Semisynthetic Cyclopamine Analogues as Potent and Orally Bioavailable Hedgehog Pathway Antagonists

Martin R. Tremblay,* Marta Nevalainen, Somarajan J. Nair, James R. Porter, Alfredo C. Castro, Mark L. Behnke, Lin-Chen Yu, Margit Hagel,† Kerry White, Kerrie Faia, Louis Grenier, Matthew J. Campbell, Jill Cushing, Caroline N. Woodward,‡ Jennifer Hoyt, Michael A. Foley,§ Margaret A. Read, Jens R. Sydor, Jeffrey K. Tong, Vito J. Palombella, Karen McGovern, and Julian Adams

Infinity Pharmaceuticals, Inc., 780 Memorial Drive, Cambridge, Massachusetts 02139

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Abstract: Herein is reported the synthesis of a novel class of hedgehog antagonists derived from cyclopamine. The acid sensitive D-ring of cyclopamine was homologated utilizing a sequence of chemoselective cyclopropanation and stereoselective acid-catalyzed rearrangement. Further modification of the A/B-ring homoallylic alcohol to the conjugated ketone led to the discovery of new cyclopamine analogues with improved pharmaceutical properties and in vitro potency (EC_{50}) ranging from 10 to 1000 nM.

The hedgehog (Hh[a]) signaling pathway is important for tissue growth and differentiation and plays a pivotal role in embryogenesis and tissue homeostasis.[1] During development, activation of the Hh-signal transduction pathway is initiated with the binding of Hh ligand to the cellular membrane receptor patched (Ptc), which relieves the Ptc-mediated inhibition of the seven-transmembrane protein smoothened (Smo). Activated Smo transduces the signal to the nucleus to regulate gene expression. The Gli transcription factors, which regulate numerous gene products involved in tissue growth and differentiation, are the nuclear executors of the Hh signaling pathway. In adulthood, the Hh signaling pathway is silent in a great majority of cells. However, there have been an increasing number of reports over the past decade documenting the implication of the Hh pathway in human diseases, such as cancer.[2,3] Indeed, aberrant Hh signaling has been described in numerous cancers such as basal cell carcinoma,[4,5] breast,[6] esophagus,[7] gastric,[7] medulloblastoma,[8] pancreatic adenocarcinoma,[9] prostate,[10] and small cell lung.[11] For these reasons, Hh pathway antagonists have been sought after as potential new treatments for cancer.[12,13]

Cyclopamine (**1**, Figure 1), a natural product isolated from *Veratrum californicum*,[14] has been a tremendously valuable pharmacological tool to validate the Hh pathway as a promising target for drug intervention in cancer. Cyclopamine was shown to be a potent antagonist of the Hh pathway, directly acting on the protein Smo[15] and has since been shown to have antitumor activity in several xenograft models of pancreatic,[9] medulloblastoma,[16] prostate,[10] small cell lung,[11] and digestive tract[7] cancers. Despite the attractive pharmacological profile of cyclopamine, its use as a systemic treatment may be limited by its poor aqueous solubility[17] (~5 μg/mL) and stability in acid. Indeed, cyclopamine readily converts to veratramine (**2**) as a result of an acid-catalyzed opening of the spirotetrahydrofuran E ring followed by rapid aromatization of the D ring.[18] Veratramine (**2**), although structurally related to cyclopamine, does not act as a Hh antagonist but has been shown to interact with several other receptors[19,20] and cause hemolysis.[21] Hence, derivatives of cyclopamine that do not have these liabilities are necessary in order to assess the full therapeutic potential of this natural product. Taipale and co-workers[22] reported potent derivatives of cyclopamine (e.g. **3**) having a hydrophobic moiety attached to the F-ring nitrogen and a 3-ketone instead of the 3-hydroxy group on the A-ring. Additionally, the 4-en-3-one cyclopamine (**10**, Scheme 3) was reported to be an orally active teratogen,[23] which mechanistically correlates with its antagonistic activity on the hedgehog pathway. These analogues and others reported[24,25] maintain the natural cyclopamine skeleton and hence are expected to be acid labile. Herein, we report the chemical synthesis and biological activity of novel skeleton-modified cyclopamine derivatives[26] with improved water solubility and chemical stability.

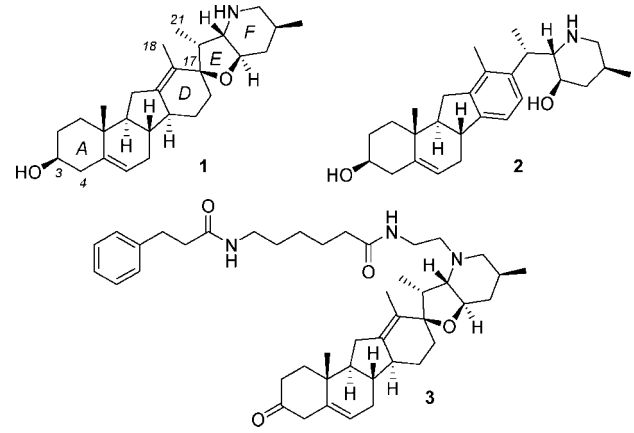

Figure 1. Structures of cyclopamine (**1**), veratramine (**2**), and **3**.

Our strategy to improve the acid stability of cyclopamine was to alter the influence of the D-ring allylic ether on the hydrolysis of the spirotetrahydrofuran E-ring, with minimal change to the D-ring geometry. It was hypothesized that the oxygen of the allylic ether could direct the addition of a zinc carbenoid from the β-face of the steroid to chemo- and stereoselectively cyclopropanate the D-ring double bond.

Cyclopamine was extracted and isolated from *V. californicum* using a slightly modified procedure originally described by Keeler[14] and more recently by Oatis.[27] Fmoc protected cyclopamine was found to be a good substrate for various cyclopropanating procedures, such as Furukawa (Et_2Zn, CH_2I_2),[28] Denmark (Et_2Zn, $ClCH_2I$),[29] and Shi (Et_2Zn, TFA or TCA, CH_2I_2)[30] protocols. For instance, *N*-Fmoc cyclopamine **4** converts to the D-ring cyclopropyl derivative **5** under Furukawa conditions effectively (55−65% yield). Deprotection of the Fmoc group of **5** gave cyclopropylcyclopamine **6** (Scheme 1). The structure of **6** was confirmed by single-crystal X-ray diffraction analysis, which definitively established that the

* To whom correspondence should be addressed. Phone: 617-453-1268. Fax: 617-682-1418. E-mail: Martin.Tremblay@infi.com.

† Current address: Avila Therapeutics, Inc., 100 Beaver Street, Waltham, MA 02453.

‡ Current address: Epix Pharmaceuticals, Inc., 4 Maguire Road, Lexington, MA 02421.

§ Current address: Broad Institute, 320 Charles Street, Cambridge, MA 02141.

[a] Abbreviations: Hh, hedgehog; Ptc, patched; Smo, smoothened; Fmoc, fluorenymethoxycarbonyl; SAR, structure−activity relationship; LC, liquid chromatography; MS, mass spectrometry; V_d, volume of distribution.

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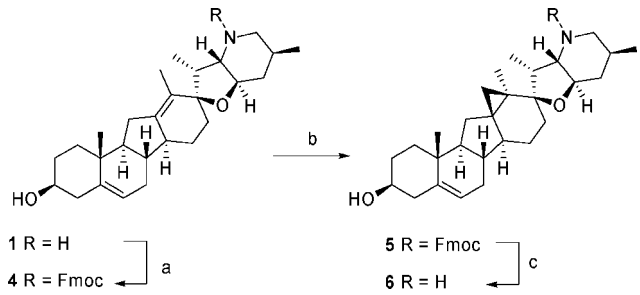
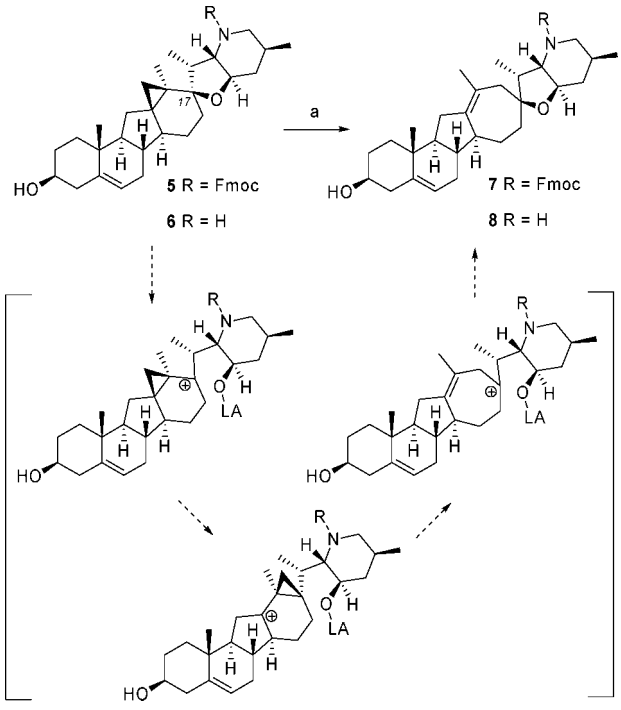
Scheme 1[a]



[a] Reagents and conditions: (a) Fmoc-OSu, CH_2Cl_2, 25 °C (80%); (b) Et_2Zn, CH_2I_2, CH_2Cl_2, 0−25 °C (55%); (c) diethylamine, CH_2Cl_2, 25 °C (90%).

Scheme 2. Acid Decomposition of **5** and **6** and Proposed Mechanism[a]



[a] Reagents and conditions: (a) BF_3-OEt_2, CH_2Cl_2, 0 °C (70%).

stereoselective incorporation of cyclopropyl occurred from the β-face of the steroid (Supporting Information).

The acid stability of cyclopropylcyclopamine derivatives **5** and **6** was studied. Interestingly, treatment of these derivatives with various Bronsted and Lewis acids caused a rearrangement forming distinct isomers, which were stable to acids. The newly formed isomers derived from **5** and **6** were isolated and characterized as the ring expanded products Fmoc protected D-homocyclopamine (**7**) and D-homocyclopamine (**8**), respectively. This novel D-homocyclopamine skeleton was subsequently confirmed by single-crystal X-ray diffraction analysis of a further modified analog (vide infra). It is noteworthy that the C17 stereocenter is conserved in the D-homocyclopamine derivatives. The acid-catalyzed ring expansion presumably occurs through the formation of a stabilized cyclopropylcarbinyl cation, subsequent rearrangement, ring opening of the cyclopropyl moiety, and finally re-formation of the spirotetrahydrofuran (Scheme 2).[31] We speculate that the formation of the C17 epimer upon reclosure of the spirotetrahydrofuran is sterically disfavored because of the C21 methyl and the C18 methyl groups. Such atypical rearrangements have been reported in the total synthesis of a few sesquiterpenes.[32−34]

Table 1. Cellular Hh Pathway Inhibition of Cyclopamine (**1**) and D-Homocyclopamine Analogues

compd	R for analogues of **9**	EC_{50} μM (N)[a]
1		0.17 ± 0.07 (3)
6		>2.0 (3)
8		>2.5 (3)
9	H	0.20 ± 0.08 (9)
14	CH_3	0.4 ± 0.1 (3)
15	Ac	0.12 ± 0.02 (3)
16	CH_2CH_2Cl	>2.5 (3)
17	$CH_2CH_2OCH_2CH_2OCH_3$	0.4 ± 0.2 (3)
18	$CH_2CH_2OCH_2CH_2OH$	>1.0 (3)
19	CH_2Ph	>2.0 (3)
20	$CH_2CH_2NHCO(CH_2)_5NHCOCH_2CH_2Ph$	0.01 ± 0.002 (3)
21	$CH_2CH_2NHCOCH_2CH_2Ph$	0.02 ± 0.01 (3)
22	OH	0.8 ± 0.1 (3)

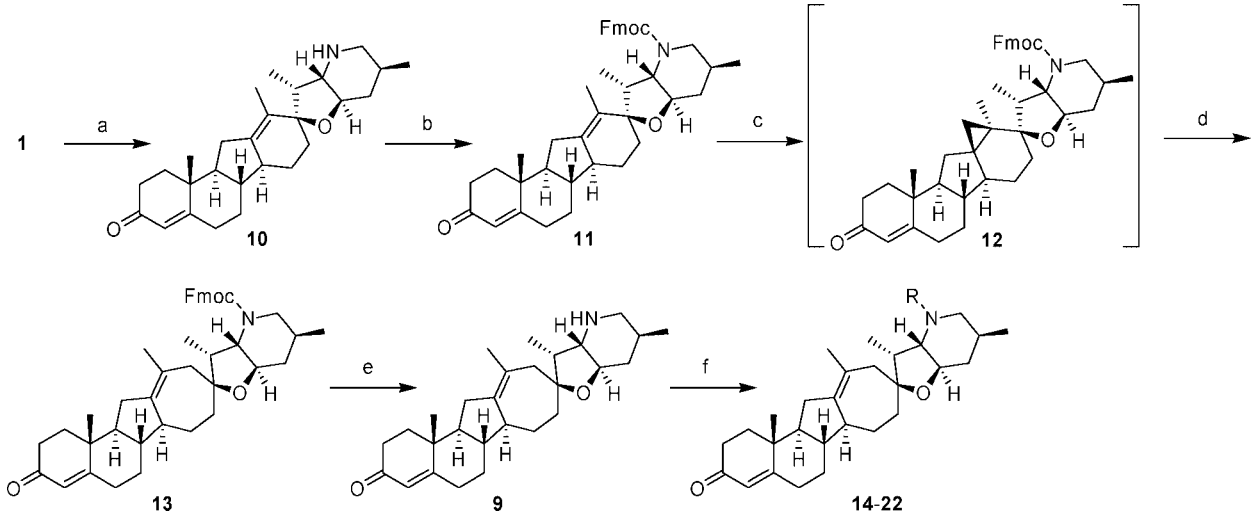
[a] The number of determinations is shown in parentheses.

The ability of D-homocyclopamine **8** to inhibit the Hh pathway was measured by its effect on oxysterol-induced differentiation of murine progenitor C3H10T1/2 cells into osteoblasts in culture. Osteoblast differentiation can be monitored by production of alkaline phosphatase, which was used as the read-out for the assay.[35] Unfortunately, D-homocyclopamine **8** was found to be weaker than cyclopamine in this Hh dependent assay (Table 1). Modifications previously reported[22,23] to improve the biological activity of cyclopamine were incorporated onto the new D-homocyclopamine scaffold. For instance, Oppenaeur oxidation of **8** yielded the enone **9**, which is equipotent to cyclopamine (Table 1). To enable further optimization of **9**, a more robust synthetic route was designed that introduced the enone prior to cyclopropanation and ring expansion (Scheme 3). This sequence took advantage of the relative inertness of the electron-poor enone toward iodoalkylzinc carbenoids and provided a better substrate for selective cyclopropanation. Oppenauer oxidation of cyclopamine gave 4-en-3-one cyclopamine **10**,[36] which was N-protected with an Fmoc group to give **11**. Subsequent cyclopropanation of the D-ring olefin of **11** was performed using an excess of bis-(iodomethyl)zinc prepared according to Denmark's procedure.[29] The crude intermediate **12** was treated with BF_3-OEt_2 to form the desired ring-expanded product **13**. Deprotection of **13** followed by silica gel chromatography and crystallization gave the desired **9** in 25−30% overall yield from cyclopamine. A single-crystal X-ray diffraction analysis confirmed the structure of **9** (Supporting Information).

With ready access to **9**, various N-substituted analogues were prepared via acylation, reductive amination, and direct alkylation. In addition to the *N*-alkyl derivatives, hydroxylamine **22** was prepared by benzoyl peroxide oxidation followed by hydrolysis of the benzoate (Scheme 3). The ability of these new analogues (**9** and **14**−**22**) to inhibit the Hh pathway was determined in C3H10T1/2 cells (Table 1).

The structure−activity relationship around enone **9** and cyclopamine was found to closely track.[37] Preservation of the basicity of the F-ring nitrogen has been thought to be a key element in determining binding to cyclopamine target. However, the *N*-acetamide derivative **15** displayed similar potency compared with the parent molecule **9**. Relatively bulky alkyl groups (e.g., CH_2CH_2Cl, **16**; Bn, **19**) are detrimental to biological activity, whereas elongated appendages, such as the glycolate analogue **17**, have potency similar to that of cyclopamine and **9**. Interestingly, the des-methyl analogue of **17**, compound **18**, has significantly reduced biological activity. More importantly,

Scheme 3[a]



[a] Reagents and conditions: (a) Al(O-*i*-Pr)$_3$, 2-butanone, 75 °C (85%); (b) Fmoc-Cl, $NaHCO_3$, THF, 25 °C (88%); (c) Et_2Zn, CH_2I_2, CH_2Cl_2, 0−25 °C; (d) BF_3−OEt_2, CH_2Cl_2, 0 °C (50%, two steps); (e) piperidine, DMF, 25 °C (75%). (f) See Table 1 for R groups and *Supporting Information* for details.

20, which has the same hydrophobic moiety on the F-ring as analogue **3**, is 20-fold more potent than its congener. A truncated version of this side chain was installed on **9** to provide analogue **21** and found to provide 10-fold increase in potency. Finally, hydroxylamine **22** displayed biological activity, albeit with 4-fold decrease in potency compared to enone **9**. In summary, D-homoanalogue **9** possesses equivalent biological activity in vitro to cyclopamine and its SAR at the F-ring nitrogen correlates well with what has been reported for cyclopamine.

The pharmaceutical properties of the free base of **9** and some of its analogues were next evaluated. Compound **9** was found to have much greater aqueous solubility than cyclopamine at pH 7.4 (>20-fold), whereas the more lipophilic analogues **20** and **21** (albeit more potent) were less soluble. In addition, stability of the newly formed D-homocyclopamine analogue **9** in simulated gastric fluid was found to be greatly enhanced in comparison to cyclopamine (98% vs 60% remaining after 60 min incubation, respectively). Encouraged by these data, pharmacokinetic properties of analogue **9** were determined. To this end, the hydrochloride of **9** was dissolved in sterile water for injection containing 30% (w/w) of 2-hydropropyl-β-cylodextrin and administered to CD-1 mice intravenously and orally. Plasma was collected at selected time points, and samples were analyzed by LC−MS/MS after protein precipitation. Compound **9** shows very good exposure (80% oral bioavailability) after oral administration in mice, is cleared from plasma with an elimination half-life of 3.2 h, and has a large V_d of about 18 L/kg.

In conclusion, synthetic modifications of cyclopamine resulted in the identification of a novel D-homocyclopamine scaffold that retains biological activity on the Hh pathway. Although various analogues were synthesized, **9** (also referred to as IPI-269609) stood out as having the most desirable pharmaceutical properties and pharmacokinetic profile. Compound **9** has shown efficacy in several xenograft models.[38,39] The full potential for the development of systemic Hh pathway antagonists related to this promising new class of compounds is currently being evaluated.

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Supporting Information Available: Detailed experimental procedures and full characterization of **4**−**22**, detailed protocols for biological tests in vitro and pharmacokinetic studies, and ORTEP representations of **6** and **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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