Targeting the Hedgehog Pathway: The development of Cyclopamine and the Development of Anti-Cancer Drugs Targeting the Hedgehog Pathway

A. Gould and S. Missailidis*

Department of Chemistry and Analytical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK

Abstract: The Hedgehog signalling pathway plays a critical role in controlling growth, especially during development, but is often over-activated in tumourigenesis. It has recently emerged as an important target for anticancer drugs, with several compounds in clinical trials. This review initially describes the Hedgehog pathway, focussing on the Patched receptor, and the Smoothened GPCR-like protein, as well as discussing the role of Cancer Stem Cells. It subsequently presents the discovery and development of drugs targeting this pathway. The initial focus is on cyclopamine – the first compound discovered that could inhibit the Hedgehog pathway – and selected cyclopamine analogues, including a review of the development of IPI-926. In addition, a number of other compounds are briefly discussed, to give an overview of current therapies in clinical development, and to indicate the possibilities for targeting different parts of the Hedgehog pathway in future. Finally, combination chemotherapy – incorporating a Hedgehog pathway inhibitor as well as another drug – is discussed from the perspective of drug resistance and effects on cancer stem cells.

Keywords: Hedgehog pathway, cancer stem cells, cyclopamine, patched receptor, smoothened protein, cyclopamine analogues.

1. INTRODUCTION

The Hedgehog signal transduction pathway is critical to stimulating and controlling growth during development. However, over-activation of this pathway is associated with tumour development. This mini-review discusses the Hedgehog pathway, drugs such as cyclopamine and others that target this pathway, and their significance in treating cancer.

Although about 40% of cancers can be cured through surgery and radiotherapy alone, chemotherapy has become increasingly important in the last 50 years [1]. Originally, the mainstay of chemotherapy has been cytotoxic drugs that target rapidly-dividing cells. However, these have been associated with significant side effects related to their impact on healthy cells, and, in the case of DNA damaging (genotoxic) drugs such as alkylating agents or topoisomerase II inhibitors, they can even lead to secondary malignancies appearing later in life [2].

However, a significant turn in drug discovery in recent years has been towards rational drug design, focused on the targeting of molecular pathways specifically responsible for cancer initiation, growth or metastasis. As it is easier to impair the function of a molecule that is over-active than it is to repair a damaged and inactivated molecule, oncoproteins rather than tumour suppressors are the principal targets. Gleevec (imatinib) is an extremely successful example of this approach, very specifically targeting the tyrosine kinase domain of the Bcr-Abl oncoprotein responsible for chronic myelogenous leukaemia (CML) [3].

*Address correspondence to this author at the Department of Chemistry and Analytical Sciences, The Open University, Walton Hall, Milton Keynes, MK7 6AA, UK; Tel: +44 1908 653199: Fax: +44 1908 858327;
E-mail: s.missailidis@open.ac.uk

Anti-cancer drugs – including Gleevec – tend to suffer from two major problems. First, the selective pressure exerted by chemotherapy favours the emergence of mutations that are drug-resistant. Second, they may fail to kill cancer stem cells (CSCs), which are the cells ultimately responsible for the growth of a tumour. Although there is a consensus that drug resistance can be addressed with combination drug therapy, the nature of CSCs and how to target them are still being actively researched [3].

The Hedgehog pathway is of interest not only because it is a specific pathway that can be over-activated in cancer and therefore rationally targeted, but also because it is implicated in CSC growth and survival [4].

In this review, we will aim to first describe the Hedgehog signalling pathway, and the role of CSCs in cancer, followed by the discovery and development of drugs targeting this pathway, with a focus on cyclopamine – the first compound discovered that could inhibit the Hedgehog pathway – and selected cyclopamine analogues. A number of other compounds will be discussed briefly, either to draw out points of general interest, or to illustrate targeting different parts of the Hedgehog pathway. Finally, combination chemotherapy – incorporating a Hedgehog pathway inhibitor together with another drug – will be discussed from the perspective of drug resistance and effects on CSCs. Readers are also directed to [5] for comprehensive coverage of Hedgehog inhibitors as anticancer drugs, and [6] for an overview of clinically interesting compounds and the activation of the Hedgehog pathway in cancer.

2. THE HEDGEHOG PATHWAY

The Hedgehog pathway is characterized by three main components. These are (1) the Patched (Ptc) receptor for the Hedgehog ligand (tumour-suppressor); (2) the Smoothened (Smo) GPCR-like protein (proto-oncoprotein); and (3) signal

1389-5575/11 $58.00+.00

transduction machinery, terminating in Gli transcription factors (proto-oncoproteins) [5].

When Hedgehog (the ligand) is absent, Ptc inhibits Smo, preventing it from transducing its signal (see Fig. **1**). But when Hedgehog is present, Ptc no longer inhibits Smo (see Fig. **2**). Smo then transduces a signal through to the nucleus, enabling Gli transcription factors. The resulting transcription and translation produces proteins that encourage growth and survival (inter alia) [5].

The Hedgehog pathway plays a critical role in early development, helping to control cell growth and tissue structure. The further cells are from a source of the Hedgehog ligand, the less stimulation they receive. Differing levels of stimulation cause different decisions to be made about cell differentiation, resulting in the different tissue structures seen along, for example, a limb. Loss of Hedgehog function can have very serious effects. In humans, for example, loss of Hedgehog function can often result in holoprosencephaly, a condition where there is insufficient separation of brain hemispheres [5]. In adults, on the other hand, Hedgehog signalling plays a supporting or dormant role, maintaining stem cell compartments in tissues, and participating in wound healing.

Over-activation of Hedgehog is associated with a variety of cancers [5, 6]. Mutations affecting genes that express components of the Hedgehog signal transduction pathway can lead to over-activation independent of Hedgehog binding, and this type of disruption is particularly associated with glioblastoma, medulloblastoma, and basal cell carcinoma (BCC). For example, most BCCs are associated with deactivation of Ptc, and some are associated with a constitutive increase in Smo's activity (i.e. regardless of inhibition from Ptc). Medulloblastoma is associated with widespread damage affecting the whole pathway, including Gli transcription factors and other signal transduction machinery as well as Ptc and Smo [5].

Alterations in regulatory pathways can cause over-expression of Hedgehog, which in turn can also lead to can-

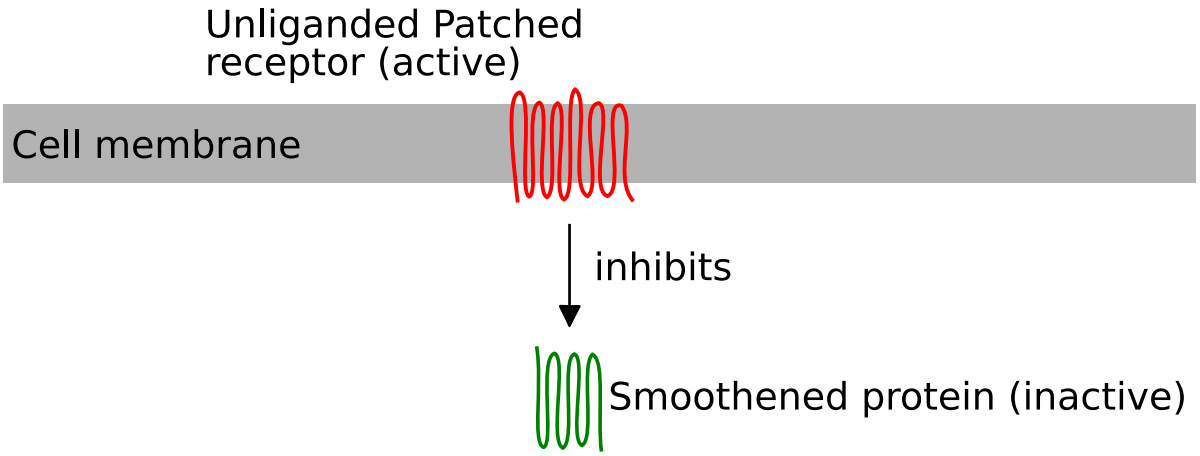

Fig. (1). Schematic of inactive Hedgehog pathway.

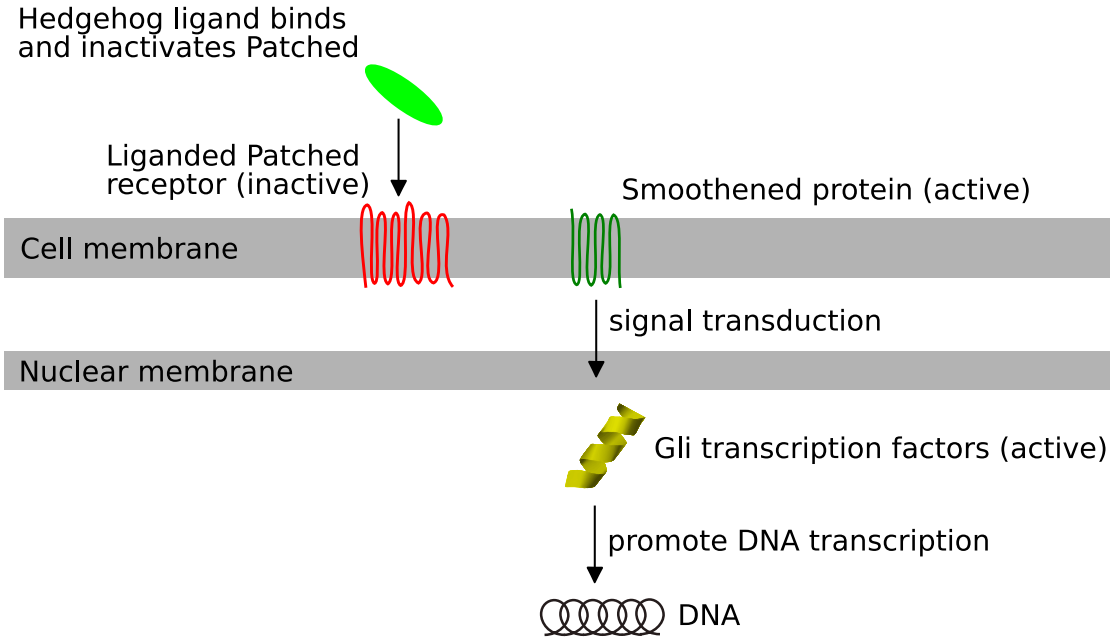

Fig. (2). Schematic of active Hedgehog pathway.

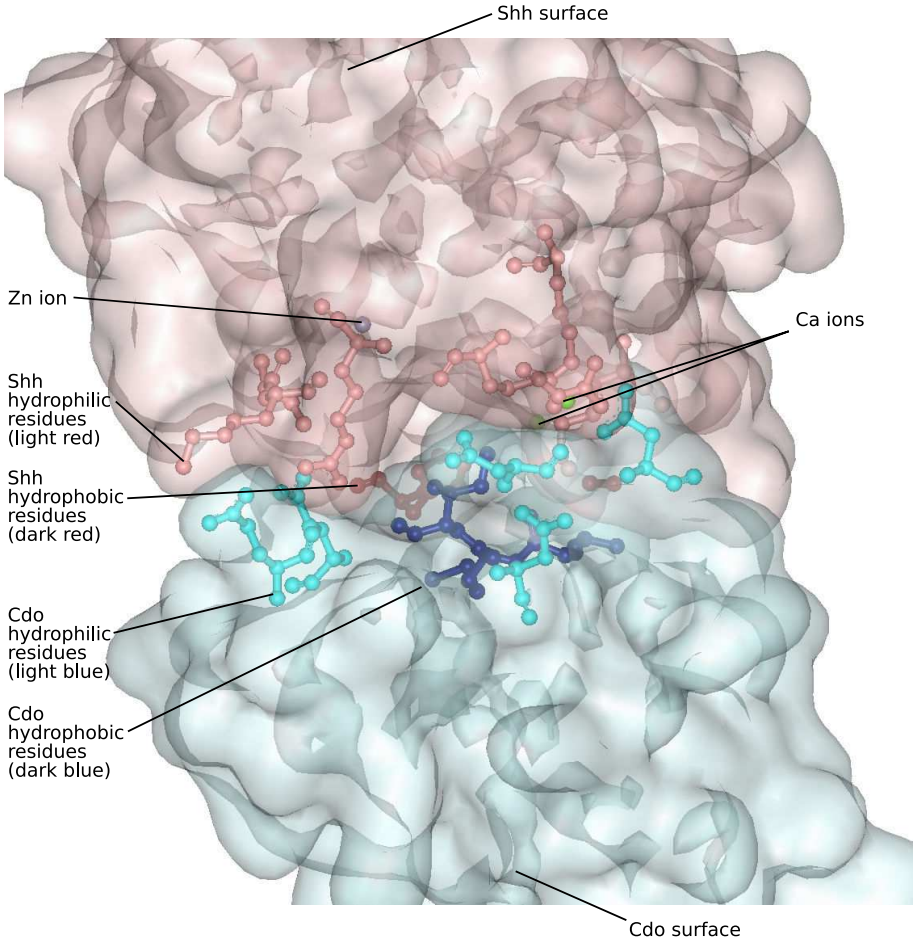

Fig. (3). Crystal structure of sonic Hedgehog bound to third fibronectin (glycoprotein) repeat of Cdo (pdb id 3D1M).

cerous growth in several other cancers, including pancreatic, prostate, lung and breast cancers. In such cases, the Hedgehog pathway itself may be intact. The over-abundant ligand can be received directly by the cancerous cells (autocrine signalling), once again over-activating the pathway. Alternatively, Hedgehog can be received by stromal cells outside the cancer, which respond with different, non-Hedgehog, growth signals that are received by cancerous cells (paracrine signalling) [6].

Appropriate drug treatments may differ depending on the underlying pathology. Where the Hedgehog pathway has been compromised, there is no point inhibiting an upstream component. However if Hedgehog is over-expressed, it may make sense to inhibit or sequester Hedgehog itself [6].

The Hedgehog pathway was discovered in Drosophila, and the basic scheme outlined in Figs. (**1** and **2**) applies to both Drosophila and mammals. Hedgehog signalling, on the other hand, is thought to be conserved within mammals, but there are important differences between Drosophila and mammals [7].

Firstly, there is more than one mammalian gene for each of Hedgehog, Ptc, and Gli. Thus, there are three Hedgehog ligands, known as Desert, Indian, and Sonic. Sonic Hedgehog (Shh) is most widely expressed and, as a result, Shh is the focus of research and drug development. Only Shh is considered in this review. Similarly, there are two mammalian Patched receptors and three different Gli genes in mammals, Gli1 – Gli3, each of which has different functions[1] [7].

Secondly, and more significantly, signal transduction between the components is quite different. Mammalian signalling is not yet well understood, and although Drosophila Hedgehog signalling is well understood, it cannot be reliably used as a guide [7].

The different parts of the pathway are discussed below, focussing primarily on Smo, because cyclopamine – and the majority of Hedgehog drugs – are Smo antagonists. Conversely, signal transduction downstream of Smo is discussed only briefly, and production and release of Shh are not presented in this review, due to the paucity of compounds targeting these areas [6].

2.1. Hedgehog and Patched

Shh is a protein, 20kD in size, and is hydrophobic, with fatty acids added to its N-terminal and cholesterol added to its C-terminal [6]. Ptc, a 12-pass integral membrane protein, was identified as the Shh receptor using co-immunoprecipitation to isolate the protein complex containing Shh bound to Ptc [8]. Binding experiments and Scatchard analysis on labelled Shh and Ptc gave a dissociation constant of

[1]In Drosophila, the Gli gene is known as Ci.

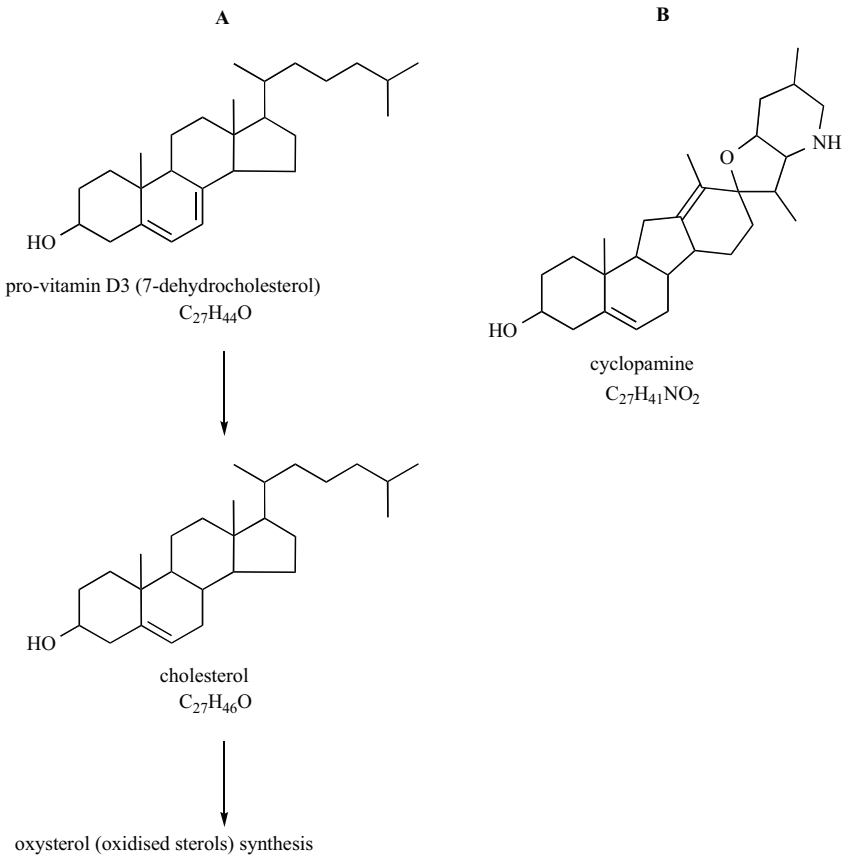

Fig. (4). A: an excerpt from the sterol synthesis pathway (adapted from [16]). **B**: cyclopamine. (Stereochemistry not shown).

1.2nM, consistent with the concentration of Shh required to activate the Hedgehog pathway *in vivo* [9].

Several cell-surface proteins participate in negative feedback loops to fine-tune the response to differing Shh concentrations: positively-acting proteins are down-regulated on pathway activation, whereas negatively-acting proteins are up-regulated. The homologues Cdo and boc facilitate Shh to Ptc binding, acting positively, whereas Hedgehog interacting protein (Hhip) inhibits Shh to Ptc binding, acting negatively[2] [10].

Ptc itself is up-regulated in a negative feedback response to Shh: increasing the quantity of unliganded Ptc increases inhibition of Smo. Ptc also acts negatively on Shh transmission to surrounding cells: once Shh binds, the Ptc-Shh complex moves from the cell membrane to lysosomes and is broken down [7].

The Shh-Cdo structure was solved in 2008 (see Fig. **3**), which highlights the hydrophilic and hydrophobic residues and metal ions required for binding. Shh-Ptc binding has not yet been solved but is thought to be similar [11].

The Shh-Hhip structure was solved in June 2009, and confirms that both Zn^{2+} and Ca^{2+} are important for binding [12]. The same group is continuing this study, now working to solve the structure of further receptors including Ptc, which would deliver new molecular targets for drug design [13].

2.2. Hedgehog and Smoothened

Whether Shh binds Smo or Ptc was once the subject of debate, particularly given the genetic sequence similarity between Smo and GPCRs [8, 9, 14]. Although it is now accepted that Shh binds to Ptc, discussion continues as to how Smo is controlled. The relationship is particularly important because cancers often disrupt Ptc inhibition of Smo [15]. If the mechanism can be elucidated, it may reveal new ways of pharmacologically inhibiting Smo.

There is general agreement that small molecules are responsible for controlling Smo, through stabilizing an active or inactive state. The principal evidence is the large number of small molecules – including cyclopamine – that are Smo antagonists or agonists [15]. Moreover, the effects of these molecules are consistent with an equilibrium between inactive and active states (as are the activating effects of up-regulation or oncogenic mutations of Smo). The similarity of Smo to GPCRs is further, circumstantial evidence [15].

A recent publication established that Hedgehog pathway activation relies on certain oxysterols. When sterol synthesis is inhibited or compromised, e.g. through mutation, Hedgehog pathway activation is attenuated. Moreover, Hedgehog activation can be rescued by supplying cholesterol or oxysterols. The similarity of cyclopamine and sterols at the hy-

[2]Cdo and boc map to iHog in Drosophila. There is no Drosophila equivalent of Hhip.

droxyl end of the molecules supports the argument for a role for sterols in modulating Smo (see Fig. **4**) [16].

In the same paper, the authors go on to suggest that Ptc may regulate Smo activity by pumping oxysterols out of the cell, unless Ptc is bound by Shh, in which case oxysterols accumulate and activate Smo. Several means of activation are suggested, including binding directly to Smo [16]. However, in a later paper, Dwyer *et al.* found that unlike known Smo agonists, oxysterols did not displace labeled cyclopamine bound to Ptc, and did not increase cyclopamine's IC_{50} for Smo. This suggests, albeit inconclusively, that oxysterols act upstream of Smo rather than binding directly to Smo [17].

The suggestion that oxysterols act upstream of Smo ties in with the hypothesis that there is an additional step in Smo activation that must occur prior to a conformational change. Oxysterols are then a candidate for promoting this first step [15]. In this two stage model, Smo must first be translocated from the cytoplasm to the plasma membrane of the primary cilium – an organelle projecting out from the cell – for signal transduction to occur [15]. Using small molecule antagonists as probes, it was confirmed that Hedgehog pathway activation requires translocation of Smo to the primary cilium, followed by activation of Smo – with Ptc somehow inhibiting the activation step [18].

However, if oxysterols do not influence Smo directly, how does Ptc inactivate Smo? One possibility is pro-Vitamin D3 (Fig. **4**). Vitamin D3 competes with cyclopamine to bind and antagonise Smo, and Ptc pumps a very similar compound out of the cell, where it may bind to Smo in the primary cilia of the releasing and neighbouring cells [19]. This unusual mechanism raises a number of questions. Furthermore, it also seems to conflict with [16], which strongly suggested that pro-Vitamin D3 does not have a role in Smo regulation. Perhaps the conflict can be resolved with the observation that [16] considered a role for pro-Vitamin D3 only as a positive regulator. Indeed, as pro-Vitamin D3 is a precursor of oxysterols, pumping it out of the cell may preserve it from consumption in the sterol synthesis pathway, and reduce the activating effects of oxysterols.

It is unclear exactly how sterols influence Hedgehog activation, and indeed how Ptc controls Smo. Nevertheless, it is clear that sterol synthesis inhibitors, e.g. statins, may be worth exploring to inhibit the Hedgehog pathway [15].

2.3. Signal Transduction Through to Glis

Although Glis are well understood, signal transduction from Smo to Glis is not. It is known that Smo signal transduction activates the G_i family of G proteins and that this is necessary but not sufficient for Gli activation [20]. Furthermore, a number of kinases are involved in transduction, but they are not pathway-specific [7]. Downstream, Suppressor of fused (Sufu) is a tumour suppressor protein also involved in the pathway [21]. Sufu's C-terminal binds all three Glis, but its N-terminal is specific to Gli1 [22]. This asymmetry is reflected in Gli functionality. All Glis have transcription activation domains and, when the Hedgehog pathway is active, they promote transcription of genes including many growth factors. However, Gli2 and Gli3 also have latent repressor domains: when the pathway is inactive they may be cleaved to form transcription repressors [7].

Glis have five 'zinc fingers', of which fingers two to five bind to DNA sequences to promote or repress transcription (see Fig. **5**) [23]. As the crystal structures for Sufu and Gli have been solved, [22, 23], mimicking Sufu or designing Gli inhibitors directly appears a promising strategy for future drug design.

3. CANCER STEM CELLS

The twentieth century revolution in genetics and molecular biology brought a new paradigm for the development of cancer: evolution. Over many generations, accumulated mutations that are most advantageous are 'naturally selected'. Similarly, drug resistance can be understood in terms of survival of those cells able to withstand chemotherapy [24].

The CSC model refines this paradigm. Like normal tissue stem cells, CSCs are small in number, but are the only cells with unlimited ability to proliferate [24]. If the surrounding tumour is eliminated, CSCs can regenerate it – and relapse times for tumours after chemotherapy can be related to the time taken for normal tissues to regenerate [4].

3.1. Inherent Drug Resistance

Tissue stem cells are better protected than ordinary cells. The same applies to CSCs, which are thought to be related to tissue stem cells [24]. CSCs proliferate infrequently: it is their non-CSC daughters that proliferate rapidly. CSCs are therefore less vulnerable to cytotoxic drugs (which target proliferating cells). CSCs also express efflux pumps – e.g.

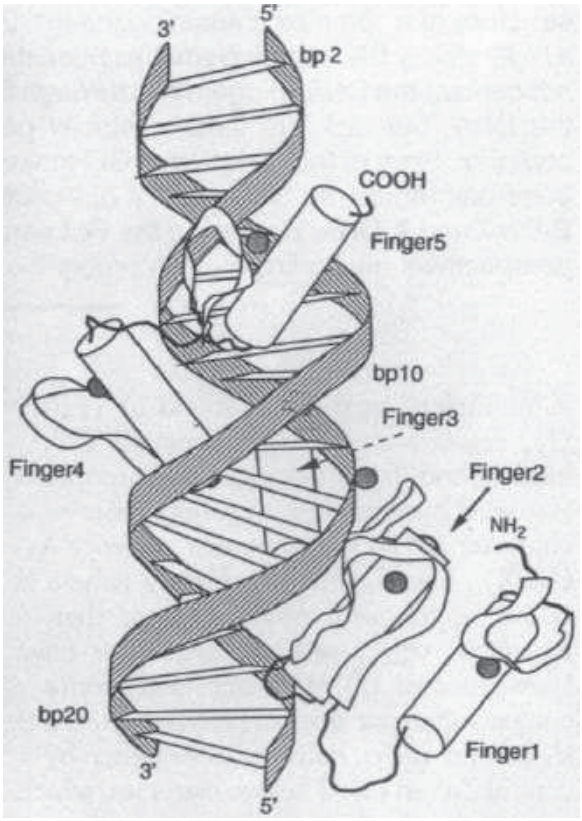

Fig. (5). Zinc finger Gli-DNA complex (taken from [23])

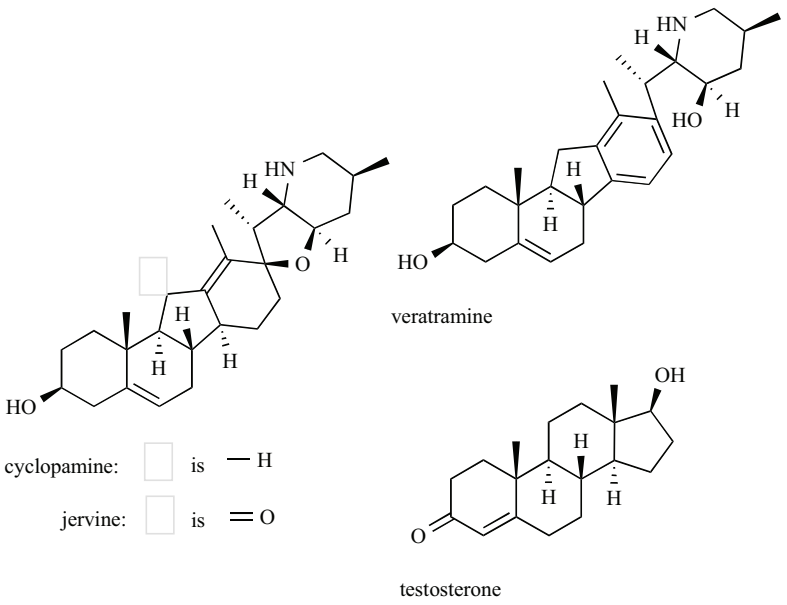

Fig. (6). Selected compounds tested for cyclopian-inducing activity in ewes. The structural formula of cyclopamine was unavailable at the time.

ATP-binding cassette (ABC) transporters – which are able to expel toxins and drugs [4].

The CSC model is supported by experiments in which human tumour cells are transplanted into immunosuppressed mice. Only those cells with stem cell surface markers are able to cause tumours in the mice [24]. However, the model isn't universally accepted, and a recent series of letters in *Science* debated whether these results could instead be explained by inter-species differences [25-27]. Moreover, CSCs cannot by themselves explain how drug resistance can spread throughout a tumour [4]. Nevertheless, the weight of so many experiments is hard to ignore (see, e.g., [28], and references within). It seems that the CSC model is useful but not in all circumstances. However, as the Hedgehog pathway is associated with tissue formation (using stem cells), the CSC model should be particularly relevant in the understanding of the Hedgehog pathway. Indeed, Hedgehog pathway activity is associated with survival and growth of CSCs in several cancers [29, 30]. Hedgehog inhibitors may be able to fill the gap left by other drugs that do not specifically target CSCs.

4. SMOOTHENED INHIBITORS

The largest and most important class of Hedgehog inhibitors involves molecules that target Smo. Thus, in this section we discuss the discovery and development of cyclopamine and selected derivatives. Finally, we offer a brief discussion of selected non-cyclopamine-based, synthetic inhibitors, to set cyclopamine-based inhibitors in context, and to draw out points of general interest in targeting Smo.

4.1. Cyclopamine and Cyclopamine Derivatives

4.1.1. Discovery of Cyclopamine

In the 1960s it was observed that ewes grazing on the corn lily *Veratrum californicum* – rich in the steroidal alkaloid cyclopamine – produced lambs with one-eye (cyclopia). In a subsequent 3-year trial of potential causative agents, the steroidal alkaloids cyclopamine, jervine and veratramine were found to have strong, weak and no cyclopian activity (respectively). Steroids, e.g. testosterone, were inactive (see Fig. **6**) [31].

The explanation for cyclopamine's effects remained a mystery until 1978, when, with its structural formula available, cyclopamine's stereochemistry was found to be critical for its action (see Fig. **7**) [32].

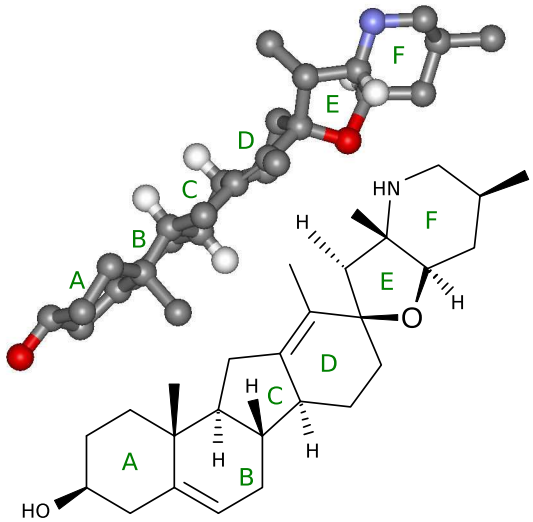

Fig. (7). Stereochemistry of cyclopamine.

The following characteristics appeared crucial for cyclopamine activity:

1. The fused furan-piperidine rings (E-F) must be at right-angles to the plane of the core steroid rings (A-D).

2. The furan ring (E) must be closed (c.f. veratramine).

3. The basic nitrogen is vital and must be some distance removed from the steroidal core.

Cyclopia (Fig. **8**) is an extreme form of holoprosencephaly. This association between cyclopamine and holoprosencephaly led to its identification as a Hedgehog pathway inhibitor in 1998 in a study using chick embryos [33].

A further study using neural cells extracted from chick embryos (explant cells) provided evidence that cyclopamine operated directly on the Hedgehog pathway, by showing that cyclopamine's effects are different to those of cholesterol synthesis inhibitors [34].

4.1.2. Initial Cyclopamine Derivatives

Cyclopamine was also shown to block the response of explant cells to Shh at concentrations of 20-100nM [34]. The same research group followed up with a structure-activity modeling assay, refining the 1978 results on the importance of cyclopamine stereochemistry [35]. Jervine (Fig. **6**) was 5 to 10× less potent than cyclopamine. Furthermore, saturating the rings of jervine to give tetrahydrojervine reduced potency threefold. Conversely, oxidising the hydroxyl group to a ketone increased activity: cyclopamine-4-en-3-one was 2× more potent than cyclopamine (see Fig. **9** and Table **1**) [35].

Fig. (8). Cyclopian (above) and normal (below) lamb skulls (taken from [31]).

This study quantitatively assessed different compounds against cyclopamine. Subsequently, molecular biology or drug development studies also adopted cyclopamine as a benchmark against which to measure biological activity or drug potency. Indeed, it is worth mentioning at this point that the division between biology and compound development in this review is rather artificial. In practice, the two go hand in hand: new biologically active compounds are used as probes to understand biology, and biological insights motivate new avenues of drug development.

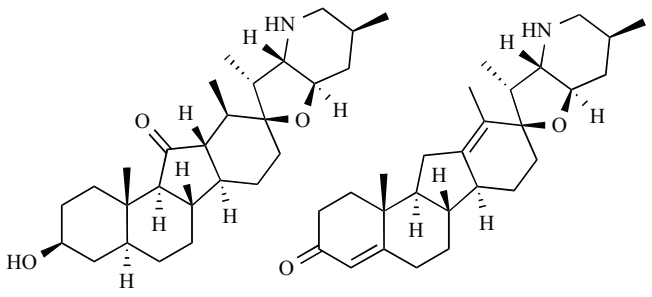

Fig. (9). Tetrahydrojervine and cyclopamine-4-en-3-one.

Table 1. Comparison of Potency of Compounds w.r.t. Cyclopamine in Blocking Explant Chick Neural Cells Response to Shh.

Compound	Relative potency
Cyclopamine-4-en-3-one	2×
Cyclopamine	1
Jervine	0.1 to 0.2×
Tetrahydrojervine	0.03 to 0.07×

A case in point is an important study which demonstrated cyclopamine's ability to address cancer. The study used a more potent cyclopamine derivative, KAAD-cyclopamine, as a probe (in addition to cyclopamine), to link cyclopamine with cancer biology, and thereby motivated further developments in the field [36]. In this study, Hedgehog activity was gauged by the quantity of light emitted by a luminescent enzyme linked to Gli-dependent cellular activity – a Gli-luciferase assay. Cyclopamine (and derivatives) blocked the activation of the Hedgehog response pathway resulting from mutations that inactivate Ptc and to a lesser extent from mutations that activate Smo, due to resistance of Smo related proteins. The study indicated that the target of cyclopamine action is likely to be a pathway component that functions between Ptch and the Gli proteins. However, cyclopamine and its derivatives failed to prevent pathway activation induced by Gli2 overexpression and mutations to Gli [36].

4.1.3. KAAD-Cyclopamine

KAAD-cyclopamine (Fig. **10**) is 10 to 20× more potent than cyclopamine. It was used to avoid cyclopamine cytotoxicity at the higher concentrations (≥10μM) required to inhibit cells with oncogenic Smo mutations [36]. KAAD-cyclopamine retains the ketone moiety of cyclopamine-4-en-3-one, and further substitutes a basic phenol-terminated side chain at the amine of the piperidine ring (F). Less basic, longer or branched chains are less active; it is suggested this may be because of cell impermeability or steric hindrance [5]. (See [5] for details of alternative substituents)

In addition, KAAD's side-chain resembles hydrophobic amino acids – e.g. isoleucine or phenylalanine – linked by peptide bonds. This resemblance may help in mimicking an endogenous ligand or in associating with the cell membrane.

If a 3d structure of Smo were available, it would be possible to evaluate such hypotheses and make refinements accordingly.

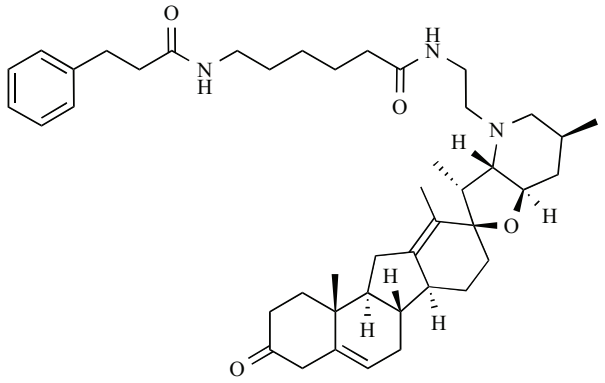

Fig. (10). KAAD-cyclopamine.

Cyclopamine is hydrophobic and might be expected to bind to a membrane protein. The above observation that cyclopamine only effectively suppressed Hedgehog signalling upstream of Smo suggested that cyclopamine bound directly to Smo. This was subsequently confirmed in a study using labelled cyclopamine [37]. In this study, the K_D of KAAD-cyclopamine was determined to be 23nM, by displacement of labelled cyclopamine [37].

4.1.4. Cyclopamine or KAAD-Cyclopamine in the Clinic?

A number of *in vitro* and *in vivo* studies have demonstrated an antitumour activity for cyclopamine and KAAD-cyclopamine. In one such study, both compounds were first shown to inhibit growth of mouse medulloblastoma cells *in vitro*. Subsequently, subcutaneously-injected cyclopamine at 50mg/kg/day for one week was shown to completely inhibit or even reverse growth of tumours resulting from the same cells grafted onto immunosuppressed mice. As a control, tumours from cells engineered with downstream, Gli over-activity were unaffected by cyclopamine [38]. No toxic effects were observed for these compounds, but toxicity was not the main focus of this study, as there were no quantitative (e.g. weight) or qualitative (e.g. autopsy) toxicity results reported [38].

Cyclopamine has been shown to have some serious disadvantages as a drug. A subsequent study observed toxicity in mice with injection or oral dosing, but not infusion, and noted poor oral bioavailability [39]. In strongly acidic conditions, such as the stomach of a mouse or human, cyclopamine's furan ring opens, converting cyclopamine to veratramine (unfortunately for sheep, cyclopamine has a good oral bioavailability in ruminating animals thanks to their less acidic stomachs) [35].

Low oral bioavailability does not necessarily preclude use in cancer chemotherapy, as injection and infusion remain common delivery methods, particularly for drugs associated with life threatening conditions [40]. But, overall, cyclopamine's relatively poor affinity and pharmacokinetic profile makes it difficult to develop as a drug.

Published accounts of *in vivo* KAAD-cyclopamine activity are not available. Like cyclopamine, KAAD-cyclopamine is hydrophobic and poorly soluble, and is unstable in acid conditions [44]. It is also bulky for a drug, which can lead to various problems: e.g. it is possible that its increased potency comes at the expense of selectivity.

Neither compound is in clinical trials [6]. Nevertheless, cyclopamine has had one documented success in humans, in which it was applied topically to BCCs, albeit only with four patients [41].

All the patients' skin tumours regressed, and after surgical removal, examination under a microscope confirmed widespread apoptosis of tumour cells [41]. Interestingly, the authors asked why apoptosis occurred, rather than a cytostatic effect. They hypothesised that cancer cells rely so heavily on the Hedgehog signalling pathway that they are no longer viable when it is removed [41]. This explanation is an example of the 'oncogene addiction' model for the success of drugs that target over-active molecular pathways in cancer [42]. Cancer cells become dependent on an earlier oncogenic mutation and when it is removed cannot tolerate the 'withdrawal symptoms'.

Although cyclopamine failed as a systemically-administered drug, it was successful in that it indicated that it is possible to treat cancer by inhibiting the Hedgehog pathway. It also acted as a starting point for further drug development. Two examples are discussed below: wrapping cyclopamine in a chemical delivery system – i.e. a prodrug – and modifying cyclopamine itself.

4.1.5. Cyclopamine Prodrugs

In metastatic prostate cancer, the Hedgehog pathway is over-activated. A simple but elegant prodrug formulation was devised to treat prostate cancer, whilst avoiding possible toxicity in healthy tissues. Cyclopamine was conjugated to two peptides that were previously shown to be specifically cleaved by Prostate Specific Antigen (PSA) at the F-ring amine, to form the prodrug. *In vivo*, the drug would be released by the prostate-specific protease prostate specific antigen (PSA), which can selectively cleave the peptide, converting the mature peptide into the active Hedgehog inhibitor within the malignant cells [43].

The lead compound, Mu-SSKYQ-cyclopamine (Fig. **11**), was hydrolysed by PSA with a half-life of 3 hours, and successfully inhibited prostate cancer cell growth *in vitro*. *In vivo* studies are ongoing [43].

These drugs would need to be injected, as degradation in and absorption from the stomach would be problematic, and metabolism of prodrugs is unpredictable. It would also be worth considering whether this prodrug strategy could extend to other, more potent cyclopamine derivatives such as cyclopamine-4-en-3-one, and the Infinity Pharmaceuticals compounds are discussed below.

4.1.6. IPI-609

Infinity Pharmaceuticals set out to improve cyclopamine's pharmacokinetic properties, starting with its acid instability. Noting that the allylic ether (–C=C–C–O–) straddling the D and E rings was susceptible to hydrolysis, they sought to reduce its reactivity without disrupting the overall

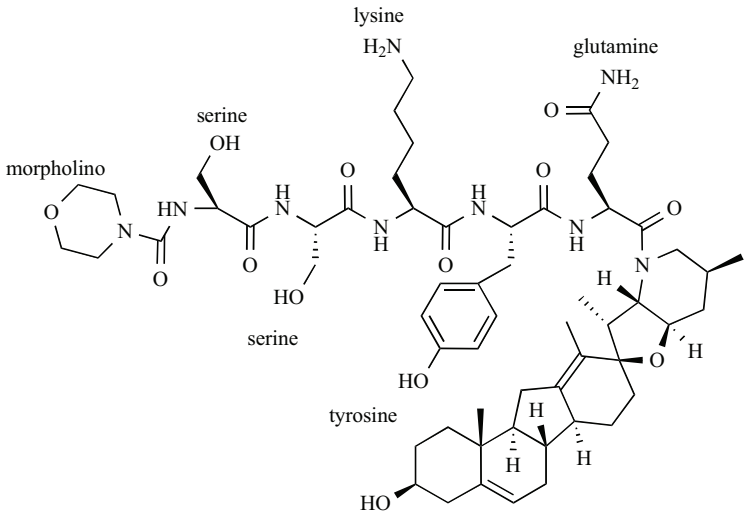

Fig. (11). Mu-SSKYQ-cyclopamine.

cyclopamine structure. The six-membered D ring was replaced by a seven-membered ring, effectively inserting a carbon atom between the double bond and oxygen (Fig. **12**). Acid stability improved, but at the cost of potency, and the ketone moiety from cyclopamine-4-en-3-one was adopted, increasing potency and solubility [44].

The resulting compound, IPI-609 (Fig. **12**), was an order of magnitude more soluble than cyclopamine at plasma pH, with 80% oral bioavailability and an elimination half-life of 3 hours in mice. KAAD-cyclopamine-style substitutions at the amine of the F-ring resulted in 10 to 20× more potency, but were rejected because of poor solubility [44].

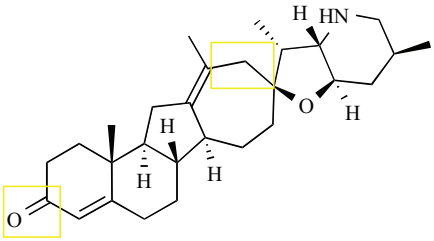

Fig. (12). IPI-609 (a.k.a. IPI-269609). Changes from cyclopamine are highlighted.

In vitro, IPI-609 and cyclopamine blocked Hedgehog signalling with an IC_{50} of 0.6μM in a Gli-luciferase assay (GliLuc). Human pancreatic cancer cells, thought to overactivate the Hedgehog pathway mainly through ligand overexpression, were surgically implanted into immunosuppressed mice. IPI-609 was given orally at 20mg/kg/day (c.f. 50mg/kg/day cyclopamine in the previously described study). It prevented the spread or metastasis of cancer to other sites – the major cause of death in pancreatic cancers (and many other cancers) [45]. However, IPI-609 did not have a statistically significant effect on the primary tumour size, although these were smaller on average. In post-mortem examinations of tumours, IPI-609 did substantially reduce the proportion of cells most able to seed new tumours. The same cells also had the most active Hedgehog pathways [45]. These results are particularly interesting. Although the drug failed by a traditional tumour size criterion, IPI-609 was arguably more therapeutically valuable. It inhibited the most serious cause of cancer mortality, i.e. metastasis, and killed the CSC-like cells that might eventually lead to a relapse (CSCs). Furthermore, no toxicity was found, using criteria such as weight and post-mortem organ examination [45]. However, IPI-609 was later rejected due to metabolic instability, as well as its relatively low potency [45, 46].

4.1.7. IPI-926

First-pass metabolism in monkeys converted IPI-609's A-ring ketone to an alcohol, which was rapidly cleared. Structure-activity relationship modelling studies were used to improve metabolic stability and potency. Metabolic stability was gauged *in vitro* using human liver P450 enzymes, and potency was measured using a cellular assay [46].

The first phase of structure-activity modelling investigated replacing the A-ring ketone, starting with a high potency, saturated A-ring *cis*-decalone analogue (**1**). Initial modelling established some general principles (see Table **2** and Fig. **13**). Consistent with these principles, the amide **7** and sulfonamide **8** were found to be metabolically stable, with **8** being the most potent [46].

The second phase of structure-activity relationship studies investigated more dramatic modifications to the A-ring, including fusing a heterocycle to it, and replacing it with a seven-membered ring. This led to the pyrazole analogue **9** and the lactam analogue **10**. As before, a hydrogen bond donor was required [46].

The pharmacokinetics and pharmacology of compounds **8**–**10** were investigated. Compounds **8** and **10** both had good oral bioavailability, but **8** stood out with a half-life of more than 8 hours in multiple species [46]. *In vivo* testing against mouse medulloblastoma also showed **8** to be superior. Oral dosing of 40mg/kg/day eliminated tumours after 9 days [46], and there were no relapses after 50 days [47]. This demonstrates the substantial improvement in metabolic stability and potency achieved by structure-activity modeling over IPI-609. Compound **8** – now renamed IPI-926 – is proceeding to phase II clinical trials [48].

Table 2. Required Properties for Substituent to Replace 3-Keto

Property	Example substitutions at 3 position	
	More potent	**Less potent**
Polarity	=O, ketone (1)	none (2)
Hydrogen bond donor	=NOH, oxime (3)	$=NOCH_3$, methyloxime (4)
R stereochemistry	OCH_3, (R)-methoxy (5)	OCH_3, (S)-methoxy (6)

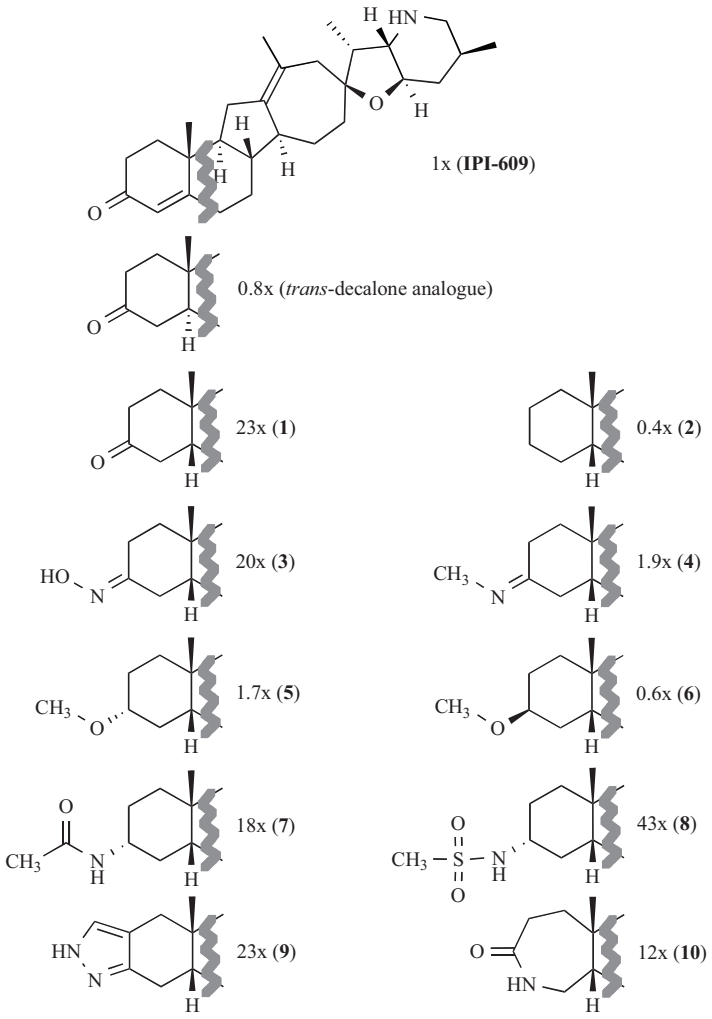

Fig. (13). Alternative substituents to replace 3-keto (compounds **1–8**), or fuse to or replace the A-ring (compounds **9–10**). Potencies are quoted relative to IPI-609.

4.2. Synthetic Inhibitors

All the other Hedgehog inhibitors in clinical trials are synthetic Smo inhibitors. XL-139 and LDE-225 (Bristol Meyers and Novartis, structures not disclosed) are in phase I trials. GDC-0449 (Genentech; Fig. **14**), with a Gli-luciferase IC_{50} of 3nM, is 200× more potent than cyclopamine and it is currently in phase II trials [49].

GDC-0449 competes with cyclopamine and therefore seems to bind to the same active site [49]. However, another inhibitor, SANT-1, (see Fig. **15**) does not appear to bind competitively [50]. It was SANT-1 which was found to block Smo translocation to the primary cilium [18]. This raises the possibility of sequentially blocking both stages of the Smo activation pathway with different drugs in combination, to counter acquired drug resistance.

Itraconazole is a drug already used as a systemic antifungal, and was identified as a Hedgehog inhibitor in a recent Gli-luciferase screen of several thousand compounds, with an IC_{50} of 800nM [51]. It operates in a similar fashion to SANT-1, and has been shown to reduce accumulation of

Smo to the primary cilium, to non-competitively inhibit Smo with respect to agonists acting at the cyclopamine binding site, and to act synergistically when used in combination with cyclopamine. Further support for developing itraconazole as an anticancer agent is gained due to its well-known toxicity profile, and its effectiveness in mouse models at similar doses to its use as an antifungal [51].

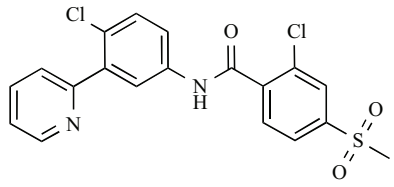

Fig. (14). The chemical structure of GDC-0449.

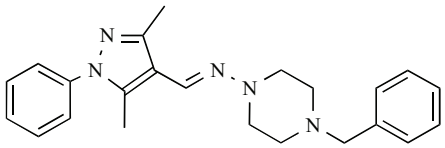

Fig. (15). The chemical structure of SANT-1.

GDC-0449 is derived from HhAntag, (Fig. **16**) which was discovered using a Gli-luciferase screen [6]. HhAntag was found to cause permanent bone defects in young mice, even with only 2 days treatment [52]. On the other hand, no serious toxicity has been found with GDC-0449 in clinical trials [49].

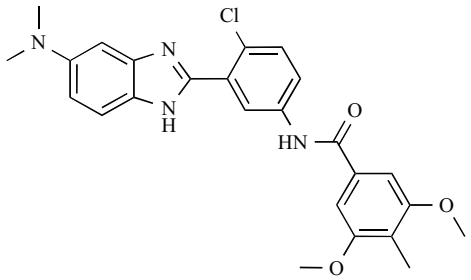

Fig. (16). The chemical structure of HhAntag.

HhAntag toxicity was similar to genetically-engineered loss of Indian Hedgehog [52]. GDC-0449 may be more specific to Sonic Hedgehog.

5. NON-SMOOTHENED INHIBITORS

Upstream of Smo, Shh binds to Ptc to activate the Hedgehog pathway. Several cell-surface proteins mediate binding, including the negative modulator Hhip, whose structure with Shh has recently been solved.

Downstream, signal transduction prior to the Gli zinc-finger transcription factors is poorly understood. The transcription factors themselves seem like the most attractive drug targets. A number of inhibitors have been developed, which are briefly described in the following sections.

5.1. Hedgehog and Patched Inhibitors

It is more common to target receptors than ligands, but Robotnikin is a small molecule that binds Shh, inhibiting Hedgehog signalling. It was recently discovered by screening thousands of small molecules (bound to a microscope slide) for Shh affinity, followed by structural optimisation (Fig. **17**) [53].

Robotnikin inhibited Hedgehog signalling only in the absence of Smo agonists (Fig. **18**), confirming it operates upstream of Smo. 30μM Robotnikin had a similar inhibitory effect to that observed with 6μM cyclopamine, making Robotnikin 5× less potent than cyclopamine [53].

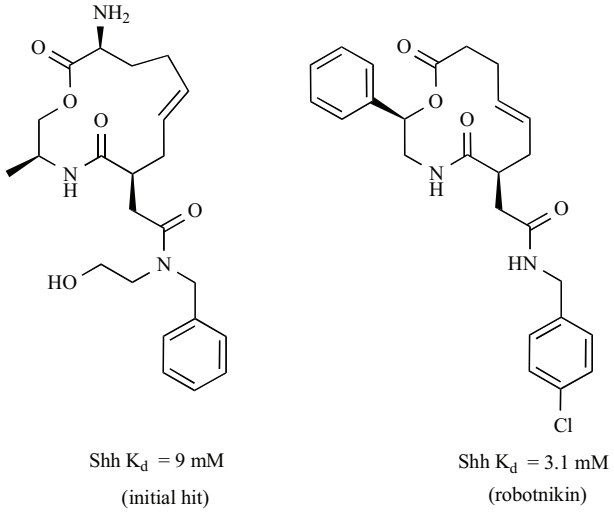

Fig. (17). Left: small-molecule microarray hit. Right: robotnikin.

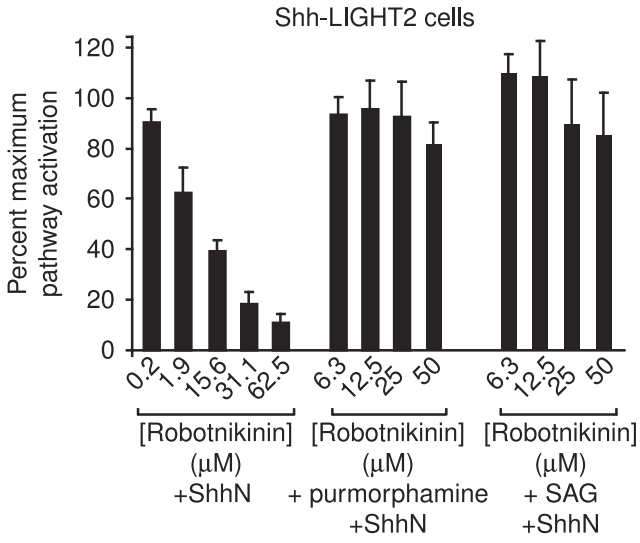

Fig. (18). In a Gli-luciferase assay, Robotnikin inhibited Shh-initiated Hedgehog signalling in a concentration-dependent manner. In the presence of Smo agonists purmorphamine and SAG, Robotnikin had little effect (taken from [53]).

An alternative to screening is to start from an endogenous molecule that binds Shh. One attractive possibility is to start from the known, Shh-Hhip structure and design a molecule to mimic the Hhip binding site [6, 54].

Antibodies have been effective in cellular assays at sequestering Shh, or binding to Ptc to block Shh [55, 56].

However, it still remains unclear whether these are being actively pursued as a therapeutic strategy.

5.2. Gli Inhibitors

Targeting transcription factors is thought to be problematic. Protein-protein interactions often involve a large area and are harder to inhibit with small molecules. Moreover, off-target effects on other transcription factors can be severe, and systemic delivery can result in on-target toxicity in healthy cells, potentially affecting several pathways [57].

Nevertheless, several approaches are underway [5]. The most promising compounds are GANT58 and GANT61, Fig. (**19**), which were discovered in a cellular screen for inhibition of Gli1 transcription (Gli1 is the transcription factor most strongly implicated in cancer) [58].

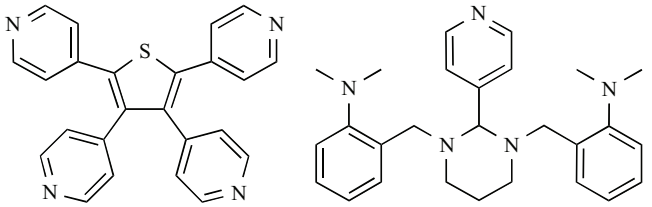

Fig. (19). The chemical structures of GANT58 and GANT61.

In cellular assays with mutations downstream of Smo, both compounds showed activity, whereas cyclopamine did not. GANT61 was effective *in vivo* against cyclopamine-resistant human prostate cancer cells grafted onto immunosuppressed mice (Fig. **20**). Their IC_{50} values in Gli-luciferase assays were 5μM, making them 10× less potent than cyclopamine [58].

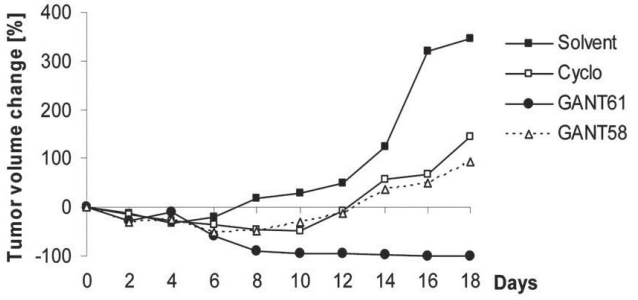

Fig. (20). Effects of subcutaneous injection of solvent control, cyclopamine, GANT58 and GANT61 on tumour growth in mice, following introduction of 22Rv1 human prostate cancer cells (taken from [58]).

Developing inhibitors of the Hedgehog pathway upstream or downstream of Smo requires breaking new pharmacological ground, but the process has begun. Robotnikin targets Shh (upstream) and the GANTs target Glis (downstream); however they are less potent than cyclopamine. Figs. (**18** and **20**) illustrate that Hedgehog and patched inhibitors are only effective for Shh over-expressing cancers, whereas Gli inhibitors can target cancers with mutations downstream of Smo.

6. COMBINATION CHEMOTHERAPY

This section begins with the inhibition of efflux pumps and also looks at the targeting of cancer stem cells, to offer a broader overlook on the role of Hedgehog inhibitors in combination therapy.

6.1. Inhibiting Efflux Pumps

In one study, cancer cell lines were treated with Shh ligand, a cytotoxic drug, or a combination of cytotoxic drug and cyclopamine. Shh protected cells against the cytotoxic drugs, whereas the combination treatment was found to enhance cytotoxicity. Radioactive labelling confirmed that the difference was due to a reduction (Shh) or increase (cyclopamine) in drug uptake [59].

Hedgehog pathway activation is associated with efflux of multiple structurally different drugs. Genetic knock-out studies showed that one mechanism underlying drug efflux is Hedgehog-activated expression of ABC transporters [59].

Other studies have found that Hedgehog inhibitors act synergistically with other drugs in assays against human cancer cell lines. E.g., GDC-0449 increased the effectiveness of the genotoxic topoisomerase II inhibitor mitoxantrone [60].

6.2. Targeting Cancer Stem Cells

These observations are consistent with a model in which Hedgehog targets CSCs, the small proportion of cells that are able to proliferate indefinitely. CSCs must be killed to eradicate a tumour, but they are inherently drug resistant because, for example, they express efflux pumps and proliferate only rarely.

The reason that Gleevec is not curative and treatment must be chronic is because Gleevec is ineffective against CSCs. A recent study shows that the CSCs' survival relies on Hedgehog signalling, as demonstrated by their susceptibility to cyclopamine. Most significantly, Hedgehog inhibitors impaired growth of Gleevec-resistant CML, both *in vitro* and *in vivo* [61].

Similarly, the results of the *in vivo* study of IPI-609 against pancreatic cancer showed effectiveness against CSCs. However, IPI-609 did not significantly decrease tumour size.

Hedgehog inhibitors have a unique – and complementary – role in cancer treatment as they can specifically target CSCs. Thus, although they may not on their own be effective at reducing tumour size in the short term, they have great potential in combination chemotherapy approaches, and hence their effectiveness should not be judged solely on this criterion [3].

Even if the CSC model is flawed, experimental results indicate that Hedgehog inhibitors can play an important role in fighting drug resistance in combination with other drugs.

CONCLUSION

Cyclopamine is the benchmark for a new class of anticancer drugs targeting the Hedgehog pathway. The four drugs in clinical trials share the same target as cyclopamine,

the GPCR-like Smoothened oncoprotein. This is surely no coincidence: cyclopamine showed that Smoothened could be inhibited to fight cancer, and drug companies had a ready supply of expertise and chemical libraries to target GPCRs.

Cyclopamine was discovered serendipitously, and, according to available information, non-cyclopamine Hedgehog inhibitors were discovered through high-throughput screening. Limited information on molecular mechanisms and structures has precluded target-based structural design and development, but powerful drugs have nonetheless been produced. An example is the cyclopamine analogue IPI-926, which is 40× more potent than cyclopamine.

Research into Hedgehog molecular biology is elucidating mechanisms and solving protein structures. These insights can be expected to guide future drug development. Upstream and downstream of Smoothened, recently-discovered compounds may act as a benchmark and a spur to develop drugs in their respective areas. However, future drug development will undoubtedly be dependent on the success or otherwise of IPI-926 and GDC-0449, the drugs most advanced in clinical trials.

The cancer stem cells model suggests a unique role for Hedgehog inhibitors in fighting drug resistance by inhibiting the regenerative core of a cancer. This is supported by experimental evidence, including results showing inhibition of Gleevec-resistant CML. Hedgehog inhibitors look set to become one prong of a combined – and more effective – chemotherapeutic attack on cancer.

ACKNOWLEDGEMENTS

Dr Missailidis would like to acknowledge the Open University for financial support.. This review article was produced based on work submitted for the Open University's S807 Master's Degree course, Molecules in Medicine. Mr Gould would like to acknowledge the teaching staff involved in S807, in particular his tutor Liz Andrews, together with Yao Xu, Dick Challand, and Simone Pitman.

REFERENCES

[1] Verweij, J.; de Jonge, M.J.A. Achievements and future of chemotherapy. *Eur. J. Cancer*, **2000**, *36*, 1479-1487.

[2] Bhatia, S.; Sklar, C. Second cancers in survivors of childhood cancer. *Nat. Rev. Cancer*, **2002**, *2*, 124-132.

[3] Weinberg, R. In: *The Biology of Cancer*, Garland Science, New York, **2007**, pp. 725-796.

[4] Dean, M.; Fojo, T.; Bates, S. Tumour stem cells and drug resistance. *Nat. Rev. Cancer*, **2005**, *5*, 275-284.

[5] Mahindroo, N.; Punchihewa, C.; Fujii, N. Hedgehog-Gli Signaling Pathway Inhibitors as Anticancer Agents. *J. Med. Chem.*, **2009**, *52*, 3829-3845.

[6] Scales, S.J.; de Sauvage, F.J. Mechanisms of Hedgehog pathway activation in cancer and implications for therapy. *Trends Pharmacol. Sci.*, **2009**, *30*, 303-312.

[7] Varjosalo, M.; Taipale, J. Hedgehog: functions and mechanisms. *Genes Dev.*, **2008**, *22*, 2454-2472.

[8] Stone, D.M.; Hynes, M.; Armanini, M.; Swanson, T.A.; Gu, Q.M.; Johnson, R.L.; Scott, M.P.; Pennica, D.; Goddard, A.; Phillips, H.; Noll, M.; Hooper, J.E.; de Sauvage, F.; Rosenthal, A. The tumour-suppressor gene patched encodes a candidate receptor for Sonic Hedgehog. *Nature*, **1996**, *384*, 129-134.

[9] Marigo, V.; Davey, R.A.; Zuo, Y.; Cunningham, J.M.; Tabin, C.J. Biochemical evidence that Patched is the Hedgehog receptor. *Nature*, **1996**, *384*, 176-179.

[10] Tenzen, T.; Allen, B.L.; Cole, F.; Kang, J.S.; Krauss, R.S.; McMahon, A.P. The cell surface membrane proteins cdo and bloc are components and targets of the Hedgehog signaling pathway and feedback network in mice. *Dev. Cell*, **2006**, *10*, 647-656.

[11] McLellan, J.S.; Zheng, X.Y.; Hauk, G.; Ghirlando, R.; Beachy, P.A.; Leahy, D.J. The mode of Hedgehog binding to Ihog homologues is not conserved across different phyla. *Nature*, **2008**, *455*, 979-984.

[12] Bishop, B.; Aricescu, A.R.; Harlos, K.; O'Callaghan, C.A.; Jones, E.Y.; Siebold, C. Structural insights into Hedgehog ligand sequestration by the human Hedgehog-interacting protein HHIP. *Nat. Struct. Mol. Biol.*, **2009**, *16*, 698-703.

[13] University of Oxford. Molecular mechanisms of the Hedgehog signalling pathway. (Accessed 14 July **2010**).

[14] van den Heuvel, M.; Ingham, P.W. Smoothened encodes a serpentine protein required for Hedgehog signalling. *Nature*, **1996**, *382*, 547-551.

[15] Rohatgi, R.; Scott, M.P. Patching the gaps in Hedgehog signalling. *Nat. Cell Biol.*, **2007**, *9*, 1005-1009.

[16] Corcoran, R.B.; Scott, M.P. Oxysterols stimulate Sonic Hedgehog signal transduction and proliferation of medulloblastoma cells. *Proc. Natl. Acad. Sci. USA*, **2006**, *103*, 8408-8413.

[17] Dwyer, J.R.; Sever, N.; Carlson, M.; Nelson, S.F.; Beachy, P.A.; Parhami, F. Oxysterols Are Novel Activators of the Hedgehog Signaling Pathway in Pluripotent Mesenchymal Cells. *J. Biol. Chem.*, **2007**, *282*, 8959-8968.

[18] Rohatgi, R.; Milenkovic, L.; Corcoran, R.B.; Scott, M.P. Hedgehog signal transduction by Smoothened: Pharmacologic evidence for a 2-step activation process. *Proc. Natl. Acad. Sci. USA*, **2009**, *106*, 3196-3201.

[19] Bijlsma, M.F.; Spek, C.A.; Zivkovic, D. Repression of smoothened by patched-dependent (Pro-)Vitamin D3 secretion. *PLoS Biol.*, **2006**, *4*, 1397-1410.

[20] Kapoor, N.; Menon, S.T.; Chauhan, R.; Sachdev, P.; Sakmar, T.P. Activation of heterotrimeric G proteins by Smoothened. *Proc. Natl. Acad. Sci. USA*, **2006**, *33*, 12607-12612.

[21] Svard, J.; Henricson, K.H.; Persson-Lek, M.; Rozell, B.; Lauth, M.; Bergstrom, A.; Ericson, J.; Toftgard, R.; Teglund, S. Genetic elimination of suppressor of fused reveals an essential repressor function in the mammalian Hedgehog signaling pathway. *Dev. Cell*, **2006**, *10*, 187-197.

[22] Merchant, M.; Vajdos, F.F.; Ultsch, M.; Maun, H.R.; Wendt, U.; Cannon, J.; Desmarais, W.; Lazarus, R.A.; de Vos, A.M.; de Sauvage, F.J. Suppressor of fused regulates Gli activity through a dual binding mechanism. *Mol. Cell Biol.*, **2004**, *24*, 8627-8641.

[23] Pavletich, N.P.; Pabo, C.O. Crystal-Structure Of A 5-Finger Gli-Dna Complex – New Perspectives On Zinc Fingers. *Science*, **1993**, *261*, 1701-1707.

[24] Weinberg, R. In: *The Biology of Cancer*, Garland Science, New York, **2007**, pp. 399-526.

[25] Kelly, P.N.; Dakic, A.; Adams, J.M.; Nutt, S.L.; Strasser, A. Tumor Growth Need Not Be Driven by Rare Cancer Stem Cells. *Science*, **2007**, *317*, p. 337.

[26] Kennedy, J.A.; Barabe, F.; Poeppl, A.G.; Wang, J.C.Y.; Dick, J.E. Comment on "tumor growth need not be driven by rare cancer stem cells''. *Science*, **2007**, *318*, p. 1772c.

[27] Adams, J.M.; Kelly, P.N.; Dakic, A.; Nutt, S.L.; Strasser, A. Response to comment on "tumor growth need not be driven by rare cancer stem cells''. *Science*, **2007**, *318*, p. 1722d.

[28] Li, C.W.; Heidt, D.G.; Dalerba, P.; Burant, C.F.; Zhang, L.J.; Adsay, V.; Wicha, M.; Clarke, M.F.; Simeone, D.M. Identification of pancreatic cancer stem cells. *Cancer Res.*, **2007**, *67*, 1030-1037.

[29] Peacock, C.D.; Wang, Q.J.; Gesell, G.S.; Corcoran-Schwartz, I.M.; Jones, E.; Kim, J.; Devereux, W.L.; Rhodes, J.T.; Huff, C.A.; Beachy, P.A.; Watkins, D.N.; Matsui, W. Hedgehog signaling maintains a tumor stem cell compartment in multiple myeloma. *Proc. Natl. Acad. Sci. USA*, **2007**, *104*, 4048-4053.

[30] Clement, V.; Sanchez, P.; de Tribolet, N.; Radovanovic, I.; Altaba, A.R.I. HEDGEHOG-GLI1 signaling regulates human glioma growth, cancer stem cell self-renewal, and tumorigenicity. *Curr. Biol.*, **2007**, *17*, 165-172.

[31] Keeler, R.F.; Binns, W. Teratogenic Compounds of Veratrum Californicum Comparison of Cyclopian Effects of Steroidal Alkaloids. *Teratology*, **1968**, *1*, 5-10.

[32] Keeler, R.F., Cyclopamine And Related Steroidal Alkaloid Teratogens - Their Occurrence, Structural Relationship, And Biologic Effects. *Lipids*, **1978**, *13*, 708-715.

[33] Cooper, M.K.; Porter, J.A.; Young, K.E.; Beachy, P.A. Teratogen-mediated inhibition of target tissue response to Shh signaling. *Science*, **1998**, *280*, 1603-1607.

[34] Incardona, J.P.; Gaffield, W.; Kapur, R.P.; Roelink, H. The teratogenic Veratrum alkaloid cyclopamine inhibits Sonic Hedgehog signal transduction. *Development*, **1998**, *125*, 3553-3562.

[35] Incardona, J.P.; Gaffield, W.; Lange, Y.; Cooney, A.; Pentchev, P.G.; Liu, S.; Watson, J.A.; Kapur, R.P.; Roelink, H. Cyclopamine inhibition of sonic Hedgehog signal transduction is not mediated through effects on cholesterol transport. *Dev. Biol.*, **2000**, *224*, 440-452.

[36] Taipale, J.; Chen, J.K.; Cooper, M.K.; Wang, B.L.; Mann, R.K.; Milenkovic, L.; Scott, M.P.; Beachy, P.A. Effects of oncogenic mutations in Smoothened and Patched can be reversed by cyclopamine. *Nature*, **2000**, *406*, 1005-1009.

[37] Chen, J.K.; Taipale, J.; Cooper, M.K.; Beachy, P.A. Inhibition of Hedgehog signaling by direct binding of cyclopamine to Smoothened. *Gene Dev.*, **2002**, *16*, 2743-2748.

[38] Berman, D.M.; Karhadkar, S.S.; Hallahan, A.R.; Pritchard, J.I.; Eberhart, C.G.; Watkins, D.N.; Chen, J.K.; Cooper, M.K.; Taipale, J.; Olson, J.M.; Beachy, P.A. Medulloblastoma Growth Inhibition by Hedgehog Pathway Blockade. *Science*, **2002**, *297*, 1559-1561.

[39] Lipinski, R.J.; Hutson, P.R.; Hannam, P.W.; Nydza, R.J.; Washington, I.M.; Moore, R.W.; Girdaukas, G.G.; Peterson, R.E.; Bushman, W. Dose- and route-dependent teratogenicity, toxicity, and pharmacokinetic profiles of the Hedgehog signaling antagonist cyclopamine in the mouse. *Toxicol. Sci.*, **2008**, *104*, 189-197.

[40] Cancer Research UK. Ways of having chemotherapy. (Accessed 14 July **2010**).

[41] Tas, S.; Avci, O. Induction of the differentiation and apoptosis of tumor cells in vivo with efficiency and selectivity. *Eur. J. Dermatol.*, **2004**, *14*, 96-102.

[42] Weinstein, I.B. Cancer: Addiction to oncogenes – The Achilles heal of cancer. *Science*, **2002**, *297*, 63-64.

[43] Kumar, S.K.; Roy, I.; Anchoori, R.K.; Fazli, S.; Maitra, A.; Beachy, P.A.; Khan, S.R. Targeted inhibition of Hedgehog signaling by cyclopamine prodrugs. *Bioorg. Med. Chem.*, **2008**, *16*, 2764-2768.

[44] Tremblay, M.R.; Nevalainen, M.; Nair, S.J.; Porter, J.R.; Castro, A.C.; Behnke, M.L.; Yu, L.C.; Hagel, M.; White, K.; Faia, K.; Grenier, L.; Campbell, M.J.; Cushing, J.; Woodward, C.N.; Hoyt, J.; Foley, M.A.; Read, M.A.; Sydor, J.R.; Tong, J.K.; Palombella, V.J.; McGovern, K.; Adams, J. Semisynthetic Cyclopamine Analogues as Potent and Orally Bioavailable Hedgehog Pathway Antagonists. *J. Med. Chem.*, **2008**, *51*, 6646-6649.

[45] Feldmann, G.; Fendrich, V.; McGovern, K.; Bedja, D.; Bisht, S.; Alvarez, H.; Koorstra, J.B.M.; Habbe, N.; Karikari, C.; Mullendore, M.; Gabrielson, K.L.; Sharma, R.; Matsui, W.; Maitra, A. An orally bioavailable small-molecule inhibitor of Hedgehog signaling inhibits tumor initiation and metastasis in pancreatic cancer. *Mol. Cancer Ther.*, **2008**, *7*, 2725-2735.

[46] Tremblay, M.R.; Lescarbeau, A.; Grogan, M.J.; Tan, E.; Lin, G.; Austad, B.C.; Yu, L.C.; Behnke, M.L.; Nair, S.J.; Hagel, M.; White, K.; Conley, J.; Manna, J.D.; Alvarez-Diez, T.M.; Hoyt, J.; Woodward, C.N.; Sydor, J.R.; Pink, M.; MacDougall, J.; Campbell, M.J.; Cushing, J.; Ferguson, J.; Curtis, M.S.; McGovern, K.; Read, M.A.; Palombella, V.J.; Adams, J.; Castro, A.C. Discovery of a Potent and Orally Active Hedgehog Pathway Antagonist (IPI-926). *J. Med. Chem.*, **2009**, *52*, 4400-4418.

[47] Infinity Pharmaceuticals. Discovery of IPI-926, a Semi-Synthetic Clinical Candidate that Targets the Hedgehog Pathway. (Accessed Wednesday 14 July **2010**).

[48] Infinity Pharmaceuticals. Product Candidate: IPI-926. (Accessed Wednesday 14 July **2010**).

[49] Shelton, C.A.; Gilmartin, A.G. Novel receptor antagonists for cancer therapy: Hedgehog pathway inhibitors. *Drug Discov. Today Ther. Strategies*, **2009**, *6*, 63-69.

[50] Chen, J.K.; Taipale, J.; Young, K.E.; Maiti, T.; Beachy, P.A. Small molecule modulation of Smoothened activity. *Proc. Natl. Acad. Sci. USA*, **2002**, *99*, 14071-14076.

[51] Kim, J.; Tang, J.Y.; Gong, R.Y.; Kim, J.; Lee, J.J.; Clemons, K.V.; Chong, C.R.; Chang, K.S.; Fereshteh, M.; Gardner, D.; Reya, T.; Liu, J.O.; Epstein, E.H.; Stevens, D.A.; Beachy, P.A. Itraconazole, a Commonly Used Antifungal that Inhibits Hedgehog Pathway Activity and Cancer Growth. *Cancer Cell*, **2010**, *17*, 388-399.

[52] Kimura, H.; Ng, J.M.Y.; Curran, T. Transient inhibition of the Hedgehog pathway in young mice causes permanent defects in bone structure. *Cancer Cell*, **2008**, *13*, 249-260.

[53] Stanton, B.Z.; Peng, L.F.; Maloof, N.; Nakai, K.; Wang, X.; Duffner, J.L.; Taveras, K.M.; Hyman, J.M.; Lee, S.W.; Koehler, A.N.; Chen, J.K.; Fox, J.L.; Mandinova, A.; Schreiber, S.L. A small molecule that binds Hedgehog and blocks its signaling in human cells. *Nat. Chem Biol.*, **2009**, *5*, 154-156.

[54] Bosanac, I.; Maun, H.R.; Scales, S.J.; Wen, X.H.; Lingel, A.; Bazan, J.F.; de Sauvage, F.J.; Hymowitz, S.G.; Lazarus, R.A. The structure of SHH in complex with HHIP reveals a recognition role for the Shh pseudo active site in signaling. *Nat. Struct. Mol. Biol.*, **2009**, *16*, 691-697.

[55] Watkins, D.N.; Berman, D.M.; Burkholder, S.G.; Wang, B.L.; Beachy, P.A.; Baylin, S.B. Hedgehog signalling within airway epithelial progenitors and in small-cell lung cancer. *Nature*, **2003**, *422*, 313-317.

[56] Nakamura, M.; Kubo, M.; Yanai, K.; Mikami, Y.; Ikebe, M.; Nagai, S.; Yamaguchi, K.; Tanaka, M.; Katano, M. Anti-patched-1 antibodies suppress Hedgehog signaling pathway and pancreatic cancer proliferation. *Anticancer Res.*, **2007**, *27*, 3743-3747.

[57] Libermann, T.A.; Zerbini, L.F. Targeting transcription factors for cancer gene therapy. *Curr. Gene Ther.*, **2006**, *6*, 17-33.

[58] Lauth, M.; Bergstrom, A.; Shimokawa, T.; Toftgard, R. Inhibition of GLI-mediated transcription and tumor cell growth by small-molecule antagonists. *Proc. Natl. Acad. Sci. USA*, **2007**, *104*, 8455-8460.

[59] Sims-Mourtada, J.; Izzo, J.G.; Ajani, J.; Chao, K.S.C. Sonic Hedgehog promotes multiple drug resistance by regulation of drug transport. *Oncogene*, **2007**, *26*, 5674-5679.

[60] Zhang, Y.M.; Laterra, J.; Pomper, M.G. Hedgehog Pathway Inhibitor HhAntag691 Is a Potent Inhibitor of ABCG2/BCRP and ABCB1/Pgp. *Neoplasia*, **2009**, *11*, 96-101.

[61] Zhao, C.; Chen, A.; Jamieson, C.H.; Fereshteh, M.; Abrahamsson, A.; Blum, J.; Kwon, H.Y.; Kim, J.; Chute, J.P.; Rizzieri, D.; Munchhof, M.; VanArsdale, T.; Beachy, P.A.; Reya, T. Hedgehog signalling is essential for maintenance of cancer stem cells in myeloid leukaemia. *Nature*, **2009**, *458*, 776-780.

Received: August 20, 2010 Revised: October 21, 2010 Accepted: November 12, 2010