetically by cyclopropanation and subsequent acid-catalyzed rearrangement. The resulting 7-membered ring cyclopamine analogs, exemplified by compound **26** (Fig. 12.6), were found to have increased chemical stability [93]. Reminiscent of the case of cyclopamine (*vide infra*), the change in oxidation state from homoallylic alcohol to the α/β-unsaturated ketone in the A/B-ring system brings significant improvement of potency and, in this case, solubility. Also, the structure–activity relationships for N-substitution on compound **26** and cyclopamine closely track, suggesting that expansion of the D-ring did not cause major changes in the binding mode. Compound **26** is equipotent to cyclopamine, yet has improved aqueous solubility and stability relative to cyclopamine. The α/β-unsaturated ketone system found in compound **26** is a very common and structurally important functionality in endogenous steroid hormones. However, this functionality on compound **26** was found to be readily metabolized to the corresponding saturated alcohol and glucuronide conjugate after oral administration in cynomolgus monkeys [64]. The conversion of the half-chair A-ring system of compound **26** to a *cis*-ring fusion system provided a remarkable improvement (approximately tenfold) in potency of

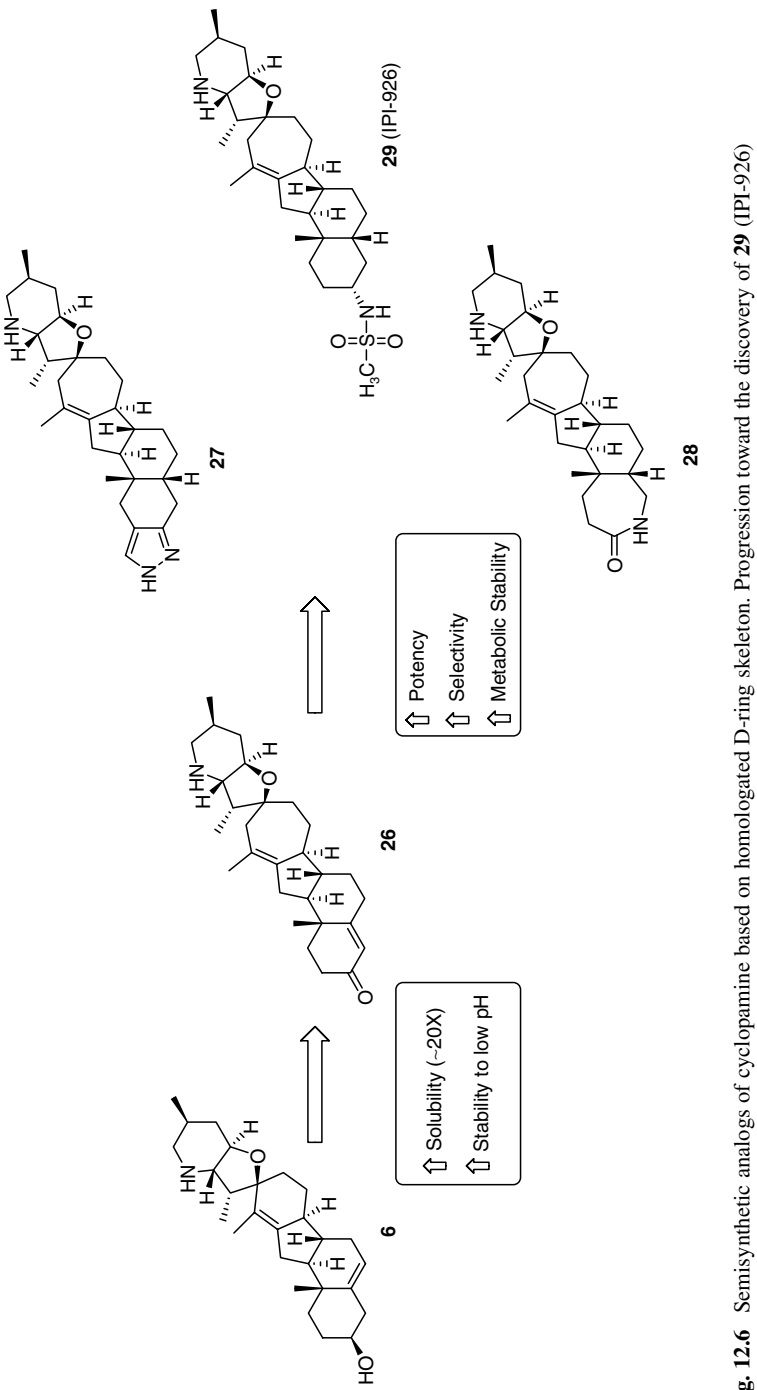


Fig. 12.6 Semisynthetic analogs of cyclopamine based on homologated D-ring skeleton. Progression toward the discovery of **29** (IPI-926)

these D-homo cyclopamine derivatives. Further modifications of the A-ring generated three new series of analogs pyrazole **27**, lactam **28**, and sulfonamide **29** with significantly greater potency and less susceptibility to metabolism than the first generation compound **26**. One key discriminating factor between the three series of analogs is their pharmacokinetic profiles. Although all three series displayed good exposure when administered orally to multiple species, the sulfonamide **29** showed a significant increase in plasma half-life due to low clearance and high tissue distribution (Table 12.1). These properties translated into greater tumor-free intervals following treatment and more robust efficacy than the two other lead compounds when studied in an Hh-dependent B837Tx tumor model, which is described in more detail below [94].

To support its clinical development, capabilities for robust large-scale production of compound **29** were established. First, sourcing of starting material and large-scale extraction and isolation of cyclopamine *Veratrum californicum* was developed and optimized. A robust process was developed to produce multiple kilograms of advanced intermediates (e.g., compound **26**) in fixed equipment. Likewise, the original conversion of cyclopamine to IPI-926 utilized multiple steps that involved potential throughput-limiting purification steps [93, 94].

Table 12.1 In vitro and in vivo profiling of cyclopamine analogs **27**–**29**[a]

Potency	**6** (cyclopamine)	**27**	**28**	**29** (IPI-926)
C3H10T1/2 (EC50)	300–400 nM	10–20 nM	30–40 nM	7–15 nM
Smo Binding (IC50)	114 nM	6 nM	6 nM	1–2 nM
DMPK	**6** (cyclopamine)	**27**	**28**	**29** (IPI-926)
Range F_{PO} (mouse, rat, dog, cynomolgus)	33%[b]	30–80%	>90%	50–100%
Range $t_{½}$ (mouse, rat, dog, cynomolgus)	4 h[b]	1–7 h	3–7 h	8–24 h
Range Vd (mouse, rat, dog, cynomolgus)	–	6–13 L/kg	2–5 L/kg	9–30 L/kg
Efficacy Ptc1$^{+/-}$/ Hic1$^{+/-}$ B837Tx allograft mouse model	**6** (cyclopamine) (40 mg/kg p.o.)	**27** (80 mg/kg p.o.)	**28** (30 mg/kg p.o.)	**29** (IPI-926) (40 mg/kg p.o.)
Tumor-free interval 21 days posttreatment	Not observed	19 days	10 days	21 days
Recurrence rate posttreatment	100%	40%	30%	0%

[a] From Tremblay et al. [94]

[b] From Lipinski et al. [59]. Species: Female C57BL/6J mice at 12–16 weeks of age

Following optimization, IPI-926 drug substance is produced without the involvement of chromatography [1]. Finally, IPI-926 drug product is a solid dosage form intended for oral administration, which was developed to support dose-escalation in the clinic. In a single molecule, IPI-926 (**29**) encompasses the structural features and pharmaceutical properties that overcome many of the deficiencies identified for cyclopamine – namely, solubility, stability, pharmacokinetic profile, and in vivo potency.

Preclinical Pharmacology of IPI-926

The activity of cyclopamine and analogs (e.g., **27**–**29**) were assessed in an assay that measures activation and inhibition of Hh pathway-dependent cellular differentiation. This assay is a Smo-mediated differentiation of the murine mesenchymal cell line C3H10T1/2; it has been demonstrated that cells exposed to either Hh ligands or oxysterols will differentiate to osteoblasts and that cyclopamine will inhibit this differentiation [18, 71, 100]. Cyclopamine inhibits the Smo-dependent differentiation of C3H10T1/2 with an IC_{50} of 300 nM. Derivatives of cyclopamine (**27**–**29**) are much more active than cyclopamine. A Smo binding competition assay was conducted with BODIPY-cyclopamine, as previously described above [12], to confirm that the inhibition of the Hh pathway is through targeting Smo. While cyclopamine blocks the binding of BODIPY-cyclopamine with an EC_{50} of 114 nM, the derivatives of cyclopamine bind more tightly (Table 12.1). IPI-926 (**29**) has an IC_{50} between 7 and 15 nM in the differentiation assay and an EC_{50} of ~2 nM in the Smo binding assay. The inhibition of Smo-driven differentiation correlates with inhibition of expression of Gli-1 regulated genes such as Gli1 and Ptch1. Similar inhibition by IPI-926 of Hh pathway gene expression is detected in the human mesenchymal cell line HEPM.

As described in previous chapters, some tumor types have mutations in Hh pathway members that lead to constitutive activity of the pathway, such as loss of Ptch function or activation of Smo. These types of mutations are found in BCC and some medulloblastoma. The activity of IPI-926 was investigated in a mouse model of medulloblastoma with the Hh pathway constitutively active due to heterozygous $Ptch1^{+/-}$. This mouse model is also heterozygous for Hic-1, the gene for *H*ypermethylated *I*n *C*ancer [8]. The B837Tx cell line was derived from a medulloblastoma in this mouse and passaged as a subcutaneous allograft in NOD/SCID mice. A single oral dose of IPI-926 leads to a dose-dependent decrease in Gli-1 in a dose-dependent manner (Fig. 12.7). A subsequent study determined the pharmacokinetic (PK) and pharmacodynamic (PD) relationship in this model. A single oral dose of either 4 or 40 mg/kg was administered to B837Tx tumor-bearing mice. Levels of Gli1 expression in the tumor and drug levels in the tumor and plasma were assessed at multiple time points postdose, taken over 7 days. The plasma drug concentration profile indicates exposure over many days after both low and high doses of IPI-926 (Fig. 12.8). Gli1 expression correlated well with tumor drug levels,

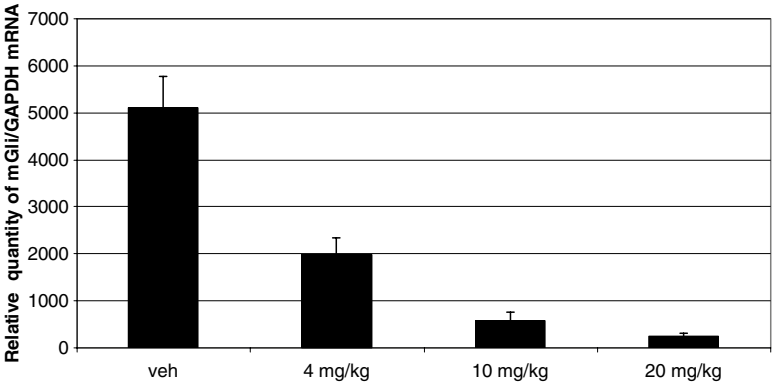


Fig. 12.7 Inhibition of Gli-1 expression at 8 h in B837Tx tumors following single oral administration of IPI-926

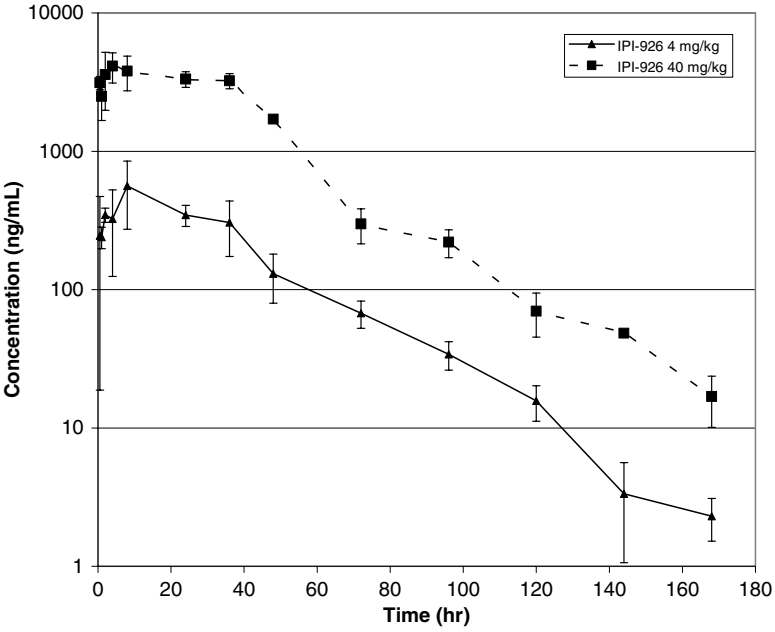


Fig. 12.8 Plasma concentration-time profile of IPI-926 following 4 and 40 mg/kg oral administration of IPI-926 to NOD/SCID mice bearing a B837Tx tumor

and a single dose of 40 mg/kg IPI-926 led to inhibition of Gli1 in the tumor beyond 6 days (Figs. 12.9 and 12.10) [78].

The antitumor activity of IPI-926 was also assessed in the medulloblastoma allograft. IPI-926 was administered orally to tumor-bearing animals daily at 4, 10, or 20 mg/kg for 21 days. During treatment, tumors regressed to undetectable levels in animals administered 10 or 20 mg/kg (Fig. 12.11), with the 4 mg/kg group showing

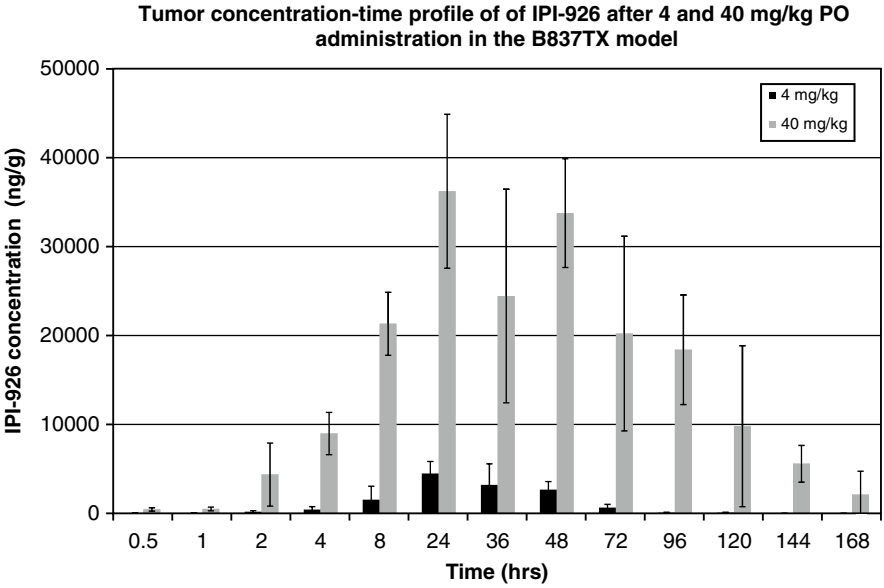


Fig. 12.9 Tumor levels of IPI-926 following single-dose administration of 4 and 40 mg/kg in B837Tx tumor-bearing mice

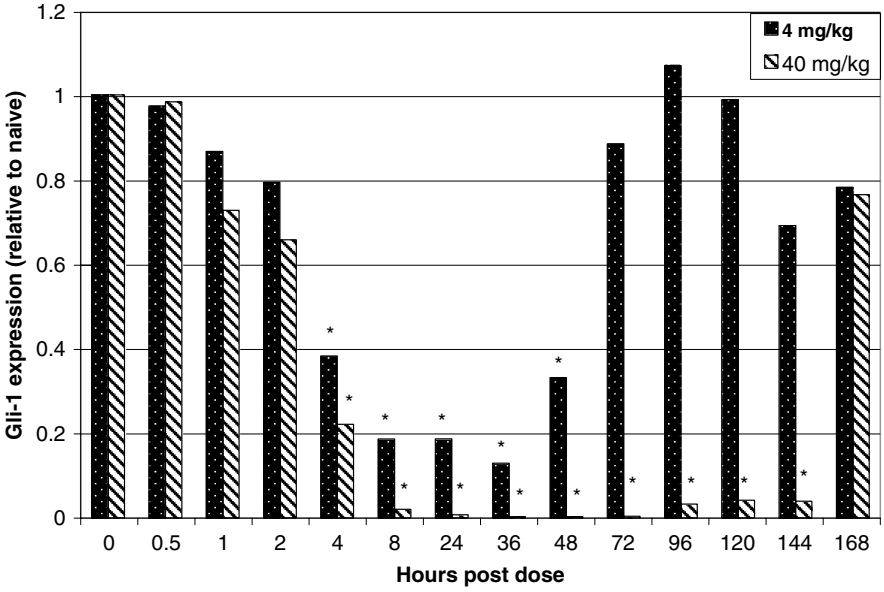


Fig. 12.10 Gli-1 mRNA expression in B837Tx tumors following administration of a single oral dose of IPI-926 (4 and 40 mg/kg); $p<0.05$

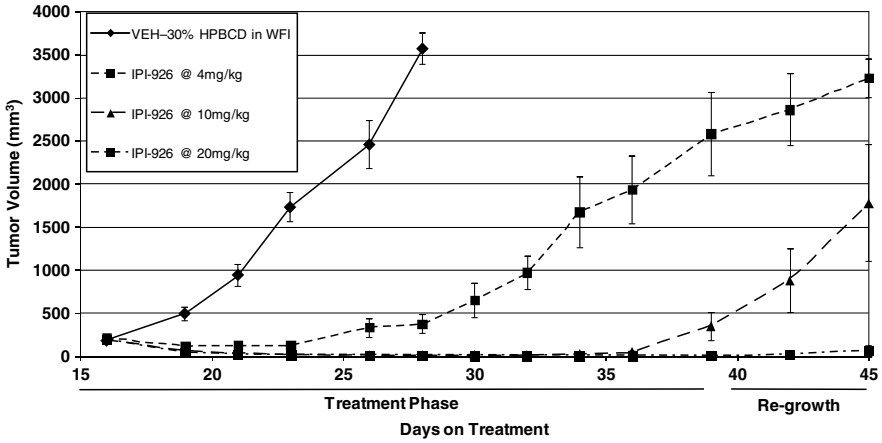
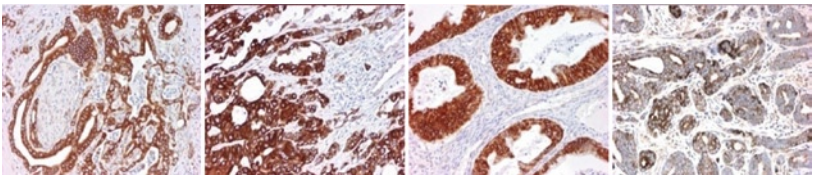


Fig. 12.11 Inhibition of B837Tx tumor growth with daily oral administration of IPI-926; $p<0.05$

50% tumor growth inhibition. In a separate study, daily administration of 40 mg/kg led to regression with no regrowth of the tumors posttreatment, thereby demonstrating that IPI-926 is active in inhibiting growth of Ptch mutant driven tumors [78].

The activity of IPI-926 was also determined in nongenetic tumors. These tumors are driven by malignant activation of the Hh pathway in a Hh ligand-dependent manner. Examples of this type of tumor include pancreatic, ovarian, prostate, and breast ([111] and also see Chaps. 7, 10, and 11). Utilizing a sensitive immunohistochemistry (IHC) method for detection of Sonic hedgehog (SHh) ligand, a high percentage of tumors of various types were found to express high levels of SHh (Fig. 12.12). In many tumor types, Hh signaling occurs with tumors secreting Hh ligand and the surrounding stroma cells responding. Blocking signaling with a Smo antagonist inhibits the Hh gene expression in the stroma and can lead to tumor growth inhibition. This has been detected in multiple tumor models with IPI-926 and other Smo inhibitors (e.g., Fig. 12.13; [112]).

An improved understanding of the mechanism of action of Hh pathway inhibition in ligand-driven tumors has been described in studies with a transgenic model of pancreatic cancer. This model is driven by activated Kras and loss of p53 [KPC] [30] and recapitulates the human disease, from PanIN lesions to the development of pancreatic adenocarcinoma (PDA) and liver metastases. The PDA tumors are highly desmoplastic, as are human PDAs, with an abundance of stromal cells producing collagen and fibronectin among the tumor cells. PDAs are not very susceptible to chemotherapy, including the standard of care, gemcitabine, in both humans and in the KPC model. It was demonstrated that treatment with IPI-926 decreased the stromal content and increased the microvascular density of the KPC tumors [74]. The effect of IPI-926 on the stroma enabled delivery of the chemotherapeutic gemcitabine to the tumor, leading to tumor growth inhibition and a doubling of the median survival of these mice. The IPI-926 plus gemcitabine-treated mice also had



Pancreas, ductal adenocarcinoma | Colon, adenocarcinoma | Ovary, cystadenocarcinoma | Prostate, adenocarcinoma

Cancer Type	Total # of Samples	SHh Positive	SHh Negative	Percent Positive
Pancreatic	92	65	27	71%
Colon	69	58	11	84%
Ovarian	68	32	36	47%
Prostate	73	56	17	77%

Fig. 12.12 Sonic Hedgehog is highly expressed in multiple tumor types. IHC for SHh shown in *top*; staining in tumor microarrays summarized in *bottom*

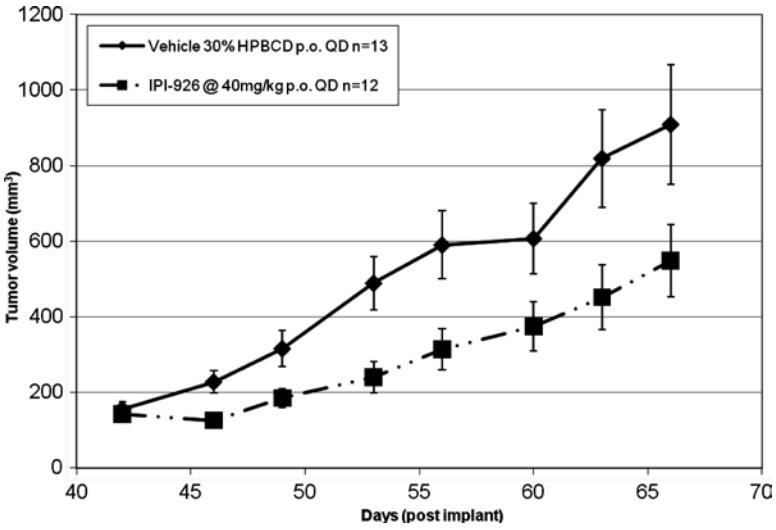


Fig. 12.13 Activity of IPI-926 in the pancreatic cancer xenograft model BxPC3, 40 mg/kg daily p.o.; $p < 0.05$

a lower incidence of liver metastases. These data provide rationale for evaluation of the combined treatment of chemotherapy and IPI-926 in pancreatic cancer.

The potential for a role for Hh pathway in tumor progenitor cells has been suggested in multiple indications (reviewed in [97]), in both hematological malignancies,

including multiple myeloma [76], acute lymphocytic leukemia [58] and chronic myeloid leukemia [17] and in solid tumors, such as glioma [3, 19] breast cancer [60] and pancreatic cancer [57]. It is believed that tumor progenitor cells are resistant to chemotherapy and therefore suspected to be responsible for disease relapse following treatment with conventional therapeutic agents. To address the role of Hh pathway in a model of minimal residual disease (MRD) postchemotherapy, IPI-926 was testing in a primary tumor model of small cell lung cancer (SCLC). Clinically, SCLC responds well to chemotherapy but then relapses within months with no further response to therapy (ref). A chemotherapy-sensitive primary tumor-derived xenograft model, LX22 was utilized to address whether IPI-926 would have an effect on time to relapse postchemotherapy [27]. The LX22 model responds well to chemotherapy and regresses, then regrows, resembling a clinical "complete response." Inhibition of Hh pathway alone in established tumors does not affect tumor growth. However, treatment with IPI-926 after SCLC tumors regress with chemotherapy leads to a significant delay in time to tumor reoccurrence (Fig. 12.14) [91]. Recently, we have determined, along with collaborators, that IPI-926 is active in additional models of minimal residual disease. Similar activity as seen in LX22 of IPI-926 in delaying tumor relapse postchemotherapy was detected in multiple primary ovarian tumor xenografts [25]. In addition, IPI-926 is also active posttumor reduction with targeted therapy, such as tyrosine kinase inhibitors (TKIs). Tumor reduction occurs in a cell line xenograft model of non-small cell lung cancer (NSCLC), NCI-H1650, with treatment with the TKI gefitinib. Maintenance therapy with

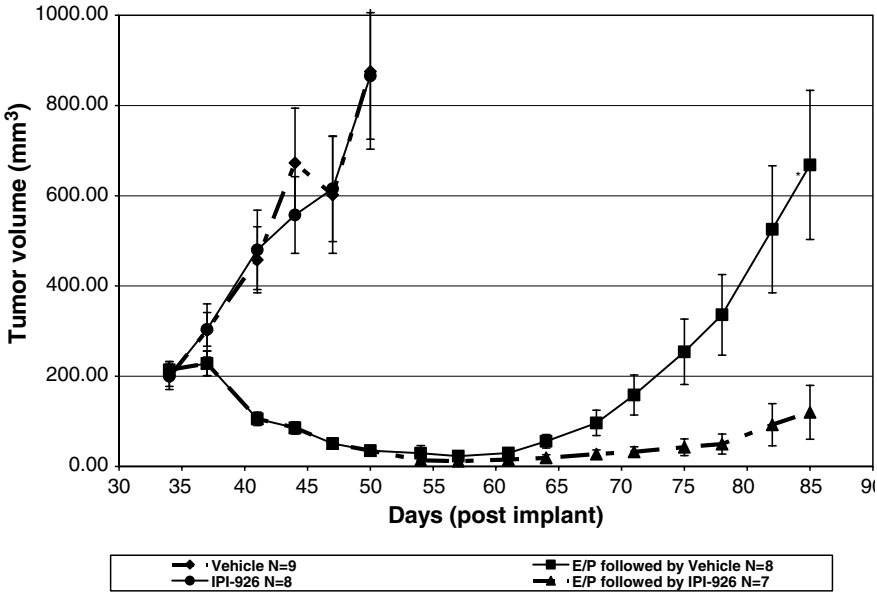


Fig. 12.14 Inhibition of the Hh pathway with IPI-926 delays LX22 SCLC growth following etoposide/carboplatin treatment. Etoposide: 12 mg/kg i.v. three consecutive doses; Carboplatin: 60 mg/kg i.v. once per week. IPI-926 40 mg/kg p.o. QD; $p = 0.0101$

IPI-926 post-gefitinib significantly delays time to tumor regrowth [63]. Finally, multiple primary tumor xenograft models of head and neck squamous cell carcinoma (HNSCC) that regress with cetuximab treatment are sensitive to IPI-926 posttumor reduction, with a delay in time to tumor regrowth [51]. These data suggests an important role for the Hedgehog pathway in tumor relapse, potentially through a cancer stem cell and provides rationale for evaluation of a Smo inhibitor such as IPI-926 in the minimal residual disease setting in the clinic.

Clinical Application

The role of the Hedgehog pathway in the preclinical settings described above suggests a broad potential for Smo antagonists in multiple clinical settings. The most straight-forward is in tumors driven by activating mutations in key Hh pathway members, such as in basal cell carcinoma and some medulloblastoma. Clinical proof of concept has been demonstrated in these indications [81, 102]. In addition, targeting the tumor microenvironment with a Hedgehog inhibitor could affect drug delivery to desmoplastic tumors such as pancreatic cancer and the studies in the KPC mouse model support evaluation of chemotherapy combined with IPI-926 in pancreatic cancer. The minimal residual disease setting is another example where the Hedgehog pathway plays a key role in relapse of tumors posttumor reduction with chemotherapy. While the precise mechanism is still being elucidated, whether Hh-dependent tumor initiating cells or a higher dependence on the microenvironment for tumor regrowth, the cells responsible for regrowth posttumor reduction are dependent on the Hh pathway and inhibited by IPI-926. There are potentially many uses for a Hh antagonist in the treatment of cancer and the trials with multiple Smo inhibitors will provide more insight into the role of Hh in these clinical settings.

Conclusion

Plant-derived natural products continue to play an important role in drug discovery for many therapeutic areas [84]. In oncology, several drugs that have greatly impacted the life of cancer patients are plant derived such as taxol, camptothecin, combrestatin, podophyllotoxin, and vinca alkaloids such as vinblastine and vincristine [16]. The discovery of the mode of action of plant-derived cyclopamine and the importance of this target in cancer has attracted the attention of many researchers to investigate the therapeutic potential of cyclopamine and its analogs. IPI-926, the focus of this chapter, is currently in clinical trials. Preclinical studies have shown that IPI-926 displays antitumor activity in both Hh ligand-independent and ligand-dependent models of malignant activation of the Hh pathway. Clinical evaluation of the first semisynthetic analog of cyclopamine will determine whether this approach will prove beneficial in an array of different clinical settings.

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