Journal Name

Dynamic Article Links ►

Cite this: DOI: 10.1039/c0xx00000x

www.rsc.org/xxxxxx

ARTICLE TYPE

Hedgehog Signalling Pathway Inhibitors as Cancer Suppressing Agents

Trieu N. Trinh,[a] Eileen A. McLaughlin,[b] Christopher P. Gordon[a] and Adam McCluskey[a]*

Received (in XXX, XXX) Xth XXXXXXXXX 20XX, Accepted Xth XXXXXXXXX 20XX
DOI: 10.1039/b000000x

Abstract: The Hedgehog (Hh) signalling pathway plays a pivotal role in the spatial and temporal regulation of cell proliferation and differentiation. By controlling the correct maturation of developing tissues and ensuring attainment of the correct size, position and the presence of fully functioning cellular structures, the Hh plays a pivotal role in development. Conversely aberrant Hh signalling is involved in Grolin syndrome, basal cell carcinoma (the most common cancer in the world), and more than one third of all human medulloblastoma cases. In all of these cases, it is believed that deregulated Hh signalling leads to increased cell proliferation and tumour formation. Inhibition of the Hedgehog signalling pathway, is a recently validated anti-cancer drug target, with Vismodegib (Erivedge™), approved by the U.S. Food and Drug Administration for the treatment of adult basal cell carcinoma.[1] In this perspective we outline the current state of Hh pathway inhibitors with a particular focus on potential limitations of upstream Hh pathway inhibition in relation to resistance mutations and crosstalk pathways. Together, these limitations indicate that inhibition of downstream components, specifically the Gli family of transcription factors, may represent a next generation approach to suppress tumours associated with aberrant Hh pathway signalling.

Introduction

The Hedgehog Signalling pathway

The Hedgehog (Hh) gene was first identified in *Drosophila melanogaster* and has subsequently been identified in numerous vertebrates, including humans.[2, 3] The Hh pathway has been shown to play a crucial role in embryogenesis by controlling cell proliferation, differentiation and tissue patterning. These important functions include correct left-right asymmetry; development of the nervous system, skeleton, skin, muscles, eyes, lungs, teeth, limbs and differentiation of sperm and cartilage.[4] In adults, the Hh pathway is significantly down-regulated and limited to the maintenance of stem cells in the hemopoietic system, neural system, mammary glands as well as tissue repair, regeneration in hair follicles and skin cells.[5]

Given the significant role the Hh pathway plays in cell proliferation, differentiation and tissue patterning, it is unsurprising that abnormalities or mutations within the Hh pathway lead to severe consequences. During embryogenesis, inadequate activation of the pathway may result in cyclopia, defects in ventral neural tube, somite, foregut patterning, severe lamb malfunction, absence of ribs, failure of lung branching and holoprosencephaly,[6-9] bone defects[10] and male infertility.[11] In contrast, aberrant up-regulation of the pathway has been shown to be a critical factor in initiating and maintaining tumour growth and survival. In both adults and children, up-regulation of the Hh pathway has been linked to Gorlin syndrome (nevoid basal cell carcinoma syndrome)[12] basal cell carcinoma,[13] medulloblastoma[14-16] and also with a wide range of other cancers including cancers of the pancreas,[17] prostate,[18] lungs,[19, 20] colon,[21] stomach,[22] breast,[23, 24] ovarian and especially, stem cell cancer.[25, 26] Consequently, Hh pathway inhibition has become an attractive chemotherapeutic target.

Hh pathway Mechanisms

The activity of the Hh pathway is characterized by its dependence on Hh ligands which are produced in secreting cells. These ligands activate or inhibit downstream signalling in receiving cells (Figure 1). In secreting cells, premature Hh proteins undergo a number of chemical transformations. This maturation process includes an autocatalytic cleavage from the precursor, an attachment of a cholesterol or endogenous steroids moieties to the C-terminal,[27] and an amide coupling of the palmytoyl-CoA to the N-terminal of the Hh protein which generate the fully active Hh ligand (Figure 1).[28] Mature Hh ligands are secreted with the aid of Disp, a trans-membrane protein on the secreting cell. Evidence suggests that the released Hh ligands reach the receiving cells via numerous mechanisms including active transport[29] and passive diffusion.[30] In the absence of Hh ligands, the ‘off state’ for receiving cells, Ptch catalytically inhibits Smo and prevents entry to the cilium where it is believed to inhibit various protein kinases, including PKA, GSK-3β and CK1 (Figure 1).[31] As a result, the Gli family of transcription factors (Gli1, 2, and 3), which are the effectors of the system, in complex with SuFu (a negative inhibitor of the vertebrate Hh pathway) are phosphorylated stepwise by a number of protein kinases (PKA, GSK-3β and CK1).[32] These phosphorylation events results in proteosomal cleavage where Gli-2 is degraded to Gli2-R,[33] Gli3 is degraded to Gli3-R (limited proteolysis) while Gli1 remains full length.[34] Simultaneously, the

inhibitory protein SuFu sequesters the remaining unprocessed cytoplasmic Gli such that only inactive Gli travels to the nucleus and inhibits the transcription of Hh target genes.[35, 36] Typical Hh target genes include the components of the pathway itself (PTCH, GLI1) and cell proliferation and differentiation (Cyclin D, E, Wnt, N-Myc),[37-39] angiogenesis (VEGF),[40] survival (BCL2),[41] epithelial-mesenchymal transition (SNAIL, ELK1, MSX2),[42, 43] invasiveness (Osteopontin),[44] and self-renewal (BMI1, NANOG) factors.[45, 46]

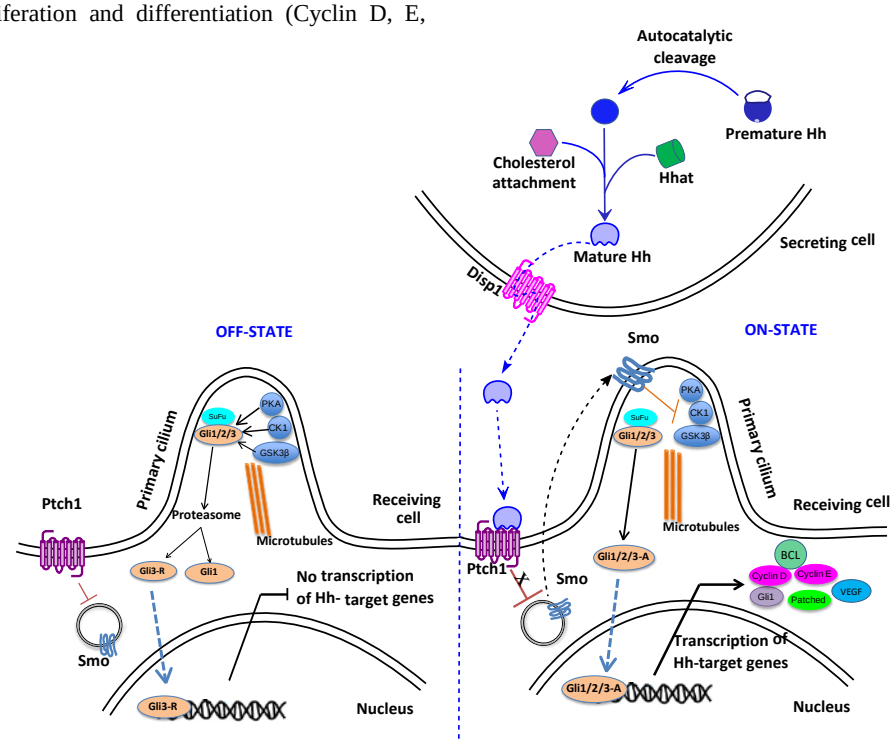

Figure 1. *The Hedgehog Signalling Pathway Mechanism.* A) In secreting cells, Hh pro-proteins undergo a number of post translational modifications. This maturation process includes an autocatalytic cleavage from the precursor, an attachment of a cholesterol or endogenous steroid moiety to the C-terminal[27] and an amide coupling of the palmytoyl-CoA to the N-terminal of the Hh protein which generate the fully active Hh ligand at the plasma membrane.[30] Mature Hh ligands are secreted with the help of Disp1, a trans-plasma membrane protein located on the secreting cell. B) In the absence of Hh-ligands (OFF-STATE), smoothend (Smo) is inhibited by Patched 1 (Ptch1) and does not enter the cilium. Consequently, the complex SuFu-Gli1,2,3 is phosphorylated by various kinases (PKA, GSK-3β, CK1) and results in truncated inactive forms (Gli2,3-R). Gli3-R travels to the nucleus and inhibits the transcription of Hh target genes. In the presence of Hh-ligands binding to and inhibiting Ptch1 (ON-STATE), Smo is released to the primary cilium, where it inhibits PKA, GSK-3β and CK1. Consequently, no phosphorylation over Gli-SuFu complex occurs and Gli1,2,3 remain in full-length active forms (Gli1,2,3-A). These active forms of Gli travel to the nucleus and induce the expression of Hh target genes.

In the presence of Hh ligands, the 'on-state', the ligands bind to Ptch resulting in Smo activation. Once activated, Smo travels to the cilium where it inhibits the phosphorylation of the Gli-SuFu complex leading to the generation of active forms of Gli (Gli1-A, Gli2-A, Gli3-A). The activated forms of Gli travel to the nucleus and induce the transcription of Hh target genes.

Hh Signalling Pathway in Cancer

Abnormally constitutive Hh pathway activation in cancer can be categorised as either: Hh ligand-independent, or Hh ligand-dependent.

Hh ligand independent (Type I) cancer is characterized by a number of mutations of different components of the Hh pathway, which results in aberrant signalling (Figure 2).

Ptch mutations

Ptch loss of function mutations have been described at high frequency in various disorders including patients with Gorlin syndrome, and those who are predisposed to basal cell carcinoma, medulloblastoma and rhabdomyosarcoma.[47-50] These mutations include deletions, insertions or nonsense mutations in Ptch proteins, which result in Ptch's inability to inhibit Smo and aberrant up-regulation of Hh signalling (Figure 2).

Smo mutations

Smo gain of function mutations up-regulate Hh signalling by continuously generating active forms of Gli, which is typically associated with sporadic basal cell carcinoma and other skin abnormalities (Figure 2).[51-53]

SuFu mutations

SuFu regulates Gli activity exogenously sequestering Gli in the cytoplasma and endogenously by repressing Gli transcription within the nucleus.[35,36,54] Multiple SuFu inactivating mutations are known to result in the nuclear accumulation of Gli proteins. This results in constitutive Hh signalling in patients, especially children, with medulloblastoma.[55-58] Alternatively, inactivated SuFu is no longer capable of retaining unprocessed full-length Gli-proteins in the cytoplasm, resulting in more frequent entry of active Gli proteins to the nucleus, and induction of aberrant constitutive the Hh pathway (Figure 2).[35]

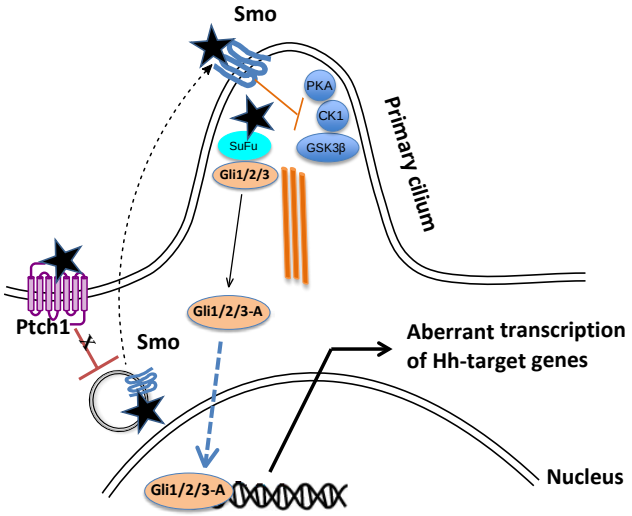

Figure 2. Type I cancer- Hh ligand independent. Loss-of-function in Ptch1 or gain-of-function in Smo mutations activate Smo prior to cilium entry and subsequent inhibition of PKA, GSK-3β and CK1 in the absence of Hh-ligands, which results in aberrant the Hh pathway activation. Loss-of-function in SuFu mutations can enable more frequent nucleus entering of active Gli to or reduce the export of active Gli out of the nucleus, all of which lead to aberrant the Hh pathway activation. Star symbol represents mutations.

Hh ligand dependent (Type II) cancer is characterized by a "typical" the Hh pathway in terms of its functioning i.e. without any mutations in the signalling components. Thus the aberrant Hh signalling results instead from the continuous receipt of Hh ligands. Depending on the Hh-ligand source, it is further subdivided into three models: *autocrine-juxtacrine*, *paracrine* and *reverse paracrine* (Figure 3). Theoretically, these models have the potential to occur independently or in combination.

Autocrine-juxtacrine model

The autocrine-juxtacrine model promotes tumour growth; typically resulting in prostate,[59] lung cancers[60] and colon cancers;[61] by having a ligand both secreted and responded to by the same/neighbouring tumour cells (Figure 3). Given that this model does not involve mutations within any of the components of the Hh pathway it is anticipated that these cancers should be effectively controlled by the use of Hh pathway inhibitors at different positions of the pathway, including Hh ligand, Smo and Gli inhibitors.

Paracrine model

The paracrine model enables the development of tumour angiogenesis, invasiveness and metastasis resulting in prostate,[62] hepatocellular carcinomas,[63] pancreatic and colorectal cancers (Figure 3).[64] Cancer cell secreted Hh ligands interact with and activate the surrounding normal stromal cells (endothelial cells, epithelial cells, fibroblasts and immune cells) activating stromal. Crucially this model does not necessarily display some standard components of the pathway, including Smo. Consequently, Smo inhibitors may not be effective against these cancers.

Reverse paracrine model

The reverse paracrine model is a variation of the paracrine type (Figure 3). It has been observed in B-cell lymphoma, multiplemyeloma and leukemia patients, where Hh ligands are excreted by the stromal microenvironment and activate the Hh pathway in cancer cells.[65, 66] This subtype highlights role of the tumour microenvironment,[67,64] and that Hh pathway inhibitor dosing should be carefully considered as they target both cancer cells and its stromal microenvironment.

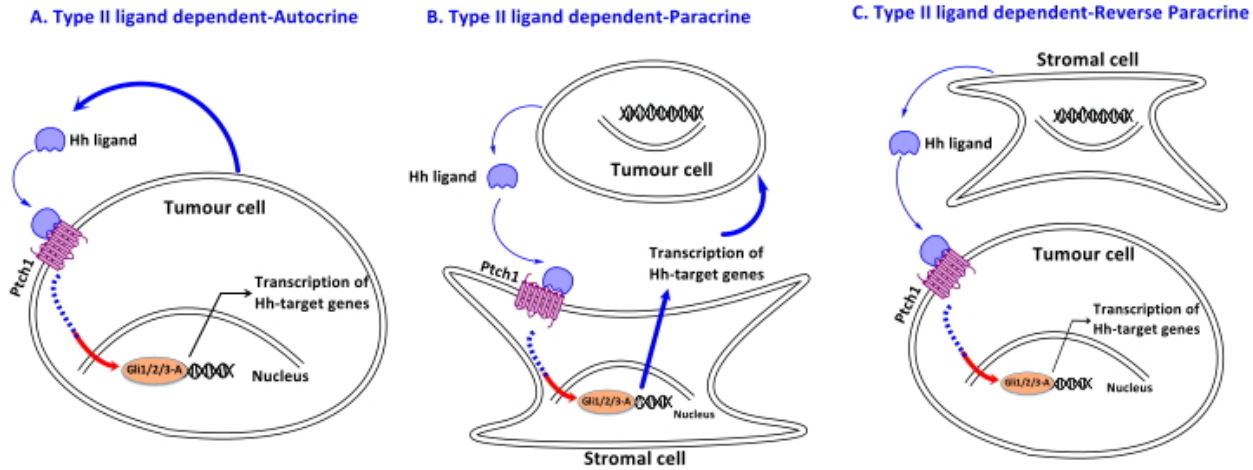

Figure 3. Type II cancers – Hh ligand dependent. **A**. *Autocrine-juxtacrine model*: Hh ligand is both produced and responded to the same cancer cell. **B**. *Paracrine model*: Tumour produces Hh ligands, which activate the Hh pathway in surrounding stromal cells. As a result, stromal cells produce necessary components back to the tumour. Evidence indicates that this results in aberrant growth, invasion and metastasis. **C**. *Reverse paracrine model*: Stromal cells secrete Hh ligands that activate the Hh pathway in tumour.

Targeting the Hh pathway in Cancers

Assessing the inhibitory activities of small molecules within the Hh pathway

Given the diverse range of cells that secrete or respond to Hedgehog proteins, and the diverse range of cancers that result from aberrant signalling, it is unsurprising that a raft of cellular models to examine the Hh pathway have been established. Whilst a complete account of currently utilised models falls outside the scope of this perspective, an overview of a number of cell types and assays techniques utilised is presented in table 1.

As outlined in table 1, a significant number of inhibitor identification programs have utilised cell viability or proliferation assays such as the MTT or the bromodeoxyuridine BrdU assays, respectively. Further, a number of luciferase reporter cell lines have been engineered such as NIH3T3 and SHH LIGHT2 cells which carry a transfected Gli-reporter construct. Moreover a homogeneous assay system that measures changes in fluorescence polarization that accompany cholesterol-dependent auto-cleavage of Hh proteins has recently been reported.[68] In contrast the luciferase assays which provide a means of means of

examining events between Smo and Gli activation, this assay provides insights to Hh auto-cleavage and esterification with cholesterol in the initial step of the pathway.

Table 1: Overview of representative cell lines and cellular assays models currently utilised to examine Hh pathway inhibition.

Cell lines	Origin	Regulator	Assay	Reference
NIH 3T3	Mouse embryonic fibroblast cells	Activated by SHh	Gli-Luciferase	69
SHH Light2	NIH/3T3 cells were co-transfected with GLI-responsive Firefly luciferase reporter and other reporters	Activated by SHh or SAG	Gli-Luciferase	69-72
C3H10/T1/2	Mouse pluripotent mesenchymal cells	Activated by SHh Hh responsive but non-dependent	Gli-Luciferase Cytotoxicity	73-75
TM3	Mouse testis Leydig cells	Activated by SHh	Luciferase assay	76
TMHh12	Not specified	Activated by SAG	Smo binding Gli-Luciferase	77
Sufu null MEFs	Mouse embryonic fibroblast without Sufu	Without Sufu, Hh target genes are highly expressed	Quantitative PCR	78
22Rv1	Prostate carcinoma	Available with elevated Gli1 level	Bromodeoxyuridine	78
PANC1	Pancreatic adenocarcinoma cells	Available with elevated Hh components, including Ptch, Sufu, Gli1,2 level	Bromodeoxyuridine Cytotoxicity	73, 78
Rh30	Rhabdomyosarcoma cell line	Available with high overexpression of Gli1 genes	Gli-Luciferase	79
HaCaT	Human keratinocyte cells	Expressing GLI1 under tetracycline control	Cytotoxicity Gli-Luciferase	73
DU145	Prostate cancer	Available with elevated Hh components, including Ptch, Sufu, Gli1,2 level	Cytotoxicity	73

Hh pathway inhibitors functioning upstream of Smo - Inhibitors of the post-translational maturation of Hh ligands

During Hh-post-translation maturation (PTM) cholesterol attaches to the newly auto-catalytically cleaved hedgehog ligand and then HHat (Hedgehog acyltransferase) mediates subsequent attachment of a palmitate molecule. Combined, this results in signalling competent hedgehog ligand.[80,81] Cholesterol attachment is required for the activation of liver X receptors (LXR).[82]

LXR activation in M2-10B4 marrow stromal cells by cholesterol analogues **1** and **2** inhibits Hh pathway activation by Shh ligands (Figure 4).[83] The depletion of cholesterol under the activation of LXR may occur on a Hh protein, and indirectly affect hedgehog ligands' maturation leading to mature hedgehog ligand deficiency. This is consistent with the observation that the same activator was not able inhibit the Hh pathway when activated by Smo agonist purmorphamine.[83] This interactivity between LXR and the Hh pathway may partially explain the anti-cancer and anti-proliferation properties of LXR activation,[84, 85] in addition to their traditional anti-atherogenic effects.[82]

The maturation of hedgehog ligands also occurs at the Hhat mediated pamitoylation step. The thieopiperidyl RU-SKI 43 (**3**) Hhat inhibitor blocks SHh palmitoylation *in vitro* and Gli activation in NIH 3T3 cells (IC_{50} = 10 μM).[69] Further investigations in SHH LIGHT2, Su^{-}/Fu^{-}, and C3H10T1/2 cell lines demonstrated that Hhat inhibitors reduced the production of mature SHh ligands.[69] The inhibitory effect of (**3**) on the Hh pathway cannot be rescued in SHH-transfected cells with SAG or SHh, suggesting the possibility of off-targets effects.[69]

Hh ligand inhibitors

Robotnikinin (**4**) inhibits the Hh pathway by binding with the hedgehog ligand which induces a conformational change thus preventing binding to Ptch1 (Figure 4). In SHH LIGHT2 cells, human primary keratinocytes, and a synthetic model of human skin, **4** displayed substantial repression of SHh-induced Gli1 and Gli2 transcripts.[72] Currently the only other reported competitive inhibitor of the Hh ligand is a monoclonal antibody, 5E1, which blocks binding of SHh ligands to Ptch1 through binding at the pseudo-active site groove of SHh.[86] The 5E1 monoclonal antibody has been largely used to elucidate the hedgehog biology.

Smo inhibitors

There has been a rapid increase in the number of Smo inhibitors identified, through targeted synthesis and high throughput screening (HTS) efforts, making them the current largest class of the Hh pathway inhibitors.

Natural product Inhibitors and Derivatives

Cyclopamine (**5**), a natural steroidal alkaloid extracted from the corn lily *Veratrum californicum*,[87] has been considered the classical inhibitor of the Hh pathway, acting by directly binding to Smo (Figure 4).[88] However, despite promising Hh antagonistic and anticancer effects in various xenograft models,[61,89,80] and in basal cell carcinoma patients,[90] the unfavourable pharmaceutical properties (poor water solubility, low pH instability), have limited the development of this compound class as clinical agents.[91] KAAD-cyclopamine (**6**), IPI609 (**7**) and IPI-926 (**8**), are representative of more drug like cyclopamine analogues (Figure 5).[92, 93] IPI-609 (**7**) inhibited SHH-induced differentiation of C3H10/T1/2 cells to osteoblasts with EC_{50} of 200 nM.[93,94] Sulfonamide substituted IPI-926 (**8**) showed superior potency to IPI-609 with improved pharmacokinetics and metabolic stability over cyclopamine. Further IPI-926 induced tumour regression in mice ligand-independent medulloblastoma,[95] as well as inhibited lung and pancreatic xenografts' growth.[96] Additionally a number of vitamin D3 analogues, such as cholecalciferol (**9**) and calcitriol (**10**), have been identified as Hh pathway antagonists.[97] In models of clear cell renal carcinoma **9**, at a concentration of 50 nM, decreased cell density in a time- and concentration- dependent manner up to 90% within 24h.[97]

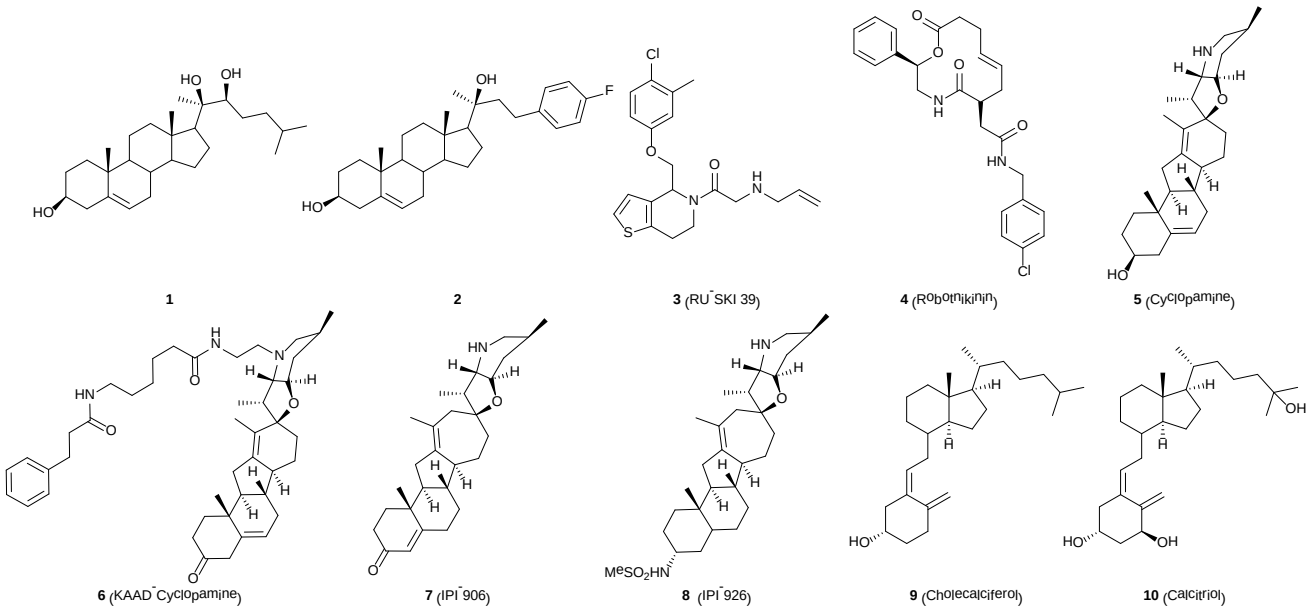

Figure 4. Hedgehog pathway inhibitors targeting upstream of Smo, the LXR's agonists (**1**) and (**2**), Hhat inhibitor (**3**), and Hh-ligand inhibitor (**4**) and the Cyclopamine scaffold of Smo inhibitors cyclopamine (**5**), KAAD-cyclopamine (**6**), IPI-906 (**7**) and IPI-926 (**8**) along with the vitamin D3 analogues cholecalciferol (**9**) and calcitriol (**10**).

Benzimidazoles, arylpyridines, pyrrolopyridine and quinazolines

From murine cell (C3H10T1/2) based HTS a series of benzimidazoles, including compound **9**, were identified as nanomolar potent the Hh pathway.[98] Lead optimization of **9** afforded GDC-0449 (Vismodegib) (**12**).[99] Vismodegib (**12**) is the first FDA approved Hh pathway inhibitor, for the treatment of adult BCC.[1] In terms of mechanism of action, Vismodegib binds to the extracellular domain of SMO and significantly inhibits downstream hedgehog signalling.[100] NVP-LDE225 (Erismodegib) (**13**, Figure 4) has also emerged as a promising Smo inhibitor displaying potent in cell activity (IC_{50} = 8 nM in TM3 cells) as well as favourable pharmacokinetics properties in animal models.[76] Further, a series of pyrrolo[3,2-*c*]pyridine were recently reported with one of these analogues TAK-441 (**14**), (Gli-luc reporter IC_{50} = 4.6 nM) is currently undergoing investigation in clinical trials.[101] In a related study a number of quinazolines were discovered including XL-139 (**15**) and the majority of compounds in this series displayed single digit nanomolar activity.[102]

Phthaladines

The phthaladines **16,17**, and **18** have been reported to be 0.1-10 μM inhibitors, via Smo targeting of the Hh pathway activated by SAG inTMHh12 cells (Figure 5).[77] LY2940680 (**18**) not only inhibits Smo in the human medulloblastoma cell line (Daoy) and murine C3H10T1/2 cell line, but also counteracts the effects in D473H, a Smo mutant.[103] Preclinical data on $Ptch^{+/-}$ $p53^{-/-}$ transgenic mice, which spontaneously develop medulloblastoma, revealed rapid anti-tumour activity and improved survival rate.[103] Second generation of phthaladine analogues such as NVP-LEQ506 (**19**) display improved potency in a Gli-luc assay, low hERG channel binding and enhanced solubility have been reported. *In vitro* analysis revealed that **19** inhibited Hh signalling in a human cell line (HEPM) as measured by Gli mRNA with an IC_{50} ~6-fold more potent than NVP-LDE225 (**13**). Moreover **19** was evaluated in C3H10T1/2 luciferase reporter cells transfected with a Smo D473H expression vector which conferred resistance to Vismodegib (**12**) in a medulloblastoma patient after an initial response. Analogue **19** retained good potency with an IC_{50} <100 nM.[104]

Piperidines and piperazines

From HTS piperidines **20**-**22** were identified as potent Smo inhibitors (Figure 8).[70] SAR development led to piperazines such as **23**-**24**, which displayed high efficacy against the Hh pathway in the SHh LIGHT2 cell line displaying IC_{50} values of 5 nM, and 25 nM, respectively. Unfortunately oxidative metabolism of the oxadizole ring resulted in high clearance rates.[70] SEN450 (**25**) is representative of an additional series of piperidine based Smo inhibitors. This compound is efficacious (IC_{50} = 23 nM), and effects reduction in tumour volume in the Hh pathway expressing glioblastoma multiforme xenograft models.[105] An additional piperazine displaying Hh pathway inhibitory activity is the triazole based antifungal agent Itraconazole (**26**) which displayed an IC_{50} of 55 nM against medulloblastoma cells.[106]

N-acylthioureas, N-acylureas and N-acylguanidines

From the lead thiourea MRT-10 (**27**) the *N*-acylurea and *N*-acylguanidine series of Smo inhibitors were developed. Amongst these were compounds **28** (IC_{50} = 60 nM)**, 29** (IC_{50} = 25 nM), and MRT-83 **(30)** (IC_{50} = 11 nM), with inhibitory activity measured against the Hh pathway SHH LIGHT2 and C3H10T1/2 cell lines (Figure 5).[107] An additional urea based analogue displaying activity in the Hh pathway is PF-04449913 (**31**). This analogue displays an IC_{50} of 5 nM (Gli-luciferase reporter C3H10T1/2) and is currently under investigation in clinical trials.[108]

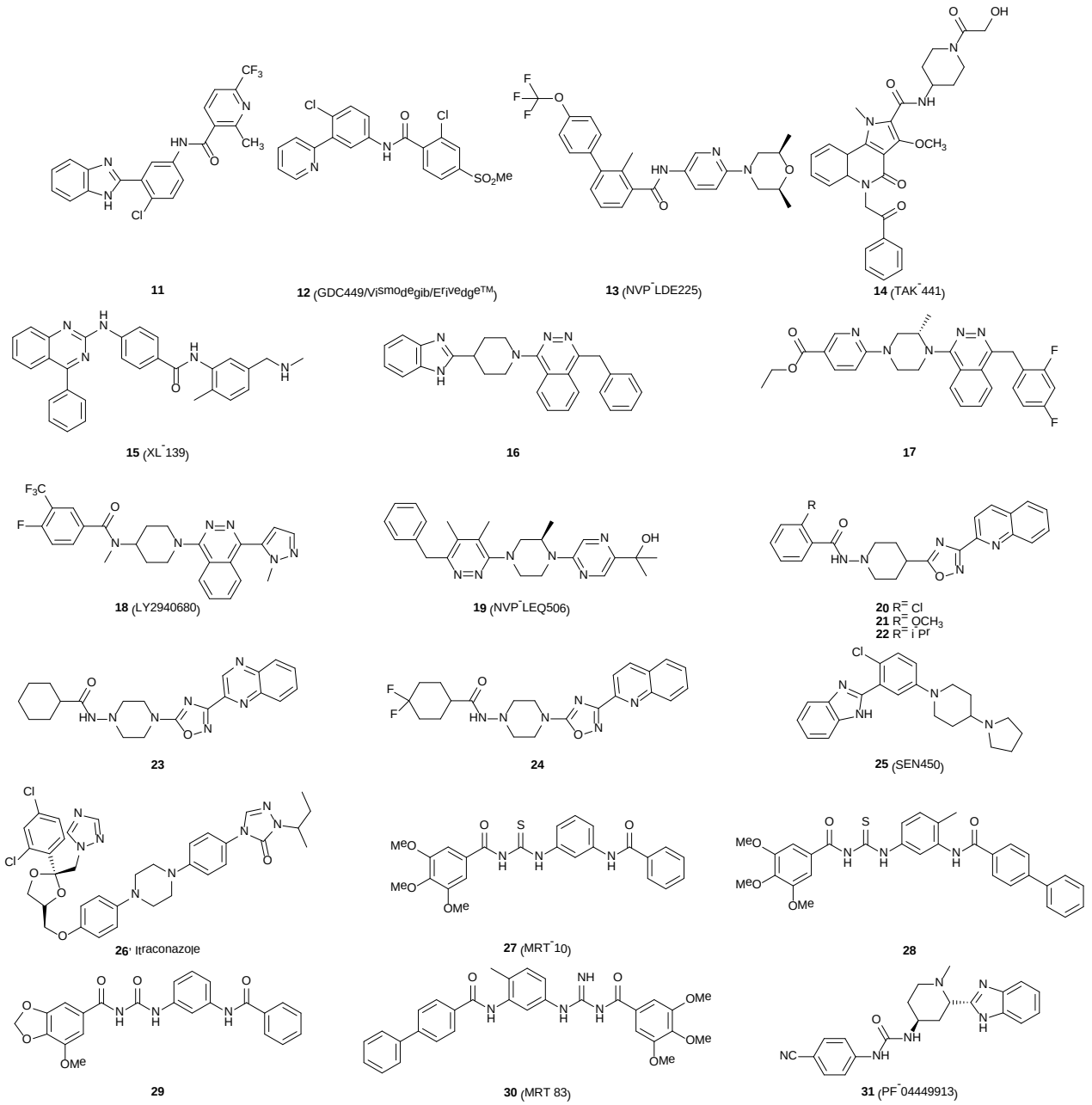

Figure 5. First generation Smo inhibitors, the benzimidazole (**11**), and pyridine (**12**) and (**13**), the phthaladines (**16**-**18**), and the second generation Smo inhibitor (**19**). Piperidines (**20**-**22**, **25**) and piperazines (23-24). The *N*-acylthioureas (**27**) and (**28**), *N*-acylurea (**29**), and *N*-acylguanidine (**30**) along with urea based analogue PF-04449913 (**31**).

Potential Limitations of Upstream Hh pathway Inhibitors

Thus, whilst upstream inhibition of the Hh pathway has afforded some promising agents, evidence is emerging that downstream components of the pathway, and other interacting pathways, can compensate for inhibitory activity elicited upstream inhibitors. For example the effectors of Hh signalling, particularly the Gli family of transcription factors, are regulated by other signalling pathways and thus the activity of Smo and Ptch inhibitors can be overridden.

Interactions of the Hh Pathway with other Signalling Pathways - The Big Crosstalk Picture

Crosstalk with the TGF-β pathway

Similar to Hh pathways, the TGF-β signalling pathway plays a crucial role in the embryonic development and both share overlapping functions including cell differentiation, cell growth control, tissue repair and regeneration.[109,110] Consequently, dysregulation of TGF-β signalling pathway also leads to the development of various cancers.[109, 110] It has been recently identified that both pathways share the same powerful effector, Gli2.[111, 112]

Gli proteins were previously regarded as effectors of the Hh pathway only, but there is increasing evidence that the TGF-β pathway controls Gli expression and activation through induction of Gli2 expression independently of the Hh pathway in human keratinocytes,[113] and by repressing PKA activity and thus indirectly elevated the number of full-length active Gli proteins in human melanoma cells (Figure 6).[114] This provides potential insights to resistance development mechanisms associated with current Hh pathway inhibitors and may be exploited to create better chemotherapeutics. This also explains how cancer cells, usually lacking of primary cilium formations, can still induce Hh pathway without mutations in the ciliary Hh components including Smo.[115]

Crosstalk with p53 and WIP1 pathways

The p53 pathway is either suppressed or subject to loss of

function mutations in multiple cancers. In the context of Hh pathway, p53 inhibits human Gli1 transcriptional activity by either reducing its nuclear localisation and protein levels or, by activating phosphorylation of Gli1 into its repressor form. Conversely, up-regulation of Gli1 represses p53 activity.[46] The oncogenic phosphatase WIP1, a p53 inhibitor, has been shown to enhance Gli1 function in human cancer cells (melanomas, breast cancer) by increasing its transcriptional activity, nuclear localisation, and protein stability. Further WIP1 has been shown to, maintain tumour growth and cancer stem cell renewal in the Hh pathway cancers.[116] WIP1 can dephosphorylate and inhibit a wide range of important targets p53, p38MAPK, ATM/ATR, Chk1/2, all of which contribute to the complete or partial inactivation of the p53 pathway (Figure 6). As a result of reduced p53 activity and the mutual inhibitory relationship between p53 and Gli1, it is likely that WIP1 modulates Gli1 activity through p53 pathway inhibition.

Crosstalk with RAS/RAF/MEK/ERK and PI3K/AKT/mTOR pathways

Given the ability to promote cancer cells survival, proliferation, invasion and inhibition of apoptosis, the abnormal activation of the RAS/RAF/MEK/ERK and PI3K/AKT/mTOR signalling pathways play an important role in the development cancers.[117, 118] The PI3K/AKT and RAS/MEK pathways share the same initiating components PGF, RTKs and RAS. These enhance Gli1 transcriptional activity, increase nuclear localisation, whilst at the same time antagonising the inhibitory effects of SuFu, PKA and GSK3β over Gli (Figure 6).[89, 119-121]

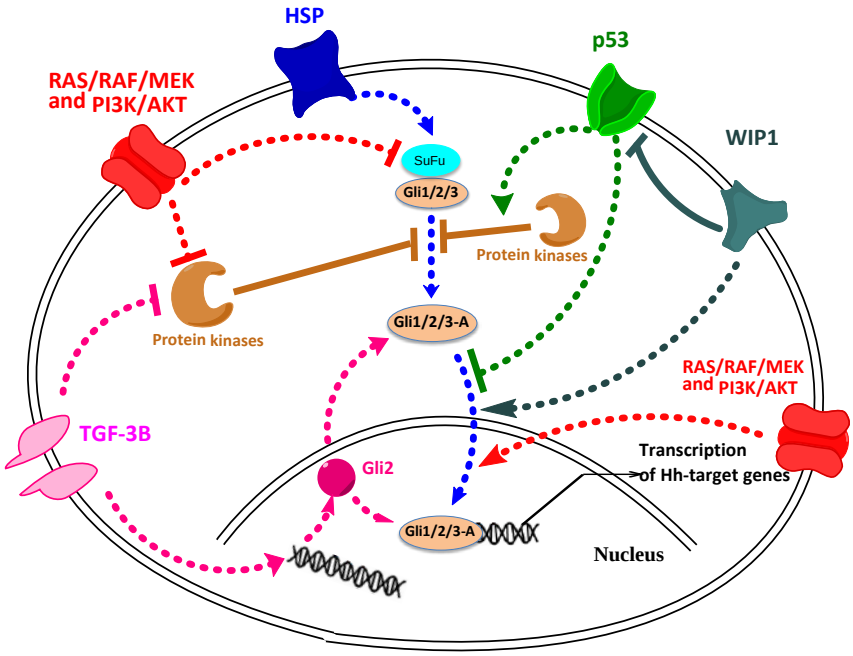

Figure 6. *Crosstalk between the Hh pathway and other oncogenic signalling pathways.* TGF-3β induces Gli2 expression in the nucleus independently of the HSP, and inhibits PKA, which results in the presence of more full- length Gli. RAS/RAF/MEK/ERK and PI3K/AKT/Mtor pathways share the same initiating components (PGF, RTKs and RAS). These pathways inhibit SuFu and the phosphorylation of PKA and GSK-3β over Gli. Evidence indicates that they enhance Gli1 transcriptional activity and increase its nuclear localisation. P53 pathway activates the phosphorylation of Gli1 into repressor form and reducing its nuclear localisation and protein levels. WIP1 increases Gli1 transcriptional activity, nuclear localisation, and protein stability, possibly by inhibiting p53.

The Need for Next Generation Hh Pathway Inhibitors

LXR activators, Hhat inhibitors, Robotnikinin and the monoclonal antibody 5E1 act as upstream antagonists at the level of hedgehog ligand production and binding. They could be a valid option for hedgehog related cancers activated by overexpressed hedgehog ligands, but not for those whose aberrant activation of the Hh pathway is due to downstream lesions of the pathway. For instance, Robotnikinin loses inhibition over the Hh pathway in cells missing Ptch1 receptors or when Smo is activated by its agonists SAG or purmorphamine.[72] Consequently, the sensitivity of these inhibitors functioning upstream of Smo is preserved only to cancers dependent on Hh ligands.

With respect to Smo inhibitors, despite being the largest class with more than 30 on going clinical trials, they possess several limitations. Firstly, from the chemical point of view, these Smo inhibitors can be categorized into a handful of structurally similar classes, including benzimidazoles, pyridines, pyridazines, piperidines, phthaladines, and piperazines. The inhibitors in each group share core structural similarities. Thus, acquired resistance against one inhibitor may result in resistance against the entire class. Secondly, most Smo inhibitors are ineffective against the ligand-dependent cancer models, in which Smo proteins are not displayed in cancer cells, but rather in the surrounding stromal microenvironment. Here, Smo inhibitors alone would be predicted to give no direct short-term tumour regression, but a long-term benefit in survival rate may be achieved due to the depletion of the Hh pathway in stromal microenvironment. This limitation, theoretically, should be overcome when combining Smo inhibitors with standard anticancer therapies. This approach is being largely applied in clinical trials. Finally, up-regulation of the Hh pathway due to any incident downstream of Smo will obviously render the tumour resistance to all of existing Smo inhibitors and further upstream inhibitors. One such example is the amplification of Gli transcription factors, which originates from the complicated crosstalk of the Hh pathway and other oncogenic signalling pathways (Figure 8). Indeed, current clinical trials are highlighting these flaws.

Clinical Experiences with Hh Pathway Inhibitors targeting Smo

Vismodegib/GDC-0449

Phase I evaluation of Vismodegib (**12**) revealed that only patients with BCC or medulloblastoma, which are Hh-ligand independent cancers, were completely or partially sensitive to Vismodegib.[122] In the same study, patients with other types of cancers, including ovarian, colorectal and pancreatic cancer displayed at best arrested cancer progression. No mutations or alterations in the Hh pathway in these patients were identified, which suggested a paracrine the Hh pathway scenario. This evidence indicated that the feedback from the surrounding stromal microenvironment supported the tumour growth regardless of the use of Vismodegib and to a greater extent, other Smo inhibitors. Phase II trial in patients with locally advanced or metastatic BCC showed a total response rate of 30% and 63% of stable disease in metastatic patients; and 43% of total response with 40% of stable disease in locally advanced BCC patients.[123] Vismodegib is the first-in-class the Hh pathway FDA approved inhibitor, but its application is strictly limited to adult patients with BCC.

Despite these promising results, Vismodegib resistance has been reported in one patient with metastatic medulloblastoma after a preliminary positive response.[124] This resistance was enacted through a D473H mutation in Smo which prevented Vismodegib-Smo binding while, maintaining the aberrant Hh signalling.[124, 125]

IPI-926 (Saridegib)

In a Phase I study of patients with locally advanced or metastatic solid tumours, IPI-926 (**8**) exhibited low levels of side effects and showed evidence of clinical activity in BCC patients.[135] Combination approaches with gemcitabine and IPI-926 in a Phase Ib study of metastatic pancreatic cancer patients were positive with good tolerance and limited toxicity.[126] However IPI-926 was less effective than gemcitabine or a placebo, terminating the metastatic pancreatic cancer Phase II study.[127] This may have been a result of Hh up-regulation in both in the tumour and stromal microenvironment. Additional clinical trials with IPI-926 are on going in patients with chondrosarcoma and myelofibrosis are on going, but the likelihood of success is questionable as these conditions are not considered to be mutation driven.[127]

NVP-LDE225

One phase I trial was conducted in patients with advanced solid tumours to determine the maximum tolerated dose of NVP-LDE225 (**13**), with additional assessments of its pharmacokinetics, pharmacodynamics, and potential efficacy.[128] NVP-LDE-225 was shown to be well tolerated up to 800 mg (mg / kg) and displayed preliminary evidence of activity of reducing Gli-1 mRNA expression in one medulloblastoma patient. However their was also evidence of several resistance mechanisms in other animal models of medulloblastoma.[129] The resistance may come from separate mechanisms, including the amplification of Gli2, an aberrant up-regulation of the PI3K signalling pathway and Smo mutations.[129]

Thus, these current clinical trials suggest that inhibition of the Hh pathway further downstream of Smo may be more effective against Smo resistant cancers and others caused by the overexpression of Hh ligands. In addition, compelling evidence of the complicated crosstalk between the Hh pathway and other oncogenic pathways highlights the crucial role of the Gli transcription factors as the unique link of the crosstalk (Figure 4). Consequently, there has been increasing interest in the creation of the new inhibitors targeting at Gli transcription level. These inhibitors are likely to be sensitive against not only Smo resistant cancers, but also others generated by several oncogenic pathways.

Next Generation the Hh pathway Inhibitors – The Gli Inhibitors

GANT-61 and GANT-58

As previously alluded to, given the Gli family are the effectors of the Hh pathway and other oncogenic pathways, Gli inhibitors pose as attractive chemotherapeutic agents. Two of the first Gli inhibitors described were GANT-61 (**32**) and GANT-58 (**33**) (Figure 6). Both compounds dependently interfere with Gli1 and Gli2-mediated transcription and suppressed Hh signalling in SHH LIGHT2 (SAG-activated) and Sufu null MEFs cell lines, suggesting their inhibitory activity lays downstream of Smo and Sufu.[78] The selectivity towards the Hh pathway was confirmed relative to other oncogenic pathways, including TNF signalling/NFκB activation, RAS/RAF/MEK/ERK, and Glucocorticoid receptor gene transactivation. GANT-61 and GANT-58 inhibited growth of Cyclopamine resistant Hh-dependent cancer cell lines PANC1 (pancreatic adenocarcinoma) and 22Rv1 (prostate carcinoma). This efficacy was reproducible in human xenografts models in mice. GANT-61 is also cytotoxic to a panel of seven human neuroblastoma cells with growth inhibition values (GI_{50}) between 5.82 - 12.4 μM. Significantly, GANT-61 inhibited neuroblastoma growth in mouse xenografts in which Smo inhibitors had no effect.[130] GANT-61 was more efficacious than the Smo inhibitor Cyclopamine in six human colon cancer cell lines, and inhibited pancreatic cancer stem cell growth in vitro and in NOD/SCID/IL2R gamma null mice xenograft models.[131, 132]

The mechanism by which GANT-61 and GANT-58 inhibit the Hh pathway at the Gli level is unknown. Current evidence suggests that GANT-61, but not GANT-58, induces modification of Gli1 and prevents it from binding to the DNA promoter.[78] As these compounds inhibit both Gli1 and Gli2, with Gli2 being intricately involved in bone development,[133, 134] possible implications for their use in therapy may include serious bone defects, especially in children.

HPI-1, HPI-2, HPI-3, HPI-4 and NanoHHI

A screen of 122,755 compounds was conducted to find candidates which could block the SAG-induced Hh pathway activation in SHH-LIGHT2 cells.[71] Four structurally diverse leads displaying IC_{50} values of < 10 μM (HPI-1 to HPI-4) (**34 - 37**) were identified (Figure 7).[71]

HPI-1, -2, -3, and -4 were active against $Ptch^{-/-}$, $Sufu^{-/-}$ cell lines consistent with Hh pathway inhibition downstream of Smo and Sufu. They were inactive against PKA and other Hh pathway associated oncogenic signalling pathways, including the PI3K/AKT/MTOR, RAS/RAF/MEK/ERK and the Wnt pathways.[71] In NIH3T3 cells, which overexpress Gli proteins, HPI-1 (**34**) and HPI-2 (**35**) inhibited Gli1 and Gli2 functions, but no significant inhibition was noted with HPI-3 (**36**) and HPI-4 (**37**).[71, 78] Each of the Hh pathway inhibitors analogues operate via a unique mechanism of action distinct from other known Gli-mediated transcription inhibitors, including GANT-61 (**32**), GANT-58 (**33**), zerumbone (**44**), arcyriaflavin C (**48**), and physalin F (**50**) (Figure 8). HPI-1 (**34**) was thought to inhibit both endogenous and exogenous Gli1/Gli2 activity independently of the primary cilium. HPI-2 (**35**) and HPI-3 (**36**) appeared to counteract the activation of Gli into active forms in the primary cilium. HPI-4 (**37**) was believed to disrupt the ciliogenesis, leading to the malfunction of Gli's ciliary processes.[71] These mechanisms are currently speculative.

Encapsulation of HPI-1 (**34**) (NanoHHI) in polymeric nanoparticles enhanced aqueous solubility and bioavailability.[135]

NanoHHI actively inhibited the Smo mutant allograft of mouse medulloblastoma, and dramatically down-regulated mGli as well as Hh target genes. NanoHHI in combination with gemcitabine significantly hampered the growth of orthotropic Pa03C pancreatic cancer when compared with gemcitabine alone.[135] This pancreatic cancer, possibly having the ligand-dependent and paracrine type of Hh pathway activation, could express resistance to the Smo inhibitor IPI-926 (**8**) as discussed above. NanoHHI resulted in no hematologic side effects or biochemical abnormalities during administration.[135]

Ketoprofen derivatives

Rationally designed from lead Gli inhibitor **38**,[136] to specifically inhibit Gli1, analogues **39** and **40** selectively inhibited Gli1-mediated transcription over that of Gli2, with IC_{50} values of 11.4 μM and 6.9 μM, respectively in C3H10T1/2 cells.[137] The phenol moiety was believed to promote metabolism in liver microsomes. Accordingly a phenol-to-indole bioisosteric replacement strategy was implemented yielding **41** and **42** with enhanced drug characteristics, including improved liver microsome stability, greater Gli1 selectivity and good membrane permeability. Both **41** and **42** inhibit exogenous and endogenous Gli1-mediated transcription in C3H10T1/2 and Rh30 cell lines, respectively.[79]

Replacement of the ketone carbonyl moiety with a ether, amide, sulphonamide, or sulfone generated several candidates with equipotent activity, including **43**, which lacked the phototoxicity linked to the ketoprofen moiety (Figure 8).[138] Thus far, **43** is the most promising candidate in this class of Hh pathway inhibitors displaying enhanced stability and low toxicity. The mechanism of action is currently unknown, but **43** does not inhibit the promotors of Gli1-mediated transcription, Dyrk1a or HDAC-1 inhibitors.[139, 140]

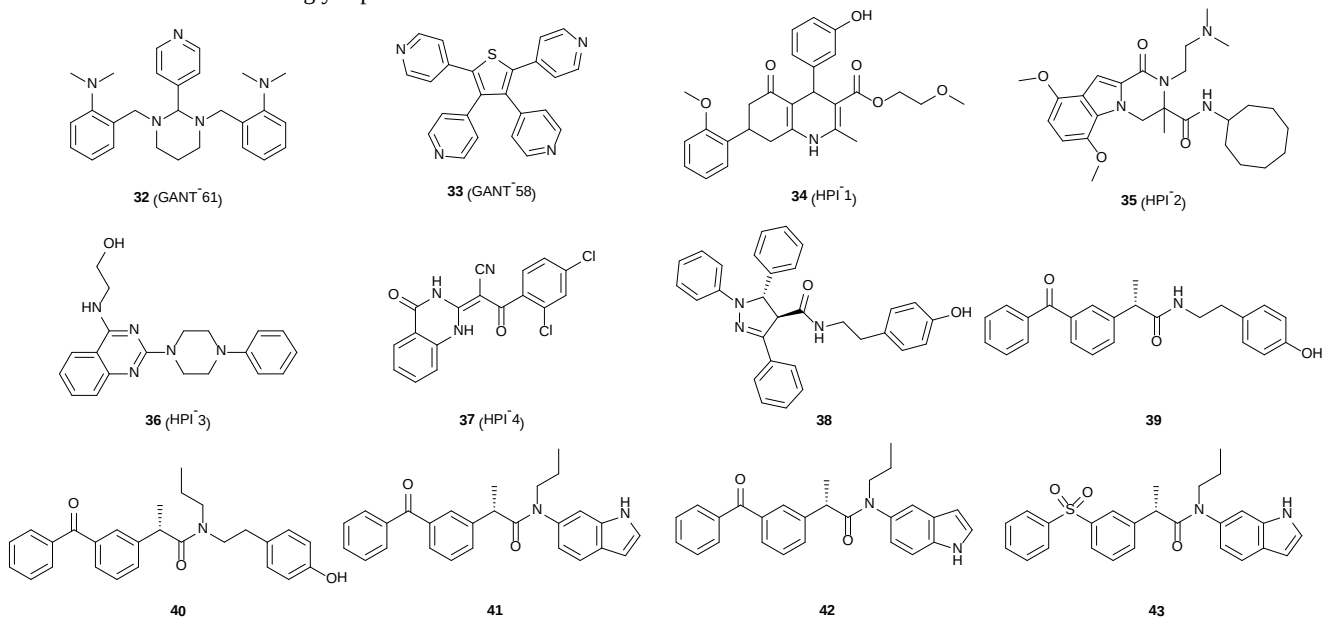

Figure 7. Chemical structures of Gli-mediated transcription inhibitors GANT-61 (**32**) and GANT-58 (**33**); the Gli-mediated transcription inhibitors HPI-1, HPI-2, HPI-3, and HPI-4 (**34**-**37**), the lead Gli inhibitor (**38**) and the Ketoprofen analogues (**39**-**43**).

Natural products displaying inhibition of Gli-mediated transcription

A wide range of natural product based Gli-mediated transcription inhibitors have been reported.[73, 141, 142] Among the first natural products Gli1 and Gli2 inhibitors identified were zerumbone (**44**), zerumbone epoxide (**45**), staurosporinone (**46**), 6-hydroxystaurosporinone (**47**), arcyriaflavin C (**48**), 6-dihydroxyarcyriaflavin A (**49**), physalin F (**50**) and B (**51**) (Figure 8).[143] These compounds inhibited the Hh pathway target proteins, including Ptch, Gl1 and Bcl2 (anti-apoptosis protein) in HaCaT and PANC1 cells, respectively. SAR studies highlighted the importance of α,β-unsaturated carbonyl group in the zerumbones (**44** and **45**),[144] as well as the indole NH moieties in **46**-**49**.[143]

The pentacyclic triterpenes colubrinic acid (**52**), betulinic acid (**53**) and alphitolic acid (**54**), were isolated from *Zizyphus cambodiana* and subsequently identified as Gli inhibitors. Compounds **52** and **53** inhibited the expression of Ptch, Gli1, Gli2 and Bcl in HaCaT and PANC1 cell lines, respectively. Further investigations on the cytotoxicity over other cell lines expressing the Hh pathway (DU145 and C3H10T1/2) demonstrated that C3H10T1/2, an Hh responsive but not reliant cell line, were less sensitive to the compounds' cytotoxic activity. This is indeed positive in terms of selectivity, as these compounds have limited effects on normal cell lines.[73, 144] Taepeenin D (**55**), (+)-drim-8-ene (**56**) and a glycoside quercetin (**57**) were isolated from *Acacia pennata* and displayed GI_{50} values of Gli-mediated transcriptional inhibition in HaCaT cells 1.6, 13.5 and 10.5 μM, respectively. These inhibitors dose dependently reduced Ptch and Bcl expression in HaCaT and PANC1 cells, but only **55** could reduce the exogenous Gli1 protein level in same cells.[33, 145] Gli inhibitors have also been identified from *Excoecaria agallocha (Euphorbiaceae)* and *Adenium obesum (Apocynaceae)*. *Excoecaria agallocha* afforded **58**-**60** as 0.5, 19.1 and 2.0 μM potent inhibitors, respectively, of Gli1-mediated transcription in HaCaT cells with selective toxicity for PANC1 and DU145 cells over the normal cells C3H10T1/2. Further, compound **58** was confirmed to inhibit the translocation of Gli1 into the nucleus, as well as the expression of Hh proteins Ptch and Bcl in PANC1 cells.[33, 145] Examination of *Adenium obesum*, identified up to 17 cardiac glycosides as potent Gli1-mediated transcriptional inhibitors with IC_{50} values from 0.11-2.4 μM. Screening against PANC1, DU145 and HaCaT cell lines indicated that these analogues were selectively cytotoxic against the PANC1 and DU145 cancer cells, while sparing the normal

cell (HaCaT). Among these, compounds **61**-**65** clearly decreased Ptch and Bcl2 proteins at 0.25 μM in PANC1 cells; and compounds **62**-**65** also decreased Ptch mRNA at 0.25μM (Figure 8).[141]

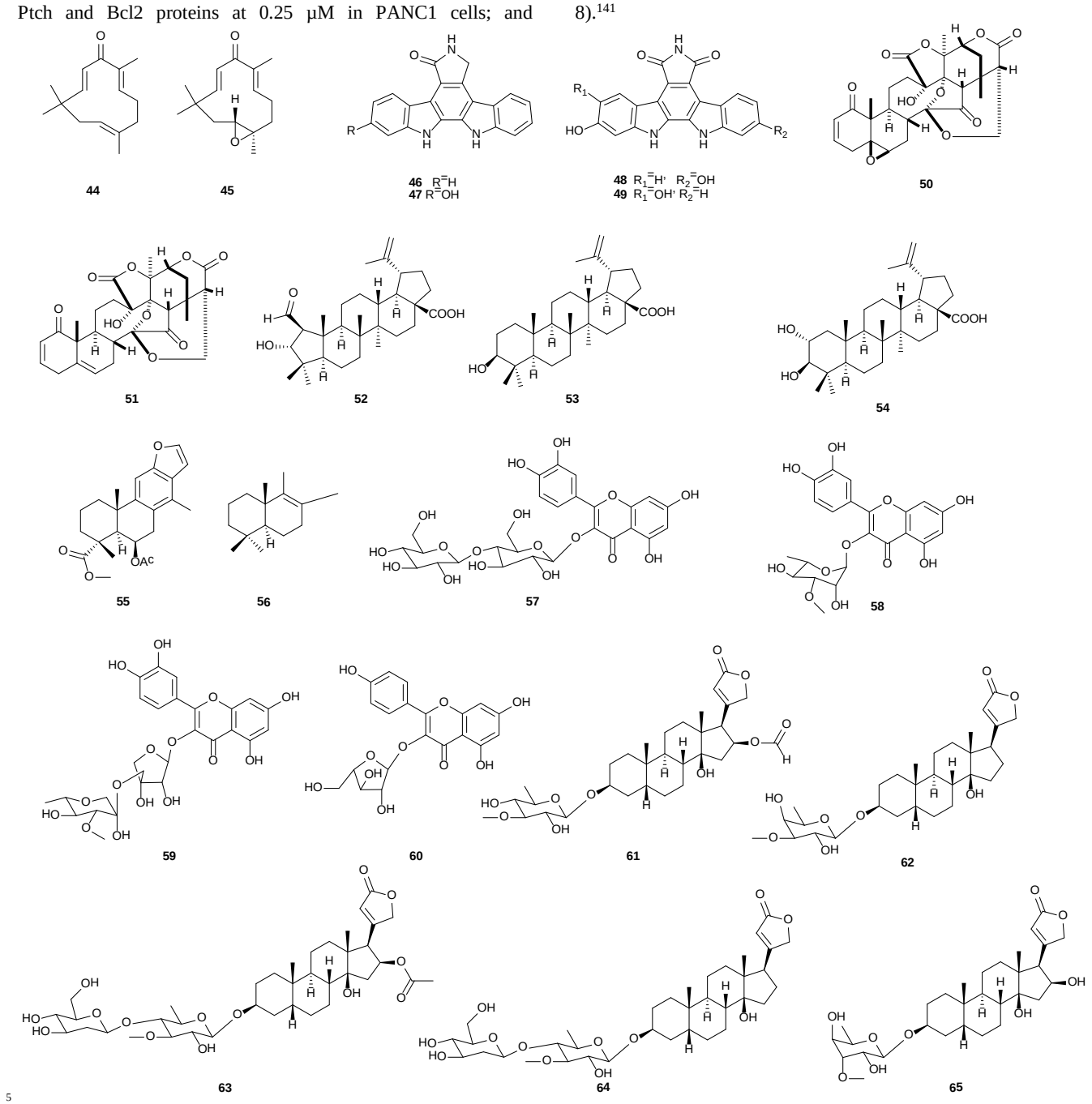

Figure 8. Structures of natural Gli-mediated transcription inhibitors (**44**-**51**) and of natural Gli-mediated transcription inhibitors (**52**-**65**).

Obstacles to the Development of Chemotherapeutics from Hh pathway Inhibitors

Acquired resistance to Hh inhibitors

One of the most important attributes of cancer cells is their unique ability to quickly generate resistance to any therapeutic agents or stressed conditions. A comparison between the genomes of a malignant melanoma and a normal cell line from the same person revealed over 33,000 nucleotide substitutions, 66 micro insertion/deletions and 37 rearrangements.[146] Not all of these mutations were anticipated to develop cancers due to the self-repairing functions. Thus, despite the highly specific molecular level targeting by Hh pathway inhibitors, they are vulnerable to resistance. Resistance against the Smo inhibitor GDC-0449 (Erivedge™), through a D473H Smo mutation, was the first such case reported.[124, 125] Point mutations in Smo and other crosstalk mechanisms resulted in Smo resistance against NVP-LDE225 via an alternative mechanism to that observed with GDC-0449.[129]

Gli transcription level inhibitors are expected to resolve a number of issues associated with the resistance of Smo inhibitors, as they are the last effector of the Hh pathway. Based on new insights into the complicated crosstalk of the Hh pathway with other oncogenic pathways, it may be appropriate to target indirect inhibition of Gli by removing/enhancing the supporting/inhibiting activity from corresponding pathways. Consistent with this is the evidence that PI3/ALK inhibitors in murine xenografts result in about a 50% reduction in the Smo and Gli protein levels. This may occur by the rescue of GSK3β-phosphorylation, as GSK3β-phosphorylation promotes proteosomal degradation of Smo and Gli proteins.[120]

Serious Side Effects

The crucial role of the Hh pathway, particularly in early

development suggests that non-physiological inhibition may give rise to significant side effects. The adult Hh pathway is less pronounced and the toxicity may be mild in those self-renewing tissues such as bone marrow, gut and skin. Common side effects of Vismodegib (Erivedge™) in adult patients include digestive disorders (diarrhoea, constipation, and decreased appetite), tiredness, hair loss, and muscle spasms. However, in children, the consequences can be very severe in the skeletal system. Indeed, experiments in young mice treated with Hh pathway inhibitors resulted in serious bone defects, including premature differentiation of chondrocytes, thinning of cortical bone, and fusion of the growth plate. Unfortunately, these bone defects could not be compensated by administering parathyroid hormone-related protein (PTHrP), whose function is to maintain chondrocytes in a proliferative state.[147]

An acceptable solution to these limitations remains elusive. However, one potential approach may be selective targeting of Gli1, instead of Gli2 and Gli3. Gli2 has been reported to induce PTHrP promoter activity, as well as PTHrP protein production, while Gli1 showed no regulation in PTHrP promoter activity.[134] Gli1 mutants are viable and normal while Gli2 and Gli3 mutants showed from severe bone, nervous system defects to lethal consequences in mice.[148] Importantly Gli2, not Gli1, interacts with and up-regulates the expression and function of Runx2, which involves in osteoblast differentiation in mesenchymal cell line (C3H10T1/2).[149] This suggests that a selective inhibition in Gli1 may minimize the defects in the skeletal system in children treated with the HPIs. Moreover HPI-1 nanoparticle incorporated NanoHHI showed no evidence of hematologic or biochemical abnormalities.[135] It is possible that the combination of nano-encapsulation and specific targeting of Gli1 may allow Hh signalling pathway inhibitors to fulfil their considerable promise as anti-cancer agents.

Conclusion

The rapid identification and development of hedgehog inhibitors has benefitted from HTS cell-based assays. However the lead optimisation is hampered at the testing stage in living systems, as it is getting more complex, possibly enhanced by multiple interactions with the microenvironment and different endocrine regulators. Furthermore, different cancers can display different types of the Hh pathway with varying crosstalk combinations, which may largely complicate the development of an effective therapeutic. As a result, despite the fact that thousands of patented and non-patented promising Hh pathway inhibitors have been developed, only one product has successfully reached the clinic (Erivedge™). Many others have been suspended in the clinical trials, when new obstacles emerged in the *in vivo* systems, significantly higher than initial expectations.

Identification of a unique target in the Hh pathway expressed in cancer cells but not in normal cell remains elusive.[150] Fortunately, in this respect, the Gli1 transcription factor has emerged as the "gold target": it is the requisite final effector of the Hh pathway but is not involved in a majority developmental processes unlike Gli2, which is a mainstay of skeletal development. Thus, selective inhibitors of the Gli1-transcription factor are expected to counteract any hedgehog-dependant cancers, irrespectively of their origin or being resistant to up-stream components' inhibitors, while displaying fewer side effects. Preliminary inhibitors of Gli1-mediated transcription have been developed with promising anti-cancer properties and their mechanisms of activity are being characterised.[71, 78, 131]

Acknowledgements

The authors thank the NHMRC (Australia) for project support and T.N.T. is the recipient of a Prime Minister's Australia Asia Postgraduate Award.

Abbreviations

BCC, Basal cell carcinoma; BCL2, B-cell CLL/lymphoma 2; BMI1, B lymphoma Mo-MLV insertion region 1 homolog; C3H10T1/2, Mesenchymal cell line; CK1, Casein kinase 1; CML, Chronic myeloid leukaemia; CSC, Cancer stem cell; Dhh, Desert Hedgehog; DU145 cell line, Prostate cancer cell line; ELK1, ETS-like gene 1; Gli1,2,3, Glioma-associated oncogene homolog 1,2,3; GSK3β, Glycogen synthase kinase 3β; Hh, Hedgehog; Hhat, Hedgehog acyltransferase; Hip, Hedgehog interacting protein; HPI, Hedgehog Signalling Pathway Inhibitor; HSP, Hedgehog Signalling Pathway; Ihh, Indian Hedgehog; LXR, Liver X receptor; MSX2, Homeobox msh-like; NANOG, Early embryo specific expression NK-type homeonbox protein; NanoHHI, HPI-1 encapsulated by nanoparticles; NFkB, Nuclear factor kappa-light-chain-enhancer of activated B cells; NIH 3T3 cell line, Mouse embryonic fibroblast cell line; N-Myc, Myelocytomatosis viral oncogene homolog; PANC1 cell line, Human pancreatic carcinoma, epithelial-like cell line; PGF, Prostaglandin F; PI3/ALK/Mtor, Phosphoinositide 3-kinase pathway; PKA, Protein kinase A; Ptch, Hedgehog ligand receptor Patched; PTM, post translational modification; RAS/RAF/MEK/ERK, Mitogen-activated protein kinases pathway; RTK, Receptor tyrosine kinases; SAG, Smothened agonist; SHh, Sonic Hedgehog; SHH LIGHT2 cell line; Smo, Smoothened protein; SNAIL, Zinc-finger transcription factors; Sufu, Suppressor of Fused; TGF-β, Transforming growth factor β; TM3 cell line, Murine testis Leydig cell line; TNF, Tumour necrosis factor; VEGF, Vascular endothelial growth factor; WIP1, Nuclear Ser/Thr phosphatase; Wnt, Wingless-related integration site.

[a] *Chemistry, Centre for Chemical Biology, The University of Newcastle, Callaghan, NSW 2308, Australia. Fax:+61 2 4921 5472; Tel: +61 2 4921 5472; E-mail: Adam.McCluskey@Newcastle.edu.au*

[2] *Biology, Priority Research Centre for Chemical Biology, The University of Newcastle, University Drive Callaghan NSW 2308, Australia.*

References

1. FDA approves new treatment for most common type of skin cancer http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm289545.htm.
2. C. Nusslein-Volhard and E. Wieschaus, *Nature*, 1980, **287**, 795-801.
3. D. Huangfu and K. V. Anderson, *Development*, 2006, **133**, 3-14.
4. P. Heretsch, L. Tzagkaroulaki and A. Giannis, *Bioorg. Med. Chem.*, 2010, **18**, 6613-6624.
5. M. Evangelista, H. Tian and F. J. de Sauvage, *Clin. Can. Res.*, 2006, **12**, 5924-5928.
6. E. Belloni, M. Muenke, E. Roessler, G. Traverso, J. Siegel-Bartelt, A. Frumkin, H. F. Mitchell, H. Donis-Keller, C. Helms, A. V. Hing, H. H. Heng, B. Koop, D. Martindale, J. M. Rommens, L. C. Tsui and S. W. Scherer., *Nat. Genet.*, 1996, **14**, 353-356.
7. C. Chiang, Y. Litingtung, E. Lee, K. E. Young, J. L. Corden, H. Westphal and P. A. Beachy, *Nature*, 1996, **383**, 407-413.
8. Y. Litingtung, L. Lei, H. Westphal and C. Chiang, *Nat. Genet.*, 1998, **20**, 58-61.
9. C. V. Pepicelli, P. M. Lewis and A. P. McMahon, *Curr. Biol.*, 1998, **8**, 1083-1086.
10. B. St-Jacques, M. Hammerschmidt and A. P. McMahon, *Genes Dev.*, 1999, **13**, 2072-2086.

11. M. J. Bitgood, L. Shen and A. P. McMahon, *Curr. Biol.*, 1996, **6**, 298-304.
12. W. Feng, I. Choi, E. Clouthier David, L. Niswander and T. Williams, *Genesis*, 2013, **51**, 677-689.
13. E. H. Epstein, *Nat. Rev. Can.*, 2008, **8**, 743-754.
14. D. G. Evans, P. A. Farndon, L. D. Burnell, H. R. Gattamaneni and J. M. Birch, *Br. J. Cancer*, 1991, **64**, 959-961.
15. T. Pietsch, A. Waha, A. Koch, J. Kraus, S. Albrecht, J. Tonn, N. Sorensen, F. Berthold, B. Henk, N. Schmandt, H. K. Wolf, A. von Deimling, B. Wainwright, G. Chenevix-Trench, O. D. Wiestler and C. Wicking, *Cancer Res.*, 1997, **57**, 2085-2088.
16. C. Raffel, R. B. Jenkins, L. Frederick, D. Hebrink, B. Alderete, D. W. Fults and C. D. James, *Cancer Res.*, 1997, **57**, 842-845.
17. S. P. Thayer, M. Pasca di Magliano, P. W. Heiser, C. M. Nielsen, D. J. Roberts, G. Y. Lauwers, Y. P. Qi, S. Gysin, C. Fernandez-del Castillo, V. Yajnik, B. Antoniu, M. McMahon, A. L. Warshaw and M. Hebrok, *Nature*, 2003, **425**, 851-856.
18. S. S. Karhadkar, G. S. Bova, N. Abdallah, S. Dhara, D. Gardner, A. Maitra, J. T. Isaacs, D. M. Berman and P. A. Beachy, *Nature*, 2004, **431**, 707-712.
19. D. N. Watkins, D. M. Berman, S. G. Burkholder, B. Wang, P. A. Beachy and S. B. Baylin, *Nature*, 2003, **422**, 313-317.
20. Z. Yuan, J. A. Goetz, S. Singh, S. K. Ogden, W. J. Petty, C. C. Black, V. A. Memoli, E. Dmitrovsky and D. J. Robbins, *Oncogene*, 2007, **26**, 1046-1055.
21. D. Qualtrough, A. Buda, W. Gaffield, A. C. Williams and C. Paraskeva, *Int. J. Cancer*, 2004, **110**, 831-837.
22. D. M. Berman, S. S. Karhadkar, A. Maitra, R. Montes De Oca, M. R. Gerstenblith, K. Briggs, A. R. Parker, Y. Shimada, J. R. Eshleman, D. N. Watkins and P. A. Beachy, *Nature*, 2003, **425**, 846-851.
23. S. Mukherjee, N. Frolova, A. Sadlonova, Z. Novak, A. Steg, G. P. Page, D. R. Welch, S. M. Lobo-Ruppert, J. M. Ruppert, M. R. Johnson and A. R. Frost, *Cancer Biol. Ther.*, 2006, **5**, 674-683.
24. M. Kasper, V. Jaks, M. Fiaschi and R. Toftgaard, *Carcinogenesis*, 2009, **30**, 903-911.
25. A. Po, E. Ferretti, E. Miele, E. De Smaele, A. Paganelli, G. Canettieri, S. Coni, L. Di Marcotullio, M. Biffoni, L. Massimi, C. Di Rocco, I. Screpanti and A. Gulino, *Embo J.*, 2010, **29**, 2646-2658.
26. M. Zbinden, A. Duquet, A. Lorente-Trigos, S.-N. Ngwabyt, I. Borges and A. Ruiz i Altaba, *Embo J.*, 2010, **29**, 2659-2674.
27. R. K. Mann and P. A. Beachy, *Biochim. Biophys. Acta*, 2000, **1529**, 188-202.
28. J. A. Buglino and M. D. Resh, in *Vitamins & Hormones*, ed. L. Gerald, Academic Press, 2012, vol. Volume 88, pp. 229-252.
29. D. Panakova, H. Sprong, E. Marois, C. Thiele and S. Eaton, *Nature*, 2005, **435**, 58-65.
30. Y. Bellaiche, I. The and N. Perrimon, *Nature*, 1998, **394**, 85-88.
31. J. Taipale, M. K. Cooper, T. Maiti and P. A. Beachy, *Nature*, 2002, **418**, 892-896.
32. R. V. Pearse, 2nd, L. S. Collier, M. P. Scott and C. J. Tabin, *Dev. Biol.*, 1999, **212**, 323-336.
33. N. Bhatia, S. Thiyagarajan, I. Elcheva, M. Saleem, A. Dlugosz, H. Mukhtar and V. S. Spiegelman, *J. Biol. Chem.*, 2006, **281**, 19320-19326.
34. H. Sasaki, Y. Nishizaki, C. Hui, M. Nakafuku and H. Kondoh, *Development*, 1999, **126**, 3915-3924.
35. P. Kogerman, T. Grimm, L. Kogerman, D. Krause, A. B. Unden, B. Sandstedt, R. Toftgard and P. G. Zaphiropoulos, *Nat. Cell Biol.*, 1999, **1**, 312-319.
36. S. Y. Cheng and S. Yue, *Adv. Cancer Res.*, 2008, **101**, 29-43.
37. A. M. Kenney and D. H. Rowitch, *Mol. Cell Biol.*, 2000, **20**, 9055-9067.
38. J. L. Mullor, N. Dahmane, T. Sun and A. Ruiz i Altaba, *Curr. Biol.*, 2001, **11**, 769-773.
39. A. M. Kenney, M. D. Cole and D. H. Rowitch, *Development*, 2003, **130**, 15-28.
40. R. Pola, L. E. Ling, M. Silver, M. J. Corbley, M. Kearney, R. B. Pepinsky, R. Shapiro, F. R. Taylor, D. P. Baker, T. Asahara and J. M. Isner, *Nat. Med.*, 2001, **7**, 706-711.
41. G. Regl, M. Kasper, H. Schnidar, T. Eichberger, G. W. Neill, M. P. Philpott, H. Esterbauer, C. Hauser-Kronberger, A.-M. Frischauf and F. Aberger, *Cancer Res.*, 2004, **64**, 7724-7731.
42. X. Li, W. Deng, C. D. Nail, S. K. Bailey, M. H. Kraus, J. M. Ruppert and S. M. Lobo-Ruppert, *Oncogene*, 2006, **25**, 609-621.
43. H. Ohta, K. Aoyagi, M. Fukaya, I. Danjoh, A. Ohta, N. Isohata, N. Saeki, H. Taniguchi, H. Sakamoto, T. Shimoda, T. Tani, T. Yoshida and H. Sasaki, *Br. J. Cancer*, 2009, **100**, 389-398.
44. S. Das, L. G. Harris, B. J. Metge, S. Liu, A. I. Riker, R. S. Samant and L. A. Shevde, *J. Biol. Chem.*, 2009, **284**, 22888-22897.
45. C. Leung, M. Lingbeek, O. Shakhova, J. Liu, E. Tanger, P. Saremaslani, M. van Lohuizen and S. Marino, *Nature*, 2004, **428**, 337-341.
46. B. Stecca and A. Ruiz i Altaba, *Embo J.*, 2009, **28**, 663-676.
47. H. Hahn, C. Wicking, P. G. Zaphiropoulous, M. R. Gailani, S. Shanley, A. Chidambaram, I. Vorechovsky, E. Holmberg, A. B. Unden, S. Gillies, K. Negus, I. Smyth, C. Pressman, D. J. Leffell, B. Gerrard, A. M. Goldstein, M. Dean, R. Toftgard, G. Chenevix-Trench, B. Wainwright and A. E. Bale, *Cell*, 1996, **85**, 841-851.
48. R. L. Johnson, A. L. Rothman, J. Xie, L. V. Goodrich, J. W. Bare, J. M. Bonifas, A. G. Quinn, R. M. Myers, D. R. Cox, E. H. Epstein Jr, M. P. Scott, *Science*, 1996, **272**, 1668-1671.
49. J. Xie, R. L. Johnson, X. Zhang, J. W. Bare, F. M. Waldman, P. H. Cogen, A. G. Menon, R. S. Warren, L.-C. Chen, M. P. Scott and E. H. Epstein, Jr, *Cancer Res.*, 1997, **57**, 2369-2372.
50. N. Soufir, B. Gerard, M. Portela, A. Brice, M. Liboutet, P. Saiag, V. Descamps, D. Kerob, P. Wolkenstein, I. Gorin, C. Lebbe, N. Dupin, B. Crickx, N. Basset-Seguin and B. Grandchamp, *Br. J. Cancer*, 2006, **95**, 548-553.
51. J. Xie, M. Murone, S.-M. Luoh, A. Ryan, Q. Gu, C. Zhang, J. M. Bonifas, C.-W. Lam, M. Hynes, A. Goddard, A. Rosenthal, E. H. Epstein, Jr. and F. J. de Sauvage, *Nature*, 1998, **391**, 90-92.
52. T. Yan, M. Angelini, B. A. Alman, I. L. Andrulis and J. S. Wunder, *Clin. Orthop. Relat. Res.*, 2008, **466**, 2184-2189.
53. F. J. De Sauvage, Mutant smoothened and methods of using the same*: US Pat.*, 20120039893, **2012**.
54. S. Y. Cheng and J. M. Bishop, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 5442-5447.
55. M. D. Taylor, L. Liu, C. Raffel, C.-C. Hui, T. G. Mainprize, X. Zhang, R. Agatep, S. Chiappa, L. Gao, A. Lowrance, A. Hao, A. M. Goldstein, T. Stavrou, S. W. Scherer, W. T. Dura, B. Wainwright, J. A. Squire, J. T. Rutka and D. Hogg, *Nat. Genet.*, 2002, **31**, 306-310.
56. L. Brugieres, G. Pierron, A. Chompret, B. Bressac-de Paillerets, F. Di Rocco, P. Varlet, A. Pierre-Kahn, O. Caron, J. Grill and O. Delattre, *J. Med. Genet.*, 2010, **47**, 142-144.
57. I. Slade, A. Murray, S. Hanks, A. Kumar, L. Walker, D. Hargrave, J. Douglas, C. Stiller, L. Izatt and N. Rahman, *Fam. Cancer*, 2011, **10**, 337-342.
58. L. Brugieres, A. Remenieras, G. Pierron, P. Varlet, S. Forget, V. Byrde, J. Bombled, S. Puget, O. Caron, C. Dufour, O. Delattre, B. Bressac-de Paillerets and J. Grill, *J. Clin. Oncol.*, 2012, **30**, 2087-2093.
59. M. Chen, M. Tanner, A. C. Levine, E. Levina, P. Ohouo and R. Buttyan, *Cell Cycle*, 2009, **8**, 149-157.
60. S. Singh, Z. Wang, F. D. Liang, K. E. Black, J. A. Goetz, R. Tokhunts, C. Giambelli, J. Rodriguez-Blanco, J. Long, E. Lee, K. J. Briegel, P. A. Bejarano , E. Dmitrovsky, A. J. Capobianco and D. J. Robbins, *Cancer Res.*, 2011, **71**, 4454-4463.
61. F. Varnat, A. Duquet, M. Malerba, M. Zbinden, C. Mas, P. Gervaz and A. Ruiz i Altaba, *EMBO Mol. Med.*, 2009, **1**, 338-351.
62. L. Fan, C. V. Pepicelli, C. C. Dibble, W. Catbagan, J. L. Zarycki, R. Laciak, J. Gipp, A. Shaw, M. L. G. Lamm, A. Munoz, R. Lipinski, J. B. Thrasher and W. Bushman, *Endocrinology*, 2004, **145**, 3961-3970.
63. I. S. Chan, C. D. Guy, Y. Chen, J. Lu, M. Swiderska-Syn, G. A. Michelotti, G. Karaca, G. Xie, L. Kruger, W. -K. Syn, B. R.

Anderson, T. A. Pereira, S. S. Choi, A. S. Baldwin and A. M. Diehl, *Cancer Res.*, 2012, **72**, 6344-6350.
64. R. L. Yauch, S. E. Gould, S. J. Scales, T. Tang, H. Tian, C. P. Ahn, D. Marshall, L. Fu, T. Januario, D. Kallop, M. Nannini-Pepe, K. Kotkow, J. C. Marsters, L. L. Rubin and F. J. de Sauvage, *Nature*, 2008, **455**, 406-410.
65. C. Dierks, J. Grbic, K. Zirlik, R. Beigi, N. P. Englund, G. -R. Guo, H. Veelken, M. Engelhardt, R. Mertelsmann, J. F. Kelleher, P. Schultz and M. Warmuth, *Nat Med*, 2007, **13**, 944-951.
66. G. V. Hegde, K. J. Peterson, K. Emanuel, A. K. Mittal, A. D. Joshi, J. D. Dickinson, G. J. Kollessery, R. G. Bociek, P. Bierman, J. M. Vose, D. D. Weisenburger and S. S. Joshi, *Mol. Cancer Res.*, 2008, **6**, 1928-1936.
67. J. Jiang and C.-C. Hui, *Dev. Cell*, 2008, **15**, 801-812.
68. S.-Q. Jiang and H. Paulus, *J. Biomol. Screen.*, 2010, **15**, 1082-1087.
69. E. Petrova, J. Rios-Esteves, O. Ouerfelli, J. F. Glickman and M. D. Resh, *Nat. Chem. Biol.*, 2013, **9**, 247-249.
70. G. Dessole, P. Jones, L. L. Bufi, E. Muraglia, J. M. Ontoria and C. Torrisi, *1,2,4-oxadiazole substituted piperidine and piperazine derivatives as Smo antagonists*. WO 2010013037 A1, **2008**.
71. J. M. Hyman, A. J. Firestone, V. M. Heine, Y. Zhao, C. A. Ocasio, K. Han, M. Sun, P. G. Rack, S. Sinha, J. J. Wu, D. E. Solow-Cordero, J. Jiang, D. H. Rowitch and J. K. Chen, *Proc. Nat. Acad. Sci. USA*, 2009, **106**, 14132-14137.
72. B. Z. Stanton, L. F. Peng, N. Maloof, K. Nakai, X. Wang, J. L. Duffner, K. M. Taveras, J. M. Hyman, S. W. Lee, A. N. Koehler, J. K. Chen, J. L. Fox, A. Mandinova and S. L. Schreiber, *Nat. Chem. Biol.*, 2009, **5**, 154-156.
73. M. A. Arai, C. Tateno, T. Hosoya, T. Koyano, T. Kowithayakorn and M. Ishibashi, *Bioorg. Med. Chem.*, 2008, **16**, 9420-9424.
74. J. L. Gunzner, D. P. Sutherlin, M. S. Stanley, L. Bao, G. Castanedo, R. L. Lalonde, S. Wang, M. E. Reynolds, S. J. Savage, K. Malesky and M. S. Dina, Pyridyl inhibitors of hedgehog signalling, WO 2009126863 A2, **2008**.
75. N. Mahindroo, C. Punchihewa and N. Fujii, *J. Med. Chem.*, 2009, **52**, 3829-3845.
76. S. Pan, X. Wu, J. Jiang, W. Gao, Y. Wan, D. Cheng, D. Han, J. Liu, N. P. Englund, Y. Wang, S. Peukert, K. Miller-Moslin, J. Yuan, R. Guo, M. Matsumoto, A. Vattay, Y. Jiang, J. Tsao, F. Sun, A. C. Pferdekamper, S. Dodd, T. Tuntland, W. Maniara, J. F. Kelleher, III, Y.-M. Yao, M. Warmuth, J. Williams and M. Dorsch, *ACS Med. Chem. Lett.*, 2010, **1**, 130-134.
77. M. Dai, F. He, R. K. Jain, R. Karki, J. Kelleher, III, J. Lei, L. Llamas, M. A. McEwan, K. Miller-Moslin, L. B. Perez, S. Peukert and N. Yusuff, *Organic compounds and their uses.* WO/2008/110611, **2008**.
78. M. Lauth, A. Bergstroem, T. Shimokawa and R. Toftgard, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 8455-8460.
79. N. Mahindroo, M. C. Connelly, C. Punchihewa, L. Yang, B. Yan and N. Fujii, *Bioorg. Med. Chem.*, 2010, **18**, 4801-4811.
80. R. K. Mann and P. A. Beachy, *Annu. Rev. Biochem.*, 2004, **73**, 891-923.
81. J. A. Buglino and M. D. Resh, *J. Biol. Chem.*, 2008, **283**, 22076-22088.
82. M. Baranowski, *J. Physiol. Pharmacol.*, 2008, **59** Suppl 7, 31-55.
83. W. -K. Kim, V. Meliton, K. W. Park, C. Hong, P. Tontonoz, P. Niewiadomski, J. A. Waschek, S. Tetradis and F. Parhami, *Mol. Endocrinol.*, 2009, **23**, 1532-1543.
84. C. -P. Chuu, J. M. Kokontis, R. A. Hiipakka and S. Liao, *J. Biomed. Sci.*, 2007, **14**, 543-553.
85. J. Belltowski, *Clin. Lipidol.*, 2011, **6**, 137-141.
86. H. R. Maun, X. Wen, A. Lingel, F. J. de Sauvage, R. A. Lazarus, S. J. Scales and S. G. Hymowitz, *J. Biol. Chem.*, 2010, **285**, 26570-26580.
87. R. F. Keeler, *Teratology*, 1970, **3**, 169-173.
88. J. K. Chen, J. Taipale, M. K. Cooper and P. A. Beachy, *Genes Dev.*, 2002, **16**, 2743-2748.
89. B. Stecca, C. Mas, V. Clement, M. Zbinden, R. Correa, V. Piguet, F. Beermann and I. A. A. Ruiz, *Proc. Natl. Acad. Sci. USA*, 2007, **104**, 5895-5900.
90. S. Tabs and O. Avci, *Eur. J. Dermatol.*, 2004, **14**, 96-102.
91. C. Mas and A. Ruiz i Altaba, *Biochem. Pharmacol.*, 2010, **80**, 712-723.
92. P. A. Beachy, J. K. Chen and A. J. Taipale, *Regulators of the Hedgehog pathway, compositions and uses related thereto*. U.S. Patent 7,098,196, **2006**.
93. M. R. Tremblay, M. Nevalainen, S. J. Nair, J. R. Porter, A. C. Castro, M. L. Behnke, L.-C. Yu, M. Hagel, K. White, K. Faia, L. Grenier, M. J. Campbell, J. Cushing, C. N. Woodward, J. Hoyt, M. A. Foley, M. A. Read, J. R. Sydor, J. K. Tong, V. J. Palombella, K. McGovern and J. Adams, *J. Med. Chem.*, 2008, **51**, 6646-6649.
94. G. van der Horst, H. Farih-Sips, C. W. G. M. Lowik and M. Karperien, *Bone*, 2003, **33**, 899-910.
95. M. R. Tremblay, A. Lescarbeau, M. J. Grogan, E. Tan, G. Lin, B. C. Austad, L. -C. Yu, M. L. Behnke, S. J. Nair, M. Hagel, K. White, J. Conley, J. D. Manna, T. M. Alvarez-Diez, J. Hoyt, C. N. Woodward, J. R. Sydor, M. Pink, J. MacDougall, M. J. Campbell, J. Cushing, J. Ferguson, M. S. Curtis, K. McGovern, M. A. Read, V. J. Palombella, J. Adams and A. C. Castro, *J. Med. Chem.*, 2009, **52**, 4400-4418.
96. V. Travaglione, presented in part at the ASMS Conference, Denver, 2008.
97. V. Dormoy, C. Beraud, V. Lindner, C. Coquard, M. Barthelmebs, D. Brasse, D. Jacqmin, H. Lang and T. Massfelder, *Carcinogenesis*, 2012, **33**, 2084-2093.
98. K. D. Robarge, S. A. Brunton, G. M. Castanedo, Y. Cui, M. S. Dina, R. Goldsmith, S. E. Gould, O. Guichert, J. L. Gunzner, J. Halladay, W. Jia, C. Khojasteh, M. F. T. Koehler, K. Kotkow, H. La, R. L. La Londe, K. Lau, L. Lee, D. Marshall, J. C. Marsters, L. J. Murray, C. Qian, L. L. Rubin, L. Salphati, M. S. Stanley, J. H. A. Stibbard, D. P. Sutherlin, S. Ubhayaker, S. Wang, S. Wong and M. Xie, *Bioorg. Med. Chem. Lett.*, 2009, **19**, 5576-5581.
99. J. Gunzner, D. Sutherlin, M. Stanley, L. Bao, G. Castanedo, R. Lalonde, S. Wang, M. Reynolds, S. Savage, K. Malesky and M. Dina, *Pyridyl inhibitors of Hedgehog signalling*, US2012/009480 A1, **2011**.
100. C. M. Rudin, *Clin. Cancer Res.*, 2012, **18**, 3218-3222.
101. T. Ohashi, Y. Oguro, T. Tanaka, Z. Shiokawa, Y. Tanaka, S. Shibata, Y. Sato, H. Yamakawa, H. Hattori, Y. Yamamoto, S. Kondo, M. Miyamoto, M. Nishihara, Y. Ishimura, H. Tojo, A. Baba and S. Sasaki, *Bioorg. Med. Chem.*, 2012, **20**, 5507-5517.
102. R. Tremblay Martin, M. Nesler, R. Weatherhead and C. Castro Alfredo, *Expert Opin. Ther. Pat.*, 2009, **19**, 1039-1056.
103. M. H. Bender, P. H. Hipskind, A. R. Capen, M. Cockman, K. M. Credille, H. Gao, J. A. Bastian, J. M. Clay, K. L. Lobb, D. J. Sall, M. L. Thompson, T. Wilson, G. N. Wishart and B. K. R Patel, *Cancer Res.*, 2011, **71** (8 suppl), 2819.
104. S. Peukert, F. He, M. Dai, R. Zhang, Y. Sun, K. Miller-Moslin, M. McEwan, B. Lagu, K. Wang, N. Yusuff, A. Bourret, A. Ramamurthy, W. Maniara, A. Amaral, A. Vattay, A. Wang, R. Guo, J. Yuan, J. Green, J. Williams, S. Buonamici, F. Kelleher Joseph, 3rd and M. Dorsch, *ChemMedChem*, 2013, **8**, 1261-1265.
105. P. Ferruzzi, F. Mennillo, A. De Rosa, C. Giordano, M. Rossi, G. Benedetti, R. Magrini, l. Pericot Mohr Gal, V. Miragliotta, L. Magnoni, E. Mori, R. Thomas, P. Tunici and A. Bakker, *Int. J. Cancer*, 2012, **131**, E33-44.
106. J. Kim, B. T. Aftab, J. Y. Tang, D. Kim, A. H. Lee, M. Rezaee, J. Kim, B. Chen, E. M. King, A. Borodovsky, G. J. Riggins, E. H. Epstein, P. A. Beachy and C. M. Rudin, *Cancer Cell*, 2013, **23**, 23-34.
107. A. Solinas, H. Faure, H. Roudaut, E. Traiffort, A. Schoenfelder, A. Mann, F. Manetti, M. Taddei and M. Ruat, *J. Med. Chem.*, 2012, **55**, 1559-1571.
108. M. J. Munchhof, Q. Li, A. Shavnya, G. V. Borzillo, T. L. Boyden, C. S. Jones, S. D. LaGreca, L. Martinez-Alsina, N. Patel, K. Pelletier, L. A. Reiter, M. D. Robbins and G. T. Tkalcevic, *ACS Med. Chem. Lett.*, 2012, **3**, 106-111.

109. R. Derynck, R. J. Akhurst and A. Balmain, *Nat. Genet.*, 2001, **29**, 117-129.
110. P. M. Siegel and J. Massague, *Nat. Rev. Can.*, 2003, **3**, 807-820.
111. D. Javelaud, M. -J. Pierrat and A. Mauviel, *FEBS Lett.*, 2012, **586**, 2016-2025.
112. C. Y. Perrot, D. Javelaud and A. Mauviel, *Pharmacol. Therap.*, 2013, **137**, 183-199.
113. S. Dennler, J. Andre, F. Verrecchia and A. Mauviel, *J. Biol. Chem.*, 2009, **284**, 31523-31531.
114. M. -J. Pierrat, V. Marsaud, A. Mauviel and D. Javelaud, *J. Biol. Chem.*, 2012, **287**, 17996-18004.
115. M. Chen, R. Carkner and R. Buttyan, *Expert Rev. Endocrinol. Metab.*, 2011, **6**, 453-467.
116. S. Pandolfi, V. Montagnani, J. Y. Penachioni, M. C. Vinci, B. Olivito, L. Borgognoni and B. Stecca, *Oncogene*, 2012.
117. M. Cully, H. You, A. J. Levine and T. W. Mak, *Nat. Rev. Can.*, 2006, **6**, 184-192.
118. M. Raman, W. Chen and M. H. Cobb, *Oncogene*, 2007, **26**, 3100-3112.
119. J. G. Monzon and J. Dancey, *OncoTargets Ther.*, 2012, **5**, 31-46.
120. B. Ramaswamy, Y. Lu, K.-y. Teng, G. Nuovo, X. Li, L. Shapiro Charles and S. Majumder, *Cancer Res.*, 2012, **72**, 5048-5059.
121. N. A. Riobo, *Proc. Nat. Acad. Sci. USA*, 2006, **103**, 4505-4510.
122. P. M. LoRusso, C. M. Rudin, J. C. Reddy, R. Tibes, G. J. Weiss, M. J. Borad, C. L. Hann, J. R. Brahmer, I. Chang, W. C. Darbonne, R. A. Graham, K. L. Zerivitz, J. A. Low and D. D. Von Hoff, *Clin. Cancer Res.*, 2011, **17**, 2502-2511.
123. A. Sekulic, M. R. Migden, A. E. Oro, L. Dirix, K. Lewis, J. D. Hainsworth, J. A. Solomon, S. Yoo, S. T. Arron, P. A. Friedlander, E. Marmur, C. M. Rudin, A. L. S. Chang, J. A. Low, A. B. Mueller, R. L. Yauch, J. C. Reddy and A. Hauschild, *Melanoma Res.*, 2011, **21**, pe9, DOI: 10.1097/01.cmr.0000399448.65869.b7.
124. C. M. Rudin, C. L. Hann, J. Laterra, R. L. Yauch, C. A. Callahan, L. Fu, T. Holcomb, J. Stinson, S. E. Gould, B. Coleman, P. M. LoRusso, D. D. von Hoff, F. J. de Sauvage and J. A. Low, *Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449*, Report 1533-4406, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University, Baltimore, MD 21231, USA, 2009.
125. R. L. Yauch, G. J. P. Dijkgraaf, B. Alicke, T. Januario, C. P. Ahn, T. Holcomb, K. Pujara, J. Stinson, C. A. Callahan, T. Tang, J. F. Bazan, Z. Kan, S. Seshagiri, C. L. Hann, S. E. Gould, J. A. Low, C. M. Rudin and F. J. de Sauvage, *Science*, 2009, **326**, 572-574.
126. D. A. Richards, J. Stephenson, B. M. Wolpin, C. Becerra, J. T. Hamm, W. A. Messersmith, S. Devens, J. Cushing, T. Schmalbach, C. S. Fuchs, *J. Clin.. Oncol.*, 2012, 30, (supp 4; abstr 213)
127. M. Allison, *Nat. Biotechnol.*, 2012, **30**, 203.
128. J. Rodon Ahnert, J. Baselga, H. A. Tawbi, Y. Shou, C. Granvil, J. Dey, M. M. Mita, A. L. Thomas, D. D. Amakye, A. C. Mita, J. *Clin*. *Oncol*. 2010, **28**, 15s (suppl; abstr 2500).
129. S. Buonamici, J. Williams, M. Morrissey, A. Wang, R. Guo, A. Vattay, K. Hsiao, J. Yuan, J. Green, B. Ospina, Q. Yu, L. Ostrom, P. Fordjour, D. L. Anderson, J. E. Monahan, J. F. Kelleher, S. Peukert, S. Pan, X. Wu, S. M. Maira, C. Garcia-Echeverria, K. J. Briggs, D. N. Watkins, Y. M. Yao, C. Lengauer, M. Warmuth, W. R. Sellers and M. Dorsch, *Sci. Transl. Med.*, 2010, **2**, 51ra70-51ra70.
130. M. Wickstroem, C. Dyberg, T. Shimokawa, J. Milosevic, N. Baryawno, O. M. Fuskevag, R. Larsson, P. Kogner, P. G. Zaphiropoulos and J. I. Johnsen, *Int. J. Cancer*, 2013, **132**, 1516-1524.
131. J. Fu, M. Rodova, S. K. Roy, J. Sharma, K. P. Singh, R. K. Srivastava and S. Shankar, *Cancer Lett.*, 2013, **330**, 22-32.
132. T. Mazumdar, J. DeVecchio, T. Shi, J. Jones, A. Agyeman and J. A. Houghton, *Cancer Res.*, 2011, **71**, 1092-1102.
133. S. Liu, G. Dontu, D. Mantle Ilia, S. Patel, N.-S. Ahn, W. Jackson Kyle, P. Suri and S. Wicha Max, *Cancer Res.*, 2006, **66**, 6063-6071.
134. J. A. Sterling, B. O. Oyajobi, B. Grubbs, S. S. Padalecki, S. A. Munoz, A. Gupta, B. Story, M. Zhao and G. R. Mundy, *Cancer Res.*, 2006, **66**, 7548-7553.
135. V. Chenna, C. Hu, D. Pramanik, B. T. Aftab, C. Karikari, N. R. Campbell, S.-M. Hong, M. Zhao, M. A. Rudek, S. R. Khan, C. M. Rudin and A. Maitra, *Mol. Cancer Ther.*, 2012, **11**, 165-173.
136. B. He, N. Fujii, L. You, Z. Xu and D. M. Jablons, Targeting GLI Proteins in human cancer by small molecules. *US Patent* 7714014, **2006**.
137. N. Mahindroo, M. C. Connelly, C. Punchihewa, H. Kimura, M. P. Smeltzer, S. Wu and N. Fujii, *J. Med. Chem.*, 2009, **52**, 4277-4287.
138. M. Actis, M. C. Connelly, A. Mayasundari, C. Punchihewa and N. Fujii, *Biopolymers*, 2011, **95**, 24-30.
139. G. Canettieri, L. Di Marcotullio, A. Greco, S. Coni, L. Antonucci, P. Infante, L. Pietrosanti, E. De Smaele, E. Ferretti, E. Miele, M. Pelloni, G. De Simone, E. M. Pedone, P. Gallinari, A. Giorgi, C. Steinkuhler, L. Vitagliano, C. Pedone, M. E. Schinin, I. Screpanti and A. Gulino, *Nat. Cell Biol.*, 2010, **12**, 132-142.
140. J. Mao, *J. Biol. Chem.*, 2002, **277**, 35156-35161.
141. M. A. Arai, C. Tateno, T. Koyano, T. Kowithayakorn, S. Kawabe and M. Ishibashi, *Org. Biomol. Chem.*, 2011, **9**, 1133-1139.
142. Y. Rifai, M. A. Arai, T. Koyano, T. Kowithayakorn and M. Ishibashi, *J. Nat. Prod.*, 2010, **73**, 995-997.
143. T. Hosoya, M. A. Arai, T. Koyano, T. Kowithayakorn and M. Ishibashi, *ChemBioChem*, 2008, **9**, 1082-1092.
144. A. Murakami, D. Takahashi, T. Kinoshita, K. Koshimizu, H. W. Kim, A. Yoshihiro, Y. Nakamura, S. Jiwajinda, J. Terao and H. Ohigashi, *Carcinogenesis*, 2002, **23**, 795-802.
145. Y. Rifai, M. A. Arai, S. K. Sadhu, F. Ahmed and M. Ishibashi, *Bioorg. Med. Chem. Lett.*, 2011, **21**, 718-722.
146. E. D. Pleasance, R. K. Cheetham, P. J. Stephens, D. J. McBride, S. J. Humphray, C. D. Greenman, I. Varela, M.-L. Lin, G. R. Ordonez, G. R. Bignell, K. Ye, J. Alipaz, M. J. Bauer, D. Beare, A. Butler, R. J. Carter, L. Chen, A. J. Cox, S. Edkins, P. I. Kokko-Gonzales, N. A. Gormley, R. J. Grocock, C. D. Haudenschild, M. M. Hims, T. James, M.-M. Jia, Z. Kingsbury, C. Leroy, J. Marshall, A. Menzies, L. J. Mudie, Z.-M. Ning, T. Royce, O. B. Schulz-Trieglaff, A. Spiridou, L. A. Stebbings, L. Szajkowski, J. Teague, D. Williamson, L. Chin, M. T. Ross, P. J. Campbell, D. R. Bentley, P. A. Futreal and M. R. Stratton, *Nature*, 2010, **463**, 191-196.
147. J. L. Brechbiel, J. M. Y. Ng and T. Curran, *Toxicol. Path.*, 2011, **39**, 478-485.
148. H. L. Park, C. Bai, K. A. Platt, M. P. Matise, A. Beeghly, C. c. Hui, M. Nakashima and A. L. Joyner, *Development*, 2000, **127**, 1593-1605.
149. A. Shimoyama, M. Wada, F. Ikeda, K. Hata, T. Matsubara, A. Nifuji, M. Noda, K. Amano, A. Yamaguchi, R. Nishimura and T. Yoneda, *Mol. Biol. Cell*, 2007, **18**, 2411-2418.
150. A. Quintas-Cardama, H. Kantarjian and J. Cortes, *Nat. Rev. Clin. Oncol.*, 2009, **6**, 535-543.